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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 $MgSO_4$. The effectiveness of the sample storage conditions was shown to be comparable to when the Quantisal™ buffer was used, with no substantial concentration loss (< 15%) for all the substances after up to 72 hours at −20º C. The method was satisfactorily validated, with limits of detection (LOD) and quantification (LOQ) ranging from 0.04 to 0.5 ng/mL and 0.1–1.5 ng/mL, respectively, and was applied to the analysis of 62 real samples. The main substances detected were 3,4-methylenedioxymethamphetamine (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.

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 replace traditional drugs of abuse, such as cocaine, cannabis, and amphetamines [1,2]. In 2021, approximately one in every 17 persons aged 15 to 64 years old 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 in 2021, following several years of stabilization [1]. 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 [3], which can even lead to fatal outcomes [4].

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 [1]. 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 [3]. A more reliable approach involves combining self-reported data with biological fluid analysis of EDM party attendees [5].

Although drug concentrations in oral fluid may not always

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accurately reflect blood concentrations [6], 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 [7]. Usually, commercial oral fluid collection devices have preservatives that prevent drug and metabolite degradation when samples are stored for long periods before analysis [8], 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 [8], 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 [9,10]. 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 [4], urine [4], oral fluid [11] and stomach content [12]. Following sample preparation, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is a common hyphenated technique for drug detection and quantification, given its ability to enhance the sensitivity, which is essential for some NPS, accommodate various classes of substances in a single chromatographic run and overcome some gas chromatography limitations (e.g., thermolabile compounds) [13].

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.

2. Experimental

2.1. Chemicals and reagents

Benzoylecgonine (BZE), N,N 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 (Round Rock, TX, USA). 25E-NBOMe, 4-chloro-alpha-pyrrolidinopropiophenone (4-chloro-alpha-PPP or 4-Cl-α-PPP;), 5-fluoro APINACA (5 F-AKB-48), ethylone (bk-MDEA), and eutylone (bk-EBDB) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Clobenzorex was acquired from LGC Standards (Toronto, Canada). 2,5-DMA, 2 C-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-ethylampheta mine (MDEA), 3,4-methylenedioxymethamphetamine (MDMA), methylenedioxypyrovalerone (MDPV), mephedrone, methadone, methamphetamine, methylone (bk-MDMA) and norketamine were donated by the United Nations Office on Drugs and Crime (UNODC, Vienna, Austria). 2 C-H, 2 C-I, 5-MeO-MIPT, AB-CHMINACA, AKB-48, α-pyrro lidino pentio 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 (Rio de Janeiro, Brazil); Amfepramone (diethylpropion) by Aché Pharmaceutical Laboratories S.A(Guarulhos, Brazil); methylphenidate by Novartis Pharma (São Paulo, Brazil). Tetrahydroharmine was synthetized and its identity and purity confirmed by mass spectrometry and Nuclear Magnetic Resonance (NMR). N-Ethylpentylone (ephylone) standard was prepared from seized material and its purity confirmed by NMR.

Twenty-two analytes were not validated in the method but include only for screening purposes: 25B-NBOH, 25 C-NBOH, 25E-NBOH, 25I-NBOH, 25B-NBOMe, 25 C-NBOMe, 25 H-NBOMe, 25I-NBOMe, 2 C-C, 2 C-E, 4-methylpentedrone, 5 F-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.

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

Acetonitrile (ACN) LC–MS grade was purchased from Scharlau (Barcelona, Spain). Methanol LC-MS grade, PSA (primary and secondary amine), 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 (Offenbach,Germany). Ultrapure water was obtained from a Milli-Q purification system (MA, USA). Quantisal™ oral fluid collection devices and elution buffer were purchased from Immunalysis (Pomona, CA, USA).

Individual stock solutions were prepared in methanol or ACN at 1 mg/mL, one mixed stock solution was prepared at final concentration of 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. The working solutions were 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 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 used for chromatographic separation. The mobile phase consisted of water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B). 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, except for anphetamine, MDA and dibutylone, for which three transitions were selected. The molecular formula, retention time (RT), respective internal standard, MRM transitions, DP, collision energy and CXP, limits of detection (LOD) and quantification (LOQ) for the 51 analytes and the 9 IS used in the method are shown in Table 1. The parameters of other 22 analytes (only screening) in LC-MS/MS system are shown in Table S1.

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. First, a quick questionnaire was applied to the participants, to have information on the dosage form and the name (or street name) of the psychoactive substance used, and the time that had elapsed between substance use and

Table 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		
2 C-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		
2 C-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		
2 C-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		
5 F-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
			329.9 → 173.0	5.8	46	39	16		
AKB-48	$C_{23}H_{31}N_3O$	THC-COOH-d3	**366.2 → 135.2**	12.5	41	23	14	0.04	0.1
			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
			213.1 → 168.0	4.3	86	33	16		
JWH-018	$C_{24}H_{23}NO$	THC-COOH-d3	**342.1 → 127.2**	11.3	101	35	26	0.04	0.1
			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		

(*continued on next page*)

Table 1 (*continued*)

Substance	Molecular formula	I.S.	Transitions (*m/z*)	RT (min.)	DP (V)	CE (V)	CXP (V)	LOD (ng/mL)	LOQ (ng/mL)
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		
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: N,N 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-methylenedioxymethamphetamine; MDEA: methylenedioxy-N-ethylamphetamine; MDPV: methylenedioxypyrovalerone; MA: methamphetamine; RT: retention time; THH: tetrahydroharmine; TH-PVP: tetramethylene-α-pyrrolidinovalerophenone.

the donation of oral fluid (expressed in minutes, hours, or days). The donors were instructed to transfer the oral fluid from the oral cavity into a 50 mL Falcon tube (non-stimulated collection). To prevent absorption of certain analytes onto the plastic surface [14], each sample was then transferred to a 2 mL glass vial, which was 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 and the same code was provided to the volunteers, who could use it to request the toxicological results, anonymously.

