Development and validation of a rapid LC-MS/MS method for the detection of 182 novel psychoactive substances in whole blood

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Abstract

Introduction: The analysis of novel psychoactive substances (NPS) represents a challenge in forensic toxicology, due to the high number of compounds characterized by different structures and physicochemical properties both among different subclasses and within a single subclass of NPS. The aim of the present work is the development and validation of a targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the detection of NPS in whole blood.

Materials and methods: A protein-precipitation based LC-MS/MS method for the detection of more than 180 NPS was developed and validated by assessing the following parameters: selectivity, linearity, accuracy, precision, limit of detection (LOD) and of quantification (LOQ) recovery, and matrix effect. Then, the method was applied to real forensic samples.

Results: The method allowed the identification of 132 synthetic cannabinoids, 22 synthetic opioids, and 28 substances among synthetic cathinones, stimulants, and other drugs. Validation was successfully achieved for most of the compounds. Linearity was in the range of 0.25–10 ng/ml for synthetic cannabinoids and 0.25–25 ng/ml for other drugs. Accuracy and precision were acceptable according to international guidelines. Three cases tested positive for fentanyl and ketamine, in the setting of emergency room administration.

Conclusions: The present methodology represents a fast, not expensive, wide-panel method for the analysis of more than 180 NPS by LC-MS/MS, which can be profitably applied both in a clinical context and in postmortem toxicology.

KEYWORDS
forensic toxicology, mass spectrometry, novel psychoactive substances, screening method, validation
1 | INTRODUCTION

The term Novel Psychoactive Substances (NPS) encompasses a high number of molecules with very different chemical characteristics, originally defined for not being covered by the United Nations International Drug Conventions 1961–1971. Since 1997, the European Monitoring Centre for Drug and Drug Addiction (EMCDDA) has been monitoring 820 NPS at the end of 2020, including synthetic cannabinoids (SCs), synthetic opioids (SOs), synthetic cathinones (SCAs), designer benzodiazepines (dBZDs), phenethylamines, and tryptamines. The peak of new compounds per year in the drug market has been reported around 2014–2015 and, even if the prevalence is still high, has then decreased, with lower diversity in the consumed substances, partially reflecting national and international legislations, such as the German act on NPS and the Chinese regulations, which appear to have a high impact on the European market. SCs were first detected around 2006; they represent the largest group of NPS and have so far dominated the market, accounting together with SCAs for 62% of NPS seizures in 2018, while in recent years, SOs and dBZDs are growing in numbers. NPS have been claimed by the suppliers as safe and legal alternatives to common drugs of abuse, and sold under codes like research chemicals, smart drugs, legal highs, dietary supplements, or bath salts, often declared to be not for human consumption. They are increasingly encountering the favor of online and physical consumers, despite their toxicity is often greater than that of the corresponding classical illicit drug.

One of the reasons for the rapid spreading of NPS across the public is the poor detectability at screening tests performed on biological fluids. Indeed, the analysis of NPS cannot be based on common immunoenzymatic methods of screening and usually requires either liquid or gas chromatography (LC or GC) coupled to mass spectrometry (MS) for both screening and confirmatory analysis.

In forensic toxicology, the bioanalysis of NPS is particularly challenging and is required when a suspicion of intoxication is coupled to a target LC-MS/MS method for the detection of more than 180 NPS in whole blood and its application to forensic cases.

2 | MATERIAL AND METHODS

2.1 | Chemicals and reagents

Standard solutions of 132 SCs, 22 SOs, and 28 among SCAs, stimulants, and other drugs were provided by the National Health Institute within the National Early Warning System; the panel of SC was kindly integrated by 98 standard solutions of SCs provided by the Forensic Toxicology Department of the Institute of Forensic Medicine, Medical Center – University of Freiburg (Panel 3). Composition of Panels 1–3 was the following (semi-systematic names).

2.1.1 | Panel 1

Standards of 3,4-dimethylmethcathinone (3,4-DMMC), 4-fluoromethcathinone (4-FMC), 4-methylethcathinone (4-MEC), AM-2201, AM-2233, AM-694, buphedrone, butylone, ethcathinone, ethylone, JWH-007, JWH-016, JWH-019, JWH-081, JWH-098, JWH-122, JWH-203, JWH-210, JWH-251, JWH-302, JWH-398, ketamine, MDPV, methcathinone (MCAT), methedrone (4-Methoxy MCAT), methylone, nordiazepam, pentylone, RCS-4, RCS-8 and WIN 48,098 (pravadoline) were provided by Comedical s.r.l. (Italy, Trento) at 0.1 mg/ml.

2.1.2 | Panel 2

Standards of (±)-cis-3-methyl norfentanyl, (±)-trans-3-methyl norfentanyl, nET, β-hydroxy fentanyl, β-hydroxythiofentanyl, β-phenyl fentanyl, 4-Acetoxy-DiPT (4-AcO-DiPT), 4-ANPP, 5-APB/6-APB, 5-CI-THJ 018, 5-EAPB, 5-FADB, 5F-APP-PICA (PX-1), 5F-APP-PINACA (PX-2), 5F-CumylPINACA, 5F-NNEI 2'-Naphthyl Isomer, 5-MAPB/6-MAPB, 5-methoxy-AMT (5-MeO-AMT), 5-methoxy-DALT (5-MeO-DALT), 5-Methoxy-DMT (5-MeO-DMT), 5-Methoxy-DPT (5-MeO-DPT), 5-Methoxy-Mipt (5-MeO-Mipt), AB-CHMINACA, AB-FUBINACA, acetyl fentanyl, acetyl norfentanyl, ADB-FUBINACA, alfentanil, APP-FUBINACA, butyryl fentanyl, butyryl fentanyl carboxyl metabolite, butyryl norfentanyl, carfentanyl, Cumyl-PGAECLOLNE (SGT-151), cyclopropylfentanyl, despropionyl para-fluorofentanyl, ethylphenidate, fentanyl, furanyl norfentanyl, JWH-018, JWH-200, JWH-250, MDBMB-CHIMICA, mephedrone (4-Methyl MCAT, 4-MMC), methoxyacetyl norfentanyl, MMB-2201 (5F-AMB-PICA), N,N-dimethylcathinone, N,N-dimethyltryptamine (DMT), norfentanyl, phenylfentanyl, phenylacetyl fentanyl, ritalinic acid and valeryl
fentanyl carboxy metabolite were provided by Comedical s.r.l. (Italy, Trento) at 0.05 mg/ml.

2.1.3 | Panel 3

4-HTMPO, 4F-MDMB-BINACA, 5F-AB-001, 5F-AB-PICA (5F-ABICA), 5F-AB-PINACA, 5F-ADB-PICA (5F-ADBICA), 5F-ADB-PINACA, 5F-AMB-PINACA, 5F-EMB-PINACA, 5F-JWH-412, 5F-MDMB-P7AICA, 5F-MDB-PICA, 5F-MDMB-PINACA (5F-ADB), 5F-PCN (5F-MN-21), 5F-PY-PICA, A-796,260, A-834,735, AB-001, AB-005, AB-005 azepane, AB-BICA, AB-CHMICA, AB-FUB7AICA (AB-7-FUBAICA), AB-FUBICA, AB-FUBINACA 2/3-fluorobenzyl isomers, AB-PICA, AB-PINACA, ADB-BICA, ADB-BINACA, ADB-CHMICA, ADB-FUBICA, ADB-PICA, ADB-PINACA, AKB-48 (APINACA), AM-1220 azepane, AM-1225, AM-1241, AM-1248, AM-1248 azepane, AM-2201 indazole carboxamide, AM-2232, AM-2233 azepane, AM-630, AM-679, AMB-CHMICA, AMB-CHMINACA, AMB-FUBICA, AMB-FUBINACA, AMB-PICA, AMB-PINACA, PB-22, Cumyl-4CN-BINACA, Cumyl-BICA, Cumyl-PICA, Cumyl-THPINACA, EG-018, EG-2201, FUB-JWH-018, FUB-NPB-22, FUB-PB-22, JWH-011, JWH-015, JWH-020, JWH-022, JWH-030, JWH-031, JWH-073, JWH-080, JWH-122 N-(4-pentenyl) analog, JWH-145, JWH-147, JWH-182, JWH-213, JWH-249, JWH-307, JWH-309, JWH-370, JWH-387, JWH-412, JWH-424, M-144, MDMB-4en-PINACA, MDMB-CHMCZCA, MDMB-CHMINACA, MDBFUBICA, MDBFUBINACA, MDMB-PICA, MDMB-PINACA, MEPIRAPIM, MMB-022 (MMB-4en-PICA), MN-25, N-Phenyl-SDB-006, NE-CHMIMO, SDB-005, THJ-2201, WIN 55,212–2, XLR-11, XLR-12 were purchased from Cayman Chemical (Ann Arbor, Michigan, USA) and kindly provided by Medical Center - University of Trento. Compounds were obtained from Sigma Aldrich (Steinheim, Germany) and kindly provided by the Forensic Toxicology Department of the Institute of Forensic Medicine, Medical Center – University of Freiburg. Compounds were diluted in methanol starting with a concentration of 0.01 mg/ml.

Internal standards (IS), nordiazepam-D5 and ketamine-D4, were obtained from Sigma Aldrich (Steinheim, Germany).

Water was obtained through a Millipore Milli-Q®. Formic acid, methanol, IPA, ACN were purchased by Merck (Germany, Darmstadt). All reagents and solvents were of LC/MS grade.

2.2 | Preparation of working solution and mobile phases

Individual methanolic solutions were used to prepare 7 working mixtures of standards.

- Panel 1, mix 1 at a concentration of 1,000 ng/ml;
- Panel 2, mix 2 at a concentration of 500 ng/ml;
- Panel 3, mixes 3–7, at a concentration of 500 ng/ml.

Internal standard mixture containing nordiazepam-D5 and ketamine-D4 was also prepared at a concentration of 0.01 mg/ml. Standards, stocks, and working solutions were stored at −20°C until their use.

Mobile phase A, 0.1% formic acid in water, and mobile phase B, 0.1% formic acid in acetonitrile were freshly prepared before the analysis. Seal wash was prepared as water/methanol 50:50 v/v. Strong wash was prepared as 0.2% formic acid in 2-propanol/acetonitrile/water/methanol (25:25:25:25 v/v/v/v). As a weak wash, mobile phase A was used.

2.3 | Sample preparation

Two samples of 500 μl of whole blood, one for SCs of Panel 3 and one for all other substances, for a total amount of 1 ml, were spiked with 10 μl of deuterated IS (final concentration: 200 ng/ml) and with a variable amount of the working solutions. After precipitation with 1.5 ml of cold acetonitrile, samples were vortexed and centrifuged (MPW Med. instruments, MPW 223e, Poland, Warsaw) at 3,000 rpm for 15 min. All the organic solvent was transferred into a 5 ml vial and evaporated under gentle nitrogen stream at 40°C. Reconstitution was performed with 150 μl of mobile phase B for SCs of Panels 1–3 and with mobile phase A/B: (80:20, v/v) for all other substances. Injection volume was 10 μl.

2.4 | UPLC–MS/MS

LC-MS/MS analysis was performed with a Waters Acquity (Ultra High-Performance Liquid Chromatography) UHPLC® (Milford, MA), coupled to a quadrupole mass detector Waters Xevo TQD, equipped with an electrospray ion source (ESI) operating in positive mode. Chromatographic separation was achieved on an Acquity UPLC® HSS C18 column (1.8 μm, 2.1 × 150 mm from Waters, Italy, Milan).

Gradient elution was as follows: Mobile phase B starting concentration was 10%, linearly increased to 40% at 8.0 min, further increased to 95% at 13.0 min, kept constant for 1.5 min, decreased to the starting conditions in 0.5 min, and kept at 10% for 2 min for equilibration. Total run time was 17 min. Flow rate was set at 0.4 ml/min. The autosampler was cooled down to 10°C. The column temperature was set to 40°C.

