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DETERMINATION OF CATHINONES AND OTHER STIMULANT, PSYCHEDELIC AND 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 interferents, 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. Methoxethamine (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

Introduction

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

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

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

also under other circumstances involving regular urine drug screening, for instance in driver's license recovery or in forensic psychiatry settings [10].

To circumvent the problematical issues of NPS identification in urine, it has been proposed to screen their presence, as parent drugs, in hair samples [4, 6, 11–13]. In hair, the parent drug usually represents the target analyte, unlike in urine, because the molecules are mostly incorporated inside the keratin matrix from the sweat, the bloodstream, and/or the sebum, before they are metabolized. The corresponding analytical strategy is facilitated by the progressively wider availability of reference standards for parent drugs, with respect to the metabolites, which in turn allows rapid upgrading of the analytical methods to detect them. Among the NPS, the prevalent group with stimulant or psychedelic activity is represented by synthetic cathinones, namely substituted phenethylamines compounds. The increasing popularity of these psychoactive drugs has created a strong demand for sensitive, robust and reliable analytical methods addressed to their identification and quantification in different matrices, including hair. In a Letter to the Editor, Torrance and Cooper reported the detection of mephedrone in hair samples at 4.2 and 4.7 ng/mg concentration with an ISO /17025 accredited method, but details on the analytical method were not included[14]. Several other methods targeting stimulant NPS were published afterwards, either using GC-MS [4, 15–19] or LC-MS/MS [4, 20–25] techniques.

In the present study, we developed and validated a new UHPLC-MS/MS analytical method devoted to the detection in hair samples of a selection of 26 stimulants and psychedelic substituted phenethylamines, including mephedrone, 3-methylmethcathinone (3-MMC), 4-methylethcathinone (4-MEC), methylone, 4-fluoroamphetamine (4-FA), 3,4-methylenedioxypyrovalerone (MDPV), pentedrone, ethcathinone, alpha-pyrrolidinovalerophenone (α-PVP), butylone, buphedrone, 25I-NBOMe, 25C-NBOMe, 25H-NBOMe, 25B-NBOMe, 2C-P, 2C-B, 1-(benzofuran-5-yl)-N-methylpropan-2-amine (5-MAPB), 5-(2-aminopropyl)benzofuran (5-APB), 6-(2-aminopropyl)benzofuran (6-APB), *para*-methoxymethamphetamine (PMMA), *para*-

methoxyamphetamine (PMA), amfepramone, bupropion, *meta*-chlorophenylpiperazine (mCPP), and trazodone plus 5 dissociative drugs, namely methoxetamine (MXE), phencyclidine (PCP), 4-methoxyphencyclidine (4-MeO-PCP), diphenidine and ketamine. The method was fully validated and applied to 23 real samples collected from proven MDMA and ketamine abusers. Furthermore, the method was applied to 54 real hair samples, randomly selected from a group of male and young (< 25 years) subjects previously tested negative within regular drug screening in driver's license recovery.

Experimental

Reagents, standards and samples

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

Sample preparation

A previously published procedure [26] was slightly modified. Briefly, about 25 mg of hair was twice-washed with dichloromethane and then methanol (2 mL, vortex mixed for 3 min). After complete removal of solvent washes, the hair was dried at room temperature by a gentle nitrogen flow and subsequently cut with scissors into 1-2 mm segments. Hair samples were ~~fortified~~ added with 3 µL of an internal standards mixture yielding a final concentration of 0.3 ng/mg. After the addition of 1.5 mL of methanol, the samples were incubated at 55 °C for 15 h without stirring. Lastly, the organic phase was collected and an aliquot of 1 µL was directly injected into the UHPLC-MS/MS system. Whenever the real samples concentrations were found to exceed the

highest calibration point, the final extracts were diluted with methanol and re-injected into the system.

