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Development of a New LC-MS/MS Screening Method for Detection of 120 NPS and 43 Drugs in Blood

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Abstract: In the last few years, liquid chromatography coupled with mass spectrometry (LC/MS) has been increasingly used for screening purposes in forensic toxicology. These techniques have the advantages of low time/resource-consuming and high versatility and have been applied in numerous new multi-analytes methods. The new psychoactive substance (NPS) phenomenon provided a great impulse to this wide-range approach, but it is also important to keep the attention on “classical” psychoactive substances, such as benzodiazepines (BDZ). In this paper, a fully validated screening method in blood for the simultaneous detection of 163 substances (120 NPS and 43 other drugs) by a dynamic multiple reaction monitoring analysis through LC-MS/MS is described. The method consists of a deproteinization of 200 μ L of blood with acetonitrile. The LC separation is achieved with a 100 mm long C18 column in 35 min. The method was very sensitive, with limits of quantification from 0.02 to 1.5 ng/mL. Matrix effects did not negatively affect the analytical sensitivity. This method proved to be reliable and was successfully applied to our routinary analytical activity in several forensic caseworks, allowing the identification and quantification of many BDZs and 3,4-methylenedioxypyrovalerone (MDPV).

Keywords: new psychoactive substances; LC-MS/MS; blood; benzodiazepine; drugs of abuse; screening



Citation: Vaiano, F.; Bertol, E.; Mineo, M.; Pietrosevoli, L.; Rubicondo, J.; Supuran, C.T.; Carta, F. Development of a New LC-MS/MS Screening Method for Detection of 120 NPS and 43 Drugs in Blood. *Separations* **2021**, *8*, 221. <https://doi.org/10.3390/separations8110221>

Academic Editor: Ivan Mikšik

Received: 18 October 2021

Accepted: 15 November 2021

Published: 17 November 2021

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1. Introduction

The increasing number and variety of substances of interest for Forensic Toxicology has required the development of new multi-analyte detection methods. These procedures are very effective tools for identifying and quantifying larger ranges of compounds through single sample extractions, with low time and resource consumption. Besides the traditional screening tests (i.e., immunoassays), which are affected by low specificity and sensitivity, gas chromatography-mass spectrometry (GC-MS) systems are the broadest used techniques for general unknown analyses [1–3]. Indeed, electron-impact full scan acquisition allows to compare the mass spectra of unknown compounds with almost “unlimited” mass spectral libraries, always updated and highly reproducible. However, GC-MS analyses are limited to thermostable compounds and require longer sample treatment procedures that often involve derivatization steps [4].

In recent years, these drawbacks of GC-MS have been overcome by the liquid chromatography with tandem MS (LC-MS/MS). Indeed, LC-MS/MS has proven to be less demanding than GC-MS for sample preparation, which can consist in a liquid–liquid extraction (LLE), in a protein precipitation (PP) or even in a dilution and direct injection without the need for removal of the aqueous phase [5–8]. The derivatization step is not

required even if it can be performed to improve the ionization efficiency (IE) [9–11]. The low IE of some compounds and unavailability of mass spectra libraries are the main issues concerning LC-MS/MS applications. However, high-resolution MS (HRMS) expanded the breadth of LC-MS applications to even non-targeted analyses, allowing the achievement of general unknown screening analyses beyond the GC-MS [12–15].

The new psychoactive substance (NPS) phenomenon has represented a great boost to these new analytical approaches [16]. According to latest European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) report [17], 830 compounds are currently being monitored, and new molecules are still emerging (46 in 2020). In Italy, the most prevalent new psychoactive substances (NPSs) are the synthetic cathinones (mephedrone, α -PHP, 3-MMC, eutylone), synthetic cannabinoids (JWH-122 and JWH-210) and opioids (ocfentanil, 2-methyl-AP-237 and carfentanyl) [18]. Besides the huge number of substances, the great chemical variability and the lack of reference standard materials make their detection an ongoing challenge for forensic toxicologists worldwide and often requires a multidisciplinary approach [19,20]. This great interest has led to the development and validation of a great number of new multi-analytes LC-MS/MS screening methods mainly focused on NPS [6,21–27].

The aim of this paper was to extend this analytical strategy also to other compounds of forensic toxicological interest, in particular benzodiazepines (BDZs) and other antidepressants.

BDZs are the most prescribed tranquilizers/antidepressants and are considered to be safe. However, they can cause serious impairments that make it hard to carry out many activities, such as driving a car [28–31]. BDZs are often co-consumed with other psychoactive substances, such as alcohol, that intensify their side effects (i.e., respiratory depression). Moreover, it is well documented that they are used to contrast the insomnia by cocaine and other stimulants, such as amphetamines, MDMA and synthetic cathinones [32,33]. In addition, new “designer BDZs” are available on the illicit drugs’ market, establishing a new class of NPSs. For these reasons, the presence of BDZs and antidepressants in biological matrices, together with the main drugs of abuse and NPSs, is worth investigating in a wide variety of forensic caseworks (i.e., driving under the influence, post-mortem analysis, acute intoxication cases). Because of their thermolability and polarity, which require extensive sample preparations for GC-MS detection, LC-MS/MS is the technique of choice for the detection of BDZs [34–36].

The new method here described could represent an important tool for a multi-analyte approach to forensic analytics that aims to reduce time and resource-consuming while keeping the high specificity and sensitivity of LC-MS/MS.

2. Materials and Methods

2.1. Chemicals and Reagents

Acetonitrile (ACN) for the PP step was purchased from Panreac Quimica S.L.U. (Castellar del Vallès, Spain). H₂O and ACN for LC-MS/MS were acquired from Biosolve Chimie SARL (Dieuze, France). Formic acid was obtained from Merck KGaA (Darmstadt, Germany). 3-OH-flunitrazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, alprazolam, amitriptyline, bromazepam, brotizolam, citalopram, clonazepam, chlordiazepoxide, delorazepam, diazepam, duloxetine, fentanyl, flunitrazepam, flurazepam, fluoxetine, halazepam (internal standard, IS), levomepromazine, lorazepam, lormetazepam, midazolam, mirtazapine, nordiazepam, oxazepam, oxcarbazepine, paroxetine, pinazepam, prazepam, promazine, quetiapine, temazepam, trazodone, triazolam, zolpidem, zopiclone, α -OH-alprazolam and α -OH-midazolam were purchased from Lipomed Inc. (Cambridge, MA, USA). Amphetamine, methamphetamine, MDA, MDMA, MDEA, ketamine and norketamine were obtained from Chemical Research 2000 s.r.l. (Rome, Italy). (\pm)-cis-3-methyl-norfentanyl, (\pm)-trans-3-methyl-norfentanyl, 2F-deschloroketamine, 2-methyl-AP-237, 3,4-MD- α -PHP, 3-MeO-PCE, 5-APB, 5-MAPB, 5Cl-AB-PINACA, 5Cl-THJ01C8, 5F-AKB48, 5F-APP-PICA, 5F-APP-PINACA, 5F-Cumyl-P7AICA, 5F-Cumyl-PeGACLONE, 5F-Cumyl-PINACA, 5F-MDMB-7-PAICA, 5F-NNEI-2'-naphtyl-isomer, 5F-

MDMB-PICA, 6-APB, 6-MAPB, AB-CHMINACA, acetyl-fentanyl, acetyl-norfentanyl, ADB-FUBINACA, alfentanil, AP-237, APP-FUBINACA, α -PHP, bentazepam, butyryl-fentanyl, butyryl-norfentanyl, carfentanyl, cinazepam, clonazolam, cumyl-PeGACLONE, cyclopropyl-fentanyl, deschloro-N-et-ketamine, diclazepam, etizolam, eutylone, flualprazolam, furanyl-norfentanyl, furanyl-fentanyl, isobutyryl-fentanyl, isotonitazene, MMB-2201, MDMB-CHMICA, MeOAc-fentanyl, MeOAc-norfentanyl, N-ethyl-pentylone, norfentanyl, ocfentanil, pF-furanyl-fentanyl, ritalinic acid, UR-144 were purchased from Comedical s.r.l. (Trento, Italy) by the Italian Early Warning System and donated to our laboratory. 1-naphyrone, 25D-NBOMe, 25H-NBOMe, 2-AI, 2C-E, 2C-N, 2F-methcathinone, 3,4-dimethyl-methcathinone (3,4-DMMC), 3-MeO-PCP, 3-methyl-methcathinone (3-MMC), 4F-amphetamine (4-FA), 4F-methcathinone, 4-MeO-PCP, 4-methyl-ethcathinone, 4-OH-DiPT, 5F-APINACA, 5-IAI, 5-MeOH-DiPT, AB-FUBINACA, ADB-PINACA, AM-2201, AM-2233, AM-694, buphedrone, butylone, BZP, CB-13, dimethylcathinone, ethcathinone, ethylone, JWH-007, JWH-016, JWH-018, JWH-019, JWH-073, JWH-081, JWH-098, JWH-122, JWH-147, JWH-200, JWH-203, JWH-210, JWH-210-d9 (IS), JWH-250, JWH-251, JWH-302, JWH-307, JWH-398, m-CPP, MDAI, MDPV, mephedrone, mephedrone-d3 (IS), methcathinone, methedrone, methoxetamine, methylone, naphyrone, pentedrone, pentylone, pravadoline, RCS-4, RCS-8, WIN 55,212-2 were purchased from LGC standards (Milan, Italy). All standards were diluted to the appropriate concentration with MeOH. Blank blood samples were collected from laboratory personnel and volunteers non-consumers of any drug.

2.2. Sample Treatment

Sample treatment was based on a previously published procedure with minimal improvement to increase the efficiency of the PP step [37]. Briefly, 200 μ L of blood was added with 700 μ L of cold ACN (0 °C) and 10 μ L of IS solutions (1 ng/ μ L). After vortex mixing and centrifugation (2500 G, 5 min), the supernatant was dried under a nitrogen stream at 40 °C and reconstituted with 100 μ L of H₂O for LC-MS/MS.