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

2.4. Sample extraction and clean-up

An extraction protocol used by our research group [4] was adapted and optimized 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 (200 µL) 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 °C for 60 days before analysis.

2.5. Method validation

The method was validated following the Standard Practices for Method Validation in Forensic Toxicology guidelines (ANSI/ASB Standard 036) [15]. The parameters evaluated included selectivity, matrix effect, linearity, recovery, bias/accuracy, repeatability (within-run precision), 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 for all 73 substances (including the 22 substances for screening) by analyzing 10 different oral fluid control 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 a pool of 10 oral fluid samples and comparing the sample normalized mean area of the post-extraction fortified sample (in-matrix) with the normalized mean area of solvent fortified samples, expressed as a

percentage. 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 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. 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.

Recovery (n=3), repeatability (n=3), bias/accuracy 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 as a percentage (n=3). Repeatability and intermediate precision were were expressed as percentage. 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) [15].

LOD of the method was defined as the lowest analyte concentration (in-matrix) that showed a peak with a signal-to-noise of μ + 3.3 s, 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 percentage 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, 5 F-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.

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 2 shows the results for matrix effect, recovery, repeatability, bias and intermediate precision for the 51 compounds analyzed during validation, and Fig. 1 shows the MRM chromatograms.

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 2), with satisfactory correlations ($r^2 \geq 0.99$). No extreme values were observed (Grubbs test).

Valen et al. [16] 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%).

The LOD and LOQ for the 51 analytes and the 9 IS used in the method are presented in Table 1, ranging from 0.04 to 0.5 ng/mL and 0.1–1.5 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 (51.9–78.0%) (Table 2). Cunha et al. [17] 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. [8] 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® collection device.

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 24 h (Table S2).

Cunha et al. [17] evaluated the long-term stability of 104 drugs of abuse using Quantisal buffer for 15, 60 and 90 days, at 25ºC, 4ºC and −20ºC, 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 h 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 h 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.

Table 2
Bias, matrix effect, recovery, repeatability and intermediate precision of the 51 substances analyzed.

