

Targeted screening and quantification of synthetic cathinones and metabolites in hair by UHPLC-HRMS

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Abstract. – **OBJECTIVE:** Synthetic cathinones (SCs) are new psychoactive substances with sympathomimetic effects, which emerged into the illegal drug market to replace controlled stimulants. Since every year more powerful and toxic substances enter the illicit market, there is the need for analytical methodologies able to detect these new compounds in conventional and non-conventional biological matrices. We sought to develop and validate a targeted screening and quantification method for thirty-two parent SCs and two metabolites in hair samples by ultra-high-performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS).

MATERIALS AND METHODS: 20 mg hair samples were soaked in 250 µL of 2 mM ammonium formate, methanol and acetonitrile mixture (50/25/25, v/v/v) and incubated overnight at 40°C. After incubation, the samples were evaporated to dryness under nitrogen stream and reconstituted with 100 µL of mobile phase mix (A:B, 80:20) and 10 µL were injected into UHPLC-HRMS. A Q Exactive™ Focus Orbitrap Mass spectrometer with full scan and targeted data-dependent MS/MS scan acquisition was used for the screening and quantitation analysis.

RESULTS: The assay was linear from 5 to 500 pg/mg hair for all the analytes under investigation. Intra-day and inter-day precision were always < 15% and matrix effect and analytical recovery were always within acceptable criteria (±25% and >50%, respectively). The developed method was applied to authentic hair samples from SCs consumers. The most prevalent found SCs were 3,4-Methylenedioxy-α-Pyrrolidinohexanophenone with a concentration range of 6.0-1,000.0 pg/mg along with α-Pyrrolidinohexiophenone (54.0 and 554.0 pg/mg, respectively), 3-Methylmetcathinone (556.0 and 5,000.0 pg/mg) and 4-Methylethcathinone (11.5 and 448.0 pg/mg)

CONCLUSIONS: The developed method showed good selectivity, specificity, an easy and low-cost sample preparation and an analysis time compatible with a high throughput laboratory.

Key Words:

Synthetic cathinones, Hair, UHPLC-HRMS, NPS.

Introduction

Synthetic cathinones (SCs) are New Psychoactive Substances (NPS) related to S-(-)-cathinone, the primary natural component of *Catha edulis* plant. Since the early 2000s, they have been notified to the EU Early Warning System as alternatives to controlled stimulants. They represent the first largest group of NPS seized within the EU^{1,2}.

SCs effects can be classified as methamphetamine-like cathinones (dopamine transporter (DAT) and norepinephrine transporter (NET) blockers, dopamine releaser); cocaine-MDMA-like cathinones (DAT blockers and serotonin releasers) and pyrovalerone cathinones (more potent and DAT selective with high addiction potential)³. Moreover, halogenated SCs (3-fluoro-α-Pyrrolidinoverophenone, 4-bromomethcathinone, flephedrone, 3-Chloromethcathinone) show higher potencies as serotonin transporters inhibitors⁴. In this concern, it has been shown that modifications of the α alkyl side chain and/or the alkyl amino group, of the aromatic ring (e.g., adding a methylenedioxy group or halogens) have led to a range of substances with different and more intense psychoactive properties³.

Several intoxications and fatalities caused by different SCs alone or in combination with other NPS have been reported in the international lit-

erature⁴⁻⁷. Currently, newest generations of SCs with not only stronger, long-lasting psychoactive effects but also more toxic side-effects keep going replacing those one already scheduled by law, making their identification in acute and chronic intoxications and fatalities cases a continuous challenge for analytical toxicologists^{8,9}.

In this concern, hair testing for SCs by liquid/gas chromatography tandem mass spectrometry has been proved as an effective tool to disclose repeated consumption of those substances in clinical and forensic toxicology^{10,11}. Nevertheless, the ability to identify and quantify newer SCs with the above-reported methodologies depends on the availability of pure chemical standards of compounds under investigation. High resolution mass spectrometry (HRMS) has become the most powerful technique used for screening, quantification, metabolism and metabolomics studies of targeted and untargeted substances, even in absence of their pure chemical standards^{12,13}. This technique has been applied to several studies¹⁴⁻¹⁹ on NPS (e.g., synthetic cannabinoids, synthetic cathinones, designer benzodiazepines, synthetic opioids) in biological matrices such as blood, plasma, urine, oral fluid and hair. Concerning this latter biological matrix, only two investigation groups^{20,21} developed screening and/or confirmation methods using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) for SCs in hair, by including a limited number of SCs in their studies.

