Received: 30 November 2018 | Revised: 6 February 2019 | Accepted: 6 February 2019

RESEARCH ARTICLE

Determination of several synthetic cathinones and an amphetamine-like compound in urine by gas chromatography with mass spectrometry. Method validation and application to real cases

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Most routine practices for drugs-of-abuse testing do not include screening procedures for new psychoactive substances, despite their increasing diffusion, preventing clear knowledge of the real consumption of these drugs in the populations. To make up for this shortcoming, a gas chromatography with mass spectrometry method was developed for the simultaneous determination of 18 synthetic cathinones and one amphetamine-like compound in human urine. The sample preparation was based on liquid–liquid extraction under alkaline condition followed by derivatization with trifluoroacetic anhydride. The separation of the 19 analytes was achieved in less than 10 min. The whole methodology was validated according to national and international guidelines. Selectivity, linearity range, limit of detection and limit of quantitation, precision and accuracy were evaluated. For all the analytes, the calibration curve was linear in the 100–1000 ng/mL concentration range. The limits of detection ranged from 10 to 30 ng/mL and limits of quantitation from 30 to 100 ng/mL. Precisions were in the ranges 0.1–10.4%, and 1.0–12.1% for low (100 ng/mL) and high (1000 ng/mL) concentration, respectively. The accuracy, expressed as bias% was within ±20% for all the analytes. The present method was successfully applied to urine samples originating from autopsies, drug abuse/withdrawal controls, clinical investigations, roadside controls, driving re-licensing, and workplace testing.

KEYWORDS
bath salts, cathinones, method validation, new psychoactive substances, urine analysis

1 | INTRODUCTION

Synthetic cathinones are sympathomimetic drugs related to cathinone, the major naturally occurring psychoactive component found in the leaves of the *Catha edulis* plant, commonly known as khat. For several years, they have been sold under the misleading name of "legal highs", "designer drugs", "bath salts", "research chemicals", "plant food" or generally labeled "not for human consumption" [1,2]. Synthetic cathinones are sold in specialized shops known as "head shops" and in online shops. In particular, today is the internet market which contributes significantly to their wide diffusion [2,3]. These compounds were initially synthesized to circumvent the existing laws on controlled

Article Related Abbreviations: MDMA, 3,4-methylenedioxymethamphetamine; NPS, New Psychoactive Substances; TBME, tert-butylmethylether; TFAA, trifluoroacetic anhydride; THC, Δ^9 -tetrahydrocannabinol.

substances, and/or to enhance the pharmacological activity of the more common amphetamines [4]. In addition to their specific stimulant and hallucinogenic properties, synthetic cathinones are abused for social and economic reasons, often serving as a replacement for 3,4-methylenedioxymethamphetamine (MDMA), cocaine, and amphetamines [4,5]. Furthermore, a growing number of studies have demonstrated synthetic cathinone adulteration of illegal drug, mainly MDMA [6] The intake of synthetic cathinones generally occurs by snorting, injection, smoking, or oral consumption [3,7]. Their effects are euphoria, rush, alertness, talkativeness, sexual arousal, focused mind, and overall positive feeling. The desired effects of synthetic cathinones occur within 30 to 45 min from administration and last from 1 to 3 h, followed by undesirable side effects that may persist from hours to days. The side effects range from neurological to cardiovascular and include anxiety, insomnia, fatigue, mydriasis, agitation, aggression, combative behavior, and panic [1,8–10].

Synthetic cathinones represent a new trend in the recreational drug market and numerous cases of abuse, dependence, intoxication, and deaths related to their consumption were described in the literature [1,5,11–14]. As a consequence, forensic and clinical laboratories worldwide are continuously requested to update their analytical procedures for the identification and quantification of these new drugs in various biological matrices.

