UNIVERSIDADE DE BRASÍLIA
FACULDADE DE CIÊNCIAS DA SAÚDE

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DETERMINAÇÃO DE SUBSTÂNCIAS PSICOATIVAS DE INTERESSE FORENSE E TOXICOLÓGICO EM MATERIAL APREENDIDO PELA POLÍCIA CIVIL DO DISTRITO FEDERAL E EM FLUIDOS BIOLÓGICOS POR GC-MS E LC-MS/MS

Brasília - DF
2024

ETTORE FERRARI JÚNIOR

Determinação de substâncias psicoativas de interesse forense e toxicológico em material apreendido pela Polícia Civil do Distrito Federal e em fluidos biológicos por GC-MS e LC-MS/MS

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Ciências da Saúde, Universidade de Brasília, como requisito parcial à obtenção do título de Doutor em Ciências Farmacêuticas.

Orientadora: Prof.ª Dra. Eloisa Dutra Caldas

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Dedico este trabalho à minha família: Letícia, Alice e Dante.

AGRADECIMENTOS

Agradeço primeiramente aos meus pais, Ettore (*in memorian)* e Graça, por acreditarem que a educação é o maior patrimônio a ser construído. Agradeço a minha esposa e companheira, Letícia por todo apoio e compreensão neste período. A família é a base sólida de toda a trajetória de nossas vidas.

Agradeço a Professora Eloisa, por acreditar e me orientar neste trabalho. Obrigado por todos os ensinamentos. É um exemplo de profissionalismo.

Aos amigos do Laboratório de Toxicologia da UnB, por toda ajuda.

Ao IML/DF, agradeço pela oportunidade de poder contribuir com a Toxicologia Forense do DF e aos amigos do Instituto de Criminalística do DF, por toda colaboração.

Por fim, agradeço a todos aqueles que de alguma forma contribuíram para a execução deste árduo trabalho.

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CAPÍTULO 4

LISTA DE ABREVIATURAS E SIGLAS

Δ-9-THC	Δ-9- tetrahidrocanabinol
2,5-DMA	2,5-Dimetoxianfetamina
6-MAM	6-monoacetilmorfina
7-AF	7-aminoflunitrazepam
ACN	Acetonitrila
AcOEt	Acetato de etila
Anvisa	Agência Nacional de Vigilância Sanitária
ATC	Antidepressivo tricíclico
BChE	Butirilcolinesterase
BZD	Benzodiazepínico
CB-1/CB-2	Receptores canabinoides
CBD	Canabidiol
CBN	Canabinol
CIT	Centro de Informação e Assistência Toxicológica
CYP	Citocromo P450
d-SPE	*Dispersive solid phase extraction*
DMT	Dimetiltriptamina
EMCDDA	*European Monitoring Centre for Drugs and Drug Addiction*
EME	Ecgonina metil éster
ESI	*Electrospray ionization*
EtOAc	*Ethyl acetate*
eV	eletron volt
GABA	*Gamma-aminobutyric acid*
GC-MS	*Gas chromatography – mass spectrometry*
IE	*Ionization energy*
HPLC	*High-performance liquid chromatography*
IML/DF	Instituto de Medicina Legal do Distrito Federal
LC-MS	*Liquid chromatography – mass spectrometry*
LLE	*Liquid-liquid extraction*
LSD	Dietilamida do ácido lisérgico
m-CPP	m-clorofenilpiperazina

MAO	Monoaminooxidase
MDA	Metilenodioxianfetamina
MDEA	Metilenodioxietiltanfetamina
MDMA	Metilenodioximetanfetamina
$MgSO_4$	Sulfato de magnésio
MRM	*Multiple Reaction Monitoring*
MS/MS	*Tandem Mass Spectrometry*
NaOAc	*Sodium acetate*
NMDA	N-metil-D-aspartato
NBOH	N-benzoilhidroxi
NBOMe	N-benzoilmetoxi
NSP	Novas substâncias psicoativas
ONS	*Office for National Statistics*
PCDF	Polícia Civil do Distrito Federal
PSA	*Primary and secondary amine*
PCP	Fenciclidina
PTV-LVI	Injetor de temperatura programada de vaporização e injeção de grandes volumes
QuEChERS	*Quick, Easy, Cheap, Effective, Rugged and Safe*
SIM	*Single ion monitoring*
SIM	Sistema de Informação de Mortalidade
SINITOX	Sistema de Informação Toxicológico-Farmacológicas
SNC	Sistema nervoso central
SLE-LTP	*Solid-liquid extraction with low-temperature partitioning*
SINAM	Sistema de Informação de Agravos de Notificação
SRM	*Single Reaction Monitoring*
t½	Tempo de meia vida
THC	Tetrahidrocanabinol
THC-COOH	11-nor-9-carboxi-delta9-tetrahidrocannabinol
THC-OH	11-hidroxi-A-tetra-hidrocanabinol
UGT	Uridina difosfato glucuronosiltransferase
UHPLC	*Ultra High Pressure Liquid Chromatography*
UNODC	*United Nations Office on Drugs and Crime*

RESUMO

FERRARI JÚNIOR, ETTORE. **Determinação de substâncias psicoativas de interesse forense e toxicológico em material apreendido pela Polícia Civil do Distrito Federal e em fluidos biológicos por GC-MS e LC-MS/MS**. Brasília, 2024. Tese de Doutorado em Ciências Farmacêuticas – Faculdade de Ciências da Saúde, Universidade de Brasília, Brasília, 2024.

Drogas de abuso são frequentemente envolvidas em casos de intoxicação exógena, incluindo casos com as novas substâncias psicoativas (NSP), como fenitilaminas, catinonas e canabinóides sintéticos. Este estudo teve como objetivos otimizar métodos por GC-MS para triagem de 25R-NBOH (R=Br, Cl, I ou etil), e desenvolver e validar métodos para análise de substâncias psicoativas por LC-MS/MS em amostras de sangue e urina coletadas no IML/DF e de fluido oral fornecida por frequentadores de festas de música eletrônica. A triagem de fenitilaminas da família 25R-NBOH por meio de GC-MS foi otimizada com colunas analíticas de 4 metros, diminuindo a degradação do composto durante a separação cromatográfica e permitindo a detecção do 25R-NBOH intacto. O método foi aplicado para análise de amostras de selos apreendidos. As amostras de sangue e urina foram extraídas por um método QuEChERS validado e analisadas por LC-MS/MS para determinação simultânea de 79 substâncias, incluindo catinonas sintéticas, fenetilaminas, canabinóides sintéticos e anfetaminas, com limites de quantificação entre 0,4 e 16 ng/mL; todas as 16 amostras de urina e 59,3% das 54 amostras de sangue foram positivas para pelo menos um analito. Catinonas sintéticas foram detectadas em 5 amostras: etilona (222 ng/mL, sangue ante-mortem), eutilona (246 e 446 ng/mL, urina) e N-etilpentilona (7,3 e 597 ng/mL, sangue ante-mortem e post-mortem, respectivamente), sendo este último referente a um caso fatal reportado no DF. As amostras de fluido oral também foram extraídas por um método QuEChERS modificado e LC-MS/MS e validado para determinação simultânea de 51 e triagem de outras 22 substâncias psicoativas. Cocaina e/ou metabólitos foram detectados em 8 amostras, com concentrações de 13,0 a 407,3 ng/mL (cocaina), 0,17 a 214,1 ng/mL (benzoilecgonina) e 1,8 a 150,1 ng/mL (ecgonina metil éster); MDMA (<0.5 - 829 ng/mL) e/ou MDA (10.1 – 829 ng/mL) foram detectadas em 27 amostras e metanfetamina (11 – 439 ng/mL) em 8 casos, junto com MDMA e MDA. Eutilona (4.7 e 24.1 ng/mL) foi encontrada em dois casos reportados como ingestão de “comprimido de MDMA”. Os métodos validados nesse estudo proporcionaram a obtenção de informações analíticas sobre o perfil de uso das substâncias psicoativas no Distrito Federal.

Palavras-chave: GC-MS, LC-MS/MS, QuEChERS, toxicologia forense, novas substâncias psicoativas.

ABSTRACT

FERRARI JÚNIOR, ETTORE. **Determination of psychoactive substances of forensic and toxicological interest in material seized by the Civil Police of the Federal District and in biological fluids by GC-MS and LC-MS/MS**. Brasília, 2023. Doctoral Thesis in Pharmaceutical Sciences – Faculty of Health Sciences, University of Brasília, Brasília, 2024.

Prescribed medicines and drugs of abuse are frequently involved in cases of exogenous intoxication, including cases with new psychoactive substances (NPS), such as phenethylamines, cathinones, and synthetic cannabinoids. This study aimed to optimize GC-MS methods for screening of 25R-NBOH (R=Br, Cl, I, or ethyl) and to develop and validate methods for the analysis of psychoactive substances by LC-MS/MS, including NPS, in blood and urine samples collected at the IML/DF and oral fluid provided by attendees of electronic music parties. The screening of 25R-NBOH family phenethylamines through GC-MS was optimized with 4-meter analytical columns, reducing compound degradation during chromatographic separation and enabling the detection of intact 25R-NBOH. The method was applied to analyze seized blotter papers. Blood and urine samples were extracted using a validated QuEChERS method and analyzed by LC-MS/MS for the simultaneous determination of 79 substances, including synthetic cathinones, phenethylamines, synthetic cannabinoids, and amphetamines, with limits of quantification ranging from 0.4 to 16 ng/mL; all 16 urine samples and 59.3% of the 54 blood samples tested positive for at least one analyte. Synthetic cathinones were detected in 5 samples: ethylone (222 ng/mL, antemortem blood), eutylone (246 and 446 ng/mL, urine), and N-ethylpentylone (7.3 and 597 ng/mL, antemortem and postmortem blood, respectively), the latter related to a fatal case reported in the DF. Oral fluid samples were also extracted using a modified QuEChERS method and LC-MS/MS and validated for the simultaneous determination of 51 substances and screening of another 22 psychoactive substances. Cocaine and/or metabolites were detected in 8 samples, with concentrations ranging from 13.0 to 407.3 ng/mL (cocaine), 0.17 to 214.1 ng/mL (benzoylecgonine), and 1.8 to 150.1 ng/mL (methyl ecgonine); MDMA (<0.5 - 829 ng/mL) and/or MDA (10.1 – 829 ng/mL) were detected in 27 samples, and methamphetamine (11 – 439 ng/mL) in 8 cases, along with MDMA and MDA. Eutylone (4.7 and 24.1 ng/mL) was found in two cases reported as "MDMA tablet" ingestion. The methods validated in this study provided analytical information on the use profile of psychoactive substances in the Federal District.

Keywords: GC-MS, LC-MS/MS, QuEChERS, forensic toxicology, new psychoactive substances.

INTRODUÇÃO

Drogas de abuso (lícitas e ilícitas)são frequentemente envolvidas em casos de intoxicação exógena no Brasil e outros países (Magalhães e Caldas, 2018a; NIH, 2023; ONS, 2023; Gummin et al., 2020). Mais recentemente, casos de overdose pelo abuso de novas substâncias psicoativas (NSP) alertaram a comunidade científica sobre o potencial risco de drogas ainda pouco estudadas (Fujita et al., 2016; Atherton et al., 2018; Costa et al., 2018; Gerace et al., 2018). O perfil do usuário de drogas é atendido em emergências hospitalares e, com frequência, o profissional de saúde e a própria vítima não sabem quais substâncias foram responsáveis pelo quadro de intoxicação, o que impossibilita uma abordagem médica mais precisa. Mesmo em casos de intoxicação fatal, a maioria dos institutos de medicina legal do país não dispõem de condições analíticas adequadas para triagem de NSP, fator limitante para o correto estabelecimento da causa da morte.

Algumas destas NSP apresentam limitações de análise por cromatografia gasosa, a mais utilizada nos laboratórios forenses. Atualmente, no Distrito Federal, não há um laboratório que realize análise em fluidos biológicos de vítimas de intoxicação por NSP, tampouco um serviço de análise toxicológica para intoxicações provenientes de emergências hospitalares.

Por meio de análises laboratoriais em amostras provenientes do IML/DF, de emergências hospitalares e dos próprios usuários de substâncias psicoativas, seria possível traçar a epidemiologia de substâncias relacionadas a eventos de intoxicação exógena e o impacto da utilização destas substâncias na sociedade local, além de dados estatísticos para subsidiar corretas ações na área da saúde e de segurança pública.

Este estudo tem como objetivos otimizar métodos por GC-MS para triagem de substâncias da família 25R-NBOH (R=Br, Cl, I ou etil), desenvolver e validar métodos por LC-MS/MS para determinação de substâncias psicoativas, incluindo NSP, em fluidos biológicos de amostras provenientes do Instituto de Medicina Legal e de doadores voluntários, usuários NSP e frequentadores de festas de música eletrônica do DF.

REVISÃO DE BIBLIOGRÁFICA

1. Apreensão de selos contendo 25R-NBOH, 25R-NBOMe e/ou LSD pela Polícia Civil do Distrito Federal

A Figura 1 mostra a estrutura molecular do LSD e exemplos de selos contendo LSD e a Tabela 1 mostra números de apreensão de selos contendo 25R-NBOH, 25R-NBOMe e/ou LSD no Distrito Federal, entre 2015 e agosto de 2022. Dados posteriores não estão disponíveis.

Figura 1: Estrutura molecular do LSD e selos apreendidos contendo LSD (Cortesia: Instituto de Criminalística -DF).

Nota-se uma tendência de substituição de substâncias da família NBOMe por NBOH no mercado ilícito local ao longo do período (Tabela 1). Em 2018, o número de apreensões de 25R-NBOMe (R=Br, Cl, etil ou I) já era bem inferior às de NBOH e LSD, sendo responsável por apenas 1,2 % do total de apreensões de selos no ano (4.474). A partir de 2019, não houve detecção de compostos desta família, e substâncias da família NBOH se consolidaram no mercado, sendo o grupo mais detectado nas apreensões desde 2017, chegando a ser detectado em 76,3 % do total de selos apreendidos entre 2019 e 2022 (69.014).

Tabela 1: Número de selos contendo 25R-NBOH, 25R-NBOMe e/ou LSD apreendidos pela Polícia Civil do Distrito Federal, de 2015 a agosto de 2022.

Classe		2015	2016	2017	2018	2019	2020	2021	2022*
NBOMe	25B	195	73	69	7	-	-	-	-
	25C	52	225	-	-	-	-	-	-
	25I	282	349	663	48	-	-	-	-
	Total NBOMe	**529**	**647**	**732**	**55**	-	-	-	-
NBOH	25B	-	-	673	-	866	-	151	2265
	25C	-	-	-	-	2.558	509	9	6
	25E	-	-	-	1.899	4.811	1.167	38.250	2.077
	25I	-	1.066	2.948	1.999	14	2	-	-
	Total NBOH	**-**	**1.066**	**3.621**	**3.898**	**8.249**	**1.678**	**38.410**	**4.348**
-	LSD	12	588	109	521	380	546	15.108	295

*até agosto de 2022. Pode haver mais de uma substância presente em selos apreendidos.

A Figura 2 mostra a estrutura molecular do 25B-NBOMe, 25C-NBOMe, 25E-NBOMe e 25I-NBOMe.

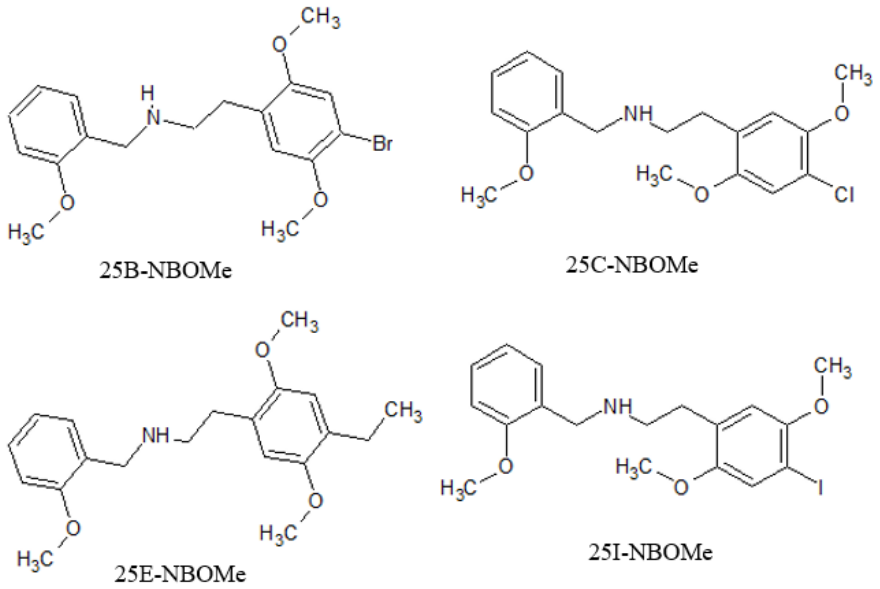


Figura 2: Estrutura molecular de 25B-NBOMe, 25C-NBOMe, 25E-NBOMe e 25I-NBOMe.

25I-NBOH foi a primeira substância da família NBOH detectada no DF (2016), sendo a substância mais encontrada nos selos apreendidos entre 2016 e 2017, até não ser mais detectada nas amostras de 2021. Em contrapartida, 25E-NBOH foi detectado em 2018 e, partir desse ano foi a substância mais encontrada nos selos. Em 2019, as

apreensões de LSD mostraram uma queda de 27,1 % em relação ao ano anterior. (Tabela 1).

O número de selos apreendidos no DF em 2021 (Tabela 1) merece destaque pela quantidade (n=53.518), maior que a de comprimidos artesanais (13.495), com um aumento do número de itens apreendidos contendo LSD e NBOH de 2.767 e 2.289 %, respectivamente, quando comparado com 2020. Em 2022, esta tendência abrupta de aumento do número de apreensões de selos não se confirmou, com apenas 4643 unidades apreendidas até agosto de 2022 (Tabela 1).

Dados estatísticos de apreensão de selos de outros Estados brasileiros também demonstraram o alto índice de detecção de 25R-NBOH e a tendência de substituição de NBOMe por NBOH no mercado de drogas (Machado et al., 2018; Souza Boff et al., 2019; Meira et al., 2021). Dados estatísticos de Minas Gerais de 2008 a 2017, evidenciaram que o LSD vinha sendo substituído pelas fenetilaminas NBOMe e NBOH (Machado et al., 2018). No Rio de Janeiro, houve um crescente número de detecções de 25R-NBOMe a partir de 2014, sendo detectado em 90,5 % dos selos apreendidos naquele ano, com declínio a partir de 2016, ano do surgimento do 25R-NBOH no mercado local, e sendo detectado em 93,7 % das apreensões de selos de 2017. De 2014 a 2019, LSD foi detectado em apenas 14,16 % das apreensoes. Os autores afirmaram que a detecção de 25I-NBOH e seus isômeros só pôde ser realizada por LC-MS/MS, devido a sua degradação a 2C-I, quando analisado por GC-MS (Meira et al., 2021). Dados de Santa Catarina também revelaram o grande número de apreensões de selos contendo de NBOH e NBOMe entre 2011 e 2017. Os NBOMes foram detectados em 10% dos selos apreendidos em 2014, chegando a 50%, em 2015. Em 2016, os NBOHs foram detectados em dois terços dos selos apreendidos, e em 50 %, no ano seguinte (Souza Boff et al., 2019). De 2017 A 2020, 52.755 selos contendo 25R-NBOMe foram apreendidos na Argentina, Chile, República Checa, Hungria e Nova Zelândia totalizaram, e não houve informação sobre detecção de 25R-NBOH (UNODC, 2023a).

O alto número de apreensões de selos no DF observado em 2021 (Tabela 1) também foi observada a nivel de mercado global de outras drogas ilícitas, afetando rotas de tráfico e o perfil de consumo de usuários, entre 2020 e 2022, sugerindo um possível reflexo do impacto da pandemia de Sars-Cov-2 (COVID-19), devido às restrições impostas de circulação e fechamento de fronteiras (Vargas, Chicauhal e Duffau, 2020, EMCDDA, 2020a, UNODC, 2021, Ferrari Júnior et al., 2024). Neste período, houve uma diminuição de disponibilidade de precursores para o preparo de algumas drogas

sintéticas, em regiões da Europa e Ásia, além da interrupção do tráfico de drogas por via aérea, pelas restrições de viagens impostas, com consequente migração para o tráfico marítimo (UNODC, 2021). No tráfico de cocaína, por exemplo, a diminuição da pureza da droga comercializada no Chile entre março e junho de 2020 (Vargas, Chicauhal e Duffau, 2020), e o aumento do preço da droga e diminuição da pureza no Reino Unido e França (UNODC, 2021) foram outros impactos observados. No Distrito Federal, apesar de um aumento de 332 % na quantidade de cocaína em pó apreendida em 2020, quando comparado a 2019, a pureza da droga comercializada nas ruas diminuiu em torno de 26 %. Em 2020, a detecção de novos diluentes de cocaína, como os aditivos plásticos Irganox 1076 e Irgafos 168, indicaram uma tendência de novos adulterantes/diluentes sendo introduzidos no mercado para mitigar a escassez local da cocaína ou até mesmo de outros adulterantes/diluentes (Ferrari Júnior et al., 2024). A análise de substâncias psicoativas ilícitas (anfetamina, cocaína, MDMA, metanfetamina e THC) em águas residuais da cidade de Novo Hamburgo (Rio Grande do Sul – Brasil) revelou que o período de confinamento imposto pela pandemia causou um menor consumo destas substâncias (Hahn et al., 2022) pela população. A mudança do perfil de consumo de algumas drogas também foi documentada na Europa, como a redução do uso de anfetaminas e cocaína, provavelmente pela paralização da vida noturna no período da pandemia. Tais informações foram confirmadas por estudos em águas residuais em várias cidades européias (EMCDDA, 2020a). Em contrapartida, o consumo do álcool aumentou consideravelmente (EMCDDA, 2020a).

1.1. Análise de substâncias da família 25R-NBOH por cromatografia gasosa acoplada à espectrometria de massas (GC-MS), em material apreendido

GC-MS é uma das técnicas analíticas mais empregadas em análise de drogas apreendidas, devido a alta sensibilidade, seletividade e capacidade de quantificação dos compostos presentes na amostra, mesmo em matrizes complexas. A comparação dos resultados obtidos com bancos de dados de espectros de massas de compostos conhecidos é outra importante característica, possibilitando o screening de substâncias potencialmente presentes na amostra (Skoog, James e West, 2015). No entanto, a técnica apresenta algumas limitações, como a necessidade de corridas cromatográficas muito longas para análise de compostos de baixa volatilidade, como alguns canabinoides sintéticos (UNODC, 2013); potencial degradação de substâncias com

baixa estabilidade térmica, como o inseticida carbamato aldicarbe (Fialkov, Gordin e Amirav, 2003; Zeeuw, 2000; Tsujikawa et al., 2013); ou que apresentam alta reatividade à coluna cromatográfica, como o esteroide anabolizante estanozolol (Rossi, Johson e Yost, 1992; Brito e Caldas, 2017).

Soluções para mitigar a reatividade e/ou termolabilidade destes compostos, sem recorrer à derivatização, incluem o ajuste de rampas de temperatura mais brandas, colunas analíticas de menor comprimento e o uso de *liner* inerte (Fialkov, Gordin e Amirav, 2003). O menor tempo de residência do analito no sistema cromatográfico, por meio do emprego de colunas analíticas menores, pode minimizar a fragmentação do analito, que geralmente ocorre no injetor ou na própria coluna, que normalmente possui 30 m de comprimento (Fialkov, Gordin e Amirav, 2003; Man et al. 2009, Zeeuw, 2009). A derivatização também é uma alternativa (Fogarty et al., 2019), entretanto, gera custo adicional, aumenta o tempo de análise, além de onerar a manutenção da instrumentação analítica.

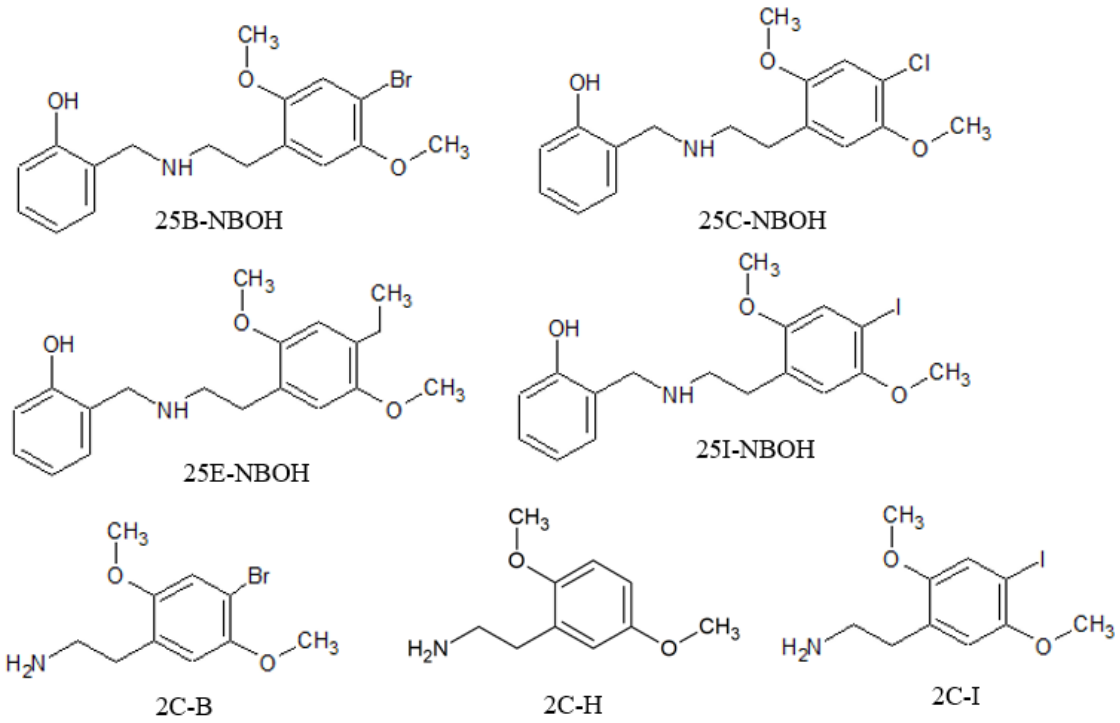


Figura 3: Estutura molecular das fenetilaminas, 25B-NBOH, 25C-NBOH, 25E-NBOH, 25I-NBOH 2C-B, 2C-H e 2C-I.

O surgimento das substâncias da família 25R-NBOH (R=Br, Cl, etil ou I) no mercado de drogas é um exemplo atual de moléculas termolábeis que desafiam a comunidade forense. Desde a primeira detecção do 25I-NBOH, em 2016, em

apreensões de selo (micropontos), esta classe de substâncias impôs a necessidade de adequação no fluxo de análise dos laboratórios, pois a aplicação do GC-MS de modo convencional ficara comprometida, naquele momento (Arantes et al., 2017).A detecção incorreta de uma droga gera dados estatísticos imprecisos, com reflexos no âmbito da toxicologia, pois dados de apreensão também são fonte de informação para o alerta de novas substâncias, para novos protocolos de ação em saúde, ou para o entendimento do perfil de consumo em uma comunidade (Machado et al., 2018; Souza Boff et al., 2019; Meira et al., 2021). A Figura 3 ilustra a estrutura molecular das fenetilaminas, 25B-NBOH, 25C-NBOH, 25E-NBOH, 25I-NBOH 2C-B, 2C-H e 2C-I.

Em 2017, foi publicada a primeira detecção de 25I-NBOH, em apreensões no Distrito Federal (Arantes et al., 2017). Quando analisado por GC-MS, 25I-NBOH foi incorretamente identificado como 2C-I (Figura 4), devido à degradação da molécula no interior do sistema cromatográfico com colunas analíticas de 30 metros de comprimento (Figura 5).

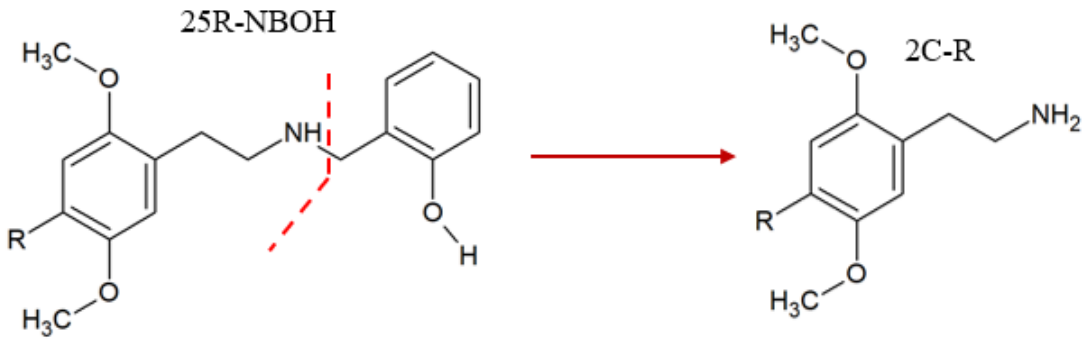


Figura 4: Moléculas de 25R-NBOH (R=Br, Cl, etil ou I) e 2C-R (R=Br, Cl, etil ou I). A linha serrilhada mostra o local onde a molécula de 25R-NBOH se cliva durante a corrida cromatográfica, utilizando GC-MS, quando analisada por coluna de 30 metros de comprimento.

Quando as amostras foram analisadas por cromatografia líquida acoplada à espectrometria de massas com analisador quadrupolo e tempo de vôo (LC–QTOF-MS), o 25I-NBOH foi detectado intacto (Arantes et al., 2017). Sendo assim, 25I-NBOH era incorretamente identificado por GC-MS como uma droga bem menos potente, o 2C-I. A dose oral recreativa de 2C-I é comumente entre 10 mg e 25 mg (Shulgin e Shulgin, 1991), massa superior ao da apresentação comercializada ilegalmente nas ruas (selo ou

microponto), que é de “microgramas”. Por isso, substâncias da família 2C-R (R=Br, Cl, etil, I) são normalmente encontradas em pó e em comprimidos e administrados por via oral ou nasal (Herman et al., 2016). Trabalhos posteriores demonstraram que a concentração média de um NBOH presente em selos é de “microgramas” (Leite, 2023; Rodrigues de Morais et al., 2020).

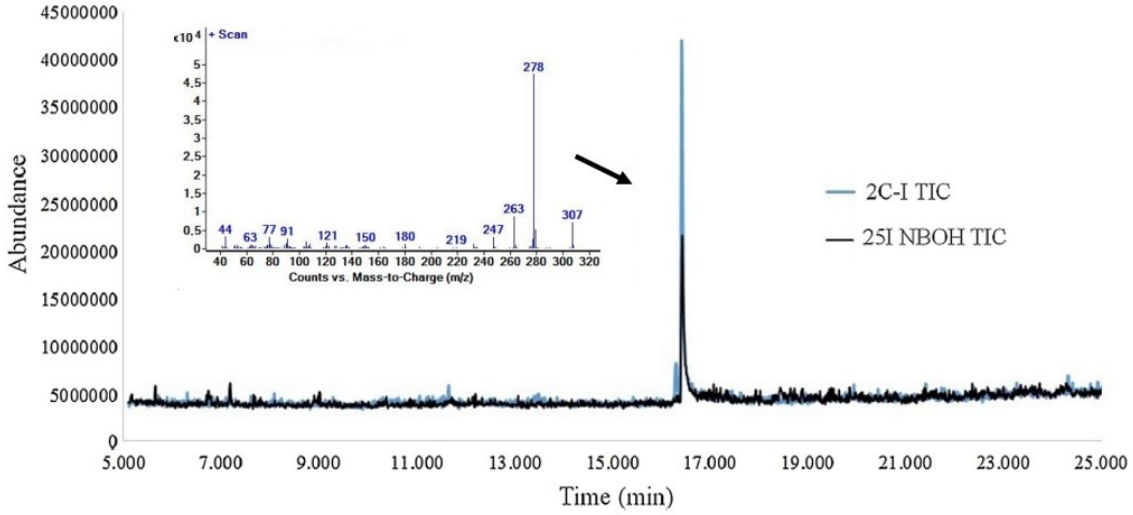


Figura 5: Cromatograma de íons totais (*total ion chromatogram*) em GC-MS. Os picos cromatográficos e o espectro de massas do 2C-I e do 25I-NBOH são indistinguíveis por GC-MS (Arantes et al., 2017).

Coelho Neto e colaboradores (2017) utilizaram GC-MS para analisar 25I-NBOH em amostras de selos apreendidos em Minas Gerais e no Distrito Federal, e demonstraram que, além da degradação do 25I-NBOH em 2C-I, havia a detecção de um pico secundário, também proveniente da clivagem do 25I-NBOH no interior do GC, e que não era detectado na análise do padrão analítico de 2C-I (Figura 6). O padrão de fragmentação deste pico secundário também variava de acordo com o tipo de solvente utilizado (metanol, isopropanol) na diluição da amostra, sugerindo uma substituição nucleofílica entre o composto detectado e o solvente utilizado. Os autores sugeriram a necessidade de avaliar esse pico secundário (2-(metoximetil)fenol) para a identificação correta de 25R-NBOH nas amostras de selos analisadas por GC-MS, utilizando coluna analíticas de 30 m de comprimento (Coelho Neto et al., 2017).

A derivatização de compostos da família 25R-NBOH também foi uma alternativa proposta para se mitigar a degradação, utilizando N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), anidrido acético (Andrade et al, 2020) ou anidrido

heptafluorobutírico (HFBA) (Lum et al., 2020). A derivatização de 25I-NBOH com MSTFA gerou dois picos cromatográficos, mono-TMS e di-TMS (Andrade et al, 2020), entretanto, não possibilitando discriminar diferentes substâncias desta família (25B-NBOH, 25C-NBOH, 25E-NBOH e 25I-NBOH) (Ferrari Jr. et al., 2020), o que foi possível com a derivatização com HFBA (Lum et al., 2020). Cabe lembrar que a derivatização utilizando anidrido acético foi avaliada apenas para o 25I-NBOH (Andrade et al., 2020).

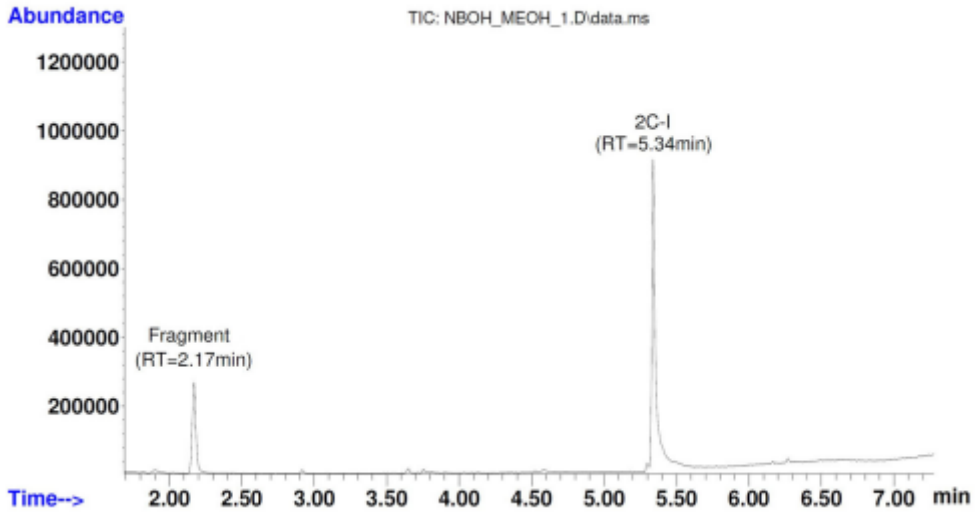


Figura 6: Cromatograma de íons totais (*total ion chromatogram*) em CG-MS. Pico cromatográfico secundário (Tr=2,17 min) e pico de 2C-I (Tr=5,34 min.), ambos provenientes da quebra do 25I-NBOH, quando analisado por GC-MS e coluna de 30 metros de comprimento (Coelho Neto et al., 2017).

Leite (2023) validou um método para quantificação de 25R-NBOH em selos apreendidos, utilizando cromatografia líquida acoplada a um detector de arranjo de diodos (HPLC-DAD), e avaliou a estabilidade de amostras contendo 25R-NBOH (R=Br, Cl, etil ou I) por até 50 dias, a 4, 25 e 50º C, armazenadas em metanol e água:acetonitrila (1:1), além de estudar a degradação de 25R-NBOH em 2C-R em selos armazenados entre 11 e 89 meses (n=156), à temperatura ambiente e protegidos da luz. Os resultados demonstraram que a degradação apresentrou uma curva exponencial (r^2 > 0,99) nas amostras armazenadas em água:acetonitrila a 50 ºC, e uma degradação linear (r^2 > 0,99), atingindo 100 % de degradação após 50 dias de armazenamento no metanol. Segundo o autor, o tempo de armazenamento não foi determinante para a formação de

2C-R e que não se pôde excluir a sua presença como sendo impureza de rota de síntese do NBOH, ou outro fator ainda não compreendido. Concluiu, ainda, que o mecanismo de degradação do 25R-NBOH é complexo e não pode ser discutido apenas como um fenômeno de degradação térmica (Leite, 2023).

Cabe lembrar que a identificação incorreta de 25I-NBOH em 2C-I trouxe implicações jurídicas graves, pois o 25I-NBOH só foi considerado uma droga proscrita em 19 de outubro de 2016, com a inclusão na Portaria SVS/MS 344/98, quando o 2C-I já era considerado uma droga proscrita (Anvisa, 2016).

2. Toxicologia analítica

A toxicologia analítica é a área responsável pela detecção, identificação e quantificação de drogas e outros xenobióticos em matrizes biológicas, no intuito de ajudar no diagnóstico, tratamento, prognóstico e prevenção de intoxicações agudas (Flanagan, 2005). Ela inclui as áreas de toxicologia forense e clínica.

Exames laboratoriais podem ser a única evidência objetiva em um caso de intoxicação aguda, guiando para a correta abordagem terapêutica no ambiente hospitalar (Helander et al., 2013) ou para a determinação da causa da morte, em laboratórios forenses (Ferrari e Caldas, 2018; Ferrari, Santos e Caldas, 2020; Launiainen e Ojanpera, 2014). Além de contribuir para a determinação da causa da morte, a toxicologia analítica proporciona resultados robustos, contribuindo para diminuição de subnotificações (Magalhães e Caldas, 2018ª) e para a produção de provas legais em uma persecução penal (Drummer, 2007; Brasil, 1941). Adicionalmente, as informações geradas podem fornecer alerta toxicológico sobre surtos epidemiológicos provocados por abuso de toxicantes e de novas drogas de abuso presentes no mercado (Klaassen, 2013; Lahti, Korpi e Vuori, 2009).

2.1. Amostras biológicas de interesse toxicológico

A Associação do Reino Unido e da Irlanda dos Laboratórios de Toxicologia Forense e dos Toxicologistas Forenses sugere uma lista de amostras biológicas que podem ser coletadas para a análise toxicológica forense, considerando inclusive, matrizes alternativas (Quadro 1).

O sangue é o principal fluido utilizado na toxicologia forense, pois a concentração sanguínea de drogas pode estar estreitamente relacionada com os efeitos

farmacológicos e tóxicos observados, fornecendo dados farmacocinéticos e proporcionando a comparação com a clínica apresentada (Woźniak et al.2017; Drummer, 2007). Na toxicologia clínica, esta correlação entre a concentração do toxicante e o evento observado é bem estabelecida para vários fármacos e drogas de abuso (Schulz et al., 2020). Entretanto, nem sempre esta correlação é possível nos casos fatais, pois logo após a morte, o sangue sofre degradação e há redistribuição dos toxicantes entre os tecidos (Drummer, 2007; Mcintyre e Escott, 2012; Yarema e Becker, 2005), o que dificulta a comparação das concentrações encontradas com valores de concentrações terapêuticas e tóxicas estabelecidas para a toxicologia clínica (Butzbach, 2010; Launiainen e Ojanpera, 2014; Schulz et al., 2020). Portanto, a publicação de casos envolvendo amostras post-mortem (Brockbals et al., 2020; Ferrari e Caldas, 2018; Ferrari, Santos e Caldas, 2020; Ketola e Kriikku, 2019; Kraemer et al., 2019; Mantinieks et al., 2020), são importantes ferramentas para a interpretação de casos em toxicologia forense.

Quadro 1: Amostras biológicas que podem ser coletadas (e quantidade sugerida) para análise toxicológica post-mortem (Elliott, Stephen e Paterson, 2018).

Material	**Quantidade**
Sangue femoral	10 mL (local especificado e adequadamente isolado)
Sangue de cavidade cardíaca	25 mL (quando a quantidade de sangue femoral for limitada)
Urina	Toda quantidade disponível
Humor vítreo	Toda quantidade disponível
Bile	10 mL
Fígado	10 – 20 g (se houver pouco sangue disponível)
Conteúdo estomacal	Toda quantidade disponível
Cérebro	10 – 20 g (pesquisa de compostos voláteis)
Pulmão	10 – 20 g (pesquisa de compostos voláteis)
Músculo esquético	10 – 20 g (se houver pouco sangue disponível)

Devido à redistribuição post-mortem, diferentes matrizes biológicas podem apresentar concentrações variadas de toxicante (Zilg et al., 2017; Yarema e Becker, 2005), o que torna a padronização do procedimento de coleta um passo importante para

a qualidade dos resultados obtidos. Atualmente, o sangue post-mortem coletado da veia femoral é a matriz biológica preconizada (Pélissier-Alicot et al., 2003, Lemaire et al, 2017). O estágio de decomposição do cadáver pode comprometer a qualidade e quantidade de matrizes biológicas disponíveis para a análise toxicológica, o que justifica a utilização de tecidos e fluidos biológicos alternativos para a pesquisa de toxicantes, como humor vítreo (Ketola e Kriikku, 2019), cabelo (Cuypers e Flanagan, 2018), conteúdo gástrico (Liang et al., 2015; Kim et al., 2014; Peres, Nascimento e Pelição, 2019) e vísceras (Drummer, 2007; Moffat et al., 2011; Orfanidis et al., 2020; Rodda et al., 2018).

A urina é o fluido biológico de escolha para triagem e monitoramento de drogas de abuso e fármacos *in vivo* , já que fornece uma janela de detecção mais longa que o sangue (Nickley, Pesce e Krock, 2017). Adicionamente, a urina apresenta outras vantagens, como o procedimento de fácil execução e não invasivo de coleta, sendo utilizada em uma rotina laboratorial para complementar os resultados pela análise sanguínea (Ambach et al., 2015; Helander et al., 2014). Entretanto, a concentração de uma substância na urina não tem boa correlação com sua concentração sanguínea (Elliott, Stephen e Paterson, 2018; Moffat et al., 2011) e não deve ser usada para interpretação do efeito farmacológico/toxicológico em humanos (Elliott, Stephen e Paterson, 2018). O fluido oral (saliva) é um fluido biológico escolhido para coletas em campo (Cunha et al., 2020; Richeval et al., 2018; Zancanaro et al., 2012), para monitorização terapêutica (Carvalho et al., 2018; Abdelshafi et al., 2018) e já foi demonstrado que NSP podem ser detectadas em saliva, como canabinoides sintéticos, piperazinas, fenetilaminas, triptaminas e opióides após exposição recente (Cunha et al., 2020; Øiestad et al., 2016; Krotulski et al., 2018). Esta matriz biológica demonstra boa correlação de concentração com o sangue (Niedbala et al., 2004; Drummer, 2006; Jenkins, Oyler e Cone, 1995) e, assim como a urina, possui vantagens de ser um procedimento não invasivo e de fácil execução, sem necessitar de ambiente próprio de coleta. Como desvantagem, os coletores de saliva apropriados para garantir a preservação dos analitos contêm conservantes, sais e outros compostos que, além de diluir a amostra, podem aumentar o efeito de matriz dos métodos cromatográficos utilizados (Langel et al., 2008; Sobczak e Gorynski, 2020), além de apresentarem baixos valores de recuperação para algumas substâncias de interesse toxicológico (Langel et al., 2008). O alto custo destes dispositivos de coleta também deve ser considerado no planejamento de uma rotina analítica.

3. Epidemiologia das intoxicações

Fármacos e drogas de abuso estão frequentemente envolvidos em casos de intoxicação exógena no Brasil (Sinitox, 2016; Magalhães e Caldas, 2018[a]; Ferrari e Caldas, 2018) e no mundo (NIH, 2023; ONS, 2023; Gummin et al., 2020).

A Tabela 2 mostra os dados estatísticos brasileiros sobre intoxicação humana, disponibilizados pelo Sistema de Informações Toxicológico-Farmacológicas (SINITOX). Fármacos (medicamentos) foram o principal agente reportado, sendo responsável por mais de 1/3 dos casos registrados, e pesticidas a classe que apresentou a maior taxa de letalidade (2,9 %). Quando medicamentos, pesticidas, raticidas e drogas de abuso são analisados em conjunto, estas classes foram responsáveis por quase a metade dos casos de intoxicação (48,5 %) e por 73,5 % dos óbitos registrados.

Tabela 2: Casos registrados no Brasil em 2016 de intoxicação humana, óbito e letalidade por agente tóxico. Disponível em https://sinitox.icict.fiocruz.br/[a].

Agente	**Intoxicação**		**Óbitos**	**Letalidade**
	n	**%**	**n**	**%**
Medicamentos	20.527	36,05	42	0,20
Domissanitários	5.660	9,94	5	0,09
Pesticidas	3.311	5,81	96	2,9
Drogas de abuso	2.745	4,82	27	0,98
Produtos químicos industriais	2.487	4,37	8	0,32
Cosméticos	1.420	2,49	-	-
Raticidas	1.014	1,78	1	0,1
Animais peçonhentos	14.884	26,1	34	0,2
Animais não peçonhentos	1.021	1,79	-	-
Desconhecido/Outros[b]	3.868	6,7	13	0,3
Total	56.937	100	226	0,4

a. Os dados mais recentes podem estar subestimados devido à falta de informações de alguns centros de informação estaduais.

b. 1217 desconhecidos; outros inclui metais, plantas, alimentos e drogas veterinárias.

Num estudo descritivo de 18247 casos de intoxicações registrados pelo Sistama de Informação de Mortalidade do Ministério da Saúde (SIM) entre 2010 e 2015,

pesticidas (23,7%), fármacos (medicamentos) (22,6%) e drogas de abuso (21,5%) foram as principais substâncias responsáveis pelos óbitos (Bochner e Freire, 2020). Outro estudo utilizou a base de dados do Sistema de Informação de Agravos de Notificação (SINAM) sobre 833.282 notificações compulsórias por intoxicação exógena no Brasil entre 2007 e 2017, 35,2% foram de ingestão voluntária, 40,3 % envolveram fármacos, 11,4% drogas de abuso, 6,9% pesticidas e 6,5% produtos veterinários/raticidas (Alvim et al., 2020). Num estudo com dados de 2564 casos de suicídio ocorridos no Rio Grande do Sul entre 2017 e 2018, 36% dos casos foram detectadas substâncias psicotrópicas no material post-mortem, e jovens apresentaram 4,5 vezes (IC95% 2,7;7,7) maior chance de serem vítimas quando havia resultados positivos para alguma substância ilícita. Em 7,3% dos óbitos, a intoxicação foi considerada a causa da morte (Franck, Monteiro e Limberger, 2020).

Dois estudos de casos de intoxicação ocorridos no DF entre 2009 e 2013 foram reportados por Magalhães e Caldas (2018a; 2018b), utilizando dados do Centro de Informação Toxicológica do DF (CIT-DF) e do SINAM-DF (Magalhães e Caldas, 2018a) e outro envolvendo também informações sobre casos fatais do SIM e Instituto Médico Legal do Distrito Federal (IML-DF) (Magalhães e Caldas, 2018b). Dados provenientes do CIT/DF e do SINAM/DF mostraram que mais de 30% dos 338 casos de intoxicação fatal analisados envolviam o uso de pesticidas, principalmente o inseticida carbamato aldicarbe. Fármacos estavam envolvidos em mais de 48 % dos casos de intoxicação, incluido os não fatais (Magalhães e Caldas, 2018a). Analisando apenas os casos fatais descritos nas 3 diferentes bases de dados, CIT/DF e do SINAM/DF e IML/DF, foram relatados 20 (6,3% de 338 casos) envolvendo abuso de cocaína, a única droga ilícita descrita. Fármacos (incluindo os de uso controlado) foram relatados em 157 casos, sendo 6 desses em associação com álcool ou pesticidas. 95 casos fatais envolviam o uso de pesticidas, e em 3 casos houve o uso concomitante com Fármacos de uso controladoou cocaína. Em 35 casos fatais (11 % do total), foi relatado o abuso de álcool (etanol) (Magalhães e Caldas, 2018b). Excetuando-se a dosagem de alcoolemia, a análise quantitativa para as demais substâncias não foi realizada em nenhum desses casos avaliados por Magalhães e Caldas (2018a,b).

Estudos com amostras de sangue post-mortem coletadas em 2018 no IML-DF trouxeram dados epidemiológicos provenientes da toxicologia analítica (Ferrari e Caldas, 2018; Ferrari, Santos e Caldas, 2020), onde foram analisadas 33 substâncias de interesse forense em 111 amostras de sangue *post-mortem* por GC-MS, das quais 49,6

% vieram de casos de homicídio, 18% de casos classificados como acidente (acidente de trabalho, afogamento, atropelamento e direção sob influência) e 16,2% de casos de suicídio. Foi detectada pelo menos uma das substâncias investigadas em 68 das 111 amostras analisadas (61,3%) (Ferrari, Santos e Caldas, 2020). O perfil das circunstâncias e as substâncias detectadas está mostrado na Figura 7. Benzodiazepínicos (diazepam, midazolam, flunitrazepam, 7-aminoflunitrazepam) foram os principais fármacos de uso controlado detectados nas amostras, correspondendo a 46% dos casos positivos (Figura 7). Outras drogas de abuso detectadas foram o MDMA, THC e cetamina (Figura 7). Cerca de 36% das amostras de sangue post-mortem de casos de homicídio analisadas neste estudo continham pelo menos uma droga ilícita. Em dois casos de suicidio, foi confirmada a presença dos pesticidas terbufos (0,03 e 0,04 µg/mL) e carbofurano (27,33 µg/mL), ambos inseticidas organofosforados. Cocaína estava presente em 17 casos (6,5%), com concentrações variando de 0,02 a 4,07 µg/mL (Ferrari, Santos e Caldas, 2020).

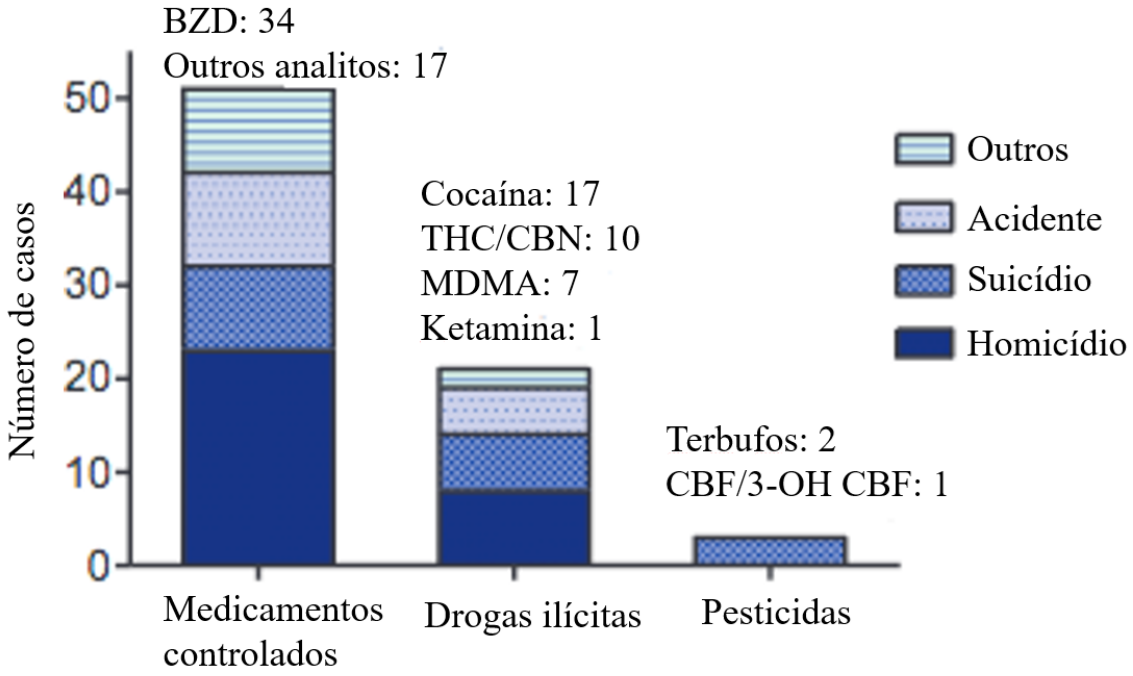


Figura 7: Distribuição das 68 amostras de casos positivos, divididos por classes de substâncias e circunstâncias envolvidas. Uma amostra pode ter mais de um analito. Outras: causa natural e indeterminada de morte. BZD: benzodiazepínicos; THC: Δ9-tetrahidrocanabinol; CBN: canabinol; MDMA: metilenodioximetanfetamina; CBF: carbofurano; 3-OH CBF: 3-hidroxicarbofurano. Outros analitos: 7-aminoflunitrazepam, tramadol, oxicodona, midazolam, flunitrazepam, propofol, levomepromazina, sertralina e bupropiona (Ferrari, Santos e Caldas, 2020; adaptado).

No final dos anos 1990, cerca de 230 substâncias psicoativas estavam sob controle internacional, e cannabis, cocaína, heroína e derivados de anfetamina (incluindo o MDMA) dominavam o mercado global (UNODC, 2023a). A partir das últimas duas décadas, as NSP, drogas que imitam as propriedades de substâncias ilícitas já conhecidas, foram introduzidas no mercado, incluindo substâncias das classes das catinonas e canabinoides sintéticos, piperazinas e fenetilaminas (UNODC, 2023a). Em 2021, o número de substâncias sob controle internacional chegou a 302, enquanto o número de NSP no mercado aumentou de 166 durante o período de 2005 a 2009, para 1127 substâncias até o final de 2021 (UNODC, 2022) (Figura 8). Segundo o Observatório Europeu da Droga e da Dependência (EMCDDA, sigla em inglês), em 2022, 41 novas substâncias foram reportadas na Europa (EMCDDA, 2023).

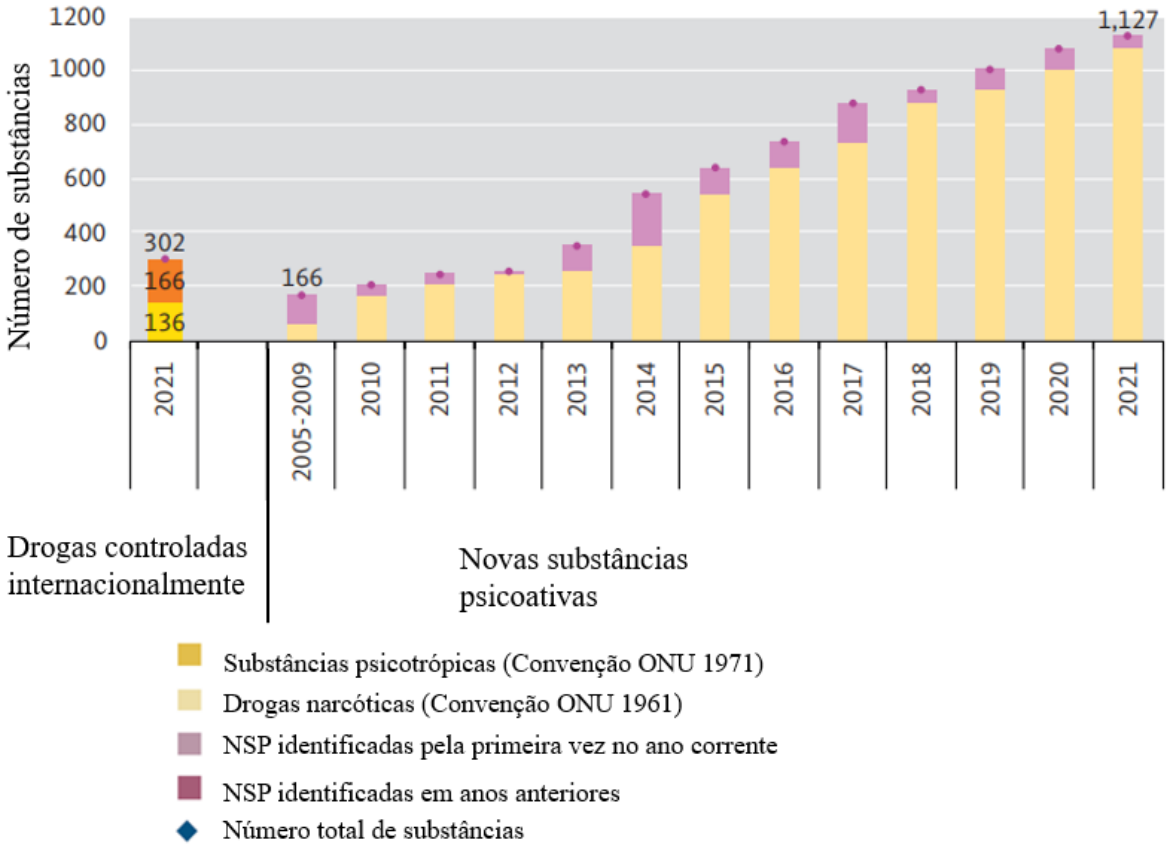


Figura 8: Número de substâncias controladas internacionalmente em 2021 e de novas substâncias psicoativas identificadas no mundo entre 2005 e 2021 (os números por ano são cumulativos) (UNODC, 2022).

O aumento do consumo de NSP nas últimas décadas (Krotulski et al., 2018; Costa et al., 2018; EMCDDA, 2023; Marchei et al., 2018) tem alterado o perfil de intoxicação por drogas de abuso, pois se tratam de substâncias pouco estudadas que podem provocar efeitos adversos inesperados, incluindo óbito (Fujita et al., 2016; Atherton et al., 2018; Costa et al., 2018; Gerace et al., 2018; Marchei et al., 2018; Adamowicz, 2016; Wikstrom et al., 2010; Maskell et al., 2011). A falta de informação sobre os novos perfis de intoxicação pode comprometer o manejo desses casos nas emergências hospitalares e sua identificação nos institutos de medicina legal.

Assim como o aumento do número de apreensões e de uso vem sendo demonstrados, o número de mortes relacionadas ao abuso de NSP segue a mesma tendência, envolvendo o consumo de catinonas sintéticas (Lee et al., 2015; Krotulski et al., 2018; Krotulski et al., 2020; Domagalskaa et al., 2020), os canabinoides sintéticos (Kraemer et al., 2019) e os derivados de opiode (Kraemer et al., 2019; McIntyre et al., 2017) e fenetilaminas (Gee et al., 2016).

No Reino Unido, houve um total de 4907 mortes por intoxicação pelo uso de drogas em 2022, dos quais 63,7% foram identificados como uso indevido (*drug* misuse), algum opioide/opiáceo foi detectado em 46,1% dos casos, e 857 mortes envolveram o uso de cocaína, um aumento de mais de 2%, quando comparado a 2021 (ONS, 2023). Em outros países da Europa, o número de mortes por overdose em 2017-2021 atribuído ao uso de opioides, incluindo heroína, fentanil e análogos merece destaque (UNODC, 2023a). Na Suécia, 90% das 539 mortes reportadas em 2017 (9,5 por 100.000 habitantes) envolveram opioides e na Alemanha, 50% dos 629 casos em 2018 envolviam o uso de opioides, associado ou não a outras classes de drogas. No mesmo ano, a Estônia registrou uma alta taxa de overdose de opioides (13 mortes por 100.000 habitantes) atribuída ao uso de fentanil e derivados e, na Finlândia (n = 200), a buprenorfina, em combinação com álcool ou benzodiazepínicos, foram as principais substâncias envolvidas nos casos de overdose. Na Alemanha, dos 295 pacientes submetidos a tratamento de desintoxicação pelo uso de drogas, cerca de 32% dos pacientes relataram o uso de canabinoides sintéticos, mas geralmente, como uso esporádico (Specka et al., 2020). No Reino Unido, dados de 2021 apontam 258 mortes envolvendo NSP, número 88,3% superior ao ano anterior (ONS, 2023). Na França, 29 % das intoxicações entre 2012 a 2017 envolveram NSP (principalmente catinonas, e canabinoides sintéticos), enquanto que derivados de anfetamina, cocaína e opioides estiveram foram responsáveis por 32; 38,5 e 52%, respectivamente (Larabi et al., 2019).

Nos Estados Unidos, casos fatais por uso de opioides (carfentanil, acetil fentanil e outros) representam um problema de saúde pública (UNODC, 2023a). Das 2048 mortes por uso de drogas de abuso (lícitas e ilícitas) em 2019, 36,8% envolveram analgésicos e destes, 83,5% eram opioides/opiáceos, além de fármacos para cardiopatias (11%) e antidepressivos (5,7%) (Gummin et al., 2020). Drogas ilícitas também foram reportadas (187 casos), principalmente metanfetamina, cocaína e MDMA (Gummin et al., 2020).

Estatística do "Global Burden of Disease Study" (GBD, 2017) estimou que 585.00 mortes em 2017 foram atribuídas ao uso de drogas, e 66% envolvendo opioides. Os dados de atendimentos em emergências hospitalares podem fornecer uma visão sobre os danos agudos relacionados com o uso de drogas e o seu impacto na saúde pública (EMCDDA, 2020b). A Figura 9 mostra as 24 principais substâncias identificadas nos 9.134 atendimentos realizados em 27 hospitais sentinela europeus em 2018, com cocaína liderando o rank, com mais de 2000 casos.

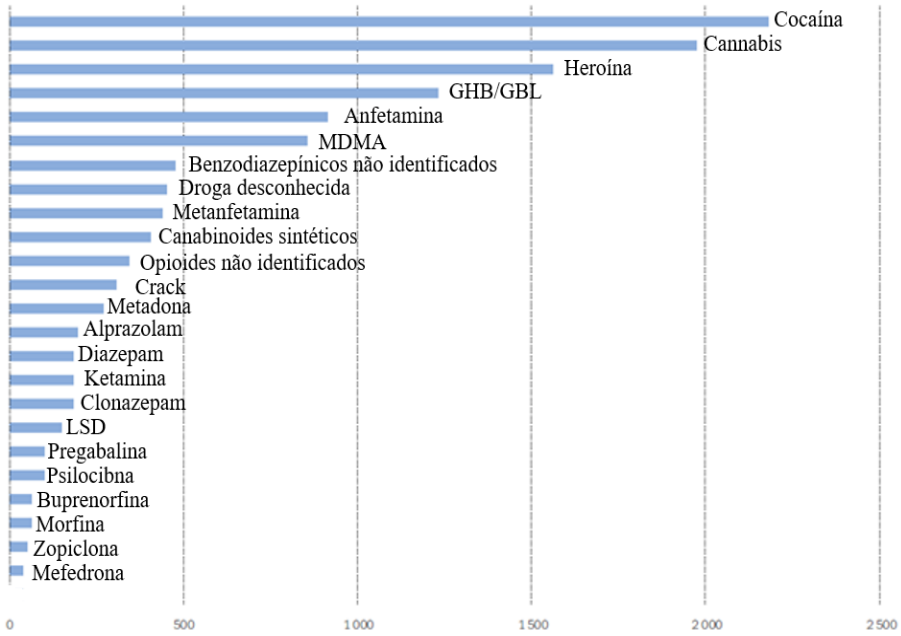


Figura 9: As 24 principais drogas registradas em 2018, em atendimentos de emergências de 27 hospitais sentinela, de 19 países europeus. Total de atendimentos: 9134. (EMCDDA, 2020b, adaptado).

No Brasil, dados recentes de apreensão de drogas revelaram a grande oferta de NSP no mercado local. Entre maio de 2008 e dezembro de 2017, em Minas Gerais, as principais classes de drogas ilícitas disponíveis eram anfetamínicos (principalmente MDMA), catinonas sintéticas e fenetilaminas (Machado et al., 2019). Em Santa

Catarina, a análise de mais de 1 milhão de drogas apreendidas na forma de selos, entre 2011 e 2017, mostrou que, além da presença de LSD, outras substâncias estavam presentes, incluindo derivados de anfetamina, opioides, fenetilaminas e canabinóides sintéticos(Souza Boff et al., 2019).

Casos de intoxicação fatal envolvendo catinona sintética tem sido reportado no Brasil (Costa et al., 2018) e no mundo (Fujita et al., 2016; Krotulski et al., 2018). No Distrito Federal um caso de intoxicação fatal por N-etilpentilona foi reportado por Ferrari e Caldas (2018), alertando a comunidade científica sobre subnotificações de casos de intoxicação envolvendo NSP na região.

4. Substâncias envolvidas em intoxicações por substâncias psicoativas

4.1.Benzodiazepínicos

Benzodiazepínicos consituem uma classe importante de fármacos com várias aplicações terapêuticas, incluindo o tratamento da convulsão, ansiedade, insônia, agitação e crises de abstinência ao álcool (Westland and Dorman, 2013). Atuam como agonistas de receptores do ácido gama-aminobutírico (GABA), aumentando a frequência de abertura do canal de cloro com consequente hiperpolarização celular (Ritter, Flower e Henderson, 2016). A Figura 10 ilustra o mecanismo de ação de um benzodiazepínico nos receptores GABA.

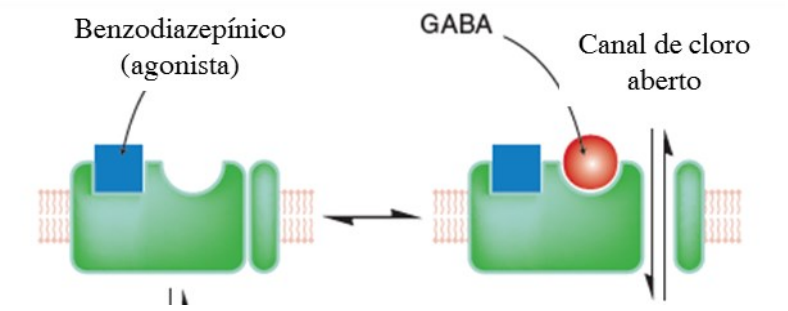


Figura 10: Modelo de interação do receptor de ácido gama-amino butírico (GABA)/benzodiazepínicos. Acedita-se que os benzodiazepínicos se ligam em local distinto da ligação do GABA (Ritter, Flower e Henderson, 2016, adaptado).

