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Green analytical toxicology method for determination of synthetic cathinones in oral fluid samples by microextraction by packed sorbent and liquid chromatography–tandem mass spectrometry

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Abstract

Purpose We developed and validated a method for quantitative analysis of ten synthetic cathinones in oral fluid (OF) samples, using microextraction by packed sorbent (MEPS) for sample preparation followed by liquid chromatography−tandem mass spectrometry (LC−MS/MS).

Method OF samples were collected with a Quantisal™ device and 200 μL was extracted using a C18 MEPS cartridge installed on a semi-automated pipette and then analyzed using LC−M/SMS.

Results Linearity was achieved between 0.1 and 25 ng/mL, with a limit of detection (LOD) of 0.05 ng/mL and a limit of quantification (LOQ) of 0.1 ng/mL. Imprecision (% relative standard deviation) and bias (%) were better than 11.6% and 7.5%, respectively. The method had good specificity and selectivity against 9 different blank OF samples (from different donors) and 68 pharmaceutical and drugs of abuse with concentrations varying between 400 and 10,000 ng/mL. No evidence of carryover was observed. The analytes were stable after three freeze/thaw cycles and when kept in the autosampler (10 °C) for up to 24 h. The method was successfully applied to quantify 41 authentic positive samples. Methylone (mean 0.6 ng/mL, median 0.2 ng/mL), N-ethylpentylone (mean 16.7 ng/mL, median 0.35 ng/mL), eutylone (mean 39.1 ng/mL, median 3.6 ng/mL), mephedrone (mean 0.5 ng/mL, median 0.5 ng/mL), and 4-chloroethcathinone (8.1 ng/mL) were quantified in these samples.

Conclusion MEPS was an efficient technique for Green Analytical Toxicology purposes, which required only 650 μL organic solvent and 200 μL sodium hydroxide, and the BIN cartridge had a lifespan of 100 sample extractions.

Keywords Microextraction by packed sorbent (MEPS) · Synthetic cathinones · Green analytical toxicology (GAT) · LC–MS/MS · Oral fluid

Introduction

Green analytical toxicology (GAT) is a new concept derived from Green Chemistry, which covers 12 principles such as reducing sample size and waste generation, minimizing sample handling, and aiming for more sustainable approaches

[1]. Microextraction by packed sorbent (MEPS) is a miniaturized sample preparation technique that aligns with the GAT concept. It requires a small volume of solvent, sample, and sorbent (1–4 mg). The MEPS cartridge has the advantage of being used in multiple samples before replacement and can be directly integrated into the extraction syringe, allowing for full automation using autosamplers [2, 3].

OF sample is an alternative matrix that has increased acceptance in toxicology laboratories. Among its advantages is included a non-invasive collection, with the possibility to be observed to avoid adulteration and no requirements for specialized places or professionals to perform it. Moreover, the on-site collection is facilitated, with more acceptance among volunteers beyond other matrix collections. The concentration of a drug in OF reflects a recent consumption once is more likely to contain parent drugs when compared

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to urine, and some drug concentrations can correlate better with blood [4, 5].

Synthetic cathinones (Fig. 1) are the second largest group of new psychoactive substances (NPS) monitored by the European Union Warning System with 162 monitored substances. Their present psychostimulant effects are structurally related to cathinone, a psychoactive substance naturally found at *Catha edulis*. In 2020, 65% of seized material (3300 kg) from the European Union was related to cathinone powders [6]. Numerous intoxication cases have been reported in the literature, including fatal ones. Pieprzyca et al. [7] reported 57 cases involving synthetic cathinones, confirmed in blood and other matrices from 2013 to 2019 in Poland. Cases were associated with intoxication, fatal intoxication, driving under the influence of drugs, and other circumstances. alpha-Pyrrolidinovalerophenone (alpha-PVP) was the cathinone most prevalent in these samples. A work published in 2022 reported a fatal case involving cardiac arrest after eutylone consumption in Japan [8]. Another cardiac arrest after consuming synthetic cathinone

(4-fluoro-3-methyl-alpha-PVP) was also published in the same year in the USA [9], demonstrating that it is still a current problem worldwide.

This work aimed to develop and validate a quantitative method for ten synthetic cathinones in OF samples using a semi-automated MEPS technique and analyzed by LC–MS/MS, following the GAT concept.

