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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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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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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

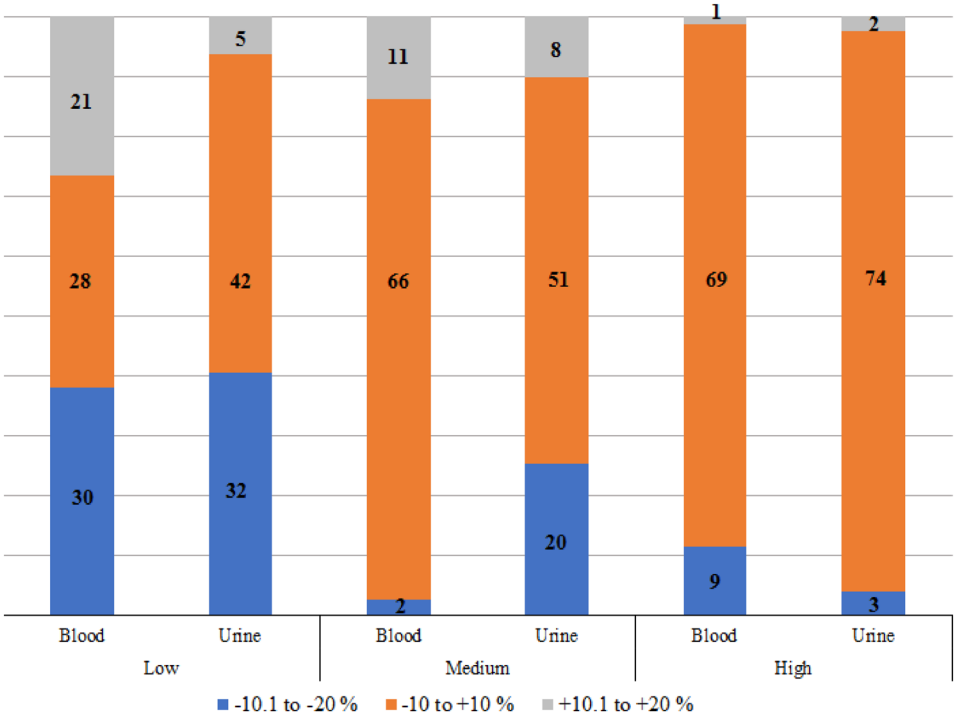

Fig. 1 Number of compounds in each range of mean bias (± %, $n = 15$) for the 79 analytes in blood and urine at the low, medium and high concentration levels, respectively: 0.4, 24 and 80 ng/mL for 25R-NBOH and 25R-NBOMe; 10, 120 and 400 ng/mL for LSD, oxycodone, 5-MAPB, AM 2201, amphetamine, codeine, *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

