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# Validation and application of a method for the quantification of 137 drugs of abuse and new psychoactive substances in hair

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ABSTRACT

*Introduction:* In the dynamic universe of new psychoactive substances (NPS), the identification of multiple and chemically diverse compounds remains a challenge for forensic laboratories. Since hair analysis represents a gold-standard to assess the prevalence of NPS, which are commonly detected together with classical drugs of abuse (DoA), our study aimed at developing a wide-screen method to detect and quantify 127 NPS and 15 DoA on hair.

*Materials and methods:* A multi-analyte ultra-high performance liquid chromatography mass spectrometry method for the identification and quantification of 127 NPS (phenethylamines, arylcyclohexylamines, synthetic opioids, tryptamines, synthetic cannabinoids, synthetic cathinones, designer benzodiazepines) and 15 DoA in hair samples was developed. A full validation was performed according to the European medicines Agency (EMA) guidelines, by assessing selectivity, linearity, accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), matrix effect and recovery. As a proof of the applicability, the method was applied to 22 authentic hair samples collected for forensic purposes.

*Results:* Successful validation was achieved, by meeting the required technical parameters, for 137 compounds (122 NPS and 15 DoA), with LOQ set at 4 pg/mg for 129 compounds, at 10 pg/mg for 6 and at 40 pg/mg for 2. The method was not considered validated for 5 NPS, as LLOQ resulted too high for a forensic analysis (80 pg/mg). Among authentic forensic samples, 17 tested positive for DoA, and 10 to NPS, most samples showing positivity for both. Detected NPS were ketamine and norketamine, 5-MMPA, ritalinic acid, methoxyacetyl fentanyl, methylone and RCS-4.

*Conclusion:* The present methodology represents an easy, low cost, wide-panel method for the quantification of 122 NPS and 15 DoA, for a total of 137 analytes, in hair samples. The method can be profitably applied by forensic laboratories. Similar multi-analyte methods on the hair matrix might be useful in the future to study the prevalence of NPS and the co-occurrence of NPS-DoA abuse.

## 1. Introduction

The term New Psychoactive Substances or NPS refers to a pool of molecules which are not covered by the United Nations International drug conventions. By the end of 2022, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has monitored over 930 substances, classified as synthetic cannabinoids, synthetic cathinones, synthetic opioids, arylcyclohexylamines (phenciclydine-type substances), phenethylamines, designer benzodiazepines, tryptamines and others [1]. Due to the variety of new compounds flooding into the NPS market, and to the dynamicity of this market itself, partially affected by national and international regulations [2], NPS represent a worldwide health problem [1,3,4] and poses several challenges to the international/national agencies and monitoring programs, as well as to forensic laboratories. Indeed, the detection of NPS cannot rely on routine screening tests, requiring target strategies, usually performed by liquid chromatography (LC) coupled with mass spectrometry (MS), or high resolution-MS screening [5–7]. However, target methods have to be kept constantly updated, to cover for the substances entering or re-entering the NPS drug market [5,7–9].

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NPS have not replaced the classical drugs of abuse (DoA) and the prevalence seems to be related to regional and national differences in drug markets, regulations and type of population or sub-population submitted to toxicological analysis. As an example, in patients admitted to drug detoxification treatment in Germany, polydrug use was common, but NPS prevalence was rather low [10]. However, NPS might be co-consumed with DoA to obtain enhanced effects, as shown in patients acutely intoxicated or with substance use disorder [11–14]. Classic illicit drugs can also be adulterated with NPS, due to their low costs, legal status (at least for not yet controlled compounds) and sometimes more potent effects, and this might give rise to health risks for the consumer, exposed to an unexpected threat [15,16]. It is therefore of paramount importance to develop multi-analyte methods to simultaneously detect not only a wide panel of NPS, but also a combination of old and new drugs.

The most commonly used matrices for forensic toxicological analyses are blood or urine, which have a relatively short detection window for DOA and for some NPS. Hair represents an alternative matrix which has gained increased popularity in forensic toxicology, because of the non-invasiveness of its collection and the opportunity to retrospectively evaluate the consumption of drugs over an extended period [17–19]. Indeed, head hair has an approximate growth rate of 1 cm/month, allowing a detection window of several months [17]. However, according to the Society of Hair Testing (SoHT) the grow rates and cycles should be considered to establish the time window represented by the analyzed scalp hair [18].

Another advantage of hair analysis is represented by the possibility of targeting the parent drug, since the incorporation takes place before metabolism and polar metabolites are usually found in lower amounts [19]. Moreover, sample collection is easy, free of biohazard risks and the samples can be stored simply at room temperature away from light [19]. On the other hand, some limitations inherent to the hair sampling are the possible external contamination, and the low concentrations often detected [17,19]. Thus, hair analysis is the method of choice for the retrospective assessment of past, chronic, sub-chronic exposure to xenobiotics and might represent an opportunity to understand the spread and the prevalence of NPS within the general population or high-risk groups [17–22].

The majority of the published methods for the detection of NPS is focused on a limited number of NPS, on a single NPS group and/or does not allow the contemporary identification of traditional illicit DoA, with a few exceptions [23].

In light of the above, the aim of the present study was to develop a wide-screen quantification method for 127 NPS (phenethylamines, arylcyclohexylamines, synthetic opioids, tryptamines, synthetic cannabinoids, synthetic cathinones, designer benzodiazepines), 15 DoA and metabolites. The method was then applied on authentic hair samples.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The National Health Institute and Comedical s.r.l. (Italy, Trento) within the national project "SNAP" provided standards of 3,4-methylmethcathinone, 4-fluoromethcathinone, 4-methylethcathinone, 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, 3,4-methylenedioxypyrovalerone, methcatinone, methedrone, methylone, pentylone, RCS-4, RCS-8 and pravadoline (WIN 48,098) at a concentration of 100 μg/ml; (±)-cis-3-methyl norfentanyl, (±)-trans-3-methyl norfentanyl, alpha-ethyl-triptamine, beta-hydroxyfentanyl, beta -hydroxythiofentanyl, beta-phenyl fentanyl, 4-AcO-DiPT, 4-ANPP, 5/6-APB, 5-Cl-THJ-018, 5-EAPB, 5F-ADB, 5F-APP-PICA (PX-1), 5F-APP-PINACA (PX-2), 5F-Cumyl-PINACA, 5F-NNEI 2'-naphthyl isomer, 5/6-MAPB, 5-MeO-AMT, 5-MeO-DALT, 5-MeO-DMT, 5-MeO-DPT, 5-MeO-MiPT, AB-CHMINACA, AB-FUBINACA, acetyl fentanyl, acetyl norfentanyl, ADB-FUBINACA, alfentanyl, APP-FUBINACA, butyryl fentanyl, butyryl fentanyl carboxy metabolite, butyryl norfentanyl, carfentanyl, Cumyl-PEGACLONE (SGT-151), cyclopropylfentanyl, despropionyl para-fluorofentanyl, ethylphenidate, fentanyl, furanyl norfentanyl, JWH-018, JWH-200, JWH-250, MDMB-CHMICA, mephedrone, methoxyacetyl norfentanyl, MMB-2201, N,N-dimethylcathinone, N,N-dimethyltryptamine, norfentanyl, phenylfentanyl, phenylacetyl fentanyl, ritalinic acid and valeryl fentanyl carboxy metabolite at a concentration of 50 μg/ml; 2-fluoro deschloroketamine, 3-methoxy PCE, deschloro-N-ethyl-ketamine, bentazepam, clonazolam, diclazepam, etizolam, 5F-Cumyl-P7AICA, 5F-Cumyl-PEGACLONE, 5F-MDMB-7-PAICA, 5-F-MDMB-PICA, UR-144, 3',4'-methylenedioxy-alpha-pyrrolidinohexiophenone, ethylone, euthylone, N-ethyl pentylone, alpha -pyrrolidinohexanophenone, furanyl fentanyl, isobutyryl fentanyl, isotonitazene, methoxyacetyl fentanyl, ocfentanyl, para-fluoro-furanyl fentanyl, 2-methyl AP-237, AP-237 at a concentration of 20 μg/ml; 5-methylmethiopropamine (5-MMPA), methoxpropamine, brorphine, butonitazene, etodesnitazene, flunitazene, metodesnitazene, metonitazene, N-pyrrolidino etonitazene (etonitazepyne), 4F-MDMB-BUTICA, 5F- Cumyl-PICA, 5F-EDMB-PICA, 5F-EMB-PICA, ADB-4en-PINACA, MDMB-4en-PICA, MDMB-4en-PINACA, MDMB-4en-PINACA butanoic acid metabolite, 3-methylmethcathinone at a concentration of 10 μg/ml; standard of amphetamine, metamphetamine, methylenedioxyamphetamine, methylenedioxymethamphetamine, cocaine, cocaethylene, ecgonine methyl ester, ketamine, norketamine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), morphine, codeine, 6-monoacetylmorphine (6-MAM), delta-9-tetrahydrocannabinol (delta-9-THC), cannabidiol at a concentration of 1 mg/ml and benzoylecgonine, internal standards (fentanyl-d5, ketamine-d4, JWH-122-d9, nordiazepam-d5) at a concentration of 100 μg/ml were obtained from Sigma Aldrich (Steinheim, Germany) and LGC Standards Ltd (Milano, Italy).

Standard compounds were stored according to supplier recommendations until their use. 2-isopropanol, acetone, acetonitrile, dichloromethane, formic acid and methanol were purchased from Sigma-Aldrich (Germany). All reagents and solvents were of LC-MS grade. Ultra-pure water was obtained from PURELAB® chorus 1, Elga Veolia.