A larga faixa de segurança desses fármacos e o alto índice terapêutico os tornam relativamente seguros, entretanto são constantemente envolvidos em acidentes devido a diminuição da capacidade de atenção proporcionada pelo seu uso (Ferrari e Caldas,

2018; Ferrari, Santos e Caldas, 2020; Van der Sluiszen et al., 2017; Orsini et al., 2017; Edvardsen et al., 2017).

Em casos de overdose, os benzodiazepínicos causam sono prolongado, sem depressão grave de respiração ou função cardiovascular, entretranto, o seu uso concomitante com outros depressores do sistema nervoso central (SNC) (álcool, drogas ilícitas, opioides/opiáceos) aumenta a possibilidade de depressão respiratória (Ritter, Flower e Henderson, 2016). Atendimentos de overdose por benzodiazepínicos em serviço de emergência normalmente são devidos a ingestão de dois ou mais fármacos (Levine e Kerrigan, 2020).

O diazepam é um benzodiazepínico de ação longa (t½ = 20 – 40h), e possui metabólitos ativos produzidos por meio de hidroxilação ou desmetilação (Luk et al., 2014; Figura 11): nordiazepam, oxazepam e temazepam, sendo os dois últimos disponíveis como medicamento comercial.

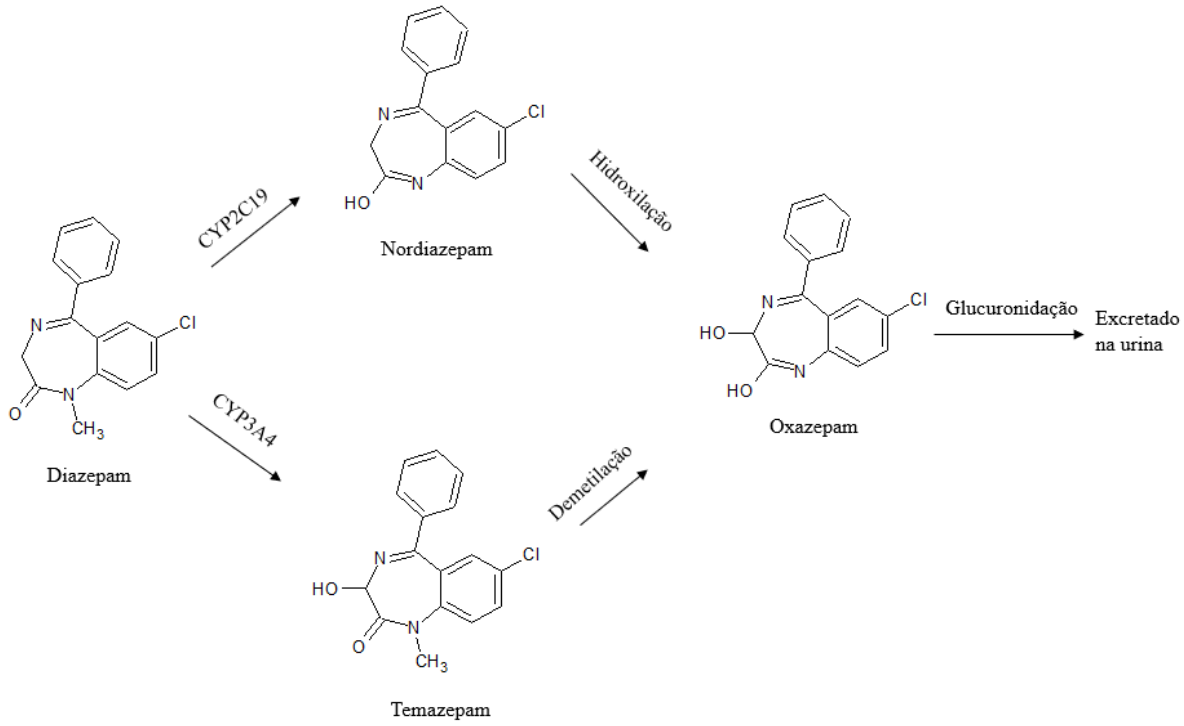


Figura 11: Metabolismo do diazepam via citocromo P450 (CYP), e seus dois metabólitos ativos temazepam e oxazepam.

Devido a diminuição da capacidade de atenção e por induzir amnésia, benzodiazepínicos são utilizados como facilitadores em casos de agressão sexual e crimes relacionados, conhecidos por “boa noite cinderela” (*date rape drug*; Skov, Holm e Linnet, 2016). O flunitrazepam é um dos utilizados para esta finalidade, cujo principal

metabólito é o 7-aminoflunitrazepam (Figura 12), sem atividade farmacológica. Em casos post-mortem, é comum apenas a detecção do metabólito no sangue (Skov, Holm e Linnet, 2016; Ferrari, Santos e Caldas, 2020).

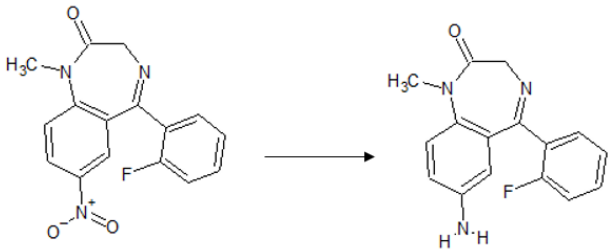


Figura 12: Estrutura molecular do flunitrazepam e de seu principal metabólito, o 7-aminoflunitrazepam.

Outros fármacos desta classe, como midazolam (Nordt e Clark, 1997), bromazepam (Fortunato et al., 2015), alprazolam (Greenblatt e Wright, 1993) e clonazepam (Zorzanelli et al., 2019) são amplamente usados e produzem efeitos semelhantes, prejudica a coordenação motora, produz relaxamento muscular, sonolência e redução do estado de alerta, sendo analitos importantes em uma análise toxicológica sistemática (Figura 13). Apesar de não possuir medicamento registrado no país, nimetazepam (Figura 13) é frequentemente relacionado a casos forenses no leste e sudeste asiático (Wang et al., 2013).

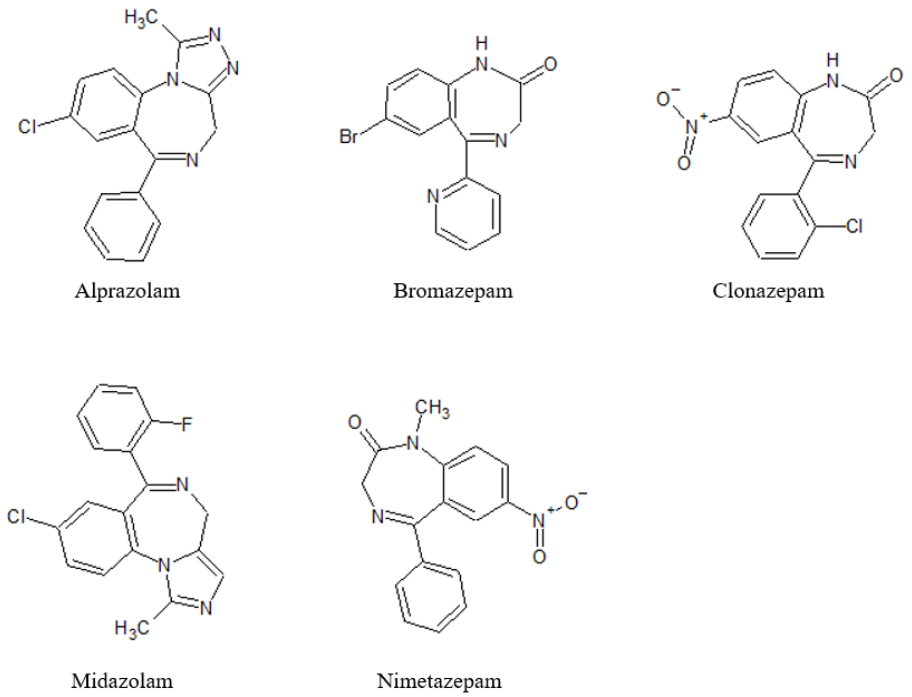


Figura 13: Estrutura molecular do alprazolam, bromazepam, clonazepam, midazolam e nimetazepam.

4.2 Antidepressivos

No geral, os fármacos antidepressivos são classificados em categorias, como os inibidores de captura de serotonina (fluoxetina, sertralina, citalopram, etc.), inibidores de noradrenalina (bupropriona, reboxetina, etc.), inibidores de de setoronina e noradrenalina (venlafaxina, duloxetina, etc.), antagonistas α2-adrenérgicos e de serotonina (mirtazapina, trazodona, etc.), inibidores da enzima monoamino-oxidase – IMAO (moclobemida, fenelzina, etc.) e os antidepressivos tricíclicos (amitriptilina, imipramina, clomipramina, etc.) (Ritter, Flower e Henderson, 2016). Os antidepressivos tricíclicos (ATC) se diferem pelas habilidades relativas no bloqueio da recaptação de neurotransmissores (Figura 14; Ritter, Flower e Henderson, 2016), em seus efeitos anticolinérgicos e anti-histamínicos e em suas propriedades sedativas (Shen, 1967; Marsh, 2007).

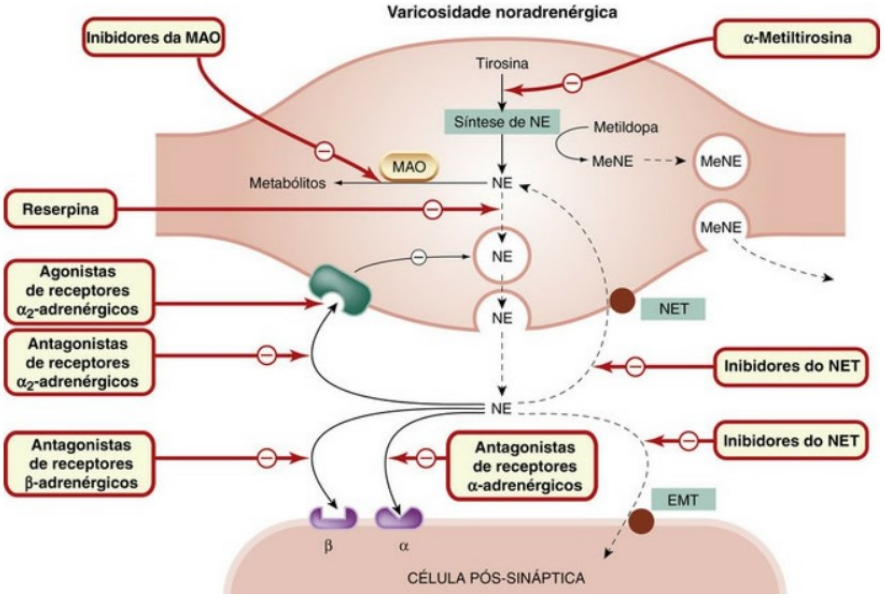
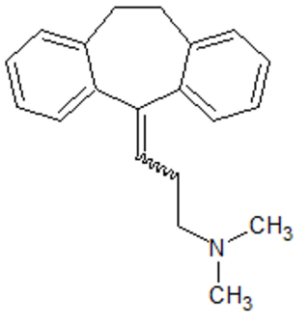


Figura 14: Terminal nervoso noradrenégico, ilustrando locais de ação de drogas e fármacos. EMT = transportador de monoamina extraneuronal; MAO = monoamina oxidase; MeNE = metilnoradrenalina; NE = noradrenalina; NET = transportador de noradrenalina neuronal (Ritter, Flower e Henderson, 2016, adapatado).

A amitriptilina (Figura 15) é um bloqueador da recaptação de monoaminas (como a noradrenalina e serotonina) não seletivo nos neurônios terminais, que também possui propriedades analgésicas (Abeyaratne et al., 2016; O'Connor et al., 2006; Chen et al., 2017; Marsh, 2007).



Amitriptilina

Figura 15: Estrutura molecular da amitriptilina.

ATCs possuem estreita janela terapêutica e casos de superdosagem, por acidente ou intencional, são comuns, com efeitos sobre o SNC e cardíaco, causando excitação, delírio e convulsões, arritmia e depressão miocárdica devido ao bloqueio dos canais de

sódio, causa mais comum na morte por uso de ATCs (Ritter, Flower e Henderson, 2016; Shen, 1967; Marsh, 2007).

A trazodona é um antagonista da serotonina e inibidor da recaptação usado como antidepressivo, ansiolítico e indutor do sono (Cantarelli e Marcolin, 2006). O seu metabólito primário, o m-CPP (m-clorofenilpiperazina) é formado pela N-dealquilação da trazodona (Patel et al., 2008), sendo um potente agonista serotoninérgico, também comercializado nas ruas como uma droga de desenho (*designer drug*) da classe das fenilpiperazinas (Gaillard et al., 2012; Meyer e Maurer, 2010). Para não haver erro de interpretação dos resultados analíticos, é importante se considerar o monitoramento de m-CPP e trazodona no mesmo método, para discriminação entre abuso de droga ou apenas a detecção de um metabólito de trazodona na terapêutica (Patel et al., 2008). A Figura 16 mostra a estrutura molecular da trazodona e do seu metabólito m-CPP.

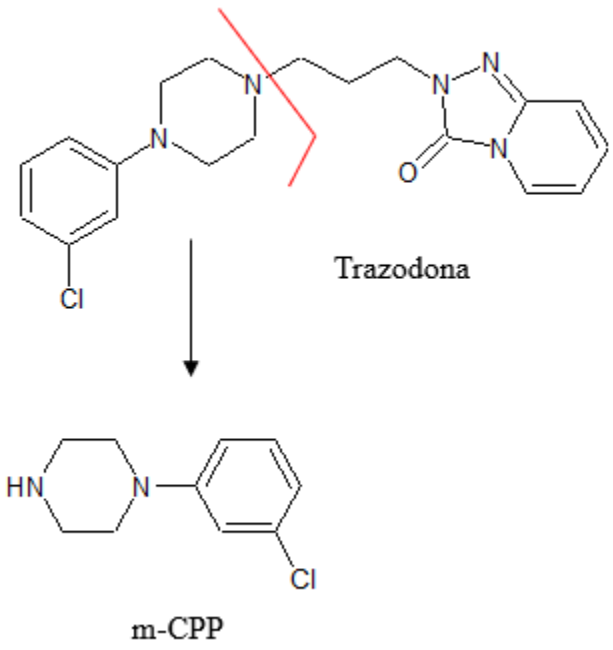


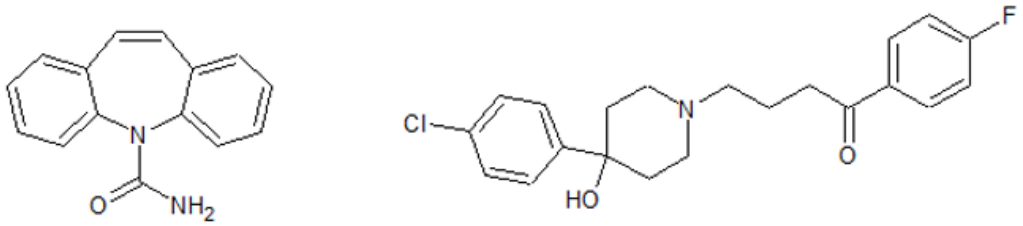
Figura 16: Trazodona e m-CPP.

4.3 Antiepiléticos e antipsicóticos

Os antiepiléticos (anticonvulsivantes) são fármacos utilizados no tratamento da epilepsia. Dentre os principais mecanismo de ação, temos a potencialização da ação do GABA (fenobarbital, benzodiazepínicos), a inibição das funções dos canais de sódio (carbamazepina, fenitoína) e inibição das funções dos canais de cálcio (valproato)

(Ritter, Flower e Henderson, 2016). A carbamazepina é estruturalmente relacionada aos ATC e seu mecanismo de ação se dá pelo bloqueio de canais de sódio nas membranas dos neurônios (Graudins, Peden e Dowsett, 2002). Os quadros de intoxicação, acidental ou intencional, são comuns devido a disponibilidade destes fármacos em residências de pacientes que os utilizam em um tratamento contínuo (Vadysinghe e Thilakarathne, 2018). A concentração sanguínea tóxica da carbamazepina gira em torno de 15 mg/L, e valores acima de 20 mg/L são descritos em intoxicações severas (Gummin et al. 2020; Jickells e Negrusz, 2008; Vallianou et al. 2017; Acikgoz et al., 2016; Karaman et al., 2017).

Os fármacos antipsicóticos são utilizados, dentre outras aplicações terapêuticas, no tratamento da esquizofrenia. São antagonistas ou agonistas de receptor de dopamina, mas também podem atuar no bloqueio de outros receptores de monoaminas. São normalmente classificados como antipsicóticos de primeira geração, ou "típicos" (clorpromazina, haloperidol, flufenazina, etc.) e de segunda geração, ou "atípicos" (clozapina, risperidona, quetiapina, etc). A distinção entre as classificações típico e atípico está relacionada ao perfil do receptor, incidência de efeitos colaterais extrapiramidais (menor nos atípicos), dentre outras características (Ritter, Flower e Henderson, 2016; de Leon et al., 2004). O haloperidol é um antipsicótico cujo t½ em uma dose oral única é de 14,5 a 36,7h, podendo chegar a até 21 dias em usuários crônicos; se liga fortemente a proteínas plasmáticas, possuindo alto volume de distribuição (de Leon et al., 2004). As concentrações sanguíneas tóxicas do haloperidol variam entre 0,01 e 0,5 mg/L (Jickells e Negrusz, 2008; Ritter, Flower e Henderson, 2016; Dekkers et al., 2017). A Figura 17 mostra a estrutura molecular dessas drogas.



Carbamazepina Haloperidol

Figura 17: Carbamazepina e haloperidol.

4.4 Opiáceos/opioides

Os opiáceos (substâncias naturais extraídas da papoula) e opioides (substâncias sintéticas ou semi-sintéticas) são agonistas dos receptores opióides altamente lipofílicos, o que lhes permite atravessar as membranas celulares para atingir os tecidos-alvo (Ritter, Flower e Henderson, 2016; Smith 2009). O metabolismo da fase 1 dessas substâncias envolve as enzimas do citocromo P450 (CYP), normalmente sujeitando a droga à oxidação ou hidrólise. A glucuronidação, catalisada pela enzima uridina difosfato glucuronosiltransferase (UGT) é o metabolismo de fase 2 mais importante (Smith 2009).

A morfina é um opiáceo utilizado no tratamento de dor severa/crônica, por meio do agonismo dos receptores opioides μ e k (Pacifici, 2016). Outras drogas *morphine-like* podem se ligar aos mesmos receptores, causando analgesia, sedação, euforia, pupilas mióticas e, em casos de overdose, depressão respiratória, quadro clínico que pode levar à morte (Ritter, Flower e Henderson, 2016). Naloxona é um antagonista dos receptores opiodes utilizado em casos de intoxicação aguda. A morfina e heroína e os opioides, como o fentanil, possuem alto risco de dependênica e se tornaram um problema de saúde pública em alguns países, principalmente os Estados Unidos (UNODC, 2023a).

A codeína é um opiáceo amplamente prescrito para o alívio de dor pós-operatória, recomendado inclusive para para uso pediátrico (Willians, Patel e Howard, 2002). É metabolizada no fígado em sua forma ativa morfina (Figura 18), que pode ser detectada na urina 30 horas após a ingestão de uma única dose (Smith, 2009). A codeína também se converte em hidrocodona (dihidrocodeína), um opioide semi-sintético utilizado no tratamento da dor leve e como antitussígeno (Ritter, Flower e Henderson, 2016).

A heroína (diacetilmorfina) é uma droga de abuso que pode ser obtida a partir da morfina (Odell, Skopec e Mccluskey, 2006). A detecção em fluidos biológicos normalmente não é possível, devido ao pequeno tempo de meia-vida, na faixa de minutos (He et al., 2008). Portanto, para avaliação da exposição ao uso de heroína, o seu metabólito 6-monoacetilmorfina (6-MAM) é preconizado, o qual, por sua vez, se converte em morfina, que é o composto ativo (He et al., 2008). Para um correto diagnóstico sobre o uso de morfina, codeína, hidrocodona e heroína, em conjunto ou em

monoterapia, é importante que um método analítico monitore todas as substâncias elencadas, cuja via metabólica é ilustrada pela Figura 18.

Outros opioides como tramadol, metadona, oxicodona, petidina (meperidina) e alfentanil (Figura 19) são amplamente utilizados na terapêutica e, com frequência relacionados a casos em toxicologia forense e em emergências hospitalares (UNODC, 2023a; EMCDDA, 2023). O tramadol possui a vantagem terapêutica de não desenvolver relevante depressão respiratória, como acontece com o uso de outros opiáceos/opioides (WHO, 2014).

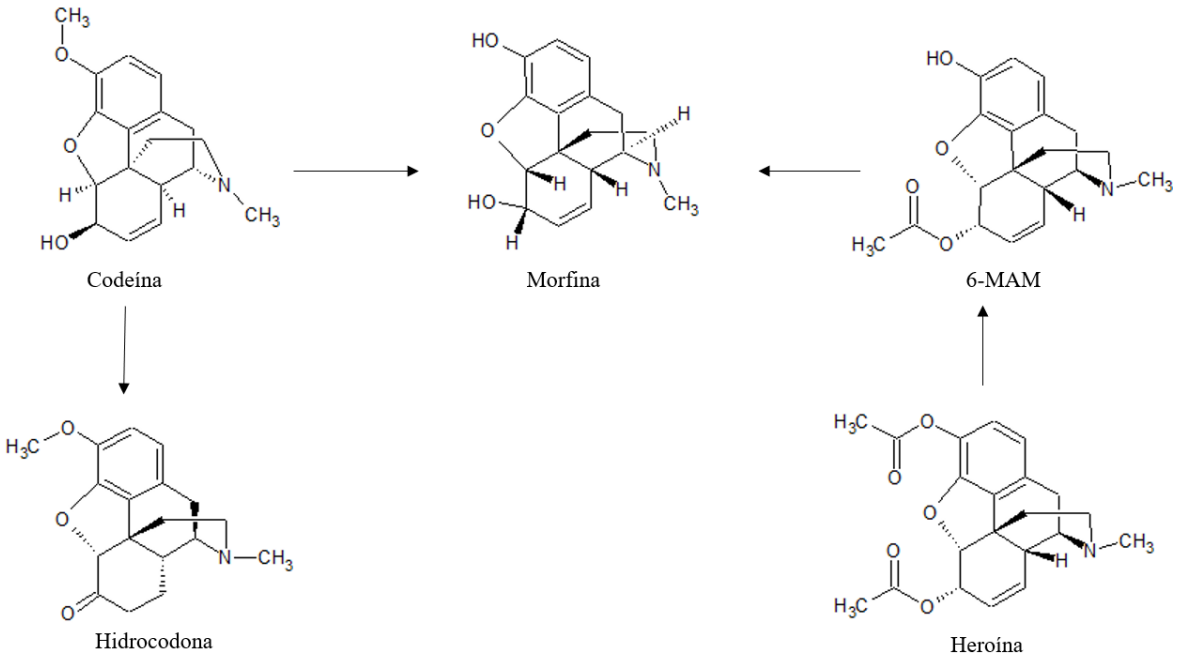


Figura 18: Via metabólica simplificada para destacar a biotransformação da codeína em hidrocodona e morfina, além da heroína à 6-monoacetilmorfina (6-MAM) e morfina (Cone et al., 2006, adaptado).

AH-7921 (Figura 19) é uma nova substância psicoativa estruturalmente relacionada aos opioides, sintetizada e patenteada na década de 1970 cujas letras "AH" se referem a "Allen e Hanburys", a empresa detentora da patente. Esta NSP é detectada no mercado desde 2012 (Katselou et al., 2015). Casos de intoxicação fatal na Europa foram relatados pelo uso de AH-7921 e, assim como ocorre em overdose por opiáceos/opioides, o principal quadro clínico associado a óbitos é o desenvolvimento de depressão respiratória (WHO, 2014).

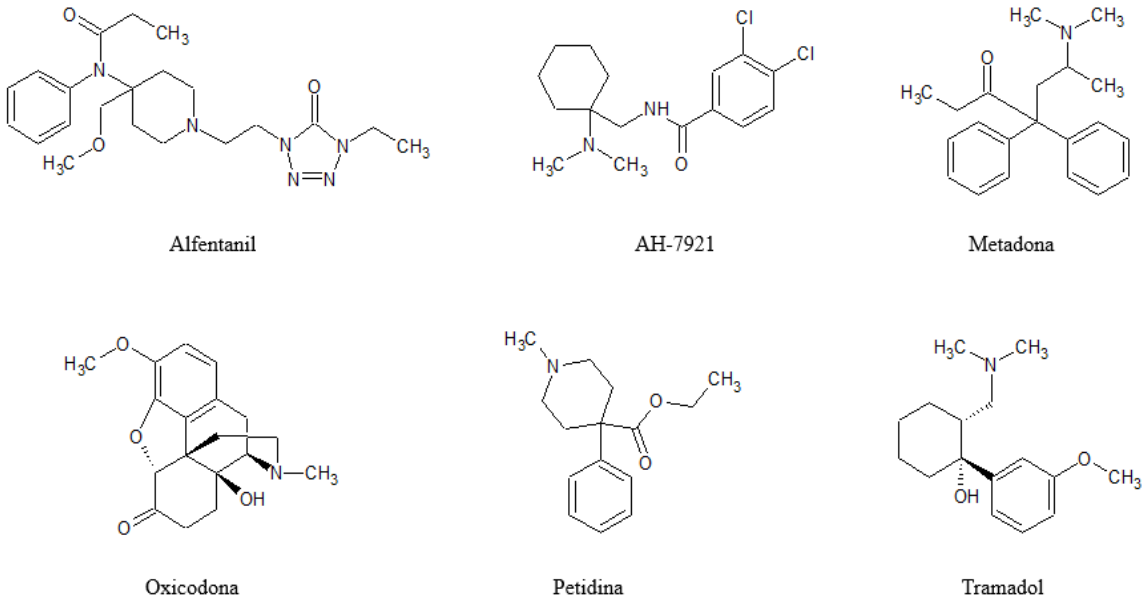


Figura 19: Estruturas moleculares do alfentanil, AH-7921, metadona, oxicodona, petidina e tramadol.

4.5 Cocaína

A cocaína é o alcaloide psicoativo da planta da coca (*Erythroxylon coca*), e considerando as diferentes formas de apresentação, ainda é uma das drogas mais consumidas por todo o mundo, com o número estimado de pessoas que já utilizaram de aproximadamente 22 milhões, até 2021 (UNODC, 2023a). A forma de sal pode ser utilizada inalada, injetada, já a forma de base livre (crack) é suficientemente volátil para ser inalada através do fumo (Ritter, Flower e Henderson, 2016).

A cocaína atua no bloqueio da captação de dopamina nos sistemas nervoso central e periférico, podendo também atuar no bloqueio da recaptação de noradrenalina e serotonina (Hummer e Unterwald, 2002) . O acúmulo de dopamina nas sinapses causa sensação de euforia, bem-estar e aumento no estado de alerta, mas também, taquicardia, hipertensão arterial e agitação motora (Brunton, Hilal-Dandan e Knollmann, 2017; Nelson et al., 2011). Desordens neurológicas e cardíacas, como isquemia, hemorragia intracraniana e infarto do miocárdio podem ocorrer em usuários crônicos (Pereira, Andrade e Valentão, 2015; Nnadi et al., 2005).

A Figura 20 mostra o metabolismo da cocaína, que envolve hidrólise do éster metílico para formar benzoilecgonina e do benzoil éster da cocaína para produzir éster

metil ecgonina (EME). A cocaína também sofre N-desmetilação pela butirilcolinesterase para formar norcocaína (Connors e Hoffman, 2013). Na urina, pequena fração é excretada inalterada e a maior parte da droga consumida é eliminada na forma de benzoilecgonina e éster metílico de ecgonina (Nickley, Pesce e Krock, 2017).

Sinais clínicos da intoxicação aguda pelo abuso de cocaína incluem danos ao sistema cardio-respiratório (infarto do miocárdio, arritmias, mortes súbitas, espasmos respiratórios), hepático, como hepatite, insuficiência hepática fulminante associada a rabdomiólise e outros sintomas graves como crises tônico/clônicas (Graziani et al., 2016; Andrews, 1997).

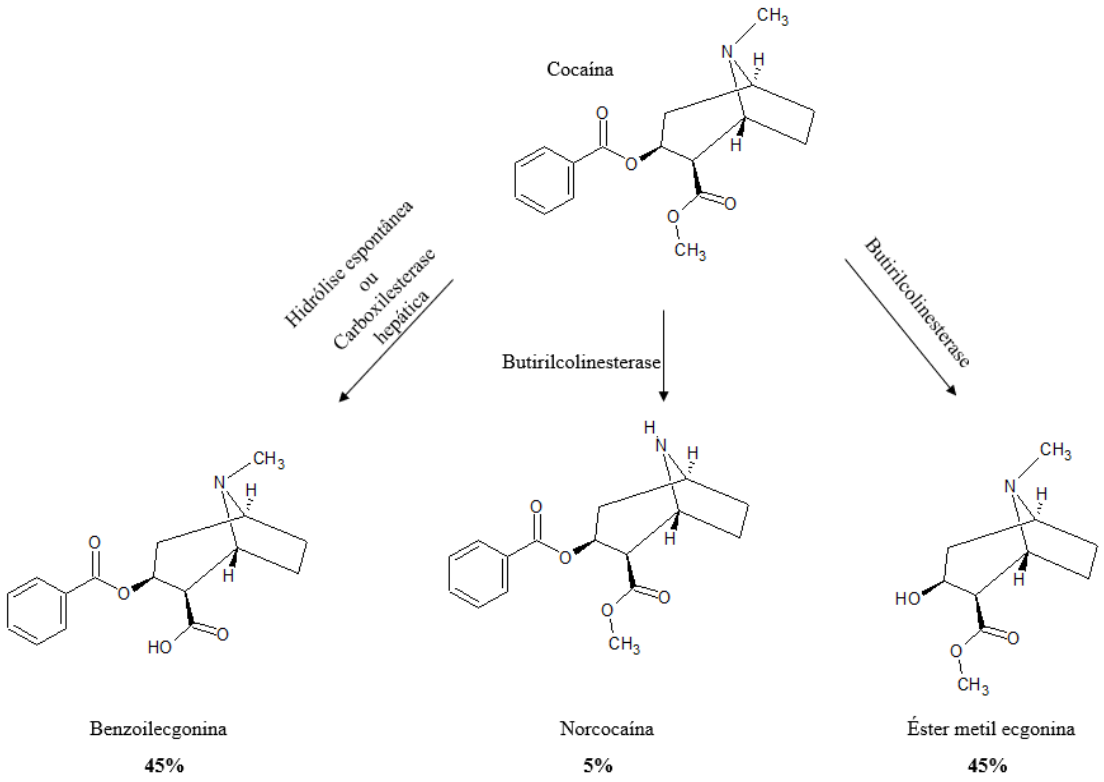


Figura 20: Três vias primárias do metabolismo da cocaína (Connors e Hoffman, 2013, adaptado).

4.6 Canabinoides naturais e sintéticos

A *Cannabis sativa L.* contém mais de 500 compostos, incluindo fitocanabinoides, sendo o Δ-9-THC (tetrahidrocanabinol) a principal substância psicoativa (Gonçalves et al., 2019). A cannabis pode ser comercializada também como

como óleo e resina (Lafaye et al., 2017), sendo a droga mais consumida no mundo, com o número estimado de usuários de aproximadamente 219 milhões em 2021 (UNODC, 2023a).

O THC é agonista de receptores canabinoides CB-1 (principalmente no SNC) e CB-2 (periféricos), causando sensação de euforia, relaxamento, com consciência sensorial aguçada (Brunton, Hilal-Dandan e Knollmann, 2017). Os efeitos farmacológicos do THC variam com a dose, via de administração, ambiente de uso e experiência do usuário. Quando fumada, os efeitos psicoativos podem durar cerca de 2 horas (Ritter, Flower e Henderson, 2016), quando efeitos prejudiciais na aprendizagem, memória e coordenação motora podem persistir, sendo também dose dependente (Kroon, Kuhns e Cousijn, 2021). A toxicidade aguda do THC é muito baixa, e casos de intoxicação são raros (Artiles et al., 2019). Em 2013, a Associação Americana de Psiquiatria incluiu a cannabis na sua lista de substâncias que podem causar desordem psiquiátricas devido ao uso (DMS 5, *Diagnostic and Statistical Manual of Mental Disorders*), principalmente alteração de humor e comportamento de intensidade leve a moderada (Bonnet e Preuss, 2017).

O THC é metabolizado principalmente no fígado, por meio das enzimas do citocromo P450 (CYP2C9, CYP2C19 e CYP3A4) com reações de hidroxilação, oxidação e descarboxilação (Gonçalces et al., 2019). Os principais metabólitos, THC-OH (11-hidroxi-A-tetra-hidrocanabinol) e THC-COOH (11-nor-9-carboxi-delta9-tetrahidrocannabinol), são utilizados para o monitoramento do uso de THC, pois proporcionam uma janela de detecção mais longa, ideal para avaliar a exposição a longo prazo (dias após o uso) (Goodwin et al., 2008). A Figura 21 mostra o THC e seus principais
metabólitos.

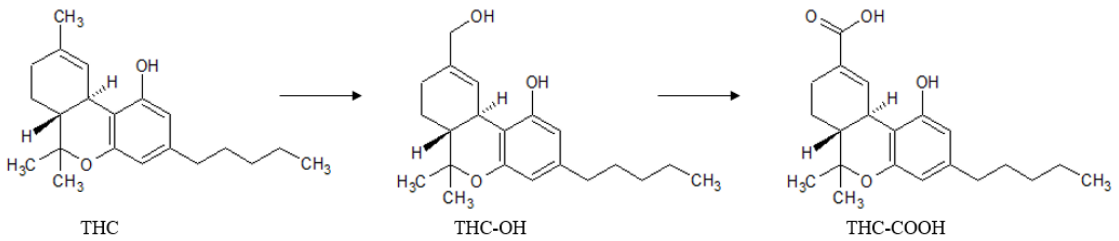


Figura 21: THC (tetrahidrocannabinol) e seus principais metabólitos, THC-OH (11-hidroxi-A-tetra-hidrocanabinol) e THC-COOH (11-nor-9-carboxi-delta9-tetrahidrocannabinol).

Nos últimos anos, canabinoides sintéticos, substâncias que imitam os efeitos do THC nos receptores canabinoides, foram introduzidos no mercado. O primeiro a se tornar popular entre os usuários foi o JWH-018, em 2008, em uma mistura para fumar chamada "Spice" (EMCDDA, 2016). Recentemente, o canabinoide semi-sintético hexahidrocanabinol passou a ser comercializado em alguns países da Europa como uma alternativa "legal" à cannabis, agravando os desafios regulamentares nesta área (EMCDDA 2023).

Os canabinoides sintéticos são uma das classes de NSP mais apreendidas no mundo (Figura 22), comercializadas, principalmente, misturada a plantas e incensos (Peters, 2014). Até 2019, estatísticas sugeriam que o Brasil não estaria acompanhando esta tendência mundial (Machado et al., 2019; Souza Boff et al., 2019), entretanto, dados recentes demonstram o aparecimento e aumento do número de apreensões de canabinoides sintéticos, principalmente no Estado de São Paulo (Araújo et al., 2023), sendo normalmente comercializados com o nome de K2, K9 e outras siglas (Brasil, 2023). No Distrito Federal, a apreensão de canabinoides sintéticos em 2023 e início de 2024 não foi expressiva, quando comparado ao número de apreensões de outras classes de NSP.

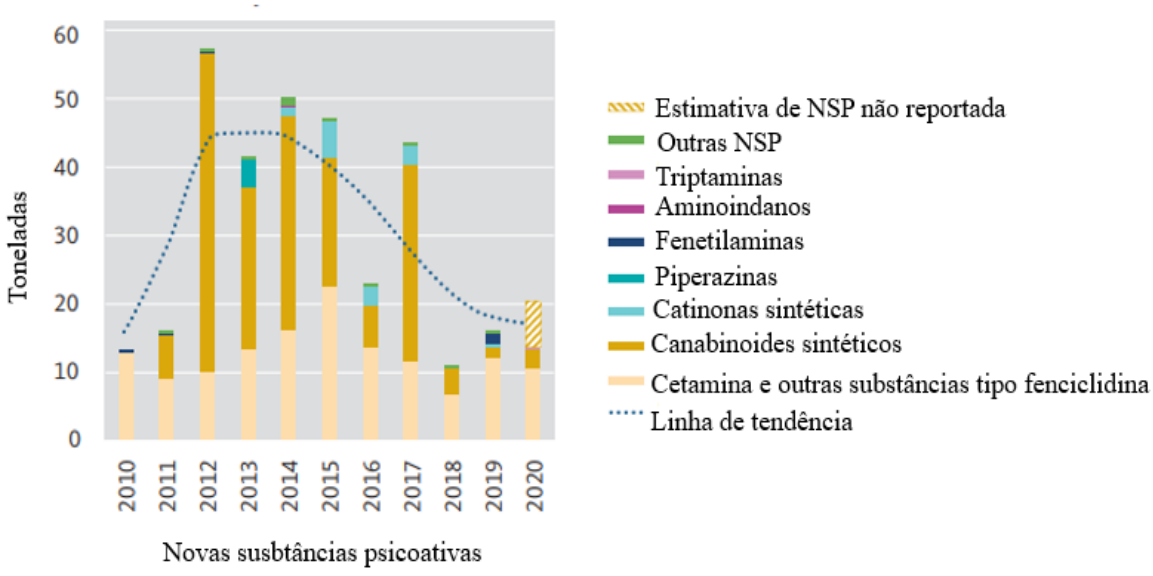


Figura 22: Série temporal da quantidade (em toneladas) de novas substâncias psicoativas (NSP) apreendidas a nível global, de 2010 a 2020 (fonte: UNODC, 2022, adaptado).

Historicamente, os canabinoides sintéticos foram sintetizadas como ferramentas para investigar o sistema de receptores de canabinoides, como potenciais agentes terapêuticos, o que trouxe uma alternativa também para o mercado cladestino de drogas ilícitas (Logan et al., 2017). Os canabinoides sintéticos recebem uma classificação química conforme a estrutura molecular envolvida: naftoilindol, naftilmetilindol, naftoilpirrol, naftilmetilindeno, fenilacetilindol, ciclohexilfenol e canabinoides clássicos (EMCDDA, 2020c). A Figura 23 mostra a estrutura molecular de alguns canabinoides sintéticos.

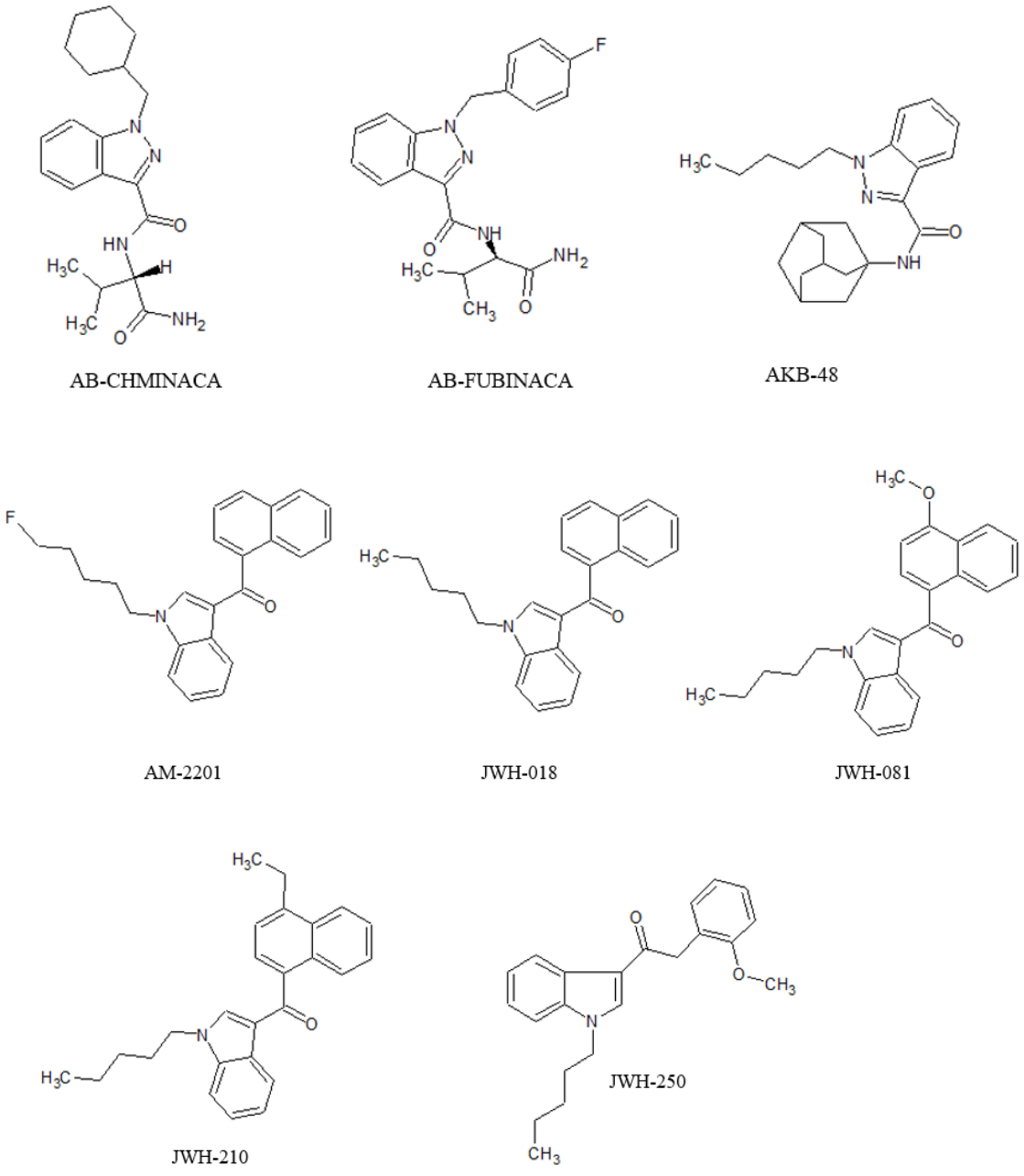


Figura 23: Canabinoides sintéticos.

Diferente dos canabinoides presentes na *Cannabis sp.*, canabinoides sintéticos estão relacionados a casos de intoxicação, incluindo casos fatais (Adamowicz, 2016). Os sintomas incluem eventos cardiovasculares, lesão renal aguda, convulsões, e, em casos mais graves, acidente vascular cerebral, infarto do miocárdio, rabdomiólise, insuficiência renal e psicose (Kronstrand et al., 2018; EMCDDA, 2023).

Quando analisados por cromatografia gasosa, alguns canabinoides sintéticos exigem métodos com rampas de alta temperatura no forno (UNODC, 2013), o que pode ocasionar a não identificação da substância presente em métodos de triagem.

4.7 LSD

A dietilamida do ácido lisérgico – LSD, potente alucinógeno, é uma das drogas mais populares no mundo, sintetizada por Albert Hoffman em 1937 a partir de um alcaloide encontrado no fungo do centeio, o *Claviceps purpúrea* (Brunton, Hilal-Dandan e Knollmann, 2017). É vendida normalmente na forma de selos (Figura 1), mas pode ter outras apresentações, como forma líquida e cápsulas.

O LSD é um agonista serotoninérgico não seletivo, que provoca as alucinações mesmo em baixas doses (1 μg/kg), mas com baixa toxicidade aguda (Brunton, Hilal-Dandan e Knollmann, 2017). Devido a baixas concentrações encontradas em materiais apreendidos, existe a necessidade da utilização de métodos com baixos limites de detecção, tanto para identificação em droga bruta (Ferrari et al., 2020), como em análise de material biológico. Um dos efeitos tardios pelo uso do LSD é o chamado efeito *flashback*, alucinações que o usuário pode experimentar meses ou anos após utilizar a droga (Passie et al., 2008).

4.8 Fenciclidina e cetamina

A fenciclidina (PCP), também chamada de pó de anjo, foi desenvolvida na década de 1950 como um anestésico, mas devido aos efeitos colaterais de confusão mental e alucinação, foi descontinuada na terapêutica (Brunton, Blumenthal e Buxton, 2008). A cetamina é um agente anestésico derivado da fenciclidina ainda utilizada como anestésico no Brasil de uso veterinário (Vasconcelos et al., 2005) e no mundo (Mazzeffi, Johnson e Paciullo, 2015), e ambas inibem os receptores N-metil-D-aspartato

(NMDA) de glutamato (Ritter, Flower e Henderson, 2016). Seu uso produz rapidamente um estado hipnótico e amnésia, um estado cataléptico que foi denominado anestesia dissociativa (Horiguchi, Huang e Meltzer, 2011). Ambas têm sido comercializadas como drogas de abuso, na forma de pó, cápsula, podendo ser fumada, inalada ou ingerida. Quadros de intoxicação pelo abuso destas substâncias podem progredir de comportamento agressivo ao coma, com pressão arterial elevada e pupilas dilatadas não reativas (Brunton, Hilal-Dandan e Knollmann, 2017).

A norcetamina é o principal metabólito ativo da cetamina (Salat et al., 2015). A estruturas moleculares da fenciclidina, cetamina e norcetamina estão mostradas na Figura 24.

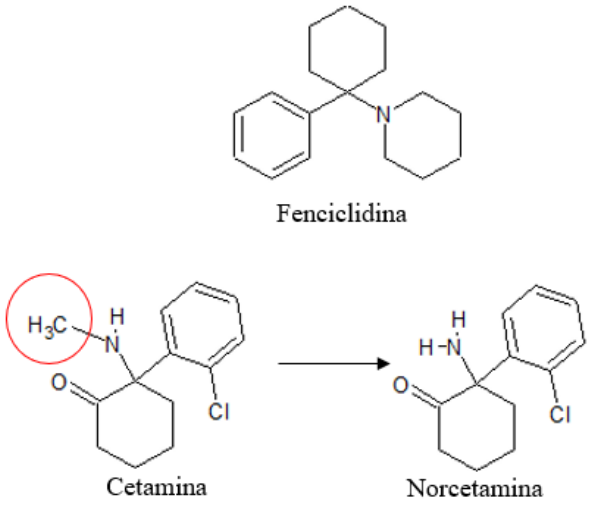


Figura 24: Estrutura molecular da fenciclidina (PCP), da cetamina e de seu principal metabólito ativo, a norcetamina.

4.9 Triptaminas e β-carbolinas

O chá de ayahuasca é uma bebida alucinógena utilizadas por muitas tribos indígenas da Amazônia. É preparada pela decocção do cipó *Banisteriopis caapi* com a mistura de folhas de Psycotria, principalmente *Psycotria viridis* (Oliveira Silveira et al., 2020; Morales-García et al., 2017; Nolli et al., 2020).

O cipó contém alcaloides que pertencem ao grupo das β-carbonilas (Figura 25), como a harmina, a harmalina e a tetrahidroharmina (Colaço et al., 2020; Nolli et al., 2020). São inibidores (inibição reversa) da enzima monoaminooxidase (MAO). O DMT (dimetiltriptamina), um potente agonista serotoninérgico, é o principal composto alucinógeno presente nas folhas de *P. Viridis* (Oliveira Silveira et al., 2020; Morales-

García et al., 2017; Nolli et al., 2020). Quando ingerido, é degradado pela MAO no trato gastrointestinal, mas na presença das β-carbolinas, o DMT (Figura 26) atinge o SNC, causando os efeitos alucinógenos desejados (Colaço et al., 2020).

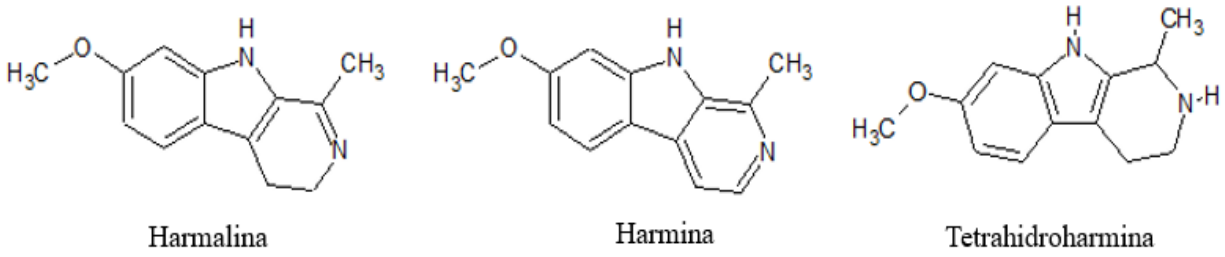


Figura 25: Estrutura molecular da harmina, harmalina e tetrahidroharmina.

Novas substâncias psicoativas derivadas de triptaminas são cada vez mais populares (UNODC, 2023a), como o 5-metoxi-N-metil-N-isopropiltriptamina (5-MeO-MiPT, figura 26), uma NSP alucinógena, agonista serotoninérgico. Em 1985, sua síntese foi descrita por Repke e colaboradores (Repke, Grotjahn e Shulgin, 1985) e Shulgin e Shulgin recomenda doses de 4 a 6 mg, para uso oral, e de 12 a 20 mg, quando fumada (Shulgin e Shulgin, 1997). Em 2017, um relato de caso no Japão descreve uma associação fatal entre 5-MeO-MiPT e metilona, uma catinona sintética (Shimizu et al., 2007).

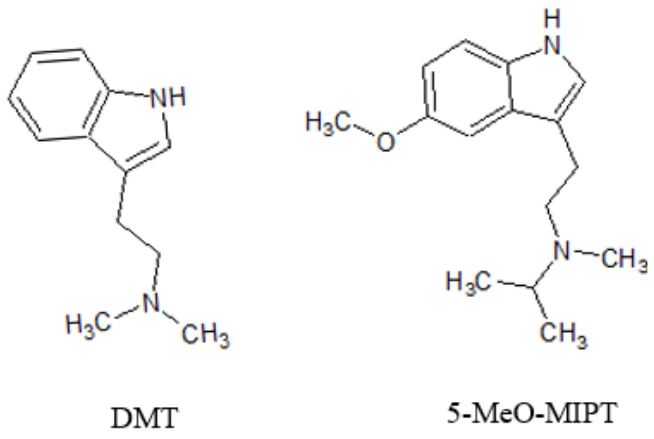


Figura 26: Estrutura molecular do DMT e do MeO-MiPT.

4.10 Catinonas sintéticas, anfetaminas, fenetilaminas e seus derivados.

Catinonas sintéticas, anfetaminas e fenetilaminas possuem estruturas moleculares semelhantes. A catinona é um análogo de β-cetona da anfetamina (Feng et al., 2017) (Figura 27A) e a classe de fenetilaminas substituídas inclui as anfetaminas substituídas (Figura 27), como substâncias com anéis substituídos, os chamados derivados "2C", anfetaminas com anéis substituídos, como os derivados "D" (DOI, DOC, etc), NBOMe (N-benzoilmetoxi), NBOH (N-benzoilhidroxi) e outras subtâncias. A Figura 27 mostra que a diferença entre as moléculas de anfetamina (Figura 27B) e de fenetilamina (Figura 27C) é de um substituinte metil ($-CH_3$).

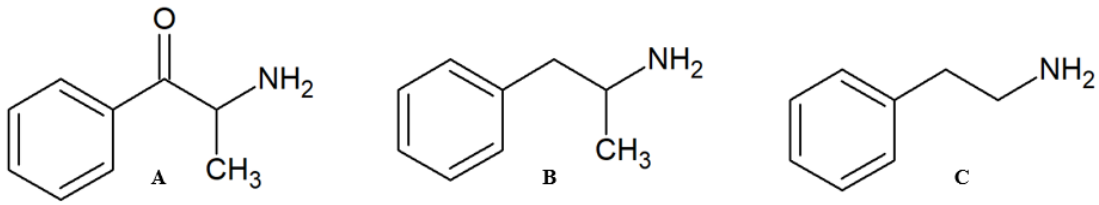


Figura 27: Estrutura molecular da catinona (A), da anfetamina (B) e da fenetilamina (C).

4.10.1 Catinonas sintéticas

O khat (*Catha edulis*, Celastraceae) é uma planta nativa da África e da Península Árabica, de onde é possível se extrair catina e catinona. Além de serem estruturalmente relacionados, catinona e anfetamina também possuem mecanismo de ação similares (Feng et al., 2017).

Os efeitos psicoativos das catinonas são alcançados por meio do aumento da concentração de monoaminas, como noradrenalina, dopamina e serotonina nas sinápses, via inibição da recaptação e/ou liberação de monoaminas, o que varia substancialmente para cada substância da classe (Dignam e Bigham, 2017). Apesar de serem conhecidas por *bath salts* (sais de banho), devido à apresentação comercial utilizada inicialmente, atualmente podem ser vendidas em forma de comprimido artesanal, pó ou cristal (Figura 28).

Efeitos adversos relacionados ao uso de catinonas sintéticas incluem efeitos cardiovasculares (infarto, dor no peito, hipertensão, palpitações, taquicardia, e vasoconstrição), no SNC (confusão, sonolência, fadiga, dor de cabeça, hipertermia, hipotermia, aumento do tônus muscular, insônia, perda de apetite, perda de

concentração, midríase, náusea, dormência, convulsões, paranoia, psicose, colapso psicótico, autoagressão e pensamentos suicidas) (Assi et al., 2017), sendo comuns casos de intoxicação fatal (Adamowicz, Zuba e Byrska, 2014; Adamowicz, 2016; Costa et al., 2018; Krotulski et al., 2018; Krotulski et al., 2020).

Figura 28: Comprimidos artesanais contendo catinonas sintéticas (cortesia: Instituto de Criminalística do Distrito Federal).

Entre as catinonas sintéticas, é comum substâncias serem isômeros, o que por vezes dificulta a identificação da molécula por meio de técnicas cromatográficas (Sakamoto e Miyagawa, 2017). A Figura 29 mostra algumas catinonas sintéticas, incluindo os isômeros dibutilona e eutilona, ambas com a fórmula química $C_{13}H_{17}NO_3$.

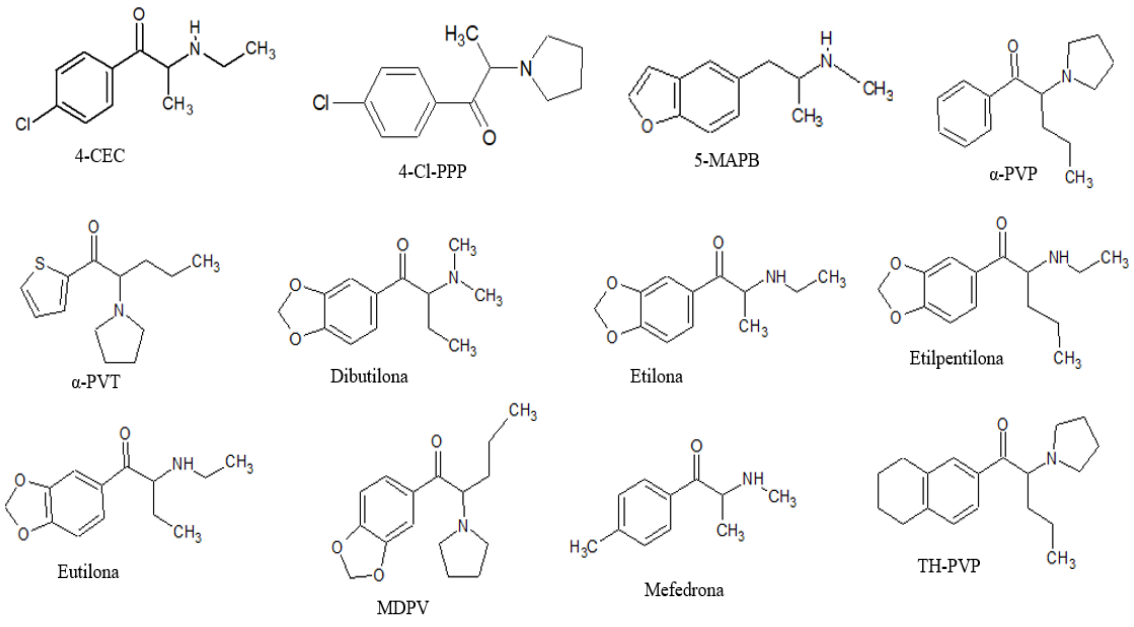


Figura 29: Estrutura molecular das catinonas sintéticas.

4.10.2 Fenetilaminas das famílas 2C, NBOMe (N-benzoilmetoxi) e NBOH (N-benzoilhidroxi)

As fenetilaminas, famílias 2C (2,5-fenetilaminas), NBOMe (N-benzoilmetoxi) e NBOH (N-benzoilhidroxi) são drogas psicoativas agonistas serotoninérgicos (Bilinski et al., 2012; Nichols, 2016; Nielsen et al., 2016). NBOMe (N-benzoilmetoxi) é uma classe de fenetilaminas substituídas que se tornou muito popular como droga recreativa (Nikolaou et al., 2014). Existem relatos de hospitalizações e fatalidades atribuídas a esses compostos (Shanks, Sozio e Behonick, 2015; Zawilska, Kacela e Adamowicz, 2020), apesar de não se saber exatamente se por uma dose letal de NBOMe, ou por associação a outras drogas. Sintomas como agitação, agressividade, taquicardia, hipertensão, e, em casos mais graves, convulsão e rabdomiólise são reações adversas que podem ocorrer com o uso abusivo de derivados de fenetilamina (Suzuki et al., 2015; Nichols, 2016).

Adição de um grupo N-benzil às fenetilaminas do grupo 2C produz um aumento de afinidade ao receptor 5-HT_2A, aumentando a potência alucinógena (Halberstadt, 2017) o que permite NBOMes e NBOHs possam ser comercializados em pequenas doses, compatíveis com selos comumente utilizados com LSD (Nichols, 2016), o que não é possível com substâncias 2C (2C-B, 2C-I, dentre outras), que requerem doses na ordem de miligramas para se atingir o efeito psicoativo. Como exemplo, 25I-NBOMe é 16 vezes mais potente que seu análogo 2C-I (Stellpflug et al., 2014).

Os compostos NBOH, além de serem comercializados como droga de abuso, também podem ser metabólitos de NBOMe (Caspar et al., 2015; Nielsen et al., 2016; Poklis et al., 2015). Conforme descrito anteriormente, por serem consideradas termolábeis, moléculas da família NBOH podem ser equivocadamente identificadas como o respectivo 2C-R quando analisadas por GC-MS em colunas analíticas de 30m de comprimento. Desde então, tentativas foram realizadas para minimizar esta degradação desses compostos no GC (Coelho et al., 2017; Andrade et al., 2020).

4.10.3 Anfetaminas

Anfetaminas e seus derivados, incluindo a 3,4-metilenodioximetanfemtamina (MDMA), vêm sendo utilizados como droga de abuso por décadas. Anfetamina e

metanfetaminas são vendidas sob o nome de rua *speed* e MDMA é a substância normalmente associada à comprimidos de ecstasy (Simmler e Liechti, 2018).

Anfetaminas e MDMA atuam tanto no bloqueio de recaptação de monoaminas, serotonina, noradrenalina e dopamina, quanto no aumento da liberação, causando aumento dos neurotransmissores na fenda sináptica (Green et al., 2003), conforme ilustrado na Figura 14 (Ritter, Flower e Henderson, 2016).

A serotonina é responsável pelos efeitos psicotomiméticos (alucinantes), a dopamina e norepinefrina pela sensação de euforia e posterior disforia (Ritter, Flower e Henderson, 2016). Os diferentes efeitos proporcionados pelo uso de anfetaminas vêm da forma como preferencialmente atuam nos neurotransmissores (Simmler e Liechti, 2018). Normalmente, as anfetaminas, incluindo o MDMA, são utilizados pela via oral, na forma de comprimidos ou pó (EMCDDA, 2023). O principal metabólito do MDMA, metilenodioxianfetamina (MDA) também é comercializado como droga de abuso, por possuir propriedades psicoestimulantes e psicodélicas (Abadinsky, 2014; Nelson et al., 2011).

Os efeitos desejados pelos usuários de anfetaminas incluem a empatia, a sociabilidade, a alegria e a autoestima (Richter, Meyer e Maurer, 2019; Hysek et al. 2014ª, Hysek et al. 2014b; Liechti, Gamma e Vollenweider, 2001). Os efeitos cardioestimulantes são comuns e incluem aumento da pressão arterial, aumento frequência cardíaca, hipertermia e hiponatremina (Haaland et al., 2017; Green et al., 2003), o que pode levar a complicações mais severas, como rabdomiólise, coagulação intravascular e a falência de órgãos, sintomas comumente envolvidos em intoxicações fatais com psicoestimulantes (Cole e Sumnall 2003). O abuso de metanfetaminas permance como um problema de saúde pública nos Estados Unidos (UNODC, 2023a), com desenvolvimento de efeitos neurotóxicos e neurocognitivos no usuário (Courtney e Ray, 2014).

Clobenzorex (N-2-cloro-benzilanfetamina) é um derivado de anfetamina e fenetilamina que já foi usado no tratamento da obesidade (Cody e Valtier, 2001) e que atualmente é utilizado como droga de abuso, como em comprimidos chamados de "rebite". Assim como a metanfetamina, o clobenzorex é metabolizado em anfetamina, o que pode gerar resultados falso-positivos em testes de imunoensaio para anfetaminas (Cody e Valtier, 2001).

A fendimetrazina é um anorexígeno que pode servir como pró-droga para a fenmetrazina, o metabólito N-desmetilado e potente liberador de dopamina/

norepinefrina, com efeitos e mecanismos de ação semelhantes aos da anfetamina (Banks et al., 2013; Solis et al., 2016).

Metilfenidato também é um derivado de anfetamina e fenetilamina, que proporciona o aumento de dopamina e norepinefrina na fenda sinaptica (Hysek et al., 2014b), sendo utilizado no tratamento de transtorno do déficit de atenção e hiperatividade (Gosfrey, 2009). Embora seja um derivado da anfetamina que produza efeitos psicoestimulantes, estudos indicam que quando utililizado em conjunto com MDMA, não há um aumento nos efeitos psicotrópicos (Hysek et al., 2014b).

Anfepramona (dietilpropiona) é uma amina simpatomimética, análogo de anfetamina, utilizada como droga supressora de apetite utilizada no controle da obesidade (Gómez-Silva, et al., 2019; Takitane et al., 2016). Atualmente, não existem medicamentos comerciais com registro vigente no país contendo anfepramona (Anvisa, 2023). A Figura 30 traz as estruturas moleculares de algumas anfetaminas e estimulantes.

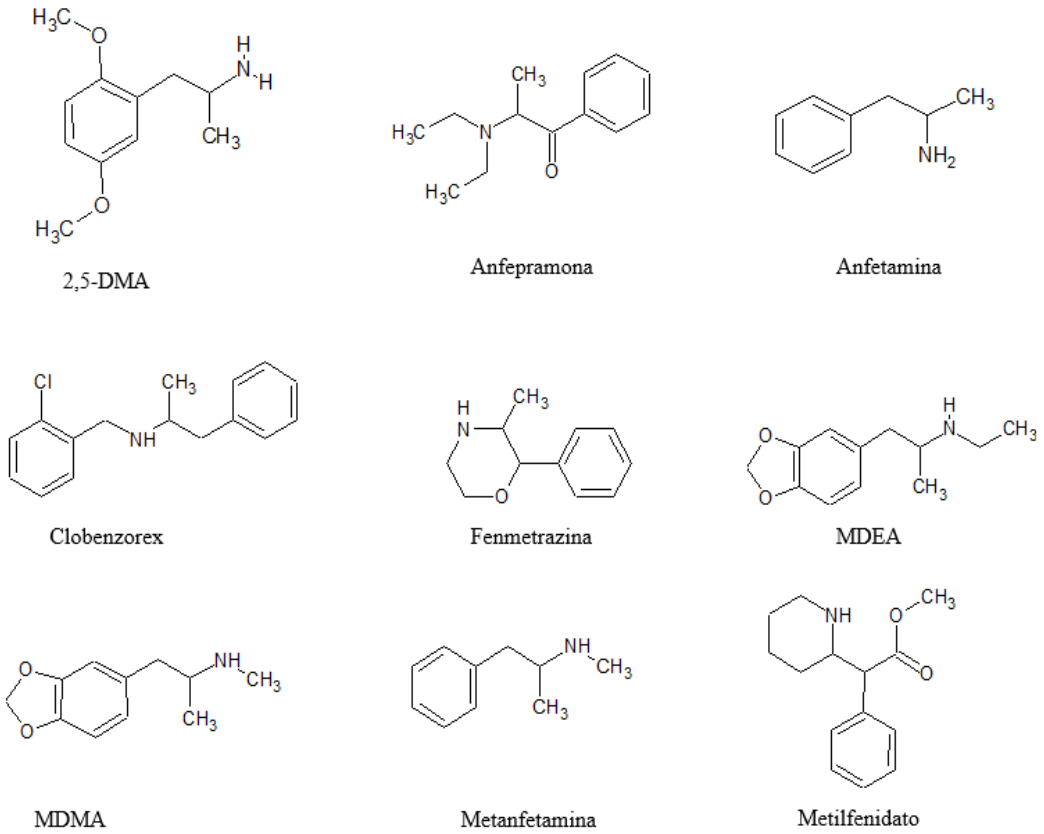


Figura 30: Estruturas moleculares das anfetaminas e derivados estudados nesta tese: 2,5-DMA, anfepramona, anfetamina, clobenzorex, fenmetrazina, MDEA, MDMA, metanfetamina e metilfenidato.

5. Determinação de drogas de abuso utilizando QuEChERS e métodos cromatográficos acoplados à espectrometria de massas

5.1 Extração em fase sólida dispersiva

O método QuEChERS (*quick, easy, cheap, effective, rugged, and safe)*, que utiliza extração em fase sólida dispersiva (d-SPE), foi utilizado por Anastassiades et al. (2003) para extração de pesticidas em alimentos, mas tem sido largamente aplicado na análise de fármacos, drogas de abuso e pesticidas em matrizes biológicas (Dulaurent et al., 2016; Plassmann et al., 2015; Usui et al., 2012; Matsuta et al., 2013; Kim et al., 2014; Kudo et al., 2014; Plossl, Giera e Bracher, 2006). Um trabalho do nosso grupo de pesquisa otimizou e validou um método utilizando d-SPE como protocolo de extração, para a determinação de 14 substâncias de interesse forense (haloperidol, diazepam, carbamazepina, bromazepam, fenobarbital, amitriptilina, MDMA, cocaína, 7-aminoflunitrazepam, carbofurano, terbufos, carbaril, metiocarbe e pirimicarbe) em sangue post-mortem, por meio da análise por CG-MS com injetor de temperatura programada de vaporização e injeção de grandes volumes – PTV-LVI/GC-MS. O método de extração se mostrou eficaz e de fácil implementação em rotina de toxicologia forense.