Here, we present the development and validation of a comprehensive method for the identification and quantification of thirty-two synthetic cathinones and two metabolites in hair with application to real samples from consumers.

Materials and Methods

Chemicals and Reagents

Pentylone, Pentedrone, N-Ethylpentylone, Naphyrone, 1-Naphyrone, Methylone, Methedrone, Methcathinone, Mephedrone (4-MMC), Methylenedioxypropylone (MDPV), 3,4-Methylenedioxy- α -pyrrolidinobutiophenone (MDPBP), Flephedrone (4-FMC), Euthylone, Ethylone, Ethcathinone, Dimethylcathinone, Butylone, Buphedrone, Bazedrone (4-MBC), α -Pyrrolidinovalephedrone (α -PVP), α -Pyrrolidinohexaphenone (α -PHP), α -Ethylaminopentiophenone (NEP), 4-methylethcathinone metabolite (4-MEC metab), 4-Methyl- α -pyrrolidinohexaphenone (MPHP), 4-Methylethcathinone (4-MEC), 4-fluoromethcathi-

none metabolite (4-FMC metab), 4-fluoro- α -Pyrrolidinohexaphenone (4-fluoro- α -PHP), 4-Ethylethcathinone (4-EEC), 4-bromomethcathinone (4-BMC), 3-Methylmethcathinone (3-MMC), 3-fluoro- α -Pyrrolidinovalephedrone (3-fluoro- α -PVP), 3-Chloromethcathinone (3-CMC), 3,4-Dimethylmethcathinone (3,4-DMMC), 3,4-Methylenedioxy- α -Pyrrolidinohexaphenone (MDPHP) and internal standard (IS) Mephedrone- d_3 , were purchased from Cayman Chemical (Ann Arbor, MI, USA). Water, acetonitrile, methanol, dichloromethane, and formic acid (>95%) were obtained from Carlo Erba (Milano, Italy). All solvents were UH-PLC and LC-MS grade. Ammonium formate buffer was acquired from Sigma-Aldrich® (Milan, Italy).

Instrumentation

UHPLC-HRMS analyses were performed on an UltiMate 3,000 liquid chromatograph coupled to a Q Exactive™ Focus mass spectrometer equipped with a heated electrospray ionization (HESI II) source (Thermo Fisher Scientific, Waltham, MA, USA).

Calibrators and Quality Control Samples

Working standard solutions at 0.1 ng/ μ L and 1 ng/ μ L containing all compounds, with the exception of 4-MMC, were prepared by appropriate methanolic dilution using stock solutions. 4-MMC working standard solutions were separately prepared and diluted at the same concentrations. IS solution of mephedrone- d_3 was separately prepared at 1 ng/ μ L diluting drug deuterium-labeled solution.

Drug-free hair samples were donated from laboratory personnel, prescreened by the developed method and methods routinely used in the laboratory to assess the absence of the analytes under investigations and of the most consumed drug of abuse (e.g., cocaine, amphetamines, opiates and benzodiazepines), and then pooled for the preparation of calibrators and quality control samples. Calibrators were prepared adding the appropriate working standard solution volumes in blank hair pool at 5.0 pg/mg hair, 10.0 pg/mg hair, 50.0 pg/mg hair, 200.0 pg/mg hair, 500.0 pg/mg hair. Low-, medium-, and high-quality control samples were set at 15.0, 250.0 and 400.0 pg/mg hair, respectively.

Hair Sample Preparation

Hair samples were decontaminated by three washing steps with dichloromethane, allowed to dry at room temperature, finely cut by scissors, and then weighed to have 20 mg specimen. This amount was placed into silanized glass vials,

soaked in 250 μ L of 2 mM ammonium formate, methanol and acetonitrile mixture (50/25/25, v/v/v) together with 1 μ L IS and incubated overnight at 40°C. After incubation, the samples were vortexed and centrifuged. The supernatant was evaporated to dryness under nitrogen stream and reconstituted with 100 μ L mobile phase mixture (A:B, 80:20), as below described, and a 10 μ L was injected into UHPLC-HRMS.

Ultra-High-Performance Liquid Chromatography

Chromatographic separation was carried out using a Thermo Scientific™ Accucore™ Phenyl-Hexyl column (100 x 2.1 mm, 2.6 μ m) maintained at 40°C. Gradient elution was performed with mobile phase A (2 mM Ammonium formate in water, 0.1% formic acid) and B (Ammonium formate 2 mM in MeOH/Acetonitrile 50/50, 0.1% formic acid) at 0.4 mL/min flow rate. The initial composition (1% B) was maintained for 0.5 min, increased from 1 to 10% B over 3.5 min, from 10 to 50% over 6 min, from 50 to 95% in 1 min, held at 95% for 1 min, and returned to initial conditions over 0.5 min. A 5.5 min equilibration followed, yielding a total run time of 18 min.