Instrumentation

All analyses were performed using an Agilent 1290 Infinity LC system (Agilent, Palo Alto, CA, USA), interfaced to a QTRAP® 4500 mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with an electrospray Turbo Ion source operated in the positive ion mode. A Zorbax Eclipse Plus C18 RRHD column (100 mm × 2.1 mm, 1.8 µm), protected by a C18 pre-column, was used for the separation of the target analytes. The column oven was maintained at 45°C and the elution solvents were water/formic acid 5 mM (solvent A) and acetonitrile/methanol 80:20 plus formic acid 5 mM (solvent B). After an initial isocratic elution at 95% A for 0.5 min, the mobile phase composition was varied by a linear gradient (A:B; v/v) from 95:5 to 45:55 in 2.5 min; then isocratic elution at 55% B was maintained for 0.5 min. The flow rate was 0.5 mL/min and the total run time was 5.5 min including re-equilibration at the initial conditions before each injection. Parameters for MS/MS detection were optimized according to our standard procedure [6]. ~~was executed in the selected reaction monitoring (SRM) mode. In order to establish appropriate SRM conditions, each analyte was individually infused into the electrospray ionization (ESI) capillary while the declustering potential (DP) was adjusted to maximize the intensity of the protonated molecular species $[M+H]^+$. The collision energy (CE) was set so as to preserve approximately 10% of precursor ion and the cell exit potentials (CXP) were also optimized.~~ The ~~SRM~~MRM transitions were monitored during a time window of ±12.5 s around the expected retention time, and the cycle time of the ~~SRM~~MRM program was 0.100 s. Optimal signals were obtained using a source block temperature of 600°C and an ion-spray voltage of 1250 V. Gas pressures were set as follows: curtain gas 38 psi, ion source gas (1) 40 psi and ion source gas (2) 25 psi. ~~SRM~~MRM transitions and potentials for the analytes and internal standards are presented in Table 1.

Method validation

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

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

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

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

i.e. 100 and 1000 pg/mg concentrations. Precision and accuracy were satisfactory when the experimental CV% and bias% lied within ±25% at LCL and ±15% at HCL with respect to the expected concentration value.

Extraction recoveries were determined by comparing the responses obtained from samples (five replicates) initially spiked with the analytes at a concentration of 1000 pg/mg and subsequently extracted and processed as usual, with the responses of blank samples in which the analytes were added at the same concentration after the extraction step. The matrix effect was calculated relatively to the ISTD, by comparing the peak area ratio between analyte and ISTD obtained from spiked hair samples, with the corresponding ratio obtained from a pure methanol solution, at the same concentrations. ~~In this case, the matrix effect is expected to be partly compensated by a well-matched internal standard, i.e. the isotopically marked analyte, whenever possible, or the one having the closest RT to the analyte, so as to undergo similar interference from the matrix.~~ The matrix effect was calculated as the mean value obtained from five different hair sources. ~~The percent difference represented either matrix suppression (values below 100%) or matrix enhancement (values above 100%).~~ The possible presence of carry-over effects was evaluated by injecting an alternate sequence of five blank head hair samples and five blank head hair samples spiked with all analytes at the maximum concentration (1000 pg/mg). ~~To ensure the absence of any carry-over effect, the signal to noise ratio had to be lower than 3 for each monitored transition~~.

Study design. Application to real samples

A total number of 77 real hair samples were considered in the present study, all arising from the samples previously analyzed in our laboratory in 2013 and 2014. All patients provided written informed consent before donating the sample, and an anonymous code was attributed to each participating subject in order to respect privacy regulations. The first group consisted of 23 real samples taken from proven MDMA and ketamine abusers (Group A). The second group was composed of 54 real hair samples selected from a group of male and young (< 25 years) subjects

and previously tested negative to conventional drugs of abuse within regular screening in driver's licence regranting protocol (Group B). These samples were re-analyzed, using the present UHPLC–MS/MS method with the aim to verify the potential presence of NPS, not previously targeted. Only the proximal 0-6 cm segment was analyzed whenever a longer head hair sample was collected. Shorter head hair, as well as pubic, axillary or chest hair samples, were analyzed in their full length.

Results and discussion

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

Validation

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

Analysis of real samples

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

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

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

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

it should be necessary to further improve the method's sensitivity in the next future, in order to verify the possible presence of NBOME-series compounds at trace level in hair, following active intake.

