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Determination of cathinones and other stimulant, psychedelic, and dissociative designer drugs in real hair samples

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(Article begins on next page)



**DETERMINATION OF CATHINONES AND OTHER STIMULANT,
PSYCHEDELIC AND DISSOCIATIVE DESIGNER DRUGS IN
REAL HAIR SAMPLES**

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3 1 **DETERMINATION OF CATHINONES AND OTHER STIMULANT, PSYCHEDELIC AND**
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5 2 **DISSOCIATIVE DESIGNER DRUGS IN REAL HAIR SAMPLES[†]**
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Abstract

The detection of new psychoactive substances (NPS) in hair proved to provide insight into their current diffusion among the population and the social characteristics of these synthetic drugs' users. Therefore, a UHPLC-MS/MS method was developed in order to determine 31 among stimulants and psychedelic substituted phenethylamines, and dissociative drugs in hair samples. The method proved to be simple, fast, specific and sensitive. The absence of matrix interferences, together with excellent repeatability of both retention times and relative abundances of diagnostic transitions, allowed the correct identification of all analytes tested. The method showed optimal linearity in the interval 10-1000 pg/mg, with correlation coefficient values varying between 0.9981 and 0.9997. Quantitation limits ranged from 1.8 pg/mg for 4-Methoxyphencyclidine (4-MeO-PCP) up to 35pg/mg for 6-(2-aminopropyl)benzofuran (6-APB). The method was applied to (i) 23 real samples taken from proven MDMA and ketamine abusers and (ii) 54 real hair samples which had been previously tested negative during regular drug screening in driver's licence recovery. Six samples tested positive for at least one target analyte. Methoxetamine (MXE) was found in 3 cases (range of concentration: 7.7-27 pg/mg); mephedrone (4-MMC) was found in 2 cases (50-59 pg/mg) while one sample tested positive to methylone at 28 pg/mg. Other positive findings included 4-methylethcathinone (4-MEC), alpha-pyrrolidinovalerophenone (α -PVP), 4-fluoroamphetamine (4-FA), 3,4-methylenedioxypyrovalerone (MDPV) and diphenidine. The present study confirms the increasing diffusion of new designer drugs with enhanced stimulant activity among the target population of poly-abuse consumers.

Keywords: cathinones, NPS, mephedrone, hair, methoxetamine

41 Introduction

42 For many decades, the spectrum of abused drugs amounted to few substances, whereas, in recent
43 years, a huge upsurge of new psychoactive substances (NPS) has been observed. These drugs, also
44 known as “legal highs”, “designer drugs”, “herbal highs” or “research chemicals”, have found a
45 wide and efficient distribution through the “e-commerce” or specialized shops [1–4]. The misuse of
46 NPS initially led agencies and governments to prohibit them as single substances, but once these
47 drugs had been banned, their chemical structure was slightly altered to create new “legal” drugs
48 with similar properties [1]. This roundabout process contributed to their proliferation. Although
49 most of the latest substances maintain their primary activity as stimulant of the central nervous
50 system, their chemical structure presents different forms, that modulate intensity, duration, and side-
51 effects.

52 The fast multiplication and wide structure variability of NPS created further problems at both
53 analytical and legislative levels. The absence of reference standards for the parent drugs, and
54 signally for their metabolites, has represented an insurmountable obstacle for a long time,
55 preventing most forensic and clinical laboratories to achieve correct identification and
56 quantification of NPS. Further serious challenge to detect their presence in biological matrices,
57 especially urine, is posed by the extensive, yet not exhaustively investigated, metabolic
58 transformation that these substances undergo once introduced into the body, and the consequent
59 limited availability of pure NPS metabolites’ standards.

60 As long as these new classes of substances are not routinely screened in roadside control and
61 workplace testing (i.e., on high-risk professionals, such as policemen, military personnel, and truck
62 drivers), an increasing risk exists that habitual drug consumers will be induced to substitute the
63 traditional Cannabis products and former stimulants (cocaine and amphetamines) with these new
64 synthetic substances [5–9]. The replacement of “old” drugs with “new” drugs appears to be fostered