Substance	Matrix effect (n=3) (%)			Recovery (n=3) (%)			Repeatability (n=3) RSD (%)			Bias (n=15)			Intermediate precision (n=15) RSD (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
2,5-DMA	-14.0	10.7	12.9	90.1	91.1	79.1	0.5	0.3	3.9	-9.7	3.2	8.4	7.0	8.9	11.6
25E-NBOMe	2.1	-23.6	-12.1	96.9	96.1	98.6	3.2	3.0	0.6	9.0	-2.0	1.9	5.1	2.5	7.4
2 C-B	10.0	0.1	-9.9	97.0	100.5	99.0	0.6	1.1	1.5	-4.7	-8.7	-5.2	6.0	3.2	9.5
2 C-H[a]	13.1	16.6	-4.3	95.3	99.9	81.5	2.1	8.4	15.6	-14.1	7.4	-4.3	17.6	20.0	18.3
2 C-I	8.5	-7.7	4.0	91.6	104.8	100.2	19.2	2.7	5.7	-12.6	1.3	-2.7	19.4	6.5	7.6
4-Cl-α-PPP	-11.4	-14.4	-1.7	77.1	87.7	78.1	9.3	11.5	8.2	7.7	-4.6	1.5	17.0	16.5	14.3
5 F-AKB-48[a]	2.8	-7.2	-1.8	97.6	86.6	89.3	8.9	2.5	0.2	3.4	2.2	-3.9	11.9	8.8	8.5
5-MAPB[a]	17.2	-19.6	-4.1	81.2	66.3	83.0	4.7	2.3	8.0	-14.4	7.4	-1.8	6.9	7.0	19.9
5-Meo-MIPT[a]	-10.7	-1.5	9.9	81.8	93.7	95.1	3.3	10.4	9.0	-15.6	-8.0	5.1	9.3	11.6	14.6
6-MAM[a]	17.3	-8.4	-8.1	103.8	98.5	97.5	1.9	1.2	2.2	3.8	-2.1	6.2	2.6	2.5	5.6
7-AF	-14.0	-10.7	-12.9	96.5	87.3	93.7	1.5	1.4	3.0	8.0	-6.5	-0.9	7.0	8.8	11.6
AB-CHMINACA	-16.4	-13.3	-14.4	84.2	96.0	96.4	10.0	1.9	2.1	-9.5	3.3	1.6	16.5	7.5	7.5
AH-7921[a]	2.5	-15.5	-10.3	88.6	95.7	80.0	8.6	3.4	4.1	13.0	-7.3	-0.1	18.1	12.3	15.9
AKB-48	0.1	-8.3	-18.1	88.3	74.4	91.9	3.3	1.4	2.4	-7.8	-8.7	-3.6	8.6	6.0	6.5
Alfa-PVT[a]	5.8	-11.1	-8.7	95.6	89.3	77.5	3.8	3.6	4.8	0.6	-1.7	2.0	5.6	6.2	8.3
AM-2201	-8.6	-3.4	2.6	88.5	100.6	98.1	1.6	0.8	0.8	2.2	-9.8	1.1	4.3	1.4	2.7
Amfepramone	19.9	-3.7	-2.8	89.2	95.5	95.7	3.1	4.0	1.9	-4.2	-6.0	2.0	4.6	7.6	3.4
Amphetamine	18.6	-13.8	-9.1	95.5	82.6	79.3	7.6	2.1	2.0	-9.8	0.2	7.9	20.0	5.1	5.7
Benzoylecgonine[a]	-17.4	9.2	-19.8	78.7	92.8	83.9	2.1	0.6	2.9	-4.0	-6.5	10.8	10.9	3.9	19.3
Benzylpiperazine	-14.2	-16.1	-17.0	72.4	71.0	90.8	5.4	3.1	4.3	4.0	2.9	-1.5	7.3	7.2	11.9
Clobenzorex[a]	18.7	6.6	-18.3	77.1	58.5	81.4	7.1	2.3	4.5	11.7	-8.7	9.8	15.6	7.1	11.8
Cocaine	19.1	-5.8	-10.5	80.2	80.3	92.5	8.9	5.1	5.2	12.4	-7.0	8.9	15.7	11.8	10.9
Dibutylone	14.7	-14.1	-11.4	99.6	98.8	97.2	4.1	2.5	2.1	-3.3	5.7	4.6	8.9	6.6	5.7
DMT[a]	3.7	9.1	-1.5	102.8	96.3	85.3	9.8	1.8	0.9	4.7	-8.8	2.0	18.9	4.2	3.0
EME[a]	-6.7	16.3	-10.6	82.9	94.5	98.1	11.8	3.7	3.4	13.6	-8.3	6.1	14.0	7.2	12.5
Ethylone[a]	-17.7	16.5	-0.7	82.5	90,0	81.1	3.9	7.2	5.6	-5.7	0.3	5.9	15.4	12.6	11.7
Eutylone[a]	-15.3	-5.9	-17.0	99.1	87.5	87.6	12.6	4.6	2.4	-7.3	7.5	8.6	12.9	7.0	7.6
Fentanyl	11.4	-11.1	-0.5	95.8	89,0	94.2	11.7	2.2	8.2	-11.4	3.9	-4.5	17.7	10.2	19.0
Flunitrazepam	17.6	-6.9	6.9	88.2	84.1	75.5	8.4	7.1	7.1	9.1	6.1	6.8	11.3	12.7	20.0
Harmaline[a]	-13.3	-7.0	-3.7	89.1	88.1	89.3	2.1	1.3	1.9	-11.4	-5.5	-1.6	4.5	4.8	4.6
Harmine[a]	-9.9	7.4	-11.8	96.9	83.1	101.1	3.1	3.4	4.1	-15.1	-3.5	4.7	8.8	6.4	8.3
JWH-018	-12.2	1.4	-0.5	85.2	99.0	101.2	1.9	1.2	0.9	-5.1	-6.0	3.4	5.1	2.1	2.9
JWH-081	-15.9	-1.6	-16.3	95.3	78.7	96.4	2.8	11.2	8.0	10.9	-0.8	6.7	13.1	16.6	19.3
JWH-210	-19.7	18.5	-14.2	75.2	91.6	82.5	4.4	0.8	2.9	-13.1	-4.3	7.1	7.1	1.5	6.9
Ketamine	-5.5	13.9	-14.1	71.1	83.4	92.3	8.4	5.5	6.9	4.6	-4.8	-4.6	12.1	9.3	13.8
Levamisole[a]	-16.5	-1.1	1.3	89.2	74.1	75.0	3.0	1.9	4.4	-7.7	-8.9	-9.1	9.0	5.1	10.1
LSD	-0.5	6.7	-11.2	97.5	96,0	88.4	1.3	2.4	2.1	-7.5	-6.1	9.6	4.15	4.3	6.9
m-CPP[a]	-17.3	14.6	3.2	87.2	85.2	89.0	3.6	2.1	3.7	-0.15	6.3	4.7	7.8	6.3	10.8
MDA	3.2	13.6	-16.0	51.9	78.0	62.7	7.4	7.6	6.3	3.8	-9.3	-3.8	15.9	15.1	16.1
MDEA	15.5	-4.6	-10.3	95.2	96.1	74.0	4.7	2.7	3.1	-3.4	-1.6	-3.2	9.3	8.6	6.6
MDMA[a]	-22.5	17.3	9.1	84.3	95.4	88.9	11.6	3.8	5.2	-3.4	-2.0	-1.9	19.8	15.1	15.3
MDPV	-18.9	14.2	-0.1	102.4	95.3	88.2	5.8	17.2	6.8	-5.0	2.8	14.4	9.0	18.3	14.5
Mephedrone	-7.8	15.1	-3.9	84.3	94.9	72.0	2.3	6.9	8.6	15.5	-2.5	1.0	18.0	15.0	18.0
Metamphetamine	13.9	-3.4	-4.1	100.1	98.6	98.7	5.6	1.5	0.9	-14.4	-1.9	0.1	7.7	3.2	3.1
Methylphenidate	-17.1	-3.3	-1.8	87.2	93.8	89.4	1.6	0.5	2.2	-14.0	1.8	-0.8	4.3	1.6	3.4
Methylone	11.5	18.6	1.4	101.3	112.7	93.0	6.5	3.7	6.2	-3.3	-4.7	8.1	9.3	9.9	12.0
N-Ethylpentylone	-15.8	1.8	-0.2	92.8	98.4	97.3	3.3	1.6	1.4	-17.2	-2.1	-5.7	3.9	2.5	3.3
Norketamine	4.6	2.6	-6.5	67.7	85.7	96.7	5.5	3.1	4.3	-11.2	-4.6	-9.0	12.1	5.8	8.7
Phenmetrazine	-18.0	-7.0	-14.0	81.0	73.9	66.6	8.1	2.9	7.5	-5.3	8.0	9.0	16.3	6.6	14.7
Tetrahydroharmine[a]	-21.6	17.9	-14.2	91.6	90.2	73.9	5.5	7.2	12.1	-9.0	-0.7	-6.8	12.7	13.0	18.5
TH-PVP	-12.1	12.0	-0.2	92.2	90.4	90.7	4.6	11.1	11.1	-1.3	-5.6	6.0	18.9	14.6	19.5