The MS was operated with positive ionization in Multiple Reaction Monitoring (MRM) mode. Specific MRM transitions and collision energies were determined by literature search, on substances tuned with the same MS-device, and a series of experiments performed on individual standards at a concentration of 1,000 ng/ml. Two characteristic transitions were chosen for each analyte. Due to the high number of analytes, two different MS methods were developed, one for substances included in Panels 1 and 2 and one for substances included in Panel 3. A total of three injections were done: extracts containing substances from Panels 1–2, reconstituted in mobile phase B and mobile phase A/B (80:20, v/v), run with the same MS method (first and second injections), followed by a third injection for substances of Panel 3 with the dedicated MS method. Extracts (containing substances of Panels 1 and 2) reconstituted with mobile phases B and A/B: (80:20, v/v) were analyzed with one MS methods,
while extracts containing substances from Panel 3 only ran with the dedicated MS method. Each method was composed of multiple detection windows containing approximately 10 compounds each, with a time ± 0.5 min from the retention time of the respective substance.

Optimized MS parameters were as follows: capillary voltage 3.50 kV, desolvation gas temperature 400°C, source gas flow (nitrogen) desolvation rate 800 L/h, cone 20 L/h, gas in collision argon, dwell time 0.01 s.

2.5 | Method validation

The method was validated according to the guidelines of the German Society of Toxicological and Forensic Chemistry (GTFCh), evaluating for all analytes the following analytical parameters: selectivity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), recovery, and matrix effect.

Selectivity was assessed by analyzing six blank blood samples from different individuals, with six blank post-mortem blood samples and with two blood samples spiked with common illicit and therapeutic drugs, by checking for interfering peaks.

Linearity was assessed using a 6-point calibration curve for the substances of Panel 3 and a 7-point calibration curve for substances included in Panels 1 and 2, by spiking appropriate amounts of each mixture (or of intermediate dilutions of the mixtures) to blank blood, resulting in the following final concentrations: 0.25, 0.5, 1.25, 2.5, 5, and 10 ng/ml for Panel 3 and 0.25, 0.5, 1.25, 2.5, 5, 10, and 25 ng/ml for Panels 1 and 2.

Six calibration batches, all including a blank whole blood sample spiked with IS only (zero sample), were analyzed on six consecutive days. Calibration curves were constructed through linear regression by plotting the area ratio of each substance with its internal standard against the concentration of the analytes. GraphPad Prism 8.2.1 was used for this task.

For the assessment of accuracy and precision, quality control (QC) samples were analyzed in two replicates for each concentration per day (intra-day precision) and on six consecutive days (inter-day precision) by spiking pooled whole blood samples to obtain the following final concentrations:

- 1 ng/ml for Panel 3 mix: QC low;
- 4 ng/ml for Panel 3 mix: QC high;
- 2 ng/ml for Panels 1 and 2 mixes: QC low;
- 12.5 ng/ml for Panels 1 and 2 mixes: QC high.

For all analytes which fulfilled identification criteria (retention time and ion ratio) at the first point of the calibration curve, LOD and LOQ were determined with an additional five-point curve, at the final concentrations of 0.06, 0.1, 0.125, 0.15, and 0.2 ng/ml, through the software Valistat 2.0 software (Arvecon GmbH, Walldorf, Germany), in accordance with the guidelines of the GTFCh. When the points were judged too few by Valistat, or when the detection of the compound was not possible at the first point of the calibration curve, LOQ was defined by the lowest concentration detectable with a signal-to-noise ratio of at least 10, accuracy ± 20% and precision ± 10%. For these substances, the LOD was assumed as 1/3 of the LOQ.

Accuracy and precision were obtained by bias calculation and relative standard errors, through Valistat software. Recovery and matrix effect were evaluated for all analytes at 2 and 4 ng/ml, by comparing absolute peak areas or the ratio between them and the IS, and by analyzing three sets of samples in duplicates. For recovery, each analyte in the QC samples (A) was compared with blood samples processed as a blank and spiked after the extraction step at the same concentration level (B). In order to assess matrix effect, that is, potential ion suppression/enhancement due to the sample matrix, B samples were compared to pure standards in a mixtures of mobile phases A and B (80:20, v/v) for all substances except for SCs, which were tested in mobile phase B (C).

2.6 | Application to real forensic cases

The validated method was applied to 10 samples of blood collected during forensic autopsies of both drug users and non-drug users and to 15 samples of blood collected in the frame of driving under the influence of drugs (DUID). Samples were stored at −20°C until analysis.

3 | RESULTS

3.1 | Method optimization

A target LC-MS/MS method was developed for the selective identification in whole blood of 182 NPS including 132 SCs, 22 SOs, and 28 among SCAs stimulants and other drugs. In Tables 1 and 2, substances, together with the IUPAC name, retention time, detection window, quantifier and qualifier ions, cone voltage, and collision energies, are shown. The total preparation of samples is achieved in approximately 30 min by protein precipitation, followed by three LC-MS runs of 17 min each, for a total of 51 min for each sample. Mobile phases were chosen on the basis of previous studies performed on psychoactive drugs, and the addition of formic acid resulted in a slight enhancement of the signal for all the analytes. On the basis of preliminary analyses, reconstitution was performed with mobile phase B for SCs, while for all the other analytes, a mixture of mobile phase A and B (80/20, v/v) was chosen. Even if this was not identical to the starting LC conditions, no retention or carry over effect was seen.