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3 65 also under other circumstances involving regular urine drug screening, for instance in driver's
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5 66 license recovery or in forensic psychiatry settings [10].
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9 67 To circumvent the problematical issues of NPS identification in urine, it has been proposed to
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11 68 screen their presence, as parent drugs, in hair samples [4, 6, 11–13]. In hair, the parent drug usually
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13 69 represents the target analyte, unlike in urine, because the molecules are mostly incorporated inside
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15 70 the keratin matrix from the sweat, the bloodstream, and/or the sebum, before they are metabolized.
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18 71 The corresponding analytical strategy is facilitated by the progressively wider availability of
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20 72 reference standards for parent drugs, with respect to the metabolites, which in turn allows rapid
21
22 73 upgrading of the analytical methods to detect them. Among the NPS, the prevalent group with
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24 74 stimulant or psychedelic activity is represented by synthetic cathinones, namely substituted
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26 75 phenethylamines compounds. The increasing popularity of these psychoactive drugs has created a
27
28 76 strong demand for sensitive, robust and reliable analytical methods addressed to their identification
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30 77 and quantification in different matrices, including hair. In a Letter to the Editor, Torrance and
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32 78 Cooper reported the detection of mephedrone in hair samples at 4.2 and 4.7 ng/mg concentration
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34 79 with an ISO 17025 accredited method, but details on the analytical method were not included[14].
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37 80 Several other methods targeting stimulant NPS were published afterwards, either using GC-MS [4,
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39 81 15–19] or LC-MS/MS [4, 20–25] techniques.
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43 82 In the present study, we developed and validated a new UHPLC-MS/MS analytical method devoted
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45 83 to the detection in hair samples of a selection of 26 stimulants and psychedelic substituted
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47 84 phenethylamines, including mephedrone, 3-methylmethcathinone (3-MMC), 4-methylethcathinone
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49 85 (4-MEC), methylone, 4-fluoroamphetamine (4-FA), 3,4-methylenedioxypropylamphetamine (MDPV),
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51 86 pentedrone, ethcathinone, alpha-pyrrolidinovalerophenone (α -PVP), butylone, buphedrone, 25I-
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53 87 NBOMe, 25C-NBOMe, 25H-NBOMe, 25B-NBOMe, 2C-P, 2C-B, 1-(benzofuran-5-yl)-N-
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55 88 methylpropan-2-amine (5-MAPB), 5-(2-aminopropyl)benzofuran (5-APB), 6-(2-
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57 89 aminopropyl)benzofuran (6-APB), *para*-methoxymethamphetamine (PMMA), *para*-
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3 90 methoxyamphetamine (PMA), amfepramone, bupropion, *meta*-chlorophenylpiperazine (mCPP),
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5 91 and trazodone plus 5 dissociative drugs, namely methoxetamine (MXE), phencyclidine (PCP), 4-
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7 92 methoxyphencyclidine (4-MeO-PCP), diphenidine and ketamine. The method was fully validated
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10 93 and applied to 23 real samples collected from proven MDMA and ketamine abusers. Furthermore,
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12 94 the method was applied to 54 real hair samples, randomly selected from a group of male and young
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14 95 (< 25 years) subjects previously tested negative within regular drug screening in driver's license
15
16 96 recovery.

19 97 **Experimental**

21 98 **Reagents, standards and samples**

23 99 The analytical standards of target analytes and the deuterated internal standards (mephedrone-d3,
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25 100 MDPV-d8, MDMA-d5, 25I-NBOMe-d3, PCP-d5, mCPP-d8) were purchased from LGC
26
27 101 Promochem (Milan, Italy) and Sigma-Aldrich (Milan, Italy). All other chemicals were purchased
28
29 102 from Sigma-Aldrich (Milan, Italy). Ultra-pure water was obtained using a Milli-Q® UF-Plus
30
31 103 apparatus (Millipore, Bedford, MA, USA). All stock standard solutions were prepared in methanol
32
33 104 at 1 mg/mL and stored at -20°C until used. Working solutions were prepared at the final
34
35 105 concentration of 100 ng/mL by dilution with methanol.

37 106 **Sample preparation**

39 107 A previously published procedure [26] was slightly modified. Briefly, about 25 mg of hair was
40
41 108 twice-washed with dichloromethane and then methanol (2 mL, vortex mixed for 3 min). After
42
43 109 complete removal of solvent washes, the hair was dried at room temperature by a gentle nitrogen
44
45 110 flow and subsequently cut with scissors into 1-2 mm segments. Hair samples were ~~fortified-added~~
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47 111 with 3 µL of an internal standards mixture yielding a final concentration of 0.3 ng/mg. After the
48
49 112 addition of 1.5 mL of methanol, the samples were incubated at 55 °C for 15 h without stirring.
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51 113 Lastly, the organic phase was collected and an aliquot of 1 µL was directly injected into the
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53 114 UHPLC-MS/MS system. Whenever the real samples concentrations were found to exceed the
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3 115 highest calibration point, the final extracts were diluted with methanol and re-injected into the
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5 116 system.