[a] = homoscedastic; the other substances were heteroscedastic (weighting factor = 1/x). 7-AF: 7-aminoflunitrazepam; α-PVT: α-pyrrolidinopentiothiophenone; DMT: N,N dimethyltryptamine; EME: ecgonine methyl ester; MDA: methylenedioxyamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; MDEA: methylenedioxy-N-ethylamphetamine; MDPV: methylenedioxypyrovalerone; TH-PVP: tetramethylene-α-pyrrolidino-valerophenone. 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.

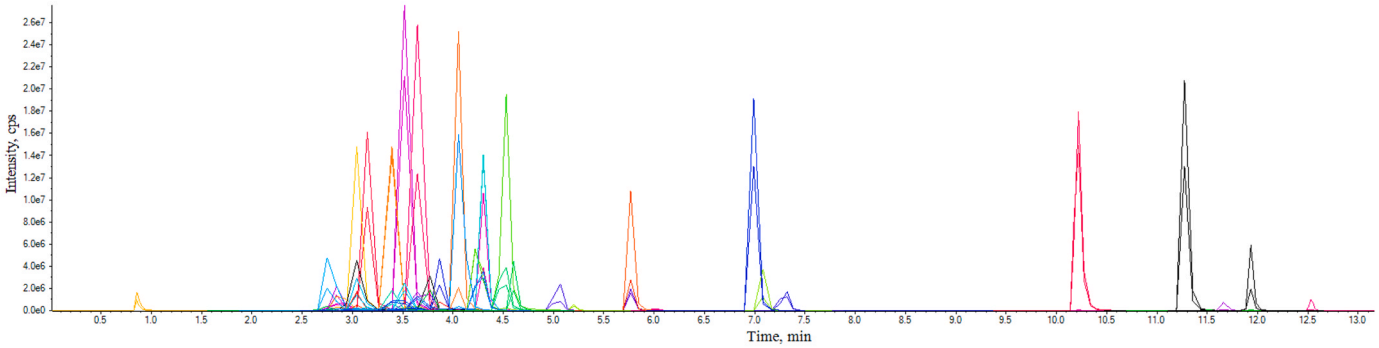

Fig. 1. Multiple Reaction Monitoring chromatogram of fortified oral fluid containing the 51 compounds validated in the method.

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 3, and Fig. 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 h was not accessed, although the extraction/purification step was conducted within the studied period.

Table 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 "< 24 h" (less than 24 hours) and ">24 h" (more than 24 h 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 one 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 [18]. In 10 cases, only MDA was detected, and in two cases, eutylone, a synthetic cathinone, was detected. It is important to point out that in 2023, eutylone was not detected in drugs seized by the Federal District Civil Police. Together with opioids and synthetic cannabinoids, synthetic cathinones are one of the most reported NPS classes in fatal cases [4,19], including cases with EDM party attendees [4].

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 [5]. In this work, excluding users who did not inform the name of the substance, in about 37% of the cases, the substance detected was different from the one reported by the user, or no substance was detected. These findings confirm the importance of reliable analytical methods for determining actual substance used, 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 [3,20], 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 LSD in the drug market, that is commonly detected in blotter papers seized in Brazil [21], which may also be a 25B-NBOMe metabolite [22]; 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. [23] 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 [24]. 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 [25].

Cocaine and/or its metabolites were identified in eight samples, at levels from 13.0 to 407.3 ng/mL for cocaine, 0.17–214.1 ng/mL for benzoylecgonine, and 1.8–150.1 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 [26], although some studies have found low validity between biological measurements and self-report [27,28]. 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 [5,29]. 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 [28]. 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 [29]. 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. [20] 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 [4] or drug seizure data [30]. Polydrug use is frequently reported in studies, which exposes drug users to a higher risk of overdose due to potential drug interactions [13]. 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. [20], 79.9%

Table 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.