Specific enzyme immunoassay kits for the detection of synthetic cathinones have been recently developed [15,16], and are available on the market. However, these kits generally allow the identification of only a limited number of cathinones. Moreover, they have a relatively high cost and questionable performances in terms of sensitivity and specificity [17]. Several screening and confirmation methods based on MS and MS/MS techniques have been published for the determination of synthetic cathinones in urine [17,18] together with an increasing number of MS protocols using a multi-target approach [19–26]. Modern forensic and clinical laboratories are required to develop fast and comprehensive methods because of the increasing number of compounds continuously introduced into the black market. Moreover, fast analytical methods are gaining importance in routine analytical laboratories, since they improve sample throughput and global productivity, as is necessary in laboratories where a considerable number of daily samples have to be processed. Therefore, we developed and validated a fast and simple GC-MS method able to identify 18 cathinone-like compounds plus 4-fluoroamphetamine in less than 10 min. The method was applied to authentic urine samples previously tested positive by Enzyme Multiplied Immunoassay Technique screening for amphetamines and methamphetamines, in order to evaluate the diffusion of several synthetic cathinones in our territory.

2 | MATERIALS AND METHODS

2.1 | Materials

3,4-Dimethylmethcathinone, 3,4-methylenedioxypyrovalerone, 4-fluoroamphetamine, 4-fluoromethcathinone, 4-methylethcathinone, 4-methylmethcathinone (Mephedrone), buphedrone, butylone (bk-MBDB), ethcathinone, methedrone, pentylone were obtained from Cayman Chemical (Ann Arbor, MI, USA); 1-naphyrone, 2-naphyrone, β-pentedrone, dimethylcathinone, ethylone, methylone, methcathinone, and amphetamine hexadeuterate were purchased from LGC Standards (Milan, Italy); 3-methylmethcathinone was purchased from Bertin Pharma (Montigny-le-Bretonneux, France); diphenylamine was purchased from Sigma-Aldrich (Milan, Italy). The purity of the analytical standards was at least ≥95%.

Stock standard solutions were stored at −20°C until used. Working solutions were prepared at the final concentration of 10 μg/mL by dilution with methanol.

Sodium hydroxide, tert-butylmethylether (TBME), and the derivatizing agent trifluoroacetic anhydride (TFAA) were purchased from Sigma-Aldrich (Milan, Italy).

2.2 | GC-MS characterization

Full scan electron ionization mass spectra of the derivatization products of parent drugs were recorded from reference standards, in order to determine the most appropriate protocol for accurate and sensitive detection of the target analytes. According to 2002/657/EC requirements [27], the relative abundances of three characteristic ions (qualifier ions vs. target ion) along with the GC peak retention time were used for the positive identification of each target compound.

2.3 | Sample preparation

Urine samples (2 mL) were added with 50 μL of internal standard mixture (amphetamine-d6 and diphenylamine) to yield a final concentration of 250 ng/mL and were subsequently alkalinized to pH 10−12 by adding few drops of sodium hydroxide 1 N solution. Liquid–liquid extraction was performed by adding 10 mL of TBME under shaking in a multimixer for 10 min. After centrifugation at 2200 rpm for 3 min, the organic layer was transferred into a vial and dried under nitrogen at room temperature. The dry residue was allowed to react with 50 μL TFAA for 30 min at 65°C. After derivatization, the excess of TFAA was evaporated under a nitrogen stream at room temperature. The dry residue was re-dissolved in 50 μL of TBME and a 1 μL-aliquot was injected into the GC system with a split ratio of 10:1.

TABLE 1 Molecular weights of the 19 cathinones and amphetamine-like compounds (TFA derivatives), GC retention times and characteristic ions (EI mode) used in selected-ion monitoring experiments

	Analyte	Derivative	Molecular weight	RT (min)	Ions (m/z)	
					Target	Others
1	4-Fluoroamphetamine (4-FA)	Mono-TFA	237	4.22	140	83, 109
2	Dimethylcathinone	Free	177	4.59	77	72, 105
3	4-Fluoromethcathinone (Flephedrone)	Mono-TFA	277	4.82	154	95, 123
4	Methcathinone	Mono-TFA	259	4.96	154	77, 105
5	Buphedrone	Mono-TFA	273	5.24	168	105, 263
6	Ethcathinone	Mono-TFA	273	5.36	140	105, 168
7	3-Methylmethcathinone (3-MMC)	Mono-TFA	273	5.38	154	91, 119
8	Mephedrone (4-MMC)	Mono-TFA	273	5.50	154	91, 119
9	β-Pentedrone	Mono-TFA	287	5.61	182	105, 287
10	4-Methylethcatinone (4-MEC)	Mono-TFA	287	5.85	140	119, 168
11	3,4-Dimethylmethcathinone	Mono-TFA	287	5.97	154	105, 133
12	Methedrone	Mono-TFA	289	6.20	154	107, 135
13	Methylone (bk-MDMA)	Mono-TFA	303	6.60	154	149, 303
14	Butylone (bk-MBDB)	Mono-TFA	317	6.80	140	149, 317
15	Ethylone (bk-MDEA)	Mono-TFA	317	6.88	140	149, 317
16	Pentylone	Mono-TFA	331	7.06	140	149, 331
17	3,4-Methylendioxypyrovalone (MDPV)	Free	275	7.84	126	121, 149
18	1-Naphyrone	Free	281	8.27	126	84, 155
19	2-Naphyrone	Free	281	8.45	126	84, 155
IS	Amphetamine-d6	Mono-TFA	237	4.14	144	123
IS	Diphenylamine	Mono-TFA	265	5.78	265	167