Material and methods

Standards and chemicals

Reference standards for 4-chloroethcathinone (4-CEC), benzedrone, butylone, eutylone, 3,4-methylenedioxy pyrovalerone (MDPV), mephedrone, methylone, *N*-ethyl pentedrone, *N*-ethylpentylone, and pentylone were purchased from Cayman Chemical (Ann Arbor, MI, USA), and MDMA-d$_5$, used as internal standard, from Cerilliant (Round Rock, TX, USA); isopropanol, methanol, and formic
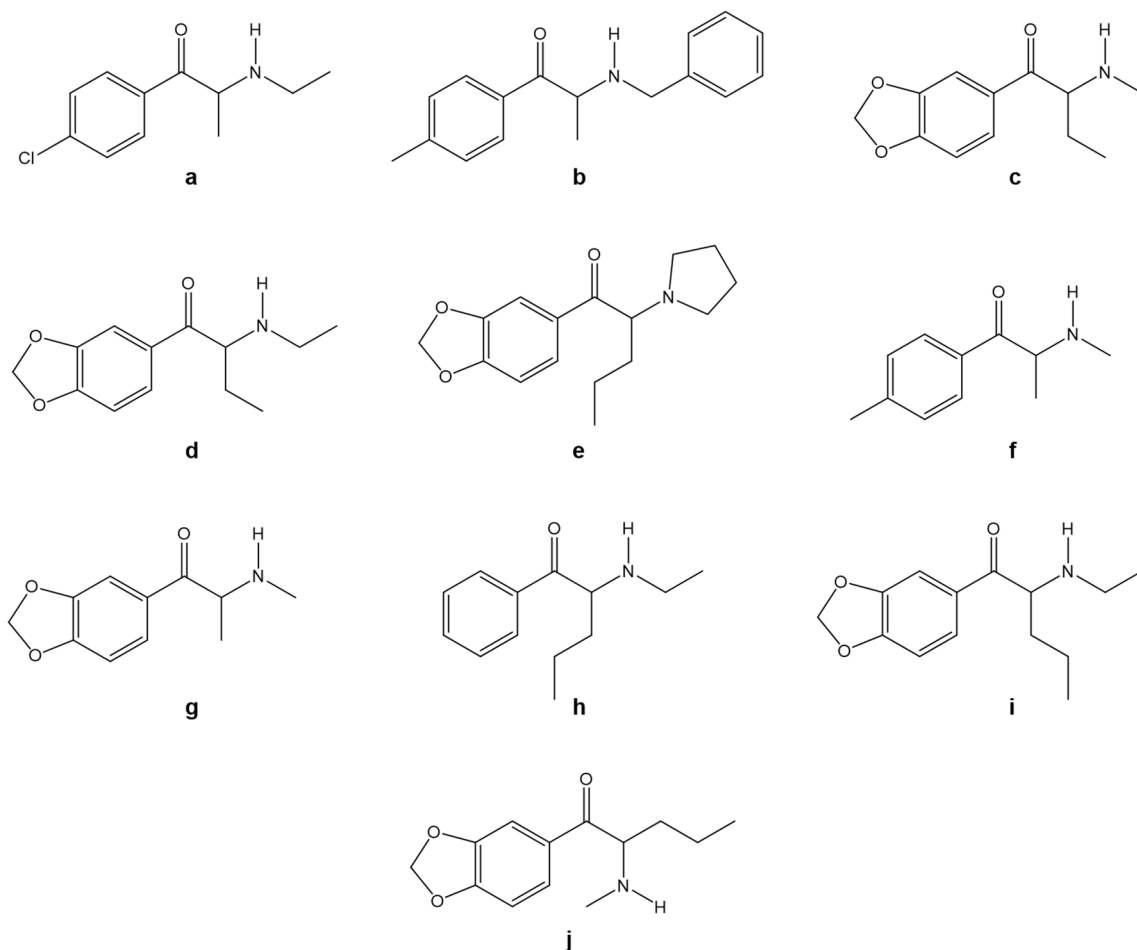

Fig. 1 Molecular structures of the 10 synthetic cathinones included in this analytical method. **a** 4-CEC, **b** benzedrone, **c** butylone, **d** eutylone, **e** MDPV, **f** mephedrone, **g** methylone, **h** *N*-ethyl pentedrone, **i** *N*-ethylpentylone, **j** pentylone

acid (98–100%) from Merck (Darmstadt, Germany); sodium hydroxide from Synth (Diadema, SP, Brazil); and ultrapure deionized water was supplied by a Milli-Q RG unit from Millipore (Billerica, MA, USA). All solvents used in the extraction procedure were HPLC grade. Quantisal™ OF collection device and its extraction buffer (phosphate buffer solution: PBS, 100 mmol/L) were purchased from Immunalysis (Pomona, CA, USA). The SGE eVol™ digital analytical syringe, a device containing a handheld digitally controlled pipette coupled to a XCHANGE™ syringe (50 μL), and C18 and M1 Barrel Insert and Needle Assembly (BIN) were purchased from SGE Analytics (Melbourne, Australia).

Calibrators, quality controls, and internal standard

Stock solutions of the substances were prepared by diluting the reference standard material in methanol. Dilutions of the stock solution in methanol were made to create the working calibrators containing all the ten synthetic cathinones at 1, 10, 50, 100, 150, 200, and 250 ng/mL. Quality control (QC) working solutions were prepared by a different analyst from the calibrator's preparation at 3, 80, and 160 ng/mL in methanol.

Internal standard solution of MDMA-d$_5$ at 10 ng/mL (ISTD) was prepared by appropriate dilution of stock solution of the reference standard in methanol. All solutions were stored in amber glass vials at −20 °C.

Sample collection

Authentic samples were collected from volunteers at parties and electronic music festivals in three different Brazilian states (São Paulo, Minas Gerais, and Bahia) from September 2018 to January 2020, using the Quantisal™ OF device. The volunteers were informed about the project aim and invited to answer an anonymous questionnaire, followed by the OF collection. The sample collections were conducted in accordance with the University of Campinas Ethical Committee (CAAE n° 88770318.0.0000.5404). The inclusion criteria were age greater than 18 years old and self-reported use of an illicit psychoactive substance in the last 24 h.

The 41 samples analyzed in this work were positive for at least one analyte covered by this method during a validated screening analysis to detect more than 104 new psychoactive substances and other drugs of abuse [10, 11]. Screening analysis was performed after each respective event, and the remained volume was stored in a cryotube at −20 °C. To 500 μL of sample, a liquid–liquid extraction was performed with 2 mL of methyl *t*-butyl ether and an evaporation step (40 °C, N$_2$/5 psi) to concentrate the extract. The analyses were performed on a Nexera UHPLC chromatographic system coupled to a LCMS8060 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan), the same used for this

method. During the period covered by the sample collection, the quantitative method was developed and validated to quantify the positive samples in a short storage period.

The synthetic cathinones chosen to be included in this method should have been detected among our authentic samples using our screening method, had been already seized in Brazil [12], or reported related to some intoxication case [13–15].

Blank samples used during method validation to create calibration curves, QC, and interference studies were obtained from volunteers from our laboratory. These blank OF samples were prepared as described below to simulate an authentic sample, where 1 mL of OF ± 10% is collected and mixed with 3 mL Quantisal™ elution buffer.

Sample preparation

The sample preparation was adapted from Rocchi et al. method [16]. To 200 μL sample [50 μL OF spiked with a working solution (1/10, v/v) and 150 μL Quantisal™ elution buffer] was added 25 μL ISTD solution, 200 μL sodium hydroxide 0.4 M, and 75 μL methanol. The sample was agitated (2500 rpm/2 min), centrifuged (12,000 rpm/5 min), and 400 μL was transferred to a new polypropylene tube to be loaded onto the MEPS BIN cartridge.

MEPS extraction procedure

Using a 50 μL syringe coupled to a semi-automated pipette, the C18 cartridge was conditioned by loading and releasing three times 50 μL methanol, followed by 3 × 50 μL ultrapure water. Sample loading was performed by eight cycles of draw–eject (50 μL) in the same polypropylene tube. The cartridge washing was performed with 2 × 50 μL ultrapure water/methanol (90:10, v/v). One draw–eject (50 μL) of sample elution was done with methanol containing 1% formic acid. This eluate was transferred to an autosampler vial with a conical glass insert, diluted with 25 μL ultrapure water, and 5 μL was injected into LC−MS/MS system.