The chromatographic conditions were optimized in order to achieve a separation of analytes with the same nominal mass and fragment ions, for example, cis- vs trans-methyl-nortorfanyl, JWH-007 vs 019, JWH-015 vs JWH-073, JWH-018 vs JWH-016, FUB-NPB-22 vs MDMB-FUBINACA, FUB-PB-22 vs MDMB-FUBICA, or butylone vs ethylene. Indeed, the chromatographic method allowed separating all isomers and analytes with the same mass by retention time, except
| N | Analyte                                                                 | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|---|-------------------------------------------------------------------------|----------|------------------------|---------------------|--------------------|--------|--------|
|   | Synthetic cannabinoids (SCs)                                            |          |                        |                     |                    |        |        |
| 1 | 5-Cl-AB-PINACA N-[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-1-(5-chloropentyl)indazole-3-carboxamide | 11.0     | 9.5–13.0               | 366                 | 249                | 25     | 24     |
| 2 | 5-Cl-THJ-018 1-(5-Chloropentyl)-1H-indazol-3-yl-[1-naphthyl] methanone     | 13.3     | 11.5–15                | 377                 | 249                | 25     | 16     |
| 3 | 5F-ADB methyl (2R)-2-[1-(5-fluoropentyl)indazole-3-carbonyl] amino]-3,3-dimethylbutanoate | 12.3     | 10.5–15                | 378                 | 233                | 20     | 20     |
| 4 | 5F-AKB-48 N-(adamantan-1-yl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide | 13.6     | 12–15.5                | 384                 | 135                | 107    | 24     |
| 5 | 5F-NNEI 2’-naphthyl isomer 1-(5-Fluoropentyl)-N-(naphthalen-2-yl)-1H-indole-3-carboxamide | 12.4     | 10.5–15                | 375                 | 232                | 22     | 20     |
| 6 | AB-CHMINACA N-[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide | 11.6     | 9.5–13.0               | 357                 | 145                | 20     | 46     |
| 7 | AB-FUBINACA N-[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-1-[(4-fluorophenyl)methyl]-1H-indazole-3-carboxamide | 10.4     | 7.5–12                 | 369                 | 253                | 20     | 20     |
| 8 | ADB-FUBINACA N-[(2S)-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-[(4-fluorophenyl)methyl]-1H-indazole-3-carboxamide | 10.9     | 9.5–13.0               | 383                 | 253                | 25     | 25     |
| 9 | AM-2201 [1-(5-fluoropentyl)indol-3-yl]-naphthalen-1-ylmethanone            | 12.5     | 10.5–15                | 360                 | 127                | 155    | 46     |
| 10| AM-2233 (2-Iodophenyl)-[1-[(1-methylpiperidin-2-yl)methyl] indol-3-yl]methanone | 7.9      | 6–8.8                  | 459                 | 98                 | 112    | 50     |
| 11| AM-694 [1-(5-fluoropentyl)indol-3-yl]-2-iodophenyl methanone               | 12.2     | 10.5–15                | 436                 | 231                | 203    | 36     |
| 12| APP-FUBINACA N-[(2S)-1-amino-1-oxo-3-phenylpropan-2-yl]-1-[(4-fluorophenyl)methyl]indazole-3-carboxamide | 10.8     | 9.5–13.0               | 417                 | 109                | 253    | 24     |
| 13| Cumyl-PEGACLONE 5-pentyl-2-(2-phenylpropan-2-yl)-2,5-dihydro-1H-pyrind}[4,3-b]indol-1-one | 13       | 11.5–15                | 373                 | 255                | 119    | 30     |
| 14| JWH-007 (2-methyl-1-pentylindol-3-yl)-naphthalen-1-ylmethanone              | 13.6     | 11.7–15                | 356                 | 127                | 155    | 40     |
| 15| JWH-016 (1-butyl-2-methylindol-3-yl)-naphthalen-1-ylmethanone              | 13.2     | 11.5–15                | 342                 | 127                | 155    | 44     |
| 16| JWH-018 (1-pentyl-1H-indol-3-yl)-1-naphthalenyl-methanone                  | 13.4     | 11.7–15                | 342                 | 127                | 155    | 44     |
| 17| JWH-019 (1-hexyl-1H-indol-3-yl)naphthalen-1-yl)methanone                   | 13.7     | 12–15.5                | 356                 | 127                | 228    | 38     |
| 18| JWH-081 (4-Methoxynaphthalen-1-yl)[1-pentyl-1H-indol-3-yl] methanone        | 13.5     | 11.7–15                | 372                 | 185                | 157    | 26     |
| N  | Analyte                                                                                      | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|----|---------------------------------------------------------------------------------------------|----------|------------------------|---------------------|-------------------|--------|--------|
| 19 | JWH-098 (4-methoxynaphthalen-1-yl)-(2-methyl-1-pentylindol-3-yl)methanone                     | 13.6     | 386                    | 185*                | 127               | 20     | 26     |
|    | JWH-122 (4-Methylnaphthalen-1-yl)(1-pentyl-1H-indol-3-yl)methanone                           | 13.7     | 11.7-15                | 185*                | 141               | 20     | 24     |
|    | JWH-200 [1-(2-morpholin-4-yl)ethylinol-3-yl]-naphthalen-1-ylmethanone                       | 8.2      | 6-8.8                  | 154*                | 155               | 20     | 46     |
|    | JWH-203 2-(2-chlorophenyl)-1-(2-methyl-1-pentyl-1H-indol-3-yl)ethanone                       | 13.2     | 11.5-15                | 154*                | 155               | 20     | 46     |
|    | JWH-210 (4-ethyl-1-naphthalenyl)(1-pentyl-1H-indol-3-yl)methanone                            | 14.0     | 12-15.5                | 183*                | 214               | 20     | 24     |
|    | JWH-250 2-(2-methoxyphenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone                               | 12.9     | 10.5-15                | 121*                | 214               | 20     | 50     |
|    | JWH-251 2-(3-methylphenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone                                | 13.2     | 11.5-15                | 105*                | 214               | 20     | 22     |
|    | JWH-302 2-(3-methoxyphenyl)-1-(1-pentylindol-3-yl)ethanone                                   | 12.7     | 10.5-15                | 214*                | 214               | 20     | 22     |
|    | MDMB-CHMICA methyl (2S)-2-[(1-(cyclohexylmethyl)-1H-indole-3-carbonyl)amino]-3,3-dimethylbutanoate | 13.0     | 11.5-15                | 240*                | 240               | 20     | 24     |
|    | MM2-2201 methyl (2S)-2-[(1-(5-fluoropentyl)-1H-indole-3-carbonyl)amino]-3-methylbutanoate   | 11.4     | 9.5-13.0               | 232*                | 232               | 34     | 12     |
|    | RCS-4 (4-methoxyphenyl)(1-pentyl-1H-indol-3-yl)methanone                                     | 12.7     | 10.5-15                | 135*                | 135               | 20     | 20     |
|    | RCS-8 1-[1-(2-cyclohexylethyl)-1H-indol-3-yl]-2-(2-methoxyphenyl)ethenone                   | 13.7     | 12-15.5                | 121*                | 121               | 20     | 24     |
|    | WIN 48.098 (4-methoxyphenyl)-(2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl)methanone        | 7.1      | 6-8.8                  | 135*                | 135               | 45     | 24     |
|    | Synthetic opioids (SOs)                                                                      |          |                        |                     |                   |        |        |
| 32 | (±)-cis-3-methyl norfentanyl N-[(3R,4S)-3-methylpiperidin-4-yl]-N-phenylpropanamide         | 4.2      | 3.0-5.5                | 247                | 69                | 25     | 29     |
| 33 | (±)-trans-3-methyl norfentanyl N-[(3R,4R)-3-methylpiperidin-4-yl]-N-phenylpropanamide      | 4.0      | 3.0-5.5                | 247                | 69                | 25     | 29     |
| 34 | β-Hydroxy fentanyl N-[1-(2-hydroxy-2-phenylethyl)piperidin-4-yl]-N-phenylpropanamide        | 5.8      | 4.5-7.2                | 353                | 204               | 35     | 38     |
| 35 | β-Hydroxythiofentanyl N-[1-(2-hydroxy-2-thiophen-2-yethyipiperidin-4-yl]-N-phenylpropanamide | 5.4      | 4.0-6.2                | 359                | 192               | 35     | 22     |
| 36 | β-Phenyl fentanyl N-(1-phenethylpiperidin-4-yl)-N,3-diphenylpropanamide                     | 9.4      | 7.5-12                 | 413                | 105               | 35     | 44     |
| 37 | 4-ANPP N-phenyl-1-(2-phenylethyl)piperidin-4-amine                                          | 6.4      | 4.5-7.2                | 281                | 105               | 42     | 30     |
| N  | Analyte                                                                 | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|----|-------------------------------------------------------------------------|----------|------------------------|---------------------|-------------------|--------|--------|
| 38 | Acetyl fentanyl N-Phenyl-N-[1-(2-phenylethyl)-4-piperidiny]-acetamide    | 5.5      | 4.0–6.2                | 323                 | 105'              | 25     | 36     |
| 39 | Acetyl norfentanyl N-phenyl-N-piperidin-4-ylacetamide                    | 2.3      | 1.0–3.0                | 219                 | 55                | 25     | 36     |
| 40 | Alfentany N-[1-[2-(4-ethyl)-5-oxotetrazol-1-y]ethyl]-4-(methoxymethyl)piperidin-4-yl-N-phenylpropanamide | 6.4      | 4.5–7.2                | 417                 | 197'              | 24     | 26     |
| 41 | Butyryl fentanyl N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]butanamide  | 7.6      | 6–8.8                  | 351                 | 105'              | 30     | 45     |
| 42 | Butyryl fentanyl carboxy metabolite 4-oxo-4-(N-[1-(2-phenylethyl)piperidin-4-yl]anilino) butanoic acid | 5.3      | 4.0–6.2                | 381                 | 105'              | 25     | 45     |
| 43 | Butyryl norfentanyl N-phenyl-N-4-piperidinyl-butanamide                  | 4.6      | 3.0–5.5                | 247                 | 55                | 25     | 36     |
| 44 | Carfentany methyl 1-(2-phenylethyl)-4-(N-propanoylanilino)piperidine-4-carboxylate | 7.4      | 6–8.8                  | 395                 | 113'              | 22     | 32     |
| 45 | Cyclopropylfentanyl N-phenyl-N-[1-[2-phenylethyl]piperidin-4-yl]cyclopropane-carboxamide | 7.1      | 6–8.8                  | 349                 | 105'              | 25     | 36     |
| 46 | Despropionyl para-fluorofentanyl N-(fluorophenyl)-1-phenethylpiperidin-4-amine | 6.7      | 4.5–7.2                | 299                 | 105'              | 25     | 38     |
| 47 | Fentanyl N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]propanamide          | 5.5      | 4.5–7.2                | 377                 | 105'              | 40     | 30     |
| 48 | Furanyl norfentanyl N-phenyl-N-piperidin-4-ylfur-2-carboxamide           | 3.6      | 2.0–4.6                | 271                 | 55'               | 16     | 38     |
| 49 | Methoxyacetyl norfentanyl 2-methoxy-N-phenyl-N-piperidin-1-ium-4-ylacetamide | 2.2      | 1.0–3.0                | 249                 | 55                | 15     | 38     |
| 50 | Norfentanyl N-phenyl-N-piperidin-4-ylpropanamide                          | 3.5      | 2.0–4.6                | 233                 | 55                | 25     | 34     |
| 51 | Phenylfentanyl N-phenyl-N-[1-(2-phenylethyl)-4-piperidyl]benzamide       | 7.9      | 6–8.8                  | 385                 | 105'              | 40     | 46     |
| 52 | Phenylacetyl fentanyl N-phenyl-N-[1-(2-phenylethyl)-4-piperidyl]benzamide | 8.8      | 7.5–12                 | 399                 | 105               | 46     | 46     |
| 53 | Valeryl fentanyl carboxy metabolite 5-oxo-5-(N-[1-(2-phenylethyl)piperidin-4-yl]anilino)pentanoic acid | 5.5      | 7.5–12                 | 395                 | 105               | 40     | 44     |
| 54 | αET 1-(1H-indol-3-yl)butan-2-amine                                        | 4.0      | 189                    | 58                  | 58                | 26     | 16     |
| 55 | 3,4-DMMC, 3,4-dimethylmethcathinone 1-(3,4-Dimethylphenyl)-2-(methylamino)propan-1-one | 4.4      | 3.0–5.5                | 192                 | 159               | 20     | 15     |
| 56 | 4-FMC, 4-Fluoromethcathinone 1-(4-fluorophenyl)-2-(methylamino)propan-1-one | 2.3      | 1.0–3.0                | 182                 | 149               | 20     | 15     |
| 57 | 4-MEC, 4-Methylethcathinone 2-(Ethylamino)-1-(4-methylphenyl)propan-1-one | 3.6      | 2.0–4.6                | 192                 | 145'              | 13     | 17     |
| N  | Analyte                                                                 | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|----|-------------------------------------------------------------------------|----------|------------------------|---------------------|--------------------|--------|--------|
| 58 | 5-APB/6-APB 1-(Benzofuran-5-yl)-propan-2-amine 1-(Benzofuran-6-yl)propan-2-amine | 3.6      | 2.0–4.6                | 176                 | 77                 | 22     | 40     |
| 59 | 5-EAPB 1-(Benzofuran-5-yl)-N-ethylpropan-2-amine                          | 4.4      | 3.0–5.5                | 204                 | 91                 | 24     | 30     |
| 60 | 5-MAPB/6-MAPB 1-(Benzofuran-5-yl)-N-methylpropan-2-amine 1-(Benzofuran-6-yl)-N-methylpropan-2-amine | 3.1      | 2.0–4.6                | 190                 | 131*               | 20     | 18     |
| 61 | 5-MeO-AMT 1-(5-methoxy-1H-indol-3-yl)propan-2-amine                        | 3.1      | 1.7–4.0                | 205                 | 147                | 22     | 20     |
| 62 | 5-MeO-DALT N-[2-[5-methoxy-1H-indol-3-yl]ethyl]-N-prop-2-enylprop-2-en-1-amine | 5.1      | 4.0–6.2                | 271                 | 110*               | 24     | 18     |
| 63 | 5-MeO-DMT 2-[5-methoxy-1H-indol-3-yl]-N,N-dimethylethylamide              | 3.0      | 1.7–4.0                | 219                 | 58*                | 20     | 46     |
| 64 | 5-MeO-DPT N-[2-[5-methoxy-1H-indol-3-yl]ethyl]-N-propylpropan-1-amine     | 5.8      | 4.5–7.2                | 275                 | 114*               | 14     | 16     |
| 65 | 5-MeO-MIPT N-[2-[5-methoxy-1H-indol-3-yl]ethyl]-N-methylpropan-2-amine    | 4.0      | 2.0–4.6                | 247                 | 86*                | 10     | 14     |
| 66 | Buphedrone 2-(methylamino)-1-phenylbutan-1-one                             | 3.2      | 1.5–3.6                | 178                 | 91                 | 20     | 26     |
| 67 | Butylone 1-(1,3-benzodioxol-5-yl)-2-(methylamino)butan-1-one              | 3.0      | 1.7–4.0                | 222                 | 174                | 27     | 19     |
| 68 | Ethcathinone 2-(ethylamino)-1-phenylpropan-1-one                          | 2.2      | 1.0–3.0                | 178                 | 72                 | 30     | 22     |
| 69 | Ethylone 1-(1,3-benzodioxol-5-yl)-2-(ethylamino)propan-1-one              | 2.6      | 1.5–3.6                | 222                 | 174*               | 27     | 19     |
| 70 | Ethylphenidate ethyl 2-phenyl-2-piperidin-2-ylacetate                     | 5.3      | 4.0–6.2                | 248                 | 56                 | 50     | 24     |
| 71 | Ketamine                                                                | 3.20     | 1.7–4.0                | 238.2               | 125.1*             | 30     | 26     |
| 72 | MDPV 1-(1,3-benzodioxol-5-yl)-2-(pyrrolidin-1-yl)pentan-1-one             | 4.7      | 3.0–5.5                | 276                 | 126*               | 30     | 25     |
| 73 | Mepedrone 2-(Methylamino)-1-[4-methylphenyl]propan-1-one                  | 1.7–4.0  | 178                   | 145                 | 160*               | 20     | 18     |
| 74 | Methactineone 2-(methylamino)-1-phenylpropan-1-one                        | 2.0      | 1.0–3.0                | 164                 | 131*               | 13     | 6      |
| 75 | Methedrone 1-(4-methoxyphenyl)-2-(methylamino)propan-1-one                | 2.7      | 1.5–3.6                | 194                 | 161                | 20     | 13     |
| 76 | Methylene 1-(1,3-benzodioxol-5-yl)-2-(methylamino)propan-1-one            | 2.2      | 1.0–3.0                | 208                 | 132                | 27     | 27     |
| 77 | N,N-Dimethylcathinone 2-(Dimethylamino)-1-phenylpropan-1-one              | 2.2      | 1.0–3.0                | 178                 | 72*                | 20     | 20     |
| 78 | N,N-DMT, N,N-Dimethyltryptamine 2-(1H-indol-3-yl)-N,N-dimethylethylamine | 2.9      | 1.5–3.6                | 189                 | 58*                | 20     | 34     |

(Continues)
for 5- and 6-APB, 5- and 6-MAPB, and the couples 5F-MDMB-PINACA/5F-ADB and 5F-EMB-PINACA/5F-AEB.

MRM transitions included in the MS/MS method were monitored in several detection windows (at least ± 0.5 min from the expected retention time of the analytes included in the respective window), which allowed to achieve a sufficient number of points to define the chromatographic peak. Analyte identification was performed by targeted MS/MS on the basis of mass of the precursor ion, two diagnostic fragments, retention time (± 0.2 min), and area ratio of quantifier and qualifier ions (±20%), fulfilling the EU Commission Decision 2002/657/EC confirmation criteria.