7 117 **Instrumentation**

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10 118 All analyses were performed using an Agilent 1290 Infinity LC system (Agilent, Palo Alto, CA,
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12 119 USA), interfaced to a QTRAP® 4500 mass spectrometer (AB Sciex, Darmstadt, Germany)
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14 120 equipped with an electrospray Turbo Ion source operated in the positive ion mode. A Zorbax
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16 121 Eclipse Plus C18 RRHD column (100 mm × 2.1 mm, 1.8 μm), protected by a C18 pre-column, was
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18 122 used for the separation of the target analytes. The column oven was maintained at 45°C and the
19
20 123 elution solvents were water/formic acid 5 mM (solvent A) and acetonitrile/methanol 80:20 plus
21
22 124 formic acid 5 mM (solvent B). After an initial isocratic elution at 95% A for 0.5 min, the mobile
23
24 125 phase composition was varied by a linear gradient (A:B; v/v) from 95:5 to 45:55 in 2.5 min; then
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26 126 isocratic elution at 55% B was maintained for 0.5 min. The flow rate was 0.5 mL/min and the total
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28 127 run time was 5.5 min including re-equilibration at the initial conditions before each injection.

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32 128 ~~Parameters for MS/MS detection were optimized according to our standard procedure [6]. was~~
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34 129 ~~executed in the selected reaction monitoring (SRM) mode. In order to establish appropriate SRM~~
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36 130 ~~conditions, each analyte was individually infused into the electrospray ionization (ESI) capillary~~
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38 131 ~~while the declustering potential (DP) was adjusted to maximize the intensity of the protonated~~
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40 132 ~~molecular species [M+H]⁺. The collision energy (CE) was set so as to preserve approximately 10%~~
41
42 133 ~~of precursor ion and the cell exit potentials (CEP) were also optimized. The SRMMRM transitions~~
43
44 134 were monitored during a time window of ±12.5 s around the expected retention time, and the cycle
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46 135 time of the SRMMRM program was 0.100 s. Optimal signals were obtained using a source block
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48 136 temperature of 600°C and an ion-spray voltage of 1250 V. Gas pressures were set as follows:
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50 137 curtain gas 38 psi, ion source gas (1) 40 psi and ion source gas (2) 25 psi. SRMMRM transitions
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52 138 and potentials for the analytes and internal standards are presented in Table 1.

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140 Method validation

141 The following parameters were investigated according to our standard procedure [6]: selectivity,
142 specificity, linearity range, detection and quantification limits (LOD and LOQ), intra-assay and
143 inter-assay precision and accuracy. Carry-over effect, recovery and matrix effects were also
144 investigated. A pool of five blank hair samples obtained from different healthy volunteers (two
145 females, three males) was prepared and analyzed as described above.

146 One qualifying SRMMRM transition was monitored, in addition to the primary fragmentation (see
147 Table 1). ~~Variations of mass transitions intensities were considered acceptable within $\pm 20\%$, with
148 respect to the corresponding control.~~ Specificity was determined on five blank head hair samples.
149 The signal-to-noise ratio (S/N) was measured on the less intense mass transition at the expected
150 analyte retention time. ~~A $S/N < 3$ was considered satisfactory in order to verify the method's
151 specificity.~~

152 The linear calibration model was checked by analyzing (two replicates) blank hair samples spiked
153 with the working solution at seven concentration levels (10, 25, 50, 100, 250, 500 and 1000 pg/mg).
154 The calibration was completed by internal standardization. The squared correlation coefficient,
155 adjusted by taking into account the number of observations and independent variables ($\text{Adj } R^2$), was
156 utilized to roughly estimate linearity. The appropriateness of the model was assessed by calculating
157 the residuals and examining the residual plots.

158 The limits of detection (LOD) were ~~estimated-calculated~~ using the Hubaux-Vox approach [27], and
159 the limits of quantitation (LOQs) were then approximated as 2 times the LOD values. The
160 ~~calculated-estimated~~ LODs were experimentally ~~verified-confirmed~~ with one blank hair sample
161 spiked at concentrations approximating these limits, verifying that the measured S/N ratio on the
162 less intense mass transition was >3 for each analyte. Intra-assay and inter-assay precision
163 (expressed as CV%) and accuracy (expressed as bias%) were evaluated by analyzing, on three days,
164 ten blank head hair samples spiked with the analytes at low (LCL) and high (HCL) calibration level,

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3 165 i.e. 100 and 1000 pg/mg concentrations. Precision and accuracy were satisfactory when the
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5 166 experimental CV% and bias% lied within $\pm 25\%$ at LCL and $\pm 15\%$ at HCL with respect to the
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7 167 expected concentration value.