High Resolution Mass Spectrometry

The HESI II operated in positive-ionization mode. Sheath gas (nitrogen) and auxiliary gas (nitrogen) flow rates were 35 and 15 a.u., respectively. Spray voltage was 3.0 kV. Capillary and source temperatures were at 320°C.

Data were acquired in full-scan (FullMS) and data-dependent MS/MS scan (DdMS²) using an inclusion list containing the exact mass (mass tolerance, 5 ppm) and acquisition windows (retention time \pm 0.80 min) of targeted analytes (Table I). FullMS acquisition was performed from m/z 100-500 with a resolution of 70,000 at full width at half maximum (FWHM) at m/z 200; automatic gain control (AGC) target was 10⁶ and maximum injection time (IT) was 200 ms. DdMS² acquisition parameters were: resolution, 17,500; isolation window, m/z 2; AGC target, 10⁵; maximum IT, 200 ms; loop count 3. MS/MS spectra were generated using stepped normalized collision energies (NCE) of 17.5, 35.0, 52.5%.

Software and Spectral Library identification

Stock solutions were injected with the above-described method for the acquisition of SCs spectra and the creation of a compound database with the respective retention times. All acquired spectra were

imported into the built-in library. Data acquisition and processing were performed with TraceFinder (v. 4.1), and the exact mass (within 5 ppm of target), retention time (within 0.8 min of target), isotope pattern (higher than 80 % match) and fragmentation pattern (presence of at least three specific fragments and library match higher than 80%) of a compound were used for positive identification to establish LOIs (Limit of Identifications).

Method Validation

The method was fully validated following the most recent criteria established by the international guidelines for the validation of analytical toxicology and bioanalytical methods^{22,23}.

Calibration points ranged from the limit of quantification (LLOQ) to 500 pg/mg for all analytes under investigation and each calibrator were analyzed in five separate runs.

Twenty different drug-free hair samples from laboratory personnel were analyzed to evaluate possible endogenous and exogenous interferences. They were firstly analyzed as blank samples to disclose eventual endogenous interferences and then analyzed after adding a mixture of 20 pg/mg hair common drugs of abuse (cocaine, benzoylecgonine, amphetamine, MDMA, THC, morphine, codeine, diazepam).

Carryover was assessed by injecting drug-free samples after the highest concentration point of the calibration curve.

Accuracy, intra-run and inter-run precision were evaluated by analyzing low, medium and high QCs in triplicate over five days (n=15). Accuracy was calculated for each QC as 100 \times gran mean of observed concentration-known concentration/known concentration (Bias %). One-way analysis of variation (ANOVA) approach was used for determination of intra-run and inter-run precision expressed as coefficient of variation (%CV). Bias values, intra-run and inter-run precision were considered acceptable when between \pm 15% and <15% CV, respectively.

In accordance with the accepted criteria for method validation in analytical toxicology²², LOD was determined with decreasing concentrations of three different drug-fortified blank hair samples and with a signal to noise ratio of 3:1.

LLOQ has been administratively chosen as the lowest calibrator and was assessed by analyzing three different blank hair sources fortified with the analyte at the concentration of the decision point and demonstrating that bias and precision met the established criteria (bias of \pm 20% and a % CV of <20%)²².

Table I. Molecular formula, exact mass of the protonated analyte, $\Delta m/z$, retention time (RT) of synthetic cathinones under investigation.

