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Microextraction by packed sorbent for the determination of selected synthetic cathinones and 2C-P in hair

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ABSTRACT

New psychoactive substances (NPS), including synthetic cathinones and phenethylamines, pose significant challenges due to their evolving chemical structures and health impacts. This study aimed to optimize and validate a methodology for detecting and quantifying a number of synthetic cathinones [methylone, ethylone, pentedrone, 4-chloroethcathinone (4-CEC), penthylone, α-pyrrolidinopentiophenone (α-PVP), 3,4-methylenedioxypyrovalerone (MDPV), 4-chloro-α-pyrrolidinovalerophenone (4-Cl-α-PVP), as well as phenethylamine 2,5-dimethoxy-4(n)-propylphenethylamine (2C-P)], in hair samples using microextraction by packed sorbent (MEPS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). We developed an environmentally friendly and cost-effective MEPS procedure, which was validated for selectivity, linearity, precision, accuracy, and recovery. Results demonstrated that the method effectively detects analytes with limits of quantification as low as 10 pg/mg. The method successfully identifies MDPV in concentrations consistent with those observed in chronic and acute drug consumption cases, proving its suitability in forensic and clinical toxicology. This approach offers a robust solution for forensic and clinical applications, by combining efficient sample clean-up with precise analytical capabilities.

Introduction

New psychoactive substances (NPS), often referred to as designer drugs or synthetic drugs, are associated with increased concerns by social and health authorities due to their ever-evolving chemical structures, unpredictable metabolism, and variable toxicity [1]. Amongst the varied group of NPS, there are synthetic cathinones ('bath salts') and phenylethylamines; these substances are manufactured in labs and have similar effects to those of amphetamines, acting on the central nervous system [2]. According to recent data, both classes are among the most detected NPS globally, largely due to their psychoactive appeal and relatively easy availability through illicit online markets. Their prevalence underscores the need for ongoing monitoring and public health interventions to mitigate associated health risks [3–5]. Included in these groups of NPS are methylone, ethylone, pentedrone, 4-chloroethcathinone (4-CEC), penthylone, α-pyrrolidinopentiophenone (α-PVP), 3,4-methylenedioxypyrovalerone (MDPV), 4-chloro-α-pyrrolidinovalerophenone (4-Cl-α-PVP), and 2,5-dimethoxy-4(n)-propylphenethylamine (2C-P) [1]. Some of these compounds have been detected in blood [6,7], urine [8,9], blood and urine [10,11], oral fluid [12–17], hair samples [18–21], as well as in other samples.

Hair testing has several benefits, including easy and non-invasive collection procedure, a wider time window of detection (from weeks to years, depending on hair length), and the convenience of room temperature storage without compromising analyte stability in the matrix.

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Reliable screening and validation processes for the assessment of these new synthetic substances in biological material are required to improve the ability and scope of drug testing, in both clinical research and forensic analysis. While offering significant benefits, hair testing comes with its own set of challenges due to the intricate composition of the hair matrix. Individual factors, such as personal characteristics, hair care treatments, and exposure to environmental elements, can influence the matrix's composition. This variability poses difficulties in standardizing analytical methods and may impact the consistency and precision of the results [22]. In addition, the fact that positive results may arise following environmental exposure (without active consumption) poses further difficulties in interpretation. As such, strict procedures need to be followed to ensure that no false positive results are issued (for instance, but not limited to, washing the samples, detection of unique metabolites or using metabolite-to-parent drug ratios) [23].

Studies have already been published where synthetic cathinones were detected in hair samples. These involved extracting the drugs by incubation with ammonium formate [24], or carbonate buffer [19], followed by clean-up using several different approaches. Along with these techniques, pretreatment of samples was performed through solid-phase extraction (SPE) or liquid-liquid extraction (LLE). These techniques involve the use of high amounts of solvents (several mL) and, in the case of SPE, the cartridges are intended for single use. Hence, in this work a more environmentally friendly and economically appealing approach was used, i.e., microextraction by packed sorbent (MEPS), where a single cartridge could be used for up to 100 times [25]. MEPS has already been reported useful as clean-up for the determination of several substances in hair, namely amphetamines [26], ketamine [27], cocaine and metabolites [28], methadone and EDDP [29], opiates [30], and ethyl glucuronide [31]. This approach has also been used for the detection of NPS in biological specimens, namely synthetic cathinones [15,32,33], piperazines, phenethylamines, synthetic cannabinoids, and metabolites [33], synthetic hallucinogens [16], phenyltropanes [14] in oral fluid, designer benzodiazepines and Z-hypnotics in plasma [34], ketamine and norketamine in hair [27], and piperazines in urine [35, 36], synthetic tryptamines in urine [9].