Conclusions

The present study proved that 31 stimulant, psychedelic and dissociative designer drugs can be determined in the keratin matrix with high sensitivity and specificity, allowing wide-range monitoring of drug intake over extended periods of time.

In general, the introduction of this UHPLC–MS/MS method within our laboratory routine drastically reduced the analysis time required for carrying out comprehensive toxicological screening, whenever requested, hence achieving a drastic increase of the overall laboratory productivity without sacrificing chromatographic resolution, accuracy and precision.

UHPLC-MS/MS methods are highly specific for very wide sets of target analytes, are rapidly adaptable to the introduction of new illicit substances, and increasingly compete with immunometric methods in terms of cheapness and high-throughput capability. This makes UHPLC-MS/MS methods ideally suited to execute comprehensive NPS screening in the forthcoming years, even for large populations, as is the case in workplace testing and driving license re-granting protocols.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

REFERENCES

1. Davidson C (2012) New psychoactive substances. Prog Neuropsychopharmacol Biol Psychiatry 39:219–220

2. King L, Kicman T (2011) A brief history of "new psychoactive substances". Drug Test Anal 3:401–403

3. Favretto D, Pascali JP, Tagliaro F (2013) New challenges and innovation in forensic toxicology: focus on the "New Psychoactive Substances". J Chromatogr A 1287:84–95

4. Kintz P, Salomone A, Vincenti M (2015) Hair Analysis in Clinical and Forensic Toxicology. Academic Press, USA

5. Vardakou I, Pistos C, Spiliopoulou C (2010) Spice drugs as a new trend: mode of action, identification and legislation. Toxicol Lett 197:157–162

6. Salomone A, Luciano C, Di Corcia D, Gerace E, Vincenti M (2014) Hair analysis as a tool to evaluate the prevalence of synthetic cannabinoids in different populations of drug consumers. Drug Test Anal 6:126–34

7. Kerwin J (2011) Doors of deception: the diaspora of designer drugs. Drug Test Anal 3:527–531

8. Papanti D, Schifano F, Botteon G, Bertossi F, Mannix J, Vidoni D, Impagnatiello M, Pascolo-Fabrici E, Bonavigo T (2013) "Spiceophrenia": a systematic overview of "spice"-related psychopathological issues and a case report. Hum Psychopharmacol 28:379–389

9. Crews Bridgit (2013) Synthetic Cannabinoids The Challenges of Testing for Designer Drugs. http://www.aacc.org/publications/cln/2013/february/Pages/Cannabinoids.aspx#.

10. Hutter M, Kneisel S, Auwärter V, Neukamm M (2012) Determination of 22 synthetic cannabinoids in human hair by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 903:95–101

11. Salomone A, Gerace E, D'Urso F, Di Corcia D, Vincenti M (2012) Simultaneous analysis of several synthetic cannabinoids, THC, CBD and CBN, in hair by ultra-high performance liquid chromatography tandem mass spectrometry. Method validation and application to real samples. J Mass Spectrom 47:604–610

12. Strano-Rossi S, Odoardi S, Fisichella M, Anzillotti L, Gottardo R, Tagliaro F (2014) Screening for new psychoactive substances in hair by ultrahigh performance liquid chromatography-electrospray ionization tandem mass spectrometry. J Chromatogr A 1372C:145–156

13. Gottardo R, Sorio D, Musile G, Trapani E, Seri C, Serpelloni G, Tagliaro F (2014) Screening for synthetic cannabinoids in hair by using LC-QTOF MS: A new and powerful approach to study the penetration of these new psychoactive substances in the population. Med Sci Law 54:22–27

14. Torrance H, Cooper G (2010) The detection of mephedrone (4-methylmethcathinone) in 4 fatalities in Scotland. Forensic Sci Int 202:e62–3

15. Barroso M, Costa S, Dias M, Vieira DN, Queiroz J, López-Rivadulla M (2010) Analysis of phenylpiperazine-like stimulants in human hair as trimethylsilyl derivatives by gas chromatography-mass spectrometry. J Chromatogr A 1217:6274–6280