### 2.2. Sample preparation

Each hair sample was rinsed with dichloromethane, methanol and acetone in consecutive 1-min steps. After drying, each sample was finely cut with scissors, and an aliquot of 25 mg was weighted in a centrifuge glass tube. Then the sample was added with 300 μl of a solution consisting of methanol and water (70:30, v/v), previously spiked with 0.1 % formic acid, then samples were incubated overnight in a thermoblock at 45 °C. Preliminary analyses were performed with different solvent mixtures, including methanol, methanol and either formic acid or chloridric acid at different concentrations (0.01–1 %) and the use of a mixed methanol-water solution was dictated by better peak shapes, avoiding fronting or tailing, as well as by better recovery data.

### 2.3. Calibration standards and quality control

Individual stock solutions of the listed standards were used to prepare one working mixture at 100 ng/ml in methanol and were stored at −20 °C until use.

Internal standards (ISs) mixture containing fentanyl-d5, ketamine-d4, JWH-122-d9, nordiazepam-d5 were prepared at a concentration of 1 μg/ml. Drug-free hair samples were obtained from laboratory staff of the Laboratory of Forensic Toxicology (Bologna, Italy) and used for the preparation of calibration curves and for matrix effect studies. Calibration samples and Quality controls (QCs) were prepared spiking aliquots of the 100 ng/ml standards working mixture on the matrix, spiking the ISs and then adding methanol to a final volume of 100 μl. Then, each point was vortexed and left to dry overnight before the extraction

**Table 1**
Retention times, Multiple Reaction Monitoring transitions (MRM) and Collision Energies (CE) of analytes of interest and their Internal Standards (ISs). RT: Retention Time.

| Compound | RT (min) | Precursor ion | Quantifier ion (CE (V)) | Qualifier ion (s) (CE (V)) | Cone (V) |
|---|---|---|---|---|---|
| **New Psychoactive Substances** | | | | | |
| (±)-cis-3-methyl Norfentanyl | 4.32 | 247 | 98.1 (18) | 69.1 (29) | 25 |
| (±)-trans-3-methyl Norfentanyl | 4.18 | 247 | 98.1 (18) | 69.1 (29) | 25 |
| (R)−5F-ADB | 12.40 | 378 | 233 (20) | 318 (14) | 45 |
| 2-F deschloroketamine | 3.02 | 222.1 | 109.0 (50) | 204.1 (20) | 38 |
| 2-methyl AP-237 | 5.61 | 287.2 | 91.1 (50) | 117.1 (16) | 28 |
| 3-MeO PCE | 6.14 | 234.2 | 121.1 (28) | 189.0 (15) | 12 |
| 3-methylmethcathinone | 3.45 | 178.1 | 160.0 (13) | 144.9 (22) | 30 |
| 3,4 MD-alpha-PHP | 6.32 | 290.1 | 135.0 (33) | 140.1(35) | 50 |
| 3,4-dimethylmethcathinone | 4.54 | 192 | 174 (13) | 159 (22) | 30 |
| 4-AcO-DiPT | 5.31 | 303 | 114 (18) | 160 (28) | 15 |
| 4-ANPP | 6.70 | 281 | 105 (22) | 188 (14) | 22 |
| 4F-MDMB-BUTICA | 12.00 | 363.2 | 144.0 (40) | 218.1 (34) | 60 |
| 4-fluoromethcathinone | 2.25 | 182 | 149 (15) | 164 (10) | 20 |
| 4-hydroxy DET | 2.69 | 233.1 | 86.1 (18) | 160 (14) | 16 |
| 4-methylethcathinone | 3.80 | 192 | 145.3 (18) | 174 (14) | 35 |
| 5Cl-AB-PINACA | 10.74 | 366 | 145 (44) | 249 (20) | 36 |
| 5Cl-THJ-018 | 13.61 | 377.2 | 248.9 (16) | 212.9 (24) | 25 |
| 5-EAPB | 4.60 | 204.1 | 131 (20) | 91 (30) | 24 |
| 5F-AKB48 (5F-APINACA) | 13.96 | 384 | 135 (24) | 106.9 (45) | 35 |
| 5F-APP-PICA | 10.80 | 396.3 | 232 (26) | 144 (44) | 25 |
| 5F-APP-PINACA | 10.68 | 397.3 | 233 (22) | 145 (46) | 20 |
| 5F-Cumyl-P7AICA | 11.95 | 368.2 | 119.1 (52) | 174.1 (36) | 62 |
| 5F-Cumyl-PEGACLONE | 11.70 | 391.2 | 119 (36) | 273.2 (18) | 50 |
| 5F-Cumyl-PICA | 12.70 | 367.2 | 249.1 (25) | 206.1 (35) | 50 |
| 5F-Cumyl-PINACA | 12.90 | 368.3 | 233 (18) | 250 (10) | 32 |
| 5F-EDMB-PICA | 12.80 | 391.2 | 232.1 (30) | 144.0 (55) | 40 |
| 5F-EMB-PICA | 12.50 | 377.2 | 144.0 (40) | 232.1 (24) | 36 |
| 5F-NNEI 2′-naphtyl isomer | 12.88 | 375.3 | 232 (20) | 144 (42) | 22 |
| 5-hydroxytryptophan | 1.13 | 221.1 | 134 (17) | 204 (11) | 28 |
| 5-MeO-AMT | 3.26 | 205.1 | 173 (22) | 147 (20) | 22 |
| 5-MeO-DALT | 5.38 | 271.2 | 110 (14) | 174 (18) | 24 |
| 5-MeO-DMT | 3.14 | 219.1 | 58 (12) | 130.1 (46) | 20 |
| 5-MeO-DPT | 6.11 | 275.2 | 114 (16) | 174 (14) | 14 |
| 5-MeO-MiPT | 4.08 | 247.1 | 86 (14) | 174 (16) | 10 |
| 5/6-APB | 3.71 | 176.2 | 91 (26) | 77 (40) | 22 |
| 5/6-MAPB | 4.10 | 190.2 | 159.1 (10) | 131 (18) | 22 |
| 5F-MDMB-P7AICA | 11.8 | 378.1 | 145.1 (40) | 233.2 (24) | 45 |
| 5F-MDMB-PICA | 12.43 | 377 | 145.1 (40) | 233.2 (20) | 36 |
| AB-CHMINACA | 12.04 | 357.4 | 145 (46) | 241.2 (28) | 38 |
| AB-FUBINACA | 10.78 | 369.3 | 253 (24) | 109 (40) | 20 |
| acetyl fentanyl | 5.72 | 323 | 105 (36) | 188 (20) | 25 |
| acetyl norfentanyl | 2.44 | 219.2 | 84.1 (18) | 55.2 (36) | 25 |
| ADB-FUBINACA | 11.33 | 383 | 253 (25) | 338 (10) | 25 |
| alpha-PHP | 6.18 | 246.2 | 91.1 (22) | 105.1 (28) | 60 |
| alfentanil | 6.65 | 417.1 | 197.1 (26) | 268.1 (16) | 24 |
| alpha-ethyltryptamine | 4.18 | 189.2 | 130 (16) | 58.1 (16) | 26 |
| AM 2201 | 12.94 | 360 | 127 (40) | 155 (28) | 45 |
| AM 2233 | 8.22 | 459 | 98 (34) | 112 (22) | 45 |
| AM-694 | 12.61 | 436 | 231 (28) | 202.7 V(40) | 45 |
| AP-237 | 5.16 | 273.2 | 117.1 (14) | 91.1(48) | 24 |
| APP-FUBINACA | 10.92 | 417.3 | 109.0 (40) | 253.0 (24) | 20 |
| bentazepam | 6.55 | 297.2 | 139.0 (38) | 166.1 (28) | 34 |
| beta-hydroxy fentanyl | 6.00 | 353 | 335 (16) | 204 (38) | 35 |
| beta-hydroxythiofentanyl | 5.62 | 359 | 192 (22) | 111 (38) | 25 |
| beta-phenyl fentanyl | 9.83 | 413.6 | 105 (44) | 188 (26) | 35 |
| brorphine | 7.23 | 400.1 | 218.1 (22) | 104 (50) | 50 |
| buphedrone | 3.38 | 178 | 160 (10) | 130.3 (32) | 30 |
| butonitazene | 9.92 | 425.2 | 100 (20) | 106.9 (52) | 46 |
| butylone | 2.71 | 222 | 204 (11) | 174 (20) | 25 |
| butyryl fentanyl | 7.70 | 351.2 | 105 (40) | 188.1 (22) | 30 |
| butyryl fentanyl carboxy metabolite | 5.49 | 381 | 105 (42) | 188 (45) | 25 |
| butyryl norfentanyl | 4.87 | 247.0 | 84 (28) | 55 (36) | 25 |
| carfentanil | 7.87 | 395.2 | 113 (32) | 105 (52) | 22 |
| clonazolam | 8.82 | 354.1 | 308 (26) | 326 (24) | 10 |
| Cumyl-PEGACLONE | 13.07 | 373.3 | 255.1 (10) | 185.1 (24) | 30 |
| cyclopropyl fentanyl | 7.45 | 349.2 | 105 (30) | 188.1 (20) | 25 |
| deschloro-N-ethyl-ketamine | 3.54 | 218.1 | 173.1(8) | 145.1 (16) | 38 |
| despropionyl para-fluorofentanyl | 7.10 | 299 | 105 (38) | 188 (16) | 15 |
| diclazepam | 11.39 | 321.1 | 89.3 (60) | 165.1 (54) | 20 |

(*continued on next page*)