2.3. LC-MS/MS

MS analysis was conducted using an HPLC Agilent 1290 Infinity system (Agilent Technologies, Palo Alto, CA, USA) interfaced with an Agilent 6460 Triple Quad MS (Agilent Technologies), equipped with an electrospray ion source (ESI) operating in positive mode. The ESI configuration was: gas temperature 325 °C; gas flow rate 10 L/min; nebulizer 20 psi; capillary 4000 V. Multiple reaction monitoring (MRM) transitions (Table 1), data acquisition and elaboration were performed using the Agilent MassHunter Workstation software package. Chromatographic separation was performed through a Zorbax Eclipse Plus C18 (2.1 \times 100 mm, 1.8 μ m, Agilent Technologies). The mobile phase initially consisted of 5 mM aqueous formic acid (A) and ACN (B) 99:1. The gradient of elution was carried out as follows: from 0–5 min, linear ramp from 0–5%B; from 5–7 min, ramp to 10%B; isocratic hold from 7 to 10 min; from 10–15 min, ramp to 20%B; from 15–20 min, ramp to 30%B; isocratic hold up to 22 min; from 22 to 25 min, ramp to 40%B; from 25 to 28 min, ramp to 50%B; from 28 to 30 min, ramp to 70%B; from 30 to 35 min to 100%B and isocratic hold to 37 min. Post-time was set at 2 min. The flow rate was 0.6 mL/min.

2.4. Validation Parameters

2.4.1. Selectivity and Specificity

Potential endogenous interfering peaks were evaluated by the measurement of 10 different blank whole blood samples. Exogenous interferences were estimated by spiking 10 different blank blood samples with 500 ng/mL of common drugs and their metabolites (including barbiturates, cannabinoids, cocaine, opioids, etc.).

Table 1. MRM transitions of each compound included in the screening method. Quantitative transitions are shown in bold.

Compound	MRM Transitions (m/z)	Retention Time (min)	Compound	MRM Transitions (m/z)	Retention Time (min)
BDZ/Antidepressants			isobutyryl-fentanyl	351: 188 , 105	18.9
3-OH-flunitrazepam	330: 311 , 284	19.9	isotonitazene	411: 100 , 72	19.8
7-aminoclonazepam	286: 222 , 121	10.3	MeOAc-fentanyl	353: 188 , 105	6.3
7-aminoflunitrazepam	284: 135 , 227	13.2	MeOAc-norfentanyl	249: 84 , 55	15.3
7-aminonitrazepam	252: 121 , 208	6.5	norfentanyl	233: 84 , 55	9.5
alprazolam	309: 281 , 205	21.9	ocfentanil	371: 188 , 105	15.7
amitriptyline	278: 91 , 105	20.9	pF-furanyl-fentanyl	393: 188 , 105	18.7
bentazepam	297: 166 , 269	17.3	Synthetic Cannabinoids		
bromazepam	316: 182 , 209	17.6	5Cl-AB-PINACA	365: 320 , 249	26.7
brotizolam	393: 314 , 279	23.5	5Cl-THJ-018	377: 249 , 145	31.9
chlordiazepoxide	300: 283 , 282	15.4	5F-AKB-48	384: 135 , 93	32.1
cinazepam	465: 347 , 319	24.7	5F-APINACA	384: 135 , 93	31.3
citalopram	325: 109 , 262	18.5	5F-APP-PICA	396: 232 , 379	26.4
clonazepam	316: 270 , 214	21.4	5F-APP-PINACA	397: 233 , 352	26.8
clonazolam	354: 308 , 280	20.4	5F-Cumyl-P7AICA	368: 250 , 145	28.4
delorazepam	305: 165 , 140	24	5F-Cumyl-PeGACLONE	391: 273 , 185	30.5
diazepam	285: 154 , 193	25	5F-Cumyl-PINACA	368: 233 , 250	30.5
diclazepam	319: 227 , 154	26.1	5F-MDMB-7PAICA	378: 318 , 145	27.8
duloxetine	298: 154 , 157	20.8	5F-MDMB-PICA	377: 232 , 144	29.7
etizolam	343: 314 , 289	23.8	5F-NNEI-2-naphtyl-isomer	375: 232 , 144	30.6
flualprazolam	327: 292 , 299	21.3	AB-CHMINACA	357: 241 , 312	28.7
flunitrazepam	314: 268 , 239	22.6	AB-FUBINACA	369: 324 , 109	26
fluoxetine	310: 148 , 117	21.8	ADB-FUBINACA	383: 338 , 253	27.3
flurazepam	388: 315 , 317	18.1	ADB-PINACA	345: 215 , 300	28.7
halazepam	353: 241 , 222	28.7	AM-2201	360: 155 , 127	30.7
levomepromazine	329: 148 , 130	25.7	AM-2233	459: 98 , 112	19.9
lorazepam	321: 275 , 303	21.5	AM-694	436: 190 , 272	31.2
lormetazepam	335: 289 , 317	24.4	APP-FUBINACA	417: 372 , 109	27.6
midazolam	326: 291 , 223	17.5	CB-13	369: 155 , 127	33.9
mirtazapine	266: 195 , 72	12.2	cumyl-PeGLACONE	373: 255 , 185	31.2
nordiazepam	271: 165 , 140	22.5	JWH-007	356: 155 , 127	32
oxazepam	287: 269 , 241	20.6	JWH-016	342: 127 , 155	31.4
oxcarbazepine	253: 208 , 236	17.4	JWH-018	342: 155 , 127	31.7
paroxetine	330: 70 , 192	20.1	JWH-019	356: 155 , 127	32.4
pinazepam	309: 241 , 269	27.2	JWH-073	328: 155 , 127	31.2
prazepam	325: 271 , 140	28.6	JWH-081	372: 185 , 157	32
promazine	285: 86 , 58	19.7	JWH-098	386: 185 , 157	32.2
quetiapine	384: 253 , 221	17.6	JWH-122	356: 169 , 141	32.2
temazepam	301: 255 , 283	23.2	JWH-147	382: 155 , 127	32.9
trazodone	372: 176 , 148	17.5	JWH-200	385: 155 , 114	21.4
triazolam	343: 308 , 315	22.6	JWH-203	340: 125 , 238	31.5
zolpidem	308: 235 , 236	14.4	JWH-210	370: 183 , 155	32.7
zopiclone	389: 217 , 245	18.3	JWH-210-d9	379: 183 , 155	32.6
α -OH-alprazolam	325: 297 , 216	20.5	JWH-250	336: 121 , 91	31.1
α -OH-midazolam	342: 168 , 203	17.7	JWH-251	320: 105 , 144	31.4
Miscellaneous			JWH-302	336: 214 , 121	30.8
2-AI	134: 117 , 115	3.7	JWH-307	386: 155 , 127	32.2
2F-deschloroketamine	222: 109 , 163	7.8	JWH-398	376: 189 , 161	32.7
3,4MD- α -PHP	290: 135 , 140	15.6	MDMB-CHMICA	385: 240 , 144	31.3
3-MeO-PCP	274: 189 , 121	16.7	MMB-2201	363: 232 , 144	28.8
3-MeO-PCE	234: 189 , 121	15.8	pravadoline	379: 135 , 114	19
4-MeO-PCP	274: 86 , 121	16.7	RCS-4	322: 135 , 77	30.8
4-OH-DiPT	261: 160 , 114	10.4	RCS-8	376: 121 , 91	32.4
5-IAI	260: 116 , 243	11.6	UR-144	312: 125 , 55	32.5
5-MeO-DiPT	275: 114 , 174	14.1	WIN 55,212-2	427: 155 , 127	28.4

Table 1. Cont.

Compound	MRM Transitions (m/z)	Retention Time (min)	Compound	MRM Transitions (m/z)	Retention Time (min)
BZP	177: 91, 65	1.9	Synthetic Cathinones		
deschloro-N-et-ketamine	218: 91, 173	8.8	1-naphyrone	282: 126, 141	17.8
ketamine	238: 125, 179	9.4	2F-methcathinone	182: 164, 149	4.8
m-CPP	197: 154, 118	10.4	3,4-DMMC	192: 174, 159	11.7
MDAI	178: 161, 103	4.9	3-MMC	178: 160, 145	7.8
methoxetamine	248: 203, 121	11.8	4F-methcathinone	182: 164, 149	4.8
norketamine	224: 125, 207	8.8	4-methyl-ethcathinone	192: 174, 144	8.8
ritalinic acid	220: 84, 56	8.6	buphedrone	178: 131, 160	6.9
α-PHP	246: 140, 91	14.6	butylone	222: 204, 174	7.7
Opioids			dimethylcathinone	178: 133, 105	4.9
(±)-cis-3-met-norfentanyl	247: 98, 69	11.7	ethcathinone	178: 160, 132	5.6
(±)-trans-3-met-norfentanyl	247: 98, 69	11.9	ethylone	222: 204, 174	6.7
2-methyl-AP-237	287: 117, 115	14.8	eutylone	236: 218, 188	8.4
acetyl-fentanyl	323: 188, 105	15.6	MDPV	276: 126, 135	13.1
acetyl-norfentanyl	219: 84, 55	6.5	mephedrone	178: 160, 145	7.7
alfentanil	417: 268, 197	17.3	mephedrone-d3	181: 148, 163	7.6
AP-237	273: 117, 115	13.8	methcathinone	164: 146, 131	3.7
butyryl-fentanyl	351: 188, 105	19.2	methedrone	194: 176, 161	6.8
butyryl-norfentanyl	247: 84, 55	13.3	methylone	208: 160, 132	5.4
carfentanyl	395: 335, 113	18.9	naphyrone	282: 141, 211	18.5
cyclopropyl-fentanyl	349: 188, 105	18.3	N-ethylpentylone	250: 202, 232	12.2
fentanyl	337: 188, 132	17.5	pentedrone	192: 174, 132	10
furanyl-fentanyl	375: 188, 105	18.2	pentylone	236: 218, 188	11.2
furanyl-norfentanyl	271: 84, 55	10.9			

2.4.2. Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) were the lower concentrations that met the identification, precision and accuracy criteria at signal-to-noise ratios (SNR) ≥ 3 and ≥ 10 , respectively [38]. The evaluation was performed for three replicates of blank specimens fortified with decreasing quantities of each substance.