This paper describes the optimization and validation of a methodology for the detection and quantification of selected substances (cathinones and phenethylamines) in hair samples, using MEPS for sample clean up and LC-MS/MS. Our research group is already experienced in developing methods for hair testing using MEPS for several different drug groups [26–30]. However, the substances included in this study are considerably different in most cases, for instance concerning their physical-chemical properties and structure. This means that the MEPS protocol must be finely optimised to maximise analyte recovery from the sample. The main differences compared to other methods are in the steps of conditioning (the second solvent), sample passage through the sorbent (the number and volume of the extracting strokes), washing (number of times and solvent composition) and elution (number of times and solvent composition). This is the first described method for the analysis of these compounds in hair using MEPS for sample cleanup.

Analytes included in the investigation were chosen based on previously published case reports of forensic intoxication involving NPS and the commercial availability of the compounds.

Material and methods

Materials, reagents and instrumentation

Analytical standards of synthetic cathinones (methylone, ethylone, pentedrone, 4- 4-CEC, penthylone, α-PVP, MDPV, 4-Cl-α-PVP, and phenethylamine 2C-P and cocaine-D3 (IS) were purchased from LGC Promochem (Barcelona, Spain). LC-MS grade quality methanol, dichloromethane, acetonitrile, and formic acid were supplied by Merck Co (Darmstadt, Germany). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Fisher Scientific (Loughborough, United Kingdom). Isopropanol (analytical grade) and ammonium hydroxide were obtained from Laborspirit, Lda, (Lisbon, Portugal), and (J.T. Baker, Deventer, Holland), respectively. Dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were supplied from Fisher Scientific (Enzymatic; Santo Antão do Tojal, Portugal). Deionised water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). VWR international provided the MEPS syringe (250 μL), and M1 extraction cartridges (4 mg; 80 % C_8 and 20 % SCX) were purchased from SGE Analytical Science (Alfragide, Portugal).

The internal standard (IS) was diluted to a final working solution of 2 μg/mL, and the working solutions of the analytical standards were produced by dilution with methanol to the final concentration of 1 and 10 μg/mL. Light protection was maintained for all stock and working solutions at 4 °C.

The statistical analysis for optimization was accomplished using Minitab Statistical Software version 17.

For this work, Waters Acquity UPLC system coupled to Waters TQD mass spectrometer (Waters® Corporation, Milford, MA, USA) was used for chromatographic analysis. The software used to acquire data in multiple reaction monitoring (MRM) mode was the Masslynx 4.1. The operational parameters of the electrospray ionization (ESI) source were operated in positive mode with the following conditions: capillary voltage (0.5 KV), extractor voltage (2.0 V), source temperature (120 °C), desolvation temperature (450 °C), flow of the drying gas (600 L/hr) and cone gas flow (20L/hr).

The chromatographic column used, ACQUITY UPLC® HSS T3 (2.1 × 100 mm; 1.8 μm), was kept at 45 °C, and the total run time was of 14 min. Although all the compounds under study elute within the first 6 min, a 14-minute chromatographic run was chosen, as this chromatographic method is used routinely in the laboratory, allowing for the detection of other drugs of abuse.

Each compound was monitored by two transitions in MRM, and one for the IS. Tables 1 and 2 contain full information on all setups and instrumental parameters. The analyses were carried out under gradient elution with binary phase A (2 mM ammonium formate, 0.1 % formic acid in methanol) and B (2 mM ammonium formate, 0.1 % formic acid in water) at a flow rate of 0.4 mL/min in accordance with the profile shown in Table 1.

Hair samples

Drug-free hair samples for MEPS optimization and method validation were provided by laboratory staff [Health Sciences Research Centre (CICS-UBI, Covilhã, Portugal)]. Authentic hair samples were collected from individuals who attended a electronic music festival, in Portugal, in July 2024. Paper envelopes containing both blank and genuine samples were kept at ambient temperature, out of direct sunlight, and in a dry environment. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee at *Universidade* da *Beira Interior* (number CE-UBI-Pj-2022–04).

Sample preparation

Hair decontamination and extraction

Hair samples were washed consecutively with dichloromethane,

Table 1
Chromatographic elution profile.

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.4	10	90
0.50		10	90
8.00		95	5
11.00		95	5
11.10		10	90
14.00		10	90

Table 2
Mass spectrometric parameters for all analytes under study.