**Table 1** (*continued*)

| Compound | RT (min) | Precursor ion | Quantifier ion (CE (V)) | Qualifier ion (s) (CE (V)) | Cone (V) |
|---|---|---|---|---|---|
| dimethylcathinone | 2.29 | 178.1 | 72 (22) | 105.3 (20) | 30 |
| ethcathinone | 2.65 | 177.7 | 72 (16) | 105.2 (22) | 30 |
| ethylone | 2.76 | 222 | 146 (26) | 204 (38) | 30 |
| ethylphenidate | 5.57 | 248.1 | 84.1 | 56 | 50 |
| etizolam | 10.18 | 343 | 138.1 (42) | 314.1 (26) | 36 |
| etodesnitazene | 3.91 | 352.2 | 100 (18) | 106.9 (40) | 52 |
| euthylone | 3.58 | 236.1 | 174 (36) | 188.1 (16) | 35 |
| fentanyl | 6.58 | 337.2 | 105.2 | 188.2 | 35 |
| flunitazene | 6.63 | 371.2 | 100 (26) | 72 (34) | 60 |
| furanyl fentanyl | 7.30 | 375.1 | 105 (38) | 188 (18) | 16 |
| furanyl norfentanyl | 3.95 | 271 | 84 (18) | 55 (38) | 16 |
| isobutyryl fentanyl | 7.90 | 351.3 | 105.1 (42) | 188.1 (22) | 30 |
| isotonitazene | 8.40 | 411.2 | 106.9 (46) | 100 (22) | 50 |
| JWH-007 | 13.55 | 356 | 127 (48) | 155 (26) | 45 |
| JWH-016 | 13.26 | 342 | 127 (44) | 155 (34) | 45 |
| JWH-018 | 13.63 | 342 | 127 (25) | 155 (34) | 45 |
| JWH-019 | 13.97 | 356 | 127 (44) | 228 (26) | 45 |
| JWH-081 | 13.91 | 372 | 185 (26) | 157.1 (40) | 45 |
| JWH-098 | 14.04 | 386 | 185 (26) | 127 (34) | 45 |
| JWH-122 | 13.76 | 356 | 169 (26) | 141 (40) | 45 |
| JWH-200 | 8.60 | 385 | 114 (46) | 155 (42) | 20 |
| JWH-203 | 13.64 | 340 | 125 (28) | 214 (22) | 45 |
| JWH-210 | 14.30 | 370 | 183 (26) | 214 (24) | 45 |
| JWH-250 | 12.98 | 336 | 91 (50) | 121 (32) | 45 |
| JWH-251 | 13.24 | 320 | 105 (22) | 214 (15) | 45 |
| JWH-302 | 12.90 | 336 | 213.9 (30) | 143.9 (44) | 45 |
| JWH-398 | 14.04 | 376 | 189 (26) | 161 (48) | 45 |
| ketamine | 3.62 | 238.2 | 125.1 (25) | 220.2 (15) | 20 |
| MDMB-4en-PICA | 12.80 | 357.21 | 212.1 (38) | 130.1 (20) | 36 |
| MDMB-4en-PINACA | 13.33 | 358.5 | 213.1 (31) | 298.19 (20) | 45 |
| MDMB-CHMICA | 13.20 | 385 | 240 (24) | 144 (46) | 20 |
| MDPV | 4.95 | 276 | 126.2 (25) | 135 (23) | 29 |
| mephedrone | 3.40 | 178.3 | 160.2 (12) | 145.1 (18) | 20 |
| methcathinone | 2.24 | 163.9 | 131 (20) | 104.8 (22) | 30 |
| methedrone | 2.83 | 194 | 176 (8) | 161 (13) | 20 |
| methoxpropamine | 5.12 | 262.2 | 203.1 (10) | 121.1 (30) | 40 |
| methoxyacetyl fentanyl | 5.57 | 353.3 | 188 (20) | 84.1 (18) | 30 |
| methoxyacetyl norfentanyl | 2.31 | 249 | 84 (14) | 55 (38) | 15 |
| methylone | 2.34 | 208 | 160.21 (15) | 132.1 (27) | 30 |
| metodesnitazene | 2.88 | 338.2 | 100.1 (22) | 72.1 (34) | 48 |
| metonitazene | 6.37 | 383.2 | 100 (22) | 121 (34) | 48 |
| MMB-2201 | 11.87 | 363.3 | 231.9 (12) | 143.9 (38) | 34 |
| N-ethyl pentylone | 4.69 | 250.2 | 202.2 (18) | 232.2 (13) | 24 |
| N-pyrrolidino etonitazene | 7.29 | 395.21 | 98.1 (22) | 107 (22) | 52 |
| N,N-dimethyltriptamine | 3.01 | 189.2 | 58 (12) | 117 (34) | 20 |
| norfentanyl | 3.66 | 233.1 | 84.3 (20) | 55.3 (34) | 25 |
| norketamine | 3.40 | 224.1 | 125 (20) | 207.1 (10) | 20 |
| ocfentanyl | 5.85 | 371.2 | 105.2 (40) | 188.2 (24) | 30 |
| para-fluoro-furanyl fentanyl | 7.68 | 393.2 | 105.1 (40) | 188.1 (22) | 25 |
| pentylone | 3.56 | 236 | 188 (18) | 218 (10) | 35 |
| phenyl fentanyl | 8.38 | 385 | 105 (46) | 188 (24) | 40 |
| phenylacetyl fentanyl | 9.28 | 399 | 188 (24) | 105 (46) | 46 |
| pravadoline (WIN 48,098) | 7.47 | 379.2 | 135 (24) | 114 (32) | 45 |
| RCS-4 | 13.25 | 322 | 135 (24) | 106.8 (40) | 20 |
| RCS-8 | 14.10 | 376 | 121 (26) | 91 (40) | 45 |
| ritalinic acid | 3.47 | 220.1 | 84.1 (20) | 56 (46) | 20 |
| UR144 | 14.27 | 312.4 | 125.1 (22) | 144.1 (38) | 42 |
| valeryl fentanyl carboxy metabolite | 5.70 | 395 | 188 (26) | 105 (44) | 40 |
| **"Classic" Drugs of Abuse (DOA)** | | | | | |
| 6-monoacetylmorphine | 2.61 | 328.1 | 165.1 (40) | 181.2 (40) | 30 |
| amphetamine | 2.65 | 136.1 | 119.1 (8) | 91.1 (15) | 20 |
| benzoylecgonine | 3.65 | 290.1 | 168.1 (20) | 105.1 (33) | 30 |
| cannabidiol | 12.01 | 315.15 | 123.1 (32) | 193.15 (20) | 35 |
| cocaethylene | 6.16 | 318.1 | 196.1 (20) | 82.1 (30) | 30 |
| cocaine | 5.11 | 304.2 | 182.26 (20) | 82.3 (28) | 30 |
| codeine | 2.16 | 300.1 | 215.1 (25) | 199.2 (27) | 30 |
| delta-9-THC | 13.5 | 315.2 | 193.1 (22) | 123 (34) | 35 |
| ecgonine methyl ester | 0.91 | 200.2 | 82.1 (23) | 182.1 (17) | 35 |
| 2-ethylidene-1.5-dimethyl-3.3-diphenylpyrrolidine | 8.18 | 278.2 | 234.2 (26) | 186.2 (35) | 50 |
| methylenedioxyamphetamine | 2.75 | 180.1 | 163.1 (10) | 133.1 (18) | 20 |
| methylenedioxymethamphetamine | 2.86 | 194.1 | 163 (14) | 133.1 (20) | 20 |
| methadone | 8.61 | 310.3 | 105.1 (32) | 265.2 (14) | 30 |
| methamphetamine | 2.57 | 150.1 | 119.1 (10) | 91.1 (12) | 20 |

(*continued on next page*)

**Table 1** (*continued*)

| Compound | RT (min) | Precursor ion | Quantifier ion (CE (V)) | Qualifier ion (s) (CE (V)) | Cone (V) |
|---|---|---|---|---|---|
| morphine | 1.32 | 286 | 165.1 (40) | 153 (40) | 35 |
| **Internal Standards (ISs)** | | | | | |
| fentanyl-d5 | 6.56 | 342.2 | 105.2 (38) | 188.2 (30) | 40 |
| JWH-122-d9 | 14.05 | 365.2 | 114.9 (35) | 169 (35) | 50 |
| ketamine-d4 | 3.28 | 242.2 | 129.1 (30) | 242 (10) | 35 |
| nordiazepam-d5 | 8.89 | 276.1 | 165.1 (28) | 213.0 (28) | 50 |

procedure. QCs were spiked with an independent working solution.

### 2.4. UHPLC–MS/MS conditions

The analysis was performed using a Waters Acquity (Ultra High Performance Liquid Chromatography) UHPLC® (Milford, MA), coupled to a quadrupole mass detector Waters Xevo TQD 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) set at 40 °C and injection volume was 2 µl. The mobile phases used were A – water 0.1 % formic acid and B – acetonitrile 0.1 % formic acid. 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 MS was operated with positive ionization in Multiple Reaction Monitoring (MRM) mode. Specific MRM transitions and collision energies were determined on the basis of the LC-MS/MS method for the detection of 182 novel psychoactive substances in whole blood already published [8] and of the literature, on substances tuned with the same MS-device. Parameter optimization was achieved through a series of experiments carried out by consecutive injection of individual standard solution at a concentration of 1 µg/ml. This also allowed to exclude inter-compounds interferences. At least two characteristic transitions were chosen for each analyte. Due to the high number of scans per chromatographic peaks, two different MS methods were developed, each involving half of the substances. A total of two injections were carried out, one per MS/MS method. Optimized MS parameters were as follows: capillary voltage 3.50 kV, desolvation gas temperature 650°C, source gas flow (nitrogen) desolvation rate 1200 l/h, cone 20 l/h, gas in collision argon.

The optimal transitions, respective cone and collision energies, retention time and ISs used for validation of all compounds are summarized in Table 1.

### 2.5. Method validation

The method was validated according to the European Medicine Academy (EMA) guidelines [24], evaluating the following analytical parameters for all analytes: selectivity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), matrix effect, recovery.