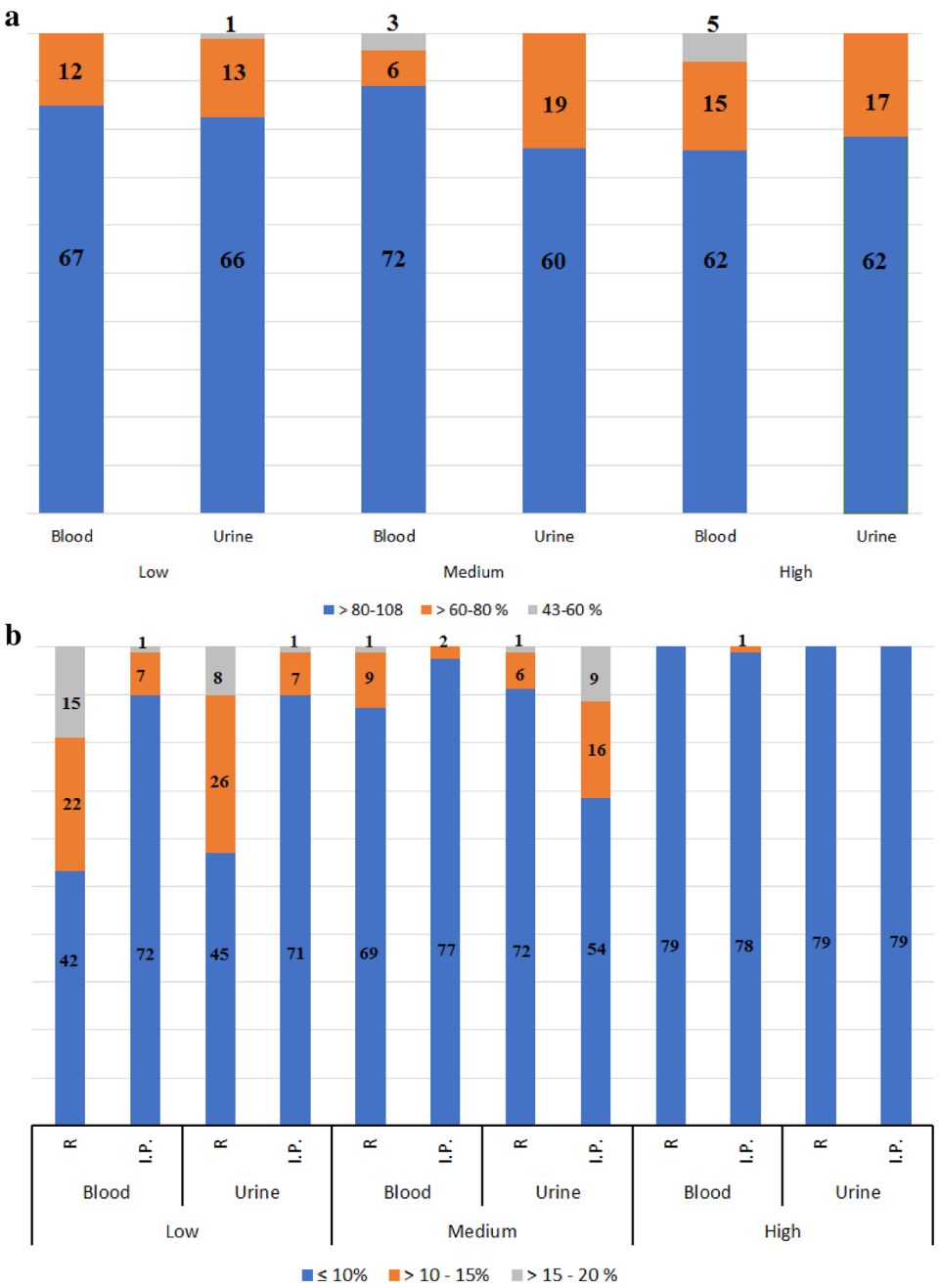

Fig. 2 Number of compounds in each range of (**a**) % mean recovery ($n=3$), (**b**) mean % RSD for repeatability (R, $n=3$) and intermediate precision (IP, $n=15$) for the 79 analytes in blood and urine at the low, medium and high concentration levels, respectively: 0.4, 24 and 80 ng/mL for 25R-NBOH 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).