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9 168 Extraction recoveries were determined by comparing the responses obtained from samples (five
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11 169 replicates) initially spiked with the analytes at a concentration of 1000 pg/mg and subsequently
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13 170 extracted and processed as usual, with the responses of blank samples in which the analytes were
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15 171 added at the same concentration after the extraction step. The matrix effect was calculated relatively
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17 172 to the ISTD, by comparing the peak area ratio between analyte and ISTD obtained from spiked hair
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19 173 samples, with the corresponding ratio obtained from a pure methanol solution, at the same
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21 174 concentrations. ~~In this case, the matrix effect is expected to be partly compensated by a well-~~
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23 175 ~~matched internal standard, i.e. the isotopically marked analyte, whenever possible, or the one~~
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25 176 ~~having the closest RT to the analyte, so as to undergo similar interference from the matrix.~~ The
26
27 177 matrix effect was calculated as the mean value obtained from five different hair sources. ~~The~~
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29 178 ~~percent difference represented either matrix suppression (values below 100%) or matrix~~
30
31 179 ~~enhancement (values above 100%).~~ The possible presence of carry-over effects was evaluated by
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33 180 injecting an alternate sequence of five blank head hair samples and five blank head hair samples
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35 181 spiked with all analytes at the maximum concentration (1000 pg/mg). ~~To ensure the absence of any~~
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37 182 ~~carry-over effect, the signal to noise ratio had to be lower than 3 for each monitored transition.~~
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184 **Study design. Application to real samples**

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47 185 A total number of 77 real hair samples were considered in the present study, all arising from the
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49 186 samples previously analyzed in our laboratory in 2013 and 2014. All patients provided written
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51 187 informed consent before donating the sample, and an anonymous code was attributed to each
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53 188 participating subject in order to respect privacy regulations. The first group consisted of 23 real
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55 189 samples taken from proven MDMA and ketamine abusers (Group A). The second group was
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57 190 composed of 54 real hair samples selected from a group of male and young (< 25 years) subjects
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3 191 and previously tested negative to conventional drugs of abuse within regular screening in driver's
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5 192 licence regranting protocol (Group B). These samples were re-analyzed, using the present UHPLC–
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7 193 MS/MS method with the aim to verify the potential presence of NPS, not previously targeted. Only
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9 194 the proximal 0–6 cm segment was analyzed whenever a longer head hair sample was collected.
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11 195 Shorter head hair, as well as pubic, axillary or chest hair samples, were analyzed in their full length.
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16 197 **Results and discussion**

18 198 The optimized UHPLC–MS/MS method allowed the simultaneous determination of 26 stimulants
19 199 and psychedelic substituted phenethylamines and 5 dissociative drugs in hair samples, plus five
20 200 internal standards. The whole chromatographic run, comprehensive of the time required for column
21 201 re-equilibration before the following injection, was completed in 5.5 min. Retention times ranged
22 202 between 1.85 min (methylone) and 3.41 min (25I-NBOMe). Due to choice of focusing the method
23 203 optimization on the high throughput requirement, compatible with common screening test
24 204 workload, two couples of isomeric cathinones could not be separated, namely mephedrone and its
25 205 isomer 3-MMC, and 6-APB and its isomer 5-APB. Thus, the mephedrone/3-MMC and 6-APB/5-
26 206 APB isomers had identical chromatographic RT and also similar fragmentation profiles, making it
27 207 impossible to discriminate them under the chromatographic conditions utilized. Consequently, the
28 208 validation experiments were carried out using only mephedrone and 6-APB as target analytes, even
29 209 though preliminary experiments showed us that very close figures-of-merits are obtained from these
30 210 isomers. Therefore, in case of real samples resulting positive for mephedrone or 6-APB, an on-
31 211 purpose confirmation method (e.g. GC-MS analysis following a derivatization step) is needed in
32 212 order to differentiate the two isomers [18, 28–30]. Figure 1 shows the **SRMMRM** chromatograms
33 213 recorded from a blank hair spiked with all analytes at 100 pg/mg concentration. It is worth noting
34 214 that the response factors for most analytes turned out quite homogeneous, due to their structural
35 215 similarities.