16. Kikura-Hanajiri R, Kawamura M, Saisho K, Kodama Y, Goda Y (2007) The disposition into hair of new designer drugs; methylone, MBDB and methcathinone. J Chromatogr B Analyt Technol Biomed Life Sci 855:121–126

17. Martin M, Muller JF, Turner K, Duez M, Cirimele V (2012) Evidence of mephedrone chronic abuse through hair analysis using GC/MS. Forensic Sci Int 218:44–48

18. Gerace E, Petrarulo M, Bison F, Salomone A, Vincenti M (2014) Toxicological findings in a fatal multidrug intoxication involving mephedrone. Forensic Sci Int 243C:68–73

19. Kim JY, Jung KS, Kim MK, Lee J Il, In MK (2007) Simultaneous determination of psychotropic phenylalkylamine derivatives in human hair by gas chromatography / mass spectrometry. Rapid Commun Mass Spectrom 1705–1720

20. Namera A, Konuma K, Saito T, Ota S, Oikawa H, Miyazaki S, Urabe S, Shiraishi H, Nagao M (2013) Simple segmental hair analysis for α-pyrrolidinophenone-type designer drugs by MonoSpin extraction for evaluation of abuse history. J Chromatogr B Analyt Technol Biomed Life Sci 942-943:15–20

21. Namera A, Urabe S, Saito T, Torikoshi-Hatano A, Shiraishi H, Arima Y, Nagao M (2013) A fatal case of 3,4-methylenedioxypyrovalerone poisoning: coexistence of α-pyrrolidinobutiophenone and α-pyrrolidinovalerophenone in blood and/or hair. Forensic Toxicol 31:338–343

22. Shah SB, Deshmukh NIK, Barker J, Petróczi A, Cross P, Archer R, Naughton DP (2012) Quantitative analysis of mephedrone using liquid chromatography tandem mass spectroscopy: application to human hair. J Pharm Biomed Anal 61:64–69

23. Rust KY, Baumgartner MR, Dally AM, Kraemer T (2012) Prevalence of new psychoactive substances : A retrospective study in hair. Drug Test Anal 4:402–408

24. Wikström M, Thelander G, Nyström I, Kronstrand R (2010) Two Fatal Intoxications with the New Designer Drug Methedrone (4-Methoxymethcathinone) Autopsy cases. J Anal Toxicol 34:594–598

25. Lendoiro E, Jiménez-Morigosa C, Cruz A, López-Rivadulla M, de Castro A (2014) O20: Hair analysis of amphetamine-type stimulant drugs (ATS), including synthetic cathinones and piperazines, by LC-MSMS. Toxicol Anal Clin 26:S13

26. Di Corcia D, D'Urso F, Gerace E, Salomone A, Vincenti M (2012) Simultaneous determination in hair of multiclass drugs of abuse (including THC) by ultra-high performance liquid chromatography–tandem mass spectrometry. J Chromatogr B 899:154–159

27. Hubaux A, Vos G (1970) Decision and detection limits for calibration curves. Anal Chem 42:849–855

28. Stanczuk A, Morris N, Gardner E., Kavanagh P (2013) Identification of (2-aminopropyl)benzofuran (APB) phenyl ring positional isomers in Internet purchased products. Drug Test Anal 5:270–276

29. Brandt SD, Daley PF, Cozzi N V. (2012) Analytical characterization of three trifluoromethyl-substituted methcathinone isomers. Drug Test Anal 4:525–529

30. Power JD, McGlynn P, Clarke K, McDermott SD, Kavanagh P, O'Brien J (2011) The analysis of substituted cathinones. Part 1: Chemical analysis of 2-, 3- and 4-methylmethcathinone. Forensic Sci Int 212:6–12