Analyte	Molecular Formula	m/z [M-H] ⁺	$\Delta m/z$ (ppm)	Retention time (min.)
1-Naphyrone	C ₁₉ H ₂₃ NO	282,1852	0.4	8.82
3,4-DMMC	C ₁₂ H ₁₇ NO	192,1387	-3.0	6.19
3-CMC	C ₁₀ H ₁₂ NOCl	198,0682	-3.0	4.58
3-fluoro- α -PVP	C ₁₅ H ₂₀ FNO	250,1603	-1.6	6.84
3-MMC	C ₁₁ H ₁₅ NO	178,1226	-0.6	4.64
4-BMC	C ₁₀ H ₁₂ NOBr	242,0177	-0.3	5.34
4-EEC	C ₁₃ H ₁₉ NO	206,1540	-0.8	6.74
4-fluoro- α -PHP	C ₁₆ H ₂₂ FNO	264,1757	-0.6	8.04
4-FMC	C ₁₀ H ₁₂ FNO	182,0978	-0.2	2.86
4-FMC metab	C ₁₀ H ₁₄ FNO	184,1133	-0.9	2.78
4-MEC	C ₁₂ H ₁₇ NO	192,1384	-1.7	5.40
4-MEC metab	C ₁₂ H ₁₉ NO	194,1545	-3.0	5.26
4-MMC	C ₁₁ H ₁₅ NO	178,1227	-1.2	4.61
Benzedrone	C ₁₇ H ₁₉ NO	254,1542	-3.00	8.37
Buphedrone	C ₁₁ H ₁₅ NO	178,1226	-0.6	4.24
Butylone	C ₁₂ H ₁₅ NO ₃	222,1123	0.0	4.98
Dimethylcathinone	C ₁₁ H ₁₅ NO	178,1226	-0.4	3.25
Ethcathinone	C ₁₁ H ₁₅ NO	178,1221	0.6	3.48
Ethylone	C ₁₂ H ₁₅ NO ₃	222,1127	-1.9	4.20
Euthylone	C ₁₃ H ₁₇ NO ₃	236,1283	-1.7	5.53
MDPBP	C ₁₅ H ₁₉ NO ₃	262,1437	-0.6	5.88
MDPHP	C ₁₇ H ₂₃ NO ₃	290,1756	-3.0	8.06
MDPV	C ₁₆ H ₂₁ NO ₃	276,1594	-0.5	6.94
Mephedrone d3	C ₁₁ H ₁₂ D ₃ NO	181,1420	-0.7	4.60
Methcathinone	C ₁₀ H ₁₃ NO	164,1069	0.3	2.77
Methedrone	C ₁₁ H ₁₅ NO ₂	194,1175	-0.1	4.10
Methylone	C ₁₁ H ₁₃ NO ₃	208,0968	-0.0	3.40
MPHP	C ₁₇ H ₂₅ NO	260,2012	-1.8	8.71
Naphyrone	C ₁₉ H ₂₃ NO	282,1852	-1.0	9.16
NEP	C ₁₃ H ₁₉ NO	206,1545	-3.0	6.33
N-Ethylpentylone	C ₁₄ H ₁₉ NO ₃	250,1443	-3.1	6.68
Pentedrone	C ₁₂ H ₁₇ NO	192,1382	-0.4	5.94
Pentylone	C ₁₃ H ₁₇ NO ₃	236,1281	-0.8	6.34
α -PHP	C ₁₆ H ₂₃ NO	246,1851	-1.9	7.86
α -PVP	C ₁₅ H ₂₁ NO	232,1698	-1.2	6.60

3,4-DMMC, 3,4-Dimethylmethcathinone; 3-CMC, chloromethcathinone; 3-fluoro- α -PVP, 3-fluoro- α -Pyrrolidinovalerophenone; 3-MMC, 3-Methylmethcathinone; 4-BMC, 4-bromomethcathinone; 4-EEC, 4-Ethylethcathinone; 4-fluoro- α -PHP, 4-fluoro- α -Pyrrolidinohexanophenone; 4-FMC, Flephedrone; 4-FMC metab, 4-fluoromethcathinone metabolite; 4-MEC, 4-Methylethcathinone; 4-MEC metab, 4-methylethcathinone metabolite; 4-MMC, Mephedrone; MDPBP, 3,4-Methylenedioxy- α -pyrrolidinobutiophenone; MDPHP, 3,4-Methylenedioxy- α -Pyrrolidinohexanophenone; MDPV; MPHP, 4-Methyl- α -pyrrolidinohexanophenone; NEP, α -Ethylaminopentiofenone); α -PHP, α -Pyrrolidinohexiophenone; α -PVP, α -Pyrrolidinovalerophenone

Ion suppression/enhancement and recovery for each analyte were measured at low and high QC concentrations. Matrix effect was calculated with the following equation: $ME = [(B/A)-1] \times 100$ where B is the peak area of the analytes in a blank sample fortified after extraction and A the peak area of the reference stan-

dards solution at the same concentration²²⁻²⁴. Recovery was calculated by Matuszewski experimental design²⁴.

Dilution integrity of samples with a concentration 5 and 10 times above the highest calibration point was evaluated verifying the curve fitting, bias and precision.