216 *Validation*

217 All the validation results are reported in Table 2 and the Supplementary Material. No carry-over
218 effect was observed under the conditions described in the experimental section. Selectivity and
219 specificity tests proved successful, i.e. SRMMRM chromatograms from negative head hair samples
220 showed no interfering signals at the retention time where the analytes were expected to elute. LOD
221 values ranged from 0.9 pg/mg for 4-MeO-PCP up to 17 pg/mg for 6-APB, while LOQ values lied
222 between 1.8 pg/mg and 35 pg/ng, respectively. Table 2 reports the Adj R² values obtained from the
223 calibration curves, that range from 0.9981 (MXE) up to 0.9997 (methylone and diphenidine) and
224 indicate good fit and linearity. The assumption of homoscedasticity was also successfully verified
225 by means of Hartley's F_{max} Test and Cochran's Test of maximum and minimum variance. Extraction
226 recoveries were mostly close to 100% and always in the interval 100±20%, as estimated from
227 samples spiked at 1000 pg/mg concentration. The hair matrix effect appeared to be significant only
228 for ethcathinone (see Table 2), for which a significant ion enhancement is evident (matrix effect >
229 +25%). Furthermore, the good linearity observed in the calibration plots supports the observation of
230 constant percent matrix effect, which in fact does not depend on the analytes' concentration.
231 Intraday and inter-day precision and accuracy were satisfactory for all analytes at low calibration
232 level (100 pg/mg). At high concentration level (1000 pg/mg), inter-day precision and accuracy were
233 satisfactory for all analytes, while modest deviation from the 15% acceptance limit was observed in
234 the evaluation of intraday precision for trazodone and interday accuracy for 4-MEC.

235 **Analysis of real samples**

236 The method was successfully applied to the analysis of real samples. Comprehensively, 5 samples
237 from Group A and one sample from Group B were found positive for at least one compound (see
238 Table 3). The molecules detected in the samples from Group A were MXE (3 samples, range of
239 concentration: 7.7-27 pg/mg), mephedrone (2 samples, respectively 50 and 59 pg/mg), while other
240 compounds were identified in one sample: 4-MEC (330 pg/mg), methylone (<LOQ), α-PVP (1040

241 pg/mg), 4-FA (55 pg/mg), MDPV (120 pg/mg) and diphenidine (4400 pg/mg). In percentage, from
242 Group A (MDMA and ketamine abusers), 5 out of 23 (21.7%) samples turned out positive for at
243 least one NPS. One subject 56-years old was found positive to both 4-MEC and mephedrone, while
244 another subject 32-years old turned out positive to as many as six NPS, namely methylone, MXE,
245 α -PVP, 4-FA, MDPV and diphenidine. Almost all Group B samples (subjects which had previously
246 been tested negative within regular drug screening in driver's licence regranting) proved to be
247 negative to NPS, with the exception of one positive result for methylone at 28 pg/mg. The other
248 analytes considered in this analytical method have not been detected in any of the samples
249 considered.

250 Among the positive samples, the measured levels for most of the drugs were interestingly in the
251 range of picograms of drug per milligrams of hair, either suggesting sporadic exposure to these
252 substances or low rate of incorporation into the keratin matrix. However, only limited literature data
253 concerning the detection of these new drugs in hair samples are currently available [4], making the
254 interpretation of NPS concentrations in hair samples still ambiguous.

255 The present method proved useful to investigate the diffusion of selected NPS among ~~selected-a~~
256 special population groupspopulations, especially in association with MDMA and ketamine. Some of
257 the detected drugs, namely mephedrone and methoxetamine, are likely the most common NPS
258 within the Italian territory in the present days. Other sporadic findings, which included 4-MEC, α -
259 PVP, methylone, 4-FA, MDPV and diphenidine, nevertheless indicate that several new substances
260 are simultaneously consumed in the local territory. Worldwide, several concerns and alerts have
261 already been raised [18, 31–34], to make forensic toxicology laboratories and emergency
262 departments in Italy aware of the increased use of for these NPS and their possible implications in
263 impairment and death cases. Interestingly, we did not detect any sample positive to NBOMe-series
264 compounds, possibly because of a delayed diffusion of these recent drugs among the Italian
265 population. On the other hand, these psychedelic phenethylamines are active at very low doses,
266 reducing the detectable levels in hair, especially in the cases of single or episodic intake. Therefore,

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3 267 it should be necessary to further improve the method's sensitivity in the next future, in order to
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5 268 verify the possible presence of NBOME-series compounds at trace level in hair, following active
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7 269 intake.
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9 270 **Conclusions**

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11 271 The present study proved that 31 stimulant, psychedelic and dissociative designer drugs can be
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13 272 determined in the keratin matrix with high sensitivity and specificity, allowing wide-range
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15 273 monitoring of drug intake over extended periods of time.
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18 274 In general, the introduction of this UHPLC–MS/MS method within our laboratory routine
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20 275 drastically reduced the analysis time required for carrying out comprehensive toxicological
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22 276 screening, whenever requested, hence achieving a drastic increase of the overall laboratory
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24 277 productivity without sacrificing chromatographic resolution, accuracy and precision.
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27 278 UHPLC-MS/MS methods are highly specific for very wide sets of target analytes, are rapidly
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29 279 adaptable to the introduction of new illicit substances, and increasingly compete with
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31 280 immunometric methods in terms of cheapness and high-throughput capability. This makes UHPLC-
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33 281 MS/MS methods ideally suited to execute comprehensive NPS screening in the forthcoming years,
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35 282 even for large populations, as is the case in workplace testing and driving license re-granting
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37 283 protocols.
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42 285 **Conflict of interest**