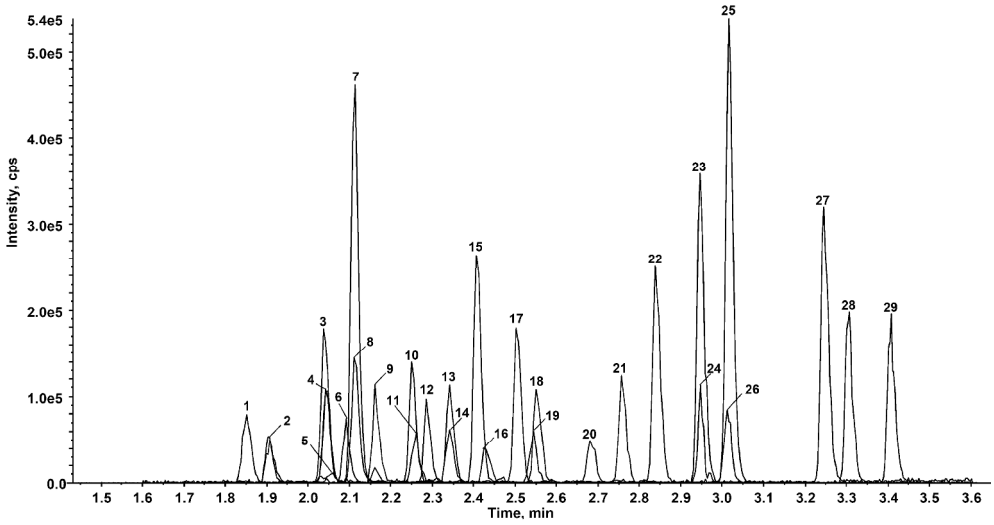
31. Marinetti LJ, Antonides HM (2013) Analysis of synthetic cathinones commonly found in bath salts in human performance and postmortem toxicology: method development, drug distribution and interpretation of results. J Anal Toxicol 37:135–46

32. Hondebrink L, Nugteren-van Lonkhuyzen JJ, Van Der Gouwe D, Brunt TM (2015) Monitoring new psychoactive substances (NPS) in The Netherlands: Data from the drug market and the Poisons Information Centre. Drug Alcohol Depend 147:109–115

33. Adamowicz P, Tokarczyk B, Stanaszek R, Slopianka M (2013) Fatal mephedrone intoxication--a case report. J Anal Toxicol 37:37–42

34. Hasegawa K, Wurita A, Minakata K, Gonmori K, Nozawa H, Yamagishi I, Watanabe K, Suzuki O (2015) Postmortem distribution of AB-CHMINACA, 5-fluoro-AMB, and diphenidine in body fluids and solid tissues in a fatal poisoning case: usefulness of adipose tissue for detection of the drugs in unchanged forms. Forensic Toxicol 33:45–53

Figure 1. SRM chromatograms recorded from blank hair sample spiked with all analytes at 100 pg/mg concentration. For each analyte, labelled by the progressive number assigned in Table 1, only the target transition is shown.



368x202mm (300 x 300 DPI)

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	261.9 260.0	47 47	244.9 -	18 -	9 -	- 242.8	- 18	- 9
20	Bupropione	2.68	240.0	52	131.0	42	12	166.0	35	5
21	Trazodone	2.76	372.1	95	176.1	35	5	148.0	46	11
22	PCP	2.84	244.1	35	86.0	16	24	159.0	20	5
23	4-MeO-PCP	2.94	274.1	23	189.1	18	9	120.9	40	8
24	Diphenidine	2.95	266.1	48	181.1	24	5	102.9	48	10
25	25H-NBOMe	3.01	302.1	36	91.0	57	9	121.0	34	6
26	2C-P	3.01	224.1	45	192.1	25	10	207.0	19	11
27	25C-NBOMe	3.25	336.1	48	121.0	24	6	91.0	61	6
28	25B-NBOMe	3.30	380.0	28	121.0	25	12	91.0	70	9
29	25I-NBOMe	3.41	428.1	76	121.0	25	6	91.0	79	8
IS1	MDMA-d5	2.04	199.1	36	165.1	16	7	-	-	-
IS2	mephedrone-d3	2.16	181.1	38	148.1	28	10	-	-	-
IS3	mCPP-d8	2.43	205.1	79	158.0	30	8	-	-	-
IS4	MDPV-d8	2.55	284.1	38	135.0	37	7	-	-	-
IS5	PCP-d5	2.84	249.1	35	86.0	16	8	-	-	-
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	LinearityRange (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

[a]LOD, limit of detection; LOQ, limit of quantitation
[b] Recovery 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	Pentedrone	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