Real Samples

The applicability of the method has been proven by analyzing eight authentic hair samples from declared SCs consumers donated by the Section of Legal Medicine storehouse (Università Politecnica delle Marche, Ancona, Italy) as discarded non-analyzed material and no data about demographics or any other information were available.

Results

A new UHPLC-HRMS method was developed and validated for the targeted screening and the quantification of 32 different synthetic cathinones and two metabolites in hair

Specifically, TraceFinder software provided simultaneous screening and quantification of SCs based on their full scan data in hair. The software permitted to identify SCs reported in the Table I through exact mass, retention time, fragment ions and isotope patterns matching, and correspondence with a compound library. As showed in the Table I, the measured exact mass error ranged between -3.1 ppm and 0.6 ppm, demonstrating a good accuracy in the exact mass determination.

Indeed, the majority of analytes under investigation had similar structure and some of them had also the same exact mass. The present method was able to chromatographically separate all the following isomers: 3,4-DMMC, 4-MEC and pentedrone; ethylone and butylone; NEP and 4-EEC; naphyrone and 1-naphyrone; 3-MMC, buphedrone, dimethylcathinone, ethcathinone; pentylone and euthylone; butylone and ethylone) with the exception of 4-MMC and 3-MMC. These latter two were only partially resolved under the established chromatographic conditions. Nevertheless, the system was able to give the right identification match, but it was not possible to quantify simultaneously the two of them, and for this reason 4-MMC has been separately validated. Figure 1 shows a representative chromatogram in overlay of all analytes under investigation.

Linearity was assessed from 5 pg/mg (LLOQ) to 500 pg/mg by least squares regression with 1/x weighting yielding to determination coefficients (R^2) in the range 0.990-0.999 and was statistically confirmed by performing residual plots test (Table II). LODs and LLOQs were 2 pg/mg and 5 pg/mg for all compounds, respectively. LOIs were determined according to the fitted parameters reported in section Software and Spectral Library

identification (Table II). No signal above LODs analytes was observed in blank matrix samples injected immediately after the highest concentration calibrator, neither additional peaks due to the presence of other tested drugs interfered with any of the analytes under investigation. Intra-day and inter-day precision and accuracy values were always acceptable (%CV < 15% and bias within $\pm 15\%$). The majority of compounds showed matrix ion suppression within -15% except for 4-MEC with ion suppression at -18.60% (Table III) while eight substances showed matrix ion enhancement within 15% and recovery ranged from 64.32 to 116.00% (Table III).

Discussion

Eight authentic hair samples from SCs consumers were tested with the validated method and results are reported in the Table IV.

Each analyzed sample was positive to more than one synthetic cathinone, with the exception of the cases H04 and H08. H04 sample was only positive for methcathinone while sample H08 contained 4-MEC and its metabolite, 4-MEC metab. Four out of eight cases tested positive to MDPHP, with a concentration range of 6.0-1,000.0 pg/mg. In addition to MDPHP, the most prevalent SCs found were α -PHP, 4-MEC and 3-MMC. Three samples (H01, H05 and H06) presented SCs with concentration out of the calibration range, so that the analysis was repeated performing a proper sample dilution. Among these, H06 sample revealed the highest 3-MMC concentration (5,000 pg/mg), along with 54.0 pg/mg α -PHP and 6.0 pg/mg MDPHP. The most frequent combination was that composed by α -PHP and 3-MMC. It has to be underlined that for its high toxic potential, 3-MMC was formally notified to EMCDDA in 2012, and due to an increase in seizures and in related harms, it has recently been added to the list of NPS under intensive monitoring²⁵.

Conclusions

LC-HRMS has become the gold standard technique for toxicological analysis and to the best of our knowledge we present here the first LC-HRMS method allowing the simultaneous screening and quantification of several last generation SCs in hair samples of consumers.