Analyte	Precursor ion (m/z)	Quantifying fragment (m/z)/Collision energy (V)	Qualifier fragment (m/z)/ Collision energy (V)	Retention time (min)	Cone voltage (V)
Methylone	208	160/16	190/23	2.56	23
Ethylone	222	174/20	204/10	2.88	30
Pentedrone	192	174/7	91/7	3.80	42
4-CEC	212	144/25	159/13	3.79	90
Pentylone	236	86/8	188/8	3.92	43
α-PVP	232	77/40	91/15	4.01	15
MDPV	276	175/15	126/15	4.15	35
4-Cl-α-PVP	266	195/20	126/20	4.95	20
2C-P	224	207/15	192/15	5.55	35
Cocaine-d3 (IS)	307	185/22	–	3.82	32

deionised water, and methanol for 15 min with agitation to eliminate excess of dirt and deposited compounds that could influence the analysis by possible external contamination. Hair samples were dried at room temperature after the decontamination step, cut into 1-mm fragments and 50 mg were weighed into glass tubes. The tubes were then firmly closed after adding 0.5 mL of NaOH (1M) for extraction. In a vortex-mixer, the tubes were gently stirred before being incubated at 45 °C overnight. Samples were let to cool at room temperature and then were neutralized with 50 μL of 10 M HCl. Subsequently, samples were centrifuged for 15 min at 3500 rpm, and a second centrifugation at 3500 rpm for 5 min was performed. Extracts were transferred to new glass tubes and 0.5 mL of 0.1 M phosphate buffer solution was added and pH was adjusted to 6, to avoid cartridge clogging and disintegration. Finally, 25 μL of IS was added prior to the MEPS procedure.

Microextraction by packed sorbent

The MEPS process was optimized beforehand [26], and the following final conditions were obtained: 250 μL of methanol 250 μL of deionised water were used for cartridge conditioning. A total of eighteen draw/eject cycles of 100 μL of the reconstituted sample were performed. There was no washing step. Lastly, elution of the retained analytes from the sorbent was accomplished with seven strokes of 100 μL of 1 % ammonium hydroxide in acetonitrile. The sorbent was washed and cleaned (reconditioned) with four times of each solvent: 100 μL of 1 % ammonium hydroxide in acetonitrile:methanol (1:1) and 100 μL of 1 % formic acid in isopropanol: deionised water (1:9). The eluted extracts were dried under a nitrogen stream, dissolved in 50 μL of methanol and a 2 μL aliquot was injected into the LC-MS/MS system.

Validation procedure

The analytical method was validated taking into consideration the recommendations from the Society of Hair Testing (SoHT) for drug analysis in hair [37,38], and according to the guiding principles for method validation from ANSI/ASB Standard 036 (1st edition)—Standard Practices for Method Validation in Forensic Toxicology [39]. Selectivity, linearity, limits, intra-, inter-day and intermediate precision and accuracy, as well as recovery and autosampler stability were the parameters assessed during a 5-day validation protocol.

Results and discussion

Method optimization

In hair testing procedures, the analytes must firstly be extracted from within the matrix. Several approaches can be used to this end, for instance solvent extraction [22–25] when the analytes are unstable under strong acidic or alkaline conditions. Synthetic cathinones are stable in alkaline moieties, so alkaline incubation was chosen for analyte extraction, the same for amphetamines [21].

Our research group has developed expertise with the MEPS procedure. Therefore, in this study, we adapted the clean-up technique as described in our previous publication [26], given the similar physical and chemical properties of the substances under investigation. The parameters that can affect the MEPS procedure were as follows, and the tested values are shown between brackets: number of strokes (6, 12, 18 or 24), washing (no wash, or wash with 50 μL of 0.1 % acetic acid in water followed by 50 μL of 10 % methanol), elution [number of cycles, 1–7]. The washing and elution steps did not yield different results from those previously obtained [26]. To evaluate the impact of the number of extraction strokes on the performance of the MEPS procedure, we conducted a series of statistical tests comparing different stroke counts (6, 12, 18, and 24 strokes; $n = 3$) for each targeted analyte. Both F-tests and *t*-tests were applied; the F-test was used to compare the variances across groups, while the *t*-test compared the means between two groups to determine if the differences were statistically significant. The results indicate that, for several compounds, the extraction efficiency was significantly influenced when 18 strokes were used. For instance, ethylone showed significant differences between 12 versus 18 strokes (*t*-test, $p = 0.003$) and between 18 versus 24 strokes (*t*-test, $p = 0.002$). In the case of 4-CEC, the comparison between 6 versus 18 strokes yielded a highly significant difference (*t*-test, $p < 0.001$). For pentylone, both the 12 versus 18 stroke comparison (*t*-test, $p = 0.0046$) and the 18 versus 24 stroke comparison (*t*-test, $p = 0.011$) were statistically significant. Similarly, α-PVP showed significant differences in the comparisons of 6 versus 18 strokes (*t*-test, $p = 0.006$) and 12 versus 18 strokes (*t*-test, $p = 0.033$), while MDPV was significant in the 6 versus 18 stroke comparison (*t*-test, $p = 0.013$), and 4-Cl-α-PVP demonstrated a significant difference in the 6 versus 18 stroke comparison (*t*-test, $p = 0.003$). These findings suggest that for ethylone, 4-CEC, pentylone, α-PVP, MDPV, and 4-Cl-α-PVP, the use of 18 strokes results in a markedly different extraction efficiency compared to other stroke numbers, whereas compounds not showing significant differences appear to be less sensitive to variations in the number of strokes.