After each sample extraction, the MEPS cartridge was cleaned by a final washing step using 3 × 50 μL methanol/isopropanol (80:20, v/v), 2 × 50 μL methanol and 2 × 50 μL ultrapure water. The draw–eject speed used during all the steps was 29.41 μL/s, corresponding to 1.7 s for a full stroke. Fig. S1 in the Supplementary material shows a workflow from the extraction protocol.

Instrumentation

The analyses were performed on a Nexera X2 UHPLC chromatographic system coupled to a LCMS8060 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The chromatographic separation was performed on a Raptor™

biphenyl column (100×2.1 mm, 2.7 µm; Restek, Bellefonte, PA, USA), maintained at 40 °C. The mobile phase consisted of ultrapure water (A) and methanol (B), both containing 0.1% formic acid (v/v) and 2 mmol/L ammonium formate. Flow rate was 0.4 mL/min, and the elution gradient was initialized with 5% B, followed by a linear increase to 100% B over 9 min, holding at 100% B for 2 min, and returning to initial conditions over 0.2 min. The system was reequilibrated for 2.3 min before the next injection, with a total chromatographic run of 13.5 min.

The mass spectrometer was equipped with an electrospray ionization source (ESI), operated in positive ion mode. The source parameters optimized were: heat block temperature at 450 °C; ion spray voltage at 2.0 kV (except for MDMA-d_5, 4.0 kV); nebulizer gas (N_2) flow at 3 L/min; desolvation line temperature at 200 °C; drying gas (N_2) flow at 5 L/min; heating gas (air) flow at 15 L/min; interface temperature at 300 °C; and collision-induced dissociation gas pressure (Ar) at 270 kPa. The analyses were performed in multiple reaction monitoring (MRM) mode. For each compound, two MRM transitions were selected, one for quantification and one qualifier for confirmation identification. Individual chromatographic retention times and MRM information are detailed in Table 1. Data were acquired and processed with the LabSolutions 5.97 software (Shimadzu).

Validation of the method

The method validation was based on guidelines for quantitative method validation in forensic toxicology published by the AAFS Standards Board (ASB) and Approved American National Standard (ANSI) [17]. Statistics and design of experiments evaluation were done using Prism software version 5.0 (GraphPad Software, San Diego, CA, USA) and Unscrambler X software version 10.3 (CAMO Software AS, Oslo, Norway).

Imprecision

The imprecision was evaluated with fortified matrix samples in triplicate at three different concentrations (low, medium, and high) over 5 days. Both intraday and interday imprecisions were calculated using the one-way analysis of variance (ANOVA, $p < 0.05$) approach. Imprecision values with %RSD lower than 15% were considered acceptable.

Table 1 MRM transitions and optimized mass spectrometer parameters to quantify ten synthetic cathinones, and MDMA-D5 as internal standard (IS) in OF samples using LC−MS/MS in positive electrospray ion mode (ESI+)

Analyte	MRM transitions (*m/z*)	Q1 pre bias (V)	CE (V)	Q3 pre bias (V)	RT (min)
4-CEC	<u>212.0 > 159.1</u>	10	20	10	4.35
	212.0 > 144.1	15	27	14	
Benzedrone	<u>254.1 > 91.0</u>	29	23	16	6.04
	254.1 > 65.1	13	55	26	
Butylone	<u>222.1 > 174.1</u>	25	18	11	4.19
	222.1 > 146.1	25	26	28	
Eutylone	<u>236.1 > 174.1</u>	12	31	11	4.53
	236.1 > 188.1	12	20	12	
MDPV	<u>276.1 > 126.1</u>	14	26	23	5.38
	276.1 > 175.1	14	23	11	
Mephedrone	<u>178.1 > 145.1</u>	20	21	30	4.04
	178.1 > 144.1	20	30	27	
Methylone	<u>208.1 > 160.1</u>	24	17	30	3.58
	208.1 > 132.1	24	27	27	
N-Ethyl pentedrone	<u>206.1 > 130.1</u>	10	32	25	4.66
	206.1 > 188.1	10	15	12	
N-Ethylpentylone	<u>250.1 > 202.1</u>	12	20	13	5.10
	250.1 > 174.1	18	30	11	
Pentylone	<u>236.1 > 188.1</u>	27	18	12	4.82
	236.1 > 175.1	12	22	11	
MDMA-d_5 (IS)	<u>199.0 > 165.0</u>	20	14	19	3.80
	199.0 > 107.0	20	25	21	

MRM multiple reaction monitoring, *CE* collision energy, *RT* retention time, Quantifier transitions are underlined

Bias

Bias was evaluated at three different concentrations, with three replicates over 5 days. The highest average acceptable bias from nominal concentration was ± 15%. Results are presented in percentages.

Limit of detection (LOD), limit of quantification (LOQ), and linearity

The LOD was determined as the lowest concentration that fully met the following identification criteria: response at least three times the signal-to-noise ratio of blank samples, retention time within ± 0.2 min of the average calibrator retention time and the ratio of the two MRM transitions within a maximum of ± 30% deviation of that established with the calibrator. Blank OF samples in triplicate were fortified at decreasing concentrations of the analytes until they yielded a reproducible instrument response that met the identification criteria after being extracted and analyzed over 5 days ($n = 15$).

Linearity was assessed over 5 days, with seven-point calibration curves (0.1, 1, 5, 10, 15, 20, and 25 ng/mL). Calibrators were required to quantify within ± 20% of each target concentration. LOQ was the lowest concentration fulfilling the identification criteria, with a signal-to-noise ratio of at least 10 and quantifying within 20% of each target concentration. A *F*-test two-sample for variances was performed to test the homoscedasticity of the linear regression analysis.

Interference studies

OF samples were fortified with 68 common pharmaceuticals and drugs of abuse/metabolites at concentrations between 200 and 10,000 ng/mL, extracted and injected into the LC–MS/MS. No peaks that satisfied the identification criteria should be visualized in each analyte's detection window. Supplementary material Table S1 includes all pharmaceuticals evaluated as potential interferents.