### 3.2 Method validation

Successful validation was achieved for the vast majority of the compounds. Validation parameters and particularly linearity ($R^2$), accuracy, precision, LOD, and LOQ are shown in Table 3. No interfering peaks due to endogenous substances were detected, except for a minimal interference in the case of 5F-EMB-PINACA, with an area 0.90% with respect to the maximum concentrations of the calibration curve.

The method produced linear calibration functions for all the analytes of interest in the tested range, with $R^2$ always better than 0.99 except for 5F-MDMB-P7AICA, AB-CHMINACA, AM-1235, MDMB-PIACA, 4-FMC, ethcathinone, methcathinone, and N,N-dimethylcathinone ($R^2$ was 0.94–0.98 with no need for a weighing factor; see the supporting information). All the analytes of interest, except for 13 SCs (5F-AKB-48, AB-001, AB-CHMINACA, ADB-CHMICA, ADB-PINACA, AKB-48, AM-1235, EG-018, JWH-016, JWH-203, JWH-210, MDMB-PICA, and MDMB-PINACA, and three stimulants (ethcathinone, methcathinone, and N,N-dimethylcathinone), showed accuracies and precisions within the guidelines of the GTFCh.18 Particularly, 5F-AKB-48 and AM-1235 did not meet the requirement for a full validation at the lower QC but showed acceptable accuracy and precision at the higher concentration. AB-001, conversely, shows better parameters at 1 ng/ml.

LOQs were in the range 0.04–0.97 ng/ml for all substances, and mostly <0.50 ng/ml, except for 5-Cl-AB-PINACA (1.25 ng/ml), 5F-AKB-48 (1.25 ng/ml), AM-1235 (1.25 ng/ml), 4-FMC (2.5 ng/ml), and mephedrone (1.25 ng/ml). Generally, higher sensitivities were achieved for SCs.

With the chosen extraction procedure, recovery and matrix effect of analytes under investigation were always higher than 75% and lower than 125% for all SOs. For SCAs, stimulants and other drugs, recovery, and matrix effect were also acceptable (>70% and <130%), with respect to the limit imposed by the GTFCh guidelines,18 except the following six compounds: 4-FMC, 5-MeO-DALT, butylone, ethcathinone, MDPV, and methcathinone. Within SCs, the number of compounds not meeting the criteria for recovery and matrix effect was higher and included the following: 5F-ADB, 5F-AKB-48, 5F-AMB-PINACA, 5F-JWH-412, 5F-PCN, A-834,735, AB-005, AB-005 azepane, AB-FUBINACA, ADB-FUBICA, AKB-48, AM-1220 azepane, AM-1235, AM-1248 azepane, AM-2201 indazole carboxamide, AM-2232, AM-2233-azepane, AM-630, AMB-CHMICA, AMB-CHMINACA, AMB-FUBINACA, AMB-PINACA, Cumyl-BICA, Cumyl-PICA, EG-018, FUB-JWH-018, FUB-NPB-22, FUB-PB-22, JWH-015, JWH-020, JWH-030, JWH-122, JWH-145, JWH-147, JWH-213, JWH-250, JWH-302, JWH-370, JWH-412, MDMB-4en-PINACA, MDMB-CHMCZCA, MDMB-CHMINACA, MDMB-FUBINACA, MDMB-PICA, MN-25, N-phenyl-SDB-006, NE-CHMIMO, THJ-2201 XLR-11, XLR-11 isomer. Matrix effect and recovery for all analytes are shown in the supporting information.

### 3.3 Application to real forensic cases

All the samples collected during forensic autopsies, with a post-mortem interval ranging from 2 to 10 days after death, tested negative for NPS, while several fatal drug intoxications were detected by applying previously validated methods for classical drugs of abuse. Among DUID samples, three tested positive for fentanyl and

| Table 1 (Continued) | N | Analyte | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|---------------------|---|---------|---------|------------------------|-------------------|------------------|-------|-------|
| 79 Nordiazepam | 9.0 | 7.5–12 | 271.1 | 140* | 165.1 | 50 | 35 |
| 80 Pentylon | 4.0 | 3.0–5.5 | 236 | 188* | 218 | 27 | 12 |
| 81 Ritalinic acid | 3.3 | 2.0–4.6 | 220 | 56 | 84* | 20 | 46 |
| Nordiazepam-D5 | 8.9 | 7.5–12 | 276 | 165* | 213 | 50 | 28 |
| Ketamine-D4 | 3.19 | 1.7–4.0 | 242.2 | 129.1 | 242 | 35 | 30 |
| N   | Analyte                                                                 | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|-----|-------------------------------------------------------------------------|----------|------------------------|---------------------|--------------------|--------|--------|
| 82  | 4-HTMPPO 4-hydroxy-3,3,4-trimethyl-1-(1-pentyl-1H-indol-3-yl) pentan-1-one | 10.7     | 10–11.7                | 330                 | 144, 214*          | 20     | 45     |
| 83  | 4F-MDMB-BINACA methyl (S)-2-(1-[4-fluorobutyl]-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate | 11.9     | 11.5–12.8              | 364                 | 219*, 304          | 36     | 24     |
| 84  | 5F-AB-001 (adamantan-1-yl)[1-(5-fluoropentyl)-1H-indol-3-yl] methanone   | 13.5     | 12.6–14                | 368                 | 79, 135*           | 36     | 40     |
| 85  | 5F-AB-PICA N-[(2S)-1-Amino-3-methyl-1-oxobutan-2-yl]-1-(5-fluoropentyl)-1H-indole-3-carboxamide | 9.8      | 9.1–11                 | 348                 | 232*, 331          | 20     | 30     |
| 86  | 5F-AB-PINACA N-[(2S)-1-Amino-3,3-dimethyl-1-oxobutan-2-yl]-1-(5-fluoropentyl)-1H-indole-3-carboxamide | 10.0     | 9.1–11                 | 349                 | 145, 233*          | 36     | 40     |
| 87  | 5F-ADB-PICA N-[(2S)-1-Amino-3,3-dimethyl-1-oxobutan-2-yl]-1-(5-fluoropentyl)-1H-indole-3-carboxamide | 10.3     | 9.1–11                 | 362                 | 144, 223*          | 36     | 40     |
| 88  | 5F-ADB-PINACA N-[(2S)-1-Amino-3,3-dimethyl-1-oxobutan-2-yl]-1-(5-fluoropentyl)-1H-indole-3-carboxamide | 10.6     | 10–11.7                | 363                 | 145, 233           | 35     | 29     |
| 89  | 5F-AMB-PINACA Methyl (2S)-2-[(1-(5-fluoropentyl)-1H-indazole-3-carbonyl)amino]-3-methylbutanoate | 12.0     | 10.8–12.5              | 364                 | 233, 304*          | 36     | 20     |
| 90  | 5F-APP-PICA N-[1-amino-1-oxo-3-phenylpropan-2-yl]-1-(5-fluoropentyl)indole-3-carboxamide | 10.4     | 7.5–12                 | 396                 | 232*, 144          | 26     | 26     |
| 91  | 5F-APP-PINACA N-[1-amino-1-oxo-3-phenylpropan-2-yl]-1-(5-fluoropentyl)indole-3-carboxamide | 10.6     | 7.5–12                 | 397                 | 233*, 145          | 22     | 34     |
| 92  | 5F-Cumyl-PINACA 1-(5-fluoropentyl)-N-(2-phenylpropan-2-yl)indazole-3-carboxamide | 12.5     | 10.5–15                | 368                 | 233*, 250          | 20     | 18     |
| 93  | 5F-EMB-PINACA/5F-MDMB-PINACA (isomers) Ethyl 2-[(1-(5-fluoropentyl)indazole-3-carbonyl) amino]-3-methylbutanoate Methyl (2S)-2-[(1-(5-fluoropentyl)-1H-indazole-3-carbonyl)amino]-3,3-dimethylbutanoate | 12.3     | 11.5–12.8              | 378                 | 233*, 145          | 20     | 18     |
| 94  | 5F-JWH-412 (4-Fluoro-1-naphthalenyl)[1-(5-fluoropentyl)-1H-indol-3-yl]methanone | 12.7     | 11.9–13.4              | 378                 | 145, 173*          | 45     | 40     |
| 95  | 5F-MDMB-P7AICA methyl (S)-2-(1-(5-fluoropentyl)-1H-pyrrolo[2,3-b]pyridine-3-carboxamido)-3,3-dimethylbutanoate | 11.1     | 10–11.7                | 378                 | 145, 233           | 45     | 40     |
| 96  | 5F-MDMB-PICA methyl (2S)-2-[(1-(5-fluoropentyl)-1H-indole-3-carbonyl)amino]-3,3-dimethylbutanoate | 11.8     | 10.8–12.5              | 377                 | 144, 232*          | 40     | 40     |
| 97  | 5F-PCN 1-(5-Fluoropentyl)-N-naphthalen-1-yl)-1H-pyrrolo[3,2-c]pyridine-3-carboxamide | 12.8     | 12.2–13.5              | 376                 | 145, 233*          | 40     | 40     |
| 98  | 5F-PY-PICA (1-(5-fluoropentyl)-1H-indol-3-yl)(pyrrolidin-1-yl) methanone | 10.6     | 10–11.7                | 303                 | 144, 232*          | 20     | 20     |