2.4.3. Linearity, Accuracy and Precision

Due to the low concentration of many available certified standards, we were not able to set up the same calibration curve for all the compounds. For most of them, an eight-point calibration curve from 1 to 500 ng/mL (1, 5, 10, 20, 50, 100, 200, 500 ng/mL) was adopted. For 5-APB, 5CI-AB-PINACA, 5CI-THJ-018, 5F-AKB48, 5F-APP-PICA, 5F-APP-PINACA, 5F-cumyl-PINACA, 5-MAPB, 6-APB, 6-MAPB, AB-CHMINACA, acetyl-fentanyl, ADB-FUBINACA, alfentanil, APP-FUBINACA, cumyl-PeGACLONE, MeOAc-norfentanyl, MMB2201 and ritalinic acid, the range was 1–200 ng/mL (1, 5, 10, 20, 50, 100, 200 ng/mL), while for (±)-cis-3-methyl-fentanyl, (±)-trans-3-methyl-fentanyl, butyryl-norfentanyl, furanyl-norfentanyl and norfentanyl, it was 1–100 ng/mL (1, 5, 10, 20, 50, 100 ng/mL). Five replicates of blank blood spiked at the proper concentrations were analyzed, and the least-squares regression procedure was applied to the data. Four quality control (QC) samples were prepared by spiking blank blood at concentration levels of 1, 15, 50 and 75 or 150 or 250 ng/mL depending on the relative calibration curve.

Accuracies and precisions were assessed by analyzing five replicates of each QC sample. Accuracy was expressed as the % mean relative error (%MRE) and the precision as the average of the relative standard deviation (%RSD). Inter-day precisions were established on the basis of 5 analyses performed over the course of one month.

2.4.4. Relative Recovery (RR), Matrix Effect (ME), Stability and Carry over

The estimation of RRs was achieved by the comparison of analytes' area from QCs prepared before and after the extraction. MEs were calculated comparing the slopes from spiked water solutions and spiked blank blood samples at QC concentrations for three replicates. The stabilities were evaluated by comparing the quantitative results obtained from five replicates of freshly fortified samples (at QC concentration levels) with those obtained from five replicates of the same sample stored at $-25\text{ }^{\circ}\text{C}$ and thawed weekly for a month. Carry over estimation was achieved by injecting the extracted blank samples into the LC-MS/MS system immediately after the highest calibrator over five runs.

3. Results and Discussion

3.1. MRM Transitions and Chromatographic Separation

MRM transitions for each compound were obtained through the Agilent Mass Hunter Optimizer, and the two most intense were used as the quantifier and qualifier (Table 1). The method includes 332 transitions. Due to the high number of compounds, acquisition was by dynamic MRM (retention time window: 1 min; max concurrent MRM: 30; dwell range: 13.17–246.50 ms). Chromatographic separation was achieved by a 100 mm long C18 column. Various gradients of elution were tested in order to obtain the best chromatographic performances in term of peaks' shapes, co-eluting compounds and isomeric pairs resolution. The final chromatographic run was 35 min long (Figures 1–3), with a retention range from 1.9 (BZP) to 33.9 min (CB-13). Unfortunately, co-elution was not completely removed even if it did not negatively affect the sensitivity and quantification of the involved substances. The isomeric pairs 1-naphyrone/naphyrone (Figure 1, #34 and #35), (\pm)-cis-3-met-norfentanyl/(\pm)-trans-3-met-norfentanyl (Figure 1, #40 and #41) and isobutyryl-fentanyl/butyryl-fentanyl (Figure 1, #54 and #55) were chromatographically baseline-separated. The separation of these opioids' couples represents a great advantage of this method since, to the best of our knowledge, no peer reviewed literature reported their chromatographic resolution to the baseline [26]. 3-MeO-PCP and 4-MeO-PCP (Figure 1, #73 and #74) had an identical retention time (16.7 min), but the effective identification can be achieved by their quantifier transitions—189 m/z for 3-MeO-PCP and 86 m/z for 4-MeO-PCP [39]. The m/z ratio can be useful for the couple 2F-methcathione and 4F-methcathinone (Figure 1, #16 and #17), whose quantifier/qualifier ratios are 2.9 and 1.1, respectively. However, this approach cannot be applied if both the molecules are present in the same sample, as the shared product ions modify the measured ratio. For the isomeric pairs 3-MMC/mephedrone (Figure 1, #25 and #26), 5/6-APB (Figure 1, #7 and #8) and 5/6-MAPB (Figure 1, #9 and #10), discrimination cannot be achieved by the MRM transitions, and the chromatographic gradient did not allow a full baseline resolution. However, when individually analyzed, these compounds can be distinguished based on the retention time.

All the other compounds sharing the $[\text{M}+\text{H}]^+$ ion and/or the MRM transitions were chromatographically identifiable.

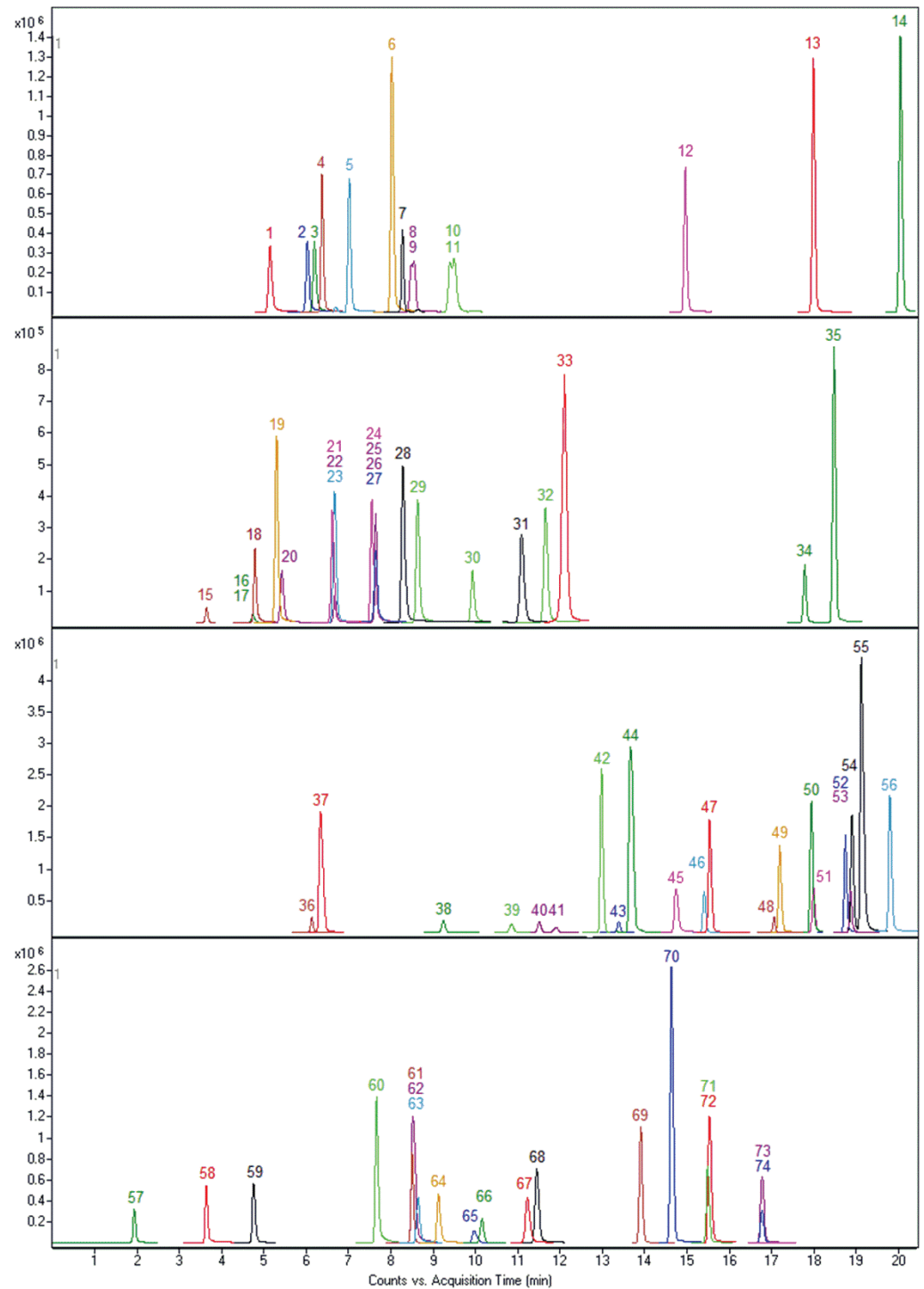


Figure 1. Chromatograms for the quantifier MRM transitions of phenethylamine, synthetic cathinones, fentanyl analogues and unclassified compounds, with a retention time within 0–20 min at 5 ng/mL. 1. MDEA; 2. amphetamine; 3. 4-FA; 4. methamphetamine; 5. MDMA; 6. 2C-N; 7. 5-APB; 8. 6-APB; 9. 5-MAPB; 10. 6-MAPB; 11. MDA; 12. 2C-E 13. 25H-NBOMe; 14. 25D-NBOMe; 15. methcathinone; 16. 2F-methcathinone; 17. 4F-methcathinone; 18. dimethylcathinone; 19. methylone; 20. ethcathinone; 21. ethylone; 22. methedrone; 23. buphedrone; 24. butylone; 25. 3-MMC; 26. Mephedrone; 27. eutylone; 28. 4-methyl-ethcathinone; 29. pentedrone; 30. pentylone; 31. 3,4-DMMC; 32. N-ethylpentylone; 33. MDPV; 34. 1-naphyrone; 35. naphyrone; 36. MeOAc-norfentanyl; 37. acetyl-norfentanyl; 38. norfentanyl; 39. furanyl-norfentanyl; 40. (±)-cis-3-met-norfentanyl; 41. (±)-trans-3-met-norfentanyl; 42. MeOAc-fentanyl; 43. butyryl-norfentanyl; 44. AP-237; 45. 2-methyl-

AP-237; 46. acetyl-fentanyl; 47. ocfentanyl; 48. alfentanyl; 49. fentanyl; 50. furanyl-fentanyl; 51. cyclopropyl-fentanyl; 52. *p*F-furanyl-fentanyl; 53. carfentanyl; 54. isobutyryl-fentanyl; 55. butyryl-fentanyl; 56. BZP; 57. 2-AI; 58. Isotonitazene; 59. MDAI; 60. 2F-deschloroketamine; 61. ritalinic acid; 62. deschloro-N-et-ketamine; 63. norketamine; 64. ketamine; 65. 4-OH-DiPT; 66. *m*-CPP; 67. 5-IAI; 68. methoxetamine; 69. 5-MeO-DiPT; 70. α -PHP; 71. 3,4MD-a-PHP; 72. 3-MeO-PCE; 73. 3-MeO-PCP; 74. 4-MeO-PCP.