3-MMC: 3-Methylmethcathinone; 4-FA: 4-fluoroamphetamine; 4-MEC: 4-Methylethcatinone; 4-MMC: Mephedrone; bk-MBDB: butylone

2.4 | Apparatus and methods

The analyses were performed using a 6890N GC, combined with a 5975 *inert* Mass Selective Detector (Agilent Technologies, Milan, Italy) with electron ionization at 70 eV. A 17 m fused-silica capillary column (J&W Scientific HP-5), with an inner diameter of 0.2 mm and a film thickness of 0.33 mm was utilized for GC separation. Helium was employed as the carrier gas at a constant pressure of 31 psi. The GC oven temperature was set at 85°C and then raised to 110°C with a 12°C/min heating rate. The oven temperature was then raised to 300°C with a 30°C/min heating rate. The oven temperature was maintained at 300°C for 1 min. The total run time was 9.9 min. The GC injector and transfer line were maintained at 230 and 250°C, respectively. Data were acquired in the selected-ion monitoring mode using each compound detection for the diagnostic ions listed in Table 1.

2.5 | Validation

The method was validated by investigating the following parameters: selectivity, linearity range, identification and quantitation limits (LOD and LOQ), precision, accuracy, and carry over. The GC-MS methods validation was carried out according to national and international guidelines [28,29]. Blank urine specimens required for the method validation were obtained from ten different healthy volunteers (four females, six males).

2.5.1 | Selectivity

Ten different blank urine samples were extracted, derivatized, and analyzed as described above. The occurrence of possible interferences from endogenous substances or derivatization by-products was tested by monitoring the selected-ion chromatograms, characteristic for each investigated compound, at the retention time interval expected for their elution.

2.5.2 | Linearity

The linear calibration model was checked by analyzing (two replicates) negative urine samples spiked with standard solutions at five concentration levels (100, 200, 500, 750, and 1000 ng/mL) for each analyte. The linear calibration parameters were obtained using the least squares