5.2 Cromatografias gasosa e líquida

A cromatografia é uma técnica de separação amplamente utilizada na área forense, tanto na análise de drogas apreendidas (Coelho Neto et al., 2017), quanto na de matrizes biológicas (Ferrari e Caldas, 2018; Costa et al., 2018; Lehmann et al., 2017).

Na cromatografia gasosa (GC), a fase móvel é composta por um gás de arraste inerte, cuja principal função é a de transportar as moléculas pela coluna cromatográfica. O hélio é o gás de arraste mais utilizado (Skoog, James e West, 2015; Harris, 2011). As colunas cromatográficas mais comuns são de 30 metros, com uma fase estacionária que normalmente é uma fase líquida imobilizada na superfície de um sólido inerte, e a ordem de eluição é determinada pelo ponto de ebulição dos eluentes (Harris, 2011, Jickells e Negrusz, 2008). Com a utilização de colunas de 30 metros de comprimento, a análise de algumas substâncias termolábeis (25R-NBOH) pode se tornar inviável, problema que pode ser mitigado com a utilização de colunas de menor comprimento,

reduzindo, assim, o tempo de residência do analito no sistema cromatográfico. A volatilização do analito acontece no injetor (Moffat et al., 2011), o que pode se tornar um problema analítico para substâncias termolábeis (Ferrari et al., 2020).

Na cromatografia líquida (LC) de fase reversa, a fase móvel é composta por solventes de alto grau de pureza (água ultrapura, metanol e acetonitrila grau HPLC) e, diferente da GC, o analito tem interação com a fase móvel, o que permite otimizações do gradiente de solventes utilizados (Skoog, James e West, 2015; Ramanathan, 2009; Lappas e Lappas, 2016; Gross, 2016; Flanagan et al., 2020).

As colunas analíticas têm normalmente comprimento de 50 mm a 100 mm e a utilização de suportes com partículas menores que 2 μm propiciam alta eficiência na separação de compostos, mas exigem o uso de bombas de alta pressão para a eluição da fase móvel devido a sua baixa permeabilidade, sistemas atualmente conhecidos por UHPLC (do inglês, *Ultra High Pressure Liquid Chromatography*) (Gross, 2016; Flanagan et al., 2020). A possibilidade de se trabalhar com estes métodos em cromatografia líquida de alta performance traz ao analista a possibilidade de rápidas corridas cromatográficas, com boa resolução (Lehmann et al., 2017; Oboardi et al. 2015; Orfanidis et al., 2020). Diferente do sistema de injeção de amostras em GC, o analito não é exposto a altas temperaturas, característica importante para análise de compostos termolábeis (Skoog, James e West, 2015).

5.3 Espectrometria de massas

A espectrometria de massas (MS) é um detector amplamente utilizado com técnicas cromatográficas (Gwak, Arroyo-Mora e Almirall, 2015; Ramanathan, 2009; Lappas e Lappas, 2016), onde o movimento de íons em campos elétricos e magnéticos é utilizado para separá-los de acordo com sua razão massa/carga (m/z) (Gross, 2016; Flanagan et al., 2020). Após a separação pela coluna cromatográfica, o fluxo do material volatilizado, junto com o gás de arraste (em GC), ou do analito junto com a fase móvel (em LC), os analitos se apresentam para a ionização do espectrômetro de massas (Skoog, James e West, 2015).

Na cromatografia gasosa acoplada à espectrometria de massas (GC-MS), a ionização por elétrons (EI) uma das fontes de íons mais comuns, a qual por meio de um fluxo de elétrons de alta energia (geralmente 70 eV) retira elétrons de moléculas a altas temperaturas, fragmentando-as (Skoog, James e West, 2015, Jickells e Negrusz, 2008).

A Figura 31 mostra o esquema de um equipamento GC-MS. Os fragmentos seguem para um analisador de massas do tipo quadrupolo, que é um filtro de massa composto por quatro hastes em que os pares opostos são conectados eletricamente, a uma voltagem com radiofreqüência de 180º, onde apenas a razões massa-carga de interesse previamente selecionadas seguem para o detector (Harris, 2011, Jickells e Negrusz, 2008).

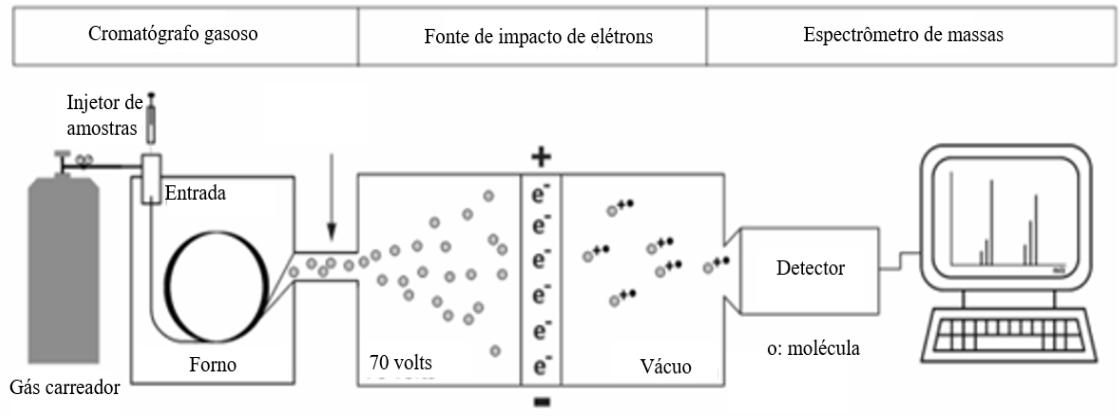


Figura 31: Representação de um GC-MS (Mbughuni, Jannetto e Langman., 2016).

As massas são varridas repetidamente no analisador de massas, durante a corrida cromatográfica, e o resultado é apresentado conforme prévia seleção de monitoramento dos íos formados. No modo Scan, o intervalo m/z sobre o qual um analisador de massa pode ser usado (ex: m/z 50 a 500) é definido e os resultados são apresentados em um espectro de massas, em função do tempo (Skoog et al., 2015). A opção de monitoramento seletivo de íons (SIM) é quando se escolhe relações massa/carga específicas que irão chegar ao detector (ex: apenas as m/z 82, 182 e 303).

Em LC, a fonte de ionização pelo processo electrospray (ESI) é considerada uma das técnicas de ionização suave, com pouca fragmentação da molécula (Ramanathan, 2009). Normalmente, a amostra vinda da cromatografia líquida é apresentada à fonte por meio de um capilar fino, onde é aplicado um campo elétrico. Os íons formados em solução são convertidos para fase gasosa por meio de uma combinação de secagem ou de ejeção de ions, sendo capaz de criar múltiplas cargas, sendo altamente eficaz para análise de moléculas grandes, como peptídeos e proteínas, o que não seria possível por GC-MS (Skoog, James e West, 2015; Lappas e Lappas, 2016; Flanagan et al., 2020).

Na cromatografia líquida acoplada a espectrometria de massas em tandem (LC-MS/MS), a análise das massas (m/z) é realizada em duas etapas seriais distintas, por

meio de um monitoramento seletivo de íons (Flanagan et al., 2020; Gross, 2016). No *Single Reaction Monitoring* (SRM), a primeira etapa é realizada pela seleção de massas (m/z) específicas de interesse (íon precursor), que será fragmentado na célula de colisão. Na segunda etapa, o produto específico do íon precursor, formado na célula de colisão, é selecionado (íon produto). Quando duas ou mais fragmentações do íon precursor são cobertas em um único ciclo, denomina-se *Multiple Reaction Monitoring* (MRM) (Flanagan et al., 2020; Gross, 2016). A técnica de MRM é ideal para métodos quantitativos, demonstrando alta seletividade (Lehmann et al., 2017; Oboardi et al. 2015; Orfanidis et al., 2020). A Figura 32 representa a análise de 25E-NBOH por MRM.

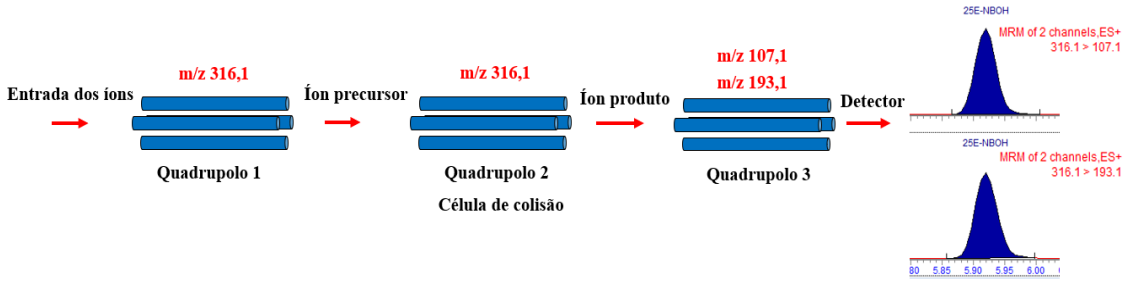


Figura 32: Representação da análise do 25E-NBOH por *Multiple Reaction Monitoring* (MRM) em um UHPLC-MS/MS.

A grande capacidade de se monitorar múltiplas transições (íons produto gerados de íons precursores conhecidos), aliado a uma cromatografia líquida de alta performance possibilita métodos de análise multianalitos, chegando a mais de 50 substâncias por corrida cromatográfica (Rago et al., 2020; Lehmann et al., 2017; Orfanidis et al., 2020).

Além do MS/MS, a cromatografia líquida possibilita outras técnicas hifenadas de alta resolução, como analisadores de massa por tempo de voo (QTOF), Orbitrap®, que podem ser utilizadas para a pesquisa de substâncias não incluídas em um método MRM, ou para metabólitos de NSP, como exemplo (Caspar et al., 2015; Kinyua et al., 2016). A Tabela 3 traz alguns métodos descritos em literatura para pesquisa de substâncias psicoativas em sangue e/ou urina, usando protocolos de QuEChERS, com modificações, e cromatografia gasosa ou líquida acoplado à espectrometria de massas.

Tabela 3: Métodos para análise de substâncias psicoativas em sangue e urina, utilizando QuEChERS, a partir de 2012.

Número e tipos de substâncias	Quantidade de amostra, solvente, extração/clean-up	Matriz biológica	Equipamento	Recuperação e LOQ (µg/mL)	Referência
13 opioides, cocaína e cocaetileno.	**Amostra**: 300uL. **Solvente**: 500 µL de EtOAc. **Extração**: NaOH 0,1 M (pH10), NaCl (50mg) e MgSO4 (100mg). Derivatização com MSTFA.	Sangue	GC-MS Íon Trap	52,4 a 95 %; 0,031 a 0,12.	Alves et al., 2017.
31 pesticidas: OF, OC e piretroides.	Amostra: 1 mL. Solvente: 3 mL EtOAc acidificado. Extração: MgSO4 (0,4g). Clean-up: PSA (50mg)	Plasma	GC–MS/MS	74 a 109 %; 0,012 a 1,35.	Srivastava et al, 2017.
Δ9-THC, 11-OH-THC e 11-COOH-THC.	**Amostra**: 350 mg. **Solvente**: ACN (650µL). **Extração**: NaCl (80mg), MgSO4 (150mg), **Clean-up**: C-18 (12,5mg). Derivatização: HMDS + TMCS	Sangue	GC–MS/MS	>50 %; 0,033 a 0,43 (ng/g).	Dybowski e Dawidowicz, 2018.
10 substâncias: 4-metoxi PV8, PV9, 4-metoxi PV9, difenidina e 6 BZDs.	**Amostra**: 0,5 mL. **Solvente**: ACN + 0,1 % de ácido acético. **Extração**: MgSO4 (6g) e NaOAc (1,5g). **Clean-up**: PSA (25 mg), C18 (25mg) e MgSO4 (150mg).	Sangue, urina e conteúdo gástrico	LC–MS/MS	85 a 94 % sangue), 75 a 88 % (urina), a 0,1 µg/mL; LOQ: NI	Kudo et al., 2015.
4 BZDs: clonazepam, diazepam, flunitrazepam e medazepam.	**Amostra**: 10 mL. **Solvente**: ACN (10 mL). **Extração**: MgSO4 (4g), NaCl (1g). **Clean-up**: MgSO4 (150mg) e GCB (25mg).	Sangue e urina	GC–MS	112 a 328 % (sangue); 51 a 134 % (urina). LOQ: NI	Westland e Doorman, 2013.
65 fármacos e drogas de abuso: (BZDs, ATCs, cocaína, MDMA, MDA, opioides, dentre outros.	**Amostra**: 0,5 mL. **Solvente**: ACN (1,5 mL) e H2O (0,5 mL). **Extração**: MgSO4 (6g) e NaOAc (1,5g). **Clean-up**: membrana de filtração para remoção de lipídeos (Captiva ND Lipids). Derivatização por acetilação.	Sangue	GC–MS	50 a 111 %; 0,1 a 1	Kudo et al., 2014.
Mais de 90 substâncias de	**Amostra**: 0,5 mL. **Solvente**: ACN + 0,1 % de ácido	Sangue	LC–MS/MS	39 a 127 %;	Usui et

Número e tipos de substâncias	Quantidade de amostra, solvente, extração/clean-up	Matriz biológica	Equipamento	Recuperação e LOQ (µg/mL)	Referência
interesse forense, dentre elas: barbitúricos, BZDs, ATC, propofol e disulfoton	acético. **Extração**: MgSO4 (6g) e NaOAc (1,5g). **Clean-up**: PSA (25 mg), C18 (25mg) e MgSO4 (150mg).			0,001 a 1	al.,2012a,b
13 substâncias, incluindo anfetaminas, BZDs, fenobarbital, clorpromazina e prometazina.	**Amostra**: 100 µL. **Solvente**: ACN (500 µL), 0,5% ácido acético. **Extração**: MgSO4 (100mg), NaCl (50mg).	Sangue	GC–MS ou LC–MS.	59 A 93 %; LOQ: NI	Matsuta et al., 2013
48 substâncias, incluindo opiodes, cocaina e metabólitos, anfetaminas, LSD e BZDs.	**Amostra**: 1 mL. **Solvente**: EtOAc. Preparo inicial: b-glucuronidase (pH 4,5-5). **Extração e clean-up**: 100 mL de solução aquosa de tampão carbonato (45 g NaHCO3, 30 g Na2CO3), MgSO4 e PSA.	Sangue	UHPLC–MS-MS	21 A 99 %; 0,002 a 0,02	Anzillotti et al. 2014.
AB-CHIMINACA metabólitos	**Amostra**: 1000 µL. **Solvente**: ACN (1000 µL) e água (1300 µL). **Extração**: 150 mg de MgSO4. **Clean-up:** Captiva ND Lipids®	Urina	LC-MS/MS	Acima de 81,2% 5ng/mL	Wurita et al., 2015
35 fármacos, drogas de abuso e metabólitos, incluindo opioides, anfetaminas, cocaína e seus metabólitos.	**Amostra**: 100 µL. **Solvente**: ACN (200 µL). **Extração**: 40 mg de MgSO4:NaCl (4:1), citrato de sódio dihidratado(1g), citrato de sódio sesqui-hidratado (0,5g).	Sangue	LC-MS/MS	34,5 A 106 %; 0,005	Dulaurent et al., 2016.
Fluoxetina, clomipramina e metabólitos	**Amostra**: 2000 µL. **Solvente**: EtOAc (2000 µL) **Extração**: Citrato dihidratado (200mg), Hidrogenocitrato de sódio (200mg), NaCl (200mg) e MgSO4 (800mg). **Clean-up**: 150 mg MgSO4, 25 mg PSA e 25mg C18	Urina	UHPLC-PDA	86 a 109% 0.1	Alves et al., 2017
MAB-CHMINACA e metabólitos	**Amostra**: 100 µL. **Solvente**: ACN (900 µL) e água (1300 µL). **Extração e Clean-up**: 150 mg MgSO4, 25 mg PSA e 25mg C18; ou 150 mg MgSO4, 25mg C18 e Captiva ND Lipids®	Urina	LC-MS/MS	96,3 a 97,7% LOD: 0,1ng/mL	Hasegawa et al., 2018

Número e tipos de substâncias	Quantidade de amostra, solvente, extração/clean-up	Matriz biológica	Equipamento	Recuperação e LOQ (µg/mL)	Referência
15 substâncias, sendo: 6 antipsicóticos, 8 antidepressivos e zolpidem.	**Amostra**: 100 µL. **Solvente**: ACN (600 µL). **Extração**: K2CO3 (5mg) e MgSO4 (150 mg). **Clean-up**: MgSO4 (150 mg) e PSA (25mg).	Plasma e sangue	UHPLC–MS-MS	85 a 113 %; 0,001 a 0,05	Pouliopoulos et al., 2018
63 compostos, incluindo 19 BZD, 10 ATC, 7 barbitúricos, 4 fenetilaminas e 14 pesticidas	**Amostra**: 200 µL. **Solvente**: ACN (1000 µL) e água (1300 µL). **Extração**: 500 mg de MgSO4: NaOAc (4:1). **Clean-up**: MgSO4 (150 mg) e C18 (50mg).	Sangue	GC-MS/MS	28 a 118%. LOQ>1,0 ng/mL	Kusano et al., 2019
28 fármacos de uso controlado	**Amostra**: 100 µL. **Solvente**: ACN (300 µL) água (300 µL). **Extração**: 100 mg de MgSO4:NaOAc (4:1)	Sangue	LC-MS/MS	>85,9% 1ng/mL	Rodrigues et al., 2020
15 fármacos de uso controlado	**Amostra**: 250 µL. **Solvente**: ACN (500 µL). **Extração**: 100 mg de MgSO4:NaCl; citrato de sódio (4:1:1)	Sangue	UPLC–MS/MS	71,9 a 87,7%; 25 ng/mL	Silva et al., 2021

Δ9-THC: Δ9-tetrahidrocannabinol; 11-COOH-THC: 11-nor-9-carboxi-Δ9-tetrahidrocannabinol; 11-OH-THC: 11-hidroxi-tetrahidrocannabinol; ACN: acetonitrila; BZD: benzodiazepínico; C-18: octadecil; EtOAc: acetato de etila; GC-MS: cromatografia gasosa acoplada à espectrometria de massas; GC–MS/MS: cromatografia gasosa acoplada à espectrometria de massas em tandem; HMDS: Hexametildisilazano; K_2CO_3: carbonato de potássio; LC–MS: cromatografia líquida acoplada à espectrometria de massas; LC–MS/MS: cromatografia líquida acoplada à espectrometria de massas em tandem; LOQ: limite de quantificação; LSD: dietilamida do ácido lisérgico; $MgSO_4$: sulfato de magnésio anidro; MSTFA:N-Metil-N-trimetilsilil-trifluoroacetamida; Na_2CO_3: carbonato de sódio; NaCl: cloreto de sódio; $NaHCO_3$: bicarbonato de sódio; NaOAc: acetato de sódio; NaOH: hidróxido de sódio; NI: não informado; OC: organoclorado; OF: organofosforado; PSA: amina primária secundária; TMCS: trimetilclorosilano; PV8: pirrolidinoheptanofenona; PV9: pirrolidinooctanofenona; UHPLC–MS-MS: cromatografia líquida de ultra eficiência acoplada à espectrometria de massas em tandem; UHPLC-PDA: cromatografia líquida de ultra eficiência acoplada ao detector de conjunto de fotodiodos. Base de dados utilizada na pesquisa: https://pubmed.ncbi.nlm.nih.gov/

OBJETIVOS

Geral:

Desenvolver métodos analíticos e monitorar o perfil de uso de substâncias psicoativas no Distrito Federal.

Específicos:

1. Otimizar um método de análise por GC-MS para triagem de novas substâncias psicoativas (NSP) e sua determinação em selos apreendidos pela Polícia Civil do Distrito Federal;
2. Fazer uma revisão de estudos publicados sobre casos fatais relacionados ao uso de NSP de 2016 a 2021;
3. Desenvolver e validar um método analítico por UHPLC-MS/MS para a determinação de drogas ilícitas e de prescrição em sangue e urina e analisar amostras reais; coletadas no IML-DF;
4. Desenvolver e validar um método analítico por LC-MS/MS, para a determinação de substâncias de interesse toxicológico em saliva e analisar amostras coletadas de doadores voluntários, usuários de substâncias psicoativas.

ESTRUTURA DA TESE

Os métodos e resultados deste trabalho serão apresentados em quatro capítulos distintos, em formato de artigo.

O primeiro capítulo (1. *Analysis of non-derivatized 2-(4-R-2,5-dimethoxyphenyl)-N-[(2-hydroxyphenyl)methyl] ethanamine using short column gas chromatography – mass spectrometry*) se refere à otimização do método proposto para análise de substâncias termolábeis. Esse estudo atende ao objetivo 1 deste trabalho, e foi publicado no periódico *Journal of Chromatography A* (Ferrari Júnior E., et al., 2020; Anexo 1).

O segundo capítulo (2. *Fatal cases involving new psychoactive substances and trends in analytical techniques*) é um artigo de revisão onde nos propusemos a estudar como casos de intoxicação fatal envolvendo o uso de novas substâncias psicoativas vem sendo elucidados pelos laboratórios de toxicologia ao redor do mundo. Esse estudo atende ao objetivo 2 deste trabalho e foi publicado no periódico *Frontiers in Toxicology* (Ferrari Júnior E., et al., 2022; Anexo 2).

O terceiro capítulo (3. *Determination of new psychoactive substances and other drugs in blood and urine by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)*) se refere à análise de 54 amostras de sangue (post-mortem e *in* vivo) e 16 amostras de urina, provenientes de 68 casos reais do IML-DF utilizando o método validado para determinação de 79 fármacos de uso controlado e drogas de abuso, incluindo novas substâncias psicoativas, além da avaliação dos resultados obtidos, junto com os achados clínicos e informações de local de crime. Esse estudo atende ao objetivo 3 deste trabalho e foi publicado no periódico *Forensic Toxicology* (Ferrari Júnior E., et al., 2022; Anexo 3).

O quarto capítulo (4. *Target analysis of drugs in oral fluid by QuEChERS extraction and LC-MS/MS)* se refere à análise de 62 amostras de fluido oral, proveniente de doadores voluntários, frequentadores de festa rave no Distrito Federal. As análises foram realizadas por meio de um método validado para determinação de 51 fármacos de uso controlado e drogas de abuso, além de triagem de outras 22 substâncias psicoativas, incluindo novas substâncias psicoativas, e os resultados foram comparados com

informações fornecidas pelos voluntários, como forma de apresentação (comprimido, pó, selo), nome da substância psicoativa, via de administração e tempo entre uso e ingestão. Os achados laboratoriais foram disponibilizados aos doadores, de modo anônimo. Esse estudo atende ao objetivo 4 deste trabalho foi submetido no periódico *Journal of Pharmaceutical and Biomedical Analysis*.

1. Analysis of non-derivatized 2-(4-R-2,5-dimethoxyphenyl)-N-[(2-hydroxyphenyl)methyl]ethanamine using short column gas chromatography – mass spectrometry

Ferrari Júnior, E.; Arantes, L.C.; Salum, L.B.; Caldas, E.D. *Journal of Chromatography A*, v. 1634, p. 461657, 2020 (Anexo I).

Abstract

The 25R-NBOH family is a group of thermally labile compounds that are relevant for forensic sciences and traditionally analyzed by GC-MS after derivatization —a step that is time consuming in a routine work. In this paper, the use of short analytical columns (4 and 10 m) showed to decrease compound degra- dation in the GC oven during chromatographic separation and to allow the analysis of non-derivatized 25R-NBOH compounds by GC-MS. A shorter column demanded a higher gas flow rate, and both factors decreased residence time of the analytes in the column and their degradation. The inlet temperature (250 °C or 280 °C) did not impact the response of25R-NBOH. A 25R-NBOH fragmentation pathway by electron ionization was also presented for the first time. The GC-MS method with a 4 m column was successfully applied to other compounds of forensic interest, and it can be tested in the analysis of bio- logical samples in toxicological investigations.

Keywords: GC-MS, short column, 25R-NBOH, thermal degradation.

2. Fatal cases involving new psychoactive substances and trends in analytical techniques

Ferrari Júnior,E.; Leite, B.H.M.; Gomes, E.B.; Vieira, T.M.; Sepulveda, P.; Caldas, E.D. *Frontiers in Toxicology*, v. 4, 2022 (Anexo II).

Abstract

New psychoactive substances (NPS) are an emerging public health issue and deaths are commonly associated with polydrug abuse. Moreover, the number of new substances available is constantly increasing, causing intoxications in low doses, characteristics that impose to toxicology and forensic laboratories to keep routine methods up to date, with high detectability and constantly acquiring new analytical standards. Likewise, NPS metabolites and respective elimination pathways are usually unknown, making it difficult the detection of the confirmation of the drug involved in the fatal case in an analytical routine. A literature search was performed on PubMed, Scopus and Web of Science databases for papers related to chromatographic analyses from fatal cases related to NPS use published from 2016 to 2021. A total of 96 papers were retrieved and reviewed in this study. Opioids, synthetic cathinones, phenethylamines/amphetamines and cannabinoids were the NPS classes most found in the fatal cases, and in many cases, multiple compounds were detected in the biological samples, including prescription and other illegal drugs. Liquid chromatography – tandem mass spectrometry, an alternative to overcome the gas chromatography – mass spectrometry limitations for some compounds, was the analytical technique most used in the studies, and high-resolution mass spectrometry was often applied to NPS metabolite investigation and structural characterization and identification of unknown compounds. Toxicological screening and quantitation methods need to be continuously updated to include new substances that are emerging on the drug market, that can be fatal at very low doses.

Keywords: new psychoactive substances, GC-MS, LC-MS/MS, HRMS, opioids, synthetic cathinones, fatal cases.

3. Determination of 79 new psychoactive substances and other drugs in blood and urine by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)

Ferrari Júnior, E.; Caldas, E.D. *Forensic Toxicology*, v. 40, p. 88-101, 2022 (Anexo III).

Abstract

Purpose This study aimed to validate a modifed QuEChERS method followed by ultra-high performance liquid chromatography–tandem mass spectrometry to determine 79 new psychoactive substances (NPS) and other drugs in blood and urine.

Methods Prescription drugs (n=23), synthetic cathinones (n=13), phenethylamines (n=11); synthetic cannabinoids (n=8), amphetamines (n=7) and other psychoactive substances (n=17) were included in the method. 500 µL of biological fuid was extracted with 2 mL of water/I (1:1), 500 mg of anhydrous $MgSO_4$/NaOAc (4:1) added, followed by a supernatant cleanup with 25 mg of primary secondary amine and 75 mg of anhydrous MgSO4. Quantifcation was done using matrix-matched calibration curves and deuterated internal standards.

Results The method was satisfactorily validated for blood and urine at limit of quantifications ranging from 0.4 to 16 ng/mL, and applied to the analysis of 54 blood (38 postmortem and 16 antemortem) and 16 antemortem urine samples from 68 forensic cases. All urine samples and 59.3% of the blood samples were positive for at least one analyte. Twenty-two analytes were detected in at least one biological sample, including the synthetic cathinones ethylone (222 ng/mL, antemortem blood), eutylone (246 and 446 ng/mL, urine), and N-ethylpentylone (597 and 7.3 ng/mL, postmortem and antemortem blood, respectively).

Conclusions The validated method was shown to be suitable for the analysis of blood and urine forensic samples and an important tool to collect information on emerging drug threats and understanding the impact of NPS and other drugs in poisoning cases.

Kewwords: drugs, new psychoactive substances (NPS), postmortem blood, urine · UHPLC–MS/MS.

4. Target analysis of psychoactive drugs in oral fluid by QuEChERS extraction and LC-MS/MS

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Abstract: This study aimed to validate a modified QuEChERS method, followed by liquid chromatography-tandem mass spectrometry, for the determination of 51 psychoactive substances and screening of 22 ones in oral fluid from electronic dance music party (EDM) attendees. Unstimulated oral fluid was collected in a polypropylene tube and stored in a glass vial at -20 ºC. The sample was extracted with acetonitrile:water and $MgSO_4$/NaOAc, followed by cleanup with primary secondary amine and MgSO4. The effectiveness of the sample storage conditions was compared with that using Quantisal collection device and showed no substantial concentration loss (< 15 %) for all the substances after up to 72 hours of storage at -20º C. The method was satisfactorily validated with LOD and LOQ ranging from 0.04 to 0.5 ng/mL and 0.1 to 1.5 ng/mL, respectively, and it was applied to the analysis of 62 real samples. The main substances detected were 3,4 methylenedioxymetamphetamine (MDMA) (<0.5 - 829 ng/mL) and/or methylenedioxyamphetamine (MDA) (10.1 – 460.6 ng/mL), found in 27 samples, and cocaine (13.0-407.3 ng/mL) and its metabolites (benzoylecgonine 0.17-214.1 ng/mL; ecgonine methyl ester 1.8-150.1 ng/mL) in eight samples. Methamphetamine (11-439 ng/mL) was detected in eight samples, along with MDMA and MDA; eutylone was detected in two cases (4.7 and 24.1 ng/mL) reported as "ecstasy" ingestion. A comparison between self-reported drug use and results of oral fluid analysis indicated that the use of illicit substances is often underreported among EDM attendees, who are often unaware of the substances they consume.

Keywords: Oral fluid, QuEChERS, LC-MS/MS, drugs, new psychoactive substances (NPS).

4.1. Introduction

The drug landscape is constantly evolving, particularly with the growing prevalence of new psychoactive substances (NPS) designed to mimic the effects of and to replace traditional drugs of abuse, such as cocaine, cannabis, and amphetamines (UNODC, 2023a; EMCDDA, 2023). In 2021, approximately one in every 17 persons worldwide had used drugs, totaling around 296 million users, marking a 23% increase from a decade earlier. The market also witnessed a surge in NPS availability, with a total of 618 identified, including 87 newly discovered substances, following several years of stabilization (UNODC, 2023a). Notably, drug use that may contain NPS is common among participants at electronic dance music parties (EDM), constituting a high-risk population due to potential associated adverse effects (Palamar and Rayes, 2020), which can even lead to fatal outcomes (Costa et al., 2018; Ferrari Jr. and Caldas, 2021).

Several risk factors contribute to the perilous nature of drug use, including the highly variable composition of illicitly sold synthetic drugs, including NPS, and that users are often unaware of which substances they have consumed (UNODC, 2023a; Togni et al., 2015). To address these challenges, a harm reduction approach using self-reported data on drug consumption history has been proposed as an alternative to estimating illicit substance consumption in a region, although it may involve incorrect information about drug use (Palamar and Rayes, 2020; Palamar et al., 2020). A more reliable approach involves combining self-reported data with biological fluid analysis of EDM party attendees (Costa et al., 2018; Palamar et al., 2020; Krotulski et al., 2018).

Although drug concentrations in oral fluid may not always accurately reflect blood concentrations (Wille et al., 2009), it is a widely used matrix for assessing recent drug intake, given its simplicity for field collection, non-invasiveness, and acceptance among EDM party attendees (Cooman et al., 2020; Mohr et al., 2021; Mohr et al., 2018). Usually, commercial oral fluid collection devices have preservatives that prevent drug and metabolite degradation when samples are stored for long periods before analysis (Langel et al., 2008; Marchei et al., 2020), an advantage that may not be necessary if the analysis is carried out within a short period after collection. Furthermore, some collection devices can dilute possible substances present in the oral fluid, which would not occur in an unstimulated oral fluid collection (Sobczak et al., 2020), in addition to being an additional cost for the analyzes.

Various extraction methods are used for drug analysis in biological samples, including liquid-liquid extraction (LLE), which can be less efficient due to matrix interference, and solid-phase extraction, which necessitates sorbent cartridges and conditioning Lehamnn et al., 2017; Lin et al., 2017; Montenarh et al., 2015). The QuEChERS extraction protocol (fast, easy, cheap, effective, robust, and safe) presents an efficient alternative for matrix removal in multi-drug analysis in various matrices such as blood (Ferrari Jr. and Caldas, 2021; Alves et al., 2016; Ferrari Jr. and Caldas, 2018; Orfanidis et al., 2021), urine (Ferrari Jr. and Caldas, 2021), oral fluid (Chinaglia et al., 2022) and stomach content (Peres et al., 2019). Following sample preparation, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is a common technique for drug detection and quantification, given its ability to achieve lower detection limits essential for some NPS and accommodate various classes of substances in a single chromatographic run (Chan et al., 2021; Ferrari Jr. et al., 2022; Trana et al., 2022).

The primary objective of this study was to optimize and validate a method for detecting drugs of abuse, including NPS, using a modified QuEChERS approach and LC–MS/MS. The method was applied for the analysis of oral fluid samples obtained from EDM party attendees in the Federal District of Brazil, who also responded a questionnaire regarding the drug used.

4.2. Experimental

4.2.1. Chemicals and reagents

Benzoylecgonine (BZE), dimethyltryptamine (DMT), ecgonine methyl ester (EME), fentanyl, harmine, harmaline, LSD, levamisole, methylenedioxyamphetamine (MDA), and standard solutions of cocaine-d3, diazepam-d5, fentanyl-d5, imipramine-d3, LSD-d3, MDA-d5, MDMA-d5, THC-COOH-d3 (internal standards, IS) were purchased from Cerilliant – Sigma Aldrich (USA). 25E-NBOMe, 4-chloro-alpha-pyrrolidinopropiophenone (4-chloro-alpha-PPP or 4-Cl-α-PPP;), 5-fluoro APINACA (5F-AKB-48), ethylone (bk-MDEA), and eutylone (bk-EBDB) were purchased from Cayman Chemical (USA). Clobenzorex was acquired from LGC Standards. 2,5-DMA, 2C-B, 5-MAPB, 6-monoacetyl morphine (6-MAM), 7-aminoflunitrazepam (7-AF), AH-7921, AM 2201, amphetamine, benzylpiperazine (BZP), cocaine, JWH-018, ketamine, meta-chlorophenylpiperazine (m-CPP), methylenedioxy-N-ethylamphetamine (MDEA),

3,4-methylenedioxymetamphetamine (3,4-MDMA), methylenedioxypyrovalerone (MDPV), mephedrone, methadone, methamphetamine, methylone (bk-MDMA) and norketamine were donated by the United Nations Office on Drugs and Crime (UNODC). 2C-H, 2C-I, 5-MeO-MIPT, AB-CHMINACA, AKB-48, α-pyrrolidinopentio thiophenone (α-PVT), dibutylone (bk-DMBDB), JWH-081, JWH-210, phenmetrazine and tetramethylene-α- pyrrolidinovalerophenone (TH-PVP), were provided by the United States Drug Enforcement Administration (DEA). Flunitrazepam was donated by INMETRO (Brazil); Amfepramone (diethylpropion) by Aché Pharmaceutical Laboratories S.A; methylphenidate by Novartis Pharma (Brazil). Tetrahydroharmine were synthetized and their identity and purity confirmed by mass spectrometry and NMR. N-Ethylpentylone (ephylone) standard was prepared from seized material and its purity confrmed by NMR.

Twenty-two analytes were not validated in the method but included only for screening purposes: 25B-NBOH, 25C-NBOH, 25E-NBOH, 25I-NBOH, 25B-NBOMe, 25C-NBOMe, 25H-NBOMe, 25I-NBOMe, 2C-C, 2C-E, 4-methylpentedrone, 5F-MDMB-PICA, α-pyrrolidinopentiophenone (α-PVP), AB-FUBINACA, ADB-BUTINACA, etizolam, femproporex, JWH-250, methylenedioxy-N-tert-butylcathinone (MDPT), methyl-α-pyrrolidinohexanophenone (MPHP), N-ethylheptedrone and pentylone.

Figure S1 (Supplementary Material) shows the chemical structure of the 73 substances monitored in the present method.

Acetonitrile (ACN) LC–MS grade was purchased from Scharlau (Spain). Methanol LC-MS grade, PSA (primary and secondary amine), anhydrous magnesium sulfate ($MgSO_4$), and sodium acetate (NaOAc) were purchased from Sigma Aldrich (USA), and formic acid was obtained from Honeywell/Fluka (Germany). Ultrapure water was obtained from a Milli-Q purification system (USA). Quantisal™ oral fluid collection devices and elution buffer were purchased from Immunalysis (USA).

Individual stock solutions were prepared in methanol or ACN at 1 mg/mL, one mixed stock solution was prepared at final concentration 10 µg/mL. One mixed working solution were prepared at 0.4 µg/mL for 25E-NBOMe, AB-CHMINACA, AB-FUBINACA AH-7921, AKB-48, BZE, EME, fentanyl, JWH-018, JWH-081, JWH-210 and LSD, and at 2 µg/mL, for the other substances. After, the working solution was diluted 10x for the optimization and validation parameters. For the internal standards

(IS), two mixed working solution were prepared at 1 µg/mL and 100 ng/mL. All solutions were kept in amber vials at -20ºC.

2.2. LC–MS/MS conditions

The analyses were performed by using a Shimadzu system (LC-20AD pumps, a SIL-20AD autosampler, and CTO-20AC column oven (Kyoto, Japan), coupled to a 6500^{+} SCIEX QTRAP® triple quadrupole mass spectrometer (Foster, USA). The software Analyst® (version 1.6) was used for control and data acquisition and the SCIEX OS® for processing the results. A Zorbax Eclipse Plus C18-column (2.1 mm ID × 100 mm, 1.8 µm, Agilent Technologies) was applied to the chromatographic separation. The mobile phase consisted of water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) and the gradient elution was performed with a constant flow rate of 0.3 mL/min and a column oven temperature of 40 °C, utilizing the following gradient: 0 min: 5 % B; 1.4 min: 30 % B; 11-12.6 min: 95 % B; 12.61-14.4 min: 5 % B. The total run time equates to 14.4 min. The injection volume was set to 3 µL. The electrospray ionization (ESI) was performed in the multiple reaction monitoring (MRM) mode with Scheduled MRM (multiple reaction monitoring) and positive ionization. Ion source optimization conditions were: curtain gas (45 psi), ion spray (5500 V), source temperature (550 °C), gas 1 and gas 2 (55 psi). For each analyte, two transitions were selected, one of quantification and one of qualification. The molecular formula, retention time (RT), respective internal standard, MRM transitions, DP, collision energy and CXP, limit of detection (LOD) and the limit of quantification for the 51 analytes and the 9 IS used in the method are shown in Table 4.1. The parameters of other 22 analytes (only screening) in LC-MS/MS system are shown in Table S1.

4.2.3. Biological samples

Method development and validation were conducted using a mix of 10 drug-free oral fluid samples provided by volunteers (matrix control). Oral fluid specimens (real samples) were generously donated by volunteers (≥18 years) who attended two electronic music events in the Federal District in September and October of 2023. These donors received instructions on how to self-collect unstimulated oral fluid into a 50 mL Falcon tube. To prevent absorption of certain analytes onto the plastic surface (Molnar et al., 2013), immediately after oral fluid collection, each sample was transferred to a 2

mL glass vial. The vials were initially stored on dry ice and subsequently transported to the laboratory for storage at -20 ºC. Each sample was identified with a code number for proper tracking.

Participants were queried about the drug dosage form, the name of the psychoactive substance, and the time that had elapsed between substance use and the donation of oral fluid (expressed in minutes, hours, or days). The study was approved by the Ethical Committee for Human Studies of the University of Brasilia, Brazil (CAAE 2936819.3.0000.0030).

Table 4.1 Molecular formula, limit of detection and limit of quantification of the 51 compounds analyzed and the 9 internal standards.

Substance	Molecular formula	I.S.	Transitions (m/z)	RT (min.)	DP (V)	CE (V)	CXP (V)	LOD (ng/mL)	LOQ (ng/mL)
2,5-DMA	$C_{11}H_{17}NO_2$	Cocaine-d3	**196.0 → 151.2**	3.7	46	23	18	0.2	0.5
			196.0 → 164.1	3.7	46	27	16		
25E-NBOMe	$C_{20}H_{27}NO_3$	Diazepam-d5	**330.1 → 121.1**	7.1	41	25	20	0.04	0.1
			330.1 → 91.0	7.1	41	61	14		
2C-B	$C_{10}H_{14}BrNO_2$	Fentanyl-d5	**308.0 → 275.9**	4.9	41	33	30	0.2	0.5
			308.0 → 260.7	4.9	41	45	30		
2C-H	$C_{10}H_{15}NO_2$	MDMA-d5	**182.1 → 150.1**	3.3	36	25	14	0.2	0.5
			182.1 → 135.0	3.3	36	37	14		
2C-I	$C_{10}H_{14}INO_2$	Diazepam-d5	**261.0 → 91.0**	5.6	46	27	12	0.2	0.5
			261.0 → 92.1	5.6	46	29	10		
4-Cl-α-PPP	$C_{13}H_{16}ClNO$	Cocaine-d3	**239.0 → 127.2**	3.5	51	29	14	0.5	1.5
			239.0 → 126.2	3.5	51	29	14		
5F-AKB-48	$C_{23}H_{30}FN_3O$	THC-COOH-d3	**384.2 → 135.9**	11.6	111	31	12	0.2	0.5
			384.2 → 92.9	11.6	111	67	15		
5-MAPB	$C_{12}H_{15}NO$	Diazepam-d5	**190.1 → 160.1**	2.7	36	25	18	0.2	0.5
			190.1 → 132.1	2.7	36	35	6		
5-MeO-MIPT	$C_{15}H_{22}N_2O$	Cocaine-d3	**247.1 → 86.1**	3.3	31	19	12	0.2	0.5
			247.1 → 174.2	3.3	31	23	12		
6-MAM	$C_{19}H_{21}NO_4$	Diazepam-d5	**328.2 → 165.2**	2.8	114	53	15	0.2	0.5
			328.2 → 211.2	2.8	114	36	15		
7-AF	$C_{16}H_{14}FN_3O$	Diazepam-d5	**284.1 → 135.1**	4.5	91	39	15	0.2	0.5
			284.1 → 226.0	4.5	91	49	15		
AB-CHMINACA	$C_{20}H_{28}N_4O_2$	Diazepam-d5	**357.2 → 312.0**	10.2	86	23	18	0.04	0.1
			357.2 → 241.0	10.2	86	35	20		
AH-7921	$C_{16}H_{22}Cl_2N_2O$	MDA-d5	**329.9 → 285.0**	5.8	46	25	26	0.04	0.1

Substance	Molecular formula	I.S.	Transitions (m/z)	RT (min.)	DP (V)	CE (V)	CXP (V)	LOD (ng/mL)	LOQ (ng/mL)
AKB-48	$C_{23}H_{31}N_3O$	THC-COOH-d3	329.9 → 173.0	5.8	46	39	16	0.04	0.1
			366.2 → 135.2	12.5	41	23	14		
			366.2 → 92.9	12.5	41	61	10		
α-PVT	$C_{13}H_{19}NOS$	Cocaine-d3	**238.1 → 126.1**	3.5	56	29	14	0.5	1.5
			238.1 → 97.0	3.5	56	31	12		
AM-2201	$C_{24}H_{22}FNO$	Diazepam-d5	**360.0 → 155.1**	10.2	101	37	28	0.2	0.5
			360.0 → 127.0	10.2	101	71	22		
Amfepramone	$C_{13}H_{19}NO$	MDA-d5	**206.1 → 105.1**	3,0	56	29	12	0.2	0.5
			206.1 → 133.1	3,0	56	23	8		
Amphetamine	$C_9H_{13}N$	MDMA-d5	**136.1 → 91.0**	2.9	68	24	15	0.5	1.5
			136.1 → 65.0	2.9	68	50	15		
			136.1 → 119.1	2.9	68	20	15		
BZE	$C_{16}H_{19}NO_4$	Diazepam-d5	**290.1 → 168.2**	3.9	80	25	15	0.04	0.1
			290.1 → 105.1	3.9	80	39	15		
BZP	$C_{11}H_{16}N_2$	MDA-d5	**178.0 → 91.1**	2.8	71	27	8	0.2	0.5
			178.0 → 65.0	2.8	71	65	16		
Clobenzorex	$C_{16}H_{18}ClN$	Cocaine-d3	**260.2 → 125.0**	5.6	80	20	15	0.2	0.5
			260.2 → 91.1	5.6	80	25	15		
Cocaine	$C_{17}H_{21}NO_4$	Cocaine-d3	**304.2 → 182.2**	3.9	86	25	15	0.2	0.5
			304.2 → 105.1	3.9	86	41	15		
Dibutylone	$C_{13}H_{17}NO_3$	MDA-d5	**236.1 → 191.1**	3.3	56	21	10	0.2	0.5
			236.1 → 161.0	3.3	56	27	18		
			236.1 → 86.1	3.3	56	27	10		
DMT	$C_{12}H_{16}N_2$	Diazepam-d5	**189.1 → 144.1**	2.8	41	25	10	0.2	0.5
			189.1 → 143.0	2.8	41	45	8		
EME	$C_{10}H_{17}NO_3$	MDMA-d5	**200.1 → 182.2**	0.8	130	20	10	0.04	0.1
			200.1 → 82.0	0.8	130	35	10		
Ethylone	$C_{12}H_{15}NO_3$	Diazepam-d5	**222.1 → 174.1**	2.9	36	25	20	0.2	0.5
			222.1 → 146.1	2.9	36	37	6		
Eutylone	$C_{13}H_{17}NO_3$	Diazepam-d5	**236.1 → 188.1**	3.4	31	25	10	0.2	0.5
			236.1 → 174.0	3.4	31	43	20		
Fentanyl	$C_{22}H_{28}N_2O$	Fentanyl-d5	**338.1 → 189.1**	5.0	101	35	54	0.04	0.1
			338.1 → 188.1	5.0	101	35	46		
Flunitrazepam	$C_{16}H_{12}FN_3O_3$	Nortryptiline-d3	**314.1 → 268.1**	7.0	150	35	15	0.2	0.5
			314.1 → 239.2	7.0	150	49	15		
Harmaline	$C_{13}H_{14}N_2O$	Cocaine-d3	**215.0 → 200.1**	4.1	71	33	14	0.2	0.5
			215.0 → 174.1	4.1	71	33	14		
Harmine	$C_{13}H_{12}N_2O$	Cocaine-d3	**213.1 → 170.1**	4.3	86	43	12	0.2	0.5

Substance	Molecular formula	I.S.	Transitions (m/z)	RT (min.)	DP (V)	CE (V)	CXP (V)	LOD (ng/mL)	LOQ (ng/mL)
JWH-018	$C_{24}H_{23}NO$	THC-COOH-d3	213.1 → 168.0	4.3	86	33	16	0.04	0.1
			342.1 → 127.2	11.3	101	35	26		
			342.1 → 155.0	11.3	101	63	55		
JWH-081	$C_{25}H_{25}NO_2$	Diazepam-d5	**372.1 → 185.1**	11.5	40	33	16	0.1	0.3
			372.1 → 157.2	11.5	40	51	10		
JWH-210	$C_{26}H_{27}NO$	Diazepam-d5	**370.1 → 183.1**	11.9	60	33	18	0.04	0.1
			370.1 → 214.1	11.9	60	33	18		
Ketamine	$C_{13}H_{16}ClNO$	LSD-d3	**238.1 → 125.0**	3.6	75	46	15	0.2	0.5
			238.1 → 220.2	3.6	75	20	15		
Levamisole	$C_{11}H_{12}N_2S$	Diazepam-d5	**205.7 → 179.0**	2.6	1	29	20	0.2	0.5
			205.7 → 92.1	2.6	1	47	10		
LSD	$C_{20}H_{25}N_3O$	LSD-d3	**324.0 → 223.1**	4.6	81	33	18	0.04	0.1
			324.0 → 281.2	4.6	81	25	16		
m-CPP	$C_{10}H_{13}ClN_2$	Cocaine-d3	**198.0 → 170.0**	4.3	181	27	18	0.2	0.5
			198.0 → 169.1	4.3	181	41	18		
MDA	$C_{10}H_{13}NO_2$	MDA-d5	**180.1 → 163.1**	3.0	128	20	15	0.5	1.5
			180.1 → 105.1	3.0	128	30	15		
			180.1 → 77.0	3.0	128	50	15		
MDEA	$C_{12}H_{17}NO_2$	MDA-d5	**208.1 → 163.2**	3.3	116	17	15	0.2	0.5
			208.1 → 135.1	3.3	116	30	15		
MDMA	$C_{11}H_{15}NO_2$	MDMA-d5	**194.1 → 163.1**	3,0	122	17	15	0.2	0.5
			194.1 → 105.1	3,0	122	34	15		
MDPV	$C_{16}H_{21}NO_3$	MDMA-d5	**276.1 → 126.1**	4.1	51	33	14	0.2	0.5
			276.1 → 205.1	4.1	51	25	12		
Mephedrone	$C_{11}H_{15}NO$	MDMA-d5	**178.2 → 160.2**	3.3	51	19	26	0.2	0.5
			178.2 → 145.1	3.3	51	27	24		
MA	$C_{10}H_{15}N$	MDMA-d5	**150.1 → 91.0**	2.9	80	27	15	0.2	0.5
			150.1 → 119.1	2.9	80	15	15		
Methylone	$C_{11}H_{13}NO_3$	MDMA-d5	**208.1 → 160.1**	2.7	60	25	12	0.2	0.5
			208.1 → 132.1	2.7	60	32	14		
Methylphenidate	$C_{14}H_{19}NO_2$	Cocaine-d3	**234.1 → 84.1**	3.9	70	55	15	0.2	0.5
			234.1 → 91.1	3.9	70	30	15		
N-ethylpentylone	$C_{14}H_{19}NO_3$	Cocaine-d3	**240.3 → 232.1**	4.1	66	19	18	0.2	0.5
			250.3 → 202.0	4.1	66	25	10		
Norketamine	$C_{12}H_{14}ClNO$	MDMA-d5	**224.1 → 125.1**	3.5	55	18	12	0.2	0.5
			224.1 → 207.1	3.5	55	32	15		
Phenmetrazine	$C_{11}H_{15}NO$	Diazepam-d5	**178.1 → 145.0**	3.2	46	27	10	0.5	1.5
			178.1 → 144.1	3.2	46	39	16		

Substance	Molecular formula	I.S.	Transitions (m/z)	RT (min.)	DP (V)	CE (V)	CXP (V)	LOD (ng/mL)	LOQ (ng/mL)
THH	$C_{13}H_{16}N_2O$	LSD-d3	**217.0 → 188.1**	3.4	46	19	14	0.2	0.5
			217.0 → 200.1	3.4	46	17	16		
TH-PVP	$C_{19}H_{27}NO$	MDA-d5	**286.2 → 145.1**	6.7	41	35	16	0.2	0.5
			286.2 → 215.1	6.7	41	27	26		
LSD-d3[1]	$C_{20}H_{22}N_3OD_3$	-	327.0 → 226.0	4.4	81	33	10	-	-
Cocaine-d3[1]	$C_{17}H_{18}D_3NO_4$	-	307.0 → 185.0	3.8	50	25	10	-	-
Diazepam-d5[1]	$C_{16}H_8D_5ClN_2O$	-	290.0 → 198.1	8.4	80	46	10	-	-
Fentanyl-d5[1]	$C_{22}H_{23}D_5N_2O$	-	342.0 → 188.0	5.0	80	20	10	-	-
THC-COOH-d3[1]	$C_{21}H_{25}D_3O_4$	-	348.0 → 330.0	11.0	80	30	10	-	-
Imipramine-d3[1]	$C_{19}H_{21}D_3N_2$	-	284.0 → 89.0	6.5	80	20	10	-	-
MDMA-d5[1]	$C_{11}H_{10}D_5NO_2$	-	199.0 → 165.0	2.9	80	20	10	-	-
Nortriptyline-d3[1]	$C_{19}H_{18}D_3N$	-	267.0 → 233.0	6.8	50	41	10	-	-
MDA-d5[1]	$C_{10}H_8D_5NO_2$	-	185.0 → 168.1	2.9	80	20	10	-	-

IS: Internal standard. 7-AF: 7-aminoflunitrazepam; α-PVT: α-pyrrolidinopentiothiophenone; BZE: benzoylecgonine; BZP: benzylpiperazine; CE: collision energy; CXP: collision cell exit potential; DMT: dimethyltryptamine; DP: declustering potential; EME: ecgonine methyl ester; I.S.: internal standard; MDA: methylenedioxyamphetamine; LOD: limit of detection: LOQ: limit of quantification; MDMA: 3,4-methylenedioxymetamphetamine; MDEA: methylenedioxy-N-ethylamphetamine; MDPV: methylenedioxypyrovalerone; MA: methamphetamine; RT: retention time; THH: tetrahydroharmine; TH-PVP: tetramethylene-α-pyrrolidinovalerophenone.

4.2.4. Sample extraction and clean-up

The previously optimized blood sample preparation method (Ferrari Jr. and Caldas, 2021) was adapted for oral fluid samples. In a 2 mL microtube, 400 µL of ACN containing IS (final concentration 20 ng/mL), 400 µL of water and 200 mg of anhydrous $MgSO_4$/NaOAc (4:1) were added to 200 µL of oral fluid. The microtube was vortexed (15 sec.) and centrifuged (3500 RPM/5 min). The supernatant was transferred to another microtube containing 10 mg of PSA and 30 mg of $MgSO_4$, vortexed and centrifuged (3500 RPM/5 min). 200 µL of the extract was dried under vacuum, reconstituted in 100 µL of water/methanol 0.1 % formic acid (1:1), and transferred to a vial for LC-MS/MS analysis.

Thirty-five samples were extracted/purified and analyzed within 72 hours of collection (September 2023). Twenty-seven samples collected in October 2023 were extracted/purified within 72 hours, but could not be immediately analyzed due to the LC-MS/MS technical problems. The extract was dried under nitrogen and kept at -20 ^{o}C for 60 days before analysis.

4.2.5. Method validation

The method was validated following the Standard Practices for Method Validation in Forensic Toxicology guidelines (ANSI/ASB Standard 036) (ANSI/ASB, 2019). The parameters evaluated included selectivity, matrix effect, linearity, recovery, bias/accuracy, repeatability (within-run precision) and intermediate precision (between-run precision), carryover, dilution integrity and sample stability. Three different sets of fortified samples were utilized during the validation: analytical standards in solvent, analytical standards added to a control matrix pre-extraction and analytical standards added to a control matrix post-extraction.

Selectivity was assessed by analyzing 10 different control matrix samples (drug-free) to investigate the presence of interferents at the MRM transitions and retention times of the analytes. Matrix effects (signal suppression or enhancement) were evaluated by analyzing pooled oral fluid samples (n = 10) and comparing the sample normalized mean area in post-extraction fortified samples (matrix-matched) with the normalized mean area in solvent fortified samples, expressed in %. Matrix effects were evaluated at the lowest, medium, and highest concentration levels, respectively: 0.1, 12 and 24 ng/mL for 25E-NBOMe, AB-CHMINACA, AH-7921, AKB-48, BZE, EME, fentanyl, JWH-018, JWH-210 and LSD; 0.3, 12 and 24 ng/mL for JWH-081; 1.5, 60 and 120 ng/mL for 4-Cl-α-PPP, α-PVT, amphetamine, MDA and phenmetrazine; and 0.5, 60 and 120 ng/mL for the other substances. The matrix effect was considered significant when it exceeded 25 %.

The linearity of the standard curve (post-extraction fortified samples) was assessed at eight different concentration levels (n = 3 at each level): 0.05, 0.1, 0.3, 2, 6, 12, 18 and 24 ng/mL for 25E-NBOMe, AB-CHMINACA, AH-7921, AKB-48, BZE, EME, fentanyl, JWH-018, JWH-081, JWH-210 and LSD; and 0.25, 0.5, 1.5, 10, 30, 30, 60, 90 and 120 ng/mL for the other substances.

The mean of normalized areas (analyte area/IS area) at each point was used for constructing the standard curve, and Grubbs test was performed to detect outliers.

Homoscedasticity of the standard curve using the least square linear regression was evaluated for each analyte by the Cochran's test, and the curve was considered homoscedastic when standard deviations were not significantly different among the tested levels (Ferrari and Caldas, 2018). For heteroscedastic standard curves, weighting factors 1/x, $1/x^2$, $1/x^{0.5}$, 1/y, $1/y^2$ and $1/y^{0.5}$ were tested to determine the best adjusted linear regression. Linearity of the standard curve was assumed when the coefficient of determination (r^2) was at least 0.99.

Bias/accuracy (n=15), recovery (n=3), repeatability (n=3), and intermediate precision (five different days, same analyst, n=15) were assessed at the lowest, medium, and highest concentration levels, as along with matrix effects. Bias/accuracy was determined as percentage of the target concentration, and recovery was calculated by comparing the normalized mean area of pre-extraction fortified samples with the normalized mean area of post-extraction fortified samples, expressed in % (n=3). Repeatability and intermediate precision were evaluated using post-extraction fortified samples. Matrix effect (suppression or enhancement of the analytical signal) was assessed by comparing the sample normalized mean area obtained in control samples with standards added post-extraction by the normalized mean area in samples with standard solutions, and expressed in % (n=3). The acceptance criteria were bias within ±20 %, recovery within the range of 80-120 %, and repeatability and intermediate precision less than 20 % RSD. Matrix effect was considered significant when exceeds 25% (suppression or enhancement) (ANSI/ASB, 2019).

LOD of the method was defined as $\mu + 3.3s$, where "μ" is the average of the signal and "s" is the standard deviation of the 10 different control samples. LOQ of the method was defined as the lowest level in which the method was validated within the acceptance criteria for bias, repeatability, and intermediate precision.

Analyte carryover (n=3) was assessed by analyzing runs of a pool of five different fortified control samples, without addition of IS, after the analysis of the highest concentration of the analytical curve; the acceptance criteria was that the mean areas of the ion at the retention time should not exceed 10 % of the ion area at the lowest curve point.

When the analyte concentration exceeds the working range of the analytical curve, the sample needs to be diluted to fit the defined working range. The dilution integrity test (n=3) was performed by diluting a control fortified sample 1:50 and 1:100

and the impact of the dilution was considered negligible when the % of initial concentration was less than 20.

Stability of the extracted samples in the LC tray (15 ºC) was assessed at the medium concentration of the standard curve, and reanalyzed after 24 h. Change in the analyte concentration after the storage period should not exceed 20 % to be considered stable.

The effectiveness of the sample storage conditions was validated by comparing the analysis results with that using the gold standard device, Quantisal™. Control oral fluid samples (200 µL) with and without Quantisal buffer (600 µL) were fortified at final concentrations of 0.8 ng/mL (25E-NBOMe, AH-7921, BZE, EME, fentanyl, JWH-018, JWH-081, JWH-210, and LSD), and 4 ng/mL for the other drugs (AB-CHMINACA, 5F-AKB-48 and AKB-48 were not tested). Samples were stored at -20 ºC for 0, 48 and 72 h and, at each time, extracted and analyzed as previously described. The change in concentration after the storage period should not exceed 20 % compared to time 0. All the analyses were performed in quadruplicate.

4.3. Results and Discussion

3.1. Method validation

No interfering peaks were observed for the MRM transitions at the chromatographic retention times of the analytes in control matrices, indicating that the method is selective. Table 4.2 shows the results for bias, matrix effect, recovery, repeatability and intermediate precision for the 51 compounds analyzed during validation, and Figure 4.1 shows the MRM chromatogram of a fortified control sample.

The highest values ion suppression values were observed for MDMA (-22.5 %), tetrahydroharmine (-21.6 %) at the lowest concentration level, and 25E-NBOMe (-23.6 %), at the medium concentration, within the acceptable level (25%). Hence, an analytical curve in solvent was used for quantification. Homoscedasticity was shown for most analytes (least squares method) and for heteroscedastic curves, a weighting factor of 1/x was applied (Table 4.2), with satisfactory correlations ($r^2 \geq 0.99$). No extreme values were observed (Grubbs test).

Table 4.2 Bias, matrix effect, recovery, repeatability and intermediate precision of the 51 substances analyzed.

Substance	Bias (n=15)			Matrix effect (n=3) (%)			Recovery (n=3) (%)			Repeatability (n=3) RSD (%)			Intermediate Precision (n=15) RSD (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
2,5-DMA	-9.7	3.2	8.4	-14.0	10.7	12.9	90.1	91.1	79.1	0.5	0.3	3.9	7.0	8.9	11.6
25E-NBOMe	9.0	-2.0	1.9	2.1	-23.6	-12.1	96.9	96.1	98.6	3.2	3.0	0.6	5.1	2.5	7.4
2C-B	-4.7	-8.7	-5.2	10.0	0.1	-9.9	97.0	100.5	99.0	0.6	1.1	1.5	6.0	3.2	9.5
2C-H[a]	-14.1	7.4	-4.3	13.1	16.6	-4.3	95.3	99.9	81.5	2.1	8.4	15.6	17.6	20.0	18.3
2C-I	-12.6	1.3	-2.7	8.5	-7.7	4.0	91.6	104.8	100.2	19.2	2.7	5.7	19.4	6.5	7.6
4-Cl-α-PPP	7.7	-4.6	1.5	-11.4	-14.4	-1.7	77.1	87.7	78.1	9.3	11.5	8.2	17.0	16.5	14.3
5F-AKB-48[a]	3.4	2.2	-3.9	2.8	-7.2	-1.8	97.6	86.6	89.3	8.9	2.5	0.2	11.9	8.8	8.5
5-MAPB[a]	-14.4	7.4	-1.8	17.2	-19.6	-4.1	81.2	66.3	83.0	4.7	2.3	8.0	6.9	7.0	19.9
5-Meo-MIPT[a]	-15.6	-8.0	5.1	-10.7	-1.5	9.9	81.8	93.7	95.1	3.3	10.4	9.0	9.3	11.6	14.6
6-MAM[a]	3.8	-2.1	6.2	17.3	-8.4	-8.1	103.8	98.5	97.5	1.9	1.2	2.2	2.6	2.5	5.6
7-AF	8.0	-6.5	-0.9	-14.0	-10.7	-12.9	96.5	87.3	93.7	1.5	1.4	3.0	7.0	8.8	11.6
AB-CHMINACA	-9.5	3.3	1.6	-16.4	-13.3	-14.4	84.2	96.0	96.4	10.0	1.9	2.1	16.5	7.5	7.5
AH-7921[a]	13.0	-7.3	-0.1	2.5	-15.5	-10.3	88.6	95.7	80.0	8.6	3.4	4.1	18.1	12.3	15.9
AKB-48	-7.8	-8.7	-3.6	0.1	-8.3	-18.1	88.3	74.4	91.9	3.3	1.4	2.4	8.6	6.0	6.5
Alfa-PVT[a]	0.6	-1.7	2.0	5.8	-11.1	-8.7	95.6	89.3	77.5	3.8	3.6	4.8	5.6	6.2	8.3
AM-2201	2.2	-9.8	1.1	-8.6	-3.4	2.6	88.5	100.6	98.1	1.6	0.8	0.8	4.3	1.4	2.7
Amfepramone	-4.2	-6.0	2.0	19.9	-3.7	-2.8	89.2	95.5	95.7	3.1	4.0	1.9	4.6	7.6	3.4
Amphetamine	-9.8	0.2	7.9	18.6	-13.8	-9.1	95.5	82.6	79.3	7.6	2.1	2.0	20.0	5.1	5.7
Benzoylecgonine[a]	-4.0	-6.5	10.8	-17.4	9.2	-19.8	78.7	92.8	83.9	2.1	0.6	2.9	10.9	3.9	19.3
Benzylpiperazine	4.0	2.9	-1.5	-14.2	-16.1	-17.0	72.4	71.0	90.8	5.4	3.1	4.3	7.3	7.2	11.9
Clobenzorex[a]	11.7	-8.7	9.8	18.7	6.6	-18.3	77.1	58.5	81.4	7.1	2.3	4.5	15.6	7.1	11.8
Cocaine	12.4	-7.0	8.9	19.1	-5.8	-10.5	80.2	80.3	92.5	8.9	5.1	5.2	15.7	11.8	10.9

Substance	Bias (n=15)			Matrix effect (n=3) (%)			Recovery (n=3) (%)			Repeatability (n=3) RSD (%)			Intermediate Precision (n=15) RSD (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
Dibutylone	-3.3	5.7	4.6	14.7	-14.1	-11.4	99.6	98.8	97.2	4.1	2.5	2.1	8.9	6.6	5.7
DMT[a]	4.7	-8.8	2.0	3.7	9.1	-1.5	102.8	96.3	85.3	9.8	1.8	0.9	18.9	4.2	3.0
EME[a]	13.6	-8.3	6.1	-6.7	16.3	-10.6	82.9	94.5	98.1	11.8	3.7	3.4	14.0	7.2	12.5
Ethylone[a]	-5.7	0.3	5.9	-17.7	16.5	-0.7	82.5	90,0	81.1	3.9	7.2	5.6	15.4	12.6	11.7
Eutylone[a]	-7.3	7.5	8.6	-15.3	-5.9	-17.0	99.1	87.5	87.6	12.6	4.6	2.4	12.9	7.0	7.6
Fentanyl	-11.4	3.9	-4.5	11.4	-11.1	-0.5	95.8	89,0	94.2	11.7	2.2	8.2	17.7	10.2	19.0
Flunitrazepam	9.1	6.1	6.8	17.6	-6.9	6.9	88.2	84.1	75.5	8.4	7.1	7.1	11.3	12.7	20.0
Harmaline[a]	-11.4	-5.5	-1.6	-13.3	-7.0	-3.7	89.1	88.1	89.3	2.1	1.3	1.9	4.5	4.8	4.6
Harmine[a]	-15.1	-3.5	4.7	-9.9	7.4	-11.8	96.9	83.1	101.1	3.1	3.4	4.1	8.8	6.4	8.3
JWH-018	-5.1	-6.0	3.4	-12.2	1.4	-0.5	85.2	99.0	101.2	1.9	1.2	0.9	5.1	2.1	2.9
JWH-081	10.9	-0.8	6.7	-15.9	-1.6	-16.3	95.3	78.7	96.4	2.8	11.2	8.0	13.1	16.6	19.3
JWH-210	-13.1	-4.3	7.1	-19.7	18.5	-14.2	75.2	91.6	82.5	4.4	0.8	2.9	7.1	1.5	6.9
Ketamine	4.6	-4.8	-4.6	-5.5	13.9	-14.1	71.1	83.4	92.3	8.4	5.5	6.9	12.1	9.3	13.8
Levamisole[a]	-7.7	-8.9	-9.1	-16.5	-1.1	1.3	89.2	74.1	75.0	3.0	1.9	4.4	9.0	5.1	10.1
LSD	-7.5	-6.1	9.6	-0.5	6.7	-11.2	97.5	96,0	88.4	1.3	2.4	2.1	4.15	4.3	6.9
m-CPP[a]	-0.15	6.3	4.7	-17.3	14.6	3.2	87.2	85.2	89.0	3.6	2.1	3.7	7.8	6.3	10.8
MDA	3.8	-9.3	-3.8	3.2	13.6	-16.0	51.9	78.0	62.7	7.4	7.6	6.3	15.9	15.1	16.1
MDEA	-3.4	-1.6	-3.2	15.5	-4.6	-10.3	95.2	96.1	74.0	4.7	2.7	3.1	9.3	8.6	6.6
MDMA[a]	-3.4	-2.0	-1.9	-22.5	17.3	9.1	84.3	95.4	88.9	11.6	3.8	5.2	19.8	15.1	15.3
MDPV	-5.0	2.8	14.4	-18.9	14.2	-0.1	102.4	95.3	88.2	5.8	17.2	6.8	9.0	18.3	14.5
Mephedrone	15.5	-2.5	1.0	-7.8	15.1	-3.9	84.3	94.9	72.0	2.3	6.9	8.6	18.0	15.0	18.0
Metamphetamine	-14.4	-1.9	0.1	13.9	-3.4	-4.1	100.1	98.6	98.7	5.6	1.5	0.9	7.7	3.2	3.1
Methylphenidate	-14.0	1.8	-0.8	-17.1	-3.3	-1.8	87.2	93.8	89.4	1.6	0.5	2.2	4.3	1.6	3.4

Substance	Bias (n=15)			Matrix effect (n=3) (%)			Recovery (n=3) (%)			Repeatability (n=3) RSD (%)			Intermediate Precision (n=15) RSD (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
Methylone	-3.3	-4.7	8.1	11.5	18.6	1.4	101.3	112.7	93.0	6.5	3.7	6.2	9.3	9.9	12.0
N-Ethylpentylone	-17.2	-2.1	-5.7	-15.8	1.8	-0.2	92.8	98.4	97.3	3.3	1.6	1.4	3.9	2.5	3.3
Norketamine	-11.2	-4.6	-9.0	4.6	2.6	-6.5	67.7	85.7	96.7	5.5	3.1	4.3	12.1	5.8	8.7
Phenmetrazine	-5.3	8.0	9.0	-18.0	-7.0	-14.0	81.0	73.9	66.6	8.1	2.9	7.5	16.3	6.6	14.7
Tetrahydroharmine[a]	-9.0	-0.7	-6.8	-21.6	17.9	-14.2	91.6	90.2	73.9	5.5	7.2	12.1	12.7	13.0	18.5
TH-PVP	-1.3	-5.6	6.0	-12.1	12.0	-0.2	92.2	90.4	90.7	4.6	11.1	11.1	18.9	14.6	19.5

[a] = homoscedastic; the factor substances were heteroscedastic (weighting factor = 1/x). 7-AF: 7-aminoflunitrazepam; α-PVT: α-pyrrolidinopentiothiophenone; DMT: dimethyltryptamine; EME: ecgonine methyl ester; MDA: methylenedioxyamphetamine; MDMA: 3,4-methylenedioxymetamphetamine; MDEA: methylenedioxy-N-ethylamphetamine; MDPV: methylenedioxypyrovalerone; TH-PVP: tetramethylene-α-pyrrolidinovalerophenone. Low, medium and high concentration levels, respectively: 0.1, 12 and 24 ng/mL for 25E-NBOMe, AB-CHMINACA, AH-7921, AKB-48, BZE, EME, fentanyl, JWH-018, JWH-210 and LSD; 0.3, 12 and 24 ng/mL for JWH-081; 1.5, 60 and 120 ng/mL for 4-Cl-α-PPP, α-PVT, amphetamine, MDA and phenmetrazine; and 0.5, 60 and 120 ng/mL for the other substances.