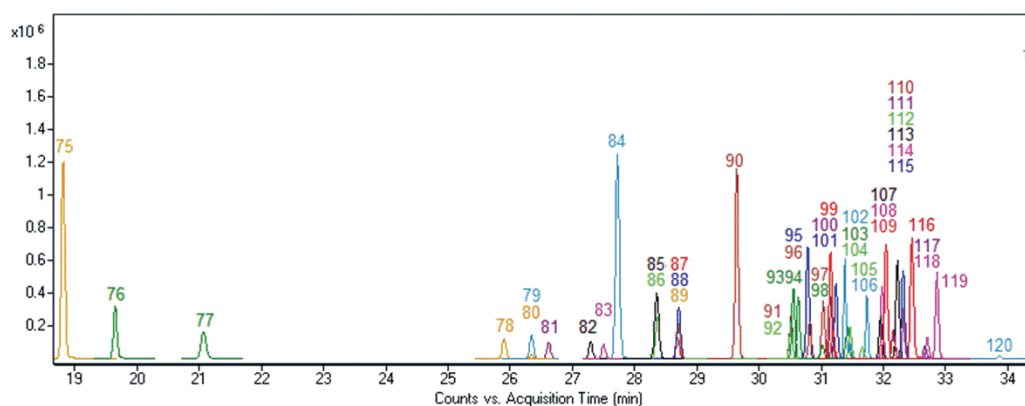


Figure 2. Chromatograms for the quantifier MRM transitions of synthetic cannabinoids compounds, with a retention time within 18–34 min at 5 ng/mL: 75. pravadoline; 76. AM-2233; 77. JWH-200; 78. AB-FUBINACA; 79. 5F-APP-PICA; 80. 5F-APP-PINACA; 81. 5CI-AB-PINACA; 82. ADB-FUBINACA; 83. APP-FUBINACA; 84. 5F-MDMB-7PAICA; 85. 5F-Cumyl-P7AICA; 86. WIN 55,212-2; 87. AB-CHMINACA; 88. ADB-PINACA; 89. MMB-2201; 90. 5F-MDMB-PICA; 91. 5F-Cumyl-PeGACLONE; 92. 5F-Cumyl-PINACA; 93. 5F-NNEI-2-naphtyl-isomer; 94. AM-2201; 95. RCS-4; 96. JWH-302; 97. MDMB-CHMICA; 98. JWH-250; 99. AM-694; 100. cumyl-PeGLACONE; 101. JWH-073; 102. 5F-APINACA; 103. JWH-251; 104. JWH-016; 105. JWH-203; 106. JWH-018; 107. 5CI-THJ-018; 108. JWH-007; 109. JWH-081; 110. 5F-AKB-48; 111. JWH-307; 112. JWH-098; 113. JWH-122; 114. JWH-019; 115. RCS-8; 116. UR-144; 117. JWH-210; 118. JWH-398; 119. JWH-147; 120. CB-13.

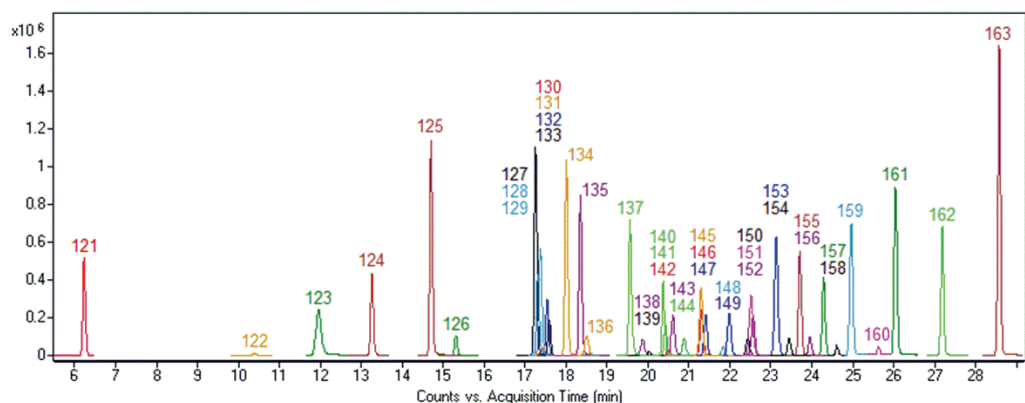


Figure 3. Chromatograms for the quantifier MRM transitions of BDZ/antidepressants, with a retention time within 6–30 min at 5 ng/mL: 121. 7-aminonitrazepam; 122. 7-aminoclonazepam; 123. mirtazapine; 124. 7-aminoflunitrazepam; 125. zolpidem; 126. chlordiazepoxide; 127. bentazepam; 128. oxcarbazepine; 129. midazolam; 130. trazodone; 131. bromazepam; 132. quetiapine; 133. α -OH-midazolam; 134. flurazepam; 135. zopiclone; 136. citalopram; 137. promazine; 138. 3-OH-flunitrazepam; 139. paroxetine; 140. clonazepam; 141. α -OH-alprazolam; 142. oxazepam; 143. duloxetine; 144. amitriptyline; 145. flualprazolam; 146. clonazepam; 147. lorazepam; 148. fluoxetine; 149. alprazolam; 150. nordiazepam; 151. flunitrazepam; 152. triazolam; 153. temazepam; 154. brotizolam; 155. etizolam; 156. delorazepam; 157. lormetazepam; 158. cinazepam; 159. diazepam; 160. levomepromazine; 161. diclazepam; 162. pinazepam; 163. prazepam.

3.2. Method Validation

The sample treatment procedure was optimized starting from two methods currently used in our laboratory for NPS and BDZ/antidepressants quantification. The first one consists of a protein precipitation with ACN, while the second procedure requires an LLE with an 8:2 mixture of dichloromethane/ethyl-acetate at pH 4.5 (phosphate buffer). Both methods were preliminarily tested at QC levels, and the evaluation was based on RR (>75%), ME (from −30 to +30%) and time/resource-consuming. LLE provided the best outcomes for BDZ/antidepressants (RR > 90%; ME from −5 to +7%) but was not suitable for most NPSs, mainly due to the low RRs (i.e., for synthetic cannabinoids, the RR was from 50–70%). On the contrary, PP resulted as very versatile with acceptable criteria met by all the compounds; moreover, it is even faster and simpler. Thus, this treatment was chosen for the new procedure and subsequently optimized at varying volumes (from 500 to 1000 μ L) and temperatures (from −25 to 0 $^{\circ}$ C) of ACN. The optimal conditions have been reported above. Compared to published procedures, which require extraction phases (LLE or solid-phase extraction), our method seemed to be simpler and faster [6,21–23,25–27].

The method proved to be highly specific and selective since no endogenous and exogenous interfering peaks were observed. The coefficient of determination (R^2) was always above 0.9900 in all three of the different tested ranges.

Sensitivity was in line with previously published methods. LOQ ranged from 0.02 ng/mL for prazepam to 1.5 ng/mL for 2F-methcathinone (Table 2). Regarding the main classes of substances, we can state that the highest sensitivities were observed among the BDZs with values always <0.5 ng/mL, except for the “designer BDZ” cinazepam and the metabolites oxazepam and 7-aminoclonazepam (1 ng/mL). Low LOQ levels were also registered for synthetic cannabinoids (0.05–0.5 ng/mL, except for ADB-FUBINACA, APP-FUBINACA and JWH-007 at 1 ng/mL) and fentanyl analogues (0.1–0.5 ng/mL, except for acetyl-norfentanyl, alfentanil and norfentanyl at 1 ng/mL). The method is adequately sensitive to detect all the included substances at recreational or sub-recreational blood concentrations [7,40–42]. At the lowest QC level, 39 substances did not meet the acceptance criteria for accuracy ($-20\% < \%MRE < 20\%$) with the highest value at 22.7% (cumyl-PeGACLONE). Accuracy improved at higher QC concentrations in the following ranges: -20.9% – $+21.7\%$ at QC2 (with 16 substances over the acceptance criteria, $-20\% < \%MRE < 20\%$); -15.0% – $+16.9\%$ at QC3 (with 7 substances over the acceptance criteria, $-15\% < \%MRE < 15\%$); -9.9% – $+9.8\%$ at QC4 (with all substances within the acceptance criteria, $10\% < \%MRE < 10\%$). In many cases, the lowest QCs corresponded to the LOQ value or were close to it, and this may explain their poor accuracy. Thus, quantification at these low concentrations should be treated with caution. None of the compounds showed poor accuracy across all concentrations. Intra-day and inter-day ranged from 1.0 to 20.0% and 2.0 to 20.9%, respectively.

As described above, ME and RR were not negatively affected by the simple and rapid PP step. The mean ME was 3.5%, with the highest values of ion suppression and ion enhancement at -31.1% (5-MeO-DiPT) and $+27.7\%$ (methcathinone), respectively. RR was always >75% and ranged from 75.3% to (4-FA) and 99.0% (5-F-APP-PINACA). Stability studies showed that storage at -25° C and the implementation of freeze/thaw cycles led to a loss <12.8%. Carry over was not observed.