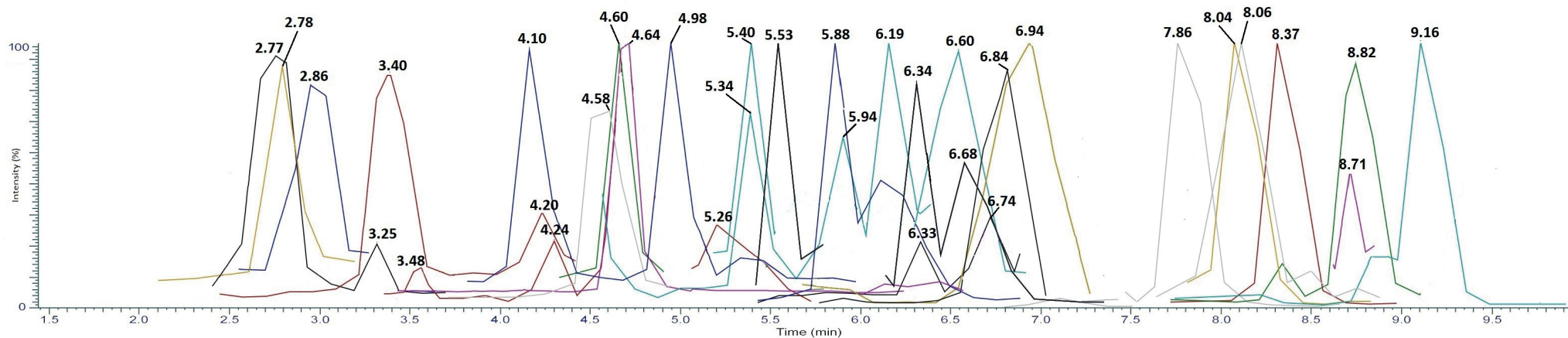


Figure 1. Overlay chromatograms of synthetic cathinones under investigation spiked at 50 pg/mg in blank hair pool. Methcathinone (2.77), 4-FMC metab (2.78), 4-FMC (2.86), dimethylcathinone (3.25), methylone (3.40), ethcathinone (3.48), methedrone (4.10), ethylone (4.20), buphedrone (4.24), 3-CMC (4.58), mephedrone d3 (4.60), 4-MMC (4.61), 3-MMC (4.64), butylone (4.98), 4-MEC metab (5.26), 4-BMC (5.34), 4-MEC (5.40), euthylone (5.53), MDPBP (5.88), pentedrone (5.94), 3,4-DMMC (6.19), NEP (6.33), pentylone (6.34), α -PVP (6.60), N-Ethylpentylone (6.68), 4-EEC (6.74), 3-fluoro- α -PVP (6.84), MDPV (6.94), α -PHP (7.86), 4-fluoro- α -PHP (8.04), MDPHP (8.06), benzedrone (8.37), MPHP (8.71), 1-Naphyrone (8.82), naphyrone (9.16).

Table II. Regression equations, determination coefficients, LOIs.

Analytes	Equation	R ²	LOI
1-Naphyrone	y=0.01789x+0.0615	0.998	4
3,4-DMMC	y=0.01656x+0.0404	0.996	3
3-CMC	y=0.01415x+0.0070	0.999	3
3-fluoro- α -PVP	y=0.01249x+0.0213	0.992	2
3-MMC	y=0.03250x+0.0135	0.999	2
4-MMC	y=0.03671x+0.0174	0.995	2
4-BMC	y=0.00823x+0.0002	0.989	4
4-EEC	y=0.01392x+0.0552	0.994	4
4-fluoro- α -PHP	y=0.06368x+0.0223	0.998	2
4-FMC	y=0.00983x+0.0679	0.998	2
4-FMC metab	y=0.02254x+0.0254	0.999	2
4-MEC	y=0.01199x+0.0575	0.999	4
4-MEC metab	y=0.00095x+0.0197	0.996	4
Benzedrone	y=0.02475x+0.0229	0.999	4
Buphedrone	y=0.01695x+0.0682	0.998	3
Butylone	y=0.01942x+0.0670	0.993	3
Dimethylcathinone	y=0.0128x-0.00187	0.995	3
Ethcathinone	y=0.0102x+0.04894	0.999	2
Ethylone	y=0.01842x+0.0531	0.999	3
Euthylone	y=0.02019x+0.0865	0.990	3
MDPBP	y=0.01466x+0.0606	0.992	3
MDPHP	y=0.03369x+0.0716	0.999	5
MDPV	y=0.06790x+0.0324	0.990	4
Methcathinone	y=0.01937x+0.1255	0.995	2
Methodrone	y=0.01803x+0.1034	0.992	3
Methylone	y=0.02206x+0.1158	0.999	2
MPHP	y=0.02002x+0.0950	0.999	4
Naphyrone	y=0.05886x+0.0355	0.990	3
NEP	y=0.01449x+0.0662	0.991	3
N-Ethylpentylone	y=0.01062x+0.0286	0.992	4
Pentredone	y=0.01408x+0.0498	0.997	3
Pentylone	y=0.01264x+0.0716	0.993	2
α -PHP	y=0.00256x+0.0346	0.994	5
α -PVP	y=0.01408x+0.0663	0.993	2