Drug-free hair samples from six different individuals were analyzed to assess selectivity, to determine the interference by endogenous hair constituents at the retention times of our analytes of interest. Absence of interfering compounds was accepted if the response was less than 20 % of the lower limit of quantification (LLOQ) for the analytes and 5 % for the IS. Immediately after the Upper Limit of Quantification (ULOQ) of every calibration curve, replicates of blank sample were analyzed to determine the carry-over. Results for blank sample following ULOQ should not be greater than 20 % of the LLOQ and 5 % for the IS. Seven-point calibration curves were prepared by spiking appropriate amounts of working solution in blank hair samples to obtain final concentrations of 4, 10, 40, 80, 160, 320 and 640 pg/mg. Each calibration batch included a blank sample spiked with IS only (zero sample), that was not included in the calibration curve. Quantification was achieved by plotting the peak area ratios of the single analyte and the coupled IS. Masslynx Software (Waters, USA) was used for this scope. Back-calculated concentrations should be within ±15 % (±20 % for the LLOQ) of the nominal concentrations and at least 75 % of the calibration points must fulfill this criterion to prove linearity [25]. The LLOQ was selected as the lowest concentration point with an accuracy and precision of ± 20 %, and a S/N > 10. To assess the LOD, the LLOQ calibrator was diluted 1:3. Three separate samples were analyzed in duplicate for at least three runs. The LOD was accepted if the analysis: 1) yields a reproducible instrument response greater than or equal to three times the noise level of the background signal from the negative samples, for both quantifier and qualifier ions and 2) achieves acceptable retention time and peaks.

Intra and inter day precision (coefficient of variation CV %) and accuracy (bias %) were determined at four concentration levels: LLOQ (4 pg/mg), QC Low (LQC, 10 pg/mg), QC Medium (MQC, 320 pg/mg), and QC High (HQC, 640 pg/mg). Intra-day assay was established processing 6 replicates of each QC and LLOQ on the same day. Inter day assay was established processing 6 replicates of each QC and LLOQ on three different days. Accuracy and precision were obtained by bias calculation and relative errors, through Masslynx software (Waters, USA). Accuracy and precision of ±15 % for QC levels and of ±20 % for LLOQ, were required.

Percent Matrix Effect (ME) and Extraction Recovery (ER) were calculated at three concentration levels (Low, Medium, and High) by means of the following equations: ME (%) =B/A x 100. ER (%) =C/B x 100. Where:

A= analyte/IS mean peak area ratio obtained by injecting *extraction solvent* (N=3) spiked at three concentration levels.

B= analyte/IS mean peak area ratio obtained by injecting *drug-free matrix extracts* (N=3) spiked at three concentration levels *after extraction*.

C= analyte/IS mean peak area ratio obtained by injecting *drug-free matrix extracts* (N=3) spiked at three concentration levels *before extraction*.

ME and RE were tested by analyzing blank hair matrices from six different sources. The CV of the ME calculated should not exceed 15 % [26].

### 2.6. Application to authentic forensic samples

As a proof of the applicability of the method, the developed and validated method was applied to 22 forensic hair samples, received from the University-Hospital of Bari, and collected from 3 women and 18 men. The age of the subjects ranged from 21 to 60 years.

Hair samples were collected from patients submitted to hair drug analysis due to a suspect of substance abuse disorder or from private subjects asking for hair drug analysis. After performing the analysis according to the forensic request, an aliquot was anonymized and used for the purpose of the study. All samples were stored at room temperature in a dark environment until analysis.

**Table 2**
Average percentage values of intra and inter day precision (coefficient of variation CV %) and accuracy (bias %). (*value that exceeded the limit required by the guidelines).