Valen et al. (2017) similarly found no relevant matrix effect for 21 psychoactive substances using LLE and two commercial devices, Intercept™ and Quantisal™ (80-139% and 86-118%, respectively). In contrast, Cunha et al.[30] reported high matrix effect values using LLE/Quantisal™ for synthetic cannabinoids (PB-22: -55.5 %, JWH-015: -40.0 %, JWH-175: -43.1 %, and JWH-122: 40.2 %).

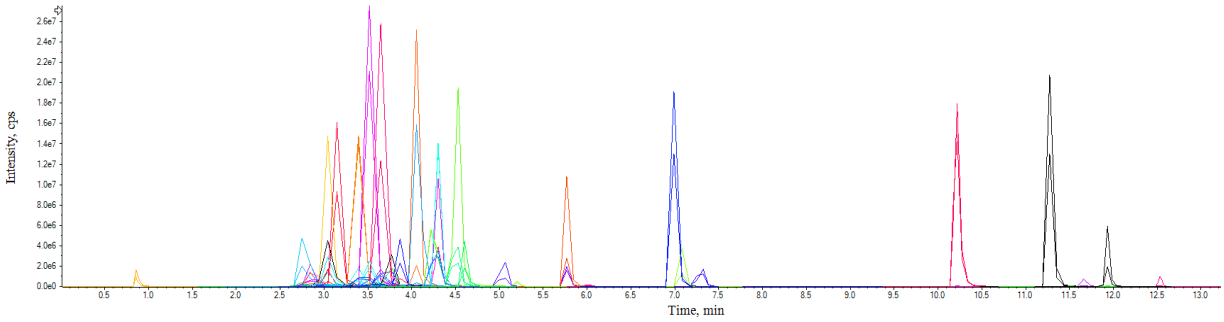


Figure 4.1. Multiple Reaction Monitoring chromatogram of fortified oral fluid containing the 51 compounds validated in the method.

The limit of detection (LOD) and limit of quantification (LOQ) for the 51 analytes and the 9 IS used in the method are presented in Table 4.1, ranging from 0.04 to 0.5 ng/mL and 0.1 to 1.5 ng/mL, respectively. Bias was within ± 20 % and recoveries were in the range of 80-120 % for most substances. Five analytes showed recovery < 80 % at two tested levels from 58.5 (clobenzorex, medium level) to 78.1 % (4-Cl-α-PPP, higher level), and MDA at all three levels (51.9 to 78.0 %). Cunha et al. (2020) validated a screening method for 104 drugs of abuse, and also found recoveries < 80 % for some compounds using LLE/Quantisal™, including JWH-081 (66.3 %), JWH-210 (64 %), amphetamine (65.3 %), MDA (67.7 %), BZP (44.2 %) and THC (63.4 %). Langel et al. (2008) evaluated the drug recovery using nine different oral fluid collection devices, including a plastic tube. The lowest recoveries were for amphetamine (51.8 %), MDMA (26.5 %), THC (< 12.5 %) and cocaine (33.3 %), using the Salivette® device collection.

Repeatability and intermediate precision were within 20 % (Table 2). Carryover results were satisfactory (data not shown), dilution tests showed RSD < 20 % for all the compounds. The results of the stability study (LC tray) showed that all analytes were stable (within ±20 % variation) after 24h (Table S2).

Cunha et al. (2020) evaluated the long-term stability of 104 drugs of abuse using Quantisal buffer for 15, 60 and 90 days, at 25ºC, 4º C and -20º C, and some

drugs/metabolites decreased the concentration after 15 days even at -20º C, such as acetyl norfentanyl (-20.4 %), HU-211 (-21.6 %), JWH-175 (-26.3 %), and JWH-176 (-33.8 %). The authors concluded that authentic sample analyses should occur as soon as possible after collection, and if stored, preferably at –20°C or lower. The results of sample stability in this study, both with and without the use of the Quantisal buffer, are shown in Table S3. All the substances, whether with or without the buffer, displayed no substantial loss (< 15 %) of concentration after up to 72 hours of storage.

In commercial collection devices, the addition of buffer and oral fluid stimulation dilutes the sample, contrary to the sampling protocol used in the present study, which increases the potential of detection. As the purpose of the study was indeed to analyze the sample within 72 hours for a rapid delivery of the results to the user, the collection protocol is well-suited for its intended purpose, with the unnecessary cost of the commercial device. To the best of our knowledge, this is the first report of a modified QuEChERS protocol followed by LC-MS/MS analysis for the determination of multiple psychoactive substances in oral fluid.

3.2. Real cases

The validated method was applied for the analysis of 62 oral fluid samples collected from volunteers who attended two EDMs. All the samples were stored in amber glass vial at -20ºC until analysis. The results are presented in Table 4.3, and Figure 4.2 shows the extracted ion chromatograms from two real cases. Due to LC-MS/MS technical problems, samples 36-62 were analyzed 60 days after collection, and the results may be underestimated as the sample stability over 72 hours was not accessed, although the extraction/purification step was conducted within the studied period.

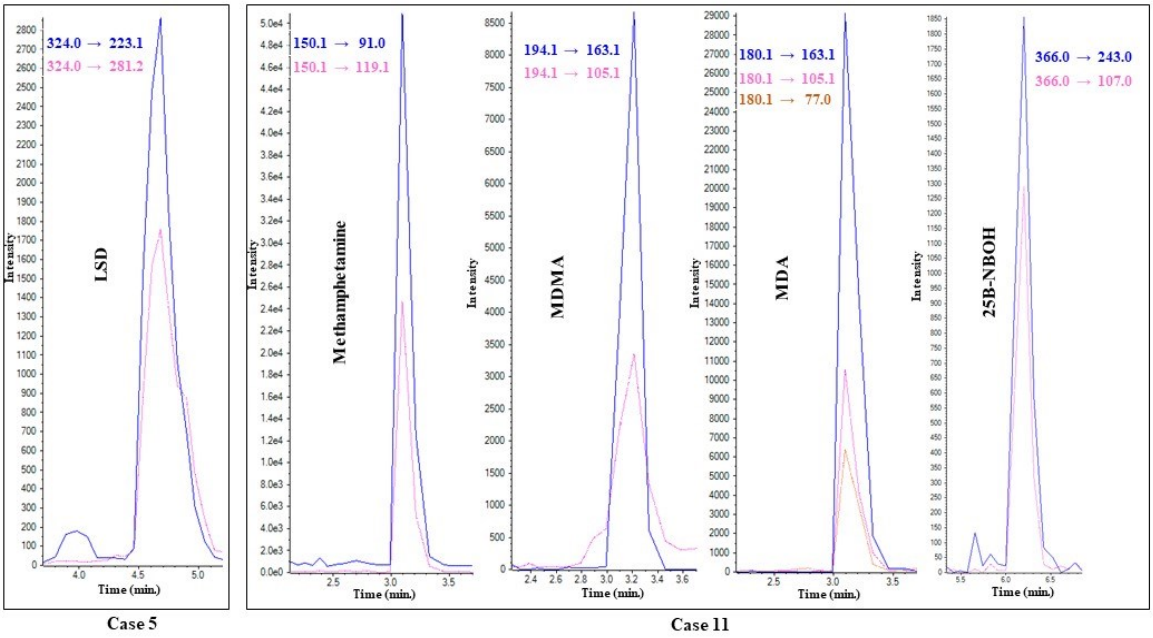


Figure 4.2. Multiple Reaction Monitoring chromatograms of real cases containing LSD (case 5), and MDMA, MDA, methamphetamine and 25B-NBOH (case 11). Case 5: LSD (0.6 ng/mL); case 11: MDMA (26.1 ng/mL), MDA (128.9 ng/mL), methamphetamine (11.0 ng/mL) and 25B-NBOH (detected).

Table 4.3 also indicates the information provided by the user about the form of the drug and the substance believed to be consumed, as well the time between consumption and sample collection. In nine cases, donors either preferred not to disclose which substance was present in the drug or were unable to provide specific information, resulting in incomplete or generic answers. This is evident in the use of terms like "< 24h" (less than 24 hours) and ">24h" (more than 24 hours or days) to describe the time elapsed between drug use and the moment of oral fluid donation.

In 52.5 % of the samples (n=32), at least on amphetamine derivative was detected. Among the participants, 36 individuals reported having consumed "ecstasy" or "MD" tablets, street names commonly used to refer to preparations believed to contain MDMA. In 9 cases, MDMA was detected along with MDA and/or methamphetamine, and all participants who had MDMA/methamphetamine detected had reported taking more than one ecstasy pill. MDA can either be a metabolite of MDMA or a psychoactive substance itself.[31] In 10 cases, only MDA was detected, and in two cases, eutylone, a synthetic cathinone, was detected. It is important to point out that no seizures of eutylone have been reported by the Federal District Civil Police in 2023.

Together with opioids and synthetic cannabinoids, synthetic cathinones are one of the most reported NPS classes in fatal cases (Ferrari Jr. and Caldas, 2021; Adamowicz et al., 2016; Krotulski et al., 2020), including cases with EDM party attendees in (Costa et al., 2018; Ferrari Jr. and Caldas, 2021).

Table 4.3. Results of 62 oral fluid samples and the reports from the volunteers: dosage form and psychoactive substance used, and time elapsed between the consumption and collection.

Sample	Dosage form	Substance reported	Time	Results (ng/mL)
1	Tablet	ni	>24h.	nd
2	Tablet	MD	<24h.	MDMA (829.0), MDA (67.6), MA (439.0), AMP (0.7)
3	White powder	Cocaine	<24h	Cocaine (407.3), BZE (162.9), EME (83.9)
4	Crack	Cocaine	<24h	BZE (65.6), EME (51.4)
5	Blotter paper	LSD	<4h.	LSD (0.6)
6	Tablet	ni	<24h	MDMA (detected; < 0.5;), MDA (191.8)
7	Tablet	MD	>24h	nd
8	Tablet	Ecstasy	<24h	MDA (274.5)
9	White powder	Cocaine	<4h	Cocaine (369.0), BZE (214.1), EME (150.1)
10	White powder	Cocaine	<2h.	Cocaine (312.9), BZE (62.1), EME (36.9)
11	Tablet;blotter paper	Ecstasy, LSD	<24h	MDMA (26.1), MDA (128.9), MA (11.0), 25B-NBOH*
12	Tablet	MD	>24h.	nd
13	Tablet	MD	<24h	MDA (265.9)
14	Tablet	ni	<24h	nd
15	Blotter paper	Acid	Minutes	LSD (68.3)
16	Tablet	MD	>24h.	nd
17	Tablet	MD	>24h.	Eutylone (4.7)
18	Tablet	ni	>24h.	nd
19	Tablet	Ecstasy	<2h.	MDMA (478.0), MDA (309.7), MA (74.8)
20	Amphetamine; Rohypnol	ni	<24h	AMP (1.8), 7-AF (2.9)
21	Tablet	ni	>24h.	nd
22	Tablet;blotter paper	MD;LSD	<24h	MDMA (112.6), MDA (339.6), MA (39.2), 25B-NBOH*
23	Blotter paper	LSD	>24h	nd
24	Tablet	ni	>24h.	nd
25	White powder	Cocaine	>24h.	Cocaine (26.8), BZE (0.17), EME (7.4)
26	Tablet	ni	>24h.	nd
27	White powder	Ket	<24h	Ketamine (375.3), norketamine (15.3)
28	Capsule	Amphetamine	<24h	AMP (detected; < 1.5)

Sample	Dosage form	Substance reported	Time	Results (ng/mL)
29	Tablet	ni	>24h.	nd
30	White powder	Ket	<24h	Ketamine (290.6), norketamine (150.2)
31	Tablet	MD	>24h.	nd
32	Cigarette	DMT	<24h	DMT (9.0)
33	Tablet	MD	<24h	nd
34	Tablet	MD	<24h	MDMA (2.8), MDA (270.3)
35	Tablet	Ecstasy	<24h	MDMA (2.2), MDA (45.3)
LC-MS/MS determination 60 days after collection.				
36	Tablet	MD	1h	MDA (272.4), MA (155.0)
37	Tablet	MD	15h	MDA (279.7)
38	Blotter paper	Acid	15 min	nd
39	Tablet	MD	15h	nd
40	Tablet	MD	1h	MDA (102.6), MA (69.1)
41	Tablet	MD	ni	MDA (176.0)
42	Tablet	MD	5 min	MDA (298.7), MA (72.7)
43	Tablet	MD	3h	MDA (39.0)
44	Tablet	MD	3h	MDA (23.9)
45	Tablet	Ecstasy	1h	MDA (200.7)
46	Tablet	MD	1h	MDA (151.0)
47	Tablet	MD	1h	Eutylone (4.1), MDA (327.6)
48	Tablet	MD	1h	AMP (35.5)
49	White powder	Cocaine	1h	Cocaine (28.5), BZE (6.3), EME (1.8)
50	Tablet	MD	40 min	MDA (84.4), MDMA (1.8)
51	Tablet	MD	4h	AMP (124.4)
52	White powder	Cocaine	ni	Cocaine (13.0), BZE (11.4), EME (2.2)
53	Tablet	Ecstasy	1h	MDA (90.5), MDMA (2.0)
54	Tablet	MD	5 min	MDA (143.0)
55	Tablet	MD	1h	MDA (10.1)
56	Blotter paper	Acid	3 min	LSD (23.4)
57	Tablet	MD	>12h	nd
58	Tablet	MD	ni	MDA (45.2)
59	Tablet	Ecstasy	ni	MDA (344.6), MDMA (4.3)
60	Tablet	MD	<30 min.	MDMA (5.9)
61	Pill/Tablet	Trazodone, ecstasy	ni	m-CPP (1.5), MDA (460.6), MA (266.0)
62	White powder	Cocaine	<24h	Cocaine (15.8), BZE (24.7), EME (2.0)

ni: not informed; nd: not detected; *screening. 7-AF: 7-aminoflunitrazepam; AMP: amphetamine; BZE: benzoylecgonine; EME: ecgonine methyl ester; MA: methamphetamine; MDA: methylenedioxyamphetamine; MDMA: methylenedioxymethamphetamine.

In a study conducted in the United States involving 223 oral fluid samples where participants reported MDMA, ecstasy or Molly (another drug slang term) use, the analytical findings did not align with the self-reported use in approximately 45% of the samples (Krotulski et al., 2018). In this work, excluding users who did not inform the name of the substance used, approximately 37% of the analyzes showed at least one substance different from the one self-reported, or no substance was detected. These findings confirm the importance of reliable analytical methods in determining actual used substance, particularly in situations where self-reported information may not be entirely accurate.

Two participants reported consuming "ket" (white powder), and the analysis revealed the presence of ketamine (ranging from 290.6 to 375.3 ng/mL) and its metabolite norketamine (ranging from 15.3 to 150.2 ng/mL). The use of ketamine is becoming more prevalent among EDM party attendees, including in Brazil Palamar and Rayes, 2020; Cunha et al., 2021), suggesting the need for continued monitoring and intervention measures to address the use of this drug in the area.

In seven samples, volunteers alleged the use of LSD/acid. In two of them, 25B-NBOH was detected in the screening (Table 3), a phenethylamine sold as a LSD in the drug market, that is commonly detected in blotter papers seized in Brazil (Ferrari Jr. et al., 2021, which may also be a 25B-NBOMe metabolite Caspar et al., 2017); in both cases, the consumption of ecstasy tablets was also reported (cases 11 and 22, Table 3). In two cases, no substance was detected and LSD was found in three samples (0.6, 23.4 and 68.3 ng/mL). Cunha et al. (2022) analyzed 42 oral fluid samples collected in EDMs in the state of São Paulo, Brazil, of which 7 had LSD at concentrations higher than 10 ng/mL. According to the authors, the highest level maybe a contamination of the oral cavity, which was confirmed by the short time (“minutes”) reported by the user, between the consumption and the oral fluid collection. It is important to note that drug levels in oral fluid must be evaluated with caution, given the weak correlation with blood concentrations for many psychoactive substances (Langel et al., 2014). Additionally, it's worth mentioning that LSD is considered safe when taken at moderate dosages (50–200 μg), and no fatal cases have been reported Nichols and Grob, 2018).

Cocaine and/or its metabolites were identified in eight samples, at levels from 13.0 to 407.3 ng/mL for cocaine, 0.17 to 214.1 ng/mL for benzoylecgonine, and 1.8 to

150.1 ng/mL for ecgonine methyl ester. Only one participant reported using crack cocaine (smoked), while the others reported using cocaine hydrochloride (inhaled).

Combining questionnaires with drug tests can provide a more comprehensive understanding of drug use compared to using just one of these methods Gjerde et al., 2011, although some studies have found low validity between biological measurements and self-report (Miller et al., 2015; Palamar et al., 2017). It is essential to recognize that drug users may underreport their usage, either due to a lack of knowledge about the specific substances they ingested or intentional omissions (Palamar et al., 2020; Krotulski et al., 2018; Gjerde et al., 2019). For example, a study in New York City, USA, found that 51.1 % of participants tested positive for at least one drug in hair samples despite not reporting drug use in their self-reports (Palamar et al., 2017). Similarly, in Norway, a study involving 1309 music festival attendees found that 5.5% reported drug use in the past 48 hours, while 10.8% tested positive for at least one substance in oral fluid (Gjerde et al., 2019). In the present study, samples were only collected from users who claimed to have used some type of psychoactive substance.

In Brazil, few studies carry out toxicological analysis of EDM party attendees. In the study by Cunha et al. (2021) conducted from 2018 to 2020, MDMA (88.5%) and Δ9-THC (73.6%) were the primary substances detected among the 462 oral fluid samples analyzed. Although only 5% of the volunteers reported recent NPS consumption, at least one NPS was detected in 181 samples (39.2 % of the total), mainly ketamine (29.4%), methylone (6.1%), and N-ethylpentylone (4.1%).

Data on NPS consumption in Brazil is scarce, limited to reported cases of intoxication (Costa et al., 2018; Ferrari Jr. and Caldas, 2021) or drug seizure data (Machado et al., 2019; Meira et al., 2021; Souza Boff et al., 2020). Polydrug use is frequently reported in studies, which exposes drug users to a higher risk of overdose due to potential drug interactions (Ferrari Jr. et al., 2022). In this work, considering MDA as an MDMA metabolite, more than one substance was detected in 7 samples (11.3 %). In the study by Cunha et al. (2021), 79.9% of the samples contained more than one psychoactive substance. Ferrari Júnior et al. (2022) reviewed 96 papers involving fatal cases due to NPS consumption, and in over 86% of the reported cases (n=83), more than one psychoactive substance was detected. A survey at EDM parties and dance festivals in New York City (USA), showed an increase in the prevalence of past-year use polydrug use, from 12.7% in 2016 to 20.5% in 2019 (Palamar and Rayes, 2020). Out of the 1270 NPS toxicology cases reported to the UNODC between December 2021 and

May 2023, 89% exclusively involved the detection of a single NPS, and among the 133 cases subjected to postmortem analysis, polydrug detection accounted for 62% of them (UNODC, 2023b). In a study conducted in Australia from 2010 to 2015 showed that regular users of psychostimulants seek NPS with properties similar to the illicit drugs they are already consuming. Poly NPS consumers were considered a particularly high-risk group, more likely to be younger, male, had overdosed on any drug in the past year, and to have engaged in criminal activity in the past month (Sutherland et al., 2017).

The use of licit drugs for non-medical purposes coupled with illicit drug consumption also highlights the risks to which EDM party attendees are exposed. Licit drugs are easily obtainable on the Brazilian illicit market. For instance, flunitrazepam (case 22, Table 2), a benzodiazepine hypnotic, is notorious for its use as a "date rape drug" (Hasegawa et al., 2015). Additionally, trazodone (case 47), an antidepressant serotonin antagonist (Patel et al., 2008), has its primary active metabolite, m-CPP, also sold as a designer drug (Gaillard et al., 2013).

A limitation of this study is the need to consider the inclusion of other synthetic cannabinoids. Recent reports suggest an increase in the number of seizures of this class of drugs in Brazil (Araujo et al., 2023) as well as globally (UNODC, 2023a). High-resolution mass spectrometry techniques are valuable for untargeted screening analysis and for the structural characterization and identification of unknown compounds (Partridge et al., 2018), and would be useful to monitor the emergence of new substances in the market, not included in the present study. Another limitation of the results was be the fact that samples some were analyzed more than 72h after collection, interfering with the interpretation of the quantitative results, which may have been underestimated.

4.4 Conclusions

The validated method and its application in this study provide valuable contributions to toxicological analysis and our understanding of drug consumption patterns among EDM party attendees in the Federal District of Brazil. The use of a modified QuEChERS protocol coupled with LC–MS/MS allows for the detection of a wide range of substances, both prescription and illegal, enhancing the comprehensiveness of the study.

The strengths of the study, including the collection of unstimulated oral fluid, rapid response to volunteers, and lower analysis costs compared to commercial

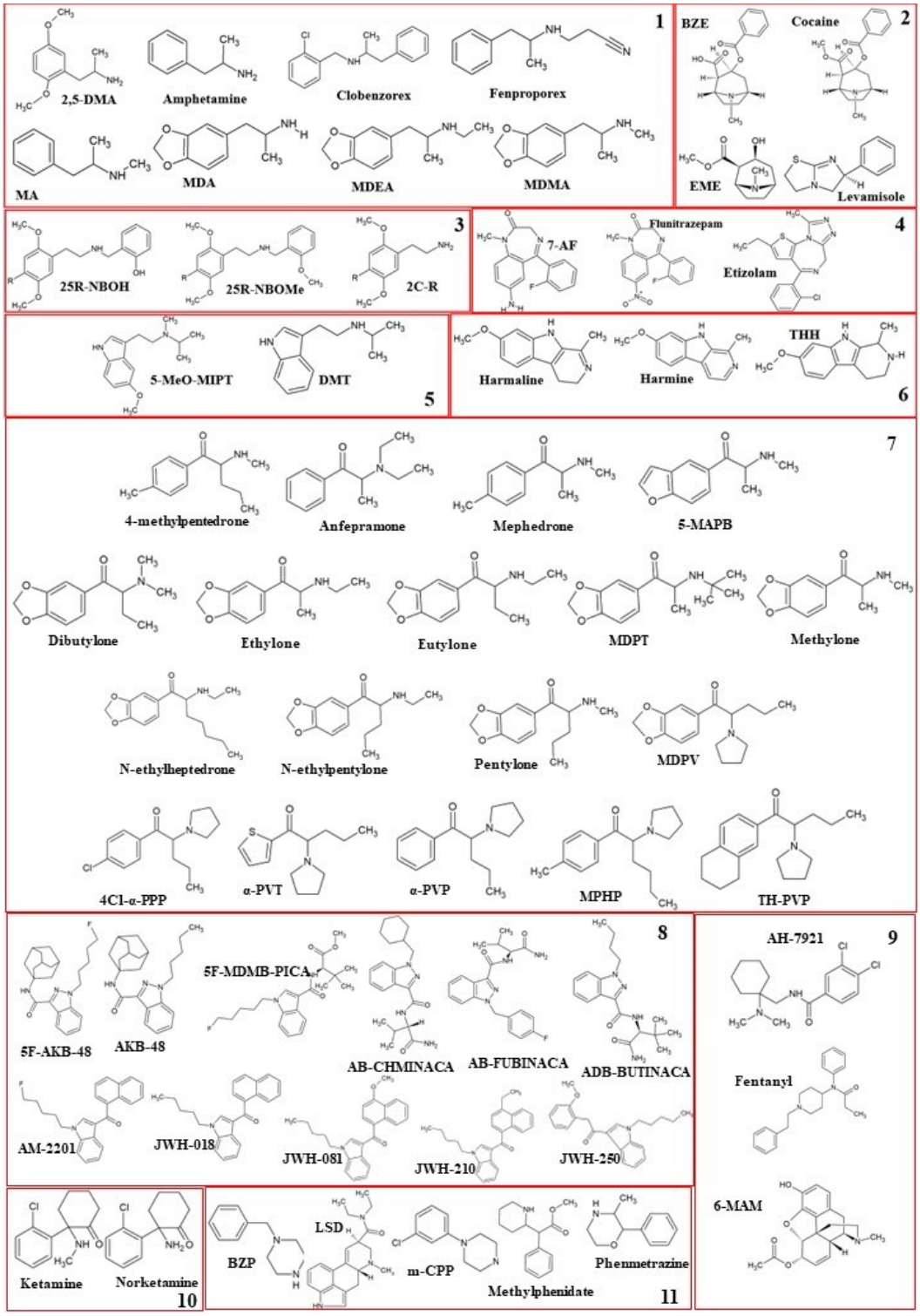
collection devices, highlight the practicality and efficiency of the proposed methodology.

The study's findings, revealing discrepancies between self-reported drug use and analytical results, emphasize the importance of reliable analytical methods in providing a more accurate picture of substance use within specific communities. The identification of substances not disclosed by participants underscores the limitations of relying solely on self-reported data and reinforces the need for objective analytical tools.

In summary, this work contributes to monitoring and addressing drug use in specific laws. The study's methodology and results may be valuable for future research, public health strategies, and regulatory efforts aimed at promoting the well-being and safety of individuals participating in electronic music events. Furthermore, the ability to compare the results with drug seizure data adds an additional layer of insight into the local drug landscape.

Supplementary material

Figure S1. Chemical structure of the 73 compounds monitored in the present method (22 substances, only screening).



1: Amphetamine derivatives; 2: Cocaine, its metabolites and the adulterant levamisole; 3: Phenethylamine derivatives 25R-NBOH (R=Br, Cl, ethyl or I), 25R-NBOMe and 2C-I (R=Br, Cl, ethyl, H or I); 4: Benzodiazepine derivatives; 5: Tryptamine derivatives; 6: Harmala alkaloids; 7: Synthetic cathinones; 8: Synthetic cannabinoids; 9: Opioids,

including the heroin metabolite 6-MAM; 10: Ketamine and its metabolite norketamine; 11: Other psychoactive substances.

Table S1. Molecular formula, MRM transitions, retention time, and respective declustering potential, collision energy and collision cell exit potential of the 22 screened compounds.

Substance	Molecular formula	Transitions (m/z)	RT (min.)	DP (V)	CE (V)	CXP (V)
25B-NBOH	$C_{17}H_{20}BrNO_3$	366.0 → 243.0	6.1	25	22	15
		366.0 → 107.0	6.1	29	30	15
25B-NBOMe	$C_{18}H_{22}BrNO_3$	381.1 → 121.1	6.5	36	25	14
		381.1 → 90.9	6.5	36	63	10
25C-NBOH	$C_{17}H_{20}ClNO_3$	323.0 → 200.0	5.9	31	29	22
		323.0 → 107.1	5.9	31	29	10
25C-NBOMe	$C_{18}H_{22}ClNO_3$	337.1 → 121.2	6.3	31	23	6
		337.1 → 90.9	6.3	31	61	8
25E-NBOH	$C_{19}H_{25}NO_3$	316.1 → 193.1	6.9	36	25	12
		316.1 → 178.1	6.9	36	37	20
25H-NBOMe	$C_{18}H_{23}NO_3$	302.1 → 121.1	5.5	61	23	12
		302.1 → 91.0	5.5	61	51	10
25I-NBOH	$C_{17}H_{20}INO_3$	414.0 → 290.9	6.4	41	31	16
		414.0 → 308.0	6.4	41	23	16
25I-NBOMe	$C_{18}H_{22}INO_3$	428.1 → 121.0	6.8	45	47	15
		428.1 → 91.0	6.8	26	26	15
2C-C	$C_{10}H_{14}ClNO_2$	216.0 → 199.0	4.3	56	17	34
		216.0 → 184.0	4.3	56	29	30
2C-E	$C_{12}H_{19}NO_2$	210.0 → 193.0	5.3	80	16	15
		210.0 → 178.0	5.3	80	24	15
4-methylpentedrone	$C_{13}H_{19}NO$	206.0 → 146.0	4.9	71	25	16
		206.0 → 144.0	4.9	71	45	14
5F-MDMB-PICA	$C_{21}H_{29}FN_2O_3$	377.0 → 232.0	9.8	71	21	14
		377.0 → 116.0	9.8	71	69	12
α-PVP	$C_{15}H_{21}NO$	232.0 → 91.0	4.1	80	30	10
		232.0 → 126.0	4.1	80	32	12
AB-FUBINACA	$C_{20}H_{21}FN_4O_2$	369.2 → 352.2	8.7	76	13	10
		369.2 → 324.3	8.7	76	21	15
ADB-BUTINACA	$C_{18}H_{26}N_4O_2$	331.1 → 201.1	9.0	55	32	10
		331.1 → 286.1	9.0	55	21	10
Etizolam	$C_{17}H_{15}ClN_4S$	345.0 → 316.0	7.8	61	31	40

Substance	Molecular formula	Transitions (m/z)	RT (min.)	DP (V)	CE (V)	CXP (V)
Fenproporex	$C_{12}H_{16}N_2$	345.0 → 261.0	7.8	61	35	32
		189.0 → 91.0	2.9	41	29	15
		189.0 → 119.0	2.9	41	15	15
JWH-250	$C_{22}H_{25}NO_2$	336.1 → 121.2	10.7	76	27	10
		336.1 → 90.9	10.7	76	59	12
MDPT	$C_{14}H_{19}NO_3$	250.0 → 194.0	3.7	61	17	12
		250.0 → 146.0	3.7	61	29	18
MPHP	$C_{17}H_{25}NO$	261.0 → 105.0	5.7	101	29	12
		261.0 → 189.0	5.7	101	23	10
N-ethylheptedrone	$C_{15}H_{23}NO$	234.0 → 146.0	5.8	45	30	15
		234.0 → 118.0	5.8	45	30	15
Pentylone	$C_{13}H_{17}NO_3$	236.0 → 188.0	3.9	80	37	15
		236.0 → 175.0	3.9	80	27	15

α-PVP: α-pyrrolidinopentiophenone; CE: collision energy; CXP: collision cell exit potential; DP: declustering potential; MDPT: methylenedioxy-N-tert-butylcathinone; MPHP: methyl-α-pyrrolidinohexanophenone; RT: retention time.

Table S2. Dilution integrity tests and sample stability of the extract samples of the 51 compounds.

Substance	Dilution integrity (n=3) (%)		Sample stability (n=3) (%)
	1:50	1:100	24 h
2,5-DMA	0.4	7.0	-1.8
25E-NBOMe	-3.6	-8.0	-1.5
2C-B	5.3	6.0	4.4
2C-H	-1.8	7.2	9.5
2C-I	-17.2	-4.4	9.6
4-Cl-α-PPP	-0.2	-6.4	-1.1
5F-AKB-48	5.3	9.4	-0.9
5-MAPB	-2.2	0.4	-13.9
5-Meo-MIPT	12.4	14.4	-15.0
6-MAM	-14.5	-13.6	-15.6
7-AF	-15.9	-10.8	-7.1
AB-CHMINACA	-2.3	-18.6	0.5
AH-7921	2.7	12.8	-17.4
AKB-48	12.5	-0.9	-2.5
Alfa-PVT	-9.7	-10.2	-6.2
AM-2201	-8.2	-7.0	-5.7

Substance	Dilution integrity (n=3) (%)		Sample stability (n=3) (%)
	1:50	1:100	24 h
Amfepramone	-7.0	-6.7	-2.9
Amphetamine	-3.8	-11.5	-16.4
Benzoylecgonine	-16.4	11.7	-5.7
Benzylpiperazine	-12.3	-19.5	-8.7
Clobenzorex	4.9	1.7	-9.4
Cocaine	-12.6	0.1	-9.9
Dibutylone	-6.7	-0.6	-5.7
DMT	8.1	10.0	-17.9
Ecgonine Methyl Ester	-1.3	17.4	-10.8
Ethylone	-7.5	-2.6	-3.6
Eutylone	4.7	1.0	-8.5
Fentanyl	4.5	8.2	-7.3
Flunitrazepam	-3.8	-16.9	-11.0
Harmaline	-0.6	6.5	-5.4
Harmine	6.4	-0.2	-17.5
JWH-018	-13.2	-9.2	-3.7
JWH-081	13.0	4.5	-9.9
JWH-210	-9.5	-13.5	-10.9
Ketamine	-0.8	-6.0	-2.7
Levamisole	-4.2	2.8	-10.8
LSD	-1.5	13.4	-10.3
m-CPP	-7.8	3.1	-19.1
MDA	-16.4	-3.0	-11.3
MDEA	-8.8	-6.8	-15.4
MDMA	-0.7	-1.8	-12.7
MDPV	7.5	3.3	-14.4
Mephedrone	1.3	4.1	-2.1
Metamphetamine	-0.4	-3.5	-8.8
Methylphenidate	-6.9	-8.0	9.9
Methylone	-5.8	-3.2	-14.1
N-Ethylpentylone	5.1	-11.0	11.5
Norketamine	4.5	-3.4	-17.0
Phenmetrazine	-2.7	12.7	-15.5
Tetrahydroharmine	-0.3	1.7	-14.9
TH-PVP	-7.4	-2.8	-4.0

Sample stability was evaluated in the LC tray (15 ºC).

Table S3. Concentration (in %) of the fortified oral fluid samples stored at -20º C and analyzed 24 h and 72 h (n=5).

Substance	Neat oral fluid		Quantisal	
	48h	72h	48h	72h
2,5-DMA	100.0 (14.7)[a]	97.4 (7.7)	97.6 (9.0)	104.8 (8.3)
25E-NBOMe	103.8 (2.4)	109.9 (4.8)	107.6 (6.3)	106.9 (10.1)
2C-B	100.0 (5.4)	97.9 (6.6)	100.0 (8.5)	95.7 (2.9
2C-H	97.7 (9.5)	95.7 (6.3)	95.7 (10.9)	93.6 (10.0)
2C-I	97.7 (4.4)	97.7 (10.4)	95.3 (9.2)	100.0 (7.7)
4-Cl-α-PPP	93.3 (14.8)	102.2 (8.0)	109.5 (6.3)	107.6 (9.2)
5-MAPB	107.7 (4.8)	100.0 (5.6)	94.9 (5.0)	105.1 (6.7)
5-Meo-MIPT	92.9 (2.8)	100.0 (12.0)	102.3 (3.1)	101.8 (13.3)
6-MAM	102.8 (5.6)	105.6 (16.1)	102.7 (7.8)	102.7 (5.6)
7-AF	104.8 (1.7)	107.1 (7.2)	111.1 (8.6)	100.0 (3.8)
AH-7921	87.5 (4.6)	100.0 (6.3)	112.5 (6.2)	100.0 (2.3)
Alfa-PVT	97.6 (7.5)	102.4 (10.9)	102.4 (4.9)	102.4 (4.3)
AM-2201	95.0 (0.7)	92.5 (3.5)	94.9 (4.2)	97.4 (4.7)
Amfepramone	97.3 (5.0)	97.3 (6.2)	111.1 (11.2)	97.2 (7.2)
Amphetamine	102.6 (6.4)	94.7 (15.6)	94.9 (5.6)	92.3 (7.9)
Benzoylecgonine	112.5 (2.9)	112.5 (4.5)	100.0 (9.4)	98.1 (12.9)
Benzylpiperazine	94.7 (7.4)	110.5 (2.8)	108.1 (9.7)	108.1 (4.2)
Clobenzorex	94.4 (5.1)	94.4 (13.0)	94.6 (7.4)	97.3 (4.3)
Cocaine	97.4 (8.1)	97.4 (7.8)	97.6 (4.5)	98.2 (3.3)

Substance	Neat oral fluid		Quantisal	
	48h	72h	48h	72h
Dibutylone	97.1 (7.5)	108.6 (13.7)	105.9 (6.0)	100.0 (3.2)
DMT	97.3 (3.3)	91.9 (9.0)	92.1 (7.0)	94.7 (6.0)
Ecgonine Methyl Ester	100.0 (6.8)	87.5 (7.1)	99.5 (6.5)	102.7 (7.3)
Ethylone	108.1 (1.4)	102.7 (10.5)	111.1 (6.5)	111.1 (2.5)
Eutylone	99.9 (9.7)	105.0 (10.9)	102.5 (3.3)	97.4 (14.3)
Fentanyl	100.0 (2.7)	99.8 (6.0)	99.7 (9.8)	100.0 (10.9)
Flunitrazepam	102.4 (0.5)	97.6 (5.5)	105.0 (4.3)	105.0 (6.5)
Harmaline	100.0 (8.0)	97.3 (8.8)	97.3 (3.8)	94.6 (1.7)
Harmine	100.0 (7.5)	94.6 (3.7)	97.2 (3.8)	102.8 (1.7)
JWH-018	100.0 (9.9)	88.9 (1.1)	102.2 (3.0)	100.0 (1.9)
JWH-081	100.3 (2.2)	87.5 (11.3)	87.5 (9.9)	100.0 (2.1)
JWH-210	100.0 (0.9)	87.5 (2.1)	93.6 (2.1)	100.0 (4.3)
Ketamine	106.7 (5.5)	104.4 (8.0)	107.1 (14.1)	109.5 (3.6)
Levamisole	97.5 (7.0)	97.5 (9.1)	90.5 (2.5)	97.6 (4.0)
LSD	100.0 (2.0)	97.4 (2.7)	100.0 (1.9)	105.0 (5.1)
m-CPP	92.5 (5.8)	90.0 (6.7)	94.7 (7.6)	94.7 (3.7)
MDA	107.0 (14.6)	104.7 (12.4)	107.7 (3.2)	105.1 (8.9)
MDEA	105.3 (2.1)	100.0 (8.6)	100.0 (5.8)	94.9 (13.2)
MDMA	100.0 (2.1)	93.0 (8.6)	100.0 (5.8)	102.4 (13.2)
MDPV	102.8 (5.6)	106.2 (6.7)	97.6 (2.8)	107.3 (3.9)

Substance	Neat oral fluid		Quantisal	
	48h	72h	48h	72h
Mephedrone	100.0 (14.0)	95.0 (3.4)	97.6 (10.2)	100.1 (14.4)
Metamphetamine	94.9 (1.3)	97.4 (2.2)	102.6 (6.2)	107.9 (11.9)
Methylphenidate	97.1 (2.2)	100.0 (4.0)	97.6 (5.1)	100.7 (6.3)
Methylone	97.3 (1.5)	94.6 (5.5)	102.5 (8.7)	95.0 (8.4)
N-Ethylpentylone	97.1 (3.6)	102.9 (10.2)	94.4 (7.2)	102.6 (3.4)
Norketamine	102.6 (5.9)	97.4 (16.5)	97.4 (7.4)	94.9 (11.2)
Phenmetrazine	95.1 (3.4)	102.4 (8.1)	107.7 (6.8)	105.1 (5.1)
Tetrahydroharmine	100.0 (6.8)	96.1 (10.0)	104.9 (10.4)	102.4 (6.6)
TH-PVP	94.9 (3.0)	102.6 (0.8)	105.6 (5.1)	102.8 (2.7)

a = standard deviation (in %). Final concentration: 0.8 ng/mL, for 25E-NBOMe, AH-7921, BZE, EME, fentanyl, JWH-018, JWH-081, JWH-210, and LSD; and 4 ng/mL for the other drugs. AB-CHMINACA, 5F-AKB-48 and AKB-48 were not tested. Concentration on day 0 is set to 100%.

CONCLUSÕES FINAIS

A otimização e validação qualitativa de um método para a detecção de substâncias da família 25R-NBOH (R=Br, Cl, I ou etil) utilizando GC-MS e coluna analítica com 4 metros de comprimento permitiu a detecção do 25R-NBOH intacto, devido a diminuição da degradação do composto por causa do menor tempo de residência das moléculas no sistema cromatográfico. Este foi o primeiro trabalho a detectar substâncias da família 25R-NBOH intactas por meio de GC-MS, sem a necessidade de uma etapa de derivatização, além da proposta do padrão do respectivo fragmentação, por meio da ionização por impacto de elétrons. O presente método foi aplicado em análise de amostras de selos apreendidos e é atualmente empregado na rotina analítica do Laboratório do IC/PCDF. O método se mostrou versátil, proporcionando corridas cromatográficas mais curtas, para compostos de baixa volatilidade, como JWH-210 E JWH-081 (canabinoides sintéticos), tadalafil e sildenafil (inibidores de PDE-5, dentre outros compostos de interesse forense. Substâncias que apresentam alta reatividade com colunas analíticas, como o estanozolol (esteroide anabolizante) apresentam picos com melhor formato e resolução.

O artigo de revisão se propôs entender sobre a abordagem que os laboratórios vêm adotando para a análise de NSP em casos de intoxicação fatal. Após a etapa de seleção dos artigos, foram avaliados 96, publicados entre 2016 e 2021. Em 12 estudos, foi detectada apenas uma substância e mortes por abuso de drogas foram comumente associadas ao uso de polidrogas. A técnica de LC-MS/MS foi uma das técnicas analíticas empregadas em 75 dos 96 artigos avaliados e emprego de técnicas de alta resolução (ex: LC-QTOF-MS, LC-HRMS – Orbitrap™) foi descrito em 36, uma alternativa interessante para elucidação estrutural de novas substâncias psicoativas e metabólitos. Apesar de normalmente apresentar limites de detecção maiores que os apresentados por cromtaógrafos líquidos modernos, o GC-MS foi utililizado em 29 trabalhos, seja em triagem ou como única técnica de escolha para elucidação da intoxicação fatal. Dentre as principais classes de substâncias envolvidas nos casos fatais, os opioides e as catinonas sintéticas foram as classes de substâncias mais encontradas, sendo notificados em 43 e 37 dos estudos, respectivamente. Canabinoides sintéticos foram a terceira classe com maior número de notificações (n=20).

Os métodos analíticos desenvolvidos e validados neste estudo envolvem extração e clean-up usando protocolos QuEChERS para determinação de 79 substâncias

psicoativas em sangue e urina, por UHPLC-MS/MS, e de 51 substâncias de interesse forense, além da triagem de outras 22 substâncias psicoativas, em fluido oral, por LC-MS/MS. Até onde sabemos, este é o primeiro estudo a realizar uma validação utilizando um método QuEChERS para a análise de multi-analitos em fluido oral. O método validado para sangue e urina foi aplicado em 68 casos da rotina do IML/DF, para análise de 54 amostras de sangue (38 post-mortem e 16 ante-mortem) e 16 amostras de urina ante-mortem. O método demonstrou ser de fácil implementação e utilização por um analista treinado. Todas as amostras de urina analisadas e 59,3% das amostras de sangue foram positivas para pelo menos um analito, incluido a detecção das catinonas sintéticas etilona (222 ng/mL, sangue ante-mortem), eutilona (246 e 446 ng/mL, urina) e N-etilpentilona (597 e 7,3 ng/mL, sangue post-mortem e ante-mortem, respectivamente). Este foi o primeiro método implementado na rotina analítica postmortem e antemorem do IML/DF para pesquisa de novas subtâncias psicoativas.

A rotina de trabalho proposta para análise de fluido oral foi avaliada por meio da análise de 62 amostras doadas por frequentadores de festa de música eletrônica no DF. Uma comparação entre o uso de drogas auto-relatado e os resultados toxicológicos no fluido oral indicou que o uso de substâncias ilícitas é frequentemente subnotificado entre os participantes da EDM, que podem estar em risco, uma vez que muitas vezes desconhecem as substâncias que consomem. O monitoramento de NSP entre usuários so mostrou uma fonte de informação toxicológica e ferramenta de conscientização de usuários sobre o risco de se utilizar estas substâncias.

Os resultados gerados nesse estudo foram importantes para uma melhor elucidação de casos reais envolvendo novas substâncias psicoativas, que não tinham sido previamente classificados como intoxicação fatal pelo médico legista. Como exemplo, a determinação de eutilona (597 ng/mL) em sangue post-mortem em um caso fatal, onde a catinona sintética foi a única substância detectada. Este foi o primeiro caso fatal reportado no Distrito Federal pelo uso de uma NSP; as altas concentrações de eutilona encontradas no sangue são compatíveis com casos fatais já reportados na literatura.

Por fim, todas os métodos otimizados e validados nesta tese de doutorado vêm gerando resultados mais confiáveis para a rotina analítica do DF, contribuindo para o melhor entendimento do impacto do consumo de NSP na nossa comunidade.

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ANEXO I

Journal of Chromatography A 1634 (2020) 461657

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Analysis of non-derivatized 2-(4-R-2,5-dimethoxyphenyl)-N-[(2-hydroxyphenyl)methyl]ethanamine using short column gas chromatography – mass spectrometry

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ARTICLE INFO

Article history:
Received 15 June 2020
Revised 22 October 2020
Accepted 26 October 2020
Available online 29 October 2020

Keywords:
GC-MS
Short column
25R-NBOH
Thermal degradation

ABSTRACT

The 25R-NBOH family is a group of thermally labile compounds that are relevant for forensic sciences and traditionally analyzed by GC-MS after derivatization — a step that is time consuming in a routine work. In this paper, the use of short analytical columns (4 and 10 m) showed to decrease compound degradation in the GC oven during chromatographic separation and to allow the analysis of non-derivatized 25R-NBOH compounds by GC-MS. A shorter column demanded a higher gas flow rate, and both factors decreased residence time of the analytes in the column and their degradation. The inlet temperature (250° C or 280°C) did not impact the response of 25R-NBOH. A 25R-NBOH fragmentation pathway by electron ionization was also presented for the first time. The GC-MS method with a 4 m column was successfully applied to other compounds of forensic interest, and it can be tested in the analysis of biological samples in toxicological investigations.

1. Introduction

The family of 2-(4-R-2,5-dimethoxyphenyl)-N-[(2-hydroxyphenyl)methyl] ethanamine (25R-NBOH, with R being a halogen or an ethyl group) is emerging as LSD alternatives in the illicit drug market [1,2], associated with blotter paper seized in Brazil [3,4] and other countries [5]. They are potent serotonin receptor agonists [6], responsible for subjective and behavioral effects [7]. 25R-NBOH compounds are also metabolites of 25R-NBOMe [8,9,10], which have been reported to be involved in human intoxications [2,11].

Until now, the thermally labile compounds of the 25R-NBOH family were considered unsuitable for GC-MS analysis unless they went through a derivatization step [12,13,14]. Other authors confirmed the presence of 25R-NBOH in blotter papers through the detection of 2C-R, which is the degradation product on GC-MS [15,16]. LC-QTOF-MS [3] and LC-MS/MS [1,17] have also been used to analyze 25R-NBOH in blotter papers. However, these equipment are not available in most forensic laboratories, which rely on GC-MS analysis for routine work. Hence, a method that allows the analysis of 25R-NBOH and other thermally labile substances by this technique would be of great use not only in the forensic scenario but also in analytical chemistry laboratories in general.

There are many factors that can affect the thermal degradation of the analyte on the GC-MS system, such as injector and oven temperatures, liner type, and contact time with the analytical column [18]. To minimize the analyte breakdown, some authors have used deactivated liners, lowered the oven temperature, and shortened the column [19–21]. The use of short column has been successfully implemented for the analysis of the thermally labile pesticide aldicarb [21,22]; the anabolic steroid stanozolol, which exhibits poor gas chromatographic behavior [21,23]; and the synthetic cannabinoids, which require extended methods and high oven temperature [24,25].

The aim of this study was to investigate the use of short columns (4 m and 10 m) for 25R-NBOH GC-MS analysis, without derivatization. Aldicarb, stanozolol, the synthetic cannabinoids JWH-081 and JWH-210 were also analyzed and used as parameter of thermal degradation behavior, column reactivity, and reduction of elution temperature.

2. Materials and methods

2.1. Chemical and reagents

Methanol, n-octadecane and N-trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) were purchased from Sigma Aldrich (USA); acetonitrile (ACN) and chloroform LC–MS grade were purchased from Scharlau (Barcelona, Spain). Certified reference standards of

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https://doi.org/10.1016/j.chroma.2020.461657
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25C-NBOH, 25B-NBOH, 25E-NBOH and 25I-NBOH were purchased from Cayman Chemical (USA); 2C-B was donated by the United Nations Office on Drugs and Crime (UNODC); 2C-I, JWH-081, and JWH-210 were provided by the United States Drug Enforcement Administration (DEA). Aldicarb was purchased from AccuStandard (USA). Stanozolol standard solution was prepared in ACN (500 µg/mL) from material seized by the Civil Police of the Federal District, Brazil, after being characterized and having its purity determined by NMR analysis. Standard solutions of 25B-NBOH, 25C-NBOH, 25E-NBOH, 25I-NBOH (200 and 400 µg/mL each), aldicarb (200 µg/mL), 2C-B, and 2C-I (400 µg/mL each) were prepared using methanol. JWH-081 and JWH-210 were diluted in acetonitrile. All standards were analyzed in triplicate. Individual blotter papers (from 36 to 100 mm^2) seized by the Civil Police of the Federal District, Brazil, were put inside a vial containing 1 mL of methanol, sonicated for 5 min and the extract analyzed by GC-MS.

2.2. Chromatographic conditions

GC–MS analyses were performed using an Agilent 7890A gas chromatograph coupled to a 5975C mass spectrometer and the system controlled by Agilent Chemstation Software version E 02.02.1431 (Agilent Technologies, Santa Clara, CA, USA). Results were also evaluated by the Agilent MassHunter Qualitative Analysis, version 10.0.

Instrumental parameters were adjusted as follows: Scan mode (40-500 m/z), 1µL injection volume, 20:1 split ratio, deactivated Ultra Inert liner without wool. Four injector port temperatures were evaluated: 150°C, 200°C, 230°C, 250°C, and 280°C. GC oven temperature programming was initiated at 50°C, followed by a ramp of 20°C/min up to 280°C, which was held for 7 min; total run time was 18.5 min and solvent delay was 2 min. MS conditions were ionization energy of 70 eV, ion source temperature at 280° C and interface heated to 280°C.

J&W Ultra Inert DB-1mscolumns (0.25 mm I.D., 0.25 μm film thickness; Agilent Technologies, # 122-0132) of 30, 10, and 4 m lengths were used. The helium gas flow was 1 mL/min for the 30 m column, 2 mL/min for the 10 m column and 4 mL/min for the 4 m column, to maintain a minimum positive pressure in the system. Thus, shortened columns have a lower pressure system compared to 30 m-column system.

2.3. Chromatographic performance of the qualitative GC-MS method for 25R-NBOH using a 4 m column

Selectivity of the method was assessed by analyzing a blank blotter paper (drug free) for any response at the 25R-NBOH retention times for possible interferences. The limit of detection (LOD) was set at a signal-to-noise ratio of 3:1 (n=3). The repeatability of the retention time was estimated by injecting each analyte 15 times (same day and different days) in the GC-MS and expressed as relative standard deviation (RSD, %). Peak width, number of theoretical plates per column, peak symmetry, tailing factor and resolution were estimated by the Agilent MassHunter Qualitative Analysis. Mass loading was assessed by determining the peak tailing [26,27] of two-fold serial dilution solutions (n=6) of 25E-NBOH (3880 to 243 µg/mL) and n-octadecane (5000 to 312 µg/mL), dissolved in methanol:chloroform (1:1).

2.4. Derivatization of 25R-NBOH

The 25R-NBOH analytical standards were derivatized with MSTFA as described previously for 25I-NBOH [13]. In summary, 100 µL of each standard (800 µg/mL) was added to a vial, the liquid evaporated under vacuum (Genevac EZ-2 series, United Kingdom) at room temperature, 150 µL of MSTFA was added, vortexed for 30s and kept at 70 °C for 1h in a dry oven. Each derivatized standard solution was directly injected into the GC-MS with a 4 m column.

3. Results

Table 1 shows the chemical structure, molecular formulas, molecular ions of the 25R-NBOH and 2C-R, in addition to their major fragments in the mass spectrometer. The fragmentation pattern of the 25R-NBOH family was described for the first time and will be discussed later in this paper.

3.1. Behavior of the analytes under different column lengths

First, the chromatographic behavior of the 25R-NBOH analytical standards (at 200 µg/mL) was assessed using a standard 30 m column. Only the degradation products (2C-R) were detected (data not shown), as it was previously reported for 25I-NBOH [3]. When the 10 m column was used, in addition to the degradants, the intact 25R-NBOH molecules were detected, but elevated baselines were also observed on the chromatograms (Fig. 1A, C, E, G). With a 4 m column, the peak area and shape of the 25R-NBOH compounds were dramatically improved, while the area of the 2C-R degradants was reduced and, in some cases, they were undetected, considering a signal-to-noise ratio of 3:1 as the LOD, even when higher 25R-NBOH concentration (400 µg/mL) was used (Fig. 1B, D, F, H).

The detectability of 2C-B and 2C-I (400 µg/mL) was confirmed using a 4 m column (Fig. 2A) and the four 25R-NBOH compounds had a satisfactory chromatographic separation in the short column (Fig. 2B). Fig. 2C illustrates the application of the method for the analysis of a seized blotter paper containing 25E-NBOH. Additionally, the proposed method is also suitable for a multi-compound forensic screening, performing well for 25R-NBOMes and was used to detect LSD in a blotter paper (Fig. S1, Supplementary Material).

Fig. 3 shows the GC-MS electron ionization mass spectra of the NBOH compounds and Fig. 4 the fragmentation pathway proposed for the drug family. Low abundance molecular ions (M^+) were observed for each compound (*m/z* 365/367, 321, 315, and 413). Five common fragmentation routes can be seen: loss of methoxy radical ($[M\text{-}OCH_3]^+$ at *m/z* 334, 290, 284, and 382 respectively), which was also observed for the 2C-R compounds (Fig. S2); cleavage of the C–N bond, leading both to the common *o*-cresol ion (*m/z* 107) and to the 2C-R fragments; cleavage of the benzylic C—C bond with hydrogen migration, leading to radical cation fragments (*m/z* 230/232, 186, 180, and 278); and the cleavage of the C–aromatic ring, producing a fragment at *m/z* 136, also a common ion to all NBOH.

As expected, synthetic cannabinoids JWH-210 and JWH-081, which are high boiling point compounds, were detected in a 30 m column using the GC conditions recommended by UNODC (240°C, for 1 min and 6°C/min to 310°C, for 8 min) [24] (Fig. S3). When the compounds were analyzed using a 10 m column and the GC-MS conditions optimized for the present study, which uses a milder oven temperature, the instrument responses were about 2 times higher, the elution times were drastically reduced, and the two JWH-081 isomers were separated (Fig. S3). The peak area and the shape of stanozolol, a synthetic steroid hormone, were drastically improved when using a 10 m chromatographic column (Fig. S4).

3.2. Effect of the inlet temperature

Lowering the inlet port temperature is one option to reduce thermal degradation of the analyte during the GC-MS analysis, but it should not compromise solvent evaporation in the inlet. For the 25R-NBOH, the inlet temperatures (250 °C or 280 °C) practically did not affect the instrument response of the parent compounds (Fig. 1B, D, F, H). Fig. 5 shows the impact of inlet tem-

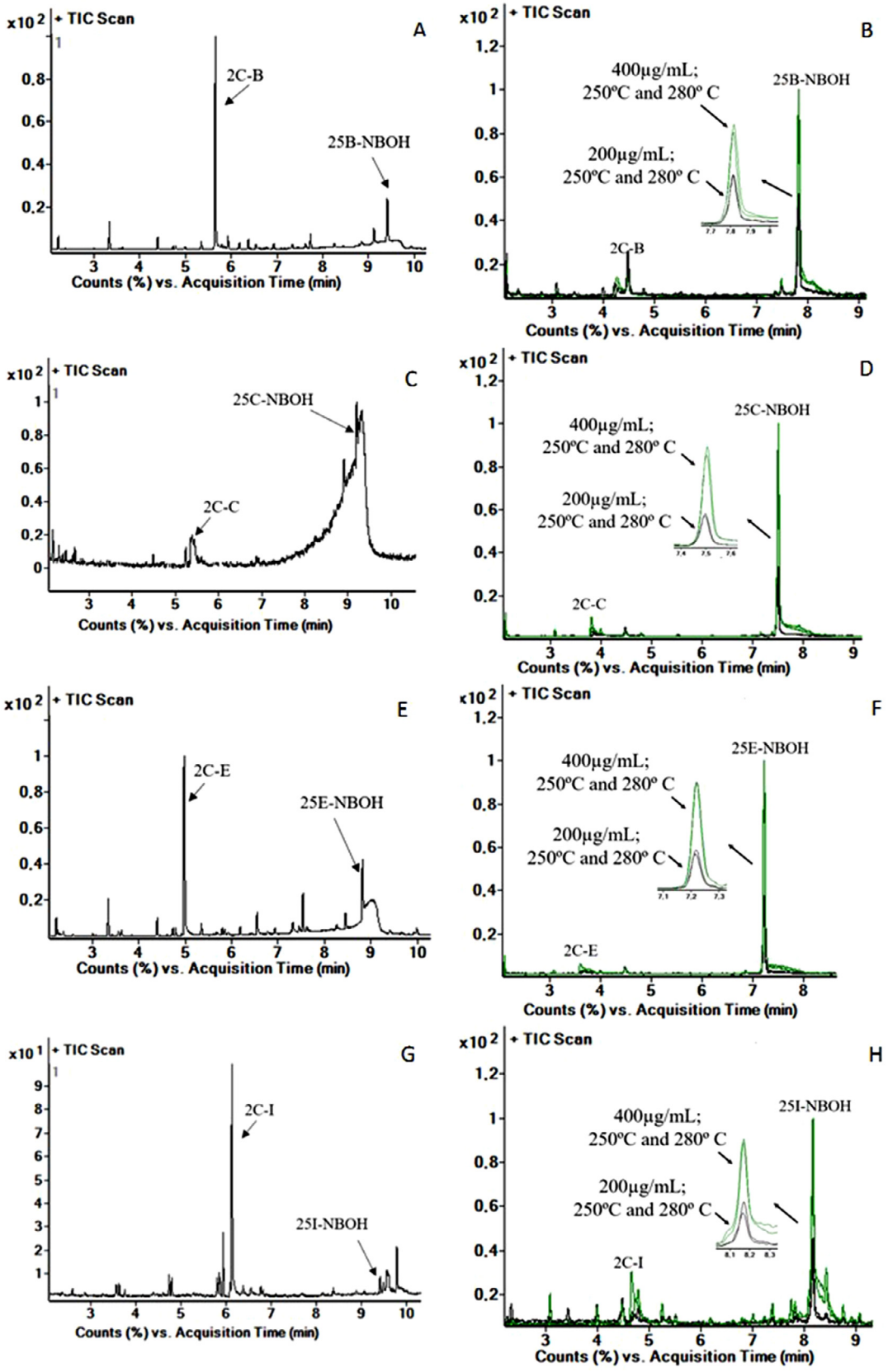
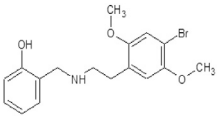
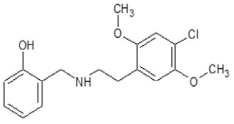
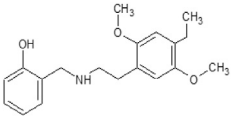
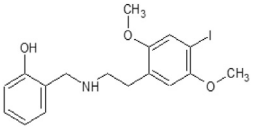
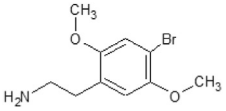
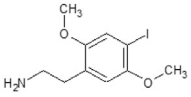
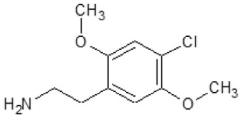
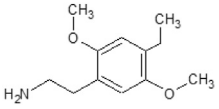


Fig. 1. GC-MS total ion chromatogram of 25R-NBOH with 10 m and 4 m columns. 25B-NBOH, A:10 m column and B: 4 m column; 25C-NBOH, C:10 m column and D: 4 m column; 25E-NBOH, E: 10 m column and F: 4 m column; 25I-NBOH, G:10 m column and H: 4 m column. Inlet temperatures 250° C and 280° C were evaluated (n=3, each) and no statistically significant differences were observed.

Table 1
Chemical structures, molecular formulas, molecular ions and the major fragments of the 25C-NBOMe, 25R-NBOH and 2C-R.

Substance	Chemical structure	Molecular formula	Molecular ion	Major fragments, m/z
25B-NBOH		$C_{17}H_{20}BrNO_3$	365	107, 136, 230-232
25C-NBOH		$C_{17}H_{20}ClNO_3$	321	107, 136, 186
25E-NBOH		$C_{19}H_{25}NO_3$	315	107, 136, 180, 165
25I-NBOH		$C_{17}H_{20}INO_3$	413	107, 136, 278
2C-B		$C_{10}H_{14}BrNO_2$	260	259-261, 230-232, 215-217
2C-I		$C_{10}H_{14}INO_2$	307	278, 262, 307
2C-C		$C_{10}H_{14}ClNO_2$	215	215, 186, 171
2C-E		$C_{12}H_{19}NO_2$	209	209, 180, 165

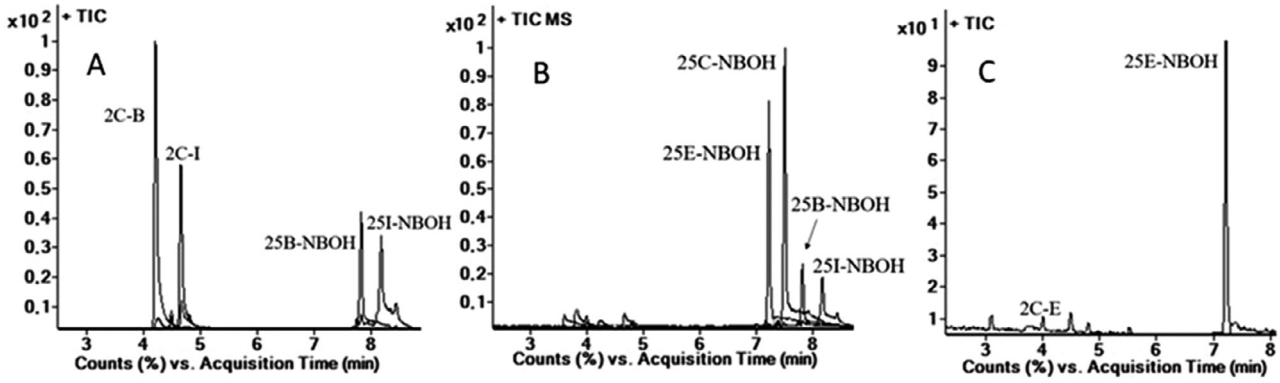


Fig. 2. GC-MS total ion chromatogram using a 4 m analytical column. A: 2C-B, 2C-I, 25B-NBOH and 25I-NBOH at 400µg/mL; B: 25R-NBOH. C: Blotter paper containing 25E-NBOH (signal-to-noise of 2C-E < 1:3).

perature (from 150 to 280 °C) on the response of 25C-NBOH, aldicarb and stanozolol, which are models for thermally labile and low volatile reactive compounds, respectively. Increasing the inlet temperature from 150 to 200 °C increased the response of all three compounds, but higher temperatures lead to the loss of aldicarb response, which disappear completely at 280 °C. While for stanozolol the response remains constant from 230 to 280 °C, the optimum inlet temperature for 25C-NBOH is reached at 250 °C (Fig. 5).