Case	Dosage form	Substance reported	Time	Results (ng/mL)
1	Tablet	ni	>24 h.	nd
2	Tablet	MD	<24 h.	MDMA (829.0), MDA (67.6), MA (439.0), AMP (0.7)
3	White powder	Cocaine	<24 h	Cocaine (407.3), BZE (162.9), EME (83.9)
4	Crack	Cocaine	<24 h	BZE (65.6), EME (51.4)
5	Blotter paper	LSD	<4 h.	LSD (0.6)
6	Tablet	ni	<24 h	MDMA (detected; < 0.5;), MDA (191.8)
7	Tablet	MD	>24 h	nd
8	Tablet	Ecstasy	<24 h	MDA (274.5)
9	White powder	Cocaine	<4 h	Cocaine (369.0), BZE (214.1), EME (150.1)
10	White powder	Cocaine	<2 h.	Cocaine (312.9), BZE (62.1), EME (36.9)
11	Tablet;blotter paper	Ecstasy, LSD	<24 h	MDMA (26.1), MDA (128.9), MA (11.0), 25B-NBOH*
12	Tablet	MD	>24 h.	nd
13	Tablet	MD	<24 h	MDA (265.9)
14	Tablet	ni	<24 h	nd
15	Blotter paper	Acid	Minutes	LSD (68.3)
16	Tablet	MD	>24 h.	nd
17	Tablet	MD	>24 h.	Eutylone (4.7)
18	Tablet	ni	>24 h.	nd
19	Tablet	Ecstasy	<2 h.	MDMA (478.0), MDA (309.7), MA (74.8)
20	Amphetamine; Rohypnol	ni	<24 h	AMP (1.8), 7-AF (2.9)
21	Tablet	ni	>24 h.	nd
22	Tablet;blotter paper	MD;LSD	<24 h	MDMA (112.6), MDA (339.6), MA (39.2), 25B-NBOH*
23	Blotter paper	LSD	>24 h	nd
24	Tablet	ni	>24 h.	nd
25	White powder	Cocaine	>24 h.	Cocaine (26.8), BZE (0.17), EME (7.4)
26	Tablet	ni	>24 h.	nd
27	White powder	Ket	<24 h	Ketamine (375.3), norketamine (15.3)
28	Capsule	Amphetamine	<24 h	AMP (detected; < 1.5)
29	Tablet	ni	>24 h.	nd
30	White powder	Ket	<24 h	Ketamine (290.6), norketamine (150.2)
31	Tablet	MD	>24 h.	nd
32	Cigarette	DMT	<24 h	DMT (9.0)
33	Tablet	MD	<24 h	nd
34	Tablet	MD	<24 h	MDMA (2.8), MDA (270.3)
35	Tablet	Ecstasy	<24 h	MDMA (2.2), MDA (45.3)
LC-MS/MS determination 60 days after collection.				
36	Tablet	MD	1 h	MDA (272.4), MA (155.0)
37	Tablet	MD	15 h	MDA (279.7)
38	Blotter paper	Acid	15 min	nd
39	Tablet	MD	15 h	nd
40	Tablet	MD	1 h	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	3 h	MDA (39.0)
44	Tablet	MD	3 h	MDA (23.9)
45	Tablet	Ecstasy	1 h	MDA (200.7)
46	Tablet	MD	1 h	MDA (151.0)
47	Tablet	MD	1 h	Eutylone (4.1), MDA (327.6)
48	Tablet	MD	1 h	AMP (35.5)
49	White powder	Cocaine	1 h	Cocaine (28.5), BZE (6.3), EME (1.8)
50	Tablet	MD	40 min	MDA (84.4), MDMA (1.8)
51	Tablet	MD	4 h	AMP (124.4)
52	White powder	Cocaine	ni	Cocaine (13.0), BZE (11.4), EME (2.2)
53	Tablet	Ecstasy	1 h	MDA (90.5), MDMA (2.0)
54	Tablet	MD	5 min	MDA (143.0)
55	Tablet	MD	1 h	MDA (10.1)
56	Blotter paper	Acid	3 min	LSD (23.4)
57	Tablet	MD	>12 h	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	<24 h	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: 3,4-methylenedioxymethamphetamine.