Nine blank samples from different individuals known to be absent of the analytes covered in this method were extracted and analyzed to evaluate possible endogenous interferences. No interfering peaks that satisfied the identification criteria should be visualized.

Recovery

The extraction efficiency was performed with two batches: the first using six replicates of blank OF samples fortified with all analytes and internal standard at the low and high QC concentrations, extracted with MEPS procedure, and injected into the LC−MS/MS; the second, using six replicates of blank OF extracted by MEPS procedure, and

the final extract was fortified with all analytes and internal standard at the low and high QC concentrations and injected into the LC−MS/MS. The average peak area of the samples fortified prior to extraction divided by the average peak area of the samples fortified after extraction is multiplied by 100 to give the extraction efficiency percentage.

Matrix effect

Matrix effects were evaluated by dividing the average peak areas of the blank OF samples fortified with all analytes and internal standard after extraction by the average peak areas of methanol fortified with all analytes and internal standard multiplied by 100. Results were expressed as percentages. A negative result indicates matrix suppression and a positive result a matrix enhancement.

Stability

Autosampler (processed sample) stability was evaluated at low and high QC concentrations. QC ($n = 3$) and calibrator samples were extracted and analyzed immediately. These samples were maintained on the autosampler at 10 °C and re-injected after 24 h. The peak areas of these stored samples were compared to those obtained immediately.

Freeze–thaw stability was performed at low and high QC samples in triplicate. On day zero, a blank OF fortified with the standard working solution was aliquoted into six independent polypropylene tubes for each studied concentration. Triplicates were quantified and the other three aliquot samples were stored at − 20 °C. After three freeze–thaw cycles (one cycle/24 h), these samples were quantified against a fresh calibration curve. The analytes were considered stable if the average concentration compared to day zero was within ± 20%.

Dilution integrity

Fortified OF samples at 100 ng/mL were diluted (1/25, v/v) in blank samples, extracted, and analyzed by LC–MS/MS in triplicate. The average concentration of the diluted sample was multiplied by the dilution factor, divided by the undiluted target concentration, and multiplied by 100. Dilution integrity is established if the bias is within ± 20% of the theoretical concentration. Intraday imprecision (%RSD) was also calculated by dividing the standard deviation by the average concentration already multiplied by the dilution factor. %RSD should be lower than ± 20%.

Carryover

A blank sample extract was injected after injecting a fortified OF sample extract with all analytes at the highest

calibrator concentration (25 ng/mL). If no analyte peak met LOD criteria, carryover was considered absent.

Results

The linearity was achieved between 0.1, 1, 5, 10, 15, 20, and 25 ng/mL for all ten synthetic cathinones ($1/x^2$ weighted linear regression, $r > 0.997$ for 5 days of calibration curves), with LOD and LOQ of 0.05 and 0.1 ng/mL, respectively.
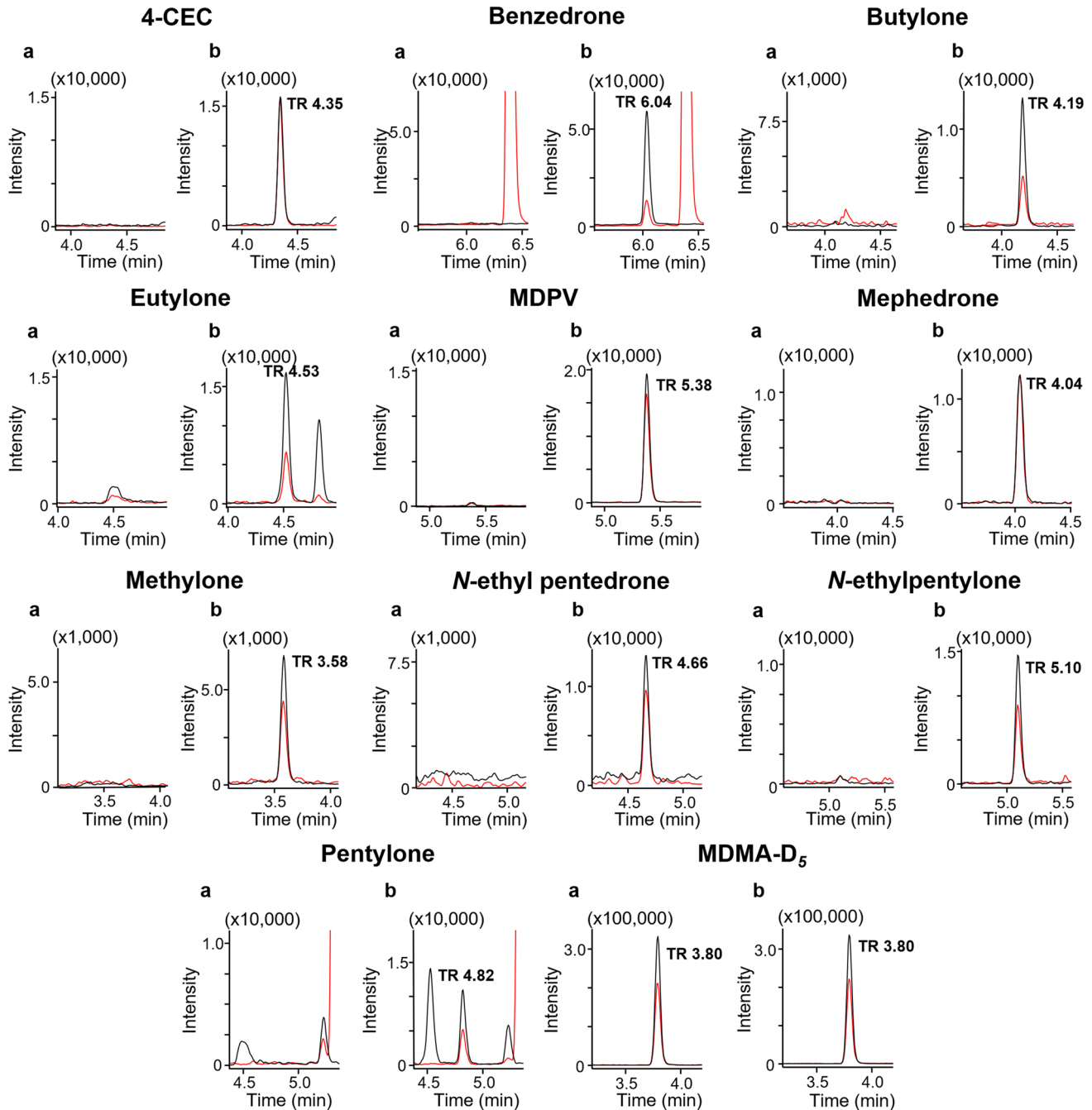

Fig. 2 MRM chromatograms for 10 synthetic cathinones extracted from OF by MEPS and analyzed by LC–MS/MS. **a** negative OF samples and **b** at 0.1 ng/mL (limit of quantification)

Extracted MRM chromatograms of blank and LOQ OF samples are shown in Fig. 2.