Table 2. Main validation parameters for each compound included in the screening method. N° of replicates: 3 for LOD, LOQ, ME and RR; 5 for linearity, accuracy and precisions. * 250 ng/mL except for: 5-APB, 5CI-AB-PINACA, 5CI-THJ-018, 5F-AKB48, 5F-APP-PICA, 5F-APP-PINACA, 5F-cumyl-PINACA, 5-MAPB, 6-APB, 6-MAPB, AB-CHMINACA, acetyl-fentanyl, ADB-FUBINACA, alfentanil, APP-FUBINACA, cumyl-PeGACLONE, MeOAc-norfentanyl, MMB2201 and ritalinic acid, 150 ng/mL; (±)-cis-3-methyl-fentanyl, (±)-trans-3-methyl-fentanyl, butyryl-norfentanyl, furanyl-norfentanyl and norfentanyl, 75 ng/mL.

Compound	LOD (ng/mL)	LOQ (ng/mL)	R ²	Accuracy (%)				Intra-Day Precision (%)				Inter-Day Precision (%)				ME (%)	RR (%)
				Q1	Q2	Q3	Q4 *	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4		
(±)-cis-3-met-norfentanyl	0.02	0.1	0.9949	-20.6	-14.8	13.5	-2.6	18.8	15.7	14.0	10.0	19.2	11.0	12.8	5.6	-0.4	78.4
(±)-trans-3-met-norfentanyl	0.01	0.1	0.9923	-19.7	-18.1	5.0	-7.9	11.0	17.2	11.7	9.4	10.8	16.7	8.1	3.7	-20.1	94.3
1-naphyrone	0.1	0.5	0.9992	20.7	19.6	-14.3	-7.9	19.6	14.0	12.0	1.0	11.8	11.4	9.5	6.6	-19.6	98.5
25D-NBOMe	0.01	0.05	0.9997	-16.5	10.7	5.5	-7.3	14.2	13.2	12.1	5.1	20.5	17.0	7.2	9.9	16.8	86.6
25H-NBOMe	0.03	0.1	0.9984	-15.1	-19.0	14.0	-5.7	19.7	19.5	5.6	9.9	18.3	18.5	14.5	10.7	16.2	90.5
2-AI	0.05	0.1	0.9909	-16.1	19.6	-14.8	-8.4	4.7	13.7	6.4	3.6	19.6	17.8	10.0	6.3	-2.2	83.6
2C-E	0.3	0.5	0.9987	20.8	-14.8	-14.8	-9.9	5.1	19.2	10.0	5.3	13.9	20.0	13.2	10.3	15.6	85.6
2C-N	0.2	0.5	0.9988	13.8	19.4	15.1	-3.5	13.7	19.0	14.3	3.8	19.8	14.3	10.9	4.8	11.6	90.6
2F-deschloroketamine	0.5	1	0.9975	-19.7	-17.5	4.1	-7.4	19.9	13.5	9.4	9.9	10.9	17.4	6.4	7.7	-5.3	98.0
2F-methcathinone	0.5	1.5	0.9961	11.0	21.2	-10.9	-2.3	9.7	19.2	2.4	1.3	19.0	18.8	12.1	8.1	-14.7	89.5
2-methyl-AP-237	0.1	0.5	0.9981	-9.5	15.5	2.2	-8.8	12.2	19.1	11.1	9.5	12.7	19.5	6.2	3.7	-14.1	87.4
3.4-DMMC	0.1	0.3	0.9973	-15.0	0.1	15.9	-7.6	19.5	20.0	9.9	3.7	19.6	15.9	15.6	10.7	-14.2	79.8
3.4MD-α-PHP	0.2	0.5	0.9997	21.4	19.0	-10.0	-7.5	19.3	14.7	15.0	6.0	18.3	19.1	15.6	7.5	-20.4	78.8
3-OH-flunitrazepam	0.01	0.05	0.9989	-11.6	11.2	-0.5	-1.8	3.9	5.1	14.7	8.1	18.1	15.0	10.5	4.2	18.1	98.8
3-MeO-PCP	0.03	0.1	0.9918	-20.3	19.2	-1.1	2.6	19.9	5.2	6.1	10.0	19.6	10.9	9.3	10.0	-3.2	92.6
3-MeO-PCE	0.2	0.5	0.9993	19.0	-11.7	12.5	7.6	7.6	15.2	14.6	9.5	11.5	10.3	9.5	6.8	-6.8	97.8
3-MMC	0.5	1	0.9947	17.8	-20.3	-8.3	8.1	11.1	19.7	14.0	10.0	17.3	11.8	15.7	5.7	-1.8	83.0
4F-amphetamine	0.1	0.5	0.9922	20.6	12.3	-0.6	-2.4	5.7	19.6	6.1	10.0	11.0	16.9	15.6	8.3	10.3	75.3
4F-methcathinone	0.1	0.3	0.9989	14.5	12.8	4.5	-4.0	19.8	19.4	13.4	1.5	19.3	10.0	13.3	3.0	-18.8	98.8
4-MeO-PCP	0.1	0.5	0.9950	19.6	-14.5	14.8	1.7	6.2	18.2	6.0	9.3	15.3	12.0	11.4	9.1	11.3	92.7
4-methyl-ethcathinone	0.1	0.3	0.9992	-11.1	14.8	15.3	-5.0	19.8	4.5	14.8	9.5	13.7	12.4	7.3	9.1	-6.7	80.9
4-OH-DiPT	0.02	0.2	0.9966	15.9	17.0	-12.3	-4.7	15.0	19.4	14.1	9.4	17.6	14.5	10.8	9.8	-16.4	80.2
5-APB	0.2	0.5	0.9992	10.2	-20.5	11.0	-9.0	7.4	19.2	14.5	5.8	14.1	13.7	12.2	2.2	-17.6	96.5
5CI-AB-PINACA	0.2	0.5	0.9932	-20.4	-17.1	-11.3	-7.3	19.0	19.6	5.7	9.8	19.0	14.9	5.9	7.7	-9.7	76.3
5CI-THJ-018	0.2	0.5	0.9969	-14.0	16.4	-14.2	-9.7	6.2	5.7	14.7	1.7	14.2	13.7	6.7	2.1	10.0	77.2
5F-AKB-48	0.05	0.2	0.9998	21.3	-11.1	14.5	-8.0	13.4	19.0	5.4	10.0	12.9	19.9	15.2	2.0	-3.6	78.5
5F-APINACA	0.05	0.1	0.9918	21.0	20.2	10.0	6.5	12.1	17.8	13.2	9.9	14.3	14.5	15.4	7.9	-17.5	90.9
5F-APP-PICA	0.2	0.5	0.9983	18.6	21.0	-14.0	9.7	19.4	2.4	9.7	10.0	18.9	17.0	5.3	2.0	20.4	87.4
5F-APP-PINACA	0.1	0.5	0.9918	22.2	-13.0	14.6	-5.1	2.2	11.5	14.3	1.6	16.0	17.9	8.6	5.9	-2.4	99.0
5F-Cumyl-P7AICA	0.2	0.5	0.9999	0.2	13.6	-3.5	-7.1	13.1	5.9	14.1	9.7	18.1	12.9	6.8	9.7	19.4	94.3
5F-Cumyl-PeGACLONE	0.05	0.2	0.9991	13.8	-17.5	-14.1	5.7	16.8	11.1	12.1	9.9	12.2	15.9	8.1	2.1	4.9	77.9
5F-Cumyl-PINACA	0.05	0.2	0.9979	21.8	16.6	-12.8	-8.5	14.0	19.7	14.4	9.5	16.2	19.9	10.2	5.8	-20.1	90.3

Table 2. Cont.