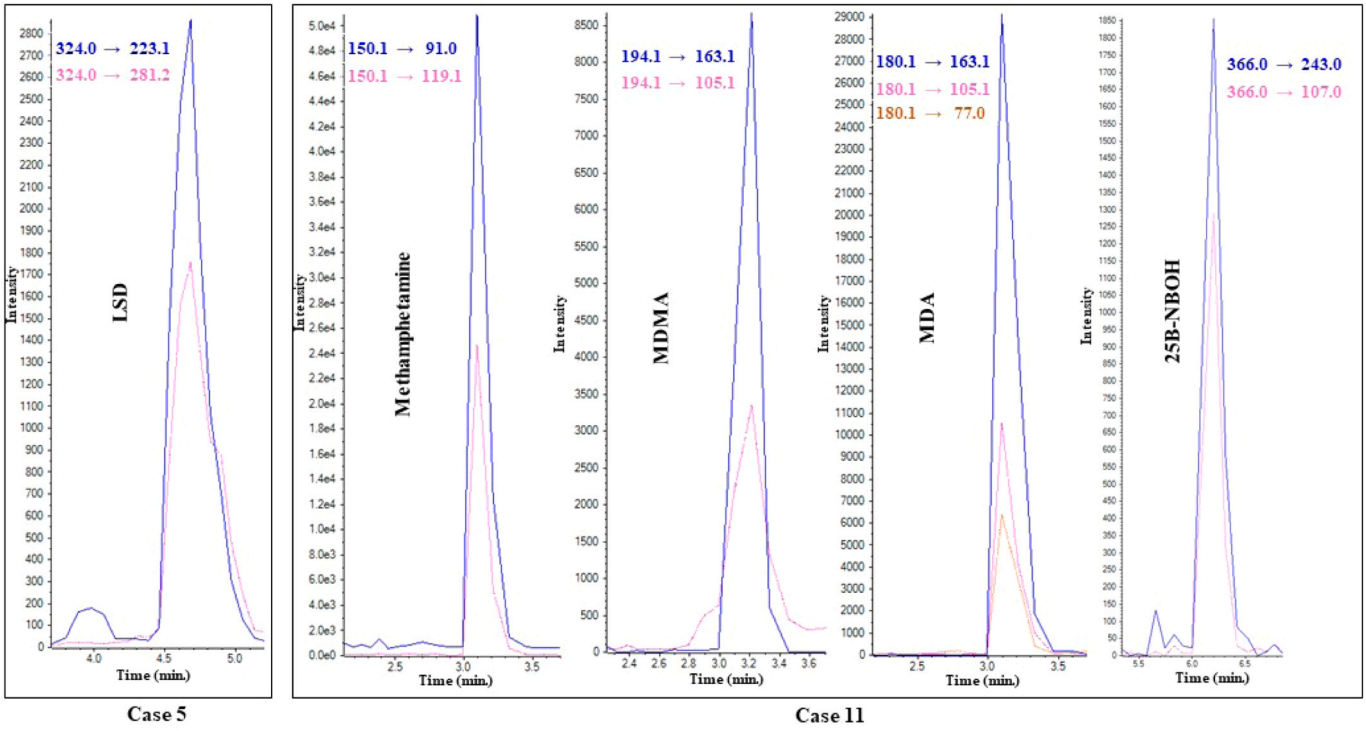

Fig. 2. Multiple Reaction Monitoring chromatograms of case 5 containing LSD (0.6 ng/mL), and case 11 containing MDMA, MDA, methamphetamine and 25B-NBOH (26.1, 128.9, 11.0 ng/mL and detected, respectively). The mass spectrometer parameters for each analyte are shown in Table 1.

of the samples contained more than one psychoactive substance. Ferrari Júnior et al. [13] 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 [3]. 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 [31]. 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 [32].

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 obtained 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" [33]. Additionally, trazodone (case 47), a serotonin antagonist antidepressant, has its primary active metabolite, m-CPP, also sold as a designer drug [34].

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 [35] as well as globally [1]. High-resolution mass spectrometry techniques are valuable for untargeted screening analysis and for the structural characterization and identification of unknown compounds [13], 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 some samples were analyzed more than 72 h after collection, interfering with the interpretation of the quantitative results, which may have been underestimated.

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 problem.

The strengths of the study, including the collection of unstimulated oral fluid, rapid response to volunteers, and its cost-effective (falcon tube) compared to commercial collection devices (e.g., Quantisal™), 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 environments. 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.

CRediT authorship contribution statement

Eloisa Dutra Caldas: Writing – review & editing, Supervision, Data curation, Conceptualization. **Agatha Souza:** Investigation, Formal analysis. **Victor Bitencourt:** Investigation, Formal analysis. **Ettore Ferrari Jr.:** Writing – original draft, Validation, Formal analysis, Data curation, Conceptualization.

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2024.116139.