In summary, better results were obtained using 24 sample strokes and the final MEPS conditions were: 24 × 100 μL sample strokes, no washing, and finally 7 × 100 μL elution cycles. The main differences relatively to other publications in which drugs of abuse were analysed in hair by MEPS, the main differences are in the number of times the sample passes through the sorbent and the elution solvents. For instance, Simão et al. [27] have developed a method to detect ketamine and norketamine. In this method 15 strokes were used, and a washing step was deemed necessary for interference removal. The analytes were eluted with 100 μL of 3 % ammonium hydroxide in methanol. Rosado *et al.* [28] developed a method to determine cocaine and metabolites, for which 21 strokes were used, as well as 50 μL deionized water and acetate buffer of pH 4 (pH 4), and the analytes were eluted with 3 × 100 μL of 2 % ammonium hydroxide in methanol. The same authors have determined methadone and EDDP [29], including sorbent conditioning with 2 % formic acid and the sample was passed with 9 strokes. The sorbent was washed with 50 μL of 3.36 % formic acid in water, and the analytes were eluted with 6 × 100 μL of 2.36 % ammonium hydroxide in methanol. The same authors have analysed opiates [30], for which the

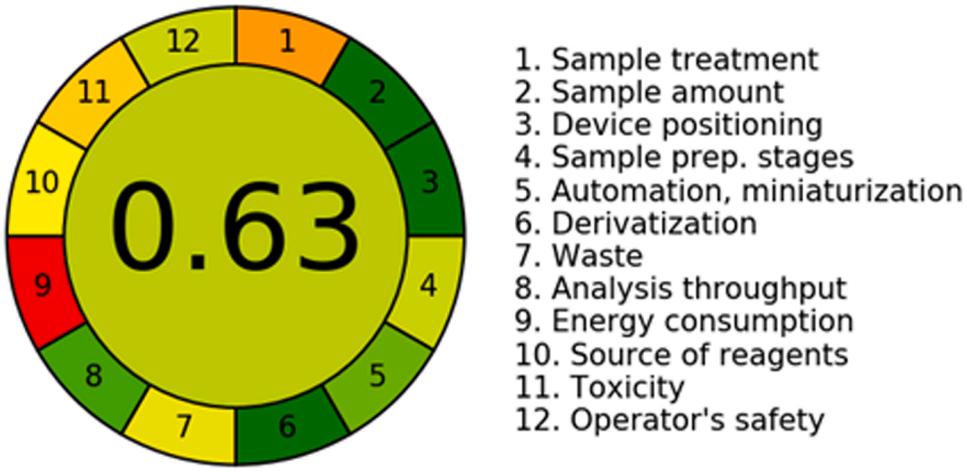

Fig. 1. Method evaluation according to the AGREE: Analytical GREEnness Metric Approach.

sorbent was conditioned with 3 × 250 μL of 2 % formic acid in water, and the sample passed through the sorbent 15 times. The cartridge was washed with 150 μL of 3.36 % of formic acid in water, and the analytes were eluted with 8 × 100 μL of 2.36 % ammonium hydroxide in methanol. Pires *et al.* [26] developed a method for amphetamine identification. Eighteen sample strokes were used, and the sorbent was not washed. Analytes were eluted with 7 × 100 μL of 2 % ammonium hydroxide in acetonitrile.

Moreover, the sample preparation and method was evaluated with the AGREE: Analytical GREEnness Metric Approach, in which all its steps are individually evaluated concerning their greenness [40]. Fig. 1 depicts a diagram

Method validation

Selectivity

Selectivity was assessed by the analysis of blank hair samples from different sources and checking for possible interferences at the retention time and selected transitions for each analyte. Blank matrix samples from ten different sources were extracted with the method described herein.

In accordance with the recommendations of the World Anti-Doping Agency [41], positive criteria for the identification of the analytes included the use of ion ratios, retention time frames, and signal-to-noise measurements. Fig. 2 shows a chromatogram of a blank hair sample, and a sample spiked at 50 pg/mg (except for pentedrone, for which 100 pg/mg was used) for all analytes under study. As a result, this method demonstrated its selectivity, since no compounds were identified in the blank hair specimens' chromatograms at the same retention times.

To evaluate specificity, three blank samples from various sources were spiked with potentially interfering compounds, consisting of a mixture of substances routinely analysed in the laboratory (drugs of abuse, pesticides, and medicinal substances) at a concentration of 200 pg/mg. No significant interferences were detected at the retention times or monitored transitions for the target compounds.