3.3. Performance of the 4 m column GC-MS qualitative method

The method was shown to be selective as no peaks were found near the 25R-NBOH eluting times. The LOD was found at 5 or 10 µg/mL, repeatability of the retention times was lower than 0.2%,

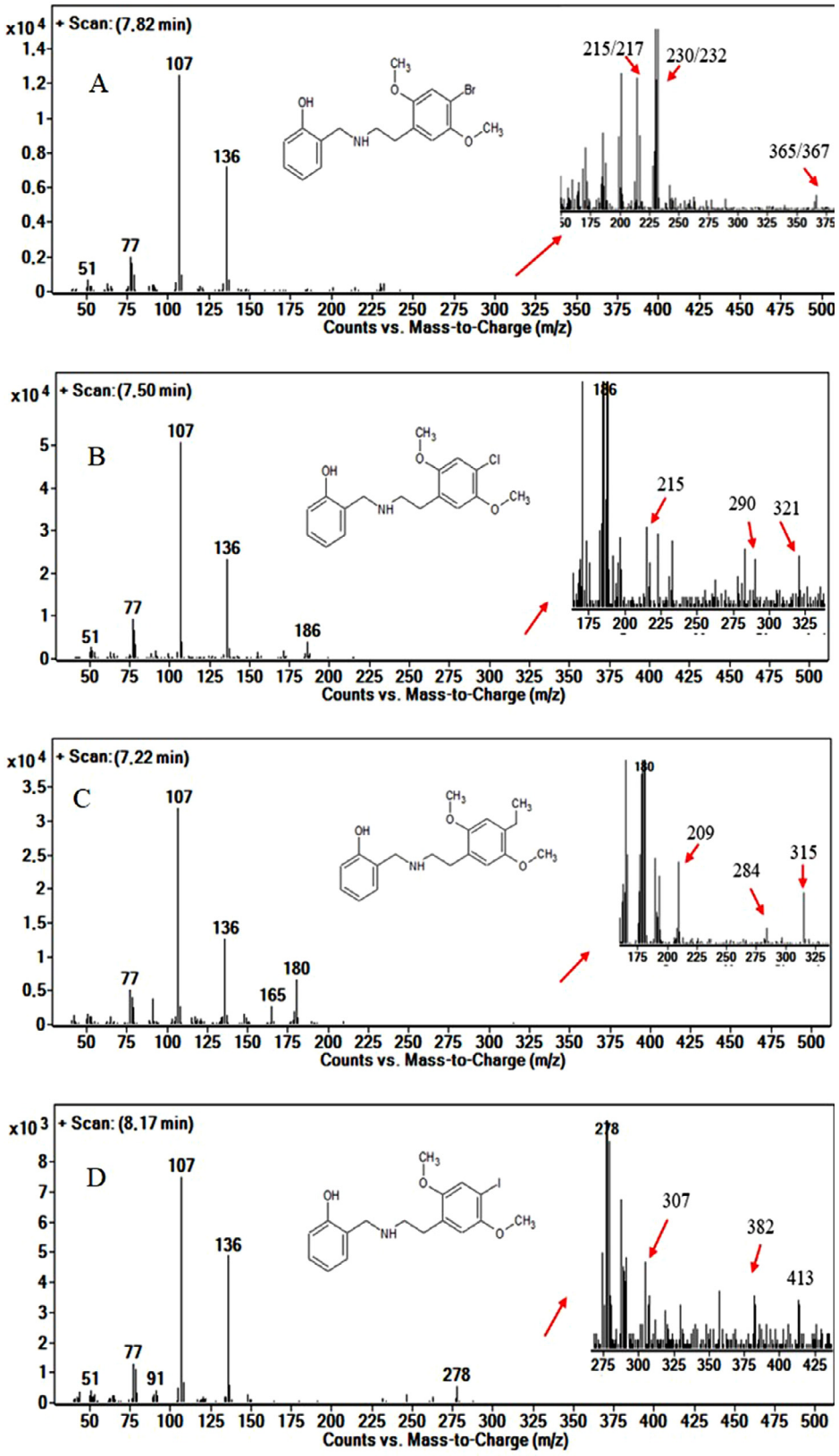


Fig. 3. Fragmentation of 25R-NBOH using a 4 m column. A: 25B-NBOH; B:25C-NBOH; C: 25E-NBOH and D: 25I-NBOH.

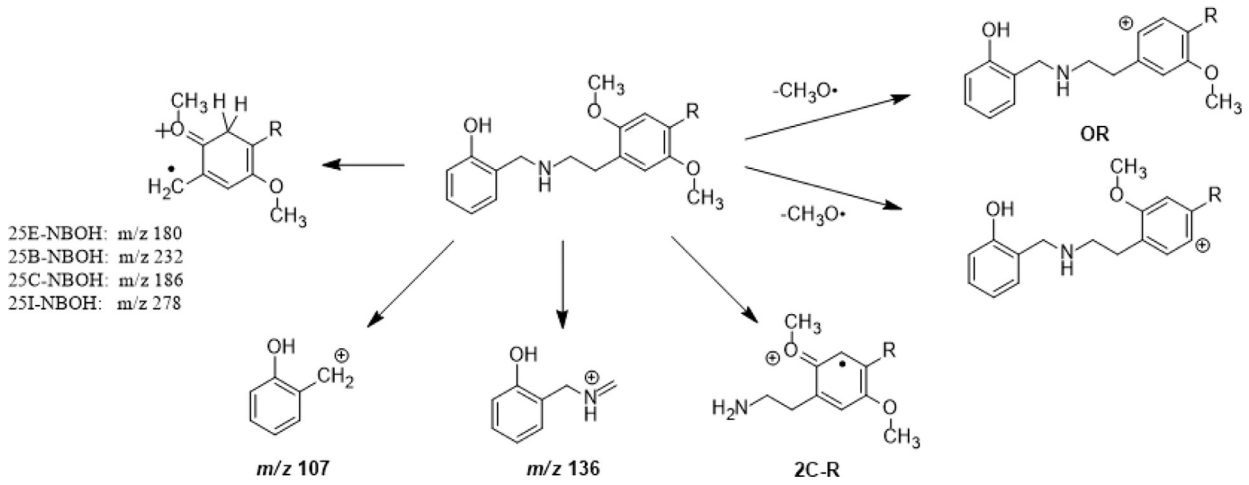


Fig. 4. Proposed fragmentation pathway for 25R-NBOH compounds using electron ionization. R = 25B-NBOH: Br; 25C-NBOH: Cl; 25E-NBOH: C_2H_5; 25I-NBOH: I. 2C-R = 2C-B (m/z 260); 2C-C (m/z 215); 2C-E (m/z 209); 2C-I (m/z 307).

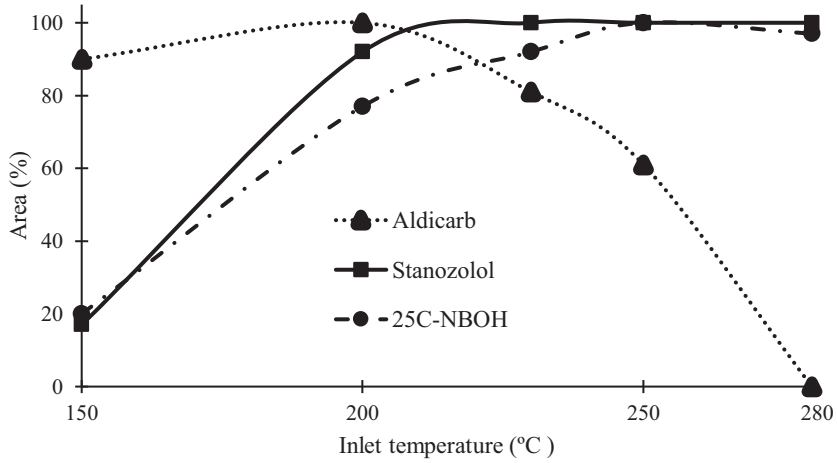


Fig. 5. Peak area percentage of aldicarb, stanozolol and 25C-NBOH using different inlet temperatures (150, 200, 230, 250 and 280 °C) in the CG-MS method (4 m column).

Table 2
Chromatographic parameters of 25R-NBOH at 200 µg/mL.

Substance	LOD (µg/mL)	RT repeatability (RSD, %)	Peak width (W, min.)	Theoretical plate (N)	Symmetry	Tailing factor
25B-NBOH	5	0.13	0.203	212372	0.96	1.7
25C-NBOH	5	0.09	0.197	228727	0.94	1.1
25E-NBOH	5	0.07	0.145	195329	0.83	1.35
25I-NBOH	10	0.06	0.227	185936	0.99	1.1

LOD: Limit of detection; RT = retention time; RSD= relative standard deviation.

peak width was below 0.3, peak symmetry higher than 0.8 and maximum tailing factor was 1.7 (Table 2). Resolution was higher than 1.5 in all cases (data not shown). Even at high mass loading of the analyte (25E-NBOH), the peak tailing was within the expected range, and similar to that of n-octadecane (Fig. S5; Supplementary Material).

3.4. Analysis of derivatized 25R-NBOH

The method performance of MSTFA derivatized 25R-NBOH was also evaluated using the 4 m column, and is illustrated in Fig. 6 for 25C-NBOH. The 2C-R degradant was not detected and the silylation generated two derivatives, mono-TMS, with m/z 73, 179 e 208 as most abundant fragments, and di-TMS, with m/z 73, 179, 280. The mono-TMS and di-TMS fragmentation pattern was quite similar for all the 25R-NBOH tested (Fig. S6, S7 and S8).

4. Discussion

Thermolabile and reactive substances exhibit poor gas chromatographic behavior and are a challenge for the analyst [18,19]. Although derivatization of the analyte before GC-MS analysis can overcome most of the problems [12], there is a burden of increasing complexity, analysis time, and cost.

Among the parameters related to sample degradation during the GC–MS analysis, those related to the residence time in the inlet, chromatographic column and oven temperature are the most important. Since the separation in the GC column is driven mainly by the analyte boiling points, temperature is normally the first parameter thought as being related to compound degradation [19]. Furthermore, the time the compound spends in the GC oven depends on the length of the column and/or the carrier gas flow rate,

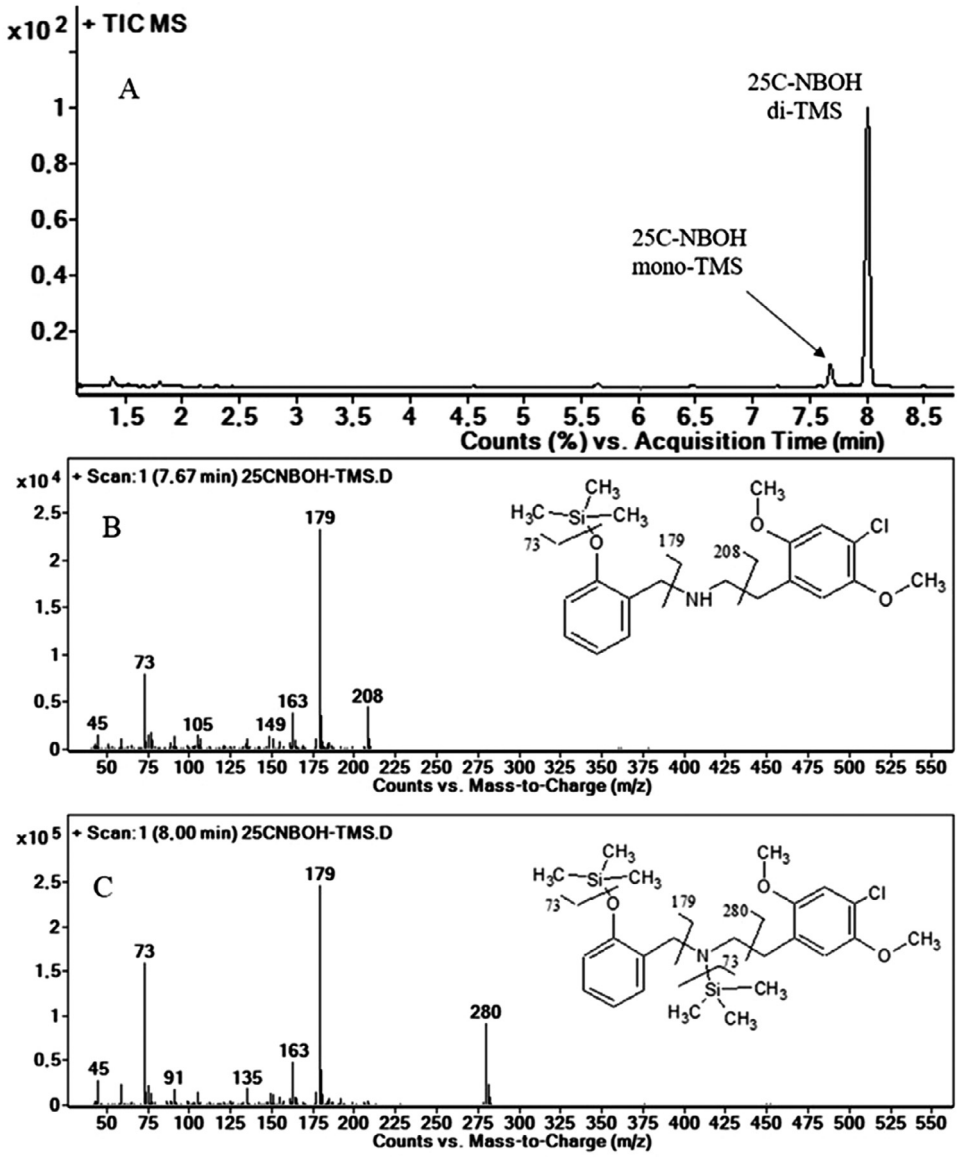


Fig. 6. A: GC-MS total ion chromatogram of 25C-NBOH derivatized with MSTFA, using a 4 m column; B: fragmentation and molecule structure of 25C-NBOH mono-TMS and C: 25C-NBOH di-TMS.

which will affect the degradation and the chromatographic performance of the analyte [22].

Thermal stability of the 25I-NBOH has been evaluated by thermogravimetric analysis and differential scanning calorimetry [13]. The authors concluded that the small temperature window between fusion and decomposition (203° C and 265° C, respectively) had a direct effect on its thermal stability. Previous work conducted by our research group has indeed showed that 25I-NBOH is not detected by GC-MS using a standard 30 m column, and the identification of this compound in blotter papers relied on the detection of its degradation derivative (2C-I) [15]. In the present study, the use of short columns to reduce the 25R-NBOH degradation during the GC separation phase was explored. Short column length reduces the elution temperature of the substances, which is important for thermolabile compound analysis [18]. With the 10 m column, the 25R-NBOH molecules were detected, but the chromatographic performance was poor, with the presence of an elevated baseline. When the NBOH compounds were analyzed with a 4 m column, the chromatographic performance improved considerably, showing a strong positive correlation between column length and analyte degradation and a negative correlation between column length and peak shape. With short columns, the gas flows were higher, which reduces the elution time and temperature of the analyte into the column [18,20].

The respective degradation products (2C-R) were drastically reduced at both 25R-NBOH concentrations tested (200 and 400 µg/mL). The detection of 2C-R compounds as 25R-NBOH degradant has been also demonstrated in “cold chromatographic” analysis. Arantes et al. [3] analyzed 25I-NBOH by LC–QTOF-MS using different fragmentor voltages and demonstrated that the fragmentation could be induced in the region between the end of the transfer capillary and the first skimmer cone, producing 2C-I on the chromatogram.

Previous studies have not only successfully analyzed synthetic cannabinoids [25], stanozolol [21,23], and aldicarb [21,22] using short columns, but also demonstrated that the use of short columns improved the peak shape and the detectability of those analytes. The combination of short column and higher gas flow

provides high signal-to-noise ratio, lower elution temperature [18], improvements that were confirmed in the present study. The use of a 10 m column and low oven temperatures did increase the instrument response for JWH-081 and JWH-210 compared to the recommended method [24], while drastically reduced the elution temperatures and retention times of these compounds.

Increasing the inlet temperature from 250 to 280 °C did not impact the 25R-NBOH response in the GC-MS, although some studies have hypothesized that this may occur [13,15]. Increasing the inlet temperature also did not impact the stanozolol response, but had a significant impact on aldicarb, which is highly thermolabile [21].

The chromatographic GC-MS parameters evaluated showed that the 4 m column method fits the purpose for a qualitative method. The method was selective, and all the substances tested were unequivocally identified. LODs of 5 µg/mL (25B-NBOH, 25C-NBOH and 25E-NBOH) or 10 µg/mL (25I-NBOH) were considered acceptable to analyze the real samples using only one blotter paper [28]. Repeatability of the retention times, tailing factor, theorical plate number, resolution and mass loading were considered suitable for a qualitative analysis [26,27,28].

The fragmentation pathway for derivatized 25R-NBOH (mono-TMS and di-TMS) was shown to be similar for all four compounds of the family and was previously reported for 25I-NBOH [13]. Although the retention time of the derivatized compounds are different, the poor structure information of the derivatized compound can be a limitation for the detection of new compounds from the same class that shows up in the market in the future. The proposed 4 m column GC-MS method was also able to detect and discriminate other compounds commonly present in blotter papers, such as LSD and the 25R-NBOMe family.

To the best of our knowledge, this is the first time that intact 25R-NBOH molecules with good chromatographic peak shapes were obtained by GC-MS analysis without the need of a derivatization step. A fragmentation pattern that is common to all compounds within the family was also proposed, and included the loss of methoxy radical and the hydroxyl of the phenol group, and cleavage of the C–N bond to form the 2C-R degradants. The fragmentation pattern is similar to that for 25C-NBOMe proposed by Zuba et al. [29], which shows the fragments m/z 121 formed by the cleavage of the C-N bond yielding 2-methoxybenzyl cation and of m/z 150 corresponding to iminium cation formed by the dissociation of the α- and β-carbon atoms. For the 25C-NBOH, these pathways lead to the m/z 107 and m/z 136 respectively, which are characteristic fragments of the family. Complementary to the m/z 107, the 2C-R compound molecular ions were detected. A fragmentation pattern of 25R-NBOH molecules was proposed by Machado et al. [16] based on the work conducted by Coelho Neto et al. [15] and Zuba et al. [29], although the intact compounds were not detected in the GC-MS system. In their proposal, the fragmentation follows the thermal degradation of 25R-NBOH into 2C-R and o-cresol, therefore different from the present proposal, which is based on the mass spectra of the intact 25R-NBOH compounds and their prominent ion fragments.

6. Conclusion

A 4 m column GC-MS method for the detection of intact non-derivatized 25B-NBOH, 25C-NBOH, 25E-NBOH, and 25I-NBOH in seized materials was developed for the first time, and their fragmentation pathways using electron ionization were proposed for the first time. The method uses regular GC-MS setup, can be easily implemented in other laboratories and can be a way to overcome the poor detectability of thermolabile, high boiling point and reactive compounds in the forensic scenario. This method can also be further applied to biological matrices in cases of toxicological investigation, after appropriate matrix preparation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ettore Ferrari Júnior: Conceptualization, Methodology, Writing - original draft. **Luciano Chaves Arantes:** Methodology, Writing - review & editing. **Lívia Barros Salum:** Investigation. **Eloisa Dutra Caldas:** Supervision, Data curation, Writing - review & editing.

Acknowledgments

The authors thank A.D. Jones for helping to elucidate the fragmentation pathway of 25R-NBOH, and B.H.M. Leite for helping with the analysis of blotter papers.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461657.

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Analysis of non-derivatized 25R-NBOH using short column gas chromatography – mass spectrometry (GC-MS)

Supplementary Material

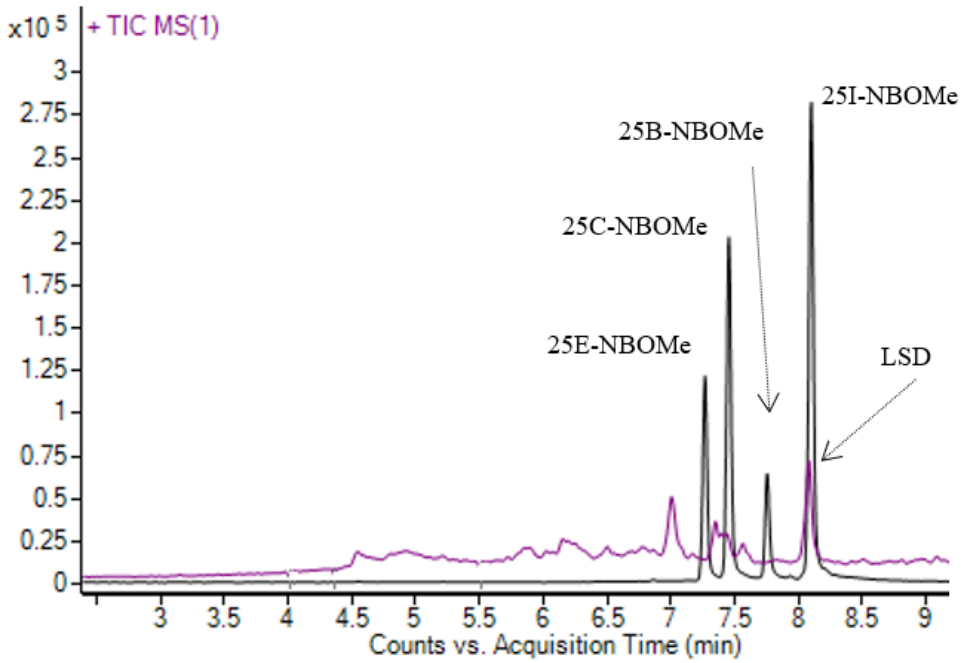

Figure S1: GC-MS total ion chromatogram of the reference standards 25E-NBOMe, 25C-NBOMe, 25B-NBOMe, 25I-NBOMe and a blotter paper containing LSD, using the proposed method (4 m column).

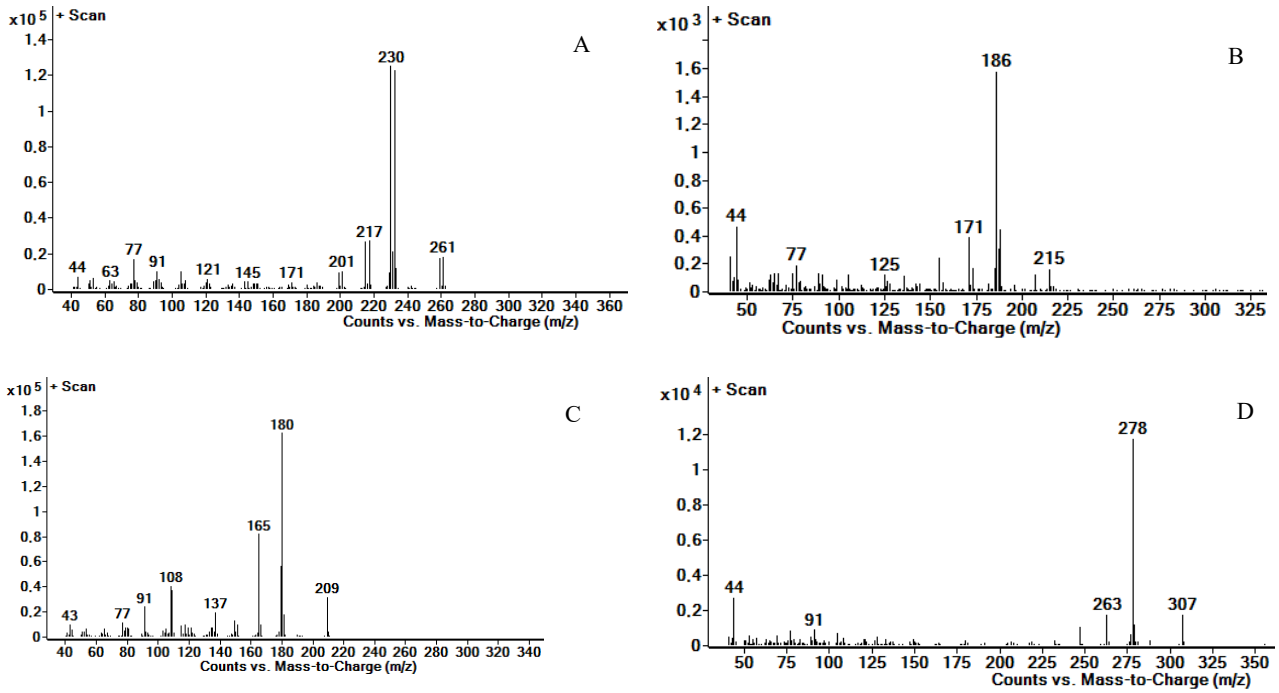

Figure S2: Fragmentation of A: 2C-B; B: 2C-C; C: 2C-E and D: 2C-I (D) under electron ionization.

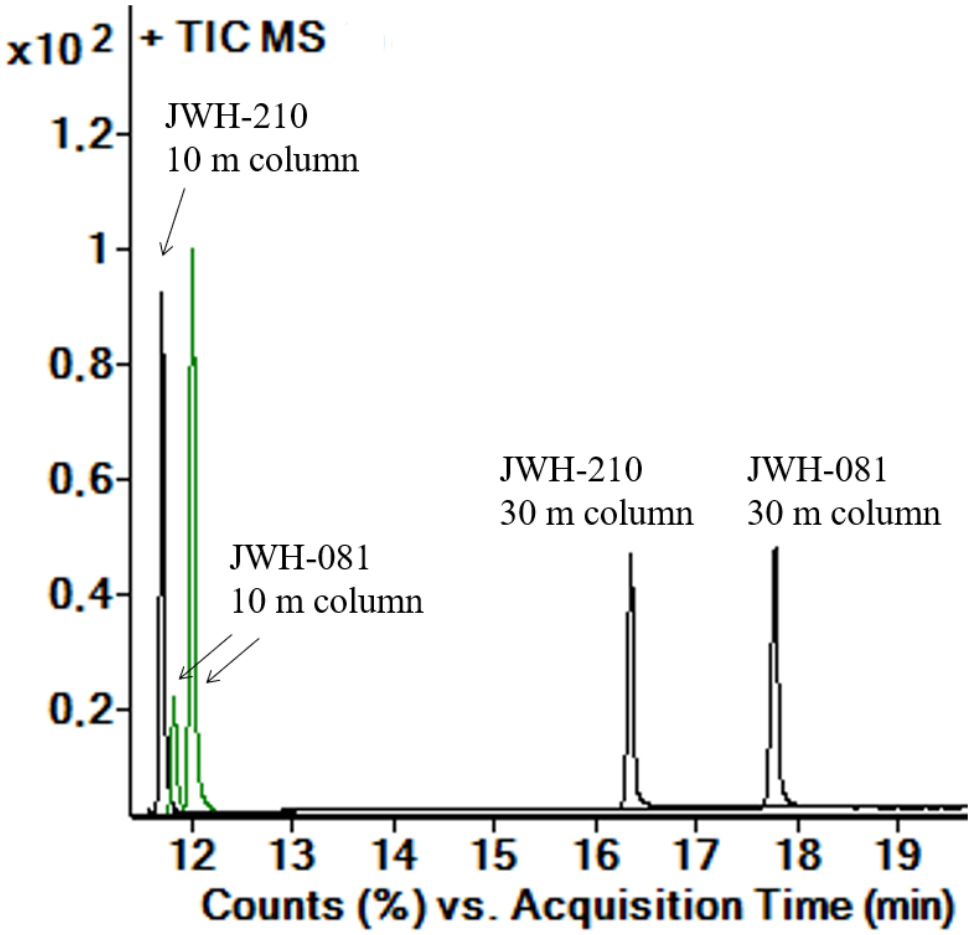

Figure S3: GC-MS total ion chromatogram of JWH-210 and JWH-081. (A) Retention times of 11.7 min for JWH-210 and 11.8 min and 12.0 min for JWH-081 (two isomers) with the 10 m column; Retention times of 16.3 min for JWH-210 and 17.8 min for JWH-081 with the 30 m column (240 °C, for 1 min; then 6 °C/min to 310 °C, for 8 min) [24].

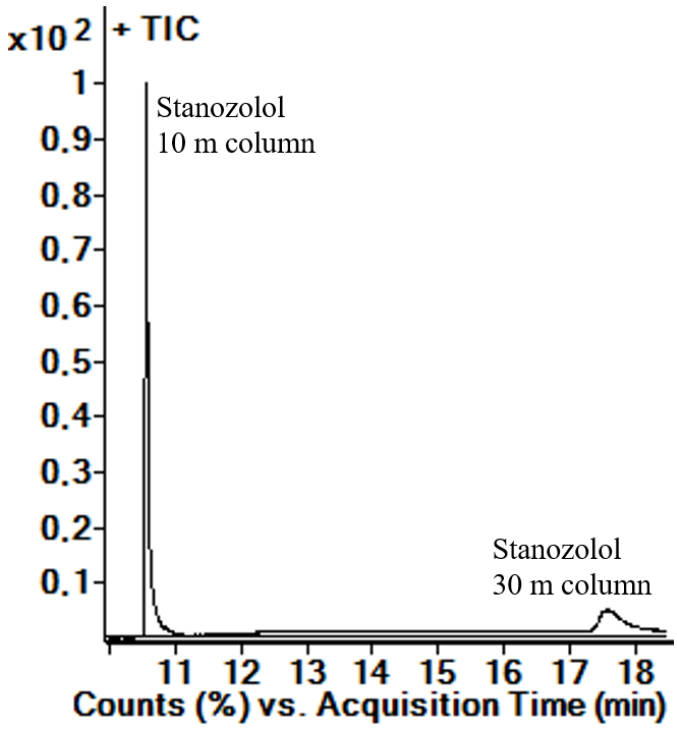

Figure S4: GC-MS total ion chromatogram of stanozolol showing retention times with the 10 m (10.58 min) and 30 m column (17.6 min).

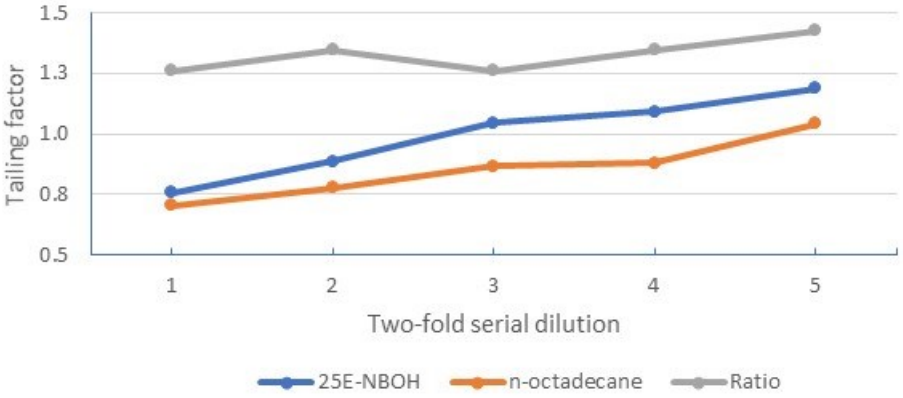

Figure S5: Tailing factor of 25E-NBOH and n-octadecane and their ratio. The concentration ranged from 3880 (serial dilution 1) to 243 (serial dilution 5) µg/mL for 25E-NBOH and from 5000 (serial dilution 1) to 312 (serial dilution 5) µg/mL for n-octadecane.

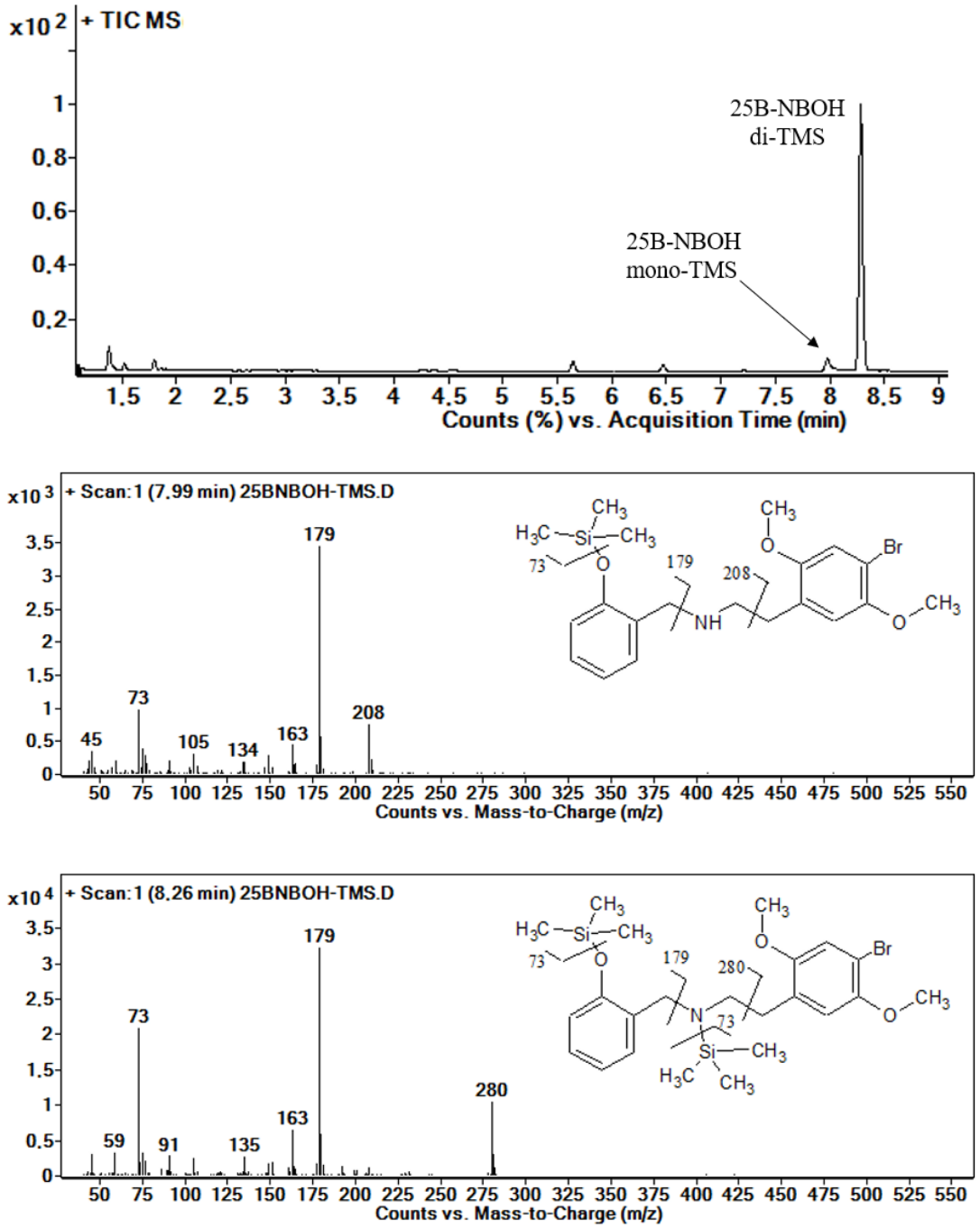

Figure S6: GC-MS total ion chromatogram of 25B-NBOH derivatized with MSTFA, fragmentation and molecule structure of 25B-NBOH mono-TMS (RT = 7.99 min) and 25B-NBOH di-TMS (RT = 8.26 min), using s 4 m column.

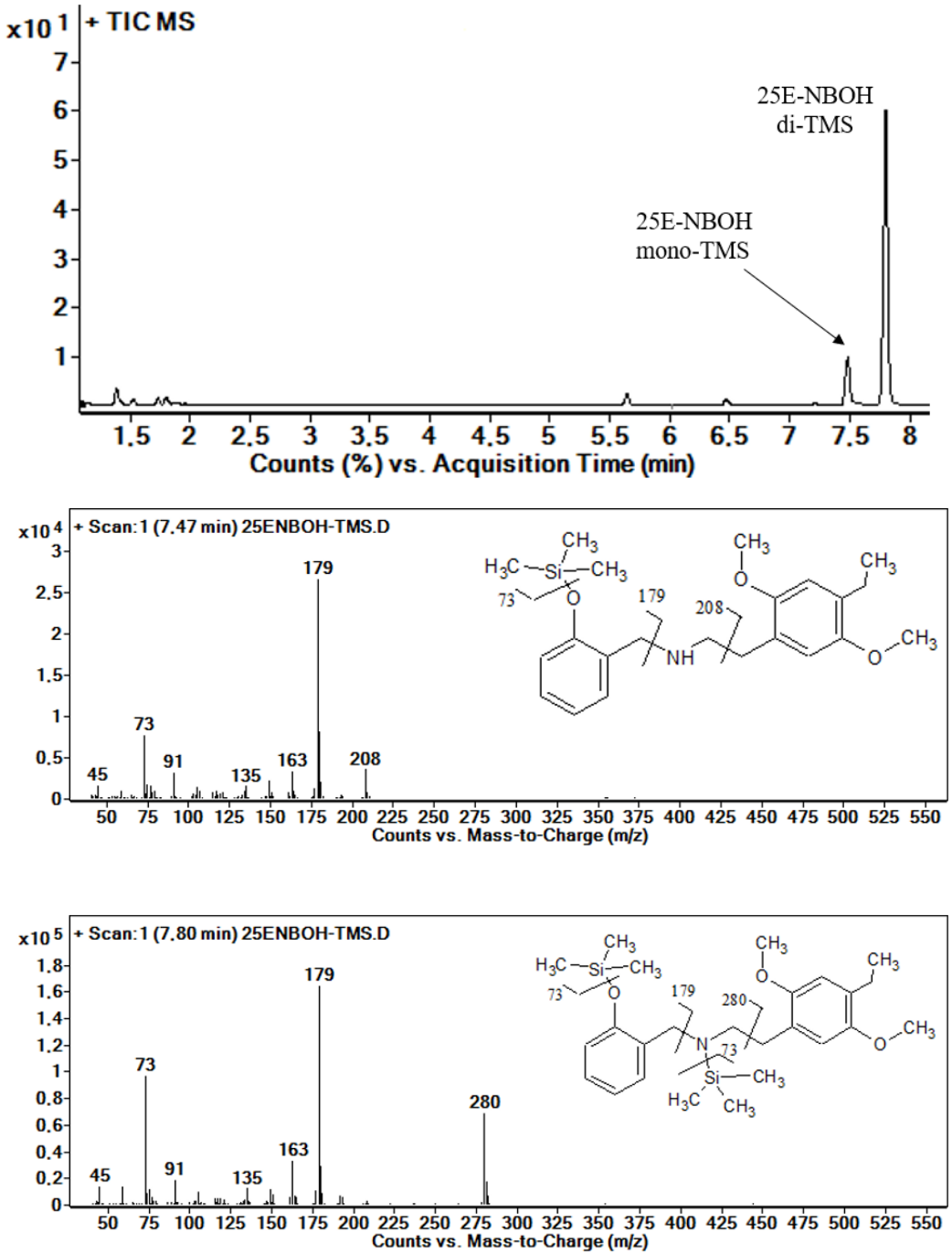

Figure S7: GC-MS total ion chromatogram of 25E-NBOH derivatized with MSTFA, fragmentation and molecule structure of 25E-NBOH mono-TMS (RT = 7.47 min) and 25E-NBOH di-TMS (RT = 7.80 min), using s 4 m column.

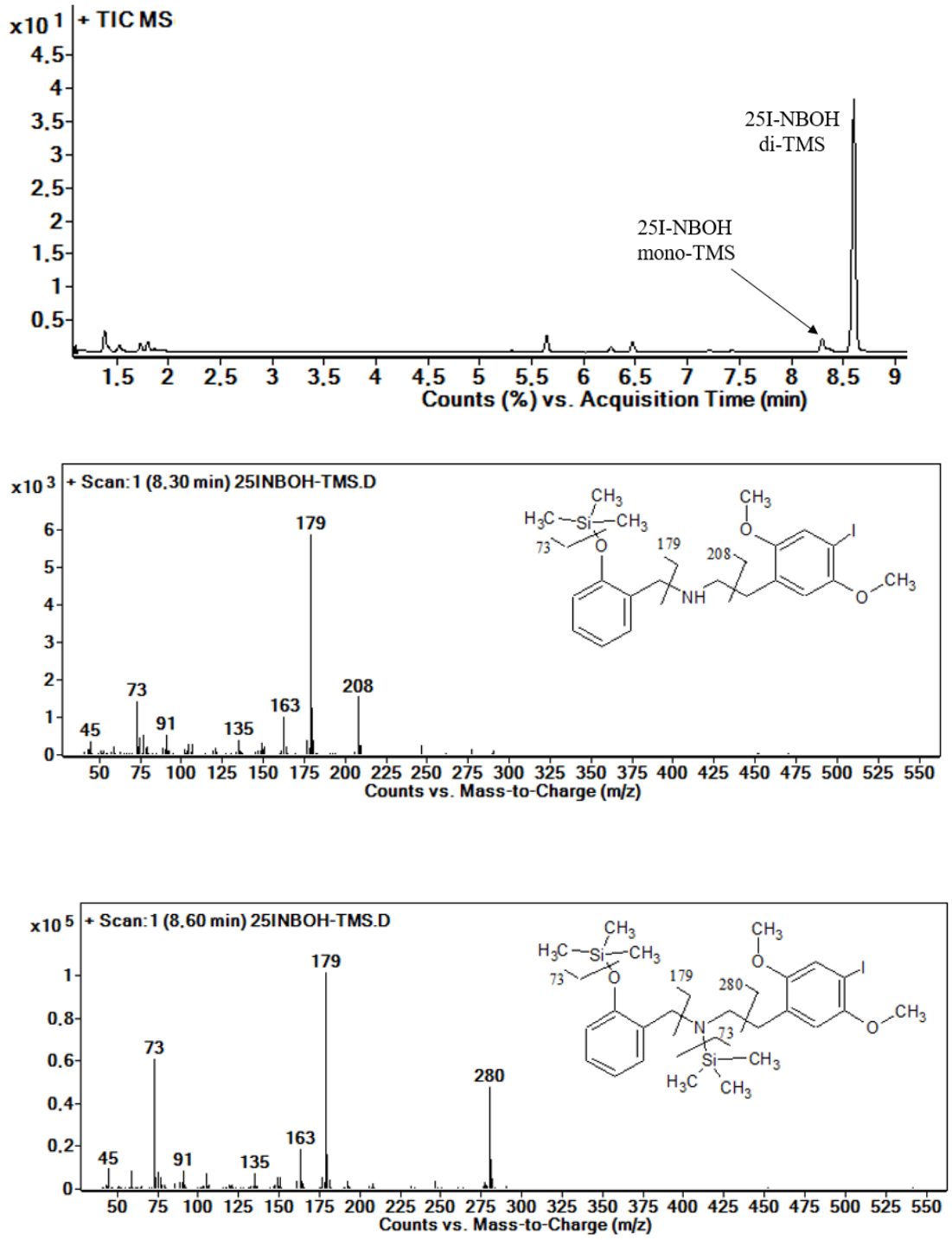

Figure S8: GC-MS total ion chromatogram of 25I-NBOH derivatized with MSTFA, fragmentation and molecule structure of 25I-NBOH mono-TMS (RT = 8.30 min) and 25I-NBOH di-TMS (RT = 8.60 min) using s 4 m column.

ANEXO II

frontiers | Frontiers in Toxicology

TYPE Review
PUBLISHED 25 October 2022
DOI 10.3389/ftox.2022.1033733

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neurotoxicology, a section of the journal Frontiers in Toxicology

RECEIVED 31 August 2022
ACCEPTED 07 October 2022
PUBLISHED 25 October 2022

CITATION
Ferrari Júnior E, Leite BHM, Gomes EB, Vieira TM, Sepulveda P and Caldas ED (2022), Fatal cases involving new psychoactive substances and trends in analytical techniques.
Frontiers in Toxicology 4:1033733.
doi: 10.3389/ftox.2022.1033733

Fatal cases involving new psychoactive substances and trends in analytical techniques

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New psychoactive substances (NPS) are an emerging public health issue and deaths are commonly associated with polydrug abuse. Moreover, the number of new substances available is constantly increasing, causing intoxications in low doses, characteristics that impose to toxicology and forensic laboratories to keep routine methods up to date, with high detectability and constantly acquiring new analytical standards. Likewise, NPS metabolites and respective elimination pathways are usually unknown, making it difficult the detection and confirmation of the drug involved in the fatal case in an analytical routine. A literature search was performed on PubMed, Scopus and Web of Science databases for papers related to chromatographic analyses from fatal cases related to NPS use published from 2016 to 2021. A total of 96 papers were retrieved and reviewed in this study. Opioids, synthetic cathinones, phenethylamines/amphetamines and cannabinoids were the NPS classes most found in the fatal cases. In many cases, multiple compounds were detected in the biological samples, including prescription and other illegal drugs. Liquid chromatography-tandem mass spectrometry, an alternative to overcome the gas chromatography–mass spectrometry limitations for some compounds, was the analytical technique most used in the studies, and high resolution mass spectrometry was often applied to NPS metabolite investigation and structural characterization and identification of unknown compounds. Toxicological screening and quantitation methods need to be continuously updated to include new substances that are emerging on the drug market that can be fatal at very low doses.

KEYWORDS

new psychoactive substances, GC-MS, LC-MS/MS, HRMS, opioids, synthetic cathinones, fatal cases

Introduction

New psychoactive substances (NPS) are drugs that are not scheduled under the 1961 United Nations Single Convention on Narcotic Drugs or the 1971 United Nations Convention on Psychotropic Substances, and are synthetized to mimic the effect of traditional drugs (UNODC, 2021a). The illicit market of NPS has been constantly changing due to introduction of new substances, which brings potential new public health problems, since little is known about their toxicology, with reporting of fatal poisoning cases worldwide (UNODC, 2020).

Some NPS are classified as a group based on structural similarity and/or psychoactive effects. Furthermore, there are also similarities among the NPS groups, as example, phenethylamines also include amphetamines, which have structures similar to cathinones. With the same quickness that the NPS appear in the market, they are replaced for other analogs to escape from the official control of illegal substances (EMCDDA, 2021a), which brings a constant challenge for forensic laboratories that uses mostly chromatographic techniques to elucidate intoxication cases. Figure 1 shows the chemical structure of the main NPS groups discussed in this review.

Gas chromatography coupled with mass spectrometer detector (GC-MS) is a robust analytical instrumentation applied to systematic toxicological analysis, which is available in most forensic laboratories, providing unequivocal molecular identification and acceptable limits of detection for the majority of compounds of toxicological interest (Rojkiewicz et al., 2016; Ellefsen et al., 2017; Atherton et al., 2018; Dwyer et al., 2018; Majchrzak et al., 2018; Ivanov et al., 2019; Tiemensma et al., 2020; Woods, 2020; Cartiser et al., 2021). However, liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods are able to overcome analytical limitations of the GC techniques, such as thermal degradation (Ferrari Júnior et al., 2020), providing lower detection limits that are needed for some compounds, as synthetic cannabinoids (Gieron and Adamowicz, 2016; Shanks and Behonick, 2016; Angerer et al., 2017; Adamowicz et al., 2019; Zawadski et al., 2020a; Hvozdovich et al., 2020; Krotulski et al., 2021a), opioids (Guerrieri et al., 2017; Krotulski et al., 2017; Krotulski et al., 2021b, 2021c; Castelino et al., 2021; Mueller et al., 2021), and phenethylamines (Kristofic et al., 2016). In some cases, the suspicion of intoxication may involve an unknown substance for the laboratory routine, a problem that can be solved using high resolution mass spectrometry (HRMS), that features high mass accuracy as a tool for untargeted screening analysis (Deville et al., 2019; Fels et al., 2019; Gaulier et al., 2019; Kovacs et al., 2019; Nash et al., 2019; Theofel et al., 2019; Yeter and Erol Öztürk, 2019; Krotulski et al., 2021d). HRMS techniques can be also applied to NPS metabolite investigation, which can be essential to confirm the use of the drugs, mainly those that are rapidly metabolized (Allibe et al., 2018; Krotulski et al., 2020a). Furthermore, the analysis of seized drugs and other materials found near the victim can bring additional information that helps to elucidate the intoxication case (Rojkiewicz et al., 2016; Strehmel et al., 2018).

The non-detection and underreporting of NPS in postmortem analysis and the absence of toxicological studies to establish possible risks caused by NPS consumption make

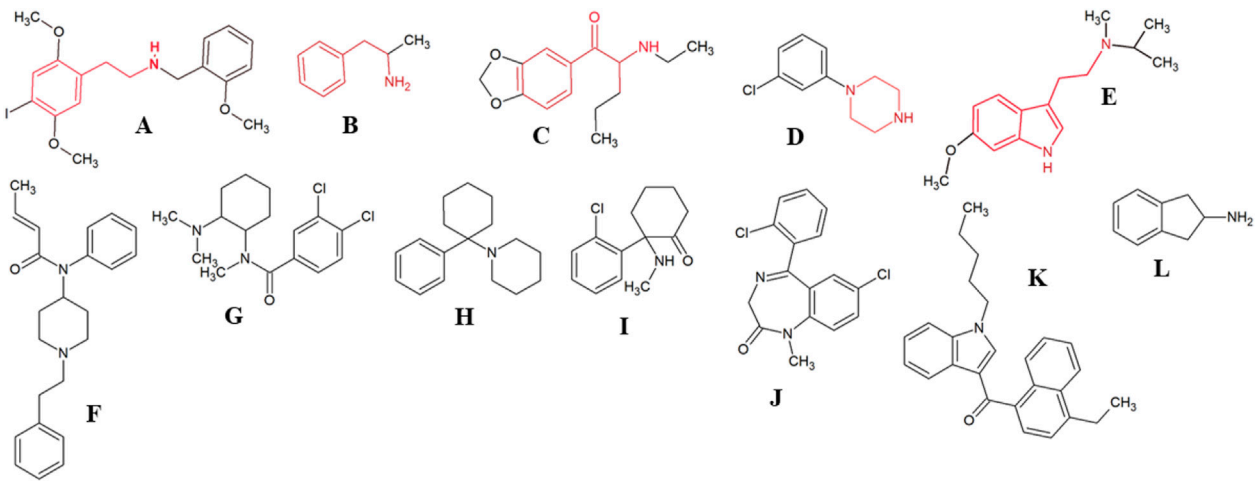

FIGURE 1
Chemical structure of the main compounds from the NPS classes discussed in this paper. 25I-NBOMe **(A)** and amphetamine **(B)**, highlighting the phenethylamine core (red); the synthetic cathinone N-ethylpentylone **(C)**, highlighting the cathinone core (red); m-CPP **(D)**, highlighting the piperazine core (red); 5-MeO-MIPT **(E)**, highlighting the tryptamine core (red); Opioids: fentanyl analog crotonylfentanyl **(F)** and U-47700 **(G)**; phencyclidine **(H)** and the analogue ketamine **(I)**; designer benzodiazepine diclazepam **(J)**; synthetic cannabinoid JWH-210 **(K)**; 2-Aminoindane **(L)**.

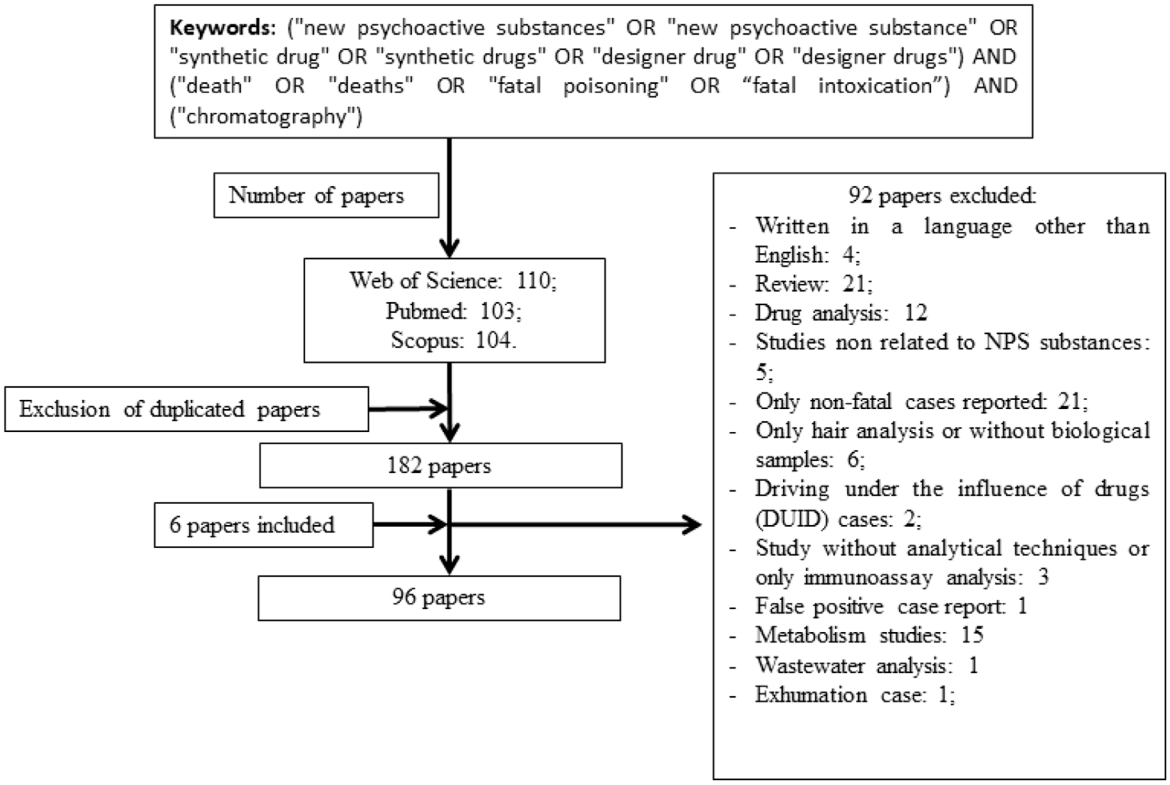

FIGURE 2
Paper selection strategy.

difficult to understand the real impact of NPS in fatal intoxication cases (EMCDDA, 2021a). Although there are some reviews on analytical techniques for NPS detection, a review that covers both the toxicological aspects of acute fatal cases and the analytical strategies used in postmortem analysis is limited in the literature.

The aim of the present paper was to review the literature published from 2016 to 2021 concerning fatal cases that involved NPS abuse and the analytical methods applied in toxicological analyses, to understand how laboratories have been dealing with those emerging drugs.

Method

A literature search was performed on PubMed, Scopus and Web of Science databases for papers related to fatal cases involving NPS using the following keywords ("new psychoactive substances" OR "new psychoactive substance" OR "synthetic drug" OR "synthetic drugs" OR "designer drug" OR "designer drugs") AND ("death" OR "deaths" OR "fatal poisoning" OR "fatal intoxication") AND ("chromatography"). Only papers published in English from January 2016 to December 2021 were considered. Additionally, six papers mentioned in some studies that escaped from our search were included. All papers were screened independently by three of the authors and only papers selected by at least two of them were included. The paper selection strategy, including the exclusion criteria, is summarized in Figure 2.

A total of 96 papers were retrieved for this review, with the highest number found in 2018 and 2019 (20 and 19 papers, respectively). Opioids and synthetic cathinones were the NPS classes most found in the fatal cases, reported in 43 and 37 of the studies, respectively (Figure 3) and this trend was observed in each year. A summary description of all papers is shown in Table 1, and include the analytical technique and extraction/cleanup methods used, the limits of detection and quantification (LOD/LOQ), the main substances found, the concentration range in blood and/or urine and the number of fatal cases. A more detailed description of the studies can be found in Supplementary Table S1.

Analytical methods applied in toxicological routine analysis

Keeping the NPS screening methods in toxicological laboratories up to date frequently involves challenges,

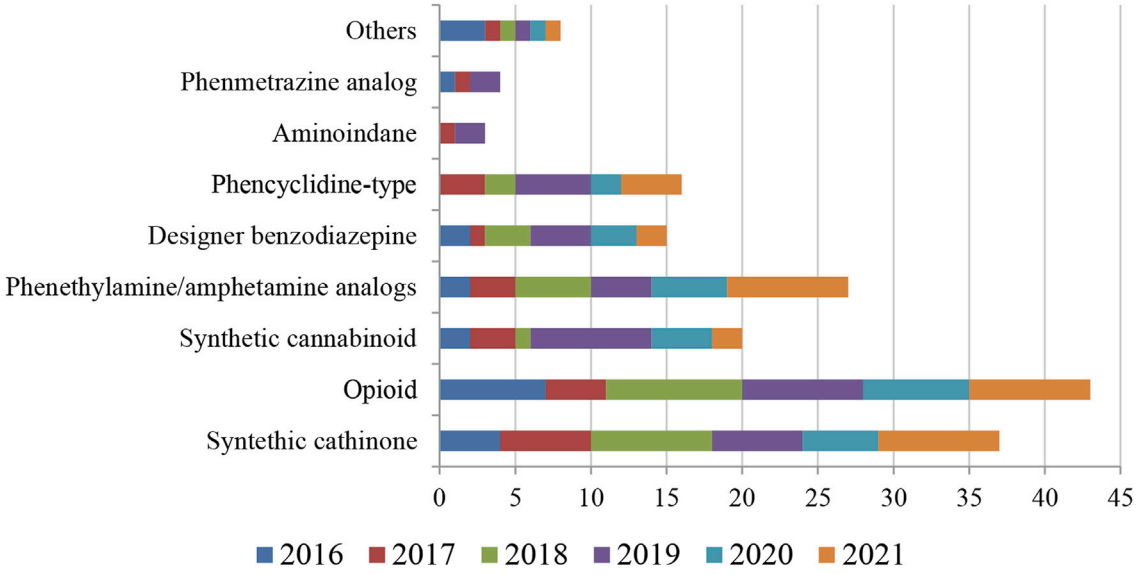

FIGURE 3
Main NPS classes reported in the publications from 2016 to 2021. Others: piperazine (2016), tryptamine (2017–2021), indole alkaloids found in kratom (2018 and 2019); methaqualone analog (2020). Amphetamine analogs were included in phenethylamine data. Opioid includes fentanyl and analogs.

including reference standard availability, method development, lack of information on new illicit drug, and limitation of immunoassays for many NPS (Partridge et al., 2018; Kriikku et al., 2019). Therefore, it is important that toxicology laboratories have different analytical techniques available to minimize possible methodological limitations.

The analytical techniques used in the studies included in this review are liquid chromatography-mass spectrometry (UHPLC-MS/MS, HPLC-MS/MS, LC-MS/MS, UPLC-MS/MS), which were used in most studies (n = 75), followed by LC- high resolution mass spectrometry (LC-QTOF-MS, LC-TOF-MS, UPLC-TOF-MS, LC-HRMS (Orbitrap™), UHPLC-HR-MS/MS, UHPLC-QTOF-MS, and UPLC-TOF-MS; n = 35), gas chromatography (GC-NPD, GC-MS or GC-MS/MS; n = 29), and LC-DAD methods (n = 5). Toxicological analysis used in 48 studies (out of 96) applied more than one technique (GC-MS, LC-DAD, LC-HRMS, LC-MS). Most studies included method validation data, which is essential to guarantee the reliability and suitability of the analytical method, and four of them used standard addition, an interesting analytical approach that overcomes the matrix effect and the need for full validation to quantify few samples (Kusano et al., 2018) (Table 1 and Supplementary Table S1).

GC-MS, available in most forensic laboratories, is a robust and easy-to-handle technique. The electron impact ionization provides reproducible information, allowing high confidence in the screening using trustable reference libraries and the selected ion monitoring (SIM) mode analysis can be applied for targeted screening and quantification (Bottinelli et al., 2017; Ballesteros et al., 2018; Dwyer et al., 2018; Brahan et al., 2021). The use of GC-MS, however, has limitations for labile compounds or those that are present at very low concentration in biological samples. Fagiola et al. (2018) related a possible misidentification of 25I-NBOH as 2C-I by GC-MS analysis, which was later found to be due to the analyte breakdown, since 25I-NBOH was detected intact using LC-QTOF-MS (Arantes et al., 2017). The breakdown of 25R-NBOH family compounds in GC-MS analysis was overcome with shortened columns (4 m-length; Ferrari Júnior et al., 2020). GC-MS was not suitable to screen for synthetic cannabinoids (Hvozdovich et al., 2020), fentanyl analogs (Poklis et al., 2016), phenethylamines (Kristofic et al., 2016), and others NPS due to low concentrations detected in intoxications involving these compounds (Ferrari Júnior and Caldas, 2021). Cathinones can exhibit a “poor fragmentation” in the GC-MS, and more specific mass spectra can be obtained using LC-MS or LC-MS/MS (Mochizuki et al., 2021). On the other hand, Arbouche et al. (2021) describe the discrimination of 3-MeO-PCP and 4-MeO-PCP using GC-MS, since they had the same retention time and transitions exhibited in the LC-MS.

LC-MS/MS methods (electrospray ionization) is indeed an important alternative to overcome the GC limitations. The electrospray ionization is considered a soft technique, with little fragmentation of the molecule when compared to electron ionization used in GC-MS (Arantes et al., 2017; Mochizuki et al., 2021). Its multiple reaction monitoring (MRM) mode is ideal for quantitative methods, demonstrating

TABLE 1 Summary of results found in the 96 papers included in this review that investigated new psychoactive substances in blood and/or urine from fatal cases. Aditional information of each study can be found in Supplementary Table S1.

References	Extraction method	Analytical techniques	LOD/LOQ (ng/mL or ng/g) [a]	Substance (class)	Blood/urine, analyte concentration and number of postmortem/death cases[a]
Adamowicz et al. (2016)[b]	LLE	LC-MS/MS	0.036/1	α-PVP (cath.)	Blood (1.1–6,200); *n* = 12
Beck et al. (2016)	PP	LC-MS/MS (ID, Q); LC-HR/MS (ID)	0.2/-	α-PVP (cath.)	Serum (62.6–304); *n* = 2
Coopman et al. (2016)[b]	LLE	UPLC-MS/MS	2.1/2.1	Ocfentanil (opioid)	Blood (15.3); *n* = 1
Fujita et al. (2016)	QuEChERS	LC-MS/MS	-	Mepirapim (SC); α-EAPP (opioid)	Blood (*n* = 1): Mepirapim (950); α-EAPP (3,100)
Gieron and Adamowicz, (2016)[b]	PP	LC-MS/MS	0.06/0.1	AB-CHMINACA (SC)	Blood (1.5); urine (0.1); *n* = 1
Kristofic et al. (2016)	SPE	LC-QTOF (SCR); LC-MS/MS (Q)	-	25C-NBOMe (PEA)	25C-NBOMe: blood (0.48–2.07), urine (1.73–27.43); 2C-C: blood (0.12), urine (0.11–0.38); *n* = 3
Liveri et al. (2016)[b]	SPE	GC-MS	LOD: Blood/urine (0.002–0.01)/LOQ: Blood (0.4–3); urine: (0.8–6)	MDPV and pentedrone (Cath)	MDPV: blood (46), urine (1,300); pentedrone (mg/L): blood (160), urine (12,000); *n* = 1
Papsun et al. (2016)[b]	LLE	LC-QTOF (SCR); LC-MS/MS (Q)	1/-	MT-45 (Piperazine); Etizolam (D-BZD)	Blood: MT-45 (520); etizolam (35); *n* = 1
Poklis et al. (2016)[b]	SPE	UPLC-MS/MS	-/1	Butyryl Fentanyl (opioid)	Butyryl fentanyl: P. blood (99–3.7), H. blood (220–9.2), urine (64–2); *n* = 2
Rojkiewicz et al. (2016)	LLE	HPLC-MS and GC-MS	7/12	4-FBF (opioid)	Blood (91–112), urine (200–414); *n* = 2
Shanks and Behonick, (2016)[b]	LLE	LC-MS/MS	0.1/0.2	5F-AMB (SC)	Blood (0.3); *n* = 1
Yonemitsu et al. (2016)[b]	QuEChERS	LC-MS/MS and GC-MS (SCR); LC-MS/MS (Q)	-	Acetyl fentanyl (opioid); 4-MeO-PV8 (Cath)	Acetyl fentanyl: F. blood (153), urine (240); 4- MeO-PV8: F. blood (389), urine (245); *n* = 1
Angerer et al. (2017)[b]	LLE	GC-MS, HPLC-MS/MS, HPLC-PDA (SCR); LC-MS/MS (Q)	0.01–0.03/0.1–0.25	5F-PB-22, AB-CHMINACA and 5F-ADB (SC)	F. blood: 5F-PB-22 (0.37), n = 1, AB-CHMINACA (4.1), *n* = 1; 5F-ADB (0.38), *n* = 1
Bottinelli et al. (2017)[b]	SPE	GC-MS, LC-DAD (SCR); GC-MS/MS (Q)	-/50	3-MMC (Cath)	P. blood (249), urine (29,694); *n* = 1
Dwyer et. Al. (2018)	LLE/SPE	GC-MS (SCR); LC-MS/MS (Q)	-	Fentanyl and acetylfentanyl (opioid)	Blood: acetylfentanyl (0.13–2,100); fentanyl (0.24–74.3); urine: only qualitative; *n* = 41
Ellefsen et al. (2017)[b]	LLE	LC-MS/MS and GC-MS	-/0.001	3-FPM (PHEN); U-47700 (opioid)	3-FPM: P. blood (2,400), aortic blood (600); U-47700: P. blood (360); *n* = 1
Guerrieri et al. (2017)[b]	LLE-LTP	LC-MS/MS	-	Acrylfentanyl (opioid)	Blood (0.01–5); *n* = 40
Johansson et al. (2017)[b]	LLE	LC-TOF-MS (SCR); LC-MS/MS (Q)	-/0.01	3-MeO-PCP (PCY)	Blood (50–180); (*n* = 6); blood (380 μg/g) in a mono-intoxication case; *n* = 1
Krotulski et al. (2017)	SPE	LC-QTOF (SCR,MI); LC-MS/MS (Q)	-	THFF and U-49900 (opioid); MeO-PCP (PCY)	Blood and urine, respectively: THFF (339; >5,000); U-49900 (1.5; 2.2); MeO-PCP (1.0; 31.8); *n* = 1
Paul et al. (2017)	NA	LC-MS/MS	0.01–2.0/0.1–2.0	AB-CHMINACA, UR-144, XLR-11 and JWH-022 (SC)	Blood: AB-CHMINACA (8.2), *n* = 1; UR-144 (12.3), XLR-11 (1.3) and JWH-022 (3), *n* = 1
Potocka-banas et al. (2017)	LLE	LC-MS/MS	1/5	α-PVP (Cath)	α-PVP: blood (174), urine (401); *n* = 1
Rojek et al. (2017)[b]	LLE	LC-MS/MS	-/0.05–10	UR-144 (SC); Pentedrone (Cath)	Blood: UR-144 (2.1), *n* = 1; UR-144 (1.4), pentedrone (2,300), (*n* = 1); UR-144 (4), pentedrone (290), *n* = 1

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TABLE 1 (*Continued*) Summary of results found in the 96 papers included in this review that investigated new psychoactive substances in blood and/or urine from fatal cases. Aditional information of each study can be found in Supplementary Table S1.