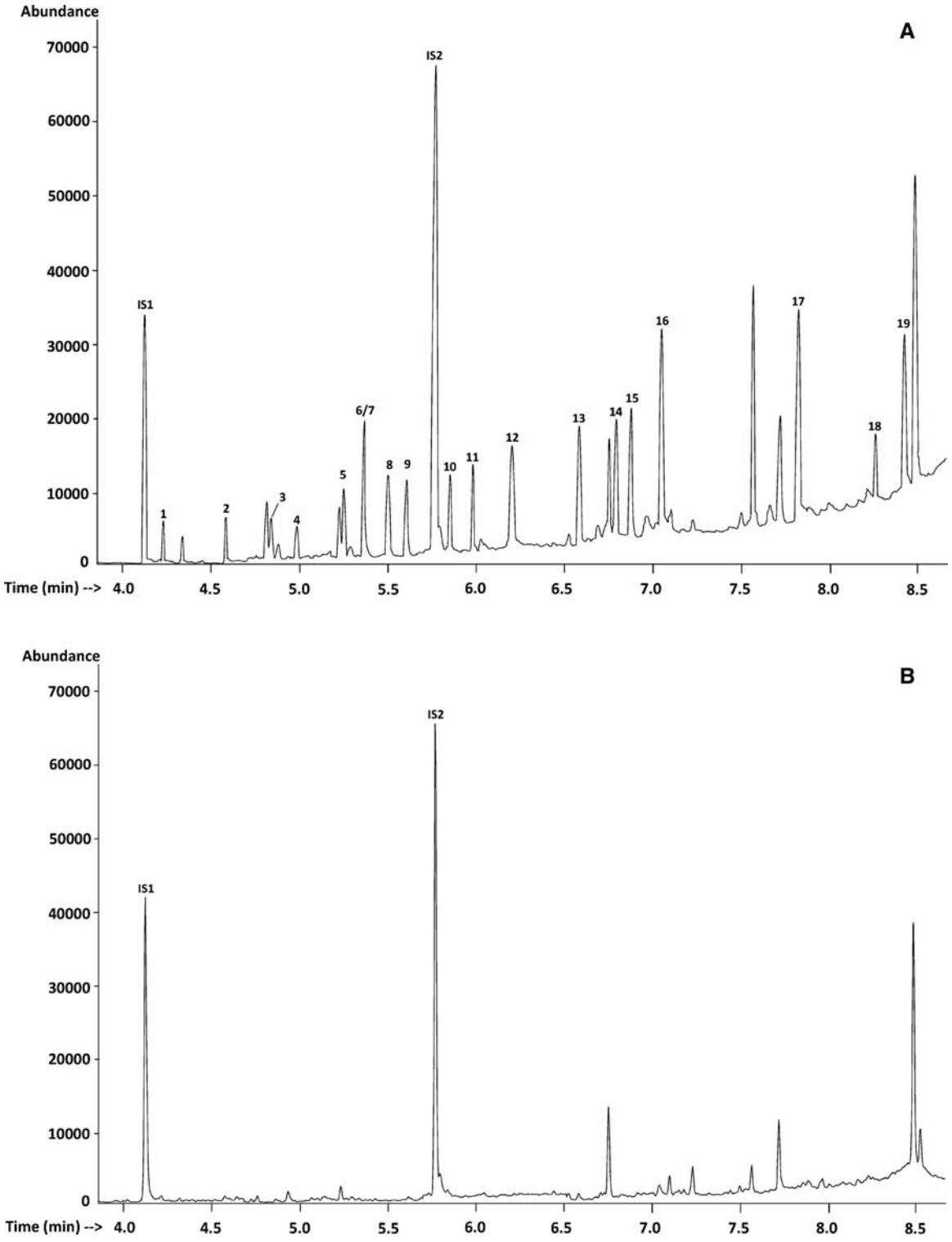

FIGURE 1 Total ion current (TIC) chromatogram from a GC-MS run of a blank urine sample spiked with all target analytes at the concentration of 100 ng/mL (panel A) in comparison with a blank urine sample (panel B). Each analyte is labelled by the progressive number assigned in Table 1

regression method, while the squared correlation coefficients (R^2) were utilized to estimate linearity. Quantitative results from area counts were corrected using the internal standard signal.

2.5.3 | LOD

The LOD was preliminarily estimated as the analyte concentration whose response provided a S/N ratio of three, as determined from the least abundant qualifier ion. The S/N ratio at

the lowest concentration of the calibration range (100 ng/mL) was used to extrapolate the theoretical LOD. These calculated LOD values were then experimentally confirmed by analyzing spiked samples at LOD concentration for all analytes. The LOQ values were initially estimated as the analyte concentration whose response provided an S/N value equal to ten. The practical LOQ was set at the lowest concentration of the calibration curves (100 ng/mL), which was positively tested for precision and accuracy requirements [30], as reported below.

2.5.4 | Precision and accuracy

Intra-assay precision (repeatability), expressed as percent variation coefficient (CV%) and accuracy evaluation (expressed as bias %), were assessed by extracting and analyzing ten replicates of negative urine samples spiked at two concentration levels (100 and 1000 ng/mL). Standard criteria designated satisfactory assay precision when CV% values were below 20% for concentrations of 100 ng/mL and below 15% for 1000 ng/mL. Satisfactory accuracy was achieved when the experimentally determined concentrations were within ±20% from the expected values at 100 ng/mL and within ±15% at 1000 ng/mL.

2.5.5 | Carry-over

The background chromatographic profiles for each analyte were monitored during the analysis of blank urine sample injected after the chromatographic run of a spiked blank urine sample containing all the analytes at 1000 ng/mL concentration. The test was repeated five times in different periods. To assure the absence of carry-over, the S/N for each transition had to be lower than three.