Linearity and limits

The ratio of each target analyte area to its corresponding IS peak area was plotted versus the compound concentration to create calibration curves. Acceptance criteria included a determination coefficient (r^2) equal to or higher than 0.99 and calibrators' accuracies within ± 15 % of the nominal value (except at the LLOQ, where ± 20 % was accepted). Linearity was evaluated using six calibrators, evenly distributed within the working range, in five different runs ($n = 5$). The method's linearity was found to be between 10 and 2500 pg/mg for most compounds, except for α-PVP (30 and 2500 pg/mg), 4-CEC and penthylone (50 and 2500 pg/mg), and pentedrone, (100 and 2500 pg/mg). The residuals analysis (sum of deviations from the calculated concentration to the nominal concentration) indicated that weighted linear regression (1/x) provided the best model for the obtained data.

The LLOQ was considered the lowest concentration that could be measured with a relative standard deviation (RSD) equal to or lower than 20 % and a relative error (RE) within ± 20 % of the nominal concentration. Considering the above, the LLOQ was 10 pg/mg for methylone, ethylone, MDPV, 4-Cl-α-PVP, and 2C-P; 30 pg/mg for α-PVP; 50 pg/mg for 4-CEC and pentylone; and 100 pg/mg for pentedrone. Table 3 summarizes calibration data.

The limits obtained were satisfactory. It is important to highlight that it is not possible to compare the results obtained with others published using the same clean-up technique, as there are no published studies using MEPS for the determination of these compounds. The only published work using microextraction techniques was that of Nzekoue *et al.* [18]. These authors developed a method using liquid-liquid microextraction for the determination of NPS in hair samples. The LLOQ results were 10 pg/mg, with solvent volumes used being approximately greater than 1 mL. Salomone *et al.* [42] e reported the same LLOQ (10 pg/mg), as well as Niebel *et al.* [24], who obtained LLOQ values close to 10 pg/mg.

Precision and accuracy

During the five-day protocol, precision and accuracy were assessed using the concentrations between LLOQ to 2500 pg/mg (four concentrations). For all concentration levels, coefficients of variation (CV) equal to or <15 % met the acceptance criteria for precision, whereas mean relative error (RE)/BIAS in the range of ± 15 % was used to measure accuracy for all concentrations except at the LLOQ (± 20 %). With respect to inter-day precision and accuracy, target analytes CVs were generally of <14 %, and the accuracy ranged from 83 % to 114 %. As for intra-day precision and accuracy, five replicates of blank hair samples spiked at 10, 30, 50, 800 and 2500 pg/mg were analysed that same day. The obtained CVs, which had an accuracy range of 97 to 109 % and were less than ± 15 %, met the established standards.

During the five-day validation phase, quality control (QC) samples at four concentration levels (10, 30, 50, 800 and 2500 pg/mg) were used in triplicate to study intermediate precision and accuracy concurrently with the calibration curve. The obtained CVs were lower than 12 % [18 % (for 4-Cl-α-PVP at LLOQ)], and accuracy was within 85 to 110 % interval. All data is presented in Table 4.

Recovery studies

To evaluate the cleanup procedure's recovery, blank hair samples spiked at three concentration levels (30, 800 and 2500 pg/mg for