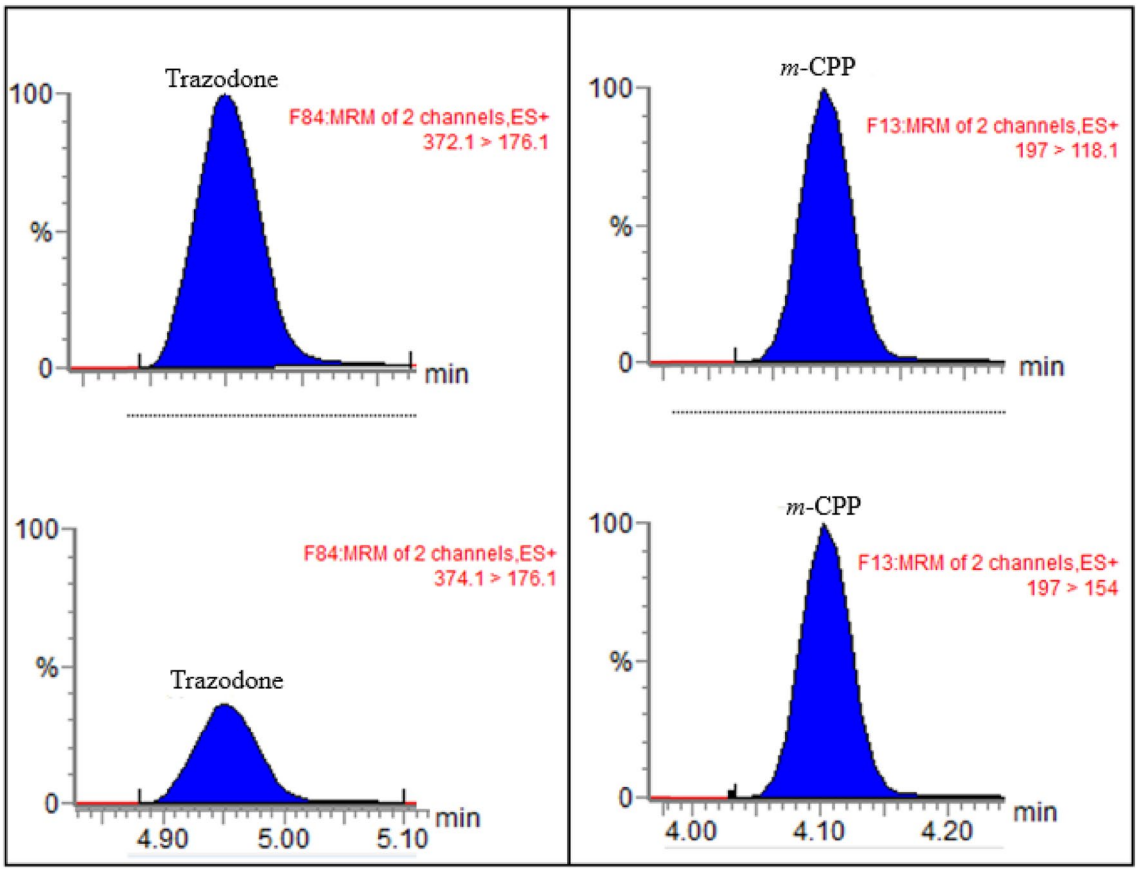

Fig. 3 Multiple reaction monitoring (MRM) chromatograms of trazodone (13,600 ng/mL) and *m*-CPP (1280 ng/mL) from a forensic postmortem blood sample, showing the two ion transitions for each compound (relative abundance)

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.

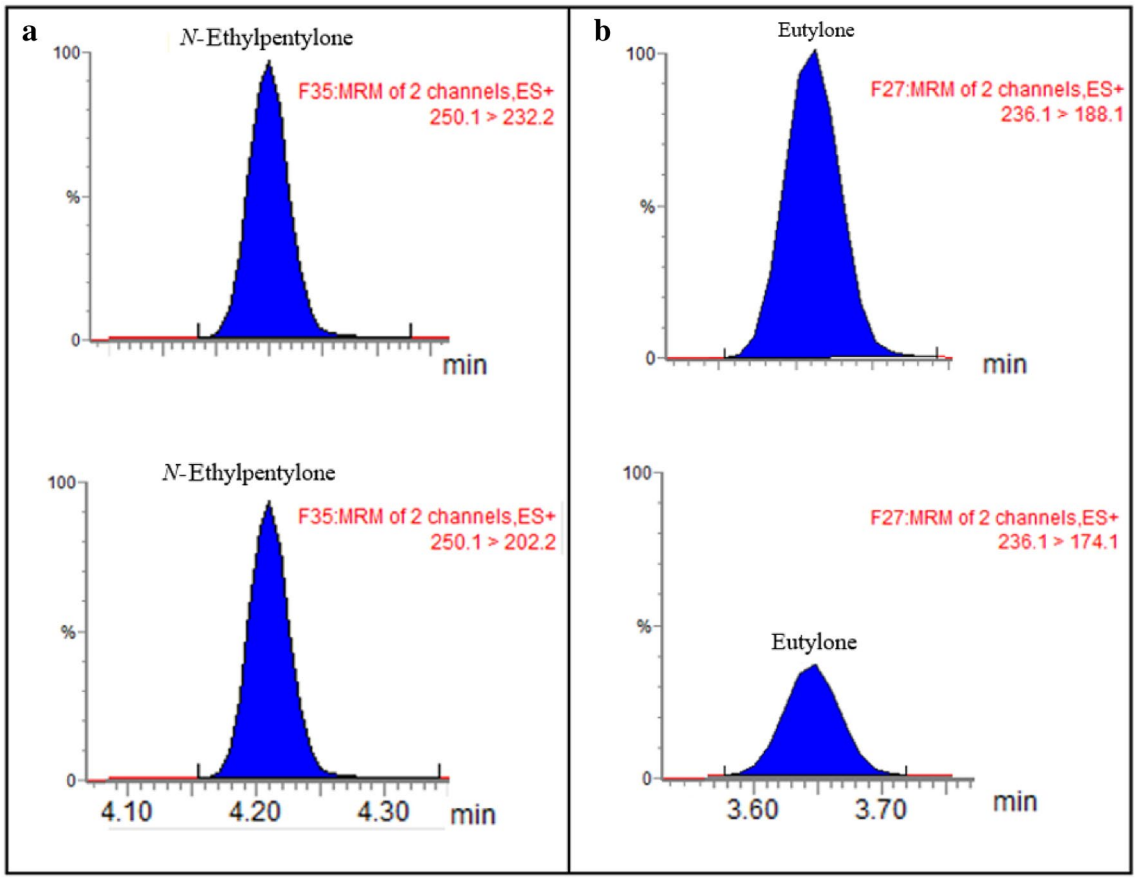

Fig. 4 MRM chromatograms of 2 forensic cases involving synthetic cathinones. (**a**) *N*-Ethylpentylone in postmortem blood (597 ng/mL) and (**b**) eutylone in antemortem urine (246 ng/mL) (relative abundance)

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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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