| Compound | Intra-day precision (Average CV %; n = 6) | | | | Inter-day precision (Average CV %; n = 18) | | | | Accuracy (Average bias %; n = 18) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LOQ | LQC | MQC | HQC | LOQ | LQC | MQC | HQC | LOQ | LQC | MQC | HQC |
| **New Psychoactive Substances** | | | | | | | | | | | | |
| (±)-cis-3-methyl Norfentanyl | 14.1 | 4.0 | 2.0 | 1.0 | 7.3 | 2.3 | 4.2 | 0.7 | 11.9 | 5.1 | 4.6 | 4.9 |
| (±)-trans-3-methyl Norfentanyl | 13.5 | 3.9 | 12.2 | 0.7 | 0.2 | 3.5 | 3.0 | 0.2 | 12.5 | 5.0 | 5.4 | 5.6 |
| (R)−5F-ADB | 14.1 | 11.5 | 13.8 | 1.7 | 8.9 | 5.8 | 4.0 | 1.3 | 18 | 3.4 | 3.6 | 4.6 |
| 2-fluoro deschloroketamine | 11.1 | 3.8 | 11.1 | 0.8 | 20.3* | 1.6 | 8.3 | 1.1 | 6.4 | 7.2 | 4.3 | 0.6 |
| 2-methyl AP-237 | 5.0 | 3.0 | 2.7 | 9.4 | 15.8 | 7.1 | 5.5 | 1.0 | 8.5 | 1.5 | 6.9 | 3.5 |
| 3-MeO PCE | 8.4 | 13.7 | 8.9 | 7.3 | 17.3 | 11.5 | 4.8 | 0.9 | 2.3 | 4.6 | 5.3 | 5.0 |
| 3-methylmethcathinone | 13.5 | 3.6 | 8.4 | 2.3 | 20.4* | 2.0 | 3.0 | 0.6 | 7.6 | 1.5 | 10.2 | 0.3 |
| 3.4 MD-alpha-PHP | 8.3 | 4.7 | 0.5 | 2.7 | 15.4 | 2.0 | 13.0 | 2.0 | 13.6 | 6.9 | 3.8 | 8.2 |
| 3.4-dimethylmethcathinone (3,4-DMMC) | 6.9 | 10.5 | 3.8 | 2.6 | 17.8 | 4.0 | 2.8 | 5.3 | 12.7 | 5.4 | 7.3 | 2.1 |
| 4-AcO-DiPT | 1.6 | 1.8 | 1.6 | 12.5 | 6.5 | 6.7 | 7.2 | 8.8 | 14.2 | 4.0 | 5.0 | 1.7 |
| 4-ANPP | 3.3 | 5.0 | 0.2 | 2.8 | 11.1 | 2.0 | 4.5 | 0.2 | 12.3 | 6.4 | 7.5 | 2.3 |
| 4 F-MDMB-BUTICA | 9.2 | 5.3 | 4.8 | 2.2 | 12.8 | 13.6 | 1.5 | 1.2 | 12.8 | 7.4 | 7.9 | 4.5 |
| 4-fluoromethcathinone (flephedrone) | 19.2 | 4.0 | 6.8 | 1.7 | 11.1 | 10.7 | 6.9 | 1.8 | 18.1 | 5.0 | 3.6 | 5.6 |
| 4-hydroxy DET | 18.6 | 2.4 | 14.5 | 3.0 | 16.9 | 8.3 | 1.3 | 10.4 | 15.9 | 4.2 | 12.6 | 7.3 |
| 4-methylethcathinone | 12.5 | 3.0 | 0.7 | 2.0 | 6.7 | 12.4 | 8.4 | 10.5 | 1.8 | 6.4 | 11.9 | 0.3 |
| 5Cl-AB-PINACA | 15.6 | 14.0 | 9.0 | 10.9 | 11.4 | 14.6 | 7.2 | 1.3 | 4.6 | 2.5 | 3.8 | 4.0 |
| 5Cl-THJ-018 | 16.6 | 11.2 | 2.5 | 9.7 | 5.0 | 1.6 | 2.0 | 15.4 | 3.9 | 4.0 | 1.4 | 0.1 |
| 5-EAPB | 14.8 | 4.8 | 12.4 | 5.6 | 7.5 | 2.9 | 2.7 | 1.7 | 15.6 | 9.7 | 2.9 | 6.6 |
| 5F-AKB48 (5 F-APINACA) | 21.2* | 3.0 | 8.6 | 9.0 | 15.1 | 2.0 | 6.4 | 2.0 | 8.7 | 13.4 | 4.6 | 9.9 |
| 5F-APP-PICA | 8.1 | 3.4 | 10.5 | 7.3 | 4.8 | 3.6 | 9.6 | 1.1 | 9.6 | 9.9 | 3.2 | 5.5 |
| 5F-APP-PINACA | 11.3 | 10.8 | 10.7 | 1.3 | 14.8 | 2.5 | 14.9 | 0.1 | 7.4 | 5.6 | 3.3 | 13.7 |
| 5F-Cumyl-P7AICA | 17.1 | 3.0 | 8.7 | 5.1 | 1.1 | 4.2 | 4.5 | 0.9 | 17.5 | 3.0 | 2.7 | 9.3 |
| 5F-Cumyl-PEGACLONE | 18.4 | 7.1 | 4.0 | 0.8 | 11.9 | 6.5 | 4.3 | 1.1 | 11.1 | 0.4 | 13.7 | 3.0 |
| 5F-Cumyl-PICA | 16.5 | 2.1 | 3.0 | 10.1 | 7.1 | 11.5 | 4.9 | 2.4 | 5.3 | 8.5 | 9.3 | 5.2 |
| 5F-Cumyl-PINACA | 11.8 | 6.7 | 6.1 | 7.2 | 16.1 | 8.6 | 1.4 | 3.5 | 24.6 | 3.4 | 8.5 | 1.1 |
| 5F-EDMB-PICA | 4.9 | 6.4 | 6.9 | 7.8 | 10.0 | 12.5 | 2.7 | 3.0 | 7.4 | 3.3 | 14.4 | 5.0 |
| 5F-EMB-PICA | 16.3 | 13.1 | 11.3 | 5.9 | 7.5 | 7.2 | 1.1 | 1.6 | 2.1 | 7.4 | 14.8 | 3.0 |
| 5F-NNEI 2′-naphtyl isomer | 11.7 | 10.6 | 2.0 | 1.1 | 9.6 | 3.0 | 12.3 | 2.0 | 8.1 | 0.6 | 4.8 | 1.4 |
| 5-hydroxytryptophan | 9.2 | 6.1 | 4.0 | 4.8 | 9.6 | 3.5 | 12.1 | 2.9 | 7.9 | 2.9 | 0.7 | 4.0 |
| 5-MeO-AMT | 15.1 | 11.4 | 3.0 | 2.6 | 9.1 | 3.0 | 4.4 | 1.7 | 9.8 | 5.0 | 0.2 | 0.8 |
| 5-MeO-DALT | 16.8 | 8.4 | 14.0 | 3.6 | 7.1 | 10.4 | 4.7 | 1.1 | 15.7 | 14.9 | 7.4 | 1.8 |
| 5-MeO-DMT | 4.0 | 4.4 | 6.4 | 9.4 | 7.0 | 1.4 | 2.8 | 1.4 | 19.2 | 14.0 | 9.1 | 0.3 |
| 5-MeO-DPT | 20.6* | 13.0 | 22.8* | 8.9 | 4.2 | 7.5 | 12.6 | 7.2 | 18.9 | 11.8 | 12.3 | 6.4 |
| 5-MeO-MiPT | 8.2 | 0.8 | 10.9 | 1.4 | 15.7 | 4.5 | 3.9 | 2.1 | 5.2 | 9.3 | 12.2 | 2.8 |
| 5/6-APB | 14.0 | 6.9 | 7.1 | 4.5 | 5.3 | 2.9 | 7.2 | 6.9 | 17.3 | 6.3 | 9.1 | 0.1 |
| 5/6-MAPB | 16.4 | 8.3 | 6.9 | 5.2 | 13.1 | 6.9 | 5.2 | 1.5 | 20.0 | 5.6 | 8.4 | 2.2 |
| 5F-MDMB-P7AICA | 9.5 | 5.9 | 14.9 | 13.1 | 13.1 | 10.6 | 15.5 | 7.6 | 19.7 | 9.7 | 0.7 | 0.3 |
| 5F-MDMB-PICA | 12.3 | 14.0 | 9.6 | 2.9 | 10.4 | 7.0 | 2.9 | 0.6 | 22.5* | 1.5 | 3.5 | 0.8 |
| AB-CHMINACA | 24.2* | 15.8 | 6.0 | 1.3 | 11.1 | 6.6 | 17.7 | 11.4 | 12.0 | 2.4 | 2.0 | 2.9 |
| AB-FUBINACA | 9.3 | 16.4 | 8.6 | 4.2 | 3.2 | 3.4 | 0.3 | 5.6 | 14.3 | 0.8 | 3.1 | 3.5 |
| acetyl fentanyl | 15.9 | 17.5 | 6.7 | 1.9 | 21.9* | 8.2 | 6.3 | 4.9 | 19.0 | 12.5 | 2.2 | 5.1 |
| acetyl norfentanyl | 1.2 | 3.0 | 5.2 | 1.2 | 10.1 | 1.9 | 9.9 | 1.1 | 15.1 | 0.6 | 1.7 | 0.9 |
| ADB-FUBINACA | 18.2 | 12.3 | 4.7 | 9.2 | 22.7* | 8.2 | 2.5 | 2.2 | 5.1 | 5.0 | 1.6 | 8.1 |
| alpha-PHP | 15.0 | 2.1 | 2.2 | 8.8 | 2.2 | 8.9 | 15.2 | 2.4 | 13.5 | 7.3 | 2.4 | 8.5 |
| alfentanil | 10.9 | 6.4 | 7.7 | 3.1 | 23.6* | 1.3 | 9.4 | 3.2 | 19.5 | 6.3 | 1.3 | 7.4 |
| alpha-ethyltryptamine | 4.6 | 5.4 | 8.2 | 5.5 | 24.1* | 17.4 | 5.5 | 1.6 | 15.0 | 4.0 | 6.3 | 0.2 |
| AM 2201 | 17.9 | 2.0 | 4.1 | 4.1 | 8.9 | 18.6 | 4.8 | 1.2 | 14.3 | 3.0 | 1.3 | 2.9 |
| AM 2233 | 8.9 | 2.9 | 4.8 | 1.2 | 1.2 | 12.1 | 14.1 | 5.9 | 7.9 | 7.2 | 9.4 | 0.2 |
| AM-694 | 13.7 | 6.1 | 5.0 | 1.8 | 16.6 | 8.8 | 11.2 | 4.8 | 17.2 | 0.2 | 1.9 | 1.5 |
| AP-237 | 3.6 | 3.8 | 12.1 | 3.1 | 10.3 | 9.7 | 6.0 | 4.9 | 16.5 | 4.3 | 0.7 | 4.0 |
| APP-FUBINACA | 10.2 | 3.7 | 12.1 | 0.7 | 2.3 | 12.3 | 1.0 | 1.3 | 20.7 | 0.5 | 4.4 | 2.3 |
| bentazepam | 1.5 | 4.0 | 9.1 | 5.4 | 9.8 | 2.2 | 8.1 | 2.9 | 10.4 | 4.7 | 0.5 | 1.7 |
| beta-hydroxy fentanyl | 1.9 | 5.6 | 2.8 | 5.2 | 9.7 | 8.0 | 14.8 | 0.3 | 5.6 | 8.7 | 6.8 | 1.7 |
| beta-hydroxythiofentanyl | 15.6 | 16.3 | 6.0 | 12.7 | 7.4 | 13.2 | 9.2 | 6.9 | 8.5 | 8.8 | 2.9 | 0.2 |
| beta-phenyl fentanyl | 7.2 | 9.2 | 7.4 | 1.3 | 9.3 | 11.5 | 13.3 | 7.2 | 15.8 | 3.2 | 5.3 | 2.6 |
| brorphine | 4.4 | 0.6 | 4.2 | 0.6 | 7.8 | 8.9 | 2.0 | 2.4 | 7.0 | 6.2 | 3.2 | 0.5 |
| buphedrone | 5.3 | 1.9 | 7.0 | 8.6 | 16.8 | 3.6 | 1.9 | 1.6 | 4.6 | 3.2 | 3.2 | 6.0 |
| butonitazene | 14.9 | 14.5 | 7.8 | 0.8 | 3.0 | 8.2 | 14.5 | 8.0 | 2.1 | 1.6 | 5.3 | 2.7 |
| butylone | 13.9 | 7.3 | 4.8 | 0.4 | 16.2 | 1.5 | 7.3 | 1.6 | 1.6 | 7.8 | 6.2 | 0.3 |
| butyryl fentanyl | 17.6 | 11.4 | 6.9 | 0.6 | 7.7 | 2.3 | 11.4 | 8.0 | 22.3* | 6.8 | 2.5 | 7.7 |
| butyryl fentanyl carboxy metabolite | 11.4 | 1.2 | 4.9 | 6.5 | 0.4 | 1.7 | 1.2 | 0.7 | 4.2 | 12.2 | 1.4 | 5.2 |
| butyryl norfentanyl | 10.6 | 9.3 | 9.7 | 1.9 | 8.5 | 13.3 | 2.5 | 2.9 | 6.0 | 9.8 | 2.7 | 11.3 |
| carfentanil | 7.5 | 8.5 | 3.1 | 1.3 | 14.8 | 3.3 | 6.1 | 1.1 | 1.5 | 2.8 | 5.5 | 5.7 |
| clonazolam | 15.8 | 13.3 | 2.6 | 2.6 | 15.0 | 9.0 | 5.2 | 6.7 | 16.0 | 1.5 | 0.6 | 2.2 |
| Cumyl-PEGACLONE | 22.4* | 6.4 | 3.6 | 1.1 | 17.3 | 2.1 | 2.7 | 1.3 | 15.1 | 5.3 | 5.4 | 0.6 |
| cyclopropyl fentanyl (hydrochloride) | 14.8 | 11.5 | 2.2 | 0.7 | 3.6 | 2.6 | 6.1 | 3.0 | 6.9 | 3.7 | 6.6 | 3.2 |

(*continued on next page*)