3 | RESULTS AND DISCUSSION

3.1 | GC-MS characterization

All target analytes were easily characterized by different fragments. The selected-ion monitoring protocol developed (see Table 1) proved efficient in discriminating the interferences.

The separation of the target analytes (as TFAA-derivatives) was achieved in less than 10 min. A total ion current GC-MS chromatogram obtained from a blank urine specimen fortified with 100 ng/mL of each compound is illustrated in Figure 1.

3.2 | Validation

3.2.1 | Selectivity

Ten blank urine samples tested for the possible occurrence of endogenous interferences provided no quantifiable analyte peaks (i.e., S/N ratio less than 3) at the expected retention time for the target analytes. It was deduced that the method was selective for the compounds tested and free from positive interference from urine components and column bleeding.

3.2.2 | Linearity and evaluation of LOD and LOQ

A calibration curve was built for all the investigated compounds in the interval 100−1000 ng/mL. Table 2 reports the resulting R^2 values, that range from 0.992 and 0.997 indicating good fit and linearity of the calibration curves within the investigated concentration range. Experimental LOD and calculated LOQ values (the latter extrapolated from S/N values of lowest concentration calibrator) are also reported in Table 2. Positive detection (S/N > 3) of analytes at the approximate LOD concentrations was confirmed experimentally. LOD values were in the range 10–30 ng/mL.

3.2.3 | Precision and accuracy

Intra-assay data on precision and accuracy are reported in Table 2. The results demonstrated satisfactory intra-assay precision at both low and high levels, as the percent variation coefficient (CV%) is lower than 15% for all analytes with the only exception of methylone (15.4% at 100 ng/mL). Also the accuracy proved satisfactory at both low and high concentration levels, since bias% values fell within ±20% for all analytes.

3.2.4 | Carry-over

The background chromatographic profiles monitored for each analyte during the analysis of blank urine injected after highly spiked samples, did not show the presence of any significant signal (i.e., the signal to noise ratio was always <3) at the retention times of the tested analytes. The presence of carry-over effect was therefore excluded.

3.3 | Real Urine Samples

Our laboratory protocol establishes for all urine samples that tested positive to amphetamines and methamphetamines by Enzyme Multiplied Immunoassay Technique to be confirmed for the presence of amphetamines, methamphetamines, and synthetic cathinones by means of a GC-MS method. The validated method presently described was applied to 594 authentic urine samples requiring confirmation for the latter class of stimulants, along with amphetamines and methamphetamines. The real samples were collected from drug abuse/withdrawal controls, clinical investigations, roadside controls, driving re-licensing, workplace testing, and autopsies.

Seven samples were found positive for at least one target analyte. Six positive samples originated from male subjects (age 20−31) while only one sample was from a female (aged 42). The final results are summarized in Table 3.

TABLE 2 Validation data for the screened molecules

Analyte		Linearity range (ng/mL)	Linearity (R^2)	Precision (CV%)		Accuracy (Bias ±%)		LOD (ng/mL)	LOQ (ng/mL)
				100 ng/mL	1000 ng/mL	100 ng/mL	1000 ng/mL		
TFAA derivative									
1	4-Fluoroamphetamine (4-FA)	100–1000	0.992	0.1	1.0	−8.5	−0.5	30	100
2	Dimethylcathinone	100–1000	0.995	6.9	9.7	+4.4	−12.0	20	70
3	4-Fluoromethcathinone (Flephedrone)	100–1000	0.992	7.5	5.9	−1.3	−5.3	30	100
4	Methcathinone	100–1000	0.993	10.9	11.5	−8.4	−11.8	10	30
5	Buphedrone	100–1000	0.990	5.1	5.3	−10.2	−5.0	30	100
6	Ethcathinone	100–1000	0.994	6.9	2.8	−9.5	−1.7	10	30
7	3-Methylmethcathinone (3-MMC)	100–1000	0.995	10.4	6.9	−13.4	+19.1	10	30
8	Mephedrone (4-MMC)	100–1000	0.991	3.8	2.3	−9.3	+6.8	10	30
9	β-Pentedrone	100–1000	0.993	3.5	4.5	−16.6	−1.2	20	70
10	4-Methylethcatinone (4-MEC)	100–1000	0.996	7.0	2.8	−9.3	+4.8	10	30
11	3,4-Dimethylmethcathinone	100–1000	0.992	4.2	4.3	−12.3	+10.5	10	30
12	Methedrone	100–1000	0.995	4.0	4.0	+3.0	+10.3	10	30
13	Methylone (bk-MDMA)	100–1000	0.997	15.4	11.5	−4.9	+16.6	20	70
14	Butylone (bk-MBDB)	100–1000	0.994	6.3	4.6	−7.3	+8.5	10	30
15	Ethylone (bk-MDEA)	100–1000	0.993	3.4	2.9	+0.8	+10.4	30	30
16	Pentylone	100–1000	0.993	2.5	4.3	+1.3	+9.0	10	30
17	3,4-Methylendioxypyrovalone (MDPV)	100–1000	0.997	6.1	6.9	−3.8	+14.7	30	100
18	1-Naphyrone	100–1000	0.995	4.8	12.1	−4.9	−8.0	30	100
19	2-Naphyrone	100–1000	0.993	4.8	6.5	−0.4	+13.7	30	100