Compound	LOD (ng/mL)	LOQ (ng/mL)	R ²	Accuracy (%)				Intra-Day Precision (%)				Inter-Day Precision (%)				ME (%)	RR (%)
				Q1	Q2	Q3	Q4 *	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4		
5F-MDMB-7PAICA	0.2	0.5	0.9991	19.5	-15.1	-10.2	-3.1	3.4	19.0	14.3	7.0	11.0	15.1	14.1	8.3	8.4	77.5
5F-MDMB-PICA	0.05	0.2	0.9985	17.3	12.6	-11.9	-9.1	13.4	10.0	14.9	7.3	15.5	15.8	13.8	3.3	3.9	93.9
5F-NNEI-2'-naphthyl-isomer	0.1	0.5	0.9998	-20.2	18.3	-11.7	-1.7	19.2	19.0	14.9	5.5	10.5	14.8	8.3	8.0	13.1	82.1
5-IAI	0.1	1	0.9945	12.3	-11.5	-11.1	2.1	1.9	17.0	14.8	3.1	19.2	11.2	14.5	4.0	7.0	81.1
5-MAPB	0.5	1	0.9990	12.1	-9.6	-10.5	7.2	9.7	6.7	15.0	4.0	20.6	11.2	6.7	8.3	21.0	94.1
5-MeO-DIPT	0.1	0.3	0.9939	-20.1	-12.2	-4.1	-8.8	17.7	8.5	8.4	1.4	13.8	19.9	5.2	2.2	-31.1	80.2
6-APB	0.2	1	0.9989	21.8	15.0	-14.2	-5.8	13.2	4.6	1.6	10.0	12.0	16.7	10.6	4.2	-2.5	88.0
6-MAPB	0.5	1	0.9999	21.1	14.3	14.7	9.5	19.0	14.6	3.8	10.0	14.5	20.2	14.0	7.0	-4.9	95.8
7-aminoclonazepam	0.5	1	0.9960	15.5	18.1	7.6	-4.4	7.0	19.2	8.7	9.0	11.5	13.1	6.1	4.3	3.5	77.2
7-aminoflunitrazepam	0.01	0.05	0.9950	17.0	0.5	3.8	9.4	17.9	9.2	6.5	9.9	16.2	14.3	14.9	9.5	-15.8	96.9
7-aminonitrazepam	0.05	0.2	0.9967	-12.1	19.3	14.6	-3.2	5.6	19.5	6.5	1.7	20.8	17.9	15.8	5.4	7.6	78.8
AB-CHMINACA	0.02	0.1	0.9985	20.0	19.2	5.9	-8.3	4.2	2.9	3.4	10.0	19.3	15.6	14.0	6.6	14.3	88.5
AB-FUBINACA	0.1	0.5	0.9941	-15.3	19.1	-2.0	-9.9	7.8	1.7	14.1	6.1	18.7	18.5	14.5	3.5	6.0	85.2
acetyl-fentanyl	0.1	0.5	0.9990	21.1	19.2	-11.8	-8.0	19.1	14.2	14.1	9.6	15.3	19.2	9.5	9.2	-0.7	76.0
acetyl-norfentanyl	0.5	1	0.9969	-10.7	-16.5	14.8	-7.9	15.0	19.4	9.8	9.0	15.0	17.1	10.0	9.7	12.2	78.2
ADB-FUBINACA	0.5	1	0.9927	-18.0	-20.4	10.7	-9.0	17.1	20.0	9.6	8.8	14.7	11.1	9.6	8.2	-8.3	92.7
ADB-PINACA	0.1	0.5	0.9991	14.5	19.0	-11.4	0.9	13.5	17.2	3.3	2.7	19.4	12.7	15.9	3.7	1.5	87.6
alfentanil	0.2	1	0.9966	-14.2	-12.2	8.0	-6.4	18.1	5.2	13.0	9.1	12.5	10.3	5.2	9.5	-0.9	93.9
alprazolam	0.05	0.1	0.9996	12.1	21.4	-13.9	-9.8	14.9	18.2	3.6	9.9	20.9	20.3	6.8	3.6	-20.1	76.4
AM-2201	0.05	0.1	0.9979	18.3	13.2	-1.3	-6.2	19.2	19.7	12.5	8.0	19.7	20.4	14.9	7.5	15.8	93.9
AM-2233	0.05	0.1	0.9997	20.0	-16.7	4.9	5.0	19.2	15.2	14.4	5.1	19.6	20.9	12.0	6.9	19.3	82.4
AM-694	0.1	0.5	0.9986	-14.8	0.3	2.7	-7.5	12.9	4.7	5.8	9.9	19.0	15.4	7.1	2.6	15.1	91.1
amitriptyline	0.5	1	0.9933	-20.6	19.8	0.7	-5.0	19.0	19.2	1.4	9.0	13.8	20.2	5.9	4.2	-2.8	80.5
amphetamine	0.1	0.5	0.9941	-18.0	-20.9	-13.9	-6.3	3.6	11.2	9.9	4.3	16.0	10.5	8.4	5.2	15.4	77.9
AP-237	0.05	0.1	0.9955	-21.4	15.3	12.2	-3.0	3.5	12.5	6.0	9.9	12.8	19.7	13.8	10.5	-2.5	95.4
APP-FUBINACA	0.5	1	0.9978	-13.6	19.5	3.5	-2.0	7.7	11.8	2.8	2.6	19.3	11.2	7.4	3.6	-19.8	77.0
benzazepam	0.2	0.5	0.9989	18.2	-16.8	-5.1	9.5	19.0	8.6	3.9	9.8	14.1	17.2	6.6	8.3	11.1	90.6
bromazepam	0.2	0.5	0.9959	20.3	-17.2	-14.6	-7.9	1.8	18.2	14.9	9.0	19.1	19.8	13.8	8.0	20.4	91.9
brotizolam	0.03	0.1	0.9982	1.0	-15.6	-8.6	-8.0	11.7	6.4	3.8	9.1	19.5	20.9	5.3	2.2	20.1	77.1
buphedrone	0.1	0.5	0.9959	15.9	19.0	-13.5	0.1	6.4	19.8	14.4	10.0	20.2	13.7	14.7	7.9	12.3	84.4
butylone	0.1	0.3	0.9973	19.8	13.5	12.2	9.5	19.6	8.2	14.9	2.3	19.1	13.7	10.3	2.8	-3.0	86.4
butyryl-fentanyl	0.1	0.5	0.9967	11.5	-16.5	7.0	-7.0	2.1	18.5	8.8	9.9	15.1	11.2	14.7	2.8	9.9	98.5
butyryl-norfentanyl	0.1	0.5	0.9970	21.6	-14.0	-1.6	-5.3	19.0	2.5	2.8	10.0	10.2	20.2	14.9	8.8	12.7	92.2
BZP	0.05	0.5	0.9989	-21.9	-17.2	-14.6	-5.4	9.9	12.6	2.6	6.0	16.5	20.3	13.3	8.9	15.5	90.5
carfentanyl	0.1	0.5	0.9952	11.4	12.2	-11.5	-9.8	19.8	5.8	14.7	9.9	16.3	11.4	11.9	10.4	-9.5	87.0

Table 2. Cont.

Compound	LOD (ng/mL)	LOQ (ng/mL)	R ²	Accuracy (%)				Intra-Day Precision (%)				Inter-Day Precision (%)				ME (%)	RR (%)
				Q1	Q2	Q3	Q4 *	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4		
CB-13	0.1	0.5	0.9983	-10.1	-11.5	13.0	-8.4	7.4	9.7	14.4	9.8	14.9	14.5	9.0	4.5	10.9	94.0
chlordiazepoxide	0.2	0.5	0.9996	12.7	-17.9	14.9	9.7	12.9	15.7	14.5	2.3	19.7	19.4	14.6	5.2	-0.7	91.6
cinazepam	0.5	1	0.9984	-20.4	-11.8	-0.6	-3.4	19.0	19.7	4.1	9.6	19.6	13.9	9.6	5.8	14.2	88.9
citalopram	0.03	0.1	0.9969	19.6	21.7	-13.3	-7.4	19.0	19.5	14.0	9.8	13.6	14.3	6.4	10.4	-6.7	94.5
clonazepam	0.1	0.5	0.9981	19.6	19.3	-9.1	-6.6	19.7	1.8	13.5	5.2	14.1	18.6	15.0	3.6	8.0	86.4
clonazolam	0.2	0.5	0.9987	-9.4	17.1	-15.0	-6.4	19.0	4.1	14.1	2.7	16.4	15.5	14.5	6.2	5.9	81.3
cumyl-PeGLACONE	0.2	0.5	0.9927	22.7	-18.2	3.6	8.0	1.7	19.2	14.4	9.5	16.6	16.4	14.9	6.6	16.2	95.4
cyclopropyl-fentanyl	0.1	0.5	0.9988	-17.2	-16.0	-14.2	6.5	17.2	19.8	14.5	9.3	19.6	20.1	8.0	3.2	6.9	80.9
delorazepam	0.1	0.5	0.9996	19.9	11.1	16.9	-4.7	1.2	19.2	4.4	9.6	10.1	12.0	9.3	9.3	-18.8	81.6
deschloro-N-et-ketamine	0.2	0.5	0.9958	-19.3	-11.5	11.1	-3.5	19.0	19.7	9.3	9.9	20.1	13.5	6.0	5.5	-7.3	96.0
diazepam	0.01	0.05	0.9980	15.9	16.8	12.3	-7.9	7.1	12.4	4.1	9.7	12.2	20.7	14.4	2.6	20.5	78.3
diclazepam	0.2	0.5	0.9908	15.1	-20.7	5.4	-2.0	19.6	18.6	14.1	7.6	10.8	19.8	9.9	8.7	20.2	98.9
dimethylcathinone	0.1	0.3	0.9987	-18.5	-20.4	-9.9	5.5	8.2	13.0	9.5	9.8	19.1	20.9	9.1	9.9	-14.4	93.9
duloxetine	0.1	0.5	0.9976	11.3	-13.8	7.2	7.4	19.8	2.9	5.5	2.4	10.0	13.2	7.7	3.0	-15.6	89.6
ethcathinone	0.1	0.3	0.9911	-20.9	16.6	14.2	-4.7	19.9	19.2	14.3	1.6	11.6	16.3	8.5	4.3	-2.2	98.8
ethylone	0.1	0.3	0.9997	-12.3	15.0	14.5	-9.0	14.6	19.7	3.7	8.6	18.2	10.9	7.2	3.2	21.5	89.5
etizolam	0.1	0.5	0.9928	15.0	-16.4	12.5	8.2	13.5	17.2	14.0	8.9	16.6	17.1	6.2	9.8	-6.8	93.8
eutylone	0.5	1	0.9997	21.6	-15.6	-11.0	-4.8	1.7	14.1	12.5	9.7	19.1	20.4	9.1	9.9	6.4	83.5
fentanyl	0.05	0.1	0.9977	14.1	-20.2	-2.6	-1.4	18.5	19.2	6.3	6.8	19.4	10.7	11.2	9.0	-1.1	90.3
flualprazolam	0.2	0.5	0.9905	-9.9	18.0	-9.9	0.6	13.8	11.8	11.9	9.9	18.3	18.5	5.8	7.2	-13.0	80.2
flunitrazepam	0.1	0.5	0.9988	11.3	-11.1	11.0	7.4	14.7	12.5	14.5	9.9	20.1	11.9	7.7	9.2	21.4	77.7
fluoxetine	0.5	1	0.9918	-20.8	19.8	13.6	-9.4	19.0	12.2	2.9	9.0	16.8	16.0	11.5	9.7	4.0	80.5
flurazepam	0.01	0.05	0.9954	-18.2	-12.2	15.1	9.5	17.1	13.7	14.7	9.7	15.7	13.8	7.3	10.4	-7.5	76.0
furanyl-fentanyl	0.1	0.5	0.9979	-20.8	18.5	14.3	0.3	19.8	19.0	2.4	10.0	15.9	11.2	14.8	9.4	10.6	76.0
furanyl-norfentanyl	0.01	0.2	0.9955	12.6	-18.0	-12.1	-6.3	18.0	19.0	14.1	9.8	11.9	13.4	9.6	9.7	1.3	86.6
isobutyryl-fentanyl	0.1	0.5	0.9912	14.1	-17.2	13.1	-8.4	3.8	15.7	15.0	6.9	15.5	19.8	12.5	7.2	4.2	87.2
isotonitazene	0.05	0.2	0.9985	20.6	-17.3	14.9	-3.9	4.9	3.7	5.3	1.5	12.3	18.0	9.1	7.7	10.6	90.6
JWH-007	0.5	1	0.9991	-13.0	21.4	-11.9	-4.9	5.2	6.7	14.6	2.7	13.7	15.5	10.1	7.6	27.4	83.7
JWH-016	0.3	0.1	0.9999	-15.0	13.3	-11.8	0.7	19.4	19.9	1.7	6.1	16.3	13.2	15.2	2.0	-4.8	80.1
JWH-018	0.3	0.1	0.9997	14.6	20.2	14.2	3.0	3.8	19.4	14.1	10.0	14.1	13.9	10.8	4.7	1.1	78.1
JWH-019	0.1	0.5	0.9913	12.7	19.8	16.4	-1.4	14.9	1.2	12.6	10.0	12.3	19.6	11.9	10.7	-19.8	76.4
JWH-073	0.1	0.5	0.9944	-16.5	15.9	14.5	-0.6	1.7	3.6	14.8	10.0	16.5	12.2	12.7	2.4	-14.6	94.7
JWH-081	0.1	0.5	0.9987	-19.5	-18.2	12.6	-0.9	19.1	7.2	3.1	9.7	11.4	14.1	12.0	7.1	21.4	93.0
JWH-098	0.1	0.5	0.9951	-13.0	17.0	-7.9	4.6	4.7	19.4	9.1	2.9	10.7	18.3	9.1	9.1	4.7	80.0
JWH-122	0.1	0.5	0.9971	-20.6	19.8	-10.4	-7.0	8.2	11.2	8.4	9.1	12.9	19.3	10.3	5.6	15.4	98.9