**Table 2** (*continued*)

| Compound | Intra-day precision (Average CV %; n = 6) | | | | Inter-day precision (Average CV %; n = 18) | | | | Accuracy (Average bias %; n = 18) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LOQ | LQC | MQC | HQC | LOQ | LQC | MQC | HQC | LOQ | LQC | MQC | HQC |
| deschloro-N-ethyl-ketamine | 16.5 | 8.5 | 6.5 | 6.1 | 11.4 | 12.5 | 10.9 | 4.2 | 11.3 | 21.8 | 10.6 | 2.4 |
| despropionyl para-fluorofentanyl | 12.9 | 8.4 | 8.6 | 3.0 | 16.6 | 1.0 | 9.2 | 1.5 | 12.0 | 1.3 | 4.2 | 5.3 |
| diclazepam | 15.1 | 6.0 | 16.3 | 6.9 | 7.0 | 11.3 | 9.4 | 5.0 | 13.0 | 13.5 | 4.0 | 4.0 |
| dimethylcathinone | 17.8 | 8.6 | 25.4 | 6.5 | 6.2 | 14.4 | 7.9 | 14.4 | 13.5 | 14.6 | 8.5 | 2.1 |
| ethcathinone | 4.6 | 8.3 | 5.6 | 12.9 | 11.0 | 10.8 | 10.2 | 0.9 | 3.2 | 5.4 | 4.3 | 0.2 |
| ethylone | 18.6 | 10.6 | 21.7 | 6.2 | 17.0 | 2.5 | 5.1 | 1.6 | 14.0 | 13.3 | 3.8 | 3.9 |
| ethylphenidate | 17.1 | 13.7 | 9.2 | 8.1 | 11.6 | 4.9 | 6.6 | 11.5 | 20.7* | 7.3 | 2.5 | 2.8 |
| etizolam | 7.0 | 4.9 | 12.8 | 3.1 | 9.9 | 4.0 | 4.8 | 5.1 | 12.4 | 12.1 | 4.0 | 0.8 |
| etodesnitazene (etazene) | 20.2 | 3.3 | 12.8 | 8.9 | 14.0 | 4.4 | 3.6 | 2.8 | 7.2 | 12.4 | 14.6 | 0.3 |
| euthylone | 7.1 | 8.6 | 13.2 | 2.8 | 5.4 | 8.1 | 5.0 | 13.3 | 12.0 | 4.1 | 5.9 | 2.9 |
| fentanyl | 17.5 | 2.3 | 4.3 | 0.9 | 14.4 | 4.4 | 3.6 | 2.8 | 5.6 | 6.1 | 1.8 | 3.8 |
| flunitazene | 16.4 | 6.3 | 11.5 | 2.8 | 16.2 | 1.7 | 3.7 | 5.5 | 8.9 | 8.4 | 7.8 | 1.6 |
| furanyl fentanyl | 2.7 | 16.5 | 4.2 | 13.5 | 3.3 | 10.1 | 12.6 | 3.8 | 14.9 | 14.7 | 1.6 | 1.7 |
| furanyl norfentanyl | 15.3 | 14.5 | 19.5 | 7.4 | 11.6 | 14.3 | 10.6 | 6.7 | 11.9 | 12.8 | 2.0 | 0.1 |
| isobutyryl fentanyl | 16.4 | 7.3 | 7.2 | 8.2 | 4.2 | 3.5 | 2.4 | 11.3 | 12.1 | 8.6 | 3.9 | 1.7 |
| isotonitazene | 3.0 | 13.5 | 11.1 | 10.3 | 4.0 | 9.3 | 8.7 | 6.0 | 5.8 | 1.5 | 3.8 | 1.4 |
| JWH-007 | 10.3 | 7.1 | 7.9 | 5.3 | 8.7 | 6.8 | 4.9 | 2.6 | 21.7* | 6.1 | 2.1 | 3.3 |
| JWH-016 | 19.2 | 3.2 | 9.7 | 2.7 | 15.6 | 14.4 | 3.6 | 9.4 | 0.6 | 3.5 | 6.8 | 0.4 |
| JWH-018 | 21.1* | 10.9 | 8.8 | 3.3 | 10.2 | 10.9 | 9.1 | 3.7 | 19.6 | 6.4 | 8.6 | 4.8 |
| JWH-019 | 2.2 | 5.0 | 8.6 | 10.8 | 7.3 | 7.3 | 5.7 | 1.9 | 8.1 | 6.2 | 2.3 | 1.4 |
| JWH-081 | 14.3 | 14.9 | 9.2 | 1.8 | 0.5 | 5.3 | 4.7 | 8.1 | 8.9 | 4.9 | 4.9 | 2.3 |
| JWH-098 | 17.9 | 1.7 | 13.7 | 11.8 | 11.9 | 6.8 | 6.2 | 5.2 | 3.7 | 5.6 | 2.7 | 2.3 |
| JWH-122 | 15.8 | 15.7 | 8.9 | 10.6 | 15.7 | 10.8 | 8.6 | 11.3 | 15.8 | 1.2 | 0.1 | 3.1 |
| JWH-200 | 6.8 | 5.9 | 3.0 | 9.0 | 4.7 | 14.6 | 7.8 | 14.4 | 13.3 | 13.8 | 6.2 | 11.3 |
| JWH-203 | 18.4 | 12.5 | 1.2 | 10.0 | 0.5 | 4.2 | 7.0 | 9.4 | 14.7 | 11.1 | 9.6 | 4.8 |
| JWH-210 | 22.4* | 15.6 | 9.9 | 7.8 | 11.6 | 6.2 | 2.1 | 22.7 | 11.8 | 1.6 | 6.6 | 0.9 |
| JWH-250 | 16.5 | 2.9 | 5.6 | 6.0 | 2.3 | 2.7 | 0.6 | 17.2 | 13.8 | 6.0 | 6.0 | 2.5 |
| JWH-251 | 13.0 | 1.1 | 13.4 | 5.9 | 2.4 | 4.7 | 1.4 | 16.5 | 7.8 | 4.3 | 2.6 | 3.4 |
| JWH-302 | 15.9 | 2.2 | 4.5 | 5.0 | 2.9 | 0.4 | 1.2 | 20.7 | 4.9 | 18.9 | 0.3 | 0.3 |
| JWH-398 | 27.1* | 11.7 | 8.1 | 1.6 | 8.2 | 9 | 14.7 | 10.4 | 8.2 | 2.7 | 4.6 | 1.2 |
| ketamine | 7.1 | 1.4 | 13.0 | 3.0 | 5.2 | 7.7 | 17.1 | 5.6 | 3.4 | 14.8 | 6.9 | 1.0 |
| MDMB-4en-PICA | 8.0 | 4.1 | 13.1 | 4.1 | 6.4 | 13.8 | 9.3 | 0.9 | 7.3 | 9.3 | 7.4 | 2.4 |
| MDMB-4en-PINACA | 16.1 | 11.2 | 5.1 | 0.7 | 13.7 | 3.1 | 0.8 | 6.5 | 7.9 | 3.7 | 1.1 | 9.7 |
| MDMB-CHMICA | 5.3 | 15.5 | 10.5 | 0.5 | 12.5 | 16.7 | 5.9 | 10.7 | 15.8 | 1.3 | 3.1 | 6.4 |
| MDPV | 9.3 | 2.6 | 20.3 | 5.0 | 12.4 | 0.3 | 8.1 | 11.1 | 3.1 | 5.3 | 7.9 | 4.3 |
| mephedrone | 2.8 | 10.1 | 12.0 | 4.4 | 1.1 | 15.7 | 13.7 | 22.3 | 6.4 | 3.9 | 9.8 | 2.2 |
| methcathinone | 12.2 | 5.1 | 2.1 | 6.8 | 11.9 | 4.1 | 13.5 | 11.0 | 0.5 | 2.4 | 10.9 | 0.8 |
| methedrone | 1.0 | 4.3 | 9.3 | 8.1 | 6.3 | 8.6 | 24.0 | 6.9 | 10.0 | 5.9 | 5.7 | 0.3 |
| methoxpropamine (mxpr) | 13.6 | 3.8 | 3.1 | 1.6 | 6.8 | 13.3 | 13.6 | 7.0 | 10.4 | 13.7 | 6.1 | 0.9 |
| methoxyacetyl fentanyl | 4.5 | 6.8 | 6.4 | 11.0 | 11.4 | 11.3 | 14.7 | 12.1 | 4.8 | 3.6 | 3.7 | 1.2 |
| methoxyacetyl norfentanyl | 18.6 | 11.6 | 0.2 | 15.0 | 8.5 | 6.1 | 7.6 | 8.5 | 3.3 | 5.0 | 1.5 | 6.1 |
| methylone | 1.0 | 3.3 | 5.9 | 1.3 | 14.5 | 6.1 | 0.8 | 8.0 | 16.5 | 12.2 | 4.1 | 1.8 |
| metodesnitazene | 0.4 | 2.4 | 12.9 | 2.4 | 1.0 | 11.9 | 6.6 | 4.8 | 14.2 | 5.5 | 2.1 | 6.5 |
| metonitazene | 2.9 | 24.6 | 5.3 | 3.9 | 2.4 | 5.9 | 13.7 | 3.6 | 0.5 | 14.3 | 7.2 | 2.4 |
| MMB-2201 | 8.4 | 10.8 | 11.0 | 0.6 | 4.2 | 10.8 | 6.2 | 14.3 | 12.5 | 11.4 | 6.0 | 1.9 |
| N-ethyl pentylone | 3.0 | 18.5 | 5.4 | 1.3 | 15.3 | 13.2 | 4.3 | 7.2 | 13.7 | 5.3 | 0.9 | 1.2 |
| N-pyrrolidino etonitazene | 17.5 | 1.3 | 5.7 | 4.5 | 15.5 | 8.0 | 5.3 | 1.9 | 10.5 | 4.8 | 1.5 | 0.2 |
| N,N-DMT | 22.8* | 16.4 | 6.6 | 1.6 | 5.1 | 7.3 | 0.4 | 3.6 | 1.8 | 11.1 | 5.1 | 2.8 |
| norfentanyl | 10.4 | 17.1 | 0.7 | 4.1 | 5.3 | 7.6 | 2.0 | 2.4 | 5.7 | 6.1 | 3.7 | 7.2 |
| norketamine | 13.8 | 12.5 | 0.7 | 3.7 | 20.0 | 2.3 | 6.6 | 14.2 | 15.5 | 15.4 | 0.6 | 1.3 |
| ocfentanyl | 13.3 | 1.9 | 3.4 | 1.6 | 10.2 | 13.3 | 3.9 | 6.5 | 7.9 | 9.1 | 13.0 | 0.2 |
| para-fluoro-furanyl fentanyl | 3.8 | 10.5 | 13.6 | 2.3 | 16.2 | 8.0 | 2.7 | 14.0 | 8.4 | 3.9 | 1.6 | 4.2 |
| pentylone | 6.9 | 14.3 | 11.3 | 9.6 | 0.2 | 0.8 | 5.2 | 11.1 | 5.0 | 1.5 | 1.2 | 0.7 |
| phenyl fentanyl | 1.9 | 14.2 | 17.2 | 6.6 | 14.4 | 4.9 | 6.3 | 10.6 | 7.5 | 9.0 | 3.2 | 4.7 |
| phenylacetyl fentanyl | 9.4 | 2.3 | 2.8 | 3.2 | 5.7 | 12.2 | 8.1 | 17.9 | 2.2 | 11.6 | 4.4 | 0.4 |
| pravadoline (WIN 48,098) | 13.1 | 14.5 | 2.3 | 0.1 | 6.4 | 11.6 | 8.1 | 11.3 | 2.6 | 5.2 | 7.6 | 4.1 |
| RCS-4 | 14.1 | 5.5 | 0.5 | 0.4 | 6.0 | 14.0 | 13.0 | 4.1 | 14.1 | 12.4 | 7.4 | 3.6 |
| RCS-8 | 20.7 | 5.8 | 0.6 | 0.1 | 6.3 | 7.3 | 8.8 | 1.5 | 7.6 | 15.2 | 7.2 | 1.5 |
| ritalinic acid | 17.2 | 11.9 | 4.5 | 2.7 | 13.8 | 4.1 | 6.9 | 4.6 | 9.9 | 5.7 | 2.6 | 1.9 |
| UR144 | 6.3 | 11.2 | 11.9 | 11.1 | 7.0 | 5.2 | 6.3 | 4.0 | 9.5 | 8.1 | 13.5 | 5.1 |
| valeryl fentanyl carboxy metabolite | 11.4 | 11.1 | 11.1 | 10.1 | 6.0 | 0.7 | 13.8 | 13.0 | 3.6 | 1.5 | 1.9 | 5.1 |
| **Classic Drugs of Abuse (DOA)** | | | | | | | | | | | | |
| 6-MAM | 15.4 | 11.4 | 11.2 | 7.3 | 13.9 | 1.3 | 5.3 | 6.1 | 2.1 | 4.3 | 2.4 | 4.3 |
| amphetamine | 17.2 | 5.9 | 12.7 | 14.3 | 1.2 | 6.3 | 8.1 | 0.3 | 12.0 | 7.1 | 4.0 | 10.6 |
| benzoylecgonine | 11.7 | 14.9 | 4.0 | 11.3 | 16.7 | 10.6 | 9.6 | 5.1 | 2.4 | 8.0 | 6.0 | 4.3 |
| cannabidiol | 15.7 | 13.2 | 10.6 | 9.6 | 8.3 | 7.5 | 2.2 | 5.5 | 8.0 | 11.1 | 6.4 | 1.0 |
| cocaethylene | 17.9 | 3.7 | 8.5 | 4.9 | 13.5 | 9.0 | 11.6 | 0.8 | 8.5 | 10.1 | 10.8 | 6.0 |
| cocaine | 4.0 | 9.8 | 12.3 | 2.6 | 8.5 | 3.1 | 5.3 | 8.9 | 2.9 | 11.9 | 4.0 | 2.8 |
| codeine | 20.8 | 13.4 | 15.8 | 6.2 | 4.3 | 4.3 | 2.4 | 9.8 | 9.6 | 3.1 | 11.4 | 6.2 |
| delta-9-THC | 15.0 | 6.7 | 6.2 | 4.9 | 13.4 | 3.7 | 2.7 | 14.0 | 15.2 | 6.4 | 3.9 | 4.4 |
| ecgonine methyl ester | 9.6 | 2.0 | 0.3 | 3.6 | 8.3 | 1.7 | 5.9 | 8.5 | 3.8 | 11.4 | 2.1 | 4.0 |
| EDDP | 18.7 | 7.9 | 6.6 | 14.7 | 17.4 | 12.2 | 4.4 | 0.2 | 15.2 | 4.7 | 6.0 | 2.3 |
| MDA | 3.3 | 11.8 | 11.1 | 6.1 | 12.7 | 3.1 | 1.3 | 4.5 | 8.3 | 0.4 | 13.3 | 7.4 |