References

[1] United Nations Office on Drugs and Crime - UNODC. World Drug Report 2023. ⟨https://www.unodc.org/unodc/en/data-and-analysis/world-drug-report-2023.html⟩ (Accessed 24 January, 2024).
[2] European Monitoring Centre for Drugs and Drug Addiction - EMCDDA. European Drug Report 2023: Trends and Developments. ⟨https://www.emcdda.europa.eu/publications/european-drug-report/2023_en⟩. (Accessed 24 January, 2024).
[3] J.J. Palamar, K.M. Keyes, Trends in drug use among electronic dance music party attendees in New York City, 2016–2019, Drug Alcohol Depend. 209 (2020) 107889, https://doi.org/10.1016/j.drugalcdep.2020.107889.
[4] E. Ferrari Júnior, E.D. Caldas, Determination of new psychoactive substances and other drugs in postmortem blood and urine by UHPLC–MS/MS: method validation and analysis of forensic samples, Forensic Toxicol. 40 (2021) 88–101, https://doi.org/10.1007/s11419-021-00600-y.
[5] A.J. Krotulski, A.L.A. Mohr, M.F. Fogarty, B.K. Logan, The detection of novel stimulants in oral fluid from users reporting ecstasy, molly and MDMA ingestion, J. Anal. Toxicol. 42 (2018) 544–553, https://doi.org/10.1093/jat/bky051.
[6] S.M.R. Wille, E. Raes, P. Lillsunde, T. Gunnar, M. Laloup, N. Samyn, A. S. Christophersen, M.R. Moeller, K.P. Hammer, A.G. Verstraete, Relationship between oral fluid and blood concentrations of drugs of abuse in drivers suspected of driving under the influence of drugs, Ther. Drug Monit. 31 (2009) 511–519, https://doi.org/10.1097/FTD.0b013e3181ae46ea.
[7] A.L.A. Mohr, M.F. Fogarty, A.J. Krotulski, B.K. Logan, Evaluating trends in novel psychoactive substances using a sentinel population of electronic dance music festival attendees, J. Anal. Toxicol. 45 (2021) 490–497, https://doi.org/10.1093/jat/bkaa104.
[8] K. Langel, C. Engblom, A. Pehrsson, T. Gunnar, K. Ariniemi, P. Lillsunde, Drug testing in oral fluid - evaluation of sample collection devices, J. Anal. Toxicol. 32 (2008) 393–401, https://doi.org/10.1093/jat/32.6.393.
[9] S. Lehmann, T. Kieliba, J. Beike, M. Thevis, K. Mercer-Chalmers-Bender, Determination of 74 new psychoactive substances in serum using automated in-line solid-phase extraction-liquid chromatography-tandem mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1064 (2017) 124–138, https://doi.org/10.1016/j.jchromb.2017.09.003.
[10] D. Montenarh, M. Hopf, S. Warth, H.H. Maurer, P. Schmidt, A.H. Ewald, A simple extraction and LC-MS/MS approach for the screening and identification of over 100 analytes in eight different matrices, Drug Test. Anal. 7 (2015) 214–240, https://doi.org/10.1002/dta.1657.
[11] K. de O. Chinaglia, A.C.F. Arantes, K.F. da Cunha, E.G. de Campos, J.M.M. Kahl, L. C. Rodrigues, J.L. Costa, Development of analytical method for the determination of methylphenidate, the analog ethylphenidate and their metabolite ritalinic acid in oral fluid samples by micro-QuEChERS and liquid chromatography–tandem mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1205 (2022) 123330, https://doi.org/10.1016/j.jchromb.2022.123330.
[12] M.D. Peres, S. Nascimento, F.S. Pelição, A new clean-up approach for stomach content toxicological analysis, Forensic Sci. Int. 302 (2019), https://doi.org/10.1016/j.forsciint.2019.109936.
[13] E. Ferrari Júnior, B.H.M. Leite, E.B. Gomes, T.M. Vieira, P. Sepulveda, E.D. Caldas, Fatal cases involving new psychoactive substances and trends in analytical techniques, Front. Toxicol. 4 (2022) 1–18, https://doi.org/10.3389/ftox.2022.1033733.
[14] A. Molnar, J. Lewis, S. Fu, Recovery of spiked Δ9-tetrahydrocannabinol in oral fluid from polypropylene containers, Forensic Sci. Int. 227 (2013) 69–73, https://doi.org/10.1016/j.forsciint.2012.11.006.
[15] ANSI/ASB. Standard practices for method validation in forensic toxicology, 2019. ⟨https://www.aafs.org/asb-standard/standard-practices-method-validation-forensic-toxicology⟩ (Accessed 22 November, 2022).
[16] A. Valen, Å.M. Leere Øiestad, D.H. Strand, R. Skari, T. Berg, Determination of 21 drugs in oral fluid using fully automated supported liquid extraction and UHPLC-MS/MS, Drug Test. Anal. 9 (2017) 808–823, https://doi.org/10.1002/dta.2045.
[17] K.F. da Cunha, K.D. Oliveira, M.A. Huestis, J.L. Costa, Screening of 104 New psychoactive substances (NPS) and other drugs of abuse in oral fluid by LC-MS-MS, J. Anal. Toxicol. 44 (2020) 697–707, https://doi.org/10.1093/jat/bkaa089.
[18] L. Hondebrink, J. Meulenbelt, M. Meijer, M. Van Den Berg, R.H.S. Westerink, High concentrations of MDMA ('ecstasy') and its metabolite MDA inhibit calcium influx and depolarization-evoked vesicular dopamine release in PC12 cells, Neuropharmacology 61 (2011) 202–208, https://doi.org/10.1016/j.neuropharm.2011.03.028.