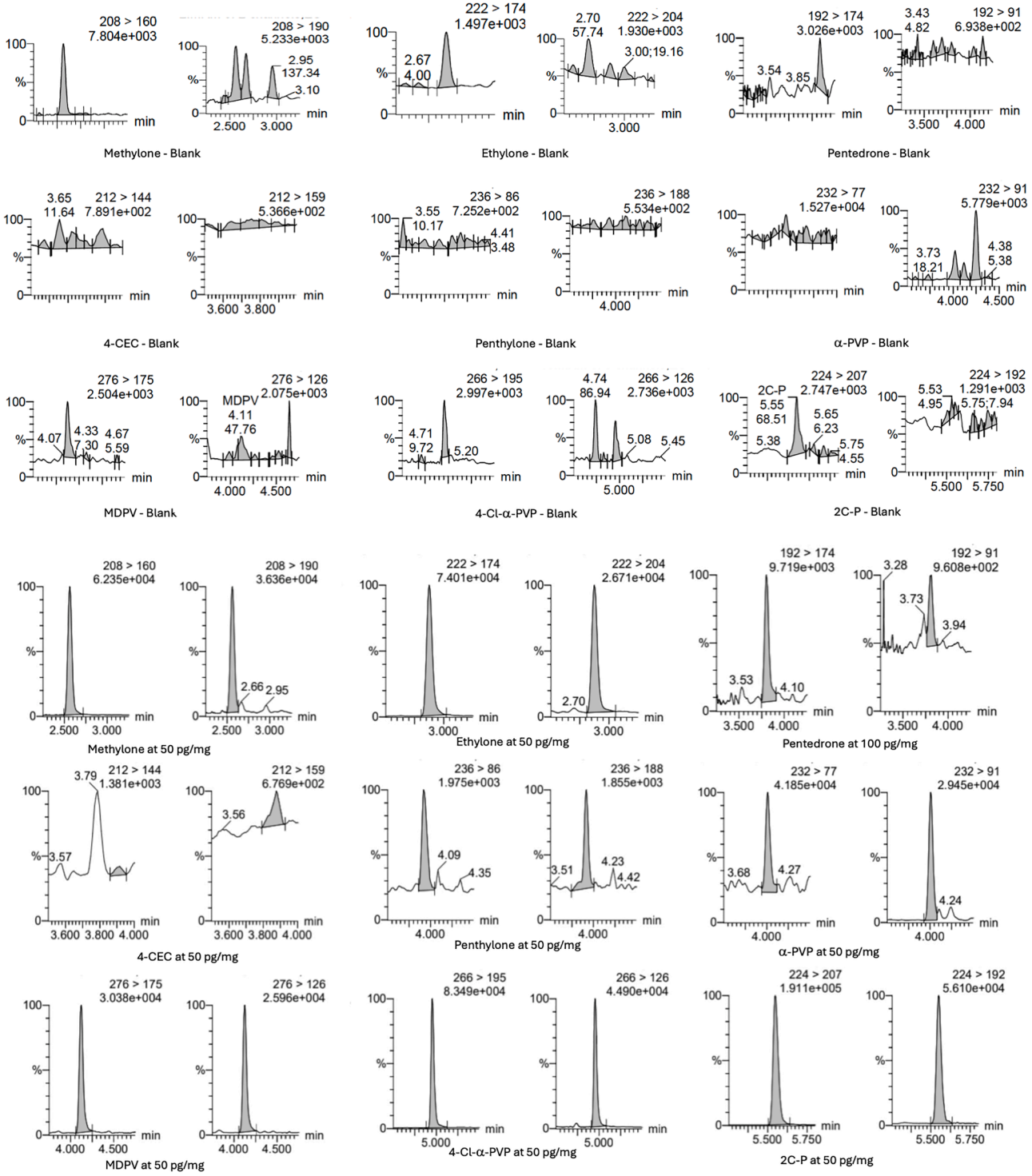

Fig. 2. Chromatogram of a blank sample and a sample fortified at 50 pg/mg, for all analytes, (except for pentedrone - 100 pg/mg).

methylone, ethylone, MDPV, 4-Cl-α-PVP, 2C-P and α-PVP; 100, 800 and 2500 pg/mg for pentedrone and, 50, 800 and 2500 pg/mg for 4-CEC and penthylone) were used to compare the extracted samples (spiked before MEPS, $n = 3$) with the unextracted samples (that represented 100 % recovery, spiked after MEPS procedure, $n = 3$). The IS was added to both sets of samples only after the MEPS process. Recoveries were computed by comparing the relative peak areas obtained in the extracted samples with those obtained for the unextracted samples. The obtained results were considered satisfactory: 8 – 34 % for methylone, 11 – 37 % for ethylone, 12 – 16 % for pentedrone, 13 – 85 % for 4-CEC, 17 – 54 % for penthylone, 17 – 41 % for α-PVP, 18 – 46 % for MDPV, 27 – 71 % for 4-Cl-α-PVP and finally, 31 – 71 % for 2C-P. Despite of the variations seen in the recoveries for some of the compounds, no significant impact was observed on the method's linearity. In addition, precision and accuracy were adequate even at low concentrations, showing that these variations had no significant impact. The thorough optimisation the method was subjected to beforehand may have contributed to this, minimising the impact of such variations, in spite of not using an isotopically labelled internal standard for each analyte.

Alvarez *et al.* [19], obtained recovery values of 87 % for MDPV,

Table 3
Linearity parameters and quantification of the method for all analytes studied.

Analyte	Linear range (pg/mg)	Linearity*		r²*	LLOQ (pg/mg) (n = 5)
		Slope	Intercept		
Methylone	10 – 2500	0.108 ± 0.012	0.005 ± 0.001	0.994 ± 0.003	10
Ethylone	10 – 2500	0.159 ± 0.033	0.005 ± 0.001	0.993 ± 0.002	10
Pentedrone	100 – 2500	0.004 ± 0.001	0.001 ± 0.0003	0.992 ± 0.002	100
4-CEC	50 – 2500	0.001 ± 0.0002	0.001 ± 0.00004	0.993 ± 0.001	50
Penthylone	50 – 2500	0.004 ± 0.0007	0.0003 ± 0.001	0.996 ± 0.0023	50
α-PVP	30 – 2500	0.073 ± 0.004	0.0037 ± 0.0019	0.995 ± 0.001	30
MDPV	10 – 2500	0.075 ± 0.029	0.002 ± 0.0005	0.996 ± 0.002	10
4-Cl-α-PVP	10 – 2500	0.143 ± 0.058	0.004 ± 0.002	0.994 ± 0.004	10
2C-P	10 – 2500	0.225 ± 0.020	0.007 ± 0.002	0.995 ± 0.002	10

* Mean values ± standard deviation.

however, the procedure of extraction was LLE, which uses more solvents, making it less eco-friendly. Recoveries in the range of 85 – 96 % were obtained for methylone, MDPV, pentedrone, 2C-P and α-PVP, by Salomone *et al.* [42].