(*continued on next page*)

**Table 2** (*continued*)

| Compound | Intra-day precision (Average CV %; n = 6) | | | | Inter-day precision (Average CV %; n = 18) | | | | Accuracy (Average bias %; n = 18) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LOQ | LQC | MQC | HQC | LOQ | LQC | MQC | HQC | LOQ | LQC | MQC | HQC |
| MDMA | 14.0 | 1.7 | 4.2 | 6.9 | 1.2 | 14.4 | 6.2 | 2.2 | 4.7 | 4.4 | 5.3 | 2.3 |
| methadone | 1.6 | 13.1 | 8.3 | 12.1 | 10.4 | 6.6 | 5.0 | 3.8 | 2.3 | 9.3 | 9.3 | 17.0 |
| methamphetamine | 18.8 | 2.7 | 0.1 | 0.5 | 13.9 | 1.5 | 15.9 | 16.3 | 8.6 | 2.1 | 5.3 | 7.3 |
| morphine | 13.5 | 0.8 | 8.8 | 12.6 | 4.6 | 14.0 | 6.0 | 3.7 | 4.9 | 2.6 | 3.1 | 1.8 |

## 3. Results

### 3.1. Method development

Successful validation was achieved for 135 analytes. Validation parameters, especially accuracy and precision are shown in Table 2. For all molecules, LOD was determined at 1/3 of the LLOQ, following the criteria listed in subsection 2.5.

Six drug-free hair samples coming from different sources were scrutinized to check for the presence of interfering peaks in MRM chromatograms where our analytes were expected to elute. No interfering peaks due to endogenous substances were detected. MRM chromatograms of drug-free hair samples running immediately after the ULOQ (640 pg/mg) showed no peak of our analytes of interest or IS, thus confirming that carry-over was negligible.

Back-calculated concentrations of calibrators were within ±15 % (±20 % for the LLOQ) for more than 75 % of the calibration points for all compounds. The method exhibited linear calibration functions for all the analytes of interest in the tested range, when a weighting factor 1/x was adopted for all the compounds. After assuming the regression model, $R^2$ was always better than 0.99 except for the 3 compounds (4-fluoromethcathinone, 5Cl-AB-PINACA, 5F-Cumyl-PINACA) which showed $R^2$=0.975–0.987. Nevertheless, the linearity of these compounds was considered acceptable. LOQs were 4 pg/mg, i.e. the lowest calibration point, for all the compounds (129), except for 13 compounds. Particularly, for 6 compounds, namely AB-CHMINACA, MDMB-CHMICA, mephedrone, methcathinone, 6-MAM and morphine, the LOQ was determined at 10 pg/mg. For 5F-MDMB-BUTICA, 5F-Cumyl-PEGACLONE, 5-hydroxytryptophan, cannabidiol, delta-9-THC, the LOQ was determined at 40 pg/mg and, finally, for 4-fluoromethcathinone and 5Cl-AB-PINACA the LOQ was at 80 pg/mg. A LLOQ of 40 pg/mg was accepted only for cannabidiol and delta-9-THC. NPS showing LLOQ from 40 pg/mg, which is higher than the expected concentration in authentic cases, were considered not validated for forensic purposes.

Intra and inter day precision (coefficient of variation CV %) and accuracy (bias %) of all analytes accordingly fit with the requirements of the EMA guidelines, with some exceptions in the LOQ controls, highlighted with "*" in Table 2. Particularly, 5 F-MDMB-PICA, butyryl fentanyl, ethylphenidate and JWH-007 showed a low accuracy at the LOQ. 5 F-AKB48, 5-MeO-DPT, AB-CHMINACA, Cumyl-PEGACLONE, JWH-018, JWH-210, JWH-398 and N,N-DMT showed low intra-day precision at the LOQ, while acetyl fentanyl, 2-fluoro deschloroketamine, 3-methylmethcathinone, ADB-FUBINACA, alfentanil and alpha-ethyltryptamine low inter-day precision at the LOQ. However, CV % or average bias was always in the range 20–25 with the only exception of the intra-day precision JWH-398.

%ME and %ER yield were calculated at three concentration levels (Low, Medium, High). With the chosen extraction procedure, matrix effect and extraction recovery of analytes under investigation were always acceptable. The matrix effect ranged from between 99 % and 113 % for LQC; 87–103 % for MQC, for 88–91 % HQC (range of the average value calculated for all the analytes). The extraction recovery was always above 80 %, ranging 82–89 % for LQC; 87–94 % for MQC, for 88–101 % HQC.

### 3.2. Application to authentic forensic samples

Considering the classical DoA, 17 samples out of 22 collected at the University of Bari tested positive (77.3 %), all but 1 being positive for cocaine and benzoylecgonine (BEC) (16 out of 22, 72.7 %). Additionally, 11 out of 22 tested positive for cocaethylene (50 %), 10 for ecgonine methyl ester (45.5 %), 6 for methadone (27.3 %), 4 of which also for 2-ethylene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (18.1 % of the total). Three samples tested positive for 6-monoacetylmorphine (6-MAM) (13.6 %), 2 for morphine and 2 for codeine (9.1 % each). MDMA, MDA and amphetamine tested positive in 3 (13.6 %), 2 (9.1 %) and 1 (4.5 %) cases. Lastly, delta-9-THC and cannabidiol were detected in 3 samples each (13.6 %).

Switching from classical DoA to NPS, 10 (45.5 %) out of 22 samples tested positive for NPS, 3 of which below the LLOQ, and the identified NPS consisted of 7 compounds. All cases positive for NPS also tested positive for DoA, except one case showing ketamine below the LLOQ and one displaying ritalinic acid. The most encountered NPS were arylalkylamines, particularly ketamine and 5-MMPA, positive each in 4 samples (18.2 %), sometimes combined. Fentanyl derivatives, particularly methoxyacetylfentanyl, was detected in 4 samples (18.2 %), although always in non-quantifiable amounts (< LLOQ). Three samples displayed ritalinic acid (13.6 %), 1 sample tested positive for a synthetic cannabinoid, RCS-4, and one for a synthetic cathinone, methylone.

More details of the positive samples among the authentic hair analyzed are shown in Table 3. Some compounds were above the ULOQ.

## 4. Discussion

Over the past years, NPS have been increasingly developed and their rapid turnover, together with the chemical diversity, represent major limits to their forensic identification [8,17]. In the present study, the choice of the psychotropic compounds to be included was based on epidemiological data related to the consumption of prescription and classic drugs of abuse in the metropolitan area of Bologna (for the DoA) [27] and by the availability of NPS certified standards provided by the National Health Institute and Comedical s.r.l. (Italy, Trento) within the national EWS project.

The choice of multi-analyte methods, with included substances pertaining to multiple classes, inherently leads to several challenges, first regarding the extraction procedure. It is well-known that the structure of hair is complex, and that drug incorporation and extraction is affected by multiple factors, including acidity/basicity and lipophilicity of compounds, as well as by melanin [20].

Indeed, several studies have suggested that melanin has a role in drug incorporation, with usual higher incorporation into pigmented hair for basic compounds like codeine, cocaine and methamphetamine. Given this binding, extraction conditions should be selected with caution to ensure release, and some enzymatic digestion/extraction method would allow to separately analyze drug content in the melanin and in the protein compartments of hairs [28,29].