3,4-DMMC, 3,4-Dimethylmethcathinone; 3-CMC, chloromethcathinone; 3-fluoro- α -PVP, 3-fluoro- α -Pyrrolidinovalerophenone; 3-MMC, 3-Methylmethcathinone; 4-BMC, 4-bromomethcathinone; 4-EEC, 4-Ethylethcathinone; 4-fluoro- α -PHP, 4-fluoro- α -Pyrrolidinohexanophenone; 4-FMC, Flephedrone; 4-FMC metab, 4-fluoromethcathinone metabolite; 4-MEC, 4-Methylethcathinone; 4-MEC metab, 4-methylethcathinone metabolite, 4-MMC, Mephedrone; MDPBP, 3,4-Methylenedioxy- α -pyrrolidinobutiophenone; MDPHP, 3,4-Methylenedioxy- α -Pyrrolidinohexanophenone; MDPV; MPHP, 4-Methyl- α -pyrrolidinohexanophenone; NEP, α -Ethylaminopentiophenone); α -PHP, α -Pyrrolidinohexiophenone; α -PVP, α -Pyrrolidinovaleropheno

The method allows the determination of 32 different SCs and two metabolites using an easy sample treatment and a total analysis time compatible with a high throughput laboratory. The strength of the method is the discrimination among isomers with the same exact mass. It should be said that under the presented chromatographic conditions, only 4-MMC and 3-MMC are partially resolved. It is possible to cor-

rectly identify them, but the partial resolution would affect the accurate quantification of the two isomers in the unlikely occurrence of co-consumption. The developed method showed a good analytical sensitivity and specificity. Finally, the FullMS-DdMS² acquisition allows to update the method by including additional SCs, which is fundamental considering the continuous evolution of the NPS phenomenon.

Table III. Intra-, inter-day precision, and accuracy values (N=15, %CV and bias%) in hair for 34 synthetic cathinones at low QC (15 pg/mg), medium QC (250 pg/mg), high QC (400 pg/mg). Matrix enhancement/suppression (ME) and analytical recovery expressed as mean values of low QC and high QC.