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45 286 We wish to confirm that there are no known conflicts of interest associated with this publication and
46
47 287 there has been no significant financial support for this work that could have influenced its outcome.
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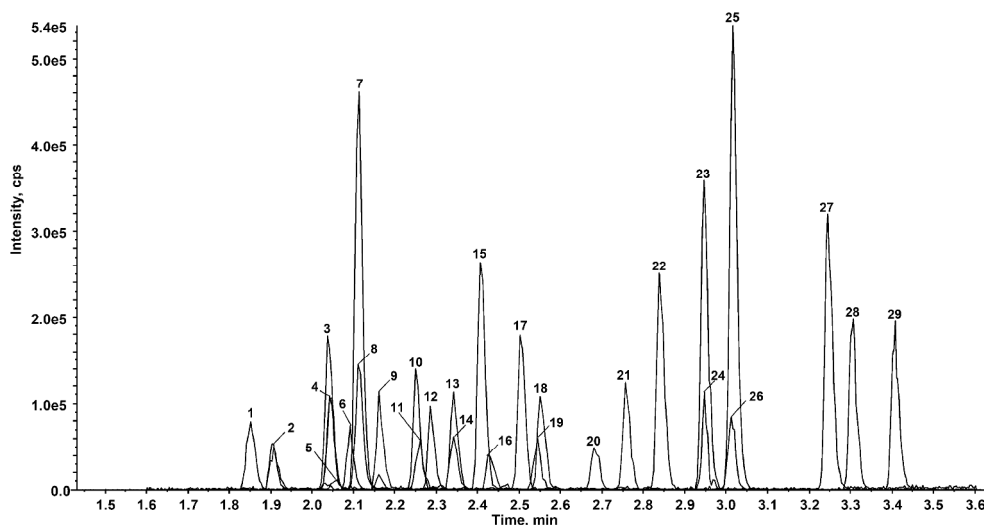
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4 **Figure 1.** SRM chromatograms recorded from blank hair sample spiked with all analytes at 100
5 pg/mg concentration. For each analyte, labelled by the progressive number assigned in Table 1,
6 only the target transition is shown.
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Table 1. MRM transitions and corresponding potentials for the target compounds and internal standard detection

	Compound	RT (min)	Precursor Ion [M+H] ⁺	DP (V)	Target			Qualifier 1		
					Fragment	CE (V)	CXP (V)	Fragment	CE (V)	CXP (V)
1	Methylone	1.85	208.0	50	160.0	25	5	132.0	38	6
2	Ethcathinone	1.90	178.1	44	160.2	17	9	130.0	41	9
3	4-FA	2.04	154.1	31	108.9	25	10	137.0	13	5
4	Buphedrone	2.04	178.0	29	131.0	31	4	160.0	18	5
5	PMA	2.04	166.1	34	149.0	26	7	121.0	14	9
6	Amfepramone	2.09	206.1	81	105.0	30	10	100.0	30	9
7	PMMA	2.11	180.1	30	149.0	16	6	120.9	26	11
8	Butylone	2.11	222.0	48	174.1	19	8	204.1	26	9
9	Mephedrone	2.16	178.1	38	145.0	28	10	160.0	18	5
10	Ketamine	2.25	238.0	38	125.0	28	10	207.1	20	9
11	6-APB	2.26	176.1	41	131.0	26	13	159.0	15	12
12	4-MEC	2.29	192.1	50	146.0	24	5	174.1	19	10
13	Pentedrone	2.34	192.1	25	132.0	25	4	161.0	17	8
14	5-MAPB	2.34	190.1	43	131.0	28	9	159.0	17	6
15	MXE	2.41	248.0	42	203.1	20	8	121.0	38	6
16	mCPP	2.43	197.1	279	154.0	30	8	118.0	44	11
17	α -PVP	2.50	232.1	83	91.0	33	9	161.0	24	5
18	MDPV	2.55	276.1	38	126.0	37	10	135.0	37	7
19	2C-B	2.54	261.9	47	244.9	18	9	-	-	-
20	Bupropione	2.68	260.0	47	-	-	-	242.8	18	9
21	Trazodone	2.76	240.0	52	131.0	42	12	166.0	35	5
22	PCP	2.76	372.1	95	176.1	35	5	148.0	46	11
23	PCP	2.84	244.1	35	86.0	16	24	159.0	20	5
24	4-MeO-PCP	2.94	274.1	23	189.1	18	9	120.9	40	8
25	Diphenidine	2.95	266.1	48	181.1	24	5	102.9	48	10
26	25H-NBOMe	3.01	302.1	36	91.0	57	9	121.0	34	6
27	2C-P	3.01	224.1	45	192.1	25	10	207.0	19	11
28	25C-NBOMe	3.25	336.1	48	121.0	24	6	91.0	61	6
29	25B-NBOMe	3.30	380.0	28	121.0	25	12	91.0	70	9
30	25I-NBOMe	3.41	428.1	76	121.0	25	6	91.0	79	8
31	IS1 MDMA-d5	2.04	199.1	36	165.1	16	7	-	-	-
32	IS2 mephedrone-d3	2.16	181.1	38	148.1	28	10	-	-	-
33	IS3 mCPP-d8	2.43	205.1	79	158.0	30	8	-	-	-
34	IS4 MDPV-d8	2.55	284.1	38	135.0	37	7	-	-	-
35	IS5 PCP-d5	2.84	249.1	35	86.0	16	8	-	-	-
36	IS6 25I-NBOMe-d3	3.41	431.1	76	124.1	25	6	-	-	-