[19] A.J. Krotulski, D.M. Papsun, C.W. Chronister, J. Homan, M.M. Crosby, J. Hoyer, B. A. Goldberger, B.K. Logan, Eutylone Intoxications—an emerging synthetic stimulant in forensic investigations, J. Anal. Toxicol. (2020), https://doi.org/10.1093/jat/bkaa113.
[20] K.F. da Cunha, K.D. Oliveira, M.S. Cardoso, A.C.F. Arantes, P.H.P. Coser, L. de N. Lima, A.C.S. Maluf, M.A. de C. Comis, M.A. Huestis, J.L. Costa, Prevalence of new psychoactive substances (NPS) in Brazil based on oral fluid analysis of samples collected at electronic music festivals and parties, Drug Alcohol Depend. 227 (2021) 108962, https://doi.org/10.1016/j.drugalcdep.2021.108962.
[21] E. Ferrari Júnior, L.C. Arantes, L.B. Salum, E.D. Caldas, Analysis of non-derivatized 2-(4-R-2,5-dimethoxyphenyl)-N-[(2-hydroxyphenyl)methyl]ethanamine using short column gas chromatography - mass spectrometry. J. Chromatogr. A 1634 (2020) 461657 https://doi.org/10.1016/j.chroma.2020.461657.
[22] A.T. Caspar, S.D. Brandt, A.E. Stoever, M.R. Meyer, H.H. Maurer, Metabolic fate and detectability of the new psychoactive substances 2-(4-bromo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25B-NBOMe) and 2-(4-chloro-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25C-NBOMe) in human and rat u, J. Pharm. Biomed. Anal. 134 (2017) 158–169, https://doi.org/10.1016/j.jpba.2016.11.040.
[23] K.F. da Cunha, J.M.M. Kahl, T.R. Fiorentin, K.D. Oliveira, J.L. Costa, High-sensitivity method for the determination of LSD and 2-oxo-3-hydroxy-LSD in oral fluid by liquid chromatography-tandem mass spectrometry, Forensic Toxicol. 40 (2022) 322–331, https://doi.org/10.1007/s11419-022-00622-0.
[24] K. Langel, H. Gjerde, D. Favretto, P. Lillsunde, E.L. Øiestad, S.D. Ferrara, A. G. Verstraete, Comparison of drug concentrations between whole blood and oral fluid, Drug Test. Anal. 6 (2014) 461–471, https://doi.org/10.1002/dta.1532.
[25] D.E. Nichols, C.S. Grob, Is LSD toxic? Forensic Sci. Int. 284 (2018) 141–145, https://doi.org/10.1016/j.forsciint.2018.01.006.
[26] H. Gjerde, E.L. Øiestad, A.S. Christophersen, Using biological samples in epidemiological research on drugs of abuse, Nor. Epidemiol. 21 (2011) 5–14, https://doi.org/10.5324/nje.v21i1.1420.
[27] P. Miller, A. Curtis, R. Jenkinson, N. Droste, S.J. Bowe, A. Pennay, Drug use in Australian nightlife settings: estimation of prevalence and validity of self-report, Addiction 110 (2015) 1803–1810, https://doi.org/10.1111/add.13060.
[28] J.J. Palamar, A. Salomone, E. Gerace, D. Di Corcia, M. Vincenti, C.M. Cleland, Hair testing to assess both known and unknown use of drugs amongst ecstasy users in the electronic dance music scene, Int. J. Drug Policy 48 (2017) 91–98, https://doi.org/10.1016/j.drugpo.2017.07.010.
[29] H. Gjerde, L. Gjersing, H. Furuhaugen, A.L. Bretteville-Jensen, Correspondence between Oral Fluid Drug Test Results and Self-reported Illicit Drug Use among Music Festival attendees, Subst. Use Misuse 54 (2019) 1337–1344, https://doi.org/10.1080/10826084.2019.1580295.
[30] Y. Machado, J. Coelho Neto, R.A. Lordeiro, M.F. Silva, E. Piccin, Profile of new psychoactive substances (NPS) and other synthetic drugs in seized materials analysed in a Brazilian forensic laboratory, Forensic Toxicol. 37 (2019) 265–271, https://doi.org/10.1007/s11419-018-0456-3.
[31] United Nations Office on Drugs and Crime - UNODC. Current NPS threats, volume 6, 2023. ⟨https://www.unodc.org/unodc/en/scientists/current-nps-threats.html⟩. (Accessed 10 January, 2024).
[32] R. Sutherland, R. Bruno, A. Peacock, S. Lenton, A. Matthews, C. Salom, P. Dietze, K. Butler, L. Burns, M.J. Barratt, Motivations for new psychoactive substance use among regular psychostimulant users in Australia, Int. J. Drug Policy 43 (2017) 23–32, https://doi.org/10.1016/j.drugpo.2016.12.021.
[33] K. Hasegawa, A. Wurita, K. Minakata, K. Gonmori, H. Nozawa, I. Yamagishi, K. Watanabe, O. Suzuki, Postmortem distribution of flunitrazepam and its metabolite 7-aminoflunitrazepam in body fluids and solid tissues in an autopsy case: usefulness of bile for their detection, Leg. Med. 17 (2015) 394–400, https://doi.org/10.1016/j.legalmed.2015.06.002.
[34] Y.P. Gaillard, A.C. Cuquel, A. Boucher, L. Romeuf, F. Bevalot, J.M. Prevosto, J. M. Menard, A fatality following ingestion of the designer drug meta-chlorophenylpiperazine (mCPP) in an asthmatic-HPLC-MS/MS detection in biofluids and hair, J. Forensic Sci. 58 (2013) 263–269, https://doi.org/10.1111/j.1556-4029.2012.02254.x.
[35] K.R.G. de Araujo, A.L. Fabris, L.F. Neves Júnior, J. de Carvalho Ponce, A.L. Soares, J.L. Costa, M. Yonamine, The mystery behind the apprehensions of the selective cannabinoid receptor type-2 agonist BZO-HEXOXIZID (MDA-19) as a drug of abuse, Forensic Toxicol. 41 (2023) 142–150, https://doi.org/10.1007/s11419-022-00646-6.