(Continues)
| N  | Analyte                                                                 | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|----|------------------------------------------------------------------------|----------|------------------------|---------------------|--------------------|--------|--------|
| 99 | A-796,260 [1-(2-morpholin-4-yl)ethyl]-1H-indol-3-yl-(2,2,3,3-tetramethylcyclopropyl) methanone | 9.0      | 6.5–10                 | 355                 | 114                | 36     | 32     |
| 100| A-834,735 [1-(oxan-4-ylmethyl)indol-3-yl]-(2,2,3,3-tetramethylcyclopropyl)methanone | 12.6     | 11.9–13.4              | 340                 | 125                | 45     | 35     |
| 101| AB-001 (adamantan-1-yl)(1-pentyl-1H-indol-3-yl)methanone                | 14.3     | 13.5–15                | 350                 | 79                 | 36     | 45     |
| 102| AB-005 [1-(1-Methylpiperidin-2-yl)methyl]-1H-indol-3-yl]-(2,2,3,3-tetramethylcyclopropyl)methanone | 9.5      | 6.5–10                 | 353                 | 112*               | 36     | 45     |
| 103| AB-005 azepane (1-[1-methylazepan-3-yl]-1H-indol-3-yl]-(2,2,3,3-tetramethylcyclopropyl)methanone | 9.6      | 6.5–10                 | 353                 | 112*               | 36     | 45     |
| 104| AB-BICA N-[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-1-benzylindole-3-carboxamide | 10.0     | 9.1–11                 | 350                 | 234*               | 20     | 20     |
| 105| AB-CHMICA N-[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-1-(cyclohexylmethyl)indazole-3-carboxamide | 11.2     | 10–11.7                | 356                 | 240*               | 35     | 20     |
| 106| AB-FUB7AICA N-[(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-pyrrolo[2,3-b]pyridine-3-carboxamide | 9.3      | 6.5–10                 | 369                 | 109*               | 36     | 40     |
| 107| AB-FUBICA N-[(25)-1-aminocarbonyl-2-methyl-1H-indole-3-carboxamide | 10.2     | 9.1–11                 | 368                 | 109                | 36     | 25     |
| 108| AB-FUBINACA 2/3-fluorobenzyl isomers N-[(1S)-1-(aminocarbonyl)-2-methyl-1H-indole-3-carboxamide | 10.5     | 9.1–11                 | 369                 | 109*               | 36     | 40     |
| 109| AB-PICA N-[(25)-1-amino-3-methyl-1-oxobutan-2-yl]-1-pentyl-1H-indole-3-carboxamide | 10.7     | 10–11.7                | 330                 | 144                | 35     | 40     |
| 110| AB-PINACA N-[(25)-1-amino-3-methyl-1-oxobutan-2-yl]-1-pentyl-1H-indole-3-carboxamide | 11.0     | 10–11.7                | 331                 | 215*               | 20     | 24     |
| 111| ADB-BICA N-[(25)-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-benzyl-1H-indole-3-carboxamide | 10.5     | 9.1–11                 | 364                 | 234*               | 20     | 24     |
| 112| ADB-BINACA N-[(25)-1-Amino-3,3-dimethyl-1-oxobutan-2-yl]-1-benzyl-1H-indole-3-carboxamide | 10.8     | 10–11.7                | 365                 | 91*                | 35     | 40     |
| 113| ADB-CHMICA N-[(aminocarbonyl)-2,2-dimethylpropyl]-1-(cyclohexylmethyl)-1H-indole-3-carboxamide | 11.9     | 10.8–12.5              | 370                 | 240                | 36     | 20     |
| 114| ADB-FUBICA N-[(25)-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-(4-fluorobenzyl)-1H-indole-3-carboxamide | 10.6     | 10–11.7                | 382                 | 252*               | 20     | 30     |
| 115| ADB-PICA/ADBICA N-[(25)-1-Amino-3,3-dimethyl-1-oxobutan-2-yl]-1-pentyl-1H-indole-3-carboxamide | 11.2     | 10.8–12.5              | 344                 | 144                | 20     | 40     |
| 116| ADB-PINACA N-[(1-amino-3,3-dimethyl-1-oxo-2-butanyl)-1-pentyl-1H-indole-3-carboxamide] | 11.6     | 10.8–12.5              | 345                 | 145                | 20     | 40     |
| N  | Analyte                                                                 | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|----|--------------------------------------------------------------------------|----------|------------------------|---------------------|--------------------|--------|--------|
| 117| AKB-48 N-(adamantan-1-yl)-1-pentyl-1H-indazole-3-carboxamide             | 14.6     | 13.5–15                | 366                 | 93*                | 36     | 40     |
| 118| AM-1220 azepane (1-[(1-methylazepan-3-yl)methyl]-1H-indol-3-yl) (naphthalen-1-yl)methanone | 8.8      | 6.5–10                 | 383                 | 98*                | 45     | 50     |
| 119| AM-1235 1-[(5-fluoropentyl)-6-nitro-1H-indol-3-yl)-(naphthalen-1-yl)methanone | 12.7     | 11.9–13.4              | 405                 | 155                | 45     | 35     |
| 120| AM-1241 (2-Iodo-5-nitrophenyl)[1-[(1-methylpiperidin-2-yl)methyl]-1H-indol-3-yl]methanone | 8.5      | 6.5–10                 | 504                 | 98*                | 45     | 35     |
| 121| AM-1248 [1-(1-methylpiperidin-2-yl)-1H-indol-3-yl][adamant-1-yl)methanone | 10.0     | 9.1–11                 | 391                 | 112                | 45     | 40     |
| 122| AM-1248 azepane adamantan-1-yl[1-[1-methylazepan-3-yl]-1H-indol-3-yl]methanone | 10.1     | 9.1–11                 | 391                 | 112*               | 45     | 40     |
| 123| AM-2201 indazole carboxamide N-(naphthalen-1-yl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide | 12.8     | 12.2–13.5              | 376                 | 213                | 45     | 24     |
| 124| AM-2232 [1-(4-cyanobutyl)-1H-indol-3-yl][naphthalen-1-yl)methanone       | 11.6     | 10.8–12.5              | 353                 | 127*               | 45     | 38     |
| 125| AM-2233 azepane (2-iodophenyl)[1-[(1-methylazepan-3-yl)lindol-3-yl]methanone | 8.0      | 6.5–10                 | 459                 | 112*               | 45     | 50     |
| 126| AM-630 [6-iodo-2-methyl-1-(2-morpholin-4-yethyl)lindol-3-yl][4-methoxyphenyl)methanone | 9.3      | 6.5–10                 | 505                 | 114                | 45     | 40     |
| 127| AM-679 (2-iodophenyl)(1-pentyl-1H-indol-3-yl)methanone                   | 13.0     | 12.2–13.5              | 418                 | 203                | 45     | 35     |
| 128| AMB-CHMICA methyl 2-[(1-cyclohexylmethyl)-1H-indol-3-yl]formamido)-3-methylbutanoate | 12.6     | 11.9–13.4              | 371                 | 144                | 20     | 24     |
| 129| AMB-CHMINACA methyl (1-cyclohexylmethyl)-1H-indazole-3-carbonyl-valinate | 13.3     | 12.6–14                | 372                 | 241*               | 36     | 20     |
| 130| AMB-FUBICA methyl (2S)-2-[(1-[(4-fluorophenyl)methyl]-1H-indole-3-carbonyl)amino)-3-methylbutanoate | 11.6     | 10.8–12.5              | 383                 | 109*               | 36     | 35     |
| 131| AMB-FUBINACA methyl (2S)-2-[(1-[(4-fluorophenyl)methyl]-1H-indazole-3-carbonyl)amino)-3-methylbutanoate | 12.1     | 11.5–12.8              | 384                 | 252                | 45     | 24     |
| 132| AMB-PICA methyl (2S)-2-[(1-pentyl-1H-indole-3-carbonyl)amino)-3-methylbutanoate | 12.2     | 11.5–12.8              | 345                 | 253*               | 44     | 24     |
| 133| AMB-PINACA methyl (2S)-2-[(1-pentyl-1H-indazole-3-carbonyl)amino)-3-methylbutanoate | 12.8     | 12.2–13.5              | 346                 | 234                | 30     | 38     |
| 134| BB-22 1-pentyl-1H-indole-3-carboxylic acid 8-quinolinyl ester             | 13.2     | 12.6–14                | 385                 | 144                | 36     | 40     |
| 135| Cumyl-4CN-BINACA 1-(4-cyanobutyl)-N-(2-phenylpropan-2-yl)-1H-indazole-3-carboxamide | 11.5     | 10.8–12.5              | 361                 | 226*               | 36     | 22     |

(Continues)
| N  | Analyte                                                                 | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|----|-------------------------------------------------------------------------|----------|------------------------|---------------------|--------------------|--------|--------|
| 136| Cumyl -BICA 1-Butyl-N-(2-phenylpropan-2-yl)-1H-indole-3-carboxamide     | 12.3     | 11.5–12.8              | 335                 | 174                | 30     | 40     |
|    |                                                                         |          |                        |                     | 217*               |        |        |
| 137| Cumyl -PICA 1-Pentyl-N(2-phenylpropan-2-yl)-1H-indole-3-carboxamide     | 12.7     | 11.9–13.4              | 349                 | 188                | 36     | 36     |
|    |                                                                         |          |                        |                     | 231*               |        |        |
| 138| Cumyl -THPINACA N(1-methyl-1-phenylethyl)-1-[(tetrahydro-2H-pyran-4-yl)methyl]-1H-indazole-3-carboxamide | 11.8     | 10.8–12.5              | 378                 | 243*               | 36     | 22     |
|    |                                                                         |          |                        |                     | 260                |        |        |
| 139| EG-018 (naphthalen-1-yl)(9-pentyl-9H-carbazol-3-yl)methane               | 14.4     | 13.5–15                | 392                 | 127*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        | 38     |
| 140| EG-2201 [9-(5-fluoropentyl)-9H-carbazol-3-yl](naphthalen-1-yl)methanone | 13.5     | 13–14.5                | 410                 | 127*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        | 38     |
| 141| FUB-JWH-018 (1-(4-fluorobenzyl)-1H-indol-3-yl)(naphthalen-1-yl)methanone | 12.7     | 11.9–13.4              | 380                 | 109*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        | 35     |
| 142| FUB-NPB-22 quinolin-8-yl 1-(4-fluorobenzyl)-1H-indazole-3-carboxylate    | 12.00    | 11.5–12.8              | 398                 | 109*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 253                |        | 20     |
| 143| FUB-PB-22 naphthalen-1-yl 1-[(4-fluorophenyl)methyl]-1H-indole-3-carboxylate | 12.1     | 11.5–12.8              | 397                 | 109*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 252                |        | 24     |
| 144| JWH-011 (1-heptan-2-yl-2-methylindol-3-yl)naphthalen-1-ylmethanone       | 14.0     | 13.5–15                | 384                 | 127*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        | 40     |
| 145| JWH-015 2-methyl-1-propyl-1H-indol-3-yl(naphthalen-1-yl)methanone         | 12.8     | 12.2–13.5              | 328                 | 127*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        | 22     |
| 146| JWH-020 (1-heptyl-1H-indol-3-yl)(naphthalen-1-yl)methanone               | 14.0     | 13.5–15                | 370                 | 127*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        | 35     |
| 147| JWH-022 naphthalen-1-yl[1-(pent-4-en-1-yl)-1H-indol-3-yl]methanone        | 13.0     | 12.2–13.5              | 340                 | 127*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        | 35     |
| 148| JWH-030 (1-hexylpyrrol-3-yl)naphthalen-1-ylmethanone                     | 12.7     | 11.9–13.4              | 292                 | 127*               | 30     | 44     |
|    |                                                                         |          |                        |                     | 155                |        | 20     |
| 149| JWH-031 (1-hexyl-1H-pyrrol-3-yl)(naphthalen-1-yl)methanone               | 13.1     | 12.6–14                | 306                 | 127*               | 45     | 44     |
|    |                                                                         |          |                        |                     | 155                |        | 20     |
| 150| JWH-073 (1-butyl-1H-indol-3-yl)(naphthalen-1-yl)methanone                 | 13.0     | 12.2–13.5              | 328                 | 127*               | 45     | 40     |
|    |                                                                         |          |                        |                     | 155                |        | 24     |
| 151| JWH-080 (1-butyl-1H-indol-3-yl)[4-methoxy-1-naphthalenyl]-methanone       | 13.1     | 12.6–14                | 358                 | 185*               | 45     | 30     |
|    |                                                                         |          |                        |                     | 200                |        | 28     |
| 152| JWH-122 N[4-(pentenyl)(4-methyl)naphthalen-1-yl][1-(pent-4-en-1-yl)-1H-indol-3-yl]methanone | 13.3     | 12.6–14                | 354                 | 141                | 45     | 40     |
|    |                                                                         |          |                        |                     | 169*               |        | 30     |
| 153| JWH-145 naphthalen-1-yl[1-pentyl-5-phenyl-1H-pyrrol-3-yl]methanone        | 13.8     | 13–14.5                | 368                 | 127                | 45     | 28     |
|    |                                                                         |          |                        |                     | 155*               |        | 30     |
| 154| JWH-147 (1-hexyl-5-phenyl-1H-pyrrol-3-yl)naphthalen-1-ylmethanone         | 14.0     | 13.5–15                | 382                 | 127*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        | 40     |
| N  | Analyte                                                                 | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|----|-------------------------------------------------------------------------|----------|------------------------|---------------------|--------------------|--------|--------|
| 155| JWH-182 (1-pentyl-1H-indol-3-yl)(4-propynaphthalen-1-yl)methanone        | 14.2     | 13.5–15                | 384                 | 141                | 45     | 45     |
|    |                                                                         |          |                        |                     | 197*               |        |        |
| 156| JWH-213 (4-ethylnaphthalen-1-yl)(2-methyl-1-pentyl-1H-indol-3-yl)methanone | 14.1     | 13.5–15                | 384                 | 155                | 45     | 40     |
|    |                                                                         |          |                        |                     | 183*               |        |        |
| 157| JWH-249 2-(2-bromophenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone             | 13.3     | 12.6–14                | 384                 | 144                | 45     | 35     |
|    |                                                                         |          |                        |                     | 169*               |        |        |
| 158| JWH-307 [5-(2-fluorophenyl)-1-pentyl-1H-pyrrol-3-yl](napthalene-1-yl)methanone | 13.6     | 13–14.5                | 386                 | 127*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        |        |
| 159| JWH-309 1-naphthalenyl[5-(1-naphthalenyl]-1-pentyl-1H-pyrrol-3-yl)methanone | 14.2     | 13.5–15                | 418                 | 127*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        |        |
| 160| JWH-370 5-(2-methylphenyl)-1-pentyl-1H-pyrrol-3-yl(naphthalen-1-yl)methanone | 14.0     | 13–14.5                | 382                 | 127*               | 45     | 45     |
|    |                                                                         |          |                        |                     | 155                |        |        |
| 161| JWH-387 4-bromonaphthalen-1-yl(1-pentyl-1H-indol-3-yl)methanone           | 14.0     | 13.5–15                | 420                 | 205                | 45     | 30     |
|    |                                                                         |          |                        |                     | 233*               |        |        |
| 162| JWH-412 (4-fluoronaphthalen-1-yl)(1-pentyl-1H-indol-3-yl)methanone        | 13.6     | 13–14.5                | 360                 | 145                | 45     | 40     |
|    |                                                                         |          |                        |                     | 173*               |        |        |
| 163| JWH-424 (8-bromonaphthalen-1-yl)(1-pentyl-1H-indol-3-yl)methanone         | 13.2     | 12.6–14                | 420                 | 205                | 45     | 35     |
|    |                                                                         |          |                        |                     | 233*               |        |        |
| 164| M-144 (1-(5-fluoropentyl)-2-methyl-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone | 13.6     | 13–14.5                | 344                 | 158                | 36     | 34     |
|    |                                                                         |          |                        |                     | 246*               |        |        |
| 165| MDMB-4en-PINACA methyl (5)-3,3-dimethyl-2-(1-(pent-4-en-1-yl]-1H-indazole-3-carboxamido)butanoate | 12.8     | 12.2–13.5              | 358                 | 145                | 36     | 40     |
|    |                                                                         |          |                        |                     | 213*               |        |        |
| 166| MDMB-CHMCZCA methyl (2S)-2-[[9-(cyclohexylmethyl)-9H-carbazole-3-carboxyl]amino]-3,3-dimethylbutanoate | 13.7     | 13–14.5                | 435                 | 290*               | 45     | 25     |
|    |                                                                         |          |                        |                     | 194                |        |        |
| 167| MDMB-CHMINACA methyl (2S)-2-[[1-(cyclohexylmethyl)-1H-indazole-3-carboxyl]amino]-3,3-dimethylbutanoate | 13.71    | 13–14.5                | 386                 | 241*               | 36     | 24     |
|    |                                                                         |          |                        |                     | 326                |        |        |
| 168| MDMB-FUBICA methyl (2S)-2-[[1-(4-fluorophenyl)methyl]-1H-indole-3-carboxyl]amino)-3,3-dimethylbutanoate | 12.0     | 11.5–12.8              | 397                 | 109*               | 45     | 40     |
|    |                                                                         |          |                        |                     | 252                |        |        |
| 169| MDMB-FUBINACA methyl (2S)-2-[[1-(4-fluorophenyl)methyl]-1H-indole-3-carboxyl]amino)-3,3-dimethylbutanoate | 12.5     | 11.9–13.4              | 398                 | 253*               | 45     | 24     |
|    |                                                                         |          |                        |                     | 338                |        |        |
| 170| MDMB-PICA methyl (2S)-3,3-dimethyl-2-[(1-pentyl-1H-indole-3-carboxyl]amino)butanoate | 13.1     | 11.9–13.4              | 359                 | 144*               | 36     | 40     |
|    |                                                                         |          |                        |                     | 233                |        |        |
| 171| MDMB-PINACA methyl (2S)-3,3-dimethyl-2-[[1-(pentyl-1H-indazole-3-carboxyl]amino]butanoate | 13.2     | 12.6–14                | 360                 | 145                | 36     | 40     |
|    |                                                                         |          |                        |                     | 215*               |        |        |
| 172| MEPIRAPIM (4-methylpiperazin-1-yl)(1-pentyl-1H-indol-3-yl)methanone       | 7.5      | 6.5–10                 | 314                 | 144                | 36     | 40     |
|    |                                                                         |          |                        |                     | 214*               |        |        |