Table 2. Range of calibration, linearity, LODs and LOQs values, recovery and matrix effect for all analytes

Compound	Linearity Range (pg/mg)	Internal Standard (IS)	Linearity (Adj R ²)	LOD ^a (pg/mg)	LOQ ^a (pg/mg)	Recovery ^b (%)	Matrix effect Mean (±%)
1 Methylone	10-1000	MDPV-d8	0.9997	3.2	6.4	85	8.3
2 Ethcathinone	10-1000	Mephedrone-d3	0.9992	3.1	6.2	88	27.4
3 4-FA	10-1000	MDMA-d5	0.9992	1.6	3.2	94	13.6
4 Buphedrone	10-1000	Mephedrone-d3	0.9992	4.2	8.4	79	11.9
5 PMA	25-1000	MDMA-d5	0.9994	8.8	18	98	16.3
6 Amfepramone	10-1000	Mephedrone-d3	0.9995	4.0	8.0	81	15.2
7 PMMA	10-1000	MDMA-d5	0.9991	1.3	2.6	97	5.8
8 Butylone	10-1000	25I-NBOMe-d3	0.9994	3.7	7.4	97	-0.5
9 Mephedrone	10-1000	Mephedrone-d3	0.9992	2.4	4.8	81	18.8
10 Ketamine	10-1000	25I-NBOMe-d3	0.9998	2.4	4.8	100	15.4
11 6-APB	50-1000	MDMA-d5	0.9991	17	35	94	3.0
12 4-MEC	10-1000	Mephedrone-d3	0.9994	3.0	6.0	86	10.2
13 Pentedrone	10-1000	Mephedrone-d3	0.9993	3.9	7.8	87	12.1
14 5-MAPB	10-1000	MDMA-d5	0.9991	4.6	9.2	100	5.6
15 MXE	10-1000	MDMA-d5	0.9981	1.0	2.0	98	7.1
16 mCPP	10-1000	mCPP-d8	0.9991	3.0	6.0	91	-0.5
17 α -PVP	10-1000	MDPV-d8	0.9993	2.0	4.0	91	3.7
18 MDPV	10-1000	MDPV-d8	0.9992	2.0	4.0	96	-4.2
19 2C-B	10-1000	25I-NBOMe-d3	0.9994	6.2	12	97	7.8
20 Bupropione	10-1000	mCPP-d8	0.9994	2.6	5.2	115	-0.3
21 Trazodone	10-1000	mCPP-d8	0.9993	1.1	2.2	94	-15.3
22 PCP	10-1000	PCP-d5	0.9995	3.6	7.2	106	-0.8
23 4-MeO-PCP	10-1000	PCP-d5	0.9991	0.9	1.8	87	3.9
24 Diphenidine	10-1000	25I-NBOMe-d3	0.9997	3.4	6.8	85	4.5
25 25H-NBOMe	10-1000	25I-NBOMe-d3	0.9994	1.0	2.0	102	0.4
26 2C-P	10-1000	25I-NBOMe-d3	0.9992	1.0	2.0	96	1.7
27 25C-NBOMe	10-1000	25I-NBOMe-d3	0.9993	1.5	3.0	100	5.6
28 25B-NBOMe	10-1000	25I-NBOMe-d3	0.9994	4.1	8.2	102	4.5
29 25I-NBOMe	10-1000	25I-NBOMe-d3	0.9991	1.5	3.0	97	6.9

^aLOD, limit of detection; LOQ, limit of quantitation^bRecovery evaluated at 1000 pg/mg.