Analytes	intra-day precision (%CV)				inter-day precision (%CV)				Accuracy (bias %)				ME (%)				Recovery (%)			
	Low	Med	High		Low	Med	High		Low	Med	High		Low	High	Low	High	Low	High		
	QC	QC	QC	QC	QC	QC	QC	QC	QC	QC	QC	QC	QC	QC	QC	QC	QC	QC		
1-Naphyrone	2.37	2.19	1.18	3.48	9.87	2.15	4.96	-5.26	-12.51	8.00	12.00	70.24	89.95							
3,4-DMMC	0.51	11.94	0.58	0.51	0.58	1.99	0.01	-0.47	4.78	-7.94	-4.01	88.00	95.99							
3-CMC	6.29	1.40	1.58	5.44	1.24	3.46	-4.50	0.85	2.08	-11.84	-2.64	64.32	68.00							
3-fluoro- α -PVP	2.05	1.43	0.75	5.17	1.65	5.05	-1.54	4.00	3.09	-2.04	-5.92	71.00	91.00							
3-MMC	2.39	2.50	2.27	2.11	4.62	2.31	0.34	-2.00	1.12	-13.24	-11.71	72.00	88.22							
4-MMC	3.32	1.09	1.84	2.74	1.44	1.60	0.99	2.17	0.21	-9.86	-13.82	83.00	94.00							
4-BMC	8.41	6.35	0.81	7.72	6.35	3.04	6.27	3.09	4.56	9.08	14.10	71.00	77.79							
4-EEC	2.77	0.18	0.77	5.93	2.46	6.27	0.12	-0.24	6.64	-12.62	-11.67	89.51	77.91							
4-fluoro- α -PHP	0.83	2.18	2.44	2.00	8.07	2.73	1.49	-4.29	-13.93	8.25	2.71	80.01	91.00							
4-FMC	5.29	1.19	0.64	5.04	3.41	0.76	2.84	1.18	-1.45	-5.00	8.00	76.71	84.22							
4-FMC metab	5.17	1.31	4.85	4.80	2.77	3.93	6.24	2.14	-12.41	-11.00	12.00	105.31	116.00							
4-MEC	1.21	1.41	3.13	0.88	1.91	2.81	0.69	1.06	1.78	-18.60	-4.32	99.13	95.19							
4-MEC metab	3.51	0.74	5.78	4.56	0.96	4.60	0.47	1.71	1.93	-8.62	-11.45	99.50	90.30							
Benzedrone	0.83	2.05	1.04	1.32	1.60	0.75	1.18	0.94	-8.83	1.00	5.00	102.58	83.83							
Buphedrone	1.92	1.35	1.60	2.58	1.49	1.22	5.03	1.45	1.18	-3.59	5.09	81.48	93.03							
Butylone	4.37	0.37	0.88	3.38	0.31	0.96	6.06	0.84	1.67	-13.67	-8.50	89.54	101.15							
Dimethylcathinone	0.80	0.52	0.18	1.49	0.37	0.70	3.12	1.35	1.08	1.00	3.00	78.69	81.09							
Ethcathinone	1.54	0.38	0.42	2.34	0.44	0.86	2.95	0.94	0.13	12.00	15.00	78.37	80.96							
Ethylone	2.68	2.77	1.09	5.05	2.30	2.82	3.57	1.68	0.74	-12.00	-9.00	91.22	85.00							
Euthylone	1.32	1.79	1.28	3.65	1.63	1.50	0.40	0.93	1.74	-10.35	-11.71	109.35	107.22							
MDPBP	3.29	0.31	3.23	6.44	1.36	2.69	0.91	2.32	2.87	-13.31	-6.52	83.51	94.87							
MDPHP	0.46	1.03	1.16	0.83	2.06	0.83	1.93	4.27	-11.02	-12.92	3.34	81.20	83.49							
MDPV	1.70	0.52	0.26	3.74	1.81	2.37	1.48	0.88	-0.01	-6.56	-2.41	96.50	99.83							
Methcathinone	0.84	0.23	2.36	3.84	0.87	2.87	6.19	2.00	-1.79	-14.00	15.00	70.00	71.34							
Methedrone	3.14	0.70	0.54	9.02	0.60	0.76	7.74	2.24	0.95	-6.00	-4.61	93.00	86.74							
Methylone	1.68	2.74	1.16	5.84	3.80	4.28	6.76	1.79	-4.94	-10.55	6.79	81.80	95.78							
MPHP	1.31	2.25	0.51	6.12	1.91	0.56	7.49	1.10	-6.99	-2.80	10.76	85.74	88.70							
Naphyrone	3.10	1.22	1.27	2.31	2.96	1.61	10.18	4.61	4.05	-1.69	-1.78	97.57	100.11							
NEP	3.85	0.74	1.99	8.07	0.56	3.64	3.29	0.03	4.32	-6.85	-10.16	78.00	86.29							
N-Ethylpentylone	3.58	0.33	0.21	3.89	0.78	1.20	1.06	1.54	3.04	-9.35	-2.04	75.42	71.26							
Pentadrone	0.71	2.85	0.73	1.20	3.75	0.88	3.04	1.78	3.42	-3.18	0.95	63.35	77.49							
Pentylone	0.95	1.84	0.85	0.72	1.37	1.41	3.93	0.60	2.22	-11.94	-8.91	70.83	76.32							
α -PHP	1.95	2.04	6.14	4.72	9.34	10.48	-0.77	-6.65	3.20	-7.82	-5.42	75.00	83.59							
α -PVP	3.84	0.63	0.69	3.28	1.23	5.18	0.42	0.74	12.43	-7.86	-8.57	73.05	75.00							

Table IV. Concentration (pg/mg) of target analytes in hair of SCs consumers.

	H01	H02	H03	H04	H05	H06	H07	H08
MDPHP	1,000.0		14.5		50.0	6.0		
3,4 DMMC	-		27.4					
4-MEC		11.5						448.0
4-MEC metab								123.0
α -PHP					554	54.0		
Butylone							176.0	
4-MMC	91.3							
Methcathinone		15.7		10.8				
3-MMC					556.0	5,000.0		
Methylone							140.0	

Conflict of Interest

The authors declare that they have no conflict of interest.

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Authors' Contributions

Conceptualization and study design: Nunzia La Maida, Giulio Mannoichi and Francesco Paolo Busardò; methodology and validation: Nunzia La Maida, Emilia Marchei, Giulio Mannoichi, Alessandro Di Giorgi and Giuseppe Basile; writing – original draft preparation, Nunzia La Maida, Simona Pichini, Alessandro Di Giorgi and Giuseppe Basile; writing – review and editing, all authors. All authors read and approved the final manuscript.

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Ethical Approval

None.

Informed Consent

None.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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