References	Extraction method	Analytical techniques	LOD/LOQ (ng/mL or ng/g) [a]	Substance (class)	Blood/urine, analyte concentration and number of postmortem/death cases[a]
Staeheli et al. (2017)[b]	LLE	LC-MS/MS	-	MDAI (AI); 2-MAPB (Cath)	P. blood: MDAI (38); 2-MAPB (21); *n* = 1
Wiergowski et al. (2017)[b]	PP/LLE	HPLC-QTOF-MS (SCR); UPLC-MS/MS (Q)	0.0053–0.0013/0.0159–4.0	25B-NBOMe (PEA); 4-CMC (Cath)	Blood. 25B-NBOMe (38.4–661), 4-CMC (0.887–2.14); *n* = 2
Allibe et al. (2018)[b]	SPE	LC-MS/MS (ID, Q); LC-HRMS (MI)	0.01/0.05	Ocfentanil (opioid)	Ocfentanil: P. blood (3.7); *n* = 1
Atherton et al. (2018)	LLE	GC-MS	-/10	N-ethylpentylone (Cath)	P. blood (31–953); *n* = 4
Ballesteros et al. (2018)[b]	SPE	LC-MS/MS and GC-MS	20/-	4-MEC and α-PVP (Cath)	α-PVP: blood (9–1,200); urine: detected 4-MEC and α-PVP; *n* = 2
Costa et al. (2018)[b]	LLE	LC-MS/MS	1 and 5/-	N-ethylpentylone (Cath)	Blood (170); *n* = 1
Fagiola et al. (2018)[b]	LLE	GC-MS or LC-MS (SCR); LC-MS/MS	2.5 (LC-MS/MS); 200 (GC–MS or LC-MS, for Cath)/-	Mitragynine and 7-OH-mitragynine; Pentylone, methylone and butylone (Cath)	Blood/urine: Mitragynine, *n* = 2; mitragynine and 7-hydroxymitragynine, *n* = 3; pentylone, methylone and butylone, *n* = 1
Gerace et al. (2018)[b]	LLE	UHPLC-MS/MS	0.6/2	U-47700 (opioid)	Blood (380); urine (10,400); *n* = 1
Koch et al. (2018)[b]	PP/LLE/SPE	LC-MS/MS	-/1	U-47700 (opioid)	Blood: 42 min (370), 9 h (37), 24 h. (6.3), 33 h (2.1), 41 h (2.3); urine (2); *n* = 1
Krpo et al. (2018)	LLE	UHPLC-QTOF-MS (SCR); UHPLC-MS/MS: (ID, Q)	-	5-APB (PEA)	P. blood (860); *n* = 1
Kusano et al. (2018)[c]	PP	LC-MS/MS (SCR, Q) LC-QTOF-MS (SCR)	0.005–0.1/-	Diphenidine (PCY); 5F-ADB (SC)	Blood (*n* = 1): 5F-ADB (0.19 ± 0.04), diphenidine (12 ± 2.6)
Lehmann et al. (2018)[b]	SPE/QuEChERS	LC-MS/MS	0.4–5/-	Methoxetamine (PCY); 4-MEC, MDPV and α-PVP (Cath)	F. blood: 4-MEC (8–118), MDPV (3–396), MXE (2–385) and α-PVP (4); *n* = 2
Maher et al. (2018)	LLE	HPLC-DAD; LC-QTOF-MS (ID); LC-MS/MS (ID, Q)	0.05–0.16/-	Cyclopropylfentanyl and crotonylfentanyl (opioid)	F. blood: (16.6–28.9); *n* = 4
Majchrzak et al. (2018)[c]	LLE	LC-MS/MS	Body fluids: 9.0–27.2; tissues: 15.0–46.0/-	N-PP (Cath)	N-PP: blood (3,100); *n* = 1
Mardal et al. (2018)[b]	LLE/PP	UHPLC-MS/MS (ID, Q); UHPLC-HR-MS/MS (MI)	-/7–68	Methoxyacetylfentanyl (opioid)	F. blood (22–56); *n* = 3
Moody et al. (2018)[b]	SPE	LC–MS/MS (Q); LC-TOF (SCR)	0.0125–0.25/0.05–0.5	4-ANPP, 2-Furanylfentanyl, carfentanil, fluorobutyrylfentanyl, U-47700, acrylfentanyl, butyrylfentanyl, fluorofentanyl, 4-methoxybutyrylfentanyl and valerylfentanyl (opioid)	Blood: 4-ANPP (0.1–410), n = 1,549; 2-furanylfentanyl (0.1–710), *n* = 1,228; carfentanil (0.1–120), *n* = 697; fluorobutyrylfentanyl (0.1–760), *n* = 563; U-47700 (0.2–3,800), *n* = 543; acrylfentanyl (0.1–29), *n* = 266; butyrylfentanyl (0.1–760), *n* = 142; p-fluorofentanyl (0.1–1), *n* = 31; o-fluorofentanyl (2.4), *n* = 1; 4-methoxybutyrylfentanyl (79), *n* = 1; valerylfentanyl (0.44), *n* = 1
Nooble et al. (2018)[b]	PP/SPE	LC-QTOF-MS (SCR); UHPLC-MS/MS: (Q)	1–5/5	Fentanyl (opioid)	Blood: fentanyl (7–39); *n* = 17
Partridge et al. (2018)[b]	LLE	LC-QTOF: (SCR, Q, MI)	0.8–3/-	U-47700 (opioid); Diclazepam and flubromazepam (D-BZD)	P. blood: U-47700 (330), diclazepam (70), flubromazepam (10); *n* = 1
Pieprzyca et al. (2018)[b]	PP	LC-MS/MS	5/10	PV8 (Cath)	PV8: blood (70–260), urine (110–130); *n* = 2

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TABLE 1 (*Continued*) Summary of results found in the 96 papers included in this review that investigated new psychoactive substances in blood and/or urine from fatal cases. Aditional information of each study can be found in Supplementary Table S1.

References	Extraction method	Analytical techniques	LOD/LOQ (ng/mL or ng/g) [a]	Substance (class)	Blood/urine, analyte concentration and number of postmortem/death cases[a]
Rohrig et al. (2017)[b]	SPE	GC-MS (SCR); GC-NPD (SCR, Q)	25/-	U-47700 (opioid)	U-47700: H. blood (260), F. blood (400), urine (4,600); *n* = 1
Strehmel et al. (2018)	PP	LC-QTOF-MS (SCR); LC-MS/MS: (Q)	-	U-47700 (opioid)	U-47700 (μg/ml): F. blood (290), H. blood (12,500), urine (240); *n* = 1
Tomczak et al. (2018)[b]	LLE	GC-MS	0.3/1	4-CMC (Cath)	Blood: (56.2–1870); *n* = 6
Adamowicz et al. (2019)[b]	PP	LC-MS/MS	-/0.1	AMB-FUBINACA and EMB-FUBINACA (SC)	AMB-FUBINACA, EMB-FUBINACA, respectively: blood (ND, ND), urine (4.7, 0.2); *n* = 1
Al-Matrouk et al. (2019)	SPE	LC–MS/MS and LC-HRMS (SCR)	-	5F-AB-PINACA, AB-PINACA, AB-CHIMICA, FUB-AMB, 5F-AB-PINACA, 5F-AKB-48, 5Cl-AKB-48, ADB-PINACA and 5F-ADB (SC)	Urine: only qualitative analysis (*n* = 6)
Ameline et al. (2019)[b]	LLE	GC-MS (SCR); UPLC-MS/MS (Q)	-	3-MeO-PCP (PCY)	P. blood (498), CAR (743), urine (16.7); *n* = 1
Chesser et al. (2019)[b]	SPE	LC-MS/MS	0.05–0.1/0.1	4-ANPP, acetylfentanyl, fentanyl, furanylfentanyl, norfentanyl and U-47700 (opioid)	Blood (femoral, cardiac, iliac, subclavian) (0.1–45; 0.1–227; 0.1–98; 0.2–89; 0.1–38; 0.4->500), for 4-ANPP, acetylfentanyl, fentanyl, furanylfentanyl, norfentanyl, U-47700, respectively; *n* = 58
De Jong et al. (2019)	SPE	UPLC-MS/MS (Q); LC-QTOF-MS (SCR)	-	3-MeO-PCP (PCY)	Serum (123), blood (152); *n* = 1
Deville et al. (2019)[b]	LLE	GC-MS and UPLC-TOF-MS (SCR, ID); HPLC-DAD (Q)	-	MDAI (AI); 5-EAPB (Cath)	MDAI, 5-EAPB, 5-MAPB, 5-APB, respectively: blood (2090, 6,450, 89, 546); urine (69,400, 14,800, 1,000, 48,800); *n* = 1
Fagiola et al. (2019)[b]	LLE	LC-MS/MS	2.5/-	Cyclopropylfentanyl (opioid)	CAR (5.6–82); *n* = 5
Fels et al. (2019)[b]	LLE/SPE	LC-QTOF-MS (ID, Q)	5/10	U-47700 (opioid)	U-47700: F. blood (27–2,200), H. blood (39–4,900), urine (100–5,400); *n* = 26
Freni et al. (2019)[b]	SPE	LC-MS/MS	0.03–0.1/-	Furanylfentanyl and 4-ANPP (opioid)	Furanyl fentanyl and 4-ANPP, respectively: CAR (11.8; 93.5), F. blood (2.7; 50.4), urine (71.3; 171.7); *n* = 1
Gaulier et al. (2019)[b]	SPE	LC-QTOF (SCR); LC-MS/MS (Q)	0.05/0.1	Carfentanil (opioid)	Blood (4.20), urine (0.40); *n* = 1
Ivanov et al. (2019)	LLE	GC-MS (ID); HPLC-UV (Q)	5F-ADB 25/-	5F-ADB and FUB-AMB (SC)	5F-ADB: blood (3.7); *n* = 1
Kovács et al. (2019)[b]	LLE	LC-MS/MS	0.01–10/-	N-ethylhexedrone (Cath); ADB-FUBINACA (SC)	Blood: NEH (285), ADB-FUBINACA (0.08); *n* = 1
Kriikku et al. (2019)[b]	SPE	UPLC-TOF-MS (SCR); GC-MS: (Q)	10/20	U-47700 (opioid)	Blood (150–2000), *n* = 10; urine (20–2,200), *n* = 12
Krotulski et al. (2019)	LLE/SPE	LC-QTOF (ID, MI)	-	4F-MDMB-BINACA (SC)	Blood and urine: qualitative analysis; *n* = 20
Lehmann et al. (2019)	SPE/QuEChERS	LC-MS/MS	-	Diclazepam and pyrazolam (D-BZD); 3-FPM (PHEN)	Diclazepam, pyrazolam, 3-FPM, respectively: F. blood (1; 28; 10), H. blood (1; 28; 9), urine (1; 500; 120); *n* = 1
Margasińska-Olejak et al. (2019)[b]	LLE	LC-MS	-	3-MMC (Cath)	Blood (800); *n* = 1
Nash et al. (2019)[b]	LLE	LC-QTOF (SCR, Q)	-	Furanylfentanyl (opioid); MMMP (Cath)	P. blood: furanylfentanyl (1.6), MMMP (6.7); *n* = 1

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TABLE 1 (*Continued*) Summary of results found in the 96 papers included in this review that investigated new psychoactive substances in blood and/or urine from fatal cases. Aditional information of each study can be found in Supplementary Table S1.

References	Extraction method	Analytical techniques	LOD/LOQ (ng/mL or ng/g) [a]	Substance (class)	Blood/urine, analyte concentration and number of postmortem/death cases[a]
Theofel et al. (2019)[b]	PP/SPE	LC-MS/MS (Q)	3/5	N-ethyldeschloroketamine (PCY)	N-ethyldeschloroketamine: urine (3,468), H. blood (2,159), F. blood (375); *n* = 1
Yeter and Erol Öztürk, (2019)[b]	SPE	LC – HRMS (ID, Q)	Blood: 0.08; urine: 0.10/blood: 0.10; urine: 0.12	5F-ADB and its methyl ester hydrolysis metabolite (SC)	Blood: 5F-ADB (0.10–1.55), 5F-ADB metabolite (0.15–23.4), *n* = 70; urine: 5F-ADB metabolite (0.28–72.2), *n* = 34
Adamowicz et al. (2020a)	LLE	LC-MS/MS	0.3/5	α-PiHP (Cath)	α-PiHP: blood (69), urine (2072); *n* = 1
Adamowicz et al. (2020b)[b]	LLE	LC-MS/MS	0.01–0.20/-	Benzylfentanyl (opioid)	Blood: Benzylfentanyl (66; 110); fentanyl (31; 32); norfentanyl (22; 41); 4-FiBF (74); despropionyl-4-FF (6.5); *n* = 3
Benedicte et al. (2020)[b]	LLE	GC-MS (SCR); LC-HRMS: (ID, MI)	0.5/1	MPHP and N-ethyl-4′methylpentedrone (Cath)	MPHP and 4-MEAP, respectively: F.blood (47; 1.6), CAR (97; 3.5), urine (2,380; 49,700); *n* = 1
Ditrana et al. (2020)[b]	PP	HPLC-MS/MS	Blood: 0.03–0.35; urine: 0.02–0.25/ blood: 0.08–1; urine: 0.06–0.5	Cyclopropylfentanyl, methoxyacetylfentanyl, furanylfentanyl, acetylfentanyl, 4-ANPP and fentanyl (opioid)	Blood (0.2–9); urine (0.2–8,900), for fentanyl derivatives; *n* = 41
Garneau et al. (2020)	SPE	GC-MS (SCR); LC-MS/MS (SCR, Q)	-	4-ANPP, furanylfentanyl, U-47700, p-fluorobutyrylfentanyl, methoxyacetylfentanyl, cyclopropylfentanyl/crotonylfentanyl, acetylfentanyl, despropionyl fluorofentanyl and N-methyl U-47931 E (opioid)	Cardiac and F. blood, respectively: 4-ANPP (33–32; 18), furanylfentanyl (14–2.4; 0.89) and U-47700 (54–45; 26); *n* = 2. Cardiac and F. blood, respectively: 4-ANPP (5.1; 9.7), p-fluorobutyrylfentanyl (31; 27), methoxyacetylfentanyl (70; 14), cyclopropylfentanyl/crotonylfentanyl (0.15; 0.1), only detected: U-47700, acetylfentanyl, despropionyl fluorofentanyl, N-methyl U-47931E; *n* = 1
Hvozdovich et al. (2020)	SPE	LC-MS/MS	-	5F-ADB, FUB-AMB, 5F-AMB, MDMB-FUBINACA, and AB-CHMINACA (SC)	Blood and/or urine: only qualitative analysis; *n* = 54.5F-ADB was the most prevalent substance
Kriikku et al. (2020)[b]	LLE	GC-NCI-MS	1/-	Flualprazolam (D-BZD)	Blood (3.0–68); *n* = 33
Krotulski et al. (2020a)	LLE/SPE	LC-QTOF-MS (SCR, MI)	-	APP-BINACA (SC)	Blood and urine: only qualitative analysis; *n* = 11
Krotulski et al. (2020b)[c]	LLE	LC-MS/MS (Q); LC-QTOF-MS (MI)	<0.02/-	Isotonitazene (opioid)	Blood (0.4–9.5), *n* = 18; urine (0.6–4.0), *n* = 6; *n* = 1
Lehmann et al. (2020)[b]	SPE	LC-MS/MS	0.4–4/5	PMMA, PMA, PMEA, 2-FA, 4-FA, 2-FMA, 3-FPM, 2-DPMP, MDEA, MDMA, MDA and methiopropamine (PEA); 3-MeO-PCP and MXE (PCY); m-CPP (piperazine); MDPBP, MDPV, 4-MEC, methedrone, methylone and α-PVP (Cath); U-47700 (opioid); pyrazolam, diclazepam; delorazepam; lormetazepam (D-BZD)	AMP and analogs (PMMA, PMA, PMEA, 4-FA, 2-FA, 2-FMA, methiopropamine, MDMA, MDA, MDEA, amphetamine, *n* = 13): 4.5–185000 (urine); 2.2–2,500 (blood). M-CPP (*n* = 1): 130 (urine), 5.3 (blood); MXE (*n* = 4): 6.6–22300 (urine), 1–390 (blood); 3-FPM (*n* = 1): 120 (urine), 5.3 (blood); U-47700 (*n* = 1): 1,500 (urine); 2-DPMP (*n* = 1): 52 (urine), 5.2 (blood); 3-MeO-PCE (*n* = 1): 3.6 (urine); BZD: (Pyrazolam, diclazepam, delorazepam, lormetazepam, *n* = 1): 1–100 (blood); Cath: (4-MEC, MDPV, methedrone, methylone, MDPBP, α-PVP, *n* = 4: 6.2–830 (urine). 3.6–340 (blood); *n* = 17

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TABLE 1 (*Continued*) Summary of results found in the 96 papers included in this review that investigated new psychoactive substances in blood and/or urine from fatal cases. Aditional information of each study can be found in Supplementary Table S1.

References	Extraction method	Analytical techniques	LOD/LOQ (ng/mL or ng/g) [a]	Substance (class)	Blood/urine, analyte concentration and number of postmortem/death cases[a]
Tiemensma et al. (2020)	NA	GC-MS and LC-MS	-	Cumyl-PEGACLONE (SC)	Blood (0.73–3.0); n = 5
Woods, (2020)[b]	LLE	GC-MS	<10/50	Mebroqualone (Meth)	F. blood (10,228; 115); *n* = 2
Zawadzki et al. (2020a)[b]	LLE	UHPLC-MS/MS	-/0.1	5F-CUMYL-P7AICA (SC)	Blood (2.8), urine (3.1); *n* = 1
Zawadzki et al. (2020b)[b]	LLE	UHPLC-MS/MS	-/1	N-ethylpentylone (Cath)	P. blood (10,600), urine (17,600); *n* = 1
Arbouche et al. (2021)	LLE	LC-MS/MS (Q); LC-HRMS (ID, MI)	-	3-MeO-PCP (PCY)	F. blood (525), urine (384); *n* = 1
Brahan et al. (2021)[b]	LLE	GC-MS/MS	-/1,000	4-MEC (Cath)	4-MEC: P. blood (14,600), CAR (43,400), urine (619,000); *n* = 1
Castellino et al. (2021)	LLE	GC-MS	1.0/-	Cyclopropylfentanyl (opioid)	Blood (14), *n* = 1; Other case: only detected, *n* = 1
Cartiser et al. (2021)[b]	SPE	GC-MS	-	4-MPD (Cath)	4-MPD: P. blood (1,285), CAR (1,128), urine (>10,000); *n* = 1
Chan et al. (2021)[b]	PP	LC-MS/MS	-	Carfentanil (opioid)	P. blood (0.5), (*n* = 1); iliac blood (0.9), *n* = 1
Ferrari Júnior and Caldas (2021)[b]	QuEChERS	UHPLC-MS/MS	4/10	N-ethylpentylone (Cath)	Blood (597); *n* = 1
Gicquel et al. (2021)[b]	SPE	LC-MS/MS (SCR); LC-HRMS (SCR, Q)	5/10	2F-DCK and 3-MeO-PCE (PCY)	2F-DCK, 3-MeO-PCE and 5-MeO-DMT, respectively: P. blood (1780; 90; 52), urine (6,100; 6,300; 2,200); *n* = 1
Hofmann et al. (2021)[b]	PP	HPLC-MS/MS	1.8–2.6/4.6–6	5-APB and 6-APB (PEA)	5-APB and 6-APB, respectively: C. blood (2,400; 660), P. blood (850; 300), urine (8,700; 3,400; *n* = 1
Kronstrand et al. (2021)[b]	PP	LC-MS/MS (Q). LC-QTOF-MS (MI)	-/2	Methoxyacetylfentanyl (opioid)	F. blood: (18–140); *n* = 10
Krotulski et al. (2021a)	LLE and PP/SPE	LC-MS/MS (Q); LC-TOF-MS (SCR); LC-QTOF-MS (MI)	-/1	Eutylone (Cath)	Blood (1,2–11000), *n* = 67; urine (60; 3,400; and >10,000), *n* = 3
Krotulski et al. (2021b)[c]	LLE	LC-MS/MS (Q); LC-QTOF-MS: (MI)	<0.1/-	Brorfine (opioid)	Blood: 0.1–10; *n* = 20
Krotulski et al. (2021c)[b]	LLE	LC-MS/MS (Q); LC-QTOF-MS (SCR, MI)	0.1/0.5	Metonitazene (opioid)	Blood (0.5–33), urine (0.6–46); *n* = 20
Krotulski et al. (2021d)	LLE/SPE	LC-QTOF-MS (ID, MI)	-	MDMB-4en-PINACA, 5F-MDMB-PICA and 4F-MDMB-BINACA (SC)	Blood: qualitative analysis; *n* = 16
Mochizuki et al. (2021)[b]	SPE	LC-LIT-MS: (ID, Q); GC-MS (ID)	0.1–1/-	4-FMC, 4-MeO-α-PVP, 4-F-α-PVP and PV8 (Cath)	4-FMC, 4-MeO-α-PVP, 4-F-α-PVP and PV8, respectively: H. blood (365; 449; 145; 218), F. blood (397; 383; 127; 167); *n* = 1
Mueller et al. (2021)[b]	SPE	UHPLC-MS/MS	0.01/0.05	Isotonitazene (opioid)	Isotonitazene: F. blood (2.28; 0.59; 0.74), CAR (1.7; 1.13; 0.7), urine (1.88; 3.37; 0.19); *n* = 3
Palazzoli et al. (2021)[b]	PP/SPE	LC-MS/MS	0.1–0.5/0.5–1	Mephedrone, DHM and NORMEP (Cath)	Mephedrone, NORMEP and DHM, respectively: F. blood: (1,088; 47.1; 15.5), C. blood (1,632; 50.2; 49.2), urine (4,443; 740.2; 171.9); *n* = 1

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TABLE 1 (*Continued*) Summary of results found in the 96 papers included in this review that investigated new psychoactive substances in blood and/or urine from fatal cases. Aditional information of each study can be found in Supplementary Table S1.

References	Extraction method	Analytical techniques	LOD/LOQ (ng/mL or ng/g) [a]	Substance (class)	Blood/urine, analyte concentration and number of postmortem/death cases[a]
Solbeck et al. (2021)[b]	SPE	LC-MS/MS (Q). LC-QTOF-MS, GC-NPD and GC-MS (SCR)	0.05/0.1	Carfentanil (opioid)	Blood (<0.1–9.2); *n* = 160
Theofel et al. (2021)[b]	NA	GC-MS and LC-QTOF-MS/MS (SCR); LC-MS/MS (Q)	-	2-MAPB (Cath)	2-MAPB: urine (167,000), H. blood (16,700), F. blood (7,300); *n* = 1
Zawadzki et al. (2021)[b]	LLE	UHPLC-MS/MS	0.05/0.1	4-FiBF (opioid)	4-FiBF: blood (76.1–257), urine (289–1,000), VH (89.9–150); *n* = 4

[a]When necessary, concentrations reported in the studies were converted to ng/mL or ng/g to facilitate the comparison among the methods.
[b]Papers that described validation procedures.
[c]Papers that described quantitation by standard addition; C-NMR: carbon-13, nuclear magnetic resonance; EI: electron impact ionization; ELISA: enzyme-linked immunoassay; FT-IR: Fourier-transform infrared spectroscopy; GC-IR: gas chromatography–infrared spectroscopy; GC-MS: gas chromatography coupled to mass spectrometry; GC-MS/MS: gas chromatography coupled to tandem mass spectrometry; GC-NCI-MS: gas-chromatography negative-chemical-ionization mass spectrometry; H-NMR: proton nuclear magnetic resonance; HPLC-DAD: high performance liquid chromatography-diode-array detector; HPLC–DAD-FLD: high performance liquid chromatography-diode-array and fluorescence detectors; HPLC-MS/MS: high performance liquid chromatography-tandem mass spectrometry; HPLC-UV: high performance liquid chromatography-ultraviolet detector; HRMS: high-resolution mass spectrometry; ID: identification; LC-DAD: liquid chromatography-diode-array detector; LC-HRMS: liquid chromatography-high-resolution mass spectrometry; LC-MS: liquid chromatography-mass spectrometry; LC-PDA: liquid-chromatography-photodiode array detector; LC-MS/MS: liquid chromatography-tandem mass spectrometry; LC-QTOF-MS: liquid chromatography-quadrupole time-of-flight mass spectrometry; LC–TOF-MS: liquid chromatography-time of flight mass spectrometry: LC-UV: liquid chromatography-ultraviolet detector; MI: metabolite investigation; MRM: multiple reaction monitoring; NMR: nuclear magnetic resonance; NPS: new psychoactive substance; Q: quantification; SCR: screening; SIM: selective ion monitoring; UHPLC-MS/MS: ultra high performance liquid chromatography-tandem mass spectrometry; UHPLC-QTOF-MS: ultra high performance liquid chromatography-quadrupole time-of-flight mass spectrometry; UPLC-MS/MS: ultra performance liquid chromatography-tandem mass spectrometry; UPLC-TOF-MS: ultra performance liquid chromatography-time-of-flight mass spectrometry; UPLC-PDA: ultra performance liquid-chromatography-photodiode array detector; UV-VIS: ultraviolet/visible spectrophotometry. **Extraction methods**: LLE: liquid-liquid extraction; LLE-LTP: liquid-liquid extraction with low-temperature partition; PP: protein precipitation; QuEChERS: quick, easy, cheap, effective, rugged, and safe; SPE: solid phase extraction. **Substances**: 2-FA: 2-Fluoroamphetamine; 2-FMA: 2-Fluoromethamphetamine; 2-Oxo-PCE: N-ethyldeschloroketamine; 3-FPM: 3-fluoro-phenmetrazine; 3-MMC: 3-methylmethcathinone; 4-FA: 4-Fluoroamphetamine; 4-FMA: 4-Fluoromethamphetamine; 4-FBF: 4-fluorobutyrfentanyl; 4-FiBF: 4-fluoroisobutyryl fentanyl; 4-MEAP: N-ethyl-4′methylpentedrone; 4-MEC: 4-methylethcathinone; 4-MPD: 4-methylpentedrone; 5F-MDMB-PINACA: 5F-ADB; α-PiHP: alpha-Pyrrolidinoisohexaphenone; AI: aminoindane; AMP: amphetamine; BZD: benzodiazepine; BZE: benzoylecgonine; Cath: synthetic cathinone; COC: cocaine; D-BZD: designer-benzodiazepine; DHM: dihydro-mephedrone; MDA: methylenedioxyamphetamine; MDMA: methylenedioxymethamphetamine; Meth: Methaqualone analog; MMMP: 2-methyl-4'-(methylthio)-2-morpholinopropiophenone; MAMP: metamphetamine; N-PP: α-propylaminopentiophenone; NA: not available; ND: non-detected; NORMEP; Nor-mephedrone; PCY: phencyclidine analog; PEA: phenethylamine; PHEN: phenmetrazine analog; PMMA: para-methoxymethamphetamine; SC: synthetic cannabinoid; THC-COOH: 11-Nor-9-carboxy-THC; THC: tetrahydrocannabinol; THFF: Tetrahydrofuranylfentanyl. **Biological fluid/tissues:** C. blood: central blood; CAR: cardiac blood; P. blood: peripheral blood; F. blood: femoral blood; H. blood: heart blood.

high sensitivity (Poklis et al., 2016; Paul et al., 2017; Staeheli et al., 2017; Pieprzyca et al., 2018; Adamowicz et al., 2020a; Chan et al., 2021) compared to LC-MS and HPLC-DAD (Adamowicz et al., 2020b).

In forensic toxicology, an extraction/cleanup protocol must guarantee the recovery of a wide range of substances with different physicochemical properties, especially when there is no suspicion of the involved substance (Ferrari Júnior and Caldas, 2018). In total, 16 studies included in this review used protein precipitation (PP) as an intermediate or only extraction step, a simple and fast protocol that presents a poor cleanup. Fifty one studies applied liquid-liquid extraction (LLE) using different solvent systems (mostly using alkaline extraction) (Rojkiewicz et al., 2016; Kriikku et al., 2020), 36 used solid phase extraction (SPE) columns, after solvent/buffer addition, enzymatic hydrolysis and/or PP (Rohrig et al., 2017; Garneau et al., 2020) and five studies used QuEChERS (quick, easy, cheap, effective, rugged, and safe) methods (Table 1), which is a combination of LLE and salts and dispersive SPE with primary and secondary amine (PSA) (Fujita et al., 2016; Ferrari Júnior and Caldas, 2021).

LOD/LOQ assessment can demonstrate if a proposed method is suitable for the analysis of NPS that cause effects at low blood concentrations, and both the extraction/cleanup protocol and the analytical instrumentation must be correctly chosen in search of a better sensitivity. Overall, the lowest LOD/LOQ were achieved by LC-MS/MS. In blood, LOQs in the reviewed studies are mostly below 1 ng/ml, such as 0.2 ng/ml for the opioid benzylfentanyl, using LLE (Adamowicz et al., 2020a), 0.05 ng/ml for isotonitazene (Mueller et al., 2021) and 0.1 ng/ml for the synthetic cannabinoid 5F-ADB, the last two using SPE (Yeter and Erol Öztürk, 2019). Chan et al. (2021) did not inform the LOQ of the LC-MS/MS method, but the authors

reported the detection of 0.5 ng/ml of the opioid in blood analysis, using protein precipitation.

Using GC-MS, regardless of the extraction protocols used, the determined LOQs in blood were generally higher, such as 10 ng/ml for N-ethylpentylone (Atherton et al., 2018), 400 ng/ml for MDPV and 3,000 ng/ml for pentedrone (Liveri et al., 2016). Solbeck et al. (2021) stated that GC-MS and GC-NPD screening demonstrated insufficient sensitivity for carfentanil, with a LOD of ~10 ng/ml in blood. Tomczak et al. (2018) reported a LOQ of 1 ng/ml for 4-CMC using LLE followed by GC-MS after derivatization, a step that is time consuming in a routine work (Ferrari Júnior et al., 2020).

Only two studies include method validation data for matrices other than blood and urine, although quantitative information was provided (Supplementary Table S1). The lack of validation is a major limitation of the reported values in gastric content and tissue samples, as they are matrices with higher complexity compared to blood and urine. Using LC-MS/MS, Chesser et al. (2019) reported LOQ of 0.01 ng/g for opioids in brain and vitreous humor and Palazzoli et al. (2021) reported LOQs of 0.5 or 1 ng/ml or ng/g in liver, kidney, bile and hair for mephedrone and its metabolites.

LC enables other high-resolution hyphenated techniques, such as quadrupole time-of-flight mass analyzers (QTOF), Orbitrap™, that features high mass accuracy being a tool for untargeted screening analysis and for structural characterization and identification of unknown compounds (Theofel et al., 2021). The full scan HRMS data may also be performed to NPS metabolite investigations, which can aid in compound identification (Wiergowski et al., 2017; Allibe et al., 2018; Mardal et al., 2018; Moody et al., 2018; Noble et al., 2018; Partridge et al., 2018; Krotulski et al., 2019; Krotulski et al., 2020a). The metabolite identification helps to understand the metabolic pathway, indicate the presence of active/toxic metabolites (e.g., cocaethylene, produced by the concomitant use of cocaine and alcohol) (Atherton et al., 2018). Sometimes, the metabolite may be the only substance detected when the ingested substance has already undergone biotransformation (Yeter and Erol Öztürk, 2019; Ferrari Júnior and Caldas, 2021).

Furthermore, high resolution techniques are important for monitoring the emergence of new substances onto the market. HRMS, however, requires well-skilled experts and it is a more expensive technique. HPLC-DAD is a good screening and quantification technique, however, it needs mass spectral analysis for compound identification (Angerer et al., 2017; Bottinelli et al., 2017; Maher et al., 2018; Deville et al., 2019; Ivanov et al., 2019). Another HPLC-DAD application would be the differentiation of isomers by the UV spectra (Botinelli et al., 2017). The presence of structural isomers is common among different NPSs, which sometimes becomes a challenge for the analyst. Mayer et al. (2018) found identical fragmentation pattern of the two isomers cyclopropylfentanyl and crotonylfentanyl, and they showed similar relative abundances by LC-MS and UHPLC-QTOF-MS. Despite the small retention time differences, UV spectral differentiation was possible using HPLC-DAD, although it would be necessary to run reference standards to mitigate any system variability. Baseline separation of the two isomer was, however, achieved by Fagiola et al. (2019) using LC-MS/MS, which was also used by DiTrana et al. (2020) to analyse cyclopropylfentanyl and its metabolite cyclopropylnorfentanyl.

The difficulty of distinguishing the 3 isomers of methylmethcathinone (2-MMC, 3-MMC and 4-MMC) in a 3-MMC intoxication case report was overcome by HPLC-DAD analysis, with each isomer showing different spectrum profiles (Botinelli et al., 2017). Theofel et al. (2021) used GC-IR and HPLC-QTOF-MS to identify the correct positional isomer of MAPB (2-MAPB, 5-MAPB or 6-MAPB) in a yellow liquid involved in a fatal case, and the results confirmed the presence of 2-MAPB. LC-QTOF-MS, in the low energy range, was also used to distinguish the isomers 3- and 4-MeO-PCP based on the different relative ratios of the fragments 189 and 274 m/z (De Jong et al., 2019). The ion ratio approach was also used by Krpo et al. (2018) to differentiate between the positional isomers 5-APB and 6-APB by UHPLC-QTOF-MS and UHPLC-MS/MS analysis to solve a fatal case.

With the emergence of new substances on the drug market, intoxication cases involving NPS may not be elucidated so quickly, which makes the reanalysis of the data previously acquired by high resolution techniques, such as LC-QTOF-MS, a mean of understanding these unresolved intoxication cases. In Finland, stored TOF-MS data of blood samples were reprocessed and showed two additional U-47700 positive cases (Kriikku et al., 2019). In Australia, initial screening analysis by LC-QTOF-MS of the postmortem peripheral blood detected methylamphetamine, amphetamine and lorazepam, and some months later, retrospective data analysis also detected U-47700, 2,5-dimethoxy-4-chloroamphetamine, diclazepam and flubromazepam, which were also confirmed in the urine samples (Partridge et al., 2018).

Non-biological material analysis

Seized drug and other materials found near the victim can be an important source of information, guiding the toxicological screening and contributing to NPS discovery. Some papers retrieved in this review did describe the analysis of these materials (e.g., Papsun et al., 2016; Yonemitsu et al., 2016; Botinelli et al., 2017; Al-Matrouk et al., 2019; Deville et al., 2019; Ivanov et al., 2019; Gicquel et al., 2021). The drug characterization is also important to alert toxicology laboratories about possible new drugs on the market. As example, the characterization of synthetic cannabinoids 4F-MDMB-BINACA (Krotulski et al., 2019) and APP-BINACA (Krotulski et al., 2020a) in seized drugs performed by GC-MS, LC-QTOF-MS and NMR, showed the presence of new

substances in the American market, which were also confirmed in biological samples.

High purity drugs found on the site is common and can help elucidating a possible accidental overdose. Mueller et al. (2021) reported isotonitazene powder (higher than 95% purity) found on the site, determined by GC–MS and proton NMR. In an intoxication case involving U-47700 abuse, the analysis of the seized powder by LC-DAD and NMR revealed a purity higher than 85% (Strehmel et al., 2018).

Due to the constant change of the NPS market, the reference standard availability is an issue for toxicology laboratories and the use of high purity seized materials can be an alternative during routine work. Rojkiewicz et al. (2016) reported that a powder from a 4-FBF fatal case, was analyzed by UV-VIS, LC-MS (ion trap MS in MS^2 and MS^3), FT-IR, GC-MS and NMR and used as a reference material for toxicological screening. Benedicte et al. (2020) used seized drugs (powders) characterized by LC–HRMS and NMR spectroscopy and showed to contain MPHP and 4-MEAP of 85% purity for the determination of these drugs in biological samples from a real case.

Fatal cases involving new psychoactive substance intake

Table 1 summarizes the concentration range of the main NPS reported in serum/blood and urine samples analyzed in the investigation of the fatal cases reviewed in this paper. In total, 28 opioids, 26 synthetic cathinones, 12 synthetic cannabinoids, 8 phenethylamine/amphetamines, 5 designer benzodiazepines and 5 phencyclidines were detected in blood samples (Table 1). Details of all studies are shown in Supplementary Table S1, including NPS detection in tissues and other matrices and all the substances found in the samples.

Blood is the most used biological fluid to evaluate the function of a drug in modifying human behavior and to investigate intoxication cases, as the blood concentration can be closely correlated with the pharmacological and toxic effects, providing pharmacokinetic data and comparison with the presented clinical signs (Elliott et al., 2018; Ferrari Júnior and Caldas, 2021). Although urine drug concentration should not be used to interpret the effect of a drug on humans, it gives a larger detection window when compared to blood (Ferrari Júnior and Caldas, 2021). Furthermore, in most studies included in this review, the drugs found in blood were detected in urine samples, which also contain the drug metabolites.

In blood and urine, synthetic cannabinoids showed concentrations below 100 ng/ml and, overall, cathinones exhibited the highest concentrations among the reported NPS classes, including eutylone and N-ethylpentylone (higher than 10,000 ng/ml) and 4-methylethcathinone (4-MEC; up to 619,000 ng/ml). Some substances presented a large concentration range in blood from the various studies, as U-47700 (0.2–3,800 ng/ml), 4-chloromethcathinone (4-CMC; 0.887–1870 ng/ml) and N-ethylpentylone (31–10600 ng/ml).

Most studies (67.4%) reported NPS detection along with other substances (Supplementary Table S1), which is very relevant as multiple drugs intake may lead to the interaction among the substances and hinder the identification of the drug or drugs that lead to fatality. Some studies of the main NPS classes are discussed further in this review.

Opioids

Opioids are a group of drugs comprising a range of substances, including opiates and their synthetic analogues that bind to opioid receptors. Morphine, codeine and thebaine are called opiates, naturally occurring alkaloids found in the opium poppy and their semi-synthetic derivatives include hydrocodone, heroin, oxycodone and buprenorphine. Opioids also include synthetic substances, as methadone, tramadol, fentanyl, and other derivatives (UNODC, 2021c). New synthetic opioids, including fentanyl analogues, have been appearing on the drug market in the last two decades and their extreme potency at very low doses leads to fatal poisonings and have become a problem for both law enforcement authorities and public health professionals, being treated in the United States as an epidemic crisis (UNODC, 2021b). Overall, synthetic opioids were the drug class most found in studies, reported in 43 papers included in this review.

Dwyer et al. (2018) reported 41 deaths involving acetyl fentanyl in Pennsylvania (United States), with the blood concentrations ranging from 0.13 to 2,100 ng/ml. In one case, only the acetyl fentanyl (170 ng/ml) was detected, but in most cases, the deaths were concluded as multiple drug toxicity, including fentanyl (26 blood samples, 0.24–60.9 ng/ml), cocaine, heroin and alcohol.

An Italian fatal intoxication case involving furanyl fentanyl was reported by Freni et al. (2019). A 53-year-old man was found dead with a needle inserted in a vein; a white powder found in the room contained the drug and N-phenetyl-piperidine (4-ANPP), a precursor of the manufacture of fentanyl-type drugs, and also a metabolite. Furanyl fentanyl levels ranged from 2.6 ng/ml in gastric content to 40.1 ng/ml in cerebrospinal fluid (CSF), and 4-ANPP levels ranged from 0.6 (CSF) to 93.5 ng/ml (cardiac blood). The presence of the substances in gastric content indicated not only intravenously but also the oral use of the product.

Maher et al. (2018) determined the synthetic opioid cyclopropylfentanyl in four fatalities that occurred in the United Kingdom, with femoral blood concentrations ranging between 16.6 and 28.9 ng/ml. Cyclopropylfentanyl was deemed to have contributed to death in all four cases, even in the presence of other drugs (not described in the paper). In Italy, cyclopropylfentanyl was detected in 7 postmortem blood

(0.8–21 ng/ml) and 11 urine samples (1.3–108 ng/ml). However, the cause of death was not concluded in the study (DiTrana et al., 2020).

Two poisoning cases involving carfentanil in Hong Kong showed blood concentrations of 0.5 and 0.9 ng/ml, and the drugs were indicated as the cause of death (Chan et al., 2021). Carfentanil was detected (>0.05 ng/ml) in 160 Canadian fatal cases, with blood concentrations reaching 9.2 ng/ml (Solbeck et al., 2021); in 156 cases, the deaths were classified as mixed drug toxicity (mainly involving cocaine and fentanyl), and in two cases, only carfentanil was detected in blood at very low concentrations (<0.1–0.84 ng/ml), indicating the high lethality of the drug.

Two studies attributed the cause of death to intoxication by methoxyacetylfentanyl alone or in combination with other drugs in United States of America (10 cases; 18–140 ng/g in blood; Kronstrand et al., 2021) and in Denmark (3 cases, 22–56 ng/g in blood; Mardal et al., 2018). In Italy, methoxyacetylfentanyl were found in postmortem blood (2.5–91 ng/ml) and urine (70–1900 ng/ml), along with its metabolite methoxyacetylnorfentanyl and other synthetic opioids (DiTrana et al., 2020) (Table 1 and Supplementary Table S1). Other studies also described blood concentrations of fentanyl derivatives, including ocfentanil (15.3 ng/ml; Coopman et al., 2016), butyryl fentanyl (99–220 ng/ml; Poklis et al., 2016), 4-fluorobutyrylfentanyl (91–112 ng/ml; Rojkiewicz et al., 2016) and 4-fluoroisobutyryl fentanyl (76.1–257 ng/ml), in addition to synthetic cathinones (N-ethylpentylone, α-PiHP and 4-CMC; Zawadzki et al., 2021).

Reports of fatal cases involving U-47700, a selective agonist of the μ-opioid receptor developed in the 1970s, were retrieved in the search. Rohrig et al. (2017) reported an acute intoxication in United States at levels of 260 and 400 ng/ml in heart and femoral blood, respectively. Vitreous humor, brain, liver and urine showed concentrations ranging from 90 to 4,600 ng/ml. In Canada, the cardiac blood concentration of U-47700 in three fatal cases ranged from 45 to 54 ng/ml (Garneau et al., 2020), along with other opioids. Other cases involving toxic blood levels of U-47700 were also related in Italy (380 ng/ml, blood; Gerace et al., 2018) and Germany (370 ng/ml, blood; Koch et al., 2018), the latter case in association of the benzodiazepine flubromazepam (830 ng/ml).

Recently, a novel opioid class, the benzimidazole derivatives, has been detected in postmortem cases. Mueller et al. (2021) reported 3 fatal cases in Switzerland involving isotonitazene, with concentrations levels ranging from 0.59 to 2.28 ng/ml in blood and from 0.19 to 3.37 ng/ml in urine. Other drugs, including benzodiazepines, were detected within the therapeutic range, and based on circumstantial evidence, autopsy, and toxicological analysis, the death cause was concluded as acute intoxication with isotonitazene. In United States, isotonitazene was found in blood samples from 18 fatal cases, with only the opioid being detected in 9 cases (Krotulski et al., 2020b). The blood concentration ranged from 0.4 to 9.5 ng/ml, similar with those found by Mueller et al. (2021), highlighting that the drug may contribute to the fatal outcome even at low concentrations. After the introduction of isotonitazene, metonitazene and brorphine emerged as potent opioids involved in fatal cases in United States, with concentration in blood (n = 20) ranging from 0.5 to 33 ng/ml (metonitazene) and from 0.1 to 10 ng/ml (brorphine) (Krotulski et al., 2021b; 2021c).

Synthetic cathinones

Khat (*Catha edulis*) is a plant native to Africa and the Arabian Peninsula that contains cathinone, a β-keto amphetamine with mechanism of action similar to amphetamines (Baumann et al., 2018). Although synthetic cathinones are traditionally known as "bath salts", due to the presentation that was initially sold, these NPS are currently sold in pills, powders, crystals and other formulations.

In France, a case of 3-MMC (3-methylmethcathinone) abuse showed blood concentrations of 249 ng/ml (peripheral) and 609 ng/ml (cardiac) (Botinelli et al., 2017). In another case, a 19-year-old woman died after consuming 3-MMC; levels of 800 ng/ml were found in blood, 153 ng/ml in vitreous humor and 5.5 mg in gastric contents (Margasińska-Olejak et al., 2019).

In Brazil, two fatal cases involving N-ethylpentylone use in rave parties were reported, with postmortem blood concentrations of 170 ng/ml (32 y, man) (Costa et al., 2018) and 597 ng/ml (19 y, woman) (Ferrari Júnior and Caldas 2021). In both cases, the cathinone was the only psychoactive substance detected. This drug has been also associated with other fatal cases worldwide. In Poland, Zawadski et al. (2020b) reported a fatal intoxication of a 30-year-old man, with levels of 10,600 ng/ml in blood and 17,600 ng/ml in urine; in addition to eutylone and four N-ethylpentylone metabolites. Two fatal cases of 34-year-old males involving N-ethylpentylone in United States were reported by Atherton et al. (2018), with levels of 121 and 953 ng/ml in blood; in the first case, other drugs were also found and the cause of death was listed as due to methamphetamine, cocaine, fentanyl, and N-ethylpentylone intoxication.

Three studies reported the detection of synthetic cathinones along with synthetic cannabinoids in fatal cases. In Japan, Fujita et al. (2016) reported serum levels of mepirapim (950 ng/ml) and α-EAPP (α-ethylaminopentiophenone, 3,100 ng/ml). In a Polish fatal case, the synthetic cannabinoid UR-144 and the cathinone pentedrone was found in blood at 4 and 290 ng/ml, respectively, and the death was directly associated with the use of the drugs; two other individuals (UR-144 blood concentration of 2.1 and 1.4 ng/ml) committed suicide, probably due to the psychiatric effects of the drug (Rojek et al., 2017). In a Hungarian fatal case (23-year-old male) involving N-ethyl-hexedrone (NEH, cathinone) and ADB-FUBINACA, showed blood levels of

285 and 0.08 ng/ml, respectively, and five ADB-FUBINACA metabolites (Kovacs et al., 2019). As ADB-FUBINACA concentration was below the toxic level, the authors hypothesized that the cause of death was NEH intoxication, with heart disease being a co-factor.

Other synthetic cathinones were determined in blood/serum from acute intoxications, as shown in Table 1, including eutylone (Krotulski et al., 2021a), N-PP (Majchrzak et al., 2018), 4-MEC (Brahan et al., 2021), α-PVP (1.1–6,200 ng/ml) (Adamowicz et al., 2016; Beck et al., 2016; Potocka-Banas et al., 2017), MPHP (Benedicte et al., 2020), and mephedrone (Palazzoli et al., 2021). A fatal poisoning (20 y, male) after multiple cathinone consumption investigated by Mochizuki et al. (2021) showed concentrations of 4-FMC, 4-MeO-α-PVP, 4-F-α-PVP and PV8 ranging from 145 to 449 ng/ml in heart blood, and from 127 to 397 ng/ml in femoral blood.

Synthetic cannabinoids

Synthetic cannabinoids are chemically manufactured substances designed to activate endogenous cannabinoids receptors and mimic the psychological effects of THC (Krotulski et al., 2021d), with many groups not structurally related to THC or other natural cannabinoids. Some are still not controlled under international drug control systems and undetected in standard drug screens, characteristics that have contributed to their popularity among drug users.

Herbal mixtures containing the drugs and intended for smoking like marijuana are commonly found in the street drug market, but are also available as bulk powders or soaked or sprayed onto paper to facilitate smuggling into prisons *via* the postal service. In the United States, blood and urine from 54 prisoner fatal overdose cases showed the presence of 5F-ADB, FUB-AMB, 5F-AMB, MDMB-FUBINACA, and AB-CHMINACA (Hvozdovich et al., 2020). Other synthetic cannabinoids were the only drugs detected in 37 cases and were listed as the proximate cause of death.

In Bulgaria, an herbal mixture found in the scene of a fatal case was shown to contain 5F-ADB and FUB-AMB. The 18-years-old victim had been using the herb for several months and overuse it during the last 48 h (Ivanov et al., 2019). Both substances were found in blood and urine, and 5F-ADB blood level was 3.7 ng/ml. The autopsy findings revealed acute respiratory distress syndrome and the authors suggested that the case report could be discussed both as drug-induced and drug-related death resulting from acute intoxication with 5F-ADB and FUB-AMB (Ivanov et al., 2019). 5F-ADB and its methyl ester metabolite was reported by Yeter and Erol Öztürk (2019) in blood (n = 70) and urine (n = 34) of fatal cases in Turkey with concentrations ranging from 0.10–1.55 ng/ml (5F-ADB, blood), 0.15–23.4 ng/ml (blood, metabolite) and 0.28–72.2 ng/ml (urine, metabolite).

Kusano et al. (2018) also reported the consumption of herbal blend containing 5F-ADB by a Japanese 53-year-old male that resulted in a fatal intoxication. Blood concentrations were 0.19 ng/ml for 5F-ADB and 12 ng/ml for diphenidine, a phencyclidine analog. Investigation of the urinary metabolites revealed pathways involving ester hydrolysis and oxidative defluorination, and further oxidation to the carboxylic acid for 5F-ADB and mono- and di-hydroxylated diphenidine metabolites. The present case demonstrates the importance of urinary metabolite screening for drugs with low blood concentrations.

In Australia, five deaths were related to Cumyl-PEGACLONE use, a synthetic cannabinoid receptor agonist with a gammacarbolinone core (Tiemensma et al., 2020). Levels in postmortem blood ranged from 0.73 to 3.0 ng/ml, but in the case with the highest concentration, the cause of death was also due to acute alcohol intoxication (BAC: 0.24%).

A 29-year-old Polish man was found dead, and the confirmed cause was asphyxia from occlusion of the upper airway by a foreign material (Zawadski et al., 2020a). 5F-CUMYL-P7AICA was detected in blood (2.8 ng/ml) and urine (3.1 ng/ml), but not in the gastric contents. It was suspected that the man smoked the dried plant mixed with the powdered synthetic cannabinoid. No other substance was detected in the screening analysis.

Paul et al. (2017) reported two deaths involving synthetic cannabinoids abuse in United States. Blood analysis found AB-CHMINACA in case 1 (8.2 ng/ml) and UR-144, XLR-11, and JWH-022, in case 2 (12.3, 1.3 and 3 ng/ml, respectively), which, according to the authors, have contributed to the death. A fatal poisoning with AB-CHMINACA and ethanol was reported by Gieron and Adamowicz (2016), with AB-CHMINACA levels ranging from 0.1 (urine) to 2.7 ng/ml (blood from lung). In United States, a herbal incense (Apollo brand) was found with a deceased 34-years-old male and showed to contain 5F-AMB (Shanks and Behonick, 2016). The drug was found at 0.3 ng/ml in blood and as no other substance of toxicological interest was detected, the death was certified as accidental due to synthetic cannabinoid toxicity.

Angerer et al. (2017) reported 3 fatal cases (25–41- year-old males) involving synthetic cannabinoids in Germany. In one case, 5F-PB-22, cannabidiol, traces of AB-CHMINACA and 5F-AKB-48 were detected in the herbal blend 'Hammer Head', and 5F-PB-22 was found in the blood at 0.37 ng/ml; the metabolites 5F-PB-22 3- carboxyindole, PB-22 5-hydroxy-pentyl, and PB-22 5-pentanoic acid were detected in the urine. In case 2, the herbal blend 'Desert Premium Potpourri 2 g' was found at the scene and shown to contain AB-CHMINACA, which was present in blood at 4.1 ng/ml, and metabolites identified in urine. In case 3, 5F-ADB was found in the seized herbal blend and in blood (0.38 ng/ml); metabolites of 5F-ADB, NE-CHMIMO and MDMB-CHMICA were detected in urine. Considering the death scene, the autopsy and the full

toxicological analysis, the authors explained the deaths as consequence of synthetic cannabinoids use, although in the two first cases relevant amounts of ethanol were found in the blood (1.45–2.6 g/kg), which might have contributed to the outcome.

Postmortem cases involving other substances

Other substances involved in fatal intoxications include phenethylamines, phencyclidine analogues and designer benzodiazepines. Phenethylamines are amphetamine analogues with a phenethylamine core in their structure (Figure 1) and also include ring substituted substances as 2C, NBOMe, NBOH compounds, benzodifurans (e.g., Bromo-Dragonfly) and others (6-APB, PMMA) (Lehmann et al., 2020; UNODC, 2021d). Phencyclidine analogues are N-methyl-D-aspartate (NMDA) receptor antagonist, and include ketamine, 3-MeO-PCP, diphenidine, methoxetamine (MXE), 2F-DCK and 3-MeO-PCE (Lehmann et al., 2018; Arbouche et al., 2021). Designer benzodiazepines include NPS that contain a benzodiazepine core, including structurally closely related compounds and are not controlled under the international drug control system (Lehmann et al., 2019; EMCDDA, 2021b).

Hofmann et al. (2021) reported a fatal case in Germany involving two stereoisomers (5- and 6-(2-aminopropyl) benzofuran), which are substituted benzofuran phenethylamines. Concentrations ranged from 300 to 2,400 ng/ml in blood and from 2,100 ng/ml in bile to 65,000 ng/ml in stomach content. No other substance was detected in the screening and the cause of death was assumed as intoxication with 5-APB/6-APB. In a Norwegian fatal case involving 5-APB, blood analysis showed levels of 860 ng/ml, which was considered the cause of death (Krpo et al., 2018).

A total of 33 fatal cases reported in Sweden and Finland were positive for flualprazolam, a designer benzodiazepine, showing median concentrations of 18.0 (3.0–68 ng/g) and poly-drug use, mainly including opioids, and flualprazolam, which were implicated as the cause of death in 13 cases (Krikku et al., 2020).

Various fatal cases were reported to be due to the use of methoxyphencyclidine (3-MeO-PCP). In Sweden, only the drug was found in femoral blood (380 ng/g) (Johansson et al., 2017), and in the Netherlands, the levels were 123 ng/ml in serum and 152 ng/ml in blood (De Jong et al., 2019). In France, a plastic bag containing 3-MeO-PCP powder was found near a 44 years-old man, and levels of 525 ng/ml were present in femoral blood and of 384 ng/ml in urine, in addition to 6 different metabolites (Arbouche et al., 2021). In another French case, powder and crystals contained 3-MeO-PCP (72.9%) and various catinones were found, and blood concentration of the deceased were 498 ng/ml (peripheral) and 743 ng/ml (cardiac) (Ameline et al., 2019). Gicguel et al. (2021) reported the detection of 3-MeO-PCE (90 ng/ml) in peripheral blood, in addition to **2**-fluorodeschloroketamine (2F-DCK) (1780 ng/ml) and a tryptamine analog, 5-MeO-DMT (52 ng/ml).

A 23-year-old male experienced severe respiratory distress and died after being detained by the police. 25C-NBOMe and 2C-C were detected at levels of 2.07 ng/ml and 27.43 ng/ml (25C-NBOMe) and of 0.12 ng/ml and 0.38 ng/ml (2C-C) in blood and urine, respectively. 25C-NBOMe concentrations in tissues ranged from 15.2 ng/g in liver to 300 ng/ml in gastric contents. Based on case history, autopsy and toxicological findings, the cause of death was 25C-NBOMe toxicity (Kristofic et al., 2016).

Wiergowski et al. (2017) reported an acute intoxication of three young men by 25B-NBOMe and 4-CMC intake. One man died after jumping off the window of the apartment, due to hallucinations; concentrations in the blood were 661 ng/ml (25B-NBOMe) and 0.887 ng/ml (4-CMC). Other man showed strong convulsions, heavy breathing and salivation before dying, and postmortem blood concentrations were 66.5 (25B-NBOMe) and 2.14 ng/ml (4-CMC). The authors concluded that the deaths were due to fatal overdose of 25B-NBOMe; O-demethylathed O, O-bis-demethylathed and glucuronidated metabolites were also found in postmortem blood (Wiergowski et al., 2017).

Conclusion

A total of 96 papers that reports fatal cases involving NPS published in the literature from 2016 to 2021 were reviewed. LC-MS/MS methods were the most used for quantification analysis, and GC-MS technique was widely used as screening and confirmation method. In addition to screening, high resolution mass spectrometry was the preferred technique used for metabolite identification.

Opioids, synthetic cathinones, phenethylamines/amphetamines and synthetic cannabinoids were the main NPS classes found in the postmortem samples, and polydrug use was reported in most studies, which exposes NPS users to a higher risk of overdose due to potential drug interactions. Furthermore, some drugs, as synthetic cannabinoids and opioids, can be fatal at low doses, making the drug detection and the toxicological evaluation an analytical challenge.

The results of this review indicate that toxicological screening and confirmation methods need to be continuously updated to include new substances that emerge on the drug market. Furthermore, results from non-biological analysis can be a source of information on the possible toxic agent, and provide the laboratory reference material to helping to discover new emerging substances.

Author contributions

EF conceptualized the idea, coordinated the literature search and wrote the first draft of the manuscript. BL, EG, TV and PS conducted the search and summarized the studies. EC critically revised the data and the manuscript, which was approved by all authors.

Funding

This work was funded by the University of Brasilia, through the call DPI/DPG NO 01/2022.

Conflict of interest

TV was employed by Brainfarma Pharmaceutical Company.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ftox.2022.1033733/full#supplementary-material

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Fatal cases involving new psychoactive substances and trends in analytical techniques

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SUPPLEMENTARY MATERIAL

Table S1 | Analytical method details in fatal/postmortem cases analysis involving new psychoactive substances, from 2016 to 2021.