Previous literature has often stated that a double or two-step extraction is fundamental, to avoid the loss of analytes and low recovery [22]. In the here-in reported method, the selection of the extraction phase was optimized according to the MRM peak shape and area, allowing to choose a single extractive procedure for all compounds, with good recovery and sensitivity. Particularly, a mixture of methanol and

**Table 3**
Forensic hair samples tested positive for NPS or DoA. Positive samples with concentrations under the LLOQ are reported as "<LLOQ (concentration in pg/mg)", given that the LOD corresponds to 1/3 of the LLOQ for all molecules. ULOQ = 640 pg/mg.

| N | NPS (pg/mg) | DoA (pg/mg) |
|---|---|---|
| #1 | ketamine 11.8 | methadone 11.5 |
| | methylone nq | EDDP <LLOQ (4) |
| | RCS-4 30.2 | cocaine >ULOQ |
| | 5MMPA nq | benzoylecgonine > ULOQ |
| | methoxyacetyl fentanyl nq | ecgonine methyl ester 265.9 |
| | | cocaethylene > ULOQ |
| | | amphetamine 69.1 |
| | | MDA 244.4 |
| | | MDMA >ULOQ |
| | | delta-9-THC <LLOQ (40) |
| #2 | ketamine 5.8 | cocaine 117.2 |
| | | benzoylecgonine 42.9 |
| | | cocaethylene <LLOQ (4) |
| | | MDMA <LLOQ (4) |
| | | delta-9-THC >ULOQ |
| | | cannabidiol <LLOQ (40) |
| #3 | ritalinic acid 18.2 | |
| #4 | ritalinic acid 21.9 | 6-MAM 28.3 |
| | 5MMPA nq | morphine 41.2 |
| | methoxyacetyl fentanyl nq | methadone <LLOQ (4) |
| | | cocaine > ULOQ |
| | | benzoylecgonine > ULOQ |
| | | ecgonine methyl ester 23.2 |
| | | cocaethylene >ULOQ |
| #5 | | cannabidiol <LLOQ (40) |
| #6 | | 6-MAM 185.1 |
| | | codeine 334.3 |
| | | morphine 38.9 |
| | | methadone <LLOQ (LOD 4) |
| | | EDDP <LLOQ (4) |
| | | cocaine > ULOQ |
| | | benzoylecgonine > ULOQ |
| | | ecgonine methyl ester 107.5 |
| | | cocaethylene 9.4 |
| #7 | ketamine nq | 6-MAM <LLOQ (10) |
| | 5MMPA nq | methadone <LLOQ (4) |
| | methoxyacetyl fentanyl nq | cocaine > ULOQ |
| | | benzoylecgonine > ULOQ |
| | | ecgonine methyl ester 15.8 |
| | | cocaethylene >ULOQ |
| | | delta-9-THC >ULOQ |
| | | cannabidiol <LLOQ (40) |
| #8 | ketamine > ULOQ | cocaine >ULOQ |
| | norketamine 204.6 | benzoylecgonine 471.8 |
| | | ecgonine methyl ester 15.8 |
| | | cocaethylene 98.2 |
| | | MDA <LLOQ (4) |
| | | MDMA 163.0 |
| #9 | ritalinic acid 15.6 | cocaine >ULOQ |
| | | benzoylecgonine >ULOQ |
| | | ecgonine methyl ester 269.0 |
| | | cocaethylene 109.2 |
| #10 | ketamine nq | |
| #12 | 5MMPA nq | cocaine >ULOQ |
| | | benzoylecgonine >ULOQ |
| | | ecgonine methyl ester 148.0 |
| | | cocaethylene 24.7 |
| #13 | | methadone 517.0 |
| | | EDDP 23.2 |
| | | cocaine 38.0 |
| | | benzoylecgonine 58.8 |
| #15 | | codeine 74.9 |
| | | cocaine 319.4 |
| | | benzoylecgonine 74.3 |
| #16 | | cocaine >ULOQ |
| | | benzoylecgonine >ULOQ |
| | | ecgonine methyl ester 49.6 |
| | | cocaethylene 127.4 |
| #17 | | cocaine 22.5 |
| | | cenzoylecgonine 12.5 |
| #18 | | cocaine >ULOQ |
| | | benzoylecgonine >ULOQ |
| | | ecgonine methyl ester 121.8 |
| | | cocaethylene >ULOQ |
| #19 | methoxyacetyl fentanyl nq | cocaine 167.1 |
| | | benzoylecgonine 72.0 |
| | | ecgonine methyl ester nq |
| | | cocaethylene 4.2 |
| #20 | | cocaine 39.9 |
| | | benzoylecgonine 13.9 |
| #21 | | methadone 11.4 |
| | | EDDP <LLOQ (4) |
| | | cocaine 50.7 |
| | | benzoylecgonine 18.7 |

water was chosen, to grant both a good extraction of synthetic cannabinoids and to provide good shape of the peaks for more hydrophilic and basic compounds [30]. Our single extraction procedure allowed to reduce the analytical time and to avoid the consumption and waste of matrix that could occur in the case of multiple preparations [8,30]. The chromatographic run was in fact completed in 17×2 minutes, which is considered efficient for routine application, given the wide range of compounds covered. In line with the literature, only 25 milligrams of matrix were used with the here-in reported method [30,31]. The multi-analyte method exhibited good linear calibration functions for 93 % of all the analytes of interest and good precision and accuracy at all concentrations evaluated, including the lowest level.

The choice of a wide-screen method for multiple NPS and DoA likely impacted the sensitivity of the method, so that it was not possible to select the lowest calibration point (4 pg/mg) as LLOQ for 7 out of 142 compounds (4.9 %), which had a LLOQ > 10 pg/mg, i.e. 5F-MDMB-BUTICA, 5F-Cumyl-PEGACLONE, 5-hydroxythriptophan, cannabidiol, delta-9-THC, 4-fluoromethcathinone and 5Cl-AB-PINACA and for these compounds the method was considered not validated for forensic purposes.

With the exception of these drugs, the LLOQs here-in determined for the vast majority of the tested compounds (4 pg/mg for 90.8 % of included substances) appear in line with those identified in similar multi-analyte hair methods [22,23,30–32], which are slightly higher compared to method targeting only one class of substances. Salomone et al. achieved LOQs in the range 0.7–4 pg/mg for most compounds, but considering only synthetic cannabinoids [33].

For delta-9-THC and cannabidiol, LLOQ are higher compared with other analytical methods, and not suitable for the identification of isolated intakes, but under the cut-off given by the Society of Hair Testing (SoHT) that enables identification of drug users [34]. Calibration ranges were selected in accordance with NPS concentrations typically found in the literature, considering both post-mortem and intra-vitam concentrations, that rarely go below 10 pg/mg [11,20,21,31,32]. Thus, the presented method is considered adequate for the measurement of NPS in hair of potential NPS users. Regarding DoA, it is not uncommon to detect very high levels of these drugs in head hair. However, the choice of cut-offs was driven mostly by needs to prove detectability and quantification at low, more than high concentration ranges. This is acknowledged as a potential limitation of the current method, although there are no established SoHT cut-offs at higher hair levels.

Taking into account the phenotype variability of the Italian population, hair of different color and structure (from curly to straight, from lighter to darker, including non-pigmented and dyed hair) were subjected to the study to assess matrix effect, always showing good results, and were also used to test the here-in developed study, proving its feasibility.

The method was applied on 22 authentic samples, and 10 out of 22 resulted positive, with the identification of 7 NPS. Despite the relatively low number of samples used for applicability, the prevalence of

ketamine and norketamine positive samples seems to be consistent with recent studies performed in Italy and substantiate the trustworthiness of the method [35]. Most of the NPS have been found to be below the LLOQ, therefore detectable only in trace amounts.

The present method has some strength and limitations. Among the limitations, it should be mentioned the use of a limited number of ISs, compared to the wide range of compounds included in the method. Nevertheless, as in a previously published multi-analyte method [8], the shortage of ISs did not prevent the achievement of good validation parameters, with particular reference to accuracy and precision. A future improvement of the method could be obtained including at least one IS for each class of NPS.

Not all compounds demonstrated high precision and accuracy, especially at the LOQ, with some compounds, especially pertaining to the group of synthetic cannabinoids, above the criteria established for validation. This is a common issue of multi-analyte methods. Nevertheless, CV % or accuracy bias $>$ 25 % was reported only for a single compound.

As a further limitation, the validated method was only applied to a limited number of forensic samples and the results here-in reported cannot be taken as an estimation of the prevalence of NPS on a local or national basis. Also, it was no possible to compare the results with self-reports from the subjects submitted to hair analysis, as suggested in the literature [36]. However, the rate of positive samples here-in shown suggests that the spreading of NPS might be relevant in Italy. Certainly, more epidemiological studies are needed to draw a clearer picture of the phenomenon, especially in high-risk populations. Future studies should be devoted to the application of the method on forensic cases with the purpose of long-term biomonitoring assessment in a clinical-forensic context but also in retrospective estimation of recent past exposure to xenobiotics in a post-mortem context.

## 5. Conclusions

The present methodology represents an easy, low cost, wide-panel method for the quantification of 137 drugs, including 122 NPS and 15 DoA, in hair samples. Given these characteristics, the here-in reported method could be profitably applied by forensic laboratories, allowing to identify the co-consumption of NPS and DoA in authentic hair samples. Similar multi-analyte methods on the keratin matrix might be useful in the future to assess the prevalence of NPS and the co-occurrence of NPS-DoA abuse.

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## CRediT authorship contribution statement

**Rossella Barone:** Formal analysis, Writing – original draft. **Guido Pelletti:** Writing – original draft, Data curation. **Arianna Giorgetti:** Data curation, Methodology, Writing – review & editing. **Francesco Introna:** Resources, Supervision. **Susi Pelotti:** Supervision. **Jennifer Paola Pascali:** Methodology. **Sara Sablone:** Resources. **Susan Mohamed:** Formal analysis, Resources.

## Declaration of Competing Interest

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

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