Table 2. Cont.

Compound	LOD (ng/mL)	LOQ (ng/mL)	R ²	Accuracy (%)				Intra-Day Precision (%)				Inter-Day Precision (%)				ME (%)	RR (%)
				Q1	Q2	Q3	Q4 *	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4		
JWH-147	0.02	0.05	0.9961	-11.7	-12.0	-4.6	-1.9	19.9	19.6	14.7	9.7	10.3	19.7	12.5	10.4	13.2	84.2
JWH-200	0.1	0.5	0.9945	-19.9	12.9	-14.6	9.1	19.6	19.5	14.6	9.9	11.4	18.7	11.8	10.4	-8.2	89.6
JWH-203	0.1	0.5	0.9908	-12.7	18.8	-13.9	-4.1	5.1	14.1	14.4	5.4	20.0	19.6	11.7	10.9	-4.1	98.1
JWH-210	0.1	0.5	0.9985	18.3	-16.2	-3.6	-6.0	19.4	11.8	5.0	10.0	17.3	17.8	11.9	9.5	10.7	95.3
JWH-250	0.1	0.5	0.9965	16.8	15.0	1.6	-4.0	5.2	19.1	19.0	10.0	20.9	18.6	15.0	9.6	-12.0	93.1
JWH-251	0.1	0.5	0.9944	-13.0	12.2	-12.4	8.8	3.2	1.4	14.7	9.6	19.2	19.8	7.7	2.3	7.7	76.2
JWH-302	0.1	0.5	0.9926	11.0	-18.8	2.2	3.6	18.0	3.0	12.6	10.0	14.0	10.0	8.8	9.5	-11.0	84.5
JWH-307	0.05	0.1	0.9934	-13.7	-18.9	-15.0	3.6	6.2	16.2	14.3	8.6	19.1	16.6	8.2	6.5	-1.0	97.1
JWH-398	0.1	0.5	0.9991	21.7	13.8	14.5	-7.1	19.9	2.0	10.9	8.0	12.6	11.1	11.3	9.9	6.3	82.9
ketamina	0.05	0.2	0.9926	12.6	-13.1	-13.8	-5.6	3.4	2.2	8.7	9.5	19.1	19.7	6.1	5.5	-1.1	84.1
levomepromazine	0.01	0.1	0.9914	-11.8	15.8	10.0	-3.1	19.4	9.3	9.1	5.7	17.6	13.5	10.9	5.8	14.6	89.1
lorazepam	0.1	0.5	0.9997	21.8	-11.0	-8.9	-8.4	2.2	19.2	8.1	2.5	12.9	15.9	7.3	2.7	-11.0	89.3
lormetazepam	0.05	0.1	0.9999	10.9	-18.4	-12.0	4.5	4.2	19.1	7.0	9.5	19.4	14.8	14.8	9.6	-9.3	92.2
m-CPP	0.1	0.5	0.9971	-12.3	-9.6	-1.0	-9.0	19.5	19.4	6.0	10.0	19.8	13.2	7.9	9.5	10.1	81.6
MDA	0.1	0.3	0.9979	19.9	19.0	-11.0	-1.0	13.2	4.0	14.9	9.7	14.9	16.6	10.2	5.6	20.7	75.6
MDAI	0.1	0.5	0.9946	12.4	-18.6	14.8	2.9	19.5	5.2	2.4	4.8	19.0	12.1	11.0	10.4	-14.5	88.9
MDEA	0.03	0.1	0.9919	12.7	0.6	-7.4	0.9	13.7	19.0	14.8	10.0	12.0	15.6	14.2	3.2	-16.9	98.4
MDMA	0.1	0.5	0.9967	-20.1	-18.6	-6.9	-1.1	19.2	15.9	14.1	10.0	19.4	13.4	6.6	6.2	-6.4	94.9
MDMB-CHMICA	0.2	0.5	0.9972	-17.8	15.6	-13.5	9.1	13.2	7.6	14.6	2.0	17.8	15.2	15.4	7.5	-3.7	80.5
MDPV	0.3	0.5	0.9935	18.5	19.5	-12.9	-6.4	14.5	12.0	11.2	9.7	11.1	17.0	13.6	9.5	-6.0	86.6
MeOAc-fentanyl	0.1	0.5	0.9942	19.8	-13.2	2.2	5.9	12.7	14.1	4.7	9.0	19.0	18.8	11.3	5.5	-18.3	96.7
MeOAc-norfentanyl	0.1	0.5	0.9998	19.7	-11.5	-14.5	1.0	1.8	13.7	14.1	9.4	20.9	20.0	9.3	6.3	-12.7	79.2
mephedrone	0.1	0.5	0.9996	15.0	13.3	5.6	5.4	19.9	12.7	14.0	10.0	20.7	17.1	6.3	9.3	14.7	85.8
methamphetamine	0.1	0.5	0.9997	-18.9	19.5	0.9	-7.2	15.5	19.8	9.7	9.8	12.2	15.4	8.1	8.5	7.9	96.4
methcathinone	0.1	0.5	0.9979	19.6	20.0	13.8	9.1	19.0	16.0	6.0	2.0	19.8	17.3	11.3	5.4	27.7	90.5
methedrone	0.1	0.5	0.9992	-14.5	14.4	-14.0	-3.5	19.0	4.0	14.6	4.5	17.6	19.1	12.2	10.2	-10.1	92.1
methoxetamine	0.1	0.5	0.9964	19.6	-18.8	14.3	0.3	18.9	2.6	1.8	9.4	15.8	11.6	7.0	5.4	-5.3	94.6
methylone	0.1	0.5	0.9941	15.0	-18.4	9.5	8.7	14.0	19.1	4.7	9.5	18.9	20.7	6.2	5.7	-13.7	89.0
midazolam	0.05	0.1	0.9955	19.8	14.6	-10.6	-2.8	11.8	14.2	14.1	10.0	16.8	13.0	7.2	2.4	8.5	79.2
mirtazapine	0.1	0.5	0.9974	-18.9	-18.1	14.9	-3.0	18.9	19.0	14.9	9.5	12.3	13.0	12.5	6.7	18.1	79.4
MMB-2201	0.1	0.5	0.9964	22.0	15.3	-7.1	-1.2	4.5	19.9	9.0	9.9	12.2	19.7	7.2	8.1	-8.9	91.4
naphyrone	0.1	0.5	0.9976	18.1	-10.2	6.2	9.6	14.4	16.8	5.1	9.6	17.3	19.8	14.0	9.4	2.8	79.6
N-ethylpentylone	0.3	0.5	0.9906	19.1	20.9	-13.8	-1.0	1.2	1.8	12.7	10.0	13.6	16.5	14.4	6.3	-5.8	98.5
nordiazepam	0.1	0.3	0.9952	14.7	-13.1	-14.7	-4.6	9.4	7.0	1.7	5.9	15.4	16.7	6.3	7.3	21.1	91.1
norfentanyl	0.5	1	0.9973	18.8	13.5	0.3	-8.1	1.2	19.0	14.7	9.8	10.7	10.0	12.0	7.2	10.3	78.2
norketamina	0.1	0.5	0.9939	12.7	-16.4	-13.1	-2.6	1.9	13.2	15.0	3.6	10.5	19.0	15.1	9.9	16.0	87.4

Table 2. Cont.