(Continues)
ketamine, administered in the emergency room. Concentrations of fentanyl and ketamine were in the range 0.65–1.67 and 570–1,000 ng/ml, respectively.

## DISCUSSION

The major challenge in the analysis of NPS resides in the diversity of structures and physicochemical properties among different NPS classes and within a single NPS class.11,16 Several methods are already available in the literature to detect and/or quantify NPS in the main biological matrices20–26 and particularly in whole blood,27–31 though methods including a high number of compounds pertaining to different classes are still scarce.22 In the present work, a LC-MS/MS screening method for the rapid determination of 182 NPS in blood, including a wide-panel of SCs and very recently emerged compounds, for example, 4F-MDMB-BINACA,32,33 as well as multiple drug classes has been developed. Protein precipitation was chosen as an easy procedure for sample preparation. As reported in the literature, SCs tend to be better extracted by liquid–liquid extraction,20,25,34,35 while both liquid–liquid and solid-phase extraction have been shown applicable for the extraction of fentanyl and its analogues,21,36 as well as for amphetamines and tryptamines.26 However, previous studies have also shown that protein precipitation could be used for SCs, SCAs, ketamine and stimulants with good efficiency.11,22,37 This type of sample preparation strongly simplifies the laboratory routine in terms of easiness and time saving, only requiring a few minutes. Moreover, it is less expensive than other extraction procedures.11,22 The use of different mobile phases for reconstitution (mobile phase B for SCs and mobile phase A/B, (80/20), (v/v) - for all other analytes) did not necessitate different chromatographic conditions, but only a total number of 3 injections per sample, with a run time of 17 minutes each.

An additional analytical challenge arises from the type of matrix to be analyzed. Serum and whole blood are certainly the preferable matrices to analyze NPS in fatal and non-fatal intoxications. Compared to serum or plasma, whole blood often requires additional steps in sample preparation, and some substances might show different concentrations in plasma or serum when compared to whole blood. However, the latter is often the only available matrix in postmortem toxicology. In fact, postmortem blood is characterized by a variable

| N | Analyte | RT (min) | Detection window (min) | Precursor ion (m/z) | Product ions (m/z) | CV (V) | Ce (V) |
|---|---------|---------|------------------------|---------------------|-------------------|--------|--------|
| 173 | MMB-022 methyl (1-(pent-4-en-1-yl)-1H-indole-3-carbonyl)-L-valinate | 11.8 | 10.8–12.5 | 343 | 144 | 212* | 36 | 38 |
| 174 | MN-25 7-methoxy-1-[2-(morpholin-4-yl)ethyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-1H-indole-3-carboxamide | 9.8 | 6.5–10 | 440 | 261* | 353 | 45 | 25 |
| 175 | N-Phenyl-SBB-006 1-pentyl-N-phenyl-1H-indole-3-carboxamide | 12.4 | 11.5–12.8 | 307 | 144 | 214* | 30 | 34 |
| 176 | NE-CHMIMO [1-(cyclohexylmethyl)-1H-indol-3-yl]-1-naphthalenyl-methanone | 13.8 | 13–14.5 | 368 | 127 | 155* | 45 | 28 |
| 177 | SDB-005 naphthalen-1-yl 1-pentyl-1H-indazole-3-carboxylate | 13.7 | 13–14.5 | 359 | 145* | 215 | 36 | 40 |
| 178 | THJ-2201 [1-(5-fluoropentyl)-1H-indazol-3-yl] (naphthalen-1-yl) methanone | 12.9 | 12.2–13.5 | 361 | 213* | 233* | 45 | 24 |
| 179 | WIN 55,212–2 (R)-[+]-[2,3-dihydro-5-methyl-3-[4-morpholinylmethyl] pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1- naphthalenylmethanone | 10.9 | 10–11.7 | 427 | 127 | 155* | 45 | 38 |
| 180 | XLR-11 [1-(5-fluoropentyl)-1H-indol-3-yl] (2,2,3,3-tetramethylcyclopropyl)methanone | 13.1 | 12.2–13.5 | 330 | 125* | 330 | 36 | 32 |
| 181 | XLR-11 isomer | 12.7 | 11.9–13.4 | 330 | 125* | 330 | 36 | 32 |
| 182 | XLR-12 (2,2,3,3-tetramethylcyclopropyl) [1-(4,4,4-trifluorobutyl)-1H-indol-3-yl]methanone | 13.1 | 12.6–14 | 352 | 125* | 254 | 36 | 32 |

**Abbreviation:** N, number.
### Table 3: Precision (relative standard deviation or RSD), accuracy (bias), linearity ($R^2$: Regression coefficient), and limit of detection (LOD) and of quantification (LOQ) of the analytes