Matrix effect

The matrix effect was calculated according to ANSI/ASB standard [39], using blank hair spiked in triplicate with 30 (100 pg/mg for pentedrone, 4-CEC and penthylone) and 2500 pg/mg. Matrix effects were assessed by comparing the analytes' peak areas with those obtained by the injection of neat standards at the same concentration (no matrix). A value of 100 % indicates no matrix effect, while values below 100 % suggest ion suppression and values above 100 % indicate ion enhancement. The matrix effects were within acceptable ranges, between 90.2 – 116 % for 30 pg/mg, 88.3 – 116.0 % for 100 pg/mg, and 80.6 – 109.4 for 2500 pg/mg, with standard deviations well under 4 % for all concentrations. These matrix effects were considered adequate, as no significant interferences from endogenous components of the hair matrix were observed.

Method applicability

The present method was applied to authentic hair samples from drug consumers. MDPV was the only analyte identified at concentrations higher than the LLOQ, with values of 36.3 pg/mg (Sample A), 72.9 pg/mg in Samples B and 73.2 pg/mg in sample C (Sample C - Fig. 3). The presence of MDPV suggests this synthetic cathinone was used by the individuals, while other substances were either not consumed or were present at levels that were too low for reliable identification and quantification. In a work by Salomone *et al.* [42], researchers were able to identify a positive sample for MDPV with a concentration level of 120 pg/mg. On a different research by Niebel *et al.* [21], the authors found three positive samples for MDPV (concentrations ranged from 20 to 800 pg/mg). It has already been suggested that a concentration of 100 pg/mg may indicate chronic consumption of this substance [19]. A study by Larabi *et al.* [20], documented a concentration of 5 pg/mg for

Table 4
Inter day (n = 5), intra-day (n = 5), intermediate precision (n = 25).

Analyte	Concentration (pg/mg)	Inter-day (%)		Intra-day (%)		Intermediate (%)	
		CV	Accuracy	CV	Accuracy	CV	Accuracy
Methylone	10	11.8	101.3	12.0	101.7	11.4	101.7
	50	4.1	101.2	2.3	101.6	4.5	101.0
	800	3.5	99.2	1.0	99.2	0.9	110.0
	2500	1.1	109.5	0.8	102.5	0.8	103.4
Ethylone	10	9.6	100.2	4.0	100.2	4.9	100.1
	50	6.6	99.0	5.4	98.8	5.0	99.0
	800	7.4	102.0	0.6	104.2	2.7	101.4
	2500	1.2	102.5	0.7	103.0	4.9	98.4
Pentedrone	100	5.0	99.5	3.9	98.4	5.1	99.5
	400	8.3	100.5	4.1	100.6	3.8	100.3
	800	2.6	100.4	2.1	100.5	3.2	100.4
	2500	0.6	99.9	0.1	96.7	0.5	100.0
4-CEC	50	13.5	82.6	11.9	97.2	11.5	85.3
	400	5.1	114.2	6.2	106.4	5.5	102.0
	800	1.9	100.9	2.0	101.1	1.9	101.4
	2500	0.9	98.1	0.7	98.4	1.0	96.9
Penthylone	50	11.3	102.1	9.9	104.4	10.8	101.4
	400	9.0	96.8	9.8	96.5	4.2	97.1
	800	1.3	100.6	1.3	100.3	0.7	100.3
	2500	1.6	96.8	1.9	98.8	1.9	95.6
α-PVP	30	6.8	100.6	1.7	100.1	7.5	100.9
	50	11.6	101.1	11.6	101.5	9.6	100.8
	800	9.7	96.6	2.2	106.7	1.9	92.2
	2500	4.0	101.1	0.3	100.1	2.1	102.0
MDPV	10	10.6	100.6	14.8	100.8	8.9	100.3
	50	6.7	87.0	5.4	102.1	5.3	86.4
	800	4.7	95.6	2.6	109.0	1.0	99.2
	2500	1.5	100.6	1.9	100.9	1.0	100.3
4-Cl-α-PVP	10	13.2	100.5	2.3	100.2	18.2	100.1
	50	8.3	100.9	7.2	101.1	8.8	101.1
	800	0.8	96.3	0.2	108.8	0.7	97.5
	2500	1.1	100.9	1.5	101.0	0.6	100.7
2C-P	10	1.8	100.1	2.4	100.1	1.4	100.1
	50	3.4	99.2	2.2	99.0	2.9	99.2
	800	3.1	106.1	2.2	101.0	3.5	101.0
	2500	1.0	100.5	0.4	100.3	1.3	100.5