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
Adamowicz et al., 2016 [2]	LC-MS/MS	LLE	0.036 / 1	α-PVP (cath.)	Blood (1.1-6200); n = 12	UR-144, BZDs, COC, THC, amphetamne, methadone, pentedrone, 3-MMC, ethcathinone
Beck et al., 2016	LC-MS/MS: identification/quantification ; LC-HR/MS: identification	PP	0.2 / -	α-PVP (cath.)	Serum (62.6-304); n=2	Opioids, BZDs, cannabis. Only α-PVP detected: 33% of the cases
Coopman et al., 2016 [2]	UPLC-MS/MS	LLE	2.1 / 2.1	Ocfentanil (opioid)	Blood (15.3); n=1	Acetaminophen, caffeine
Fujita et al., 2016	LC-MS/MS	QuEChERS	-	Mepirapim (SC); α-EAPP (opioid)	Blood: Mepirapim (950); α-EAPP (3100); n=1	ND

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
Gieron and Adamowicz, 2016 [2]	LC-MS/MS	PP	0.06 / 0.1	AB-CHMINACA (SC)	Blood (1.5); blood from brain (2.2); blood from lung (2.7); blood from liver (0.3); blood from kidney (1.3); blood from intestines (1.0); urine (0.1); n=1	Ethanol
Kristofic et al., 2016	LC-QTOF: screening; LC-MS/MS: quantification	SPE	-	25C-NBOMe (PEA)	25C-NBOMe: blood (0.48-2.07), urine (1.73-27.43), brain (19.10), spleen (27.13), lung (25.21), liver (15.20), kidney (25.06); 2C-C: blood (0.12), urine (0.11-0.38); n=3	Blood and urine: 25C-NBOMe, 25C-NBOH, 2C-C
Liveri et al., 2016 [2]	GC-MS	SPE	LOD: Blood/urine (0.002 - 0.01) / LOQ: Blood (0.4-3); urine: (0.8-6)	MDPV and pentedrone (Cath)	MDPV: blood (46), urine (1300); pentedrone (mg/L): blood (160), urine (12000); n=1	Blood and urine: Etizolam, ephedrine, olanzapine, mirtazapine
Papsun et al., 2016 [2]	LC-QTOF: screening; LC-MS/MS: quantification	LLE	1 / -	MT-45 (Piperazine); Etizolam (D-BZD)	Blood: MT-45 (520); etizolam (35); n=1	ND
Poklis et al., 2016 [2]	UPLC-MS/MS	SPE	- / 1	Butyryl Fentanyl (opioid)	Butyryl fentanyl: P. blood (99-3.7), H. blood (220-9.2), VH (32-9.8), GSC (590-4000), brain (93-63), liver (41-39), bile (260-49), urine (64-2); n=2	Acetyl fentanyl, alprazolam
Rojkiewicz et al., 2016	HPLC-MS and GC-MS	LLE	7 / 12	4-FBF (opioid)	Blood (91-112), urine (200-414), liver (902-411), kidney (136-197); n=2	ND
Shanks and Behonick, 2016 [2]	LC-MS/MS	LLE	0.1 / 0.2	5F-AMB (SC)	Blood (0.3); n=1	ND
Yonemitsu et al., 2016 [2]	LC-MS/MS and GC-MS: screening; LC-MS/MS: quantification	QuEChERS	-	Acetyl fentanyl (opioid); 4-MeO-PV8 (Cath)	Acetyl fentanyl: F. blood (153), urine (240), GSC (880); 4-methoxy PV8: F. blood (389), urine (245), GSC (500); n=1	7-aminonitrazepam, phenobarbital,

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
						methylphenidate, chlorpromazine, risperidone
Angerer et al., 2017 [2]	GC-MS, HPLC-MS/MS and HPLC-PDA: screening; LC-MS/MS: quantification	LLE	0.01-0.03 / 0.1-0.25	5F-PB-22, AB-CHMINACA and 5F-ADB (SC)	F. blood: 5F-PB-22 (0.37), n=1, AB-CHMINACA (4.1), n=1; 5F-ADB (0.38), n=1	Metabolites of 5F-ADB, NE-CHMIMO and MDMB-CHMICA; olanzapine, trimipramine
Bottinelli et al., 2017 [2]	GC-MS, LC-DAD: screening; GC-MS/MS: quantification	SPE	- / 50	3-MMC (Cath)	3-MMC: P. blood (249), CAR (609), VH (2988), bile (1291), urine (29694); n=1	ND
Dwyer et. Al. 2017	GC-MS: screening; LC-MS/MS: quantification	LLE/SPE	-	Fentanyl and acetylfentanyl (opioid)	Blood: acetylfentanyl (0.13–2100); fentanyl (0.24-74.3); urine: only qualitative; n=41	Ethylone, ketamine, BZDs, COC, heroin and other opioids
Ellefsen et al., 2017 [2]	LC-MS/MS and GC-MS	LLE	- / 0.001	3-FPM (PHEN); U-47700 (opioid)	3-FPM: P. blood (2400), aortic blood (600); U-47700: P. blood (360); n=1	Amitriptyline, nortriptyline, methamphetamine, amphetamine, Flubromazolam, delorazepam and others BZD
Guerrieri et al., 2017 [2]	LC-MS/MS	LLE-LTP	-	Acrylfentanyl (opioid)	Blood (0.01-5); n=40	4-MeO-α-POP, MO CHMINACA, amphetamines, BZDs, 4Cl-α-PVP, N-etylnorhexedron, 4Cl-isobutylfentanyl, MDMA. THC
Johansson et al., 2017 [2]	LC-TOF-MS: screening; LC-MS/MS: quantification	LLE	- / 0.01	3-MeO-PCP (PCY)	Blood (50 to 180); (n=6); blood (380 µg/g) in a mono-intoxication case; n=1	Buprenorphine, 5-MeO-MIPT, fentanyl, tramadol
Krotulski et al., 2017	LC-QTOF: screening and metabolite investigation; LC-MS/MS: quantification	SPE	-	THFF and U-49900 (opioid); MeO-PCP (PCY)	Blood and urine, respectively: THFF (339; >5000); U-49900 (1.5; 2.2); MeO-PCP (1.0; 31.8); n=1	alprazolam, paroxetine, topiramate, zolpidem, trazodone, aripiprazole, chlorpheniramine,

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
						dextro/levomethorphan, promethazine
Paul et al., 2017	LC-MS/MS	NA	0.01-2.0 / 0.1-2.0	AB-CHMINACA, UR-144, XLR-11 and JWH-022 (SC)	Blood: AB-CHMINACA (8.2), n=1; UR-144 (12.3), XLR-11 (1.3) and JWH-022 (3), n=1	ND
Potocka-banas et al., 2017	LC-MS/MS	LLE	1 / 5	α-PVP (Cath)	α-PVP: blood (174), urine (401), brain (92), liver (190), kidney (122), GSC (606); n=1	Midazolam, metoclopramide
Rojek et al., 2017 [2]	LC-MS/MS	LLE	- / 0.05-10	UR-144 (SC); Pentedrone (Cath)	Blood: UR-144 (2.1), n=1; UR-144 (1.4), pentedrone (2300), (n=1); UR-144 (4), pentedrone (290), n=1	ND
Staeheli et al., 2017 [2]	LC-MS/MS	LLE	-	MDAI (AI); 2-MAPB (Cath)	P. blood: MDAI (38); 2-MAPB (21); n=1	diphenhydramine, morphine
Wiergowski et al., 2017 [2]	HPLC-QTOF-MS: screening; UPLC-MS/MS: quantification	PP/LLE	0.0053-0.0013 / 0.0159-4.0	25B-NBOMe (PEA); 4-CMC (Cath)	Blood. 25B-NBOMe (38.4-661), 4-CMC (0.887-2.14); n=2	THC
Allibe et al., 2018 [2]	LC-MS/MS: identification/ and quantification; LC-HRMS (QTOF): metabolite investigation	SPE	0.01/ 0.05	Ocfentanil (opioid)	Ocfentanil: P. blood (3.7), CAR (3.9), VH (2.0), bile (8.4) , GSC (2.5), nasal swabs (nd); n=1	Caffeine, acetaminophen, heroin and other opioids
Atherton et al., 2018	GC-MS	LLE	- / 10	N-ethylpentylone (Cath)	P. blood (31-953); n=4	Fentanyl, COC, hydrocodone, alprazolam
Ballesteros et al., 2018[2]	LC-MS/MS and GC-MS: detection	SPE	20 / -	4-MEC and α-PVP (Cath)	α-PVP: blood (9-1200); urine: detected 4-MEC and α-PVP; n=2	Amphetamine, MDMA, MDA, lormetazepam and other BZD, THC-COOH
Costa et al., 2018 [2]	LC-MS/MS	LLE	1 and 5 / -	N-ethylpentylone (Cath)	Blood (170); n=1	ND

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
Fagiola et al., 2018 [2]	GC-MS or LC-MS: screening; LC-MS/MS	LLE	2.5 (LC-MS/MS); 200 (GC–MS or LC-MS, for cathinones) / -	Mitragynine and 7-OH-mitragynine; Pentylone, methylone and butylone (Cath)	Blood/urine: Mitragynine, n=2; mitragynine and 7-hydroxymitragynine, n=3; pentylone, methylone and butylone, n=1	Synthetic opioid
Gerace et al., 2018 [2]	UHPLC-MS/MS	LLE	0.6 / 2	U-47700 (opioid)	Blood (380); urine (10400); pubic hair (5700) n=1	ND
Koch et al., 2018 [2]	LC-MS/MS	PP/LLE/ SPE	- / 1	U-47700 (opioid)	Blood: 42 min (370), 9 h. (37), 24h. (6.3), 33 h. (2.1), 41 h. (2.3); urine (2); n=1	Flubromazepam and other BZD, lidocaine, pregabalin
Krpo et al., 2018	UHPLC-QTOF-MS: screening; UHPLC-MS/MS: confirmation and quantification	LLE	-	5-APB (PEA)	P. blood (860); n=1	Ethanol, THC
Kusano et al., 2018 [3]	LC-MS/MS: screening and quantification LC-QTOF-MS: screening	PP	0.005-0.1 / -	Diphenidine (PCY); 5F-ADB (SC)	Blood: 5F-ADB (0.19 ± 0.04), diphenidine (12 ± 2.6); n=1	ND
Lehmann et al., 2018 [2]	LC-MS/MS	SPE/ QuEChERS	0.4-5 / -	Methoxetamine (PCY); 4-MEC, MDPV and α-PVP (Cath)	F. blood: 4-MEC (8 to 118), MDPV (3 to 396), MXE (2 to 385) and α-PVP (4); H. blood, P. fluid, bile, stomach content, brain, liver, lung, kidney, muscle, urine: 4-MEC (8 to 901), MDPV (3 to 1202), MXE (1 to 1391); n=2	ND
Maher et al., 2018	HPLC-DAD; LC-QTOF-MS: identification; LC-MS/MS: identification/ quantification	LLE	0.05- 0.16 / -	Cyclopropylfentanyl and crotonylfentanyl (opioid)	F. blood: (16.6-28.9); n=4	ND
Majchrzak et al., 2018[3]	LC-MS/MS	LLE	Body fluids: 9.0-27.2; tissues:	N-PP (Cath)	N-PP: blood (3100), eyeball fluid (4400), liver (5900), kidney (5400), brain (2300); n=1	ND

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
			15.0-46.0 / -			
Mardal et al., 2018 [2]	UHPLC-MS/MS: identification and quantification; UHPLC-HR-MS/MS: metabolite investigation	LLE/ PP	- / 7- 68	Methoxyacetylfentanyl (opioid)	F. blood (22), brain (74), n=1; F. blood (23), urine (120), n=1; F. blood (56), n=1	Oxycodone
Moody et al., 2018 [2]	LC–MS/MS: quantification; LC-TOF: screening	SPE	0.0125-0.25 / 0.05-0.5	4-ANPP, 2-Furanylfentanyl, carfentanil, fluorobutyrylfentanyl, U-47700, acrylfentanyl, butyrylfentanyl, fluorofentanyl, 4-methoxybutyrylfentanyl and valerylfentanyl (opioid)	Blood: 4-ANPP (0.1-410), n=1549; 2-furanylfentanyl (0.1-710), n=1228; carfentanil (0.1-120), n=697; fluorobutyrylfentanyl (0.1-760), n=563; U-47700 (0.2-3800), n= 543; acrylfentanyl (0.1-29), n=266; butyrylfentanyl (0.1-760), n=142; p-fluorofentanyl (0.1-1), n=31; o-fluorofentanyl (2.4), n=1; 4-methoxybutyrylfentanyl (79), n=1; valerylfentanyl (0.44), n=1	ND
Nooble et al., 2018 [2]	LC-QTOF-MS: screening; UHPLC-MS/MS: quantification	PP/SPE	1-5 / 5	Fentanyl (opioid)	Blood: fentanyl (7–39); n=17	ND
Partridge et al., 2018 [2]	LC-QTOF: screening, quantification and metabolite investigation	LLE	0.8-3 / -	U-47700 (opioid); Diclazepam and flubromazepam (D-BZD)	P. blood: U-47700 (330), diclazepam (70), flubromazepam (10); n=1	Methamphetamine, amphetamine, lorazepam, DOC
Pieprzyca et al., 2018 [2]	LC-MS/MS	PP	5 / 10	PV8 (Cath)	PV8: blood (70-260), urine (110 to 130), liver (20-40), kidney (10-40); n=2	Clindamycine, paracetamol, metamizole, lidocaine, dextromethorphan, drotaverine
Rohrig et al., 20180 [2]	GC-MS: screening; GC-NPD: screening and quantification	SPE	25 / -	U-47700 (opioid)	U-47700: H. blood (260), F. blood (400), VH (90), brain (380), liver (280), urine (4600); n=1	THC

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
Strehmel et al., 2018	LC-QTOF-MS: screening; LC-MS/MS: quantification	PP	-	U-47700 (opioid)	U-47700 (µg/ml): F. blood (290), H. blood (12500), liver (9900), urine (240), GSC (570), bile (2300), CSF (400); n=1	Caffeine, nicotine, oxycodone, theobromine, theophylline
Tomczak et al., 2018 [2]	GC-MS	LLE	0.3 / 1	4-CMC (Cath)	Blood: (56.2-1870); n= 6	Diazepam, MDMA, MDA, THC, amphetamine, 3-MMC, Estazolam, COC metabolites
Adamowicz et al., 2019 [2]	LC-MS/MS	PP	- / 0.1	AMB-FUBINACA and EMB-FUBINACA (SC)	AMB-FUBINACA, EMB-FUBINACA, respectively: blood (ND, ND), urine (4.7, 0.2), urine hydrolyzed (8.2, 0.1), kidney tissue (0.2, 0.4), kidney (bloody fluid) (0.1, 0.1), liver tissue (0.2, 0.2), liver (bloody fluid) (0.8, ND), stomach tissue (0.9, 2.7), stomach content (5.8, 36.2), intestine tissue (0.8, 3.5), intestine (bloody fluid) (0.1, 0.2), lung tissue (ND, 1.4), lung (bloody fluid) (0.1, ND), brain (0.6, 0.6); n=1	Lorazepam, haloperidol, lidocaine
Al-Matrouk et al., 2019	LC–MS/MS and LC-HRMS: screening	SPE	-	5F-AB-PINACA, AB-PINACA, AB-CHIMICA, FUB-AMB, 5F-AB-PINACA, 5F-AKB-48, 5Cl-AKB-48, ADB-PINACA and 5F-ADB (SC)	Urine: only qualitative analysis (n=6)	ND
Ameline et al., 2019 [2]	GC-MS: screening; UPLC-MS/MS: quantification	LLE	-	3-MeO-PCP (PCY)	P. blood (498), CAR (743), urine (16.7), hair (15600); n=1	ND
Chesser et al., 2019 [2]	LC-MS/MS	SPE	0.05-0.1/0.1	4-ANPP, acetylfentanyl, fentanyl, furanylfentanyl, norfentanyl and U-47700 (opioid)	Blood (femoral, cardiac, iliac, subclavian) (0.1 - 45; 0.1-227; 0.1-98; 0.2-89; 0.1-38; 0.4->500); VH (0.1-28; 0.1-45; 0.2-68; 0.3-14; 0.1-19; 0.1-328); brain (ND; 0.1->600; 0.3-176; 0.4-167;	ND

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
					0.4-22; 1->600), for 4-ANPP, acetylfentanyl, fentanyl, furanylfentanyl, norfentanyl, U-47700, respectively; n=58	
De Jong et al., 2019	UPLC-MS/MS: quantification; LC-QTOF-MS: screening	SPE	-	3-MeO-PCP (PCY)	Serum (123), blood (152); n=1	amphetamine
Deville et al., 2019 [2]	GC-MS and UPLC-TOF-MS: screening and identification; HPLC-DAD: quantification	LLE	-	MDAI (AI); 5-EAPB (Cath)	MDAI, 5-EAPB, 5-MAPB, 5-APB, respectively: blood (2090, 6450, 89, 546); urine (69400, 14800, 1000, 48800); n=1	Oxazepam
Fagiola et al., 2019 [2]	LC-MS/MS	LLE	2.5 / -	Cyclopropylfentanyl (opioid)	CAR (5.6-82); n=5	BZD, COC, opioids, methamphetamine, despropionyl fentanyl, THC-COOOH
Fels et al., 2019 [2]	LC-QTOF-MS: identification and quantification	LLE/SPE	5 / 10	U-47700 (opioid)	U-47700: F. blood (27–2200), H. blood (39–4900), liver (72–8400), urine (100-5400), VH (14-11000), P. fluid (43-4600), GSC (630-180000), putrefaction fluid (61-320); n=26	Fentanyl and analogs, amphetamine, methamphetamine, MDMA, opioids, flubromazepam and others BZD, N-Ethylpentylone and others cathinones, 3-MeO-PCP and others phencyclidine analogs, SCs, 3-FPM, MDAI, mitragynine
Freni et al., 2019 [2]	LC-MS/MS	SPE	0.03-0.1 / -	Furanylfentanyl and 4-ANPP (opioid)	Furanyl fentanyl and 4-ANPP, respectively: CAR (11.8± 0.7; 93.5 ± 7.6), F. blood (2.7± 0.1; 50.4 ± 2.9), urine (71.3 ±3.3; 171.7 ± 13.8), bile (7.7 ± 0.8; 41.9 ± 1.6), CSF (2.6± 0.2; 10.2 ± 0.6), GSC (40.1 ± 11.2; 24.2 ± 2.0); n=1	ND

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
Gaulier et al., 2019 [2]	LC-QTOF: screening; LC-MS/MS: quantification	SPE	0.05/ 0.1	Carfentanil (opioid)	Blood (4.20), urine (0.40); n=1	Diclazepam and others BZD, heroin and others opioids, COC, MDMA, benzoylfentanyl and 4-fluobutyrylfentanyl, ethylhexedrone, AB-FUBINACA, MAM 2201, methoxetamine
Ivanov et al., 2019	GC-MS: detection; HPLC-UV: quantification	LLE	5F-ADB 25 / -	5F-ADB and FUB-AMB (SC)	5F-ADB: blood (3.7); n=1	ND
Kovács et al., 2019 [2]	LC-MS/MS	LLE	0.01-10 / -	N-ethylhexedrone (Cath); ADB-FUBINACA (SC)	Blood: NEH (285), ADB-FUBINACA (0.08); n=1	THC, THC-COOH
Kriikku et al., 2019 [2]	UPLC-TOF-MS: screening; GC-MS: quantification	SPE	10 / 20	U-47700 (opioid)	Blood (150–2000), n=10; urine (20-2200), n=12	m-CPP, phenazepam and others BZD, buprenorphine, pregabalin, THC, α-PVP, amphetamine
Krotulski et al., 2019	LC-QTOF: qualitative analyses and metabolite identification	LLE/ SPE	-	4F-MDMB-BINACA (SC)	Blood and urine: qualitative analysis; n = 20	5F-MDMB-PINACA (5F-ADB). 4F-MDMB-BINACA
Lehmann et al., 2019	LC-MS/MS	SPE/ QuEChERS	-	Diclazepam and pyrazolam (D-BZD); 3-FPM (PHEN)	Diclazepam, pyrazolam, 3-FPM, respectively: F. blood (1; 28; 10), H. blood (1; 28; 9), urine (1; 500; 120), P. fluid (1; 11; 16), CSF (4; 45; 13), bile (17; 340; 190), brain (23; 100; 76), liver (34; 92; 160), lung (21; 98; 89), kidney (45; 160; 94), muscle (19; 88; 56), stomach contents (16; 380; 84); n=1	2-FA, 2-FMA, methiopropamine, amphetamine, caffeine, lorazepam
Margasińska-Olejak et al., 2019 [2]	LC-MS	LLE	-	3-MMC (Cath)	Blood (800), VH (153), GSC (5.5 mg); n=1	

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
Nash et al., 2019 [2]	LC-QTOF: screening and quantification	LLE	-	Furanylfentanyl (opioid); MMMP (Cath)	P. blood: furanylfentanyl (1.6), MMMP (6.7); n=1	THC, mirtazapine, paliperidone, quetiapine, 4-ANPP
Theofel et al., 2019 [2]	LC-MS/MS: quantification	PP/SPE	3 / 5	N-ethyldeschloroketamine (PCY)	N-ethyldeschloroketamine: liver (6137), urine (3468), bile fluid (3290), GSC (3086), H. blood (2159), liquor (1564), F. blood (375); n=1	Deschloroketamine, metamizole, opioids, ibuprofen, venlafaxine
Yeter and Erol Öztürk, 2019 [2]	LC - HRMS: identification and quantification	SPE	Blood: 0.08; urine: 0.10 / blood: 0.10; urine: 0.12	5F-ADB and its methyl ester hydrolysis metabolite (SC)	Blood: 5F-ADB (0.10-1.55), 5F-ADB metabolite (0.15-23.4), n=70; urine: 5F-ADB metabolite (0.28-72.2), n=34.	AMB-FUBINACA, ADB-FUBINACA, 5F-MDMB-PICA, JWH-018, MAB-CHMINACA, AB-CHMINACA, CUMYL-4CN-BINACA, cannabis, MDMA, COC, heroin, amphetamine, methamphetamine
Adamowicz et al., 2020a	LC-MS/MS	LLE	0.3 / 5	α-PiHP (Cath)	α-PiHP: blood (69), urine (2072) and bile (341), solid tissues (7–478); n=1	4-CMC, N-ethylhexedrone, BZE, MDMA
Adamowicz et al., 2020b [2]	LC-MS/MS	LLE	0.01-0.20 / -	Benzylfentanyl (opioid)	Blood: Benzylfentanyl (66; 110); fentanyl (31; 32); norfentanyl (22; 41);4-FiBF (74); despropionyl-4-FF (6.5); n=3	α-PHP, N-Ethylhexedrone, 5-APB (or 6-APB), 4-FMA, 4-FA,(α-PiHP), THC-COOH
Benedicte et al., 2020 [b]	GC-MS: screening; LC-HRMS: confirmation and metabolite identification	LLE	0.5 / 1	MPHP and N-ethyl-4′methylpentedrone (Cath)	MPHP and 4-MEAP, respectively: F.blood (47; 1.6), CAR (97; 3.5), urine (2380; 49700); n=1	THC, 4′-carboxi-PHP
Ditrana et al., 2020 [2]	HPLC-MS/MS	PP	Blood: 0.03-0.35; urine: 0.02-0.25 / blood: 0.08-1; urine: 0.06-0.5	Cyclopropylfentanyl, methoxyacetylfentanyl, furanylfentanyl, acetylfentanyl, 4-ANPP and fentanyl (opioid)	Blood (0.2-9); urine (0.2-8900), for fentanyl derivatives; n=41	Opioids

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
Garneau et al., 2020	GC-MS: screening; LC-MS/MS: screening and quantification	SPE	-	4-ANPP, furanylfentanyl, U-47700, p-fluorobutyrylfentanyl, methoxyacetylfentanyl, cyclopropylfentanyl/ crotonylfentanyl, acetylfentanyl, despropionyl fluorofentanyl and N-methyl U-47931 E (opioid)	Cardiac and F. blood, respectively: 4-ANPP (33-32; 18), furanylfentanyl (14-2.4; 0.89) and U-47700 (54-45; 26); n=2. Cardiac and F. blood, respectively: 4-ANPP (5.1; 9.7), p-fluorobutyrylfentanyl (31; 27), methoxyacetylfentanyl (70; 14), cyclopropylfentanyl/ crotonylfentanyl (0.15; 0.1), only detected: U-47700, acetylfentanyl, despropionyl fluorofentanyl, N-methyl U-47931 E; n=1	Amphetamine, metamphetamine, COC, methadone, THC, BZDs
Hvozdovich et al., 2020	LC-MS/MS	SPE	-	5F-ADB, FUB-AMB, 5F-AMB, MDMB-FUBINACA, and AB-CHMINACA (SC)	Blood and/or urine: only qualitative analysis; n=54. 5F-ADB was the most prevalent substance	Ketamine, morphine, and others
Kriikku et al., 2020 [2]	GC-NCI-MS	LLE	1 / -	Flualprazolam (D-BZD)	Blood (3.0-68); n=33	ND
Krotulski et al., 2020a	LC-QTOF-MS: screening and metabolite investigation	LLE/SPE	-	APP-BINACA (SC)	Blood and urine: only qualitative analysis; n=11	4F-MDMB-BINACA, 5F-MDMB-PICA, 5F-MDMB-PINACA, fentanyl, etizolam, THC, opioids
Krotulski et al., 2020b [3]	LC-MS/MS: quantification; LC-QTOF-MS: metabolite investigation	LLE	<0.02 / -	Isotonitazene (opioid)	Blood (0.4-9.5), n=18; urine (0.6-4.0), n=6; VH (0.1), n=1	4-ANPP, and U-47700, etizolam, COC
Lehmann et al., 2020 [2]	LC-MS/MS	SPE	0.4–4 / 5	PMMA, PMA, PMEA, 2-FA, 4-FA, 2-FMA, 3-FPM, 2-DPMP, MDEA, MDMA, MDA and methiopropamine (PEA); 3-MeO-PCP and MXE (PCY); m-CPP (piperazine); MDPBP, MDPV, 4-MEC,	Amphetamine and analogs (PMMA, PMA, PMEA, 4-FA, 2-FA, 2-FMA, methiopropamine, MDMA, MDA, MDEA, amphetamine, n=13): 4.5-185000 (urine); 2.2-2500 (blood). M-CPP (n=1): 130 (urine), 5.3 (blood); MXE (n=4): 6.6-22300 (urine), 1-390 (blood), 810 (kidney); 3-FPM (n=1): 120 (urine), 5.3 (blood); U-47700 (n=1): 1500 (urine);	Femoral blood and urine: 2-DPMP, MXE, 3-MeO-PCE, PMMA, PMA, PMEA, methylone, metamphetamine,amphetamine, MDMA, methedrone, MDEA,4-MEC, methadone, EDDP, 2-FA, 2-FMA, MDPV, 3-FPM, U-47700

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
				methedrone, methylone and α-PVP (Cath); U-47700 (opioid); pyrazolam, diclazepam; delorazepam; lormetazepam (D-BZD)	2-DPMP (n=1): 52 (urine), 5.2 (blood); 3-MeO-PCE (n=1): 3.6 (urine); Synthetic benzodazepines (Pyrazolam, diclazepam, delorazepam, lormetazepam, n=1): 1-100 (blood); Cathinones (4-MEC, MDPV, methedrone, methylone, MDPBP, α-PVP, n=4: 6.2-830 (urine). 3.6-340 (blood), 53-230 (kidney); n=17	
Tiemensma et al., 2020	GC-MS and LC-MS	NA	-	Cumyl-PEGACLONE (SC)	Blood (0.73-3.0); n=5	5F-Cumyl-P7AICA, 5F-Cumyl-PEGACLONE, lignocaine, paliperidone, THC
Woods, 2020 [2]	GC-MS	LLE	<10 / 50	Mebroqualone (Meth)	F. blood (10228; 115); n=2	Lorazepam, oxycodone, diphenhydramine, amphetamine, methamphetamine
Zawadzki et al., 2020a [2]	UHPLC-MS/MS	LLE	- / 0.1	5F-CUMYL-P7AICA (SC)	Blood (2.8), urine (3.1); n=1	ND
Zawadzki et al., 2020b [2]	UHPLC-MS/MS	LLE	- / 1	N-ethylpentylone (Cath)	P. blood (10600), urine (17600); n=1	Eutylone
Arbouche et al., 2021	LC-MS/MS: quantification; LC-HRMS: confirmation and metabolite investigation	LLE	-	3-MeO-PCP (PCY)	F. blood (525), urine (384); n=1	Methadone, THC
Brahan et al., 2021 [2]	GC-MS/MS	LLE	- / 1000	4-MEC (Cath)	4-MEC: P. blood (14600), CAR (43400), urine (619000), VH right and left (2900, 4400), bile (43500), GSC (28200); n=1	Hydroxyzine
Castellino et al., 2021	GC-MS	LLE	1.0 / -	Cyclopropylfentanyl (opioid)	Blood (14), n=1; Other case: only detected, n=1	alcohol, COC, oxycodone

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
Cartiser et al., 2021 [2]	GC-MS	SPE	-	4-MPD (Cath)	4-MPD: P. blood (1285), CAR (1128), urine (>10,000), bile (1187), VH left and right (734; 875); n=1	COC, sildenafil, bromazepam, nevirapine
Chan et al., 2021 [2]	LC-MS/MS	PP	-	Carfentanil (opioid)	P. blood (0.5), (n=1); iliac blood (0.9), n=1	Naproxen, desloratadine, olopatadine, zolpidem
Ferrari Jr. and Caldas, 2021 [2]	UHPLC-MS/MS	QuEChERS	4 / 10	N-ethylpentylone (Cath)	Blood (597); n=1	ND
Gicquel et al., 2021 [2]	LC-MS/MS: screening; LC-HRMS (QTOF): screening and quantification	SPE	5 / 10	2F-DCK and 3-MeO-PCE (PCY)	2F-DCK, 3-MeO-PCE and 5-MeO-DMT, respectively: P. blood (1780; 90; 52), urine (6100; 6300; 2200), bile (1200; 3500; 1700), VH (1500; 66; 155); n=1	Amphetamine, COC, THC, levamisole, lorazepam
Hofmann et al., 2021 [2]	HPLC-MS/MS	PP	1.8-2.6 / 4.6-6	5-APB and 6-APB (PEA)	5-APB and 6-APB, respectively: C. blood (2400; 660), P. blood (850; 300), urine (8700; 3400), stomach content (65000; 4500), bile (4700; 2100), muscle (1400; 370), brain (7700; 1700), kidney (380; 64), liver (6900; 1600), lung (3300; 760); n=1	ND
Kronstrand et al., 2021[2]	LC-MS/MS: quantification. LC-QTOF-MS: metabolite investigation	PP	- / 2	Methoxyacetylfentanyl (opioid)	F. blood: (18-140); n=10	Opioids, BZDs
Krotulski et al., 2021a	LC-MS/MS: quantification; LC-TOF-MS: screening; LC-QTOF-MS: metabolite investigation	LLE and PP/SPE	- / 1	Eutylone (Cath)	Blood (1,2-11000), n=67; urine (60; 3400; and >10000), n=3; brain (6.2), n=1; liver (10000), n=1.	Blood: Fentanyl, MDMA, methamphetamine, etizolam and pther benzodiazpines, ketamine, COC, opioids, THC and prescribed medicines; urine: 5F-MDMB- PICA 3,3-dimethylbutanoic acid

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
Krotulski et al., 2021b [3]	LC-MS/MS: quantification; LC-QTOF-MS: metabolite investigation	LLE	<0.1 / -	Brorfine (opioid)	Blood: 0.1-10; n=20	Opioids, BZDs, gabapentin, THC, cyclobenzaprine, COC
Krotulski et al., 2021c [2]	LC-MS/MS: quantification; LC-QTOF-MS: screening and metabolite investigation	LLE	0.1 / 0.5	Metonitazene (opioid)	Blood (0.5-33), urine (0.6-46); n=20	Fentanyl, BZDs, opioids, amphetamine, metamphetamine, THC
Krotulski et al., 2021d	LC-QTOF-MS: identification and metabolite investigation	LLE/SPE	-	MDMB-4en-PINACA, 5F-MDMB-PICA and 4F-MDMB-BINACA (SC)	Blood: qualitative analysis; n=16	COC metabolites, phenytoin, amphetamine, methamphetamine, opioids, THC
Mochizuki et al., 2021 [2]	LC-LIT-MS: detection and quantification; GC-MS: identification	SPE	0.1-1 / -	4-FMC, 4-MeO-α-PVP, 4-F-α-PVP and PV8 (Cath)	4-FMC, 4-MeO-α-PVP, 4-F-α-PVP and PV8, respectively: H. blood (365; 449; 145; 218), F. blood (397; 383; 127; 167); n=1	ND
Mueller et al., 2021 [2]	UHPLC-MS/MS	SPE	0.01 / 0.05	Isotonitazene (opioid)	Isotonitazene: F. blood (2.28; 0.59; 0.74), CAR (1.7; 1.13; 0.7), urine (1.88; 3.37; 0.19), humor vitreous (0.36; 0.12; 0.65), pericardiac fluid (6.7; 5.01; 2.66), lung (0.52; 17.9; 2.39), liver (0.04; 0.04; 0.02), kidney (1.61; 1.02; 0.67), heart (7.74; 2.17; ND), brain (18.6; 2.72; 4.45), spleen (4.4; 3.44; 2.62), muscle (1.15; 2.08; 1.0), CSF (ND; 0.88; ND), hair (75; 182; 32/35); n=3	ND
Palazzoli et al., 2021 [2]	LC-MS/MS	PP/SPE	0.1-0.5 / 0.5-1	Mephedrone, DHM and NORMEP (Cath)	Mephedrone, NORMEP and DHM, respectively: F. blood: (1088; 47.1; 15.5), C. blood (1632; 50.2; 49.2), urine (4443; 740.2; 171.9), right lung (1808; 10.1; 15.4), left lung (1368; 29.6; 40.6), brain (1596; 15.6; ND), liver (1080; 9.5; 169.2), kidney (1468; 15.2; 39.2), bile (752; ND; ND); n=1	COC

Reference	Analytical techniques	Extraction Method	LOD/LOQ (ng/mL or ng/g) [1]	Substance (class)	Biological fluid/tissue, analyte concentration and number of postmortem/death cases [1]	Other substances detected (mainly NPS)
Solbeck et al., 2021 [2]	LC-MS/MS: quantification. LC-QTOF-MS, GC-NPD and GC-MS: screening.	SPE	0.05 / 0.1	Carfentanil (opioid)	Blood (< 0.1-9.2); n = 160	COC, fentanyl, acetaminophen, BZD, metamphetamine, amphetamine, opioids
Theofel et al., 2021 [2]	GC-MS and LC-QTOF-MS/MS: screening; LC-MS/MS: quantification	NA	-	2-MAPB (Cath)	2-MAPB: urine (167000), GSC (98900), bile (30800), liver (22200), H. blood (16700), F. blood (7300); n=1	N-demethyl-2-MAPB and hydroxy-2-MAPB, diazepam, fephedrone, 2C-B, THC
Zawadzki et al., 2021 [2]	UHPLC-MS/MS	LLE	0.05 / 0.1	4-FiBF (opioid)	4-FiBF: blood (76.1- 257), urine (289-1000), VH (89.9-150), bile (1100-5410), brain (94.5-176), kidney (388-811), liver (1400-2040), stomach wall (1900) and GSC (2280-3990); n=4	N-ethylpentylone, 4-CMC, α-PiHP, amphetamine, tramadol

[1] when necessary, concentrations reported in the studies were converted to ng/mL or ng/g to facilitate the comparison among the methods; [2] papers that described validation procedures; [3] papers that described quantitation by standard addition; C-NMR: carbon-13 nuclear magnetic resonance; EI: electron impact ionization; ELISA: enzyme-linked immunoassay; FT-IR: Fourier-transform infrared spectroscopy; GC-IR: gas chromatography – infrared spectroscopy; GC-MS: gas chromatography coupled to mass spectrometry; GC-MS/MS: gas chromatography coupled to tandem mass spectrometry; GC-NCI-MS: gas-chromatography negative-chemical-ionization mass spectrometry; H-NMR: proton nuclear magnetic resonance; HPLC-DAD: high performance liquid chromatography-diode-array detector; HPLC–DAD-FLD: high performance liquid chromatography-diode-array and fluorescence detectors; HPLC-MS/MS: high performance liquid chromatography- tandem mass spectrometry; HPLC-UV: high performance liquid chromatography-ultraviolet detector; HRMS: high-resolution mass spectrometry; LC-DAD: liquid chromatography-diode-array detector; LC-HRMS: liquid chromatography-high-resolution mass spectrometry; LC-MS: liquid chromatography- mass spectrometry; LC-PDA: liquid-chromatography-photodiode array detector; LC-MS/MS: liquid chromatography- tandem mass spectrometry; LC-QTOF-MS: liquid chromatography-quadrupole time-of-flight mass spectrometry; LC–TOF-MS: liquid chromatography-time of flight mass spectrometry: LC-UV: liquid chromatography-ultraviolet detector; MRM: multiple reaction monitoring; NMR: nuclear magnetic resonance; NPS: new psychoactive substance; SIM: selective ion monitoring; UHPLC-MS/MS: ultra high performance liquid chromatography- tandem mass spectrometry; UHPLC-QTOF-MS: ultra high performance liquid chromatography- quadrupole time-of-flight mass spectrometry; UPLC-MS/MS: ultra performance liquid chromatography- tandem mass spectrometry; UPLC-TOF-MS: ultra performance liquid chromatography-time-of-flight mass spectrometry; UPLC-PDA: ultra performance liquid-chromatography-photodiode array detector; UV-VIS: ultraviolet/visible spectrophotometry. **Extraction methods**: LLE: liquid-liquid extraction; LLE-LTP: liquid-liquid extraction with low-temperature partition; PP: protein precipitation; QuEChERS: quick,

easy, cheap, effective, rugged, and safe; SPE: solid phase extraction. **Substances**: 2-FA: 2-Fluoroamphetamine; 2-FMA: 2-Fluoromethamphetamine; 2-Oxo-PCE: N-ethyldeschloroketamine; 3-FPM: 3-fluoro-phenmetrazine; 3-MMC: 3-methylmethcathinone; 4-FA: 4-Fluoroamphetamine; 4-FMA: 4-Fluoromethamphetamine; 4-FBF: 4-fluorobutyrfentanyl; 4-FiBF: 4-fluoroisobutyryl fentanyl; 4-MEAP: N-ethyl-4′methylpentedrone; 4-MEC: 4-methylethcathinone; 4-MPD: 4-methylpentedrone; 5F-MDMB-PINACA: 5F-ADB; α-PiHP: alpha-Pyrrolidinoisohexaphenone; AI: aminoindane; AMP: amphetamine; BZD: benzodiazepine; BZE: benzoylecgonine; Cath: synthetic cathinone; COC: cocaine; D-BZD: designer-benzodiazepine; DHM: dihydro-mephedrone; MDA: Methylenedioxyamphetamine; MDMA: Methylenedioxymethamphetamine; Meth: Methaqualone analog; MMMP: 2-methyl-4'-(methylthio)-2-morpholinopropiophenone; MAMP: metamphetamine; N-PP: α-propylaminopentiophenone; NA: not available; ND: non-detected; NORMEP; Nor-mephedrone; PCY: phencyclidine analog; PEA: phenethylamine; PHEN: phenmetrazine analog; PMMA: para-methoxymethamphetamine; SC: synthetic cannabinoid; THC-COOH: 11-Nor-9-carboxy-THC; THC: tetrahydrocannabinol; THFF: Tetrahydrofuranylfentanyl. **Biological fluid/tissues:** GSC: gastric content; C. blood: central blood; CAR: cardiac blood; P. blood: peripheral blood; F. blood: femoral blood; H. blood: heart blood; P. fluid: pericardial fluid; VH: vitreous humor; CSF: cerebrospinal fluid.

ANEXO III

Forensic Toxicology
https://doi.org/10.1007/s11419-021-00600-y

ORIGINAL ARTICLE

Determination of new psychoactive substances and other drugs in postmortem blood and urine by UHPLC–MS/MS: method validation and analysis of forensic samples

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Received: 7 July 2021 / Accepted: 22 September 2021

Abstract

Purpose This study aimed to validate a modified QuEChERS method followed by ultra-high performance liquid chromatography–tandem mass spectrometry to determine 79 new psychoactive substances (NPS) and other drugs in blood and urine.

Methods Prescription drugs ($n=23$), synthetic cathinones ($n=13$), phenethylamines ($n=11$); synthetic cannabinoids ($n=8$), amphetamines ($n=7$) and other psychoactive substances ($n=17$) were included in the method. 500 µL of biological fluid was extracted with 2 mL of water/ACN (1:1), 500 mg of anhydrous $MgSO_4$/NaOAc (4:1) added, followed by a supernatant cleanup with 25 mg of primary secondary amine and 75 mg of anhydrous $MgSO_4$. Quantification was done using matrix-matched calibration curves and deuterated internal standards.

Results The method was satisfactorily validated for blood and urine at limit of quantifications ranging from 0.4 to 16 ng/mL, and applied to the analysis of 54 blood (38 postmortem and 16 antemortem) and 16 antemortem urine samples from 68 forensic cases. All urine samples and 59.3% of the blood samples were positive for at least one analyte. Twenty-two analytes were detected in at least one biological sample, including the synthetic cathinones ethylone (222 ng/mL, antemortem blood), eutylone (246 and 446 ng/mL, urine), and *N*-ethylpentylone (597 and 7.3 ng/mL, postmortem and antemortem blood, respectively).

Conclusions The validated method was shown to be suitable for the analysis of blood and urine forensic samples and an important tool to collect information on emerging drug threats and understanding the impact of NPS and other drugs in poisoning cases.

Keywords Drugs · New psychoactive substances (NPS) · Postmortem blood · Urine · UHPLC–MS/MS

Introduction

New psychoactive substances (NPS) are synthetized to mimic the effect of traditional drugs, with new compounds continually being introduced in the market. The number of NPS worldwide rose from 166 substances over the period 2005–2009 to 950 substances by the end of 2019 [1], and its abuse is a potential risk for users [2].

Early Warning Systems (EWS) have been implemented worldwide to rapidly detect and monitor the use and impact of NPS. In 2020, the EWS of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported that cathinone and synthetic cannabinoids were the main classes of NPS detected in seized material, accounting for 36% and 28% of the total number of seizures, respectively [3]. In Brazil, data on NPS are scarce, but include seizure data [4, 5] and some postmortem cases [6]. Furthermore, prescription drugs are among the major causes of fatal poisonings in the world, and their concomitant use with illegal drugs is common [7–9].

Blood is the most used biological material to evaluate the role of a drug in modifying human performance and behavior [10] and to investigate intoxication cases [7, 11]. Urine has a larger detection window when compared to other biological specimens, and is the main matrix used in different

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Published online: 05 October 2021

areas of analytical toxicology, such as doping, workplace drug testing, and screening analysis in clinical and forensic toxicology [12]. However, urine drug/metabolite concentrations should not be used to interpret the effect of a drug on human behavior [10]. Analyzing urine and/or blood on a toxicological investigation can provide information on drug intoxications and help to understand the risks of drug abuse [11].

Many extraction/clean-up methods have been used in forensic toxicology for the analysis of a wide range of compounds in biological matrices, including liquid–liquid extraction (LLE) [13], solid phase extraction (SPE) [14, 15] and dispersive solid phase extraction, including QuEChERS (quick, easy, cheap, effective, rugged, and safe) [16–18]. LLE is a simple, not expensive technique, although its application to complex biological matrices, such gastric content, is limited due to high matrix effects and limit of detection (LOD) [13]. SPE can be automated, but has a high analysis cost and maybe time consuming, in addition to difficulties for compounds with different physicochemical properties [15]. QuEChERS has been applied for the analysis of a wide range of compounds, including common drugs and NPS in different matrices. This technique requires a lower amount of sorbents and solvents, it does not need cartridges and column conditioning, yielding an efficient matrix removal [16–18].

The aim of this study was to optimize and validate a method for the determination of prescription drugs and other drugs of abuse, including NPS, in blood (antemortem and postmortem), and urine, using a modified QuEChERS method and ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS), and to analyze samples from forensic cases.

Materials and methods

Chemicals and reagents

Certified reference standards of 25C-NBOH, 25B-NBOH, 25E-NBOH, 25I-NBOH, 25E-NBOMe, 4-chloro-α-PPP (4-Cl-PPP; 4-chloro-α-pyrrolidinopropiophenone), ethylone (bk-MDEA), eutylone (bk-EBDB), and 4-chloroethcathinone (4-CEC) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Clobenzorex was purchased from LGC Standards (Manchester, NH, USA). Δ^9-Tetrahydrocannabinol (THC), 11-nor-9-carboxy-THC (THC-COOH), 2,5-dimethoxyamphetamine (2,5-DMA), 6-monoacetyl morphine (6-MAM), 7-aminoflunitrazepam (7-AF), AH-7921, temazepam, norketamine, nimetazepam, morphine, methylone (bk-MDMA), meta-chlorophenylpiperazine (*m*-CPP), 3,4 methylenedioxy-*N*-ethylamphetamine (MDEA), 3,4-methylenedioxymethamphetamine (3,4-MDMA), methylenedioxypyrovalerone (MDPV), mephedrone, methadone, methamphetamine, LSD, ketamine, JWH-018, AM 2201, cocaine, codeine, benzoylecgonine (BZE), amphetamine, alprazolam, 2C-B, 25B-NBOMe, 25C-NBOMe, and 25I-NBOMe were donated by the United Nations Office on Drugs and Crime (UNODC). 5-MeO-MIPT, AB-CHMINACA, AB-FUBINACA, AKB-48, α-pyrrolidinopentiophenone (α-PVP), α-pyrrolidino pentiothiophenone (α-PVT), dibutylone (bk-DMBDB), tetramethylene-α-pyrrolidinovalerophenone (TH-PVP), JWH-081, JWH-210, JWH-250, phenmetrazine, 2C-H, 2C-I, JWH-081, and JWH-210 were donated by the United States Drug Enforcement Administration (DEA). Standards of sibutramine, midazolam, alfentanil, clonazepam, haloperidol, diazepam, carbamazepine (CBZ), bromazepam, and amitriptyline hydrochloride were kindly donated by the Brazilian Pharmacopeia. Flunitrazepam was donated by INMETRO (Duque de Caxias, RJ, Brazil); Amfepramone (diethylpropion, diethylcathinone) by Aché Pharmaceutical Laboratories S.A (Guarulhos, SP, Brazil); methylphenidate by Novartis Pharma (São Paulo, SP, Brazil). Nitrazepam, meperidine, phencyclidine, trazodone, and hydrocodone were donated by Agilent Technologies (Santa Clara, CA, USA) and tramadol was purchased from Cristália Pharmaceutical (Itapira, SP, Brazil). Harmine, harmaline, and standard solutions of 1 mg/mL cocaine-d_3, diazepam-d_5, LSD-d_3, and MDMA-d_5 (internal standards, IS) were purchased from Cerilliant–Sigma Aldrich (Round Rock, TX, USA). Dimethyltryptamine (DMT) and tetrahydroharmine were synthetized and their identity and purity confirmed by mass spectrometry and NMR [19]. *N*-Ethylpentylone (ephylone) standard was prepared from seized material.

Acetonitrile (ACN) LC–MS grade was purchased from Scharlau (Barcelona, Spain). Primary and secondary amine (PSA), anhydrous magnesium sulfate ($MgSO_4$), and sodium acetate (NaOAc) were purchased from Sigma Aldrich (St. Louis, MO, USA), and formic acid was obtained from Honeywell/Fluka (Düsseldorf, Germany). Ultrapure water was obtained from a Milli-Q purification system (Millipore; Bedford, MA, USA).

Individual stock solutions were prepared in methanol or ACN. THC-COOH and AM 2201 were prepared at 0.1 mg/mL, LSD at 0.025 mg/mL, nitrazepam, meperidine, phencyclidine, trazodone and hydrocodone at 0.001 mg/mL, and the other analytes at 1 mg/mL. Mixed working solutions were prepared at final concentration 150 ng/mL for 25R-NBOH and 25R-NBOMe, with R being a halogen or an ethyl group (C = Cl, B = Br, I = I, E = ethyl), and at 750 ng/mL for the other 71 compounds. Secondary mixed working solutions at 100 ng/mL and 15 ng/mL containing 25R-NBOH and 25R-NBOMe (mix 1) and the other analytes (mix 2) were prepared in addition to a mixed working solution containing all ISs, cocaine-d_3, diazepam-d_5,

LSD-d_3, and MDMA-d_5, at 400 ng/mL. All solutions were kept in amber vials at – 20 °C.

UHPLC–MS/MS conditions

A Waters Acquity UHPLC H-Class Plus system (Waters; Milford, MA, USA) was used for chromatographic separation (Acquity UHPLC BEH C18-column, 2.1 mm i.d. × 100 mm, 1.7 µm particle size), coupled with a Xevo TQ-S Micro tandem-quadrupole mass spectrometer (Waters; Manchester, UK) equipped with a Z-spray electrospray interface was used. Different flow rates (0.4, 0.5, 0.6 mL/min) and injection volumes (0.5, 1, 3 and 5 µL) were evaluated to assess the best peak shape and sensitivity for most compounds and the parameters were established as follows: the mobile phase consisted of water with 0.1% formic acid (A) and ACN with 0.1% formic acid (B). Gradient elution was performed with a constant flow rate of 0.5 mL/min and a column oven temperature of 40 °C, utilizing the following gradient: 0–0.5 min: 1% B; 4 min: 30% B; 7 min: 60% B; 9 min: 70% B; 10–12 min: 99% B; 12.1 min: 1% B. Subsequently, B was held at 1% for 2 min for column equilibration. The total run time equates to 14.1 min. The injection volume was set to 1 µL.

The mass spectrometer with electrospray ionization (ESI) was operated in positive multiple reaction monitoring (MRM) mode. The capillary voltage was set to 3.0 kV. The source block temperature was 150 °C, and the desolvation gas (nitrogen) was heated to 550 °C and delivered at a flow rate of 1100 L/h. The cone gas (nitrogen) was set to 150 L/h, and argon was used as the collision gas. The sample tuning for optimal cone voltage and collision energy was done for each analyte (400 ng/mL) individually and using the IntellStart software (Waters): 10 µL/min flow of each analyte solution was introduced to the mass spectrometer in combination with a LC flow of 0.2 mL/min and 50% of mobile phase B. System operation and data acquisition were controlled using Mass Lynx 4.2 software (Waters). All data were processed with the Target Lynx (Waters).

The molecular formula, retention time (RT), MRM transitions, cone voltage, and collision energy for the 79 analytes and the 4 ISs used in the method are shown in Table S1 (Supplementary Material). The analytes were identified by comparing the RT, the MRM transitions, and the ratio between the two product ions of the corresponding standards. Tramadol was the only substance for which only one transition was included in the method. The relative standard deviation of the RTs was < 2.5% ($n = 15$).

Biological samples

Method development and validation were conducted with drug free postmortem blood and urine samples provided by the Forensic Medical Institute of the Federal District of Brazil (IML/DF), and drug-free urine samples donated by the researcher. A total of 70 samples involved in 68 forensic cases analyzed in this study were also provided by the IML/DF for analysis: 38 postmortem blood samples, 16 antemortem blood samples and 16 antemortem urine samples.

Postmortem blood samples were collected from the femoral vein or cardiac cavity during necropsy. Antemortem blood samples were collected by venipuncture and antemortem urine samples were obtained from the laboratorial routine of the IML/DF. All the blood samples (antemortem and postmortem) were collected in grey-top tubes containing sodium fluoride and potassium oxalate and the urine samples, without any preservative. All the samples collected were stored at – 50 °C until the analysis and analyzed within 15 days after collection. Postmortem blood samples were collected from violent death cases (homicide, suicide, car accident). Antemortem blood and urine samples were collected from individuals in criminal actions under the effect of psychoactive substances (robbery, homicide), drug abuse, drivers suspected to be under influence of drugs, among other circumstances.

Sample extraction and clean-up

Four extraction protocols were tested based on previous work conducted by our research group [7], varying the amount of water and ACN (– 20 °C): protocol 1 (P1) (1 mL of ACN and 1 mL of water); protocol 2 (P2) (0.5 mL of ACN and 1 mL of water); protocol 3 (P3) (1 mL of ACN and 0.5 mL of water); and protocol 4 (P4) (0.5 mL of ACN and 0.5 mL of water). In all cases, 500 µL of biological fluid (urine or blood) and 20 µL of the IS mix were added to a 15 mL falcon-type tube (two glass beads were also added to the tubes containing blood). After adding water and ACN according to each protocol (P1–P4), the tubes were vortexed (15 s.), 500 mg of a mixture of anhydrous $MgSO_4$/NaOAc (4:1) added, vortexed (15 s.), and centrifuged (3430 × *g*/5 min). The supernatant was transferred to a 2 mL microtube containing 25 mg of PSA and 75 mg of anhydrous $MgSO_4$, vortexed (15 s.) and centrifuged (3430 × *g*/5 min). 400 µL of the extract was dried under vacuum (Genevac EZ-2 series, United Kingdom) at 30 °C, reconstituted to 200 µL with mobile phase B (ACN with 0.1% formic acid) and transferred to a vial.

Method validation

The method was validated for selectivity, matrix effect, linearity, recovery, bias/accuracy, repeatability (within-run precision) and intermediate precision (between-day precision), carryover, dilution integrity and sample stability [20]. Each parameter was validated for blood and urine. Three different sets of fortified samples were used during the validation procedure: analytical standards in solvent, analytical standards added to a control matrix pre-extraction and analytical standards added to a control matrix post-extraction.

Selectivity was evaluated by analyzing 10 different blank matrix samples (postmortem blood and antemortem/postmortem urine) to investigate the presence of interferents at the analyte RTs and the MRM transitions chosen in the method.

Matrix effects (interference of other substances leading to suppression or enhancement of the analytical signal) were evaluated by analyzing pooled blood and urine samples ($n = 10$, each) and comparing the sample mean area in post-extraction fortified samples (matrix-matched) with the mean area in solvent fortified samples, and expressed in %. Matrix effects were evaluated for each analyte and matrix at the lowest, medium, and highest concentration level of the calibration curve, and were considered significant when exceeds 25%.

Linearity of the matrix-matched calibration curve was evaluated at five different concentration levels ($n = 3$ at each level): 0.4, 8, 24, 48 and 80 ng/mL for 25R-NBOH and 25R-NBOMe; 10, 40, 120, 240 and 400 ng/mL for LSD, oxycodone, 5-MAPB, AM 2201, amphetamine, codeine, *N*-ethylpentylone, hydrocodone, MDEA and trazodone; 16, 40, 120, 240 and 400 ng/mL for morphine; 10, 16, 40, 120 and 240 ng/mL for THC-COOH; and 4, 40, 120, 240 and 400 ng/mL for the other analytes.

The mean of normalized areas at each point was used for constructing the calibration curve, and Grubbs test was performed to detect outliers. Homoscedasticity of the calibration curve using the least square linear regression was evaluated for each analyte by the Cochran's test, and the curve was considered homoscedastic when standard deviations were not significantly different among the tested levels [21]. For heteroscedastic calibration curves, weighting factors $1/x$, $1/x^2$, $1/x^{0.5}$, $1/y$, $1/y^2$ and $1/y^{0.5}$ were tested to determine the best adjusted linear regression. Linearity of the calibration curve was assumed when the coefficient of determination (r^2) was at least 0.99.

Recovery and repeatability ($n = 3$), bias/accuracy and intermediate precision (triplicate analysis in five different days, same analyst, $n = 15$) were evaluated at the lowest, medium, and highest concentration levels, respectively: 0.4, 24 and 80 ng/mL for 25R-NBOH and 25R-NBOMe; 10, 120 and 400 ng/mL for LSD, oxycodone, 5-MAPB, AM 2201, amphetamine, codeine, *N*-ethylpentylone, hydrocodone, MDEA, and trazodone; 16, 120 and 400 ng/mL for morphine; 10, 40 and 240 ng/mL for THC-COOH; and 4, 120 and 400 ng/mL for the other analytes.

Recovery was calculated by comparing the normalized mean area of pre-extraction fortified samples with the normalized mean area of post-extraction fortified samples, expressed in % ($n = 3$). Bias/accuracy ($n = 15$) was determined as percentage of the target concentration (±%), repeatability ($n = 3$) and intermediate precision ($n = 15$) as relative standard deviation (% RSD) [20]. The acceptance criteria were recovery within the range of 80–120%, bias/accuracy within ± 20%, and repeatability and intermediate precision less than 20% [20].

LOD of the method was defined for each analyte in each matrix as μ + 3.3 s, when "μ" is the average of the noise signal of the blank samples and "s" is the standard deviation of the 10 different blank samples. Limit of quantification (LOQ) of the method was defined for each analyte and each matrix as the lowest level in which the method was validated within the acceptance criteria for bias, recovery, repeatability, and intermediate precision.

The carryover was evaluated by analyzing runs of a pool of five different blank samples of each matrix (postmortem blood and antemortem/postmortem urine) after running the highest calibrator. The analysis was done in triplicate and the acceptance criterion was that the mean area of the quantifier ion at the analyte RT should not exceed 10% of the area of the lowest calibrator [20].

Dilution of the sample is sometimes necessary for forensic samples to fit the calibration curve range. A dilution integrity test was performed for each matrix by diluting with a blank matrix a fortified sample 1:10 and 1:50 (150 ng/mL for 25R-NBOH and 25R-NBOMe, and 750 ng/mL for the other compounds). The impact of the dilution was considered negligible when the estimated concentration of the diluted samples was less than 20% that of the non-diluted sample ($n = 3$).

Stability of the extracted samples was evaluated under different laboratory conditions. Vials containing control fortified samples at medium and high concentrations ($n = 3$) were left in the LC–MS/MS tray (10 °C) or in a dry oven (30 °C) and reanalyzed after 24 h. Change in the concentration after the storage period should not exceed 20% for the analyte to be considered stable under laboratory conditions.

Results

Optimization of the extraction

The method was optimized and validated using postmortem blood samples, to represent the worst-case situation as it is a

Table 1 Internal standard (IS) used, coefficient of determination (r^2), weighting factor (WF), limit of detection (LOD) and limit of quantification (LOQ) of the 79 analytes in blood and in urine

Compound	IS	Blood		Urine		LOD (ng/mL)	LOQ (ng/mL)
		r^2	WF*	r^2	WF*		
2.5-DMA	COC-d_3	0.995	1	0.994	1	1	4
25B-NBOH	LSD-d_3	0.997	1	0.994	1/x	0.1	0.4
25B-NBOMe	LSD-d_3	0.996	1	0.996	1	0.1	0.4
25C-NBOH	LSD-d_3	0.995	1	0.994	1/x	0.1	0.4
25C-NBOMe	LSD-d_3	0.996	1/x^0.5	0.994	1/x^0.5	0.1	0.4
25E-NBOH	COC-d_3	0.995	1	0.996	1/x^0.5	0.1	0.4
25E-NBOMe	LSD-d_3	0.995	1/x^0.5	0.996	1/x^0.5	0.1	0.4
25I-NBOH	LSD-d_3	0.997	1	0.995	1/x^0.5	0.1	0.4
25I-NBOMe	COC-d_3	0.995	1/x	0.995	1/x^0.5	0.1	0.4
2C-B	MDMA-d_5	0.994	1	0.987	1	1	4
2C-H	COC-d_3	0.994	1	0.997	1	1	4
2C-I	COC-d_3	0.998	1/x	0.996	1/x	1	4
4-Cl-PPP	COC-d_3	0.989	1	0.997	1	1	4
4-CEC	COC-d_3	0.991	1	0.990	1	1	4
5-MAPB	COC-d_3	0.998	1	0.996	1	1	4
5-Meo-MIPT	COC-d_3	0.987	1	0.995	1/x	1	4
6-MAM	LSD-d_3	0.996	1	0.997	1	1	4
7-AF	MDMA-d_5	0.996	1	0.998	1	1	4
AB-Chminaca	DIA-d_5	0.997	1/x^0.5	0.996	1/x	0.5	0.8
AB-Fubinaca	DIA-d_5	0.996	1	0.996	1/x	0.5	0.8
AH-7921	COC-d_3	0.995	1	0.988	1	1	4
AKB-48	MDMA-d_5	0.995	1/x^0.5	0.994	1	0.5	0.8
Alfa-PVP	LSD-d_3	0.997	1	0.989	1	1	4
Alfa-PVT	MDMA-d_5	0.996	1	0.997	1	1	4
Alfentanil	MDMA-d_5	0.996	1/x^0.5	0.991	1	1	4
Alprazolam	DIA-d_5	0.995	1/x	0.996	1/x	4	10
AM-2201	COC-d_3	0.994	1	0.992	1	5	10
Amitriptyline	COC-d_3	0.995	1/x^0.5	0.998	1	1	4
Amfepramone	COC-d_3	0.995	1/x^0.5	0.996	1/x^0.5	1	4
Amphetamine	MDMA-d_5	0.987	1	0.990	1	4	10
Benzoylecgonine	COC-d_3	0.994	1/x^0.5	0.993	1/x^0.5	1	4
Bromazepam	DIA-d_5	0.989	1	0.995	1	1	4
Carbamazepine	DIA-d_5	0.990	1	0.994	1/x^0.5	1	4
Clobenzorex	MDMA-d_5	0.989	1	0.997	1	1	4
Clonazepam	DIA-d_5	0.994	1/x^0.5	0.987	1/x^0.5	1	4
Cocaine	COC-d_3	0.995	1/x^0.5	0.987	1	1	4
Codeine	DIA-d_5	0.986	1	0.993	1	5	10
Diazepam	DIA-d_5	0.996	1/x^0.5	0.989	1	1	4
Dibutylone	MDMA-d_5	0.989	1	0.997	1	1	4
DMT	LSD-d_3	0.995	1	0.994	1/x^0.5	1	4
Ethylone	MDMA-d_5	0.996	1	0.997	1	1	4
Eutylone	MDMA-d_5	0.995	1	0.997	1	1	4
Flunitrazepam	DIA-d_5	0.992	1	0.986	1	1	4
Haloperidol	COC-d_3	0.997	1/x^0.5	0.997	1/x^0.5	0.5	0.8
Harmaline	MDMA-d_5	0.994	1/x^0.5	0.996	1	1	4
Harmine	MDMA-d_5	0.993	1	0.994	1	1	4
Hydrocodone	MDMA-d_5	0.995	1	0.992	1	4	10
JWH-018	DIA-d_5	0.995	1/x^0.5	0.995	1/x	0.5	0.8
JWH-081	DIA-d_5	0.997	1/x^0.5	0.996	1/x^0.5	1	4

Table 1 (continued)

Compound	IS	Blood		Urine		LOD (ng/mL)	LOQ (ng/mL)
		r^2	WF*	r^2	WF*		
JWH-210	MDMA-d_5	0.996	1/*x*^0.5	0.995	1	0.5	0.8
JWH-250	MDMA-d_5	0.996	1/*x*^0.5	0.990	1	0.5	0.8
Ketamine	COC-d_3	0.998	1	0.995	1/*x*^0.5	1	4
LSD	LSD-d_3	0.994	1	0.996	1	4	10
m-CPP	MDMA-d_5	0.987	1	0.997	1	1	4
MDEA	MDMA-d_5	0.988	1	0.999	1	4	10
MDMA	MDMA-d_5	0.996	1/*x*	0.999	1	1	4
MDPV	MDMA-d_5	0.993	1	0.996	1	1	4
Mephedrone	MDMA-d_5	0.996	1/*x*^0.5	0.998	1	1	4
Meperidine	MDMA-d_5	0.997	1/*x*^0.5	0.998	1/*x*	4	10
Methadone	COC-d_3	0.996	1/*x*	0.999	1/*x*	1	4
Methanphetamine	MDMA-d_5	0.997	1/*x*	0.999	1	4	10
Methylphenidate	COC-d_3	0.996	1	0.997	1/*x*^2	1	4
Methylone	MDMA-d_5	0.996	1/*x*^0.5	0.999	1	1	4
Midazolam	LSD-d_3	0.994	1/*x*^0.5	0.995	1	1	4
Morphine	MDMA-d_5	0.991	1	0.979	1	10	16
N-Ethylpentylone	MDMA-d_5	0.996	1/*x*^0.5	0.998	1	4	10
Nimetazepam	DIA-d_5	0.996	1/*x*^0.5	0.986	1	1	4
Norketamine	DIA-d_5	0.986	1	0.992	1	1	4
Oxycodone	DIA-d_5	0.996	1/*x*^0.5	0.995	1/*x*^0.5	4	10
PCP	LSD-d_3	0.996	1/*x*	0.996	1/*x*^0.5	4	10
Phenmetrazine	COC-d_3	0.993	1	0.987	1	1	4
Sibutramine	COC-d_3	0.991	1	0.997	1/*x*^0.5	1	4
Temazepam	DIA-d_5	0.998	1/*x*	0.995	1/*x*^0.5	5	10
Tetrahydroharmine	LSD-d_3	0.994	1	0.987	1	1	4
THC	MDMA-d_5	0.996	1/*x*	0.997	1/*x*^0.5	1	4
THC-COOH	COC-d_3	0.995	1/*x*^0.5	0.986	1	4	10
TH-PVP	MDMA-d_5	0.989	1	0.997	1	1	4
Tramadol	COC-d_3	0.995	1	0.994	1	1	4
Trazodone	COC-d_3	0.996	1/*x*^2	0.998	1/*x*^2	4	10

7-AF 7-aminoflunitrazepam, *COC-d_3* cocaine-d_3, *DIA-d_5* diazepam-d_5

*1 = homoscedastic; other values = heteroscedastic

more complex matrix than antemortem sample [7, 19]. The extraction protocols for both blood and urine that used the smaller amount of ACN (P2 and P4) did not form enough supernatant, which made them unsuitable to proceed to the next sample preparation step. Therefore, only P1 (1 mL ACN and 1 mL water) and P3 (1 mL ACN and 0.5 mL of water) protocols were evaluated for the matrix effect, recovery, and repeatability.

All the analytes in both extraction protocols showed acceptable repeatability (RSD < 20%). In urine, 6-MAM and oxycodone showed ion suppression effect (> 25%) in both protocols; in P3, morphine showed ion enhancement (27.7%) and phenmetrazine, ion suppression (29.5%). In blood, codeine showed ion enhancement (26.2%) and 2,5-DMA, ion suppression (25.0%) in P3 protocol (data not shown). P1 protocol showed recovery ranging from 80 to 120% for 76 analytes in urine and for 71 analytes in blood. Using P3, recovery for 71 analytes were within the optimum range for urine and 67 analytes for blood (data not shown). Considering matrix effects and recovery, the P1 extraction procedure was chosen for validation of the 79 analytes in urine and blood.

UPLC–MS/MS method validation

No interfering peaks were observed for the MRM transitions at the RTs of the analytes in blank matrices (blood and urine), indicating that the method is selective. Matrix effects for the 79 compounds in blood and urine are shown in Table S2. In urine, ion suppression was observed for amphetamine, codeine, methamphetamine, and morphine (28.8–37.1%) at the lowest concentration level and for 6-MAM and AM 2201 at medium level (36.1 and 35.1%, respectively). In blood, significant ion suppression (28.0–37.2%) was found at the lowest level for 6-MAM, benzoylecgonine, codeine, methamphetamine, and morphine. As significant matrix effects (> ± 25%) were found for 12 matrix-analyte combinations (Table S2), a matrix-matched calibration curve was used for quantification [20].

Table 1 shows the respective IS used for quantification, the coefficient of determination (r^2) and the adjusted weighting factor of the matrix-matched calibration curve for each analyte in blood and urine (equal to 1 for homoscedastic and different from 1 for heteroscedastic compounds). All four IS were tested for all compounds and the one that gave the best matrix-matched calibration curve linearity was used for quantification. Most analytes had the best $r^2 \geq 0.99$ and only for morphine in urine, the r^2 was less than 0.98 (0.979).

Summary of bias/accuracy in blood and urine is shown in Fig. 1, and of recovery, repeatability/intermediate precision of the compounds are shown in Fig. 2. Detailed information is shown in Tables S3 to S5 (Supplementary Material). Bias for both matrices was within ± 20%, and recoveries were in the range of 80–120% for most substances. Repeatability and intermediate precision for both matrices were within 20%, except for 5-MAPB (22%) at the low concentration level of the repeatability in blood. LOD ranged from 0.1 to 10 ng/mL and LOQ, from 0.4 to 16 ng/mL (Table 1).

Carryover results were within the proposed accepted range (data not shown). The dilution tests showed RSD < 10% for all the compounds and the post-processing stability study showed that all analytes were stable at 10 °C (LC tray) and at 30 °C after 24 h. Furthermore, Figure S1 (Supplementary Material) shows that the variation of the concentration results (%) at medium and high concentration levels at different storage temperature for urine and blood are also within the accepted range (± 20%).

Forensic cases

The validated method was used for the analysis of 16 urine antemortem samples and 54 blood samples (antemortem and postmortem) from 68 forensic cases. Twenty-two blood samples (31.4% of the analyzed samples) did not contain any of the investigated analytes. At least one substance was detected in samples from 46 cases (16 urine and 32 blood samples) and 27.8% of the 79 analytes investigated in this study were detected in at least one biological sample. The analyte concentration found in each case/matrix are shown in Table 2 for antemortem samples

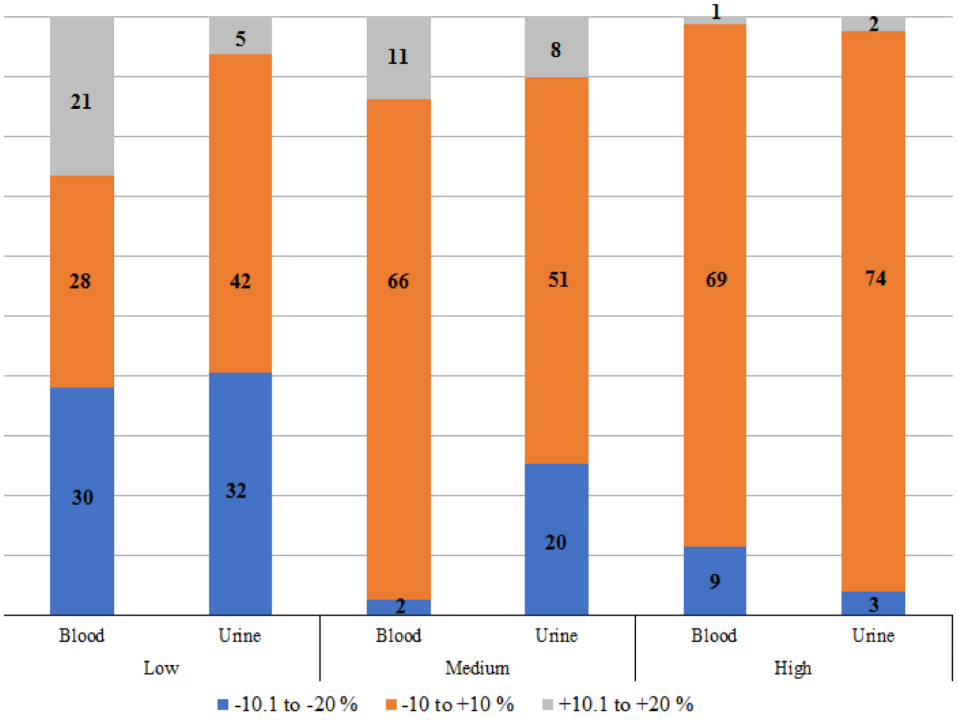

Fig. 1 Number of compounds in each range of mean bias (± %, $n = 15$) for the 79 analytes in blood and urine at the low, medium and high concentration levels, respectively: 0.4, 24 and 80 ng/mL for 25R-NBOH and 25R-NBOMe; 10, 120 and 400 ng/mL for LSD, oxycodone, 5-MAPB, AM 2201, amphetamine, codeine, *N*-ethylpentylone, hydrocodone, MDEA and trazodone; 16, 120 and 400 ng/mL for morphine; 10, 40 and 240 ng/mL for THC-COOH; and 4, 120 and 400 ng/mL for the other analytes. Detailed information is shown in Table S3

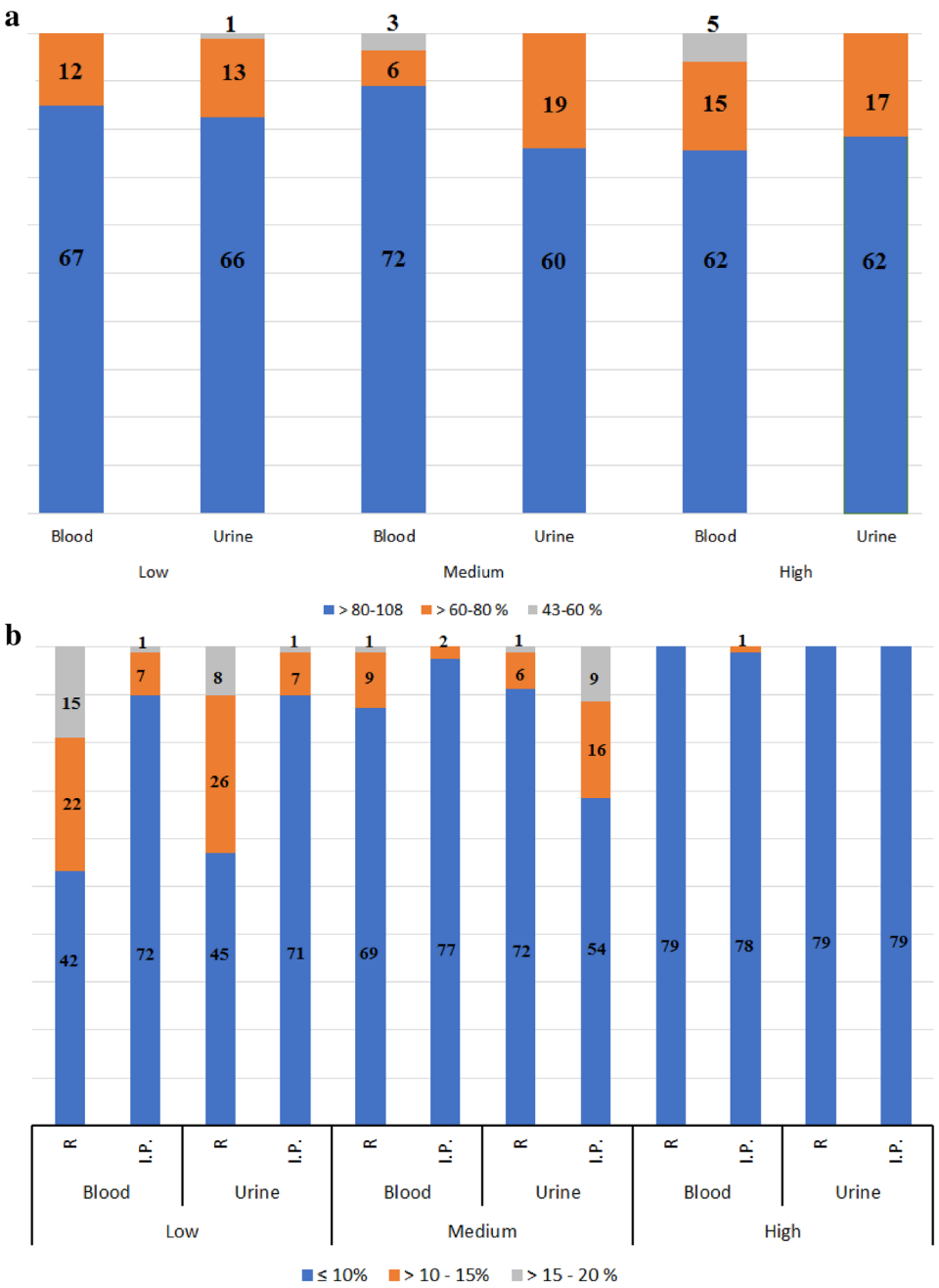

Fig. 2 Number of compounds in each range of (**a**) % mean recovery ($n=3$), (**b**) mean % RSD for repeatability (R, $n=3$) and intermediate precision (IP, $n=15$) for the 79 analytes in blood and urine at the low, medium and high concentration levels, respectively: 0.4, 24 and 80 ng/mL for 25R-NBOH and 25R-NBOMe; 10, 120 and 400 ng/mL for LSD, oxycodone, 5-MAPB, AM 2201, amphetamine, codeine, *N*-ethylpentylone, hydrocodone, MDEA and trazodone; 16, 120 and 400 ng/mL for morphine; 10, 40 and 240 ng/mL for THC-COOH; and 4, 120 and 400 ng/mL for the other analytes. Detailed information are shown in Tables S4 and S5

(blood and urine) and in Table 3 for postmortem blood samples, and the concentration ranges for each analyte are shown in Table 4. Samples that contained the analyte at a concentration higher than the calibration range were diluted for the quantification. Quality control samples fortified at the medium level (urine and blood) were included in each batch of 15–20 samples and gave satisfactory bias/accuracy (within ± 20%).