| Analyte | QC low | QC high | $R^2$ | LOD – LOQ |
|---------|-------|---------|-------|-----------|
|         | Intraday (RSD %) | Interday (RSD %) | Accuracy (bias %) | Intraday (RSD %) | Interday (RSD %) | Accuracy (bias %) |
| Synthetic cannabinoids (SCs) | | | | | | |
| 4-HTMIPPO | 6.01 | 7.13 | 6.8 | 4.20 | 5.94 | −0.86 | 0.990 | 0.06-0.15 |
| 4F-MDMB-BINACA | 2.96 | 4.36 | 8.0 | 4.70 | 5.12 | 0.96 | 0.997 | 0.04-0.09 |
| 5-CI-AB-PINACA | 10.82 | 10.82 | 1.8 | 5.40 | 6.87 | −6.19 | 0.997 | 0.42-1.25 |
| 5-CI-THJ-018 | 14.98 | 14.98 | −1.1 | 3.60 | 6.26 | −5.46 | 0.995 | 0.17-0.5 |
| 5F-AB-001 | 3.28 | 9.36 | 1.10 | 5.60 | 5.62 | −4.60 | 0.991 | 0.14-0.94 |
| 5F-AB-PICA | 10.51 | 10.79 | −2.9 | 5.40 | 6.19 | −5.09 | 0.994 | 0.09-0.39 |
| 5F-AB-PINACA | 3.97 | 7.42 | 6.6 | 6.00 | 6.26 | −1.57 | 0.996 | 0.17–0. |
| 5F-ADB | 7.42 | 7.42 | 3.2 | 6.50 | 6.53 | −4.67 | 0.999 | 0.14–0.97 |
| 5F-ADB-PICA | 2.08 | 7.61 | 2.5 | 6.00 | 6.37 | −4.16 | 0.993 | 0.13–0.85 |
| 5F-ADB-PINACA | 4.05 | 8.94 | 1.8 | 6.00 | 6.22 | −6.46 | 0.995 | 0.05–0.14 |
| 5F-AMB-PINACA | 5.35 | 5.39 | 7.8 | 4.90 | 5.74 | −4.13 | 0.999 | 0.10–0.51 |
| 5F-APP-PICA | 11.41 | 11.41 | −1.1 | 4.40 | 4.39 | 0.09 | 0.997 | 0.08–0.25 |
| 5F-APP-PINACA | 10.29 | 10.29 | −2.3 | 7.80 | 8.47 | −3.04 | 0.998 | 0.08–0.25 |
| 5F-Cumyl-PINACA | 11.85 | 12.25 | −2.3 | 4.40 | 5.40 | −0.57 | 0.998 | 0.01–0.04 |
| 5F-EMB-PINACA/5F-MDMB-PINACA (isomers) | 4.72 | 7.35 | 9.0 | 2.20 | 5.40 | −1.55 | 0.994 | 0.03–0.08 |
| 5F-JW-412 | 8.23 | 8.23 | 5.8 | 2.60 | 4.70 | 0.31 | 0.997 | 0.08–0.25 |
| 5F-MDMB-P7AICA | 8.88 | 9.63 | 5.4 | 7.16 | 7.16 | −5.1 | 0.984 | 0.03–0.08 |
| 5F-MDMB-PICA | 3.01 | 4.68 | 9.9 | 2.20 | 4.98 | −3.0 | 0.993 | 0.04–0.10 |
| 5F-NNEI 2’-naphthyl isomer | 13.59 | 13.59 | −2.8 | 8.70 | 10.23 | −0.76 | 0.997 | 0.04–0.11 |
| 5F-PCN | 12.09 | 12.09 | 3.0 | 6.50 | 6.5 | 3.94 | 0.996 | 0.17–0.5 |
| 5F-PY-PICA | 5.5 | 5.5 | 9.8 | 4.80 | 8.42 | 2.53 | 0.999 | 0.04–0.10 |
| A-796,260 | 5.23 | 6.05 | 6.2 | 6.40 | 6.81 | −0.51 | 0.994 | 0.05–0.13 |
| A-834,735 | 8.92 | 8.92 | 4.6 | 9.30 | 9.33 | −2.80 | 0.996 | 0.17–0.5 |
| AB-005 | 4.93 | 4.93 | 9.4 | 4.90 | 6.24 | 2.44 | 0.990 | 0.08–0.26 |
| AB-005 azepeane | 6.30 | 8.95 | 7.1 | 6.80 | 7.31 | −1.34 | 0.991 | 0.07–0.20 |
| AB-BICA | 8.54 | 8.54 | 0.6 | 6.20 | 8.01 | −4.67 | 0.990 | 0.13–0.86 |
| AB-CHIMICA | 6.84 | 7.48 | 5.1 | 9.60 | 9.56 | 2.84 | 0.993 | 0.09–0.40 |
| AB-FUB7AICA | 7.37 | 9.76 | 0.0 | 6.70 | 6.90 | −1.44 | 0.992 | 0.17–0.5 |
| AB-FUBICA | 6.80 | 8.48 | 6.2 | 9.30 | 9.33 | −1.86 | 0.993 | 0.02–0.07 |
| AB-FUBINACA | 5.50 | 6.98 | −1.8 | 6.50 | 9.77 | −8.72 | 0.996 | 0.08–0.25 |
| AB-FUBINACA 2 fluorobenzyl/3 fluoro | 9.34 | 9.34 | 6.6 | 7.60 | 7.58 | −1.99 | 0.995 | 0.04–0.11 |
| AB-PICA | 9.94 | 9.94 | 4.4 | 5.40 | 8.00 | −3.63 | 0.990 | 0.05–0.12 |
| AB-PINACA | 3.79 | 7.32 | 3.0 | 5.00 | 5.36 | −1.23 | 0.995 | 0.03–0.07 |
| ADB-BICA | 7.22 | 7.22 | 0.5 | 4.10 | 6.72 | −2.92 | 0.998 | 0.05–0.15 |
| ADB-BINACA | 12.01 | 12–01 | −0.6 | 1.90 | 3.38 | −2.74 | 0.992 | 0.04–0.11 |
| ADB-FUBICA | 10.17 | 10.17 | 5.5 | 2.80 | 5.94 | −4.21 | 0.998 | 0.06–0.19 |
| ADB-FUBINACA | 14.48 | 14.48 | −0.5 | 8.40 | 8.42 | −4.08 | 0.999 | 0.08–0.25 |
| ADB-PICA/ADBICA | 8.60 | 8.60 | 5.7 | 4.90 | 5.42 | −3.41 | 0.991 | 0.02–0.05 |
| AM-1220-azepane | 6.99 | 8.33 | 1.3 | 5.00 | 7.78 | −4.10 | 0.996 | 0.17–0.5 |
| AM-1241 | 6.83 | 6.83 | 7.3 | 2.30 | 6.79 | −0.37 | 0.995 | 0.07–0.21 |
| AM-1248 | 3.19 | 3.69 | 5.7 | 4.70 | 8.68 | −2.83 | 0.995 | 0.06–0.19 |
| AM-1248 azepane | 5.05 | 5.30 | 9.2 | 5.00 | 5.20 | −0.92 | 0.992 | 0.04–0.10 |

(Continues)
| Analyte                     | QC low | QC high |
|----------------------------|--------|---------|
|                            | Intray (RSD %) | Interday (RSD %) | Accuracy (bias %) | Intray (RSD %) | Interday (RSD %) | Accuracy (bias %) | R² | LOD - LOQ |
| AM-2201                    | 6.25   | 6.76   | 0.4 | 5.30 | 7.09 | 2.97 | 0.999 | 0.17-0.5 |
| AM-2201 indazol carboxamide| 9.80   | 9.80   | -1.6 | 2.90 | 3.64 | -3.39 | 0.994 | 0.17-0.5 |
| AM-2232                    | 7.34   | 7.34   | 7.6 | 5.50 | 6.27 | -2.92 | 0.991 | 0.05-0.13 |
| AM-2233                    | 8.98   | 9.45   | -3.6 | 2.50 | 2.92 | 0.67 | 0.999 | 0.06-0.19 |
| AM-2233 azepane            | 9.38   | 10.33  | 4.5 | 4.40 | 4.44 | -2.47 | 0.996 | 0.05-0.14 |
| AM-630                     | 6.32   | 7.47   | 2.5 | 7.60 | 7.85 | -3.40 | 0.992 | 0.07-0.23 |
| AM-679                     | 8.52   | 8.52   | 4.1 | 6.00 | 6.04 | -7.76 | 0.993 | 0.06-0.15 |
| AM-694                     | 12.29  | 12.29  | -5.5 | 3.90 | 6.48 | 2.62 | 0.999 | 0.17-0.5 |
| AMB-CHMIC                 | 4.75   | 4.85   | 7.9 | 5.40 | 5.36 | -1.23 | 0.998 | 0.13-0.78 |
| AMB-CHMINAC                | 11.04  | 11.04  | 4.4 | 6.20 | 6.78 | -4.13 | 0.993 | 0.17-0.5 |
| AMB-FUBICA                | 6.42   | 6.42   | 9.6 | 3.80 | 6.84 | -1.27 | 0.993 | 0.06-0.17 |
| AMB-FUBINACA              | 4.44   | 6.78   | 6.2 | 5.90 | 5.92 | -2.59 | 0.998 | 0.07-0.24 |
| AMB-PICA                  | 6.96   | 8.90   | 5.3 | 3.50 | 7.42 | -2.16 | 0.994 | 0.09-0.33 |
| AMB-PINACA                | 6.28   | 7.13   | 0.1 | 5.00 | 5.31 | -2.63 | 0.996 | 0.17-0.5 |
| APP-FUBINACA              | 11.34  | 13.49  | 0.0 | 13.60 | 14.54 | -2.64 | 0.997 | 0.02-0.05 |
| BB-22                     | 6.77   | 7.10   | 4.9 | 4.90 | 8.26 | -0.18 | 0.990 | 0.05-0.13 |
| Cumyl-4CN-BINACA          | 6.75   | 7.18   | 5.1 | 3.60 | 4.79 | 1.4 | 0.998 | 0.08-0.28 |
| Cumyl-BICA                | 6.72   | 7.99   | 0.8 | 3.10 | 5.26 | 2.64 | 0.999 | 0.10-0.48 |
| Cumyl-PEGACLONE           | 9.12   | 9.12   | 5.4 | 2.90 | 7.45 | -1.11 | 0.996 | 0.10-0.48 |
| Cumyl-PICA                | 6.67   | 8.01   | 6.3 | 4.30 | 4.86 | 5.24 | 0.998 | 0.08-0.33 |
| Cumyl-THPINACA            | 6.46   | 6.46   | 8.8 | 6.50 | 7.63 | -0.65 | 0.999 | 0.11-0.57 |
| EG-2201                   | 4.94   | 9.55   | 3.4 | 7.60 | 7.63 | -4.09 | 0.999 | 0.10-0.43 |
| FUB-JWH-018               | 5.28   | 6.13   | 7.8 | 7.80 | 7.84 | -3.27 | 0.994 | 0.04-0.10 |
| FUB-NPB-22                | 3.33   | 3.91   | 9.6 | 3.80 | 4.31 | 1.63 | 0.999 | 0.08-0.32 |
| FUB-PB-22                 | 11.34  | 11.34  | 3.4 | 4.20 | 5.16 | 3.86 | 0.999 | 0.08-0.25 |
| JWH-007                   | 6.82   | 8.39   | -2.6 | 5.00 | 4.98 | -3.63 | 0.999 | 0.17-0.5 |
| JWH-011                   | 9.35   | 9.35   | -3.7 | 6.80 | 8.87 | -3.10 | 0.991 | 0.04-0.09 |
| JWH-015                   | 4.51   | 6.65   | 9.5 | 4.00 | 4.69 | 3.22 | 0.998 | 0.09-0.38 |
| JWH-018                   | 9.93   | 9.93   | -2.6 | 9.00 | 8.98 | -2.76 | 0.994 | 0.17-0.5 |
| JWH-019                   | 5.27   | 11.96  | 2.0 | 10.16 | 10.17 | -0.38 | 0.999 | 0.03-0.09 |
| JWH-020                   | 14.91  | 14.91  | 0.2 | 3.70 | 4.45 | 3.52 | 0.994 | 0.06-0.18 |
| JWH-022                   | 6.47   | 6.47   | 9.3 | 4.80 | 4.84 | -4.90 | 0.992 | 0.05-0.12 |
| JWH-030                   | 11.33  | 11.33  | 4.8 | 6.71 | 8.24 | -1.81 | 0.999 | 0.06-0.16 |
| JWH-031                   | 8.21   | 8.21   | 7.9 | 5.40 | 5.38 | -3.05 | 0.993 | 0.17-0.5 |
| JWH-073                   | 5.41   | 3.40   | 9.6 | 5.30 | 5.53 | -3.09 | 0.995 | 0.03-0.09 |
| JWH-080                   | 2.89   | 9.78   | 2.4 | 2.50 | 6.03 | -4.43 | 0.992 | 0.17-0.5 |
| JWH-081                   | 5.85   | 10.27  | 0.2 | 5.80 | 6.07 | -3.87 | 0.999 | 0.13-0.79 |
| JWH-098                   | 6.42   | 6.42   | -3.6 | 8.10 | 8.12 | -2.89 | 0.999 | 0.17-0.5 |
| JWH-122                   | 10.42  | 10.42  | -5.3 | 4.20 | 4.21 | -4.00 | 0.999 | 0.03-0.09 |
| JWH-122 N-(4-pentenyl)    | 10.25  | 10.25  | 4.8 | 4.10 | 6.12 | 0.70 | 0.991 | 0.03-0.07 |
| JWH-145                   | 5.10   | 9.27   | 2.5 | 5.70 | 5.67 | -3.72 | 0.993 | 0.03-0.08 |
| JWH-147                   | 11.19  | 11.19  | -1.9 | 8.70 | 9.19 | -0.89 | 0.993 | 0.07-0.23 |
| JWH-182                   | 9.63   | 9.69   | -0.4 | 3.10 | 5.60 | -1.40 | 0.990 | 0.08-0.25 |
| JWH-200                   | 9.17   | 9.17   | -2.8 | 1.70 | 4.24 | -5.37 | 0.994 | 0.17-0.5 |
| Analyte                       | QC low         | QC high        |
|------------------------------|----------------|----------------|
|                              | Intraday (RSD %) | Interday (RSD %) | Accuracy (bias %) | Intraday (RSD %) | Interday (RSD %) | Accuracy (bias %) | R²   | LOD – LOQ |
| JWH-213                      | 9.79           | 9.79           | 1.5              | 2.20             | 2.21             | 0.69              | 0.991 | 0.06-0.18 |
| JWH-249                      | 7.41           | 8.92           | 2.2              | 4.10             | 4.11             | -1.28             | 0.992 | 0.05-0.12 |
| JWH-250                      | 10.67          | 10.67          | -2.4             | 3.10             | 7.13             | -5.55             | 0.995 | 0.11-0.53 |
| JWH-251                      | 5.95           | 5.95           | 0.6              | 5.30             | 5.31             | -6.23             | 0.998 | 0.17-0.5  |
| JWH-302                      | 0.83           | 0.86           | -9.0             | 4.40             | 5.76             | -4.41             | 0.999 | 0.04-0.10 |
| JWH-307                      | 14.08          | 14.08          | -0.1             | 7.90             | 7.92             | -1.18             | 0.992 | 0.02-0.07 |
| JWH-309                      | 12.53          | 12.27          | -2.5             | 2.40             | 2.44             | -7.50             | 0.992 | 0.10-0.48 |
| JWH-370                      | 14.37          | 14.37          | -1.1             | 3.20             | 3.24             | -3.20             | 0.993 | 0.10-0.49 |
| JWH-387                      | 10.21          | 10.21          | -7.9             | 4.60             | 7.03             | -2.14             | 0.991 | 0.13-0.82 |
| JWH-412                      | 13.71          | 13.71          | -4.3             | 3.40             | 3.43             | -0.31             | 0.994 | 0.07-0.20 |
| JWH-424                      | 11.49          | 11.49          | -1.0             | 6.00             | 6.04             | -5.65             | 0.993 | 0.08-0.32 |
| M-144                        | 6.45           | 8.65           | 3.0              | 5.10             | 5.12             | -4.01             | 0.994 | 0.17-0.5  |
| MDMB-4en-PINACA              | 6.42           | 7.52           | 1.7              | 5.20             | 5.22             | -4.04             | 0.999 | 0.17-0.5  |
| MDMB-CHMCZCA                 | 7.05           | 7.05           | -4.0             | 11.09            | 11.10            | -3.33             | 0.999 | 0.07-0.22 |
| MDMB-CHMICA                  | 6.09           | 6.68           | -8.9             | 3.80             | 4.88             | -7.19             | 0.998 | 0.17-0.5  |
| MDMB-CHMINACA                | 7.64           | 10.77          | 2.1              | 4.60             | 4.62             | -4.32             | 0.998 | 0.17-0.5  |
| MDMB-FUBICA                  | 2.65           | 6.86           | 8.1              | 4.30             | 4.94             | -0.91             | 0.999 | 0.11-0.54 |
| MDMB-FUBINACA                | 7.88           | 7.88           | 3.3              | 9.40             | 9.38             | -3.15             | 0.997 | 0.09-0.36 |
| MEPIRAPIM                    | 6.74           | 6.74           | 2.9              | 4.00             | 3.99             | 1.47              | 0.996 | 0.07-0.23 |
| MMB-022                      | 6.94           | 7.72           | 1.7              | 5.10             | 5.34             | 1.23              | 0.999 | 0.17-0.5  |
| MMB-2201                     | 4.97           | 4.97           | -5.2             | 7.00             | 8.88             | -2.43             | 0.998 | 0.17-0.5  |
| MN-25                        | 7.93           | 7.93           | 4.1              | 2.50             | 5.13             | 1.62              | 0.996 | 0.05-0.12 |
| N-phenyl-SDB-006-            | 4.82           | 5.38           | 7.2              | 4.00             | 6.14             | -0.95             | 0.992 | 0.06-0.17 |
| NE-CHIMIMO                   | 5.27           | 5.27           | 2.3              | 8.40             | 8.37             | 0.30              | 0.995 | 0.08-0.26 |
| RCS-4                        | 11.11          | 11.11          | -2.0             | 4.10             | 5.38             | -2.95             | 0.997 | 0.12-0.67 |
| RCS-8                        | 4.97           | 9.91           | -5.4             | 5.80             | 5.83             | -4.19             | 0.998 | 0.07-0.19 |
| SDB-005                      | 3.32           | 6.95           | 7.4              | 5.10             | 5.78             | -1.07             | 0.991 | 0.08-0.25 |
| THJ-2201                     | 7.08           | 7.08           | 0.8              | 4.60             | 4.56             | -3.34             | 0.999 | 0.17-0.5  |
| WIN 48.098                   | 10.73          | 10.73          | -4.7             | 2.90             | 5.32             | -6.07             | 0.996 | 0.07-0.20 |
| WIN 55.212-2                 | 5.08           | 5.21           | 5.9              | 3.20             | 5.08             | 0.05              | 0.995 | 0.08-0.27 |
| XLR-11                       | 4.04           | 4.04           | 5.9              | 2.70             | 2.67             | -3.28             | 0.999 | 0.08-0.27 |
| XLR-11 isomer                | 4.08           | 4.08           | 9.4              | 5.50             | 5.58             | -1.06             | 0.996 | 0.08-0.27 |
| XLR-12                       | 7.68           | 7.68           | 6.1              | 5.00             | 5.73             | -1.12             | 0.995 | 0.09-0.33 |