Intra- and interday imprecisions (%RSD) were lower than 7.1% and 11.6%, respectively (Table 2); while, bias (%) was not higher than 7.5% in all three evaluated concentrations (0.3, 8, and 16 ng/mL). Matrix effects lower than 16.2% were observed at low and high QC concentrations ($n = 6$). MDMA-d$_5$ presented a matrix effect of 2.0 and −5.6% for low and high QC concentrations.

Recovery varied between 8.3 and 56.3% for methylone and benzedrone, respectively, and between 9.8 and 10.6% for MDMA-d$_5$. Besides the high variability in recovery results, the method was high enough to quantify all analytes with precision and accuracy, even in low concentrations.

All analytes were stable after the sample extracts were kept in the autosampler (10 °C) for 24 h (peak areas variation lower than −4.4%, including the ISTD). The stability was also observed after three freeze–thaw cycles at low and high QC concentrations (1 cycle/24 h). The highest concentration variation was observed for benzedrone at 0.3 ng/mL (−14.1%). Detailed information regarding imprecision, bias, matrix effects, recovery and stability is available in Table 2.

Table 2 Method validation results for **i**ntra- and interday imprecisions, biases, matrix effects, recovery rates and stabilities for analyses of ten synthetic cathinones in OF samples collected using Quantisal™ device, extracted by MEPS and identified and quantified by LC–MS/MS

Analyte	QC	Intraday imprecision (%RSD) ($n = 3$)	Interday imprecision (%RSD) ($n = 15$)	Bias (%) ($n = 15$)	Matrix effect (%) ($n = 6$)	Recovery (%) ($n = 6$)	Stability (%) F/T (3 cycles)	24 h-autosampler (10 °C)
4-CEC	Low	4.2	7.3	−0.5	−3.8	32.5	−10.9	0.6
	Medium	5.4	10.1	−2.7	NA	NA	NA	2.3
	High	3.0	8.4	−2.0	−9.8	26.3	4.7	−0.8
Benzedrone	Low	4.3	7.7	−3.2	−10.0	56.3	−14.1	−0.6
	Medium	7.1	9.3	−3.7	NA	NA	NA	1.9
	High	5.6	7.1	−1.4	−1.2	47.4	4.0	−1.3
Butylone	Low	3.1	5.8	3.5	0.5	17.6	−8.3	1.2
	Medium	3.3	7.1	1.9	NA	NA	NA	1.8
	High	2.4	7.0	3.9	−8.0	17.0	1.2	−1.8
Eutylone	Low	4.6	6.4	2.8	−4.6	25.1	−5.9	−0.4
	Medium	3.8	8.2	−0.1	NA	NA	NA	2.1
	High	2.4	8.9	2.7	16.2	21.1	2.4	−1.2
MDPV	Low	4.2	7.7	1.5	−8.7	43.2	−11.7	−0.1
	Medium	5.7	8.5	−1.5	NA	NA	NA	1.9
	High	3.5	8.1	1.0	−2.7	38.7	4.2	−1.2
Mephedrone	Low	4.6	5.6	6.2	9.7	20.8	−12.9	−4.4
	Medium	4.0	7.1	1.9	NA	NA	NA	0.7
	High	2.9	6.5	3.2	−9.3	19.3	3.5	−1.2
Methylone	Low	2.5	5.6	7.5	8.5	8.4	−3.3	−2.3
	Medium	2.9	6.8	4.1	NA	NA	NA	1.8
	High	2.8	6.9	4.9	−7.5	8.3	−1.9	−1.9
N-Ethyl pentedrone	Low	4.9	9.6	5.3	−5.7	39.9	−5.2	−2.7
	Medium	5.5	11.6	−3.1	NA	NA	NA	2.3
	High	3.0	11.5	1.3	−8.3	29.9	3.5	−1.1
N-Ethylpentylone	Low	4.0	6.7	2.4	8.8	38.4	−11.5	0.0
	Medium	5.7	10.7	−2.0	NA	NA	NA	1.8
	High	2.9	9.9	−0.2	−8.2	30.6	5.4	−1.1
Pentylone	Low	4.2	6.2	2.4	−10.7	29.8	9.6	−0.7
	Medium	5.6	9.7	−1.0	NA	NA	NA	1.7
	High	3.9	10.6	0.3	−10.2	24.9	0.6	−0.4

Stability results are presented as %difference

%RSD percent relative standard deviation, *NA* not evaluated, *QC* quality control, *Low* 0.3 ng/mL, *Medium* 8 ng/mL, *High* 16 ng/mL, *F/T* freeze/thaw

Dilution integrity was evaluated in triplicate with a 1/25 dilution at 100 ng/mL. Bias (%) and %RSD results were lower than -18.4% for benzedrone and 10.2% for *N*-ethyl pentedrone (Table 3).

There were no endogenous interferences when 9 blank OF samples were analyzed against this method. Also, no exogenous interferences were observed fortifying blank OF with 68 pharmaceuticals and drugs of abuse, divided into eight standard mixtures at concentrations ranging from 200 to 10,000 ng/mL (Supplementary material Table S1). No carryover was observed injecting a negative sample after the highest calibrator concentration (25 ng/mL).

The BIN cartridge was cleaned and reused during the sample validation and authentic sample analyses until the sorbent clogging led to a loss of performance, which decreased the time for all 50 μL extract to pass through the syringe. No response variations were observed in the analytes during the method validation, only a physical issue. The average lifespan observed for this method was 100 samples until a new BIN was necessary.