In two cases, both antemortem blood and urine samples were analyzed (cases 3 and 16; Table 2). In case 16, trazodone and clonazepam were found in the blood and *m*-CPP, the main trazodone metabolite, in urine (Table 2). Figure 3 shows MRM chromatograms of a postmortem blood of another suicide case (39-year-old woman, case 43) containing trazodone and *m*-CPP, which was also detected in other four autopsy cases (Table 3).

Table 2 Results of 22 antemortem positive samples from 20 forensic cases investigated by the Civil Police of Federal District (6 blood and 16 urine samples)

Case	Gender (age)	Specimen	Substances detected (concentration, ng/mL)
1	M (22)	Urine	7-AF (16.8), THC-COOH (165)
3	M (70)	Blood	Amitriptyline (433)
		Urine	Amitriptyline (25.6)
4	M (22)	Urine	Amitriptyline (444), BZE (13,900), clonazepam (32.7), cocaine (653), THC-COOH (798)
7	M (31)	Urine	Amitriptyline (17.8), BZE (970)
9	M (24)	Urine	BZE (203)
12	M (34)	Urine	BZE (936), THC-COOH (776)
14	F (23)	Blood	Carbamazepine (7920), diazepam (808)
16	F (30)	Blood	Clonazepam (5.3), trazodone (226)
		Urine	*m*-CPP (6.5)
17	M (23)	Urine	BZE (47,800), cocaine (494), THC-COOH (227)
26	F (30)	Urine	*m*-CPP (290), MDMA (90.9), trazodone (41.7)
29	M (23)	Urine	MDMA (2730)
34	M (27)	Urine	THC-COOH (694)
35	M (27)	Urine	THC-COOH (755)
36	M (32)	Urine	THC-COOH (363)
37	M (50)	Urine	THC-COOH (379)
38	M (27)	Blood	BZE (59.1), ethylone (222)
40	H (22)	Blood	*N*-Ethylpentylone (7.3)
41	M (NI)	Urine	Eutylone (415)
42	M (NI)	Urine	Eutylone (246)
44	M (26)	Blood	MDMA (21.3)

M male, *F* female, *NI* not informed, *7-AF* 7-aminoflunitrazepam, *BZE* benzoylecgonine, *MDMA* 3,4-methylenedioxymetamphetamine, *THC-COOH* 11-nor-9-carboxy-THC, *m-CPP* meta-chlorophenylpiperazine

Cocaine was detected in two urine samples, and in only one postmortem blood sample (Tables 2 and 3). Levels of benzoylecgonine (BZE), the main cocaine metabolite, ranged from 11.3 to 112 ng/mL in four postmortem blood sample, and reached 47,800 ng/mL in one urine sample (the highest drug level found in the study) (Table 4). THC was only found in one postmortem blood sample, but its metabolite THC-COOH was found in three postmortem blood samples and eight urine samples (Tables 2 and 3).

Out of the 14 synthetic cathinones included in the method, three were detected in the samples (Table 4). *N*-Ethylpentylone was detected at 597 ng/mL level in a postmortem blood sample (Fig. 4a), and in an antemortem blood sample at much lower level (7.3 ng/mL). Ethylone (222 ng/mL, antemortem blood) and eutylone in two urine samples 246 ng/mL (Fig. 4b) and 446 ng/mL were also detected.

Discussion

In this study, a lower number of compounds with significant matrix effect was found in protocol P1, probably due to the higher extract dilution (2 mL) compared to the other protocols (1.5 mL) which, indeed, is one of the tools to overcome matrix effects [22]. The main matrix effect found was ion suppression, which is commonly observed in LC–MS/MS methods. Orfanidis et al. [18] reported up to 29.4% ion suppression for 6-MAM and norfentanyl, Lehmann et al. [15] for PCP in serum (89%), Odoardi et al. [23] for synthetic cannabinoids in whole blood (up to 39%) and Yang et al. [24] in urine for amphetamine (29.3%) and morphine (39.4%). Ion enhancement was found for some compounds (up to 18%) and was also reported by other authors. Lehmann et al. [15] showed ion enhancement for methylphenidate (71%) and 4-AcO-MET (290%) in serum, and Gaunitz et al. [25] found up to 85%

Table 3 Results of 26 postmortem blood positive samples from 26 forensic **c**ases investigated by the Civil Police of the Federal District

Case	Gender (age)	Substances detected (concentration, ng/mL)
2	F (66)	Amitriptyline (6440), diazepam (808), temazepam (218)
5	M (40)	Amitriptyline (5220), BZE (11.3), diazepam (337), temazepam (16.1)
6	F (31)	Amitriptyline (127), clonazepam (8.4)
8	F (19)	Amitriptyline (11,500)
10	M (38)	BZE (112)
11	M (36)	BZE (27.4)
13	M (43)	BZE (99.3)
15	M (22)	Clonazepam (39.4), codeine (71.2), THC-COOH (134)
18	M (34)	Cocaine (176)
19	F (84)	Diazepam (12.7), *m*-CPP (18.8), midazolam (2210)
20	F (36)	Diazepam (278), temazepam (44.4)
21	M (27)	Diazepam (53.0)
22	M (55)	Ketamine (1830), norketamine (855)
23	F (72)	Midazolam (186)
24	M (38)	Morphine (137)
25	F (91)	*m*-CPP (10.4), methylphenidate (5.7), midazolam (218), trazodone (78.1)
27	F (54)	Midazolam (144)
28	M (31)	MDMA (149)
30	M (23)	MDMA (1590), THC (31.9), THC-COOH (795)
31	F (74)	Midazolam (118)
32	F (95)	Morphine (78.4)
33	F (54)	THC-COOH (106)
39	M (21)	Clonazepam (120), diazepam (205), temazepam (9.4), midazolam (439)
43	F (39)	*m*-CPP (1280), trazodone (13,600)
45	M (33)	Midazolam (464)
46	F (19)	*N*-Ethylpentylone[a] (597)

[a]A fatal case (year 2018)

ion enhancement for synthetic cannabinoid metabolites in urine.

Nine analytes in urine and 7 analytes in blood showed recoveries less than 80% for 2 or 3 tested levels (range of 57–79% for urine; 43–79% for blood). Low recoveries have also been reported by Lehmann et al. [15] for 51 of the 74 investigated drugs in blood using in-line SPE-LC–MS/MS (27–69%), and by Odoardi et al. [23] for the 57 compounds evaluated (5–68%) using a dispersive liquid–liquid microextraction. Gaunitz et al. [25] obtained low recoveries (43%–69%) for 9 of the 61 synthetic cannabinoid metabolites in urine, using a SPE extraction. Orfanidis et al. [18] found recoveries ranging from 75.2 to 114.9% for the 84 analytes in blood, including cathinones, amphetamines, opioids and prescription drugs using a QuEChERS protocol, and Yang et al. [24] obtained recoveries between 71.1 and 99.6% for 10 compounds in urine, including amphetamines, opioids, ketamine, and norketamine, using SPE extraction/clean-up and LC–MS/MS.

The group of NBOHs and NBOMes had the lowest LOQ (0.4 ng/mL) and morphine the highest (16 ng/mL) among all analytes included in the method, values that were enough for detecting drugs at therapeutic blood concentration [26] and drug intoxication cases, including phenethylamine derivatives, such as 25R-NBOMe [27], cathinones [14] and morphine [26].

The list of substances included in the validated method is in accordance with recent data from Brazilian drug seizures. In the state of Minas Gerais, the main seized synthetic drugs from 2008 to 2017 were amphetamines (mainly MDMA), cathinones, and phenethylamines [5]. The analysis of more than 1 million of blotter papers seized from 2011 to 2017 in the state of Santa Catarina showed phenethylamines and synthetic cannabinoids, in addition

Table 4 Concentration range of the analytes found in 22 antemortem samples (6 blood and 16 urine) and 26 postmortem blood samples from 46 forensic cases

Analyte	Postmortem blood. ng/mL (*n*)	Antemortem blood, ng/mL (*n*)	Antemortem urine, ng/mL (*n*)
7-Aminofluni-trazepam	–	–	16.8 (1)
Amitriptyline	127–11,500 (4)	433 (1)	17.8–444 (3)
Benzoylecgonine	11.3–112 (4)	59.1 (1)	203–47,800 (5)
Carbamazepine	–	7920 (1)	–
Clonazepam	8.4–120 (3)	5.3 (1)	32.7 (1)
Cocaine	176 (1)	–	494–653 (2)
Codeine	71.2 (1)	–	–
Diazepam	53.0–808 (6)	808 (1)	–
Ethylone	–	222 (1)	–
Eutylone	–	–	246–415 (2)
Ketamine	1830 (1)	–	–
m-CPP	10.4–1280 (3)	–	6.5–290 (2)
MDMA	149–1590 (2)	21.3 (1)	90.9–2730 (2)
Methylphenidate	5.7 (1)	–	–
Midazolam	149–2210 (7)	–	–
Morphine	78.4–137 (2)	–	–
N-Ethylpenty-lone	597 (1)	7.3 (1)	–
Norketamine	855 (1)	–	–
Temazepam	9.4–218 (4)	–	–
THC	31.9 (1)	–	–
THC-COOH	106–795 (3)	–	165–798 (8)
Trazodone	78.1–13,600 (2)	226 (1)	41.7 (1)

n number of positive samples

to LSD, amphetamines, and opioids [4]. Four compounds of the *N*-benzylphenethylamine class (25R-NBOMe) were included in the present study, in addition to four 25R-NBOHs. NBOMe compounds have been available since 2010 in the on-line market, resulting in various toxicity and fatal cases [28], and have been detected in seized materials analyzed in Brazil [4, 5]. The 25R-NBOH compounds are another emerging drug family in the illicit drug market [29], and are also 25R-NBOMe metabolites [30]. The inclusion of metabolites in a systematic toxicological analysis is important to elucidate phenethylamines poisoning in forensic cases. Low blood concentrations involving NBOMe ingestion have been reported in the literature, including 25I-NBOMe (0.25 ng/mL) [27] and 25B-NBOMe (0.16 ng/mL) [31].

Synthetic cathinones are an important class of NPS, with central nervous system-stimulant properties similar to cocaine and conventional amphetamines. The molecular structure of these substances is related to cathinone, a psychoactive of natural origin present in Khat (*Catha edulis*) [6, 32]. The validated method includes 14 synthetic cathinones, such as the structural isomers eutylone and dibutylone, which showed good chromatographic separation. Concomitant consumption of synthetic drugs and prescribed drugs is frequently related, which can lead to overdose due to pharmacological interactions [3]. Hence, besides the illicit drugs, 23 prescription drugs (benzodiazepines, antidepressants, opioids, and others) were also included in the method.

About 28% of the 79 analytes investigated in this study were detected in at least one biological sample. Prescription drugs were detected in 52% of the 46 positive samples (urine and/or blood), mainly amitriptyline (8 cases) and benzodiazepines (12 cases), alone or in combination with illegal drugs. Overdose with amitriptyline was the cause of death of a 19-year-old woman, which was confirmed by the very high level of the drug in postmortem blood. Trazodone and *m*-CPP, the main trazodone metabolite, were found together in three forensic cases (blood and/or urine). Trazodone is a serotonin antagonist and reuptake inhibitor used as an antidepressant [33], and *m*-CPP is also sold as a designer drug [34]. Hence, it is important to differentiate *m*-CPP found as a trazodone metabolite from its intake as a designer drug to help to interpretate the forensic case. THC (mainly as its metabolite THC-COOH; 11 cases) and cocaine (mainly as its metabolite benzoylecgonine; 9 cases) were the main illegal drugs found in the samples. In a previous work conducted in the Federal District, cocaine was the main illegal drug found in the postmortem blood samples, present in 15% of the analyzed samples (up to 3130 ng/mL), and benzodiazepines the main prescription drugs; however, NPS were not included in the study [8].

Three synthetic cathinones were detected in five forensic cases, including a 19-year-old female case who died after taking *N*-ethylpentylone in a rave party. Synthetic cathinones have also been reported in biological samples by other authors. *N*-Ethylpentylone concentrations in postmortem blood ranged from 7 to 170 ng/mL in Brazilian cases [6] and from 12 to 1200 ng/mL in USA [35, 36]. Lee et al. [32] reported seven postmortem cases with ethylone detected in blood, ranging from 38 to 2572 ng/mL and Krotulski et al. [37] reported 22 postmortem cases involving eutylone, with blood levels ranging from 1.2 to 11,000 ng/mL and two urine samples (60 and > 10,000 ng/mL).

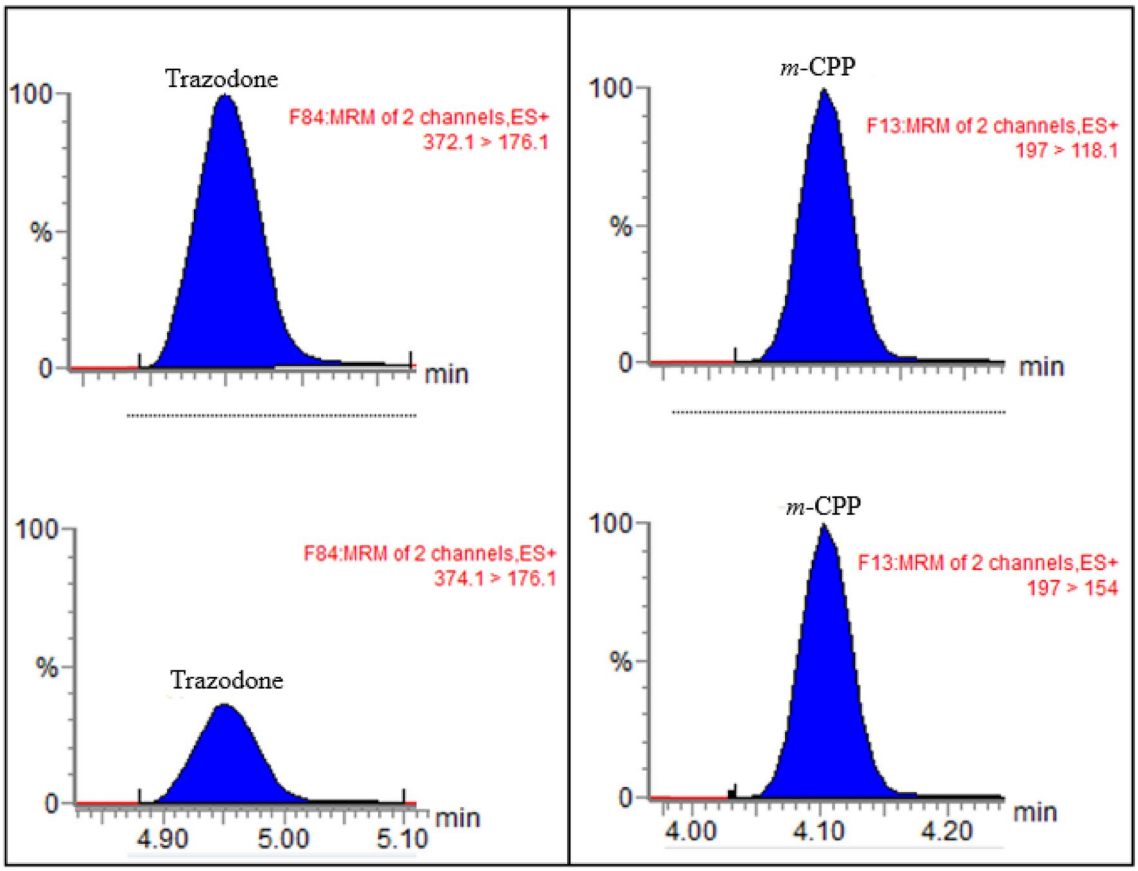

Fig. 3 Multiple reaction monitoring (MRM) chromatograms of trazodone (13,600 ng/mL) and *m*-CPP (1280 ng/mL) from a forensic postmortem blood sample, showing the two ion transitions for each compound (relative abundance)

To the best of our knowledge, this is the first study that uses a QuEChERS method for the analysis of a large number of analytes that included 25R-NBOMe and 25R-NBOH family compounds in blood and urine. The method is easy to implement, showing to be well-suited for toxicological analysis. Accuracy and precision obtained were comparable or better than those methods that used LLE [13] or SPE [15]. One limitation of this work is that some metabolites that could be detected in urine samples are not included in the method. For example, an enzymatic hydrolysis step should be included in the sample preparation to detect phase II metabolites from opioids and synthetic cannabinoids.

Conclusions

A modified QuEChERS protocol followed by UHPLC–MS/MS method was validated for toxicological analysis of antemortem urine and blood, and postmortem blood for the determination of prescription and illegal drugs, including NPS of various classes, such as synthetic cathinones and cannabinoids, phenethylamines, tryptamines, amphetamines, opioids and others. The method is simple and fast to execute in a forensic laboratory and it was successfully applied to the analysis of forensic case samples. The main advantages over most published methods are the large scope, with 79 analytes from different chemical classes (NPS, such as 25R-NBOMe and 25R-NBOH, prescription and common drugs) and its application in two biological matrices. Sample analysis from forensic medical institutes is important for monitoring and understanding the impact of NPS and other drugs in poisoning cases. Analyzing different biological specimens showed to be an interesting approach to collect information on emerging drug threats.

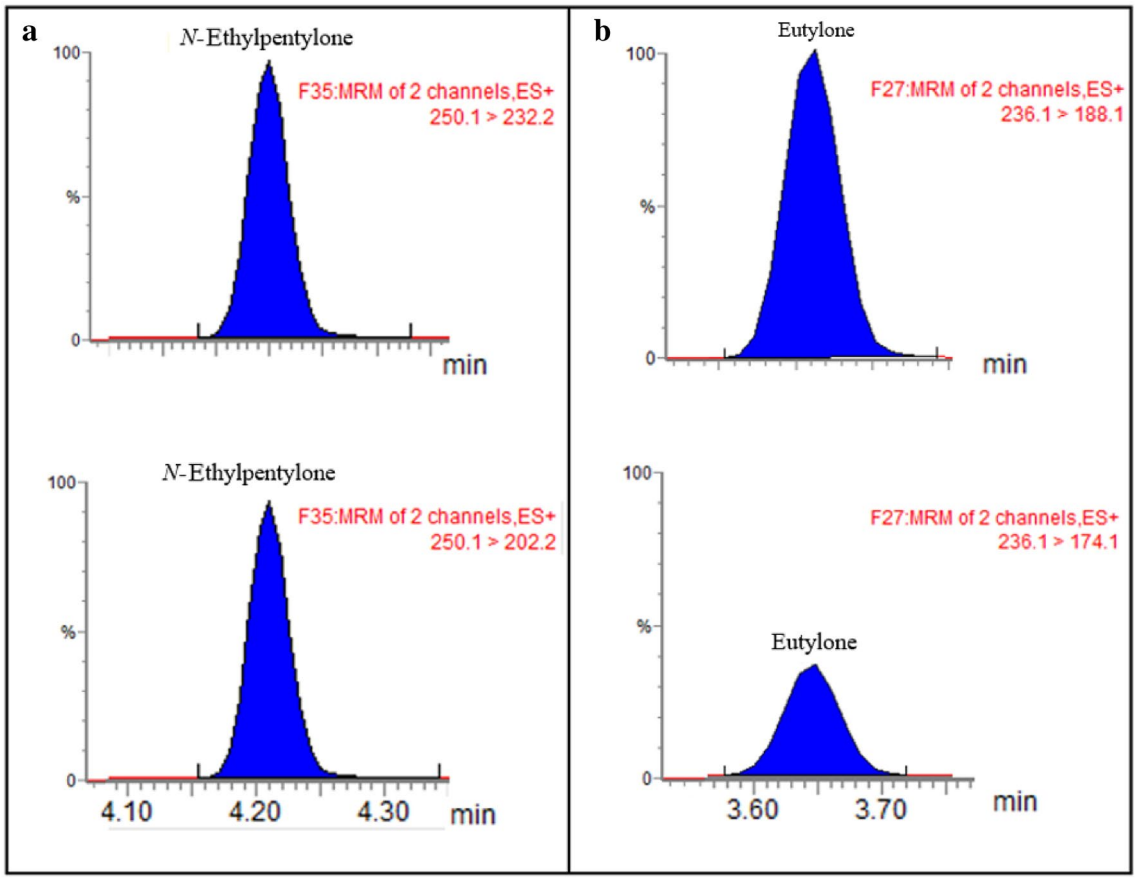

Fig. 4 MRM chromatograms of 2 forensic cases involving synthetic cathinones. (**a**) *N*-Ethylpentylone in postmortem blood (597 ng/mL) and (**b**) eutylone in antemortem urine (246 ng/mL) (relative abundance)

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11419-021-00600-y.

Acknowledgements The authors thank Tales Mateus Vieira da Rocha for the help with sample preparation.

Declarations

Conflict of interest There are no financial or other relations that could lead to a conflict of interest to disclose.

Ethical approval This study was approved by the Ethical Committee for Human Studies of the University of Brasilia, Brazil (CAAE 2936819.3.0000.0030).

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Supplementary material

Determination of new psychoactive substances and other drugs in postmortem blood and urine by UHPLC–MS/MS: method validation and analysis of forensic samples

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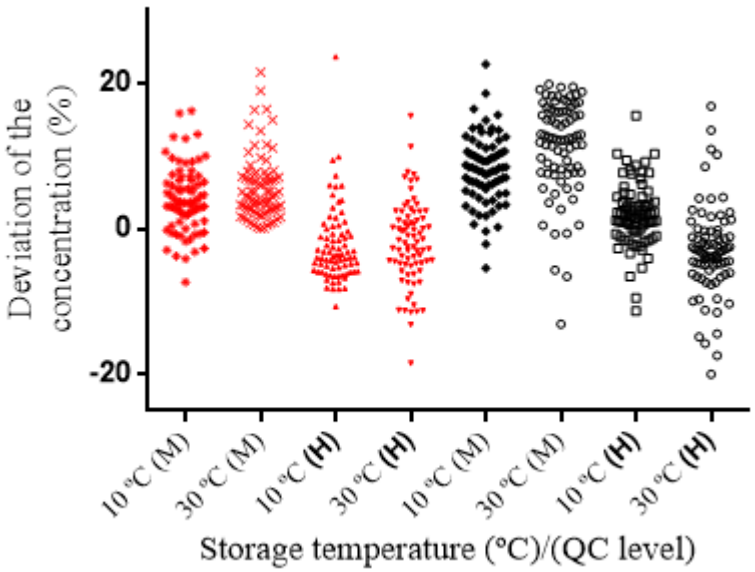

Fig. S1 Concentration deviations (%) of the analytes at the storage temperatures 10ºC and 30ºC for the QC medium and high concentrations for blood and urine (n = 3, each). M = QC medium. H = QC high. Red symbols are the blood tests, and the black ones are the urine tests. Medium and high concentrations for each analyte: 24 and 80 ng/mL, for 25R-NBOH and 25R-NBOMe; 40 and 240 ng/mL for THC-COOH; 120 and 400 ng/mL for the other analytes

Table S1 Retention time (RT), multiple reaction monitoring transitions and respective cone voltage and collision energy of the 79 compounds analyzed and the 4 internal standards

Compound	Molecular formula	RT (min)	Transition (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
2,5-DMA	$C_{11}H_{17}NO_2$	3.71	**196 > 121**	10	25
			196 > 179	10	10
25B-NBOH	$C_{17}H_{20}BrNO_3$	5.73	**366 > 107**	25	30
			366 > 243	25	22
25B-NBOMe	$C_{18}H_{22}BrNO_3$	6.24	**380 > 121**	25	48
			380 > 91.2	25	20
25C-NBOH	$C_{17}H_{20}ClNO_3$	5.59	**322.2 > 107.1**	40	25
			322.2 > 199	40	20
25C-NBOMe	$C_{18}H_{22}ClNO_3$	6.11	**336.9 > 121**	25	20
			336.9 > 91	25	49
25E-NBOH	$C_{19}H_{25}NO_3$	6.15	**316.1 > 107.1**	20	35
			316.1 > 193.1	20	22
25E-NBOMe	$C_{20}H_{27}NO_3$	6.65	**330.1 > 121.1**	20	20
			330.1 > 91.1	20	65
25I-NBOH	$C_{17}H_{20}INO_3$	5.97	**414.1 > 107.1**	30	20
			414.1 > 291	30	32
25I-NBOMe	C18H22INO3	6.47	**428.1 > 121**	30	47
			428.1 > 91	30	26
2C-B	$C_{10}H_{14}BrNO_2$	4.9	**260 > 243**	30	15
			260 > 228	30	20
2C-H	$C_{10}H_{15}NO_2$	3.38	**182 > 165**	10	10
			182 > 150	10	19
2C-I	$C_{10}H_{14}INO_2$	4.73	**307.9 > 290.9**	30	15
			307.9 > 275.9	30	23
4-Cl-PPP	$C_{13}H_{16}ClNO$	4.15	**238 > 139**	30	26
			238 > 167	30	20
4-CEC	$C_{11}H_{14}ClNO$	3.98	**212 > 159**	25	20
			212 > 139	25	30
5-MAPB	$C_{12}H_{15}NO$	4.17	**190 > 131**	25	20
			190 > 159	25	10
5-Meo-MIPT	$C_{15}H_{22}N_2O$	3.93	**247.1 > 174**	30	20
			247.1 > 159.1	30	32
6-MAM	$C_{19}H_{21}NO_4$	3.15	**328 > 165**	40	36
			328 > 211	40	25
7-AF	$C_{16}H_{14}FN_3O$	4.33	**284.1 > 135**	45	27
			284.1 > 227	45	27
AB-CHMINACA	$C_{20}H_{28}N_4O_2$	8.06	**357.1 > 312**	30	16
			357.1 > 241	30	26

Compound	Molecular formula	RT (min)	Transition (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
AB-FUBINACA	$C_{20}H_{21}FN_4O_2$	7.18	**369.1 > 324**	30	15
			369.1 > 109	30	40
AH-7921	$C_{16}H_{22}Cl_2N_2O$	5.55	**329 > 284**	30	15
			329 > 95	30	30
AKB-48	$C_{23}H_{31}N_3O$	11.18	**366.2 > 135.2**	30	26
			366.2 > 93.1	30	55
α-PVP	$C_{15}H_{21}NO$	4.28	**232 > 91**	25	30
			232 > 126	25	22
α-PVT	$C_{13}H_{19}NOS$	3.84	**238.6 > 97**	30	25
			238.6 > 126	30	20
Alfentanil	$C_{21}H_{32}N_6O_3$	5.33	**417 > 99**	30	40
			417 > 165	30	36
Alprazolam	$C_{17}H_{13}ClN_4$	6.35	**309.1 > 205.1**	20	38
			309.1 > 281.1	20	24
AM-2201	$C_{24}H_{22}FNO$	9.17	**360.2 > 155**	30	25
			360.2 > 127	30	46
Amitriptyline	$C_{20}H_{23}N$	6.3	**278.2 > 105.1**	50	20
			278.2 > 91.1	50	20
Amfepramone	$C_{13}H_{19}NO$	3.37	**206 > 100**	25	29
			206 > 105	25	29
Amphetamine	$C_9H_{13}N$	3.03	**136.1 > 119**	20	7
			136.1 > 91.1	20	14
Benzoylecgonine	$C_{16}H_{19}NO_4$	3.59	**290.1 > 168**	20	40
			290.1 > 104.8	20	30
Bromazepam	$C_{14}H_{10}BrN_3O$	5.37	**316 > 182**	35	32
			316 > 209.1	35	26
Carbamazepine	$C_{15}H_{12}N_2O$	5.79	**237.1 > 194.1**	50	20
			237.1 > 220	50	13
Clobenzorex	$C_{16}H_{18}ClN$	5.5	**260 > 91.1**	20	45
			260 > 125	20	30
Clonazepam	$C_{15}H_{10}ClN_3O_3$	6.21	**316.1 > 270.1**	20	35
			316.1 > 214	20	25
Cocaine	$C_{17}H_{21}NO_4$	4.42	**304.1 > 182**	20	18
			304.1 > 81.9	20	30
Cocaina-d_3[a]	$C_{17}H_{18}D_3NO_4$	4.4	**307 > 185.1**	20	25
Codeine	$C_{18}H_{21}NO_3$	2.86	**300.1 > 165**	40	38
			300.1 > 215	40	23
Diazepam	$C_{16}H_{13}ClN_2O$	7.14	**285.1 > 154.1**	20	25

Compound	Molecular formula	RT (min)	Transition (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
			285.1 > 193.2	20	30
Diazepam-d_5[a]	$C_{16}H_8D_5ClN_2O$	7.1	**290 > 154**	30	30
Dibutylone	$C_{13}H_{17}NO_3$	3.5	**236.1 > 161**	30	17
			236.1 > 133.1	30	22
DMT	$C_{12}H_{16}N_2$	3.26	**189.1 > 58**	25	12
			189.1 > 144	25	21
Ethylone	$C_{12}H_{15}NO_3$	3.16	**222.1 > 174.1**	20	18
			222.1 > 204.1	20	12
Eutylone	$C_{13}H_{17}NO_3$	3.64	**236.1 > 174.1**	20	30
			236.1 > 188.1	20	15
Flunitrazepam	$C_{16}H_{12}FN_3O_3$	6.47	**314.1 > 268**	30	24
			314.1 > 239	30	34
Haloperidol	$C_{21}H_{23}ClFNO_2$	5.74	**376 > 165.1**	30	32
			376 > 123.1	30	52
Harmaline	$C_{13}H_{14}N_2O$	4.12	**215 > 172**	30	40
			215 > 200	30	40
Harmine	$C_{13}H_{12}N_2O$	4.14	**213 > 170**	30	40
			213 > 198	30	40
Hydrocodone	$C_{18}H_{21}NO_3$	3.24	**300.1 > 199.1**	40	29
			300.1 > 128.1	40	58
JWH-018	$C_{24}H_{23}NO$	10.3	**342.1 > 127.1**	40	45
			342.1 > 155	40	27
JWH-081	$C_{25}H_{25}NO_2$	10.8	**372.2 > 185.1**	50	23
			372.2 > 157.1	50	40
JWH-210	$C_{26}H_{27}NO$	10.8	**370.2 > 183.1**	20	25
			370.2 > 214.1	20	23
JWH-250	$C_{22}H_{25}NO_2$	9.63	**336.1 > 121**	30	21
			336.1 > 91	30	40
Ketamine	$C_{13}H_{16}ClNO$	3.71	**238.1 > 207**	20	12
			238.1 > 124.9	20	25
LSD	$C_{20}H_{25}N_3O$	4.69	**324.1 > 223.1**	30	23
			324.1 > 208.1	30	30
LSD-d_3[a]	$C_{20}H_{22}N_3OD_3$	4.69	**327 > 226**	30	31
m-CPP	$C_{10}H_{13}ClN_2$	4.13	**197 > 118.1**	35	34
			197 > 154	35	18
MDEA	$C_{12}H_{17}NO_2$	3.57	**208.1 > 163**	20	11
			208.1 > 105	20	26

Compound	Molecular formula	RT (min)	Transition (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
MDMA	$C_{11}H_{15}NO_2$	3.3	**194.1 > 163**	20	10
			194.1 > 105	20	22
MDMA-d_5[a]	$C_{11}H_{10}D_5NO_2$	3.29	**199 > 165**	30	15
MDPV	$C_{16}H_{21}NO_3$	4.38	**276.1 > 175**	40	23
			276.1 > 205	40	18
Mephedrone	$C_{11}H_{15}NO$	3.55	**178.1 > 145.1**	25	18
			178.1 > 91	25	30
Meperidine	$C_{15}H_{21}NO_2$	4.51	**248.1 > 220.1**	20	20
			248.1 > 174.1	20	18
Methadone	$C_{21}H_{27}NO$	6.37	**310.2 > 265.1**	20	14
			310.2 > 104.9	20	28
Methanphetamine	$C_{10}H_{15}N$	3.23	**150.1 > 119**	20	9
			150.1 > 91	20	15
Methylphenidate	$C_{14}H_{19}NO_2$	4.16	**234 > 84.2**	25	28
			234 > 56	25	62
Methylone	$C_{11}H_{13}NO_3$	2.9	**208 > 160.1**	30	18
			208 > 132.1	30	28
Midazolam	$C_{18}H_{13}ClFN_3$	5.47	**326.1 > 291.2**	20	25
			326.1 > 249.1	20	34
Morphine	$C_{17}H_{19}NO_3$	2.04	**286.2 > 165.1**	50	35
			286.2 > 201.1	50	23
N-Ethylpentylone	$C_{14}H_{19}NO_3$	4.22	**250.1 > 232.2**	40	14
			250.1 > 202.2	40	17
Nimetazepam	$C_{16}H_{13}N_3O_3$	6.52	**296 > 221**	30	35
			296 > 250	30	25
Norketamine	$C_{12}H_{14}ClNO$	3.6	**224 > 207**	20	10
			224 > 125	20	22
Oxycodone	$C_{18}H_{21}NO_4$	3.09	**316.1 > 241**	20	27
			316.1 > 298.1	20	17
PCP	$C_{17}H_{25}N$	5.13	**244.1 > 86**	20	9
			244.1 > 91	20	25
Phenmetrazine	$C_{11}H_{15}NO$	3.16	**178 > 117**	30	20
			178 > 91.1	30	20
Sibutramine	$C_{17}H_{26}NCl$	6.58	**280.1 > 153**	30	13
			280.1 > 125	30	31
Temazepam	$C_{16}H_{13}ClN_2O_2$	6.63	**301.1 > 255.1**	20	21
			301.1 > 228.1	20	22
Tetraharmine	$C_{13}H_{16}N_2O$	3.75	**217 > 188**	30	19

Compound	Molecular formula	RT (min)	Transition (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
			217 > 173	30	35
THC	$C_{21}H_{30}O_2$	10.8	**315.2 > 193.1**	30	20
			315.2 > 122.9	30	31
THC-COOH	$C_{21}H_{28}O_4$	9.15	**345.1 > 299**	25	20
			345.1 > 327	25	15
TH-PVP	$C_{19}H_{27}NO$	6.35	**286.2 > 145**	30	22
			286.2 > 126.1	30	30
Tramadol	$C_{16}H_{25}NO_2$	4.1	**264>58.1**	25	25
Trazodone	$C_{19}H_{22}ClN_5O$	4.95	**372.1 > 176.1**	20	23
			374.1 > 176.1	20	23

Bold type is used for quantifiers

[a] Internal standard

Table S2 Mean matrix effect (%) of the 79 analytes in blood and urine

Analyte	Blood (*n* = 3)			Urine (*n* = 3)		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
2,5-DMA	10.8	-4.3	0.3	14.7	-14.2	-3.0
25B-NBOH	-19.5	10.4	2.1	-21.8	4.5	0.9
25B-NBOMe	4.3	13.3	14.0	-24.6	-8.4	0.9
25C-NBOH	8.3	9.3	0.7	-2.8	-8.1	2.7
25C-NBOMe	-21.7	-17.1	-1.9	-2.8	16.9	3.4
25E-NBOH	-1.1	1.5	-9.5	-7.9	0.3	6.3
25E-NBOMe	9.1	1.0	1.5	2.7	-4.9	1.3
25I-NBOH	-22.2	-6.8	-15.2	-3.5	-4.3	7.2
25I-NBOMe	-4.1	0.4	-9.8	-10.1	-6.1	1.4
2C-B	-10.7	-8.4	6.5	-19.5	-9.8	5.6
2C-H	-10.1	5.7	5.6	9.0	-2.0	-2.7
2C-I	-4.6	-0.7	3.1	-15.4	-10.6	1.3
4-Cl-PPP	-0.2	-8.3	1.2	-3.7	0.3	-5.4
4-CEC	14.2	0.2	-4.8	7.7	-11.1	-5.1
5-MAPB	-6.8	-19.8	-16.3	3.3	2.5	-1.4
5-Meo-MIPT	10.8	0.6	3.9	-3.5	-13.8	-0.6
6-MAM	-37.2	9.0	-22.5	11.1	-36.1	1.7
7-AF	-19.1	-6.9	2.8	-5.5	-15.0	7.7
AB-Chminaca	-1.4	-2.6	4.9	-2.5	9.6	1.9
AB-Fubinaca	1.8	-1.9	1.4	7.9	-0.1	1.4
AH-7921	2.6	-1.2	1.5	-5.3	-5.1	0.8
AKB-48	5.8	0.1	-6.2	-13.2	-16.6	-4.1
α-PVP	11.1	3.1	5.9	4.3	-0.7	-3.6
α-PVT	7.6	-2.9	4.4	6.1	-7.3	10.1
Alfentanil	5.4	-3.8	6.4	5.5	-7.7	7.1
Alprazolam	-22.2	-1.4	-1.6	-18.9	-4.5	2.6
AM-2201	-13.5	0.1	-19.0	8.0	-35.1	-0.5
Amitriptyline	2.2	4.7	7.7	-2.9	-6.2	-5.4
Amfepramone	1.9	4.6	-12.1	4.8	3.8	-0.4
Amphetamine	-11.8	-13.6	4.9	-35.4	-2.9	12.2
Benzoylecgonine	-35.1	-5.9	7.1	7.2	-1.9	2.7
Bromazepam	11.2	7.3	13.0	-9.4	-3.7	11.8
Carbamazepine	1.6	-3.9	0.9	13.9	-14.0	-0.9
Clobenzorex	-7.8	-3.2	5.9	9.6	-6.9	9.5
Clonazepam	-17.5	-0.4	2.6	7.0	-0.7	-2.7
Cocaine	11.4	19.1	-2.9	-18.8	12.5	-15.5
Codeine	-32.6	10.9	-4.5	-28.8	-5.4	-7.0
Diazepam	1.5	-2.0	0.2	4.9	3.3	-1.1
Dibutylone	-2.4	1.0	3.9	-2.6	-5.5	3.8
DMT	0.5	-4.6	0.3	0.9	-0.2	-0.3
Ethylone	-4.1	-2.9	0.2	2.7	-9.5	9.3

Analyte	Blood (n = 3)			Urine (n = 3)		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
Eutylone	7.4	1.2	1.0	1.9	5.2	14.6
Flunitrazepam	1.4	-2.6	1.5	4.4	-19.0	-18.7
Haloperidol	-5.1	3.4	-18.3	-1.5	-0.2	-4.4
Harmaline	-24.2	-10.5	7.3	-1.7	-8.6	7.9
Harmine	-5.3	9.4	9.9	-14.1	9.3	-14.1
Hydrocodone	2.4	-7.9	1.6	3.6	2.3	11.0
JWH-018	2.7	-5.9	1.7	3.7	-6.3	2.6
JWH-081	-18.0	-7.5	-1.3	6.0	-2.7	-3.8
JWH-210	-3.6	-2.9	1.9	-7.8	-7.4	6.6
JWH-250	-4.6	-3.0	3.5	-6.6	-8.1	8.4
Ketamine	8.2	10.0	-11.7	9.3	-14.9	-2.5
LSD	-16.5	-12.3	-5.6	-21.7	-0.3	1.5
m-CPP	-4.1	-0.7	7.5	-4.7	14.8	18.0
MDEA	-20.2	-2.8	0.4	-18.0	-7.1	6.5
MDMA	6.5	-1.1	5.5	5.5	-5.3	6.5
MDPV	4.7	-14.0	9.8	4.0	-9.1	6.7
Mephedrone	1.4	-0.3	3.9	-4.1	-6.4	10.5
Meperidine	0.1	-12.5	0.2	6.2	-5.1	7.1
Methadone	-6.1	5.0	-8.2	4.7	-14.9	-1.3
Metamphetamine	-28.0	-0.4	-3.3	-37.1	-4.5	10.8
Methylphenidate	9.1	0.5	-8.3	-9.6	-4.2	0.9
Methylone	-4.4	-4.3	3.7	-9.5	-6.2	9.1
Midazolam	-3.1	-1.4	-6.3	6.1	4.8	2.2
Morphine	-29.5	-6.7	-25.1	-28.9	-6.5	-16.1
N-Ethylpentylone	-13.9	-1.5	-0.7	-16.4	-6.6	8.5
Nimetazepam	7.4	-1.7	-0.3	-6.2	-20.9	-3.2
Norketamine	-9.9	5.9	2.4	4.4	2.9	1.6
Oxycodone	0.4	3.3	0.4	-8.5	-0.2	-0.9
PCP	-13.9	9.2	-1.9	-18.9	-6.8	-1.3
Phenmetrazine	-4.6	6.8	-8.1	-9.3	-4.1	-10.6
Sibutramine	-13.3	5.3	-4.1	-8.5	-8.7	-1.9
Temazepam	-15.3	-3.4	-0.9	4.0	-16.1	-0.1
Tetrahydroharmine	-12.2	-10.1	-6.8	-6.4	-8.9	1.7
THC	-18.5	-9.6	0.9	-1.5	3.0	10.7
THC-COOH	-10.1	-21.8	-11.6	-7.5	-12.8	4.8
TH-PVP	5.1	-0.6	6.6	-3.4	-7.2	5.9
Tramadol	-19.0	-0.5	-10.1	-7.4	-19.3	-2.8
Trazodone	-6.8	-5.3	-7.5	-12.9	-0.1	3.0

Low, medium and high concentration levels, respectively: 0.4, 24 and 80 ng/mL for 25R-NBOH and 25R-NBOMe; 10, 120 and 400 ng/mL for LSD, oxycodone, 5-MAPB, AM 2201, amphetamine, codeine, *N*-ethylpentylone, hydrocodone, MDEA and trazodone; 16, 120 and 400 ng/mL for morphine; 10, 40 and 240 ng/mL for THC-COOH; and 4, 120 and 400 ng/mL for the other analytes

Table S3 Mean bias/accuracy (± %) of the 79 analytes in blood and urine

Analyte	Blood (*n* = 15)			Urine (*n* = 15)		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
2,5-DMA	-17.8	-8.6	2.9	-3.1	6.1	-1.6
25B-NBOH	-10.6	-5.9	2.2	15.1	4.1	-2.5
25B-NBOMe	-17.3	-1.8	2.3	5.4	-3.9	1.2
25C-NBOH	14.3	-7.8	1.7	-15.6	2.5	2.7
25C-NBOMe	8.7	-5.4	1.4	5.4	13.6	1.5
25E-NBOH	6.9	-5.7	1.7	-6.2	14.8	-2.5
25E-NBOMe	-17.3	3.0	-5.7	-10.1	-7.8	1.9
25I-NBOH	5.3	-4.5	1.3	4.2	-15.6	5.5
25I-NBOMe	-19.6	7.5	2.7	-17.3	3.0	1.9
2C-B	-15.0	-2.4	3.7	9.3	-12.1	4.2
2C-H	19.0	-3.6	3.0	-3.7	2.3	3.2
2C-I	10.1	1.7	-1.3	-2.6	5.4	-1.5
4-Cl-PPP	-5.1	-5.4	2.4	-18.1	-3.3	2.8
4-CEC	-11.5	-1.9	9.0	-3.4	-15.1	4.7
5-MAPB	4.2	2.4	2.9	3.0	13.5	2.3
5-Meo-MIPT	-9.9	-6.2	2.4	-2.8	11.6	-3.7
6-MAM	-19.3	1.7	1.6	-7.5	-5.8	-4.3
7-AF	-19.0	2.1	2.5	-18.9	-9.1	2.2
AB-Chminaca	-18.1	5.4	13.3	-15.2	8.4	12.7
AB-Fubinaca	-15.0	13.1	1.4	-10.8	12.3	7.3
AH-7921	7.3	-7.6	2.3	1.8	-4.1	-2.1
AKB-48	-8.3	2.9	-9.9	-18.1	4.6	-2.1
α-PVP	4.2	3.9	1.4	-15.1	-4.4	-14.0
α-PVT	7.0	-6.4	1.9	-19.9	-5.2	2.0
Alfentanil	12.1	-7.2	1.7	-14.7	-13.6	8.3
Alprazolam	-9.3	14.8	-9.4	-15.3	9.5	-12.1
AM-2201	-16.0	-5.0	-1.9	-9.2	-11.9	2.4
Amitriptyline	-14.5	10.5	-10.7	11.0	2.8	-5.6
Amfepramone	-4.2	-9.3	3.2	-1.8	7.0	-2.1
Amphetamine	10.2	-9.0	2.6	-15.7	-17.5	1.3
Benzoylecgonine	11.5	3.1	4.3	12.9	-12.0	-8.0
Bromazepam	14.9	1.4	2.3	6.7	6.6	2.2
Carbamazepine	-15.1	-7.8	2.9	2.3	-10.5	-2.1
Clobenzorex	10.0	-7.9	2.2	-5.6	-2.8	-1.6
Clonazepam	11.0	-6.9	-6.4	2.3	-12.4	8.0
Cocaine	13.6	3.2	-2.4	3.1	13.5	-4.8
Codeine	-12.0	13.0	-11.6	2.0	-2.3	3.4
Diazepam	-8.5	7.6	-10.6	-19.1	7.6	3.2
Dibutylone	10.7	-7.2	1.9	-14.7	-12.4	2.2
DMT	-19.7	5.2	-8.5	2.5	16.1	3.6
Ethylone	10.8	-6.8	1.8	-13.3	-7.8	2.1

Analyte	Blood (n = 15)			Urine (n = 15)		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
Eutylone	11.0	-8.0	2.1	-16.3	-11.3	1.2
Flunitrazepam	6.6	-12.6	-8.8	-12.6	-4.7	8.8
Haloperidol	-5.2	-8.4	3.0	-8.2	-5.0	2.1
Harmaline	-15.5	-5.1	1.9	-2.6	2.4	-1.9
Harmine	-6.7	2.7	-12.5	-12.6	-12.8	1.4
Hydrocodone	10.1	-4.6	2.3	-8.3	-14.6	2.7
JWH-018	-12.7	9.5	-11.8	-15.1	5.4	13.4
JWH-081	-13.9	5.7	-6.6	9.1	-7.8	7.1
JWH-210	15.7	-6.8	2.4	-3.3	-10.2	4.9
JWH-250	13.5	-8.4	2.0	-3.9	-19.6	4.6
Ketamine	-13.5	-2.8	1.5	-6.1	8.6	-7.2
LSD	9.6	-8.7	2.4	-16.2	-10.5	7.3
m-CPP	13.0	-8.2	2.1	-13.4	3.3	3.6
MDEA	10.2	-8.2	2.3	-6.4	-7.0	4.5
MDMA	-14.9	12.2	-7.1	-19.5	-2.7	2.6
MDPV	10.1	-11.3	3.4	-15.5	-8.9	-11.2
Mephedrone	11.8	-7.5	-2.9	-8.1	5.5	3.9
Meperidine	-10.0	6.3	-5.5	-6.0	-5.9	5.1
Methadone	-1.8	12.8	-6.8	-4.9	5.1	9.2
Metamphetamine	-8.8	12.2	-7.0	-4.8	-2.9	1.6
Methylphenidate	-11.9	2.7	-8.6	-17.4	-2.4	3.7
Methylone	-11.6	9.9	-9.3	-9.5	-3.7	-2.7
Midazolam	-18.6	2.0	-5.1	-12.2	9.7	-5.4
Morphine	-16.0	-7.3	8.0	-9.5	-14.2	6.7
N-Ethylpentylone	2.9	12.4	-9.9	11.9	-5.4	4.9
Nimetazepam	3.2	2.0	-8.2	8.5	-16.9	7.5
Norketamine	-14.0	2.8	-11.0	-9.5	4.5	4.8
Oxycodone	-5.1	6.0	-10.7	-16.6	3.5	-3.2
PCP	-7.1	10.5	-7.8	6.4	-16.8	5.2
Phenmetrazine	15.5	-7.4	1.5	-14.5	2.4	3.8
Sibutramine	-18.7	-7.4	2.1	-16.8	-3.1	4.7
Temazepam	11.8	-2.0	3.2	14.6	-18.0	3.8
Tetrahydroharmine	-16.1	8.6	-11.3	6.3	-9.9	3.2
THC	-9.1	5.2	-6.1	-17.9	6.3	-1.5
THC-COOH	-11.8	10.8	-3.5	-15.2	12.1	-3.4
TH-PVP	-8.1	13.8	-7.5	-17.3	-4.3	2.5
Tramadol	-17.6	2.5	-6.8	-10.8	-6.7	1.3
Trazodone	9.2	4.8	-10.4	-4.3	6.0	-2.1

Low, medium and high concentration levels, respectively: 0.4, 24 and 80 ng/mL for 25R-NBOH and 25R-NBOMe; 10, 120 and 400 ng/mL for LSD, oxycodone, 5-MAPB, AM 2201, amphetamine, codeine, *N*-ethylpentylone, hydrocodone, MDEA and trazodone; 16, 120 and 400 ng/mL for morphine; 10, 40 and 240 ng/mL for THC-COOH; and 4, 120 and 400 ng/mL for the other analytes

Table S4 Mean recovery (%), repeatability and intermediate precision (RSD, %) of the 79 analytes in blood

Analyte	Recovery (*n* = 3)			Repeatability (*n* = 3)			Intermediate precision (*n* = 15)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
2,5-DMA	83	69	71	7.8	8.7	0.5	5.2	4.5	7.4
25B-NBOH	105	83	98	8.2	3.9	3.8	4.9	4.3	2.2
25B-NBOMe	93	86	86	16.1	2.0	2.8	3.9	10.3	8.1
25C-NBOH	96	88	103	7.8	1.1	2.1	4.4	2.5	2.0
25C-NBOMe	85	81	91	5.4	5.0	6.2	6.2	5.1	1.6
25E-NBOH	97	83	99	13.7	14.7	3.6	2.8	4.2	1.5
25E-NBOMe	93	82	87	3.8	1.5	1.3	6.3	5.1	6.3
25I-NBOH	75	95	89	18.9	2.1	3.9	10.0	2.2	8.4
25I-NBOMe	102	87	93	11.2	3.8	3.0	4.0	4.2	6.0
2C-B	102	97	50	3.9	4.3	2.3	6.5	5.0	4.9
2C-H	93	96	91	10.9	1.4	2.8	5.5	4.0	5.2
2C-I	94	51	71	8.0	10.2	5.0	4.5	3.7	4.8
4-Cl-PPP	80	99	85	7.3	5.2	7.4	6.8	7.7	3.6
4-CEC	82	91	68	15.2	1.4	7.0	7.3	2.1	3.6
5-MAPB	80	83	69	22.1	5.9	2.0	5.1	2.2	5.1
5-Meo-MIPT	95	100	100	10.1	4.4	7.5	5.2	4.9	3.5
6-MAM	100	101	99	3.9	9.6	4.9	3.7	6.1	2.1
7-AF	94	101	88	2.3	1.3	2.6	2.1	3.6	3.2
AB-Chminaca	89	98	98	11.1	1.6	4.4	3.4	9.8	2.0
AB-Fubinaca	88	94	96	5.2	3.5	2.0	5.6	3.0	2.2
AH-7921	84	102	101	6.9	0.6	2.4	4.4	4.4	7.3
AKB-48	87	99	93	18.2	7.6	5.4	3.9	3,0	3.2
α-PVP	86	81	96	18.5	3.9	0.5	4.5	7.3	2.3
α-PVT	87	86	84	6.8	3.4	3.3	5.4	4.5	1.5
Alfentanil	90	96	102	8.1	8.3	4.3	5.0	3.5	1.2
Alprazolam	92	91	92	7.7	5.1	5.4	13.0	5.7	1.7
AM-2201	99	99	93	7.7	19.5	2.1	7.3	12.8	10.1
Amitriptyline	85	92	101	17.4	7.7	5.3	2.1	3.3	3.0
Amfepramone	77	86	83	11.4	2.3	5.9	5,0	4.5	5.8
Amphetamine	87	53	43	11.7	7.8	3.5	3.4	2.8	2.8
Benzoylecgonine	92	99	78	18.3	10.6	8.4	12.1	5.1	6.4
Bromazepam	73	99	95	3.4	4.0	1.1	6.9	2.9	7.4
Carbamazepine	85	101	97	5.6	1.3	4.0	3.8	5.6	3.5
Clobenzorex	80	82	85	18.5	2.9	3.6	4.2	2.2	2.7
Clonazepam	79	83	86	16.3	8.5	6.0	3.7	4.8	2.3
Cocaine	93	89	83	0.8	0.6	1.7	4.5	3.9	7.9
Codeine	99	94	95	7.0	3.2	2.4	12.6	4.6	3.6
Diazepam	89	102	94	3.1	1.8	3.6	6.8	6.0	2.2
Dibutylone	87	89	93	7.0	10.4	0.5	4.8	2.0	3.4

Analyte	Recovery (*n* = 3)			Repeatability (*n* = 3)			Intermediate precision (*n* = 15)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
DMT	83	95	99	0.7	2.1	1.1	2.6	2.8	1.3
Ethylone	95	93	77	3.2	4.8	0.9	2.1	3.8	2.5
Eutylone	95	94	86	7.0	10.4	0.5	3.1	7.7	5.4
Flunitrazepam	86	85	89	6.1	3.2	4.1	3.8	8.4	1.8
Haloperidol	91	85	100	0.2	3.4	1.5	4.2	1.3	3.9
Harmaline	89	91	67	10.8	3.4	9.2	9.9	4.5	4.8
Harmine	85	90	99	16.5	8.1	0.5	8.7	6.6	2.5
Hydrocodone	73	96	99	3.8	14.1	1.2	2.4	5.0	3.8
JWH-018	87	95	84	11.3	1.8	1.6	1.6	5.1	2.1
JWH-081	84	95	86	15.8	6.9	6.7	6.0	5.0	2.3
JWH-210	92	89	91	7.6	7.8	1.7	3.4	3.6	3.4
JWH-250	90	98	94	7.9	8.6	3.0	1.8	3.2	3.9
Ketamine	78	89	93	17.4	0.2	1.2	10.1	4.0	5.3
LSD	98	88	96	16.7	2.2	5.5	9.6	2.1	7.7
m-CPP	100	84	64	4.8	6.6	5.2	9.4	3.8	3.6
MDEA	98	99	83	10.4	4.7	2.5	4.2	3.1	3.2
MDMA	81	100	79	4.1	6.6	2.8	2.5	3.5	3.0
MDPV	100	90	79	11.5	11.1	1.3	5.0	3.9	2.3
Mephedrone	93	71	78	4.1	6.3	1.9	5.8	3.2	2.5
Meperidine	101	95	84	15.8	6.7	6.4	6.1	2.1	1.1
Methadone	60	96	99	10.6	0.9	7.5	6.2	2.3	6.2
Metamphetamine	87	71	59	9.4	6.3	3.2	16.3	3.2	1.4
Methylphenidate	84	86	98	12.3	0.7	1.5	7.7	3.2	4.9
Methylone	88	60	44	4.8	8.6	4.0	3.5	4.1	2.3
Midazolam	93	94	98	13.2	1.4	6.3	3.2	7.3	4.1
Morphine	100	108	103	12.5	5.3	9.5	5.3	9.0	4.1
N-Ethylpentylone	90	92	83	8.1	12.7	1.0	6.2	2.3	2.4
Nimetazepam	84	88	93	8.9	4.8	2.5	8.5	3.6	1.5
Norketamine	83	82	98	0.2	2.1	2.0	10.1	6.0	2.4
Oxycodone	85	98	95	8.0	4.2	2.5	9.2	7.8	2.5
PCP	102	86	95	11.3	1.9	6.5	3.7	2.5	1.6
Phenmetrazine	85	84	78	12.7	4.1	6.1	2.7	3.4	6.4
Sibutramine	75	93	93	10.3	6.0	1.8	6.7	4.6	4.0
Temazepam	79	85	82	10.9	4.5	3.7	5.9	6.0	2.5
Tetrahydroharmine	87	93	94	6.3	2.6	1.0	4.7	2.5	1.8
THC	73	88	96	10.4	10.0	4.5	11.0	3.9	3.8
THC-COOH	82	47	57	11.8	14.7	3.4	4.9	4.7	7.2
TH-PVP	99	92	85	4.1	8.8	4.1	3.4	3.1	2.2
Tramadol	86	97	84	10.6	1.4	5.6	7.8	2.5	4.1
Trazodone	88	100	97	7.4	2.2	4.9	12.5	4.2	6.6

Low, medium and high concentration levels, respectively: 0.4, 24 and 80 ng/mL for 25R-NBOH and 25R-NBOMe; 10, 120 and 400 ng/mL for LSD, oxycodone, 5-MAPB, AM 2201, amphetamine, codeine, *N*-ethylpentylone, hydrocodone, MDEA and trazodone; 16, 120 and 400 ng/mL for morphine; 10, 40 and 240 ng/mL for THC-COOH; and 4, 120 and 400 ng/mL for the other analytes

Table S5 Mean recovery (%), repeatability and intermediate precision (RSD, %) of the 79 analytes in urine

Analyte	Recovery (*n* = 3)			Repeatability (*n* = 3)			Intermediate precision (*n* = 15)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
2,5-DMA	98	76	99	12.3	3.7	7.2	3.2	5.4	4.9
25B-NBOH	90	97	77	11.5	6.9	1.6	5.7	12.4	4.5
25B-NBOMe	65	77	98	12.9	4.4	1.4	4.2	5.4	5.1
25C-NBOH	101	97	75	10.0	1.2	1.8	3.6	5.0	4.4
25C-NBOMe	81	81	96	13.7	5.1	2.7	5.4	3.3	6.2
25E-NBOH	88	76	95	14.9	2.5	2.8	7.6	2.6	2.9
25E-NBOMe	101	80	81	6.1	7.9	3.3	8.4	5.3	4.5
25I-NBOH	94	80	78	10.4	2.5	5.9	12.0	2.7	6.0
25I-NBOMe	80	78	89	3.7	1.5	1.3	9.4	5.1	1.9
2C-B	84	91	94	9.1	13.4	6.5	3.4	10.6	3.8
2C-H	91	80	79	8.9	3.0	5.2	2.9	11.1	2.4
2C-I	64	76	97	10.0	3.3	8.5	8.8	9.2	2.0
4-Cl-PPP	78	90	89	11.0	3.5	0.5	7.6	19.1	3.7
4-CEC	89	85	90	14.7	1.6	5.5	12.4	12.5	3.0
5-MAPB	89	89	96	8.9	3.1	3.4	3.8	13.1	1.8
5-Meo-MIPT	82	79	89	12.4	4.0	1.6	4.7	15.7	3.2
6-MAM	98	88	86	8.3	4.2	4.6	3.1	16.4	7.7
7-AF	84	99	89	2.8	1.7	1.2	8.7	15.7	1.8
AB-Chminaca	88	87	93	9.6	2.0	5.3	6.0	2.9	4.2
AB-Fubinaca	90	87	91	19.3	0.5	6.3	3.8	15.9	3.3
AH-7921	76	89	75	15.9	0.9	1.6	5.4	8.6	3.7
AKB-48	89	91	86	7.5	8.2	2.4	2.2	5.6	1.9
α-PVP	68	91	81	4.6	4.1	1.2	3.3	4.1	6.0
α-PVT	93	88	99	14.2	3.5	3.0	12.0	3.1	2.6
Alfentanil	96	93	89	11.1	5.7	2.7	4.0	14.6	1.2
Alprazolam	81	84	87	15.6	3.4	8.0	9.9	10.8	3.9
AM-2201	85	81	79	20.4	4.1	4.8	3.6	18.7	3.6
Amitriptyline	93	80	95	6.3	1.2	2.2	4.9	10.3	2.4
Amfepramone	88	80	91	17.3	4.4	3.0	3.8	14.0	3.5
Amphetamine	89	97	89	9.0	4.6	2.3	11.7	3.4	3.2
Benzoylecgonine	96	79	99	14.8	8.8	5.8	7.8	15.3	6.6
Bromazepam	90	99	84	10.4	6.3	1.1	2.2	1.4	4.6
Carbamazepine	97	96	84	1.2	10.4	5.2	3.7	1.6	4.8
Clobenzorex	87	89	92	10.3	7.6	3.3	7.3	8.0	3.3
Clonazepam	67	82	91	6.8	6.0	5.4	5.4	0.9	4.5
Cocaine	74	97	82	10.5	3.4	2.9	3.9	2.5	3.0
Codeine	81	73	78	12.5	9.9	2.8	5.6	4.1	3.7
Diazepam	69	85	88	12.1	3.2	5.1	4.5	5.8	3.8
Dibutylone	90	93	90	9.5	5.0	2.9	10.3	3.2	1.7

Analyte	Recovery (*n* = 3)			Repeatability (*n* = 3)			Intermediate precision (*n* = 15)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
DMT	89	76	76	2.7	2.8	3.0	5.7	3.1	7.3
Ethylone	91	98	94	4.4	5.1	3.4	4.1	4.0	0.9
Eutylone	94	87	100	9.5	1.2	2.9	4.4	8.7	5.2
Flunitrazepam	93	85	92	7.5	1.1	7.9	5.7	2.2	2.8
Haloperidol	92	92	80	5.6	2.1	8.7	3.7	13.5	1.2
Harmaline	92	82	83	4.5	15.5	1.6	5.7	6.2	5.2
Harmine	87	88	73	4.4	2.3	2.5	5.2	3.1	1.8
Hydrocodone	95	95	97	2.7	5.6	3.0	5.0	15.4	1.8
JWH-018	90	84	94	6.8	7.2	6.8	5.3	1.2	3.9
JWH-081	98	85	94	14.3	6.6	5.7	2.8	6.1	3.9
JWH-210	94	94	91	6.9	12.5	2.6	2.5	7.2	1.9
JWH-250	92	95	91	7.0	6.8	2.5	1.7	17.1	0.8
Ketamine	88	84	70	11.6	4.7	2.3	7.2	8.4	3.4
LSD	83	79	76	4.0	1.4	2.6	13.1	3.3	5.0
m-CPP	95	89	80	11.3	6.5	5.6	6.6	12.0	1.5
MDEA	92	86	94	12.3	1.3	0.9	7.4	6.9	3.2
MDMA	85	92	89	7.9	0.7	2.7	8.2	5.5	1.2
MDPV	95	89	86	14.0	5.9	1.8	3.1	4.7	2.6
Mephedrone	92	99	91	3.9	6.9	3.3	3.6	8.0	2.3
Meperidine	97	88	99	15.7	8.3	6.3	10.9	6.9	1.2
Methadone	63	88	74	13.8	2.9	6.1	4.7	5.3	2.5
Metamphetamine	88	89	87	10.0	7.1	1.5	17.9	12.4	2.0
Methylphenidate	86	73	99	2.5	5.8	3.4	9.8	12.7	3.4
Methylone	87	86	87	4.7	1.5	2.1	4.6	4.5	1.8
Midazolam	95	79	91	5.6	7.5	3.5	5.6	3.5	4.3
Morphine	101	73	77	5.9	12.9	9.2	7.8	10.6	4.8
N-Ethylpentylone	86	90	71	5.8	3.6	2.1	7.3	4.8	1.3
Nimetazepam	94	90	85	2.6	4.7	6.3	5.4	1.9	2.9
Norketamine	57	94	81	18.3	5.3	4.2	5.5	6.4	5.6
Oxycodone	69	91	77	20.2	11.6	6.4	4.6	14.3	2.5
PCP	81	88	97	8.8	11.8	2.5	1.7	5.5	4.5
Phenmetrazine	84	77	93	3.4	1.1	8.1	7.6	11.8	3.0
Sibutramine	87	90	85	5.9	5.8	7.3	6.4	6.6	4.0
Temazepam	75	83	87	14.0	6.0	5.0	9.3	0.5	2.9
Tetrahydroharmine	91	88	76	7.7	7.9	2.6	9.0	3.0	4.5
THC	88	88	97	4.7	5.5	7.4	9.8	8.7	2.5
THC-COOH	90	77	90	10.2	9.3	5.4	6.6	8.1	8.0
TH-PVP	97	92	91	5.2	6.7	6.2	9.8	4.4	1.6
Tramadol	93	81	94	9.4	3.0	5.4	8.8	8.7	4.0
Trazodone	87	78	89	7.8	3.0	2.0	5.9	6.9	2.4

Low, medium and high concentration levels, respectively: 0.4, 24 and 80 ng/mL for 25R-NBOH and 25R-NBOMe; 10, 120 and 400 ng/mL for LSD, oxycodone, 5-MAPB, AM 2201, amphetamine, codeine, *N*-ethylpentylone, hydrocodone, MDEA and trazodone; 16, 120 and 400 ng/mL for morphine; 10, 40 and 240 ng/mL for THC-COOH; and 4, 120 and 400 ng/mL for the other analytes