Table 3. Synoptic summary of real samples positive to synthetic cannabinoids

Case	Group	Age	Gender	Hair Type	4-MEC (pg/mg)	Mephedrone (pg/mg)	MXE (pg/mg)	α -PVP (pg/mg)	Methylone (pg/mg)	4-FA (pg/mg)	MDPV (pg/mg)	Diphenidine (pg/mg)	Other findings
1	A	56	Male	Hair	330	50	-	-	-	-	-	-	MDMA
2	A	26	Male	Hair	-	59	-	-	-	-	-	-	Ketamine
3	A	43	Female	Hair	-	-	7.7	-	-	-	-	-	MDMA
4	A	33	Female	Hair	-	-	28	-	-	-	-	-	MDMA
5	A	32	Male	Hair	-	-	27	1040	< LOQ	55	120	4400	MDMA
6	B	28	Male	Hair	-	-	-	-	28	-	-	-	-

Table supplementary material. Intraday/Interday precision (CV%) and accuracy (bias%) for each analyte tested

Compound	Low level (100 pg/mg)				High Level (1000 pg/mg)				
	Intraday (n=10)		Interday (n=30)		Intraday (n=10)		Interday (n=30)		
	Precision (CV%)	Accuracy (bias%)	Precision (CV%)	Accuracy (bias%)	Precision (CV%)	Accuracy (bias%)	Precision (CV%)	Accuracy (bias%)	
1	Methylone	9.2	4.9	14.0	21.2	11.7	1.6	11.7	4.1
2	Ethcathinone	7.2	2.0	12.4	6.3	7.5	12.9	10.4	13.9
3	4-FA	9.0	7.0	16.3	20.5	6.2	1.5	17.9	12.9
4	Buphedrone	8.6	6.0	12.0	12.1	6.3	8.4	10.4	4.5
5	PMA	17.6	13.1	18.5	17.4	5.6	-2.7	12.7	0.8
6	Amfepramone	11.3	12.9	11.8	17.3	6.6	14.4	12.2	10.8
7	PMMA	16.8	14.7	20.4	6.2	7.0	2.4	7.3	1.4
8	Butylone	11.4	3.7	12.2	6.7	5.8	-0.5	14.0	-0.5
9	Mephedrone	10.5	6.5	18.8	13.0	6.5	12.2	10.5	10.2
10	Ketamine	3.0	-7.3	5.1	-5.3	1.9	+1.3	4.0	+3.1
11	6-APB	8.2	1.5	12.7	13.2	5.8	9.9	10.3	3.2
12	4-MEC	11.3	-5.5	15.8	8.9	8.2	6.5	15.3	29.5
13	Pentadrone	7.2	4.8	12.9	11.9	7.8	6.0	11.9	8.0
14	5-MAPB	6.9	7.7	10.6	7.1	7.1	5.0	10.1	7.1
15	MXE	16.7	12.2	19.1	8.2	7.0	4.4	7.3	2.2
16	mCPP	12.9	-1.1	12.4	8.0	10.2	7.1	9.5	8.6
17	α -PVP	5.8	13.5	10.1	7.9	6.6	3.3	7.2	3.7
18	MDPV	8.3	5.0	11.7	10.2	7.2	3.7	8.5	2.5
19	2C-B	8.6	-0.4	12.9	9.8	7.2	-9.0	14.2	7.1
20	Bupropion	8.4	10.9	15.3	4.8	9.9	14.4	10.6	12.6
21	Trazodone	12.7	8.2	24.4	-3.4	14.2	5.2	23.6	-2.6
22	PCP	7.7	-7.9	7.8	-7.9	6.7	-0.9	7.5	+2.5
23	4-MeO-PCP	9.9	-0.6	21.6	-1.1	7.5	2.6	11.6	-0.7
24	Diphenidine	5.6	6.4	12.1	1.6	4.4	8.0	7.9	7.4
25	25H-NBOMe	11.7	1.0	15.6	4.2	7.9	1.6	14.2	6.6
26	2C-P	8.4	9.1	10.7	10.7	6.4	2.9	9.1	4.0
27	25C-NBOMe	17.6	16.4	20.5	2.7	7.3	0.4	9.4	2.0
28	25B-NBOMe	17.5	14.4	17.1	9.2	6.7	-0.5	7.5	-2.8
29	25I-NBOMe	16.8	11.8	18.9	2.4	6.4	0.2	6.9	-1.0