From 462 OF samples collected at parties and electronic music festivals in Brazil [11], 41 samples (8.9%) known as positive for synthetic cathinone were applied to this quantitative method. In these samples, 1 was positive for 4-CEC (2.4%), 2 for mephedrone (4.9%), 4 for eutylone (9.8%), 12 for *N*-ethylpentylone (29.3%), and 26 for methylone (63.4%) (Fig. 3). Five of these samples presented one cathinone with a concentration lower than our established LOQ, but still higher than LOD (0.05 ng/mL) and were still reported. Four samples (9.8%) showed the presence of two different cathinones. One eutylone sample had a concentration reported as higher than the upper limit of quantification (ULOQ) after the concentration level was kept above the calibration range, even after a 1/25 sample dilution. Table 4 presents all exact concentration values.

Table 3 Bias and imprecision results obtained for ten synthetic cathinones when OF samples at 100 ng/mL were submitted to a 1/25 dilution, extracted by MEPS and identified and quantified by LC–MS/MS

Analyte	Bias (%, $n=3$)	Imprecision (%RSD, $n=3$)
4-CEC	− 6.4	6.9
Benzedrone	− 10.2	7.1
Butylone	− 11.2	6.9
Eutylone	2.3	1.8
MDPV	− 11.2	4.4
Mephedrone	− 2.3	4.7
Methylone	− 18.4	4.3
N-Ethyl pentedrone	− 10.5	9.6
N-Ethylpentylone	− 0.9	4.8
Pentylone	− 14.7	10.2

%RSD relative standard deviation

Discussion

Optimization of the source parameters is recommended to ensure low LOD. The optimum conditions were chosen considering the average absolute peak areas obtained for each analyte after several injections ($n=5$) of a standard mixture solution. The parameters with the most significant impact on analytes detectability were ion spray voltage, heating and drying gases, with an average increase in analytes response of 41.9% (40.4–44.5%).

Spiked OF samples at 1 ng/mL ($n=5$) were extracted using C18 and M1 cartridges and the relative areas were analyzed using a *t*-test ($p < 0.05$) to observe any statistical significance in the studied analytes. C18 cartridge showed statistical significance over M1 for all analytes, except for methylone. Thus, the C18 cartridge was chosen once higher relative areas were presented for most analytes (Supplementary material Fig. S2). Important to note that methylone is the first analyte to elute during chromatographic analysis, with the lowest extraction recovery rate (Table 2) using the C18 cartridge, which may explain why it had a better performance with the mixed-mode M1 cartridge (C8 + SCX). Benzedrone is the last analyte to elute during chromatographic analysis, with the highest recovery rate using the C18 cartridge.

Also, a t-test was performed but considering the sample loading step. We compared the draw–eject cycles in the same sample vial or discharging the volume in a waste vial. 4-CEC, MDPV, mephedrone, and benzedrone results were statistically significant using a different vial. However, for most analytes, the %RSD for the relative area was higher in this condition (Supplementary material Fig. S3). Thus, we kept the draw–eject cycle being performed in the same sample vial.

A two-level five-factor half fractional factorial design (2^{6-1}_{VI}) was used to cover the main factors that could influence the extraction. Two levels (low, high) and respective factors were: addition of formic acid to methanol—sample preparation (without, 1%); addition of formic acid to methanol—elution step (without, 1%); number of sample aspirations through the cartridge (4, 8); speed draw-injection (2, 8); BIN dryness before elution step (without, 3×); and elution volume (50, 150 μL). The elution volume was the most influencing factor for all analytes. No statistical significance was observed for BIN dryness and this step was removed from the protocol.

The LOD objective was to reach the same value obtained for our screening method [10], which would be used to filter the authentic samples to be quantified under this described methodology. For the screening method, a liquid–liquid extraction protocol is used with 500 μL sample and its reach LOD value of 0.05 ng/mL, except
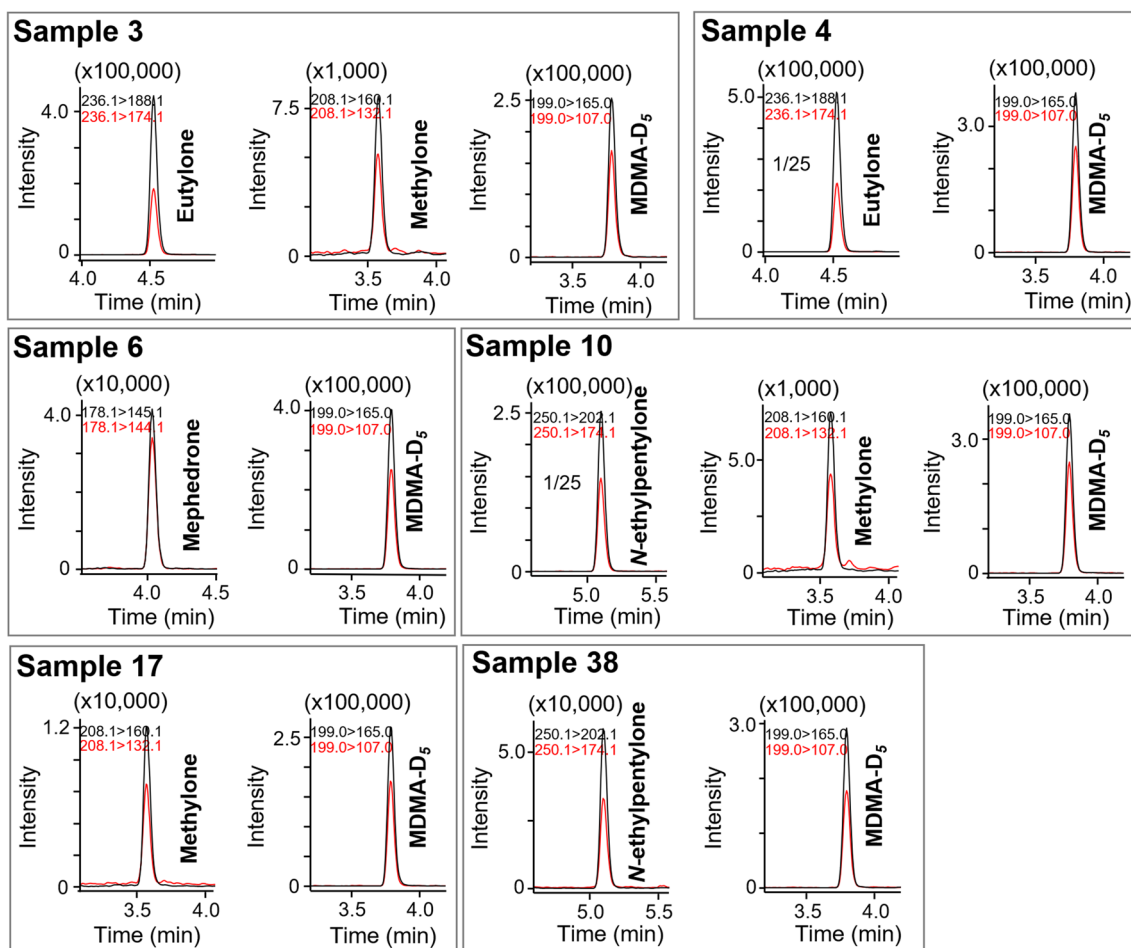
Fig. 3 Extracted ion chromatograms of 6 authentic OF samples positive for four different synthetic cathinones, collected with Quantisal™ device, and extracted using MEPS