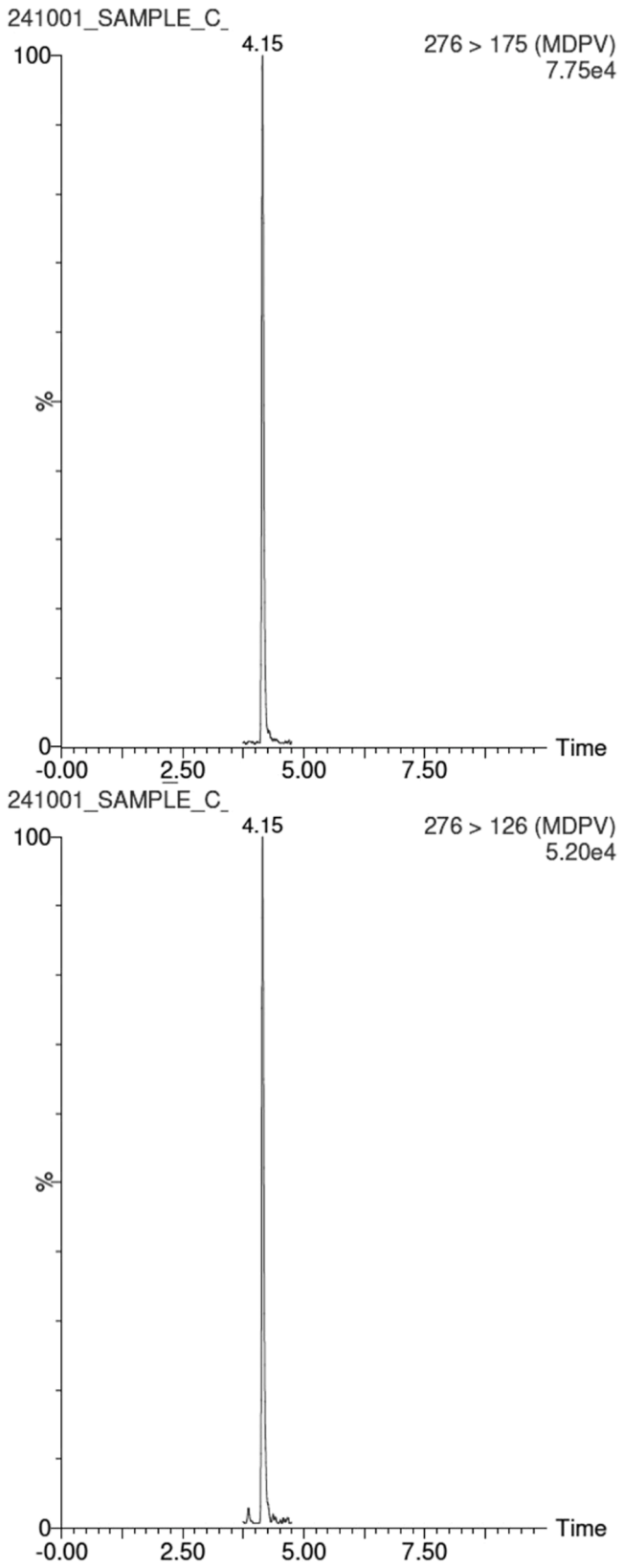

Fig. 3. Chromatogram of authentic sample (MDPV at 73.20 pg/mg).

MDPV in hair following a single exposure in the context of a drug-facilitated sexual assault. The concentrations found in our work are approximately 16 times higher, suggesting the possibility of frequent use of MDPV. No other compound was detected in these samples.

Conclusions

In this study, a reliable method for the detection and quantification of selected synthetic cathinones and 2C-P in hair samples using a miniaturized extraction technique, MEPS, coupled with LC-MS/MS was successfully developed and validated. The MEPS procedure was straightforward, providing satisfactory precision and accuracy for all analytes. Following ANSI/ASB guidelines, the methodology was thoroughly validated and optimized, with all parameters falling within acceptable limits and offering advantages in terms of environmental sustainability. The method was successfully applied to real samples, with three authentic samples testing positive for MDPV at concentrations ranging from 36 to 73 pg/mg. These values align with chronic and occasional exposure levels reported in the literature, supporting the method's applicability in assessing both acute and chronic drug exposure for MDPV. Future research could extend this work by adapting MEPS for additional NPS analytes, further expanding the potential of hair analysis as a long-term monitoring tool for substance use assessment.

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CRediT authorship contribution statement

Ana Y. Simão: Writing – original draft, Methodology, Investigation, Formal analysis. **Luana M. Rosendo:** Investigation, Formal analysis. **Pedro Dinis:** Methodology, Investigation. **Cláudia Margalho:** Methodology, Investigation, Formal analysis. **Maristela Andraus:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Mário Barroso:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Eugenia Gallardo:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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