Compound	LOD (ng/mL)	LOQ (ng/mL)	R ²	Accuracy (%)				Intra-Day Precision (%)				Inter-Day Precision (%)				ME (%)	RR (%)
				Q1	Q2	Q3	Q4 *	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4		
ocfentanil	0.2	0.1	0.9994	18.2	14.0	6.1	9.7	19.6	19.2	4.5	9.9	20.0	16.2	5.9	4.5	-6.4	87.9
oxazepam	0.5	1	0.9966	-20.4	15.3	10.1	9.4	18.9	19.0	13.7	7.5	19.2	11.8	9.9	9.3	3.8	92.1
oxcarbazepine	0.5	1	0.9988	-11.5	19.9	12.8	9.8	2.4	6.2	5.4	9.4	19.7	14.6	5.6	6.8	18.4	89.3
paroxetine	1	2	0.9986	22.4	13.8	12.8	-4.1	19.1	11.2	14.1	9.6	18.2	20.7	7.1	2.9	-0.7	80.8
pentedrone	0.1	0.3	0.9934	-10.4	-18.6	-13.6	7.1	13.6	19.7	3.6	9.4	19.6	19.7	13.4	8.8	-5.1	79.9
pentylone	0.1	0.3	0.9966	17.2	-18.6	14.0	-2.8	19.0	4.5	12.5	9.8	10.3	10.5	14.8	2.1	-12.7	85.0
pF-furanyl-fentanyl	0.01	0.2	0.9981	19.6	-9.7	-3.6	-9.1	4.7	20.0	1.5	9.5	16.0	11.9	7.5	8.1	2.4	90.0
pinazepam	0.01	0.05	0.9983	18.0	-9.1	-6.0	-7.6	12.6	15.7	1.5	4.5	10.2	10.9	7.9	7.6	20.6	79.7
pravadoline	0.02	0.1	0.9946	0.4	16.1	5.5	9.6	18.4	2.0	1.1	3.5	20.7	20.1	13.9	10.6	-16.3	82.6
prazepam	0.01	0.02	0.9981	-10.5	19.5	1.0	9.2	5.4	19.0	14.1	2.9	11.3	17.8	12.4	9.9	8.3	92.0
promazine	0.1	0.5	0.9944	-20.2	-17.0	-6.6	-0.1	4.2	19.8	14.6	1.3	19.7	16.1	13.9	7.4	21.8	91.7
quetiapine	0.1	0.5	0.9987	18.0	-20.4	6.9	6.4	19.0	4.2	9.7	2.0	19.8	12.0	11.2	10.3	20.1	87.2
RCS-4	0.05	0.1	0.9994	16.8	11.4	16.8	6.0	8.8	19.6	2.4	9.0	16.8	12.1	11.7	3.5	8.5	80.2
RCS-8	0.02	0.05	0.9922	19.4	-18.3	5.4	1.0	1.2	10.0	6.1	9.1	11.3	17.3	11.2	2.1	17.0	79.0
Ritalinic acid	0.2	1	0.9965	-14.2	-11.8	8.2	0.5	14.9	18.2	12.1	6.7	14.3	17.4	8.1	4.8	15.0	76.9
temazepam	0.1	0.5	0.9978	22.0	-19.0	-11.6	-8.9	19.0	19.9	14.0	9.6	16.3	13.1	9.9	9.5	21.3	85.6
trazodone	0.1	0.5	0.9986	18.8	19.3	10.9	9.7	13.9	12.4	9.4	4.8	19.6	19.5	6.8	8.0	1.2	93.6
triazolam	0.03	0.1	0.9984	21.5	-15.8	-14.0	1.9	2.4	14.2	14.3	10.0	15.5	11.8	15.5	4.7	7.1	86.6
UR-144	0.2	0.5	0.9993	19.2	14.7	13.2	-6.0	6.8	14.1	7.9	9.8	13.4	19.7	12.1	3.4	-8.8	84.1
WIN 55.212-2	0.05	0.1	0.9985	-18.8	19.6	-13.9	-9.3	18.3	19.2	2.5	9.9	11.9	11.5	12.0	8.7	-17.2	97.9
zolpidem	0.05	0.1	0.9932	-17.0	-14.8	13.9	-9.0	15.2	19.6	11.7	9.9	18.5	19.9	12.3	5.5	-3.2	91.8
zopiclone	0.05	0.1	0.9991	22.0	-18.7	14.8	-5.4	4.5	19.1	14.5	2.6	12.5	13.2	9.5	7.5	16.7	94.4
α-OH-alprazolam	0.05	1	0.9964	-17.1	-12.5	11.6	5.6	19.0	12.4	14.1	9.9	11.8	14.6	8.9	4.5	-3.8	80.9
α-OH-midazolam	0.05	0.1	0.9977	-18.7	14.5	-12.0	-7.2	9.4	19.7	14.8	9.7	10.3	11.2	9.4	10.1	-7.6	89.9
α-PHP	0.5	0.1	0.9967	11.6	18.0	8.1	9.8	19.4	9.8	12.1	9.8	18.5	14.7	14.8	7.1	10.6	93.6

3.3. Application to Real Samples

Before validating this new method, BDZ/antidepressants and NPSs were quantified by means of two separated procedures from two aliquots of blood, entailing double time, resource and sample consuming [43]. The application of this new method significantly improved our analytical activity, especially when a wide-range detection strategies are required. In the last few months, it has been successfully applied in cases of acute intoxications ($n = 2$), post-mortem analysis ($n = 1$) and the evaluation of driving under the influence (DUID) for drivers involved in road accidents ($n = 48$). In DUID cases, the most detected compounds were BDZs: diazepam and its main metabolites nordiazepam, temazepam and oxazepam ($n = 5$), alprazolam ($n = 3$) and lorazepam ($n = 2$), and they were always within or below the therapeutic concentrations (Table 3) [40]. Midazolam, fentanyl and ketamine were also detected when administrated by the healthcare personnel at the emergency department to induce sedation. Moreover, ketamine and its main metabolite (norketamine) were also quantified in two DUID cases (Figure 4). MDPV was found at 42.3 ng/mL in a blood sample from a 23-year-old man who was hospitalized due to severe agitation. None of the newest NPSs was detected.

Table 3. Concentrations found in real cases of consumption.

Case	Forensic Casework	Compound	Concentration (ng/mL)
#1	DUID	alprazolam	50.39
		α -OH-alprazolam	3.32
#2	DUID	diazepam	346.07
		nordiazepam	70.82
		temazepam	35.56
		oxazepam	15.77
#3	DUID	lorazepam	85.30
#4	DUID	ketamina	246.37
		nor-ketamina	177.43
#5	DUID	diazepam	1046.36
		nordiazepam	638.16
		temazepam	18.68
		oxazepam	80.93
#6	DUID	diazepam	28.89
		nordiazepam	74.53
		temazepam	10.58
		oxazepam	<LOQ
#7	DUID	alprazolam	31.30
		α -OH-alprazolam	<LOQ
#8	DUID	ketamina	305.01
		nor-ketamina	198.77
#9	DUID	alprazolam	7.27
		α -OH-alprazolam	<LOQ
#10	DUID	diazepam	38.25
		nordiazepam	30.78
		temazepam	29.11
		oxazepam	26.89
#11	DUID	diazepam	1830.17
		nordiazepam	44.00
		temazepam	19.02
		oxazepam	<LOQ
#12	DUID	lorazepam	178.12
#13	Acute intoxication	MDPV	42.3

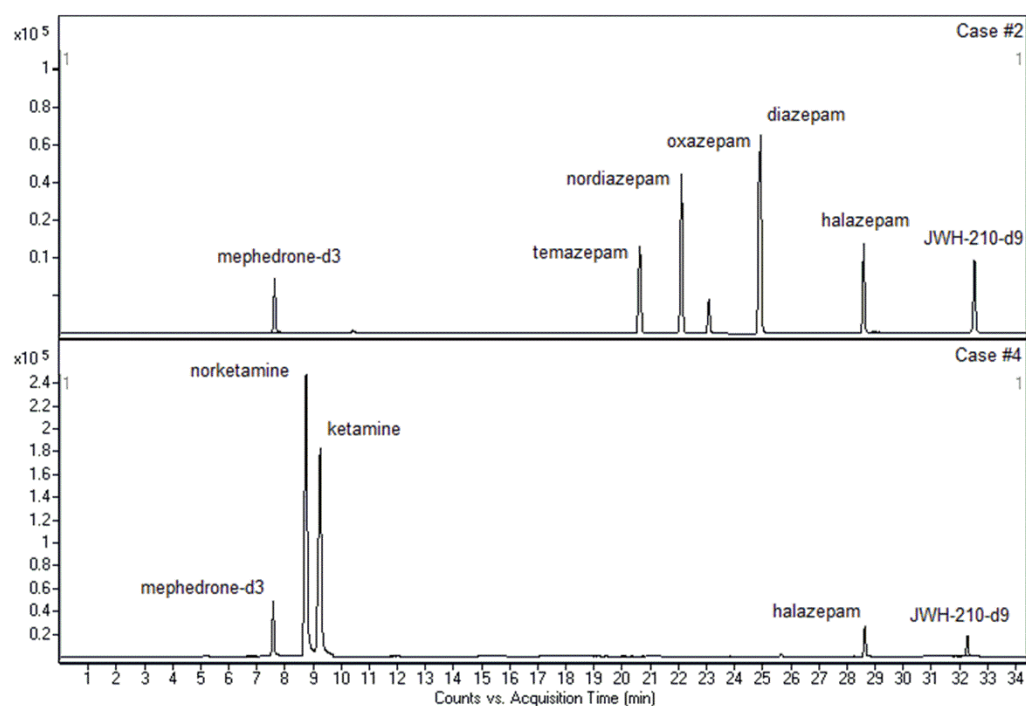


Figure 4. Chromatograms for real case #2 and #4.

4. Conclusions

An LC-MS/MS-based multi-analyte method was fully validated. The here described procedure employs a simple sample preparation (PP) and requires a small sample volume (200 μ L of blood) for the detection of 120 NPS and 43 drugs of great forensic-toxicological interest, such as BDZs. The short preparation time represents this analytical method's great advantage, as well as its high chromatographic efficiency. Moreover, the high specificity and sensitivity make this methodology suitable for all cases that require the identification and quantification of a wide range of compounds. Implementation in our routinary activity was actual proof of the advantages of this method, as well as its efficacy and reliability. In the future, the new method will be used to study the prevalence of BDZ/antidepressants in road accidents.

Author Contributions: Conceptualization, F.V. and F.C.; methodology, F.V.; software, F.V.; validation, F.V. and F.C.; formal analysis, F.V., M.M., L.P. and J.R.; investigation, F.V., M.M., L.P. and J.R.; resources, F.V.; data curation, F.V. and M.M.; writing—original draft preparation, F.V. and F.C.; writing—review and editing, F.V. and F.C.; visualization, C.T.S. and E.B.; supervision, C.T.S. and E.B.; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflict of interest.

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