Synthetic opioids (Sos)

(±)-cis-3-methyl norfentanyl
3.31 9.56 2.7 8.00 8.54 -4.03 0.998 0.09-0.33
(±)-trans-3-methyl norfentanyl
6.02 8.46 7.0 3.70 4.94 -4.16 0.996 0.05-0.13
β-Hydroxy fentanyl
5.40 5.66 5.2 4.00 3.95 -0.72 0.995 0.04-0.10
β-Hydroxythiofentanyl
3.87 4.97 8.9 4.10 4.70 -3.81 0.996 0.03-0.08
β-Phenyl fentanyl
5.34 5.85 6.8 2.40 4.04 -0.58 0.993 0.07-0.22
4-ANPP
9.24 10.21 4.7 6.10 7.47 -1.61 0.992 0.17-0.5
Acetyl fentanyl
0.36 6.12 1.4 4.20 6.50 -5.05 0.997 0.03-0.08
Acetyl norfentanyl
0.84 7.22 3.1s 2.90 6.40 -5.50 0.996 0.08-0.30
Alfentanyl
2.08 9.45 4.7 4.20 4.23 -6.16 0.994 0.08-0.31
Butyl fentanyl
7.40 7.40 9.6 3.40 6.24 -4.54 0.993 0.04-0.10

(Continues)
grade of hemolysis, preventing serum or plasma separation. In the living subject, when an intoxication is suspected or in cases of suspected driving under the influence (DUI), blood and/or urine samples are often collected in hospitals. When the separation of serum is not performed directly in the hospital where the blood is taken, the vials are sometimes frozen and sent to a forensic laboratory, where separation of the hemolyzed material is no more achievable.

As for the amount of whole blood, Adamowicz and Tokarczyk used 0.2 ml of blood, though the method was only a qualitative
screening with LODs ranging from 0.01 to 3.09 ng/ml. Other studies using the same amount of blood or serum showed higher sensitivity, though only analyzing a limited number of compounds.22,37 In the method here presented, the use of a higher volume, similarly to previous studies,20,22,26 provided a high sensitivity despite the high number of included substances. Nevertheless, future studies to reduce the needed volume of whole blood are encouraged.

Since the legislation on NPS is based on a substance-by-substance (individual listing) basis or on generic or analogue control, rather than on define biological concentrations,46 literature data on previous NPS analytical methods and on intoxications were used to establish the linearity ranges of the present study and to verify whether the sensitivity was acceptable. According to the literature, SCAs and stimulants in blood tend to be quantified mostly at few dozen/hundred nanograms per milliliter after recreational use and even higher levels are to be expected in cases of acute toxicity.11,12,22,41,42 Tryptamines, fentanyl and SOs are also typically characterized by high concentrations in post-mortem or intoxication samples,13,24,43,44 while expected concentrations of SCs in blood are generally lower.13,14,44–46 Indeed, in the method of Kneisel and Auwärter,20 the calibration points were in the range 0.01–2.0 ng/ml and the LODs in the range 0.001–0.1 ng/ml. However, concentrations up to 190 ng/ml have been reported.47 Therefore, the LODs obtained with presented method are satisfactory for the purpose and provided sufficient sensitivity for all NPS classes.

Accuracy and precision were studied for all selected analytes at different concentrations and the criteria required for validation were met by 165 substances, which can be considered validated for quantitative purposes. The presented approach provides a very useful tool for the combined targeted analysis and broad screening of NPS in whole blood. Moreover, the method can be easily extended to include novel compounds, allowing for a quick adaption to the dynamic development of the NPS market.

The major limit of the present method resides in the recovery and, particularly, in the matrix effect for some molecules. As already shown in previous studies,22 4-FMC might be particularly problematic with regard to matrix effect. For SCs, in the study of Kneisel and Auwärter,20 conducted on serum samples, most analytes were affected by remarkable matrix effects, and recovery was in the range 5.7–56%. Similarly, significant matrix effects were highlighted by methods involving protein precipitation, since this has been described to lead to large amounts of endogenous compounds in the injected sample, enhancing or reducing the signals.11,32 Indeed, whole blood is a complex matrix, and it is very likely that the type of sample, as well as the employment of precipitation provoked matrix effects. However, the influence of such parameters, whenever linearity, accuracy and precision remain acceptable, is a matter of debate. Taking into account solely the analytes which showed acceptable recoveries and matrix effects, the method can be considered as a fully validated tool for 138 analytes of interest.

The difficulties related to ion suppression/enhancement have recently been shown in cases of analysis of whole blood samples with a method validated for serum.38 Keeping in mind that the matrix effect could be severe, a standard addition method was suggested by the authors to provide a more precise quantification.

Another acknowledged limitation is represented by the use of only two internal standards. Though nordiazepam-D5 and ketamine-D4, which are widely available in most forensic laboratories, have proven satisfactory for the evaluation of accuracy and precision. Nevertheless, better results could be expected by using specific standards with more chemical similarity to the various NPS subclasses. On the other hand, the use of a limited number of broadly available internal standards can be seen as a strength of the method, in terms of costs and applicability in many forensic laboratories. On the basis of the chosen internal standard and due to its relevance as metabolite or co-consumed drug in NPS intoxications, nordiazepam was also included in the present method.

Finally, the presented method has so far only been applied to a very limited set of real-case samples. Despite the limitedness of the case study and the absence of positive findings regarding NPS intended “in a strict sense,” the application of the method allowed the detection and quantification of ketamine and fentanyl. Online surveys have so far demonstrated a limited use of NPS in Italy in comparison to traditional drugs, with a prevalent consumption of phenethylamines and cathinones once/twice in lifetime.49 Ketamine is one of the most cited NPS substances in the Italian mass media and its use was reported in online surveys by 66.7% of respondents,49 while fentanyl is largely used in the emergency setting. In the literature, methods for NPS detection are usually applied only to a limited number of real cases, due to difficulties in retrieving a wider casuistry22,50 and the absence of broad-panel methods has so far hampered a thorough knowledge of the NPS prevalence in Italy. Even though the limited sample is certainly a drawback of the study, an extensive application of the method was beyond the scope of our research and future applications on a wider scale would be desirable to provide more comprehensive epidemiological data regarding NPS consumption.

5 | CONCLUSIONS

In the highly dynamic world of novel psychoactive substances (NPS), characterized by the ongoing emergence of multiple and chemically diverse compounds on the market, several challenges arise for the analysis of NPS. Since methods to simultaneously detect different classes of NPS are still scarce, the present methodology represents an easy, low cost, wide-panel method for the detection of more than 180 novel psychoactive substances, including 132 synthetic cannabinoids, 22 synthetic opioids, 28 among synthetic cathinones, stimulants and other drugs.

The developed method can be profitably applied both in a clinical context, with 17 × 3 min run time and a broad screening for multiple compounds, and in postmortem toxicology, where the multi-analyte method is advantageous by reducing time and costs of analysis. When considering real forensic cases and a quantitative analysis is requested, the matrix effect should be taken into consideration, and a multidisciplinary case-by-case evaluation, including an assessment of circumstantial, clinical, post-mortem, and toxicological data, is necessary.
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