for eutylone (0.1 ng/mL) and *N*-ethyl pentedrone (1 ng/mL) – analytes added to the scope after publication. In this method, we used 200 μL sample, MEPS as extraction protocol, and we could reach a LOD of 0.05 ng/mL and LOQ of 0.1 ng/mL for all 10 analytes. Once the chromatographic resolution can be an important issue in NPS analysis with the presence of isomeric analytes occasionally, the gradient elution was the same in both methods. Considering the 10 analytes covered by this developed method, we had two position isomers: eutylone (retention time: 4.53 min) and pentylone (retention time: 4.82 min). The method also presents chromatographic resolution between two other isomers: *N*-ethylpentylone (included in this method, retention time: 5.10 min) and dipentylone (not included in this method, [10]).

One of the limitations of the method was to use only one internal standard to represent all the ten analytes, and also the MDMA-d_5, with a structure close to the synthetic cathinones and eluting close to them (retention time for MDMA-d_5 was 3.8 min and synthetic cathinones eluted between

3.6 and 6.0 min). However, despite the limitation, all the analytes demonstrated good results regarding imprecision and bias during the validation process, as shown in Table 2.

Despite using a semi-automated pipette in this method, which contains all the extraction steps and speed saved in its memory but still requires human resources to operate it, the MEPS technique is possible to be incorporated into the equipment to perform the sample extraction without any human supervision after the sample preparation step [18]. Since the BIN can be reused up to 100 times, it is expected to reduce costs, extraction time, and waste when compared to existing methods such as solid-phase extraction.

Rocchi et al. [16] developed a method for OF samples using MEPS (C18 cartridge) to quantify 31 NPS using UHPLC−MS/MS. Despite the 90 μL sample, the collection was performed by passive drool, while we used a dispositive containing an elution buffer to prevent analyte degradation during transportation. Thus, even using 200 μL sample, this corresponds to only 50 μL of OF. Four analytes are present in both methods with LODs between 0.05 and 0.39 ng/mL.

Table 4 Forty-one authentic OF samples collected with Quantisal™ device at parties and electronic music festivals and quantified using the developed method

Sample number	4-CEC	Eutylone	Mephedrone	Methylone	N-Ethylpentylone[a]	Other detected substances[b]
1	8.1				>LOD	MDMA, THC, ketamine
2		0.4		2.1		MDMA, MDA, MDEA, THC, LSD, cocaine, levamisole, ketamine
3		3.6		0.1		MDMA, MDA, THC, cocaine, 25I-NBOH
4		113.2				MDMA, MDA, THC
5		>ULOQ				MDMA, MDA, MDEA, cocaine, levamisole, methamphetamine, amphetamine
6			0.3			MDMA, MDA, THC
7			0.7			MDMA, MDA, MDEA, THC, amphetamine
8				>LOD		MDMA, MDA, THC
9				>LOD		MDMA, MDA, MDEA, THC, ketamine
10				>LOD	59.3	MDMA, MDA
11				0.1		MDMA, MDA, THC
12				0.1		MDMA, MDA, MDEA, THC, LSD, cocaine, levamisole
13				0.1		MDMA, MDA, MDEA, THC, LSD, cocaine, levamisole
14				0.1		MDMA, MDA, MDEA, THC, quetiapine
15				0.1		MDMA, MDA, MDEA, THC, methylphenidate, venlafaxine
16				0.1		MDMA, MDA, MDEA, methamphetamine
17				0.2		MDMA, MDA, THC, ketamine
18				0.2		MDMA, MDA, MDEA, THC, LSD, 5-MeO-MiPT
19				0.2		MDMA, MDA, MDEA, THC, LSD, cocaine, levamisole, methamphetamine, ketamine
20				0.2		MDMA, MDA, MDEA, THC, LSD, ketamine
21				0.2		MDMA, MDA, MDEA, THC
22				0.2		MDMA, MDA, MDEA, THC, ketamine
23				0.3		MDMA, MDA, MDEA, THC, LSD, cocaine, ketamine
24				0.3		MDMA, MDA, THC, LSD, psilocin
25				0.3		MDMA, MDA, MDEA, THC, 25C-NBOH
26				0.3		MDMA, MDA, MDEA, THC, psilocin
27				0.4		MDMA, MDA, MDEA, THC, LSD
28				0.8		MDMA, MDA, MDEA, THC
29				2.1		MDMA, MDA, MDEA
30				2.2		MDMA, MDA, MDEA
31				3.1		MDMA, MDA, MDEA, THC
32					>LOD	MDMA, MDA, THC, cocaine, levamisole, ketamine
33					0.1	MDMA, MDA, THC
34					0.2	MDMA, THC, LSD
35					0.2	MDMA, THC
36					0.3	MDMA, MDA, MDEA, THC
37					0.3	MDMA, MDA, MDEA
38					0.4	MDMA, MDA, ketamine, citalopram
39					1.1	MDMA, MDA, MDEA, THC, LSD, ketamine
40					21.9	MDMA, MDA, THC, LSD
41					82.9	MDMA, MDA, MDEA, THC, ketamine

>LOD concentrations between 0.05 and 0.1 ng/mL, >ULOQ concentration higher than the upper limit of quantification (even with a 1/25 dilution)

[a]Concentrations above the ULOQ were obtained after a 1/25 sample dilution to fit in the calibration range

[b]Detected during the screening analysis [10]

Ares et al. [19] also developed a MEPS (M1 cartridge) method to extract OF samples and quantify 21 analytes using ultra-performance liquid chromatography–tandem mass spectrometry. The samples were collected using Salivette® device, which does not dilute the sample with any preservative. LOD for 4 cathinones in both methods was 0.25 ng/mL using a 300 μL sample.

Sorribes-Soriano et al. [20] developed a method to quantify methylone in OF samples using MEPS (C18) and ion mobility spectrometry. OF was collected by spitting in a polypropylene tube and 90 μL was used for extraction protocol and obtained a LOD of 4 ng/mL.

Bianchi et al. [21] developed a quantitative method for ten analytes in OF samples using MEPS (C18) and analyzed by desorption electrospray ionization high-resolution mass spectrometry, being the mephedrone the only included synthetic cathinone. OF was collected directly into polypropylene tubes and 50 μL was used for the extraction, and the LOQ was 50 ng/mL for mephedrone. This work involved 40 authentic samples collected at parties and one of them was positive for mephedrone (5.8 μg/mL).

All the methods involving OF and MEPS technique include a prior sample preparation to remove potential interferents, such as proteins and food debris, and avoid sorbent clogging. Diluting samples during the sample preparation could have increased the BIN lifespan and be responsible for matrix effects results lower than the ideal ± 25% [13].

Most published extraction methods to analyze synthetic cathinones in OF samples by LC–MS are based on liquid–liquid extraction or solid-phase extraction [10, 22–25]. Comparing those available published works analyzing synthetic cathinones in OF samples by MEPS with the method described above (Table 5), this work is the first to include samples obtained using Quantisal™ OF collection device, which guarantees a quantitative collection of 1 mL OF ± 10% mixed with 3 mL of elution buffer. This work obtained the lowest LOQ if we considered the correspondent OF volume used for the extraction (50 μL). Also, this method required low organic volume (650 μL) only behind a work that uses 600 μL [20].

Considering the finding regarding authentic OF samples and synthetic cathinones, Di Di Trana et al. [26] collected 56 blood, urine, and OF samples, and quantified them by HPLC−MS/MS. OF samples from authentic antemortem cases collected by spitting were donated as discarded material by the Polytechnique University of Marche, Italy and 100 μL was used. Fourteen OF samples were positive, and butylone (LOD 0.15 ng/mL) was quantified in three samples, with concentrations of 0.1, 1, and 120 ng/mL. Over a 4-year study, Krotulski et al. [27] collected 223 OF samples from volunteers attending electronic music festivals in the USA and indicated recent ecstasy use in the survey. The extraction used 500 μL sample collected with Quantisal™ device and confirmed by LC–MS/MS. A total of 352 samples were positive for MDMA and/or some synthetic cathinone, being methylone ($n = 36$), butylone ($n = 25$), eutylone ($n = 3$), pentylone ($n = 2$), and N-ethyl pentylone ($n = 22$) coincident in both methods, but only concentrations above 4 ng/mL were described. The obtained median was 973, 206, and 31 ng/mL for methylone, butylone, and N-ethyl pentylone, respectively. Six samples containing the survey responses had more than one synthetic cathinone identified in the toxicological analysis.

Until recently, there were no data involving synthetic cathinones pharmacokinetics. Poyatos et al. [28] studied the methylone pharmacokinetics in humans after controlled

Table 5 Available literature on the analysis of synthetic cathinones in oral fluid samples by MEPS

Analytes	Number of SC/metabolites	OF collection device	Sample volume (μL)	Organic solvent volume (μL)[a]	SC LOD (ng/mL)	SC LOQ (ng/mL)	Authentic SC samples	References
10	10	Quantisal™ (OF and buffer 1:3)	200 (50 OF)	650	0.05	0.1	Yes	This work
31 new psychoactive substances	10	Salimetrics™ (passive drool)	90	2650	0.05–0.83	0.13–2.50	No	[16]
21 drugs of abuse/metabolites	11	Salivette™ (passive drool)	300	1040[b]	0.25	0.5	No	[19]
1	1	Passive drool	90	600	4	14	No	[20]
10	1	Passive drool	400	2650	NA	50	Yes	[21]

SC synthetic cathinones, *OF* oral fluid, *NI* not available

[a]Considering all solutions containing some organic solvent during the sample pre-treatment and extraction, and number of cycles and syringe volume

[b]Evaporation step required

increasing doses. Sprega et al. [29] reported methylone and two of its metabolite concentration in OF samples in correlation with plasma concentration. Methylone maximum concentration (C_{max}) in OF and plasma was obtained 2 h after each administered dose. A proportional linear correlation value between the methylone dose and C_{max} between 100 and 200 mg was observed in OF samples, and an inversely proportional linear correlation value between methylone dose and pH values. OF concentration was significantly higher than in plasma, with OF/plasma of 3.6, 16.4, 37.7 and 33.9 after 50, 100, 150, and 200 mg methylone, respectively.

Even finding authentic samples with more than one cathinone in this work, biological matrix analysis cannot distinguish whether multiple drugs in the same sample were due to the consumption of one substance with several adulterants or polydrug use of several substances over a short period.

Conclusion

A fast and semi-automated MEPS extraction method to quantify ten synthetic cathinones was developed and fully validated, considering GAT principles. A small volume of sample (200 μL) and organic solvents were required during sample preparation and extraction (up to 650 μL), added to 200 μL sodium hydroxide. The BIN lifespan was around 100 samples and each extraction was performed in 3.5 min.

To 200 μL sample collected with Quantisal™ device, LOD and LOQ of 0.05 and 0.1 ng/mL were achieved, respectively. Forty-one authentic samples were quantified under this method, with 5 different synthetic cathinones being reported.

Declarations

Conflict of interest The authors declare that they have no conflict of interest associated with this manuscript.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the University of Campinas committee (Comitê de Ética em Pesquisa da UNI-CAMP—CEP, CAAE n° 88770318.0.0000.5404), and with the ethical standards as laid down in the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was available for all individual participants included in the study.

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