TABLE 3 Synoptic summary of real samples positive for target analytes

Positive sample	Sex	Age	Origin	Result	Concentration (ng/mL)	Other Findings
1	F	42	RC	Butylone	1780	MDMA, THC metabolites
2	M	29	RC	Butylone	760	MDMA
3	M	20	CI	Butylone	8390	MDA, MDMA
4	M	24	RC	Butylone	5510	MDA, MDMA
5	M	31	RC	4-Fluoroamphetamine	110	Amphetamine, Ketamine, Cocaine metabolites
6	M	24	A	Mephedrone	80550	Cocaine metabolites
7	M	20	RC	3-MMC	42700	THC metabolites

RC: roadside control; CI: clinical investigation; A: Autopsy; THC; Δ^9 -tetrahydrocannabinol

Synthetic cathinones were detected in six samples including butylone (four cases; range 760–8390 ng/mL); mephedrone (one case; 80550 ng/mL), and 3-methylmethcathinone (one case; 42700 ng/mL). In one case, the amphetamine-like compound 4-fluoroamphetamine was found at 850 ng/mL concentration. In five cases, the samples originated from roadside controls, while in two cases the samples were obtained, respectively, from a clinical and a post-mortem investigation.

Besides cathinones, all tested samples were found positive to at least one traditional drug of abuse (and/or their metabolites) including amphetamine, MDMA, cocaine, ketamine, and Δ^9-tetrahydrocannabinol. This finding strongly suggests that new psychoactive substances (NPS) consumers are typically poly-abuser of both traditional and new drugs. It is noteworthy that all four urine samples that tested positive to butylone were also positive to MDMA. As a consequence, it cannot be excluded that butylone may have been unknowingly or unintentionally consumed after deceitful trade, as already reported in recent studies [31,32]. As a matter of fact, several cathinones including methylone, ethylone, and butylone are

likely present as adulterants or in replacements for drugs sold as ecstasy [33–37], due to their psychoactive effects somehow similar to MDMA. In conclusion, it is not unlikely that most of the positive results that we found could be unexpected by the users themselves and can be attributed to the unaware intake of NPS.

4 | CONCLUDING REMARKS

Due to the wide range of NPS present in the black market, the chance to develop and implement effective analytical strategies to detect their presence in biological samples has become extremely challenging. The fast appearance of new NPS with distinctive chemical structures and metabolites makes the immunochemical screenings scarcely available, quite unspecific, and rapidly outdated, with the risk of underestimating the real consumption of these new drugs in the population and their potential involvement in traffic or work-related accidents. Among the various NPS classes, cathinones are among the most widely abused, especially by a percentage of electronic music events participants and regular disco clients [31,32].

In the present study, a rapid GC-MS method for the simultaneous determination of a wide range of synthetic cathinones was developed and validated, with the objective of introducing its application within the regular routine task of confirming the results arising from immunoassay screening. The method is particularly suited for routine analysis, especially when a considerable number of samples have to be analyzed, since about 6 injections/h could be performed after a rapid sample preparation step spending around 1 h. Furthermore, considering the use of a simple instrumentation based on a single quadrupole GC-MS system, available in most of forensic and clinical laboratories, the proposed method is easily reproducible and affordable. The method showed good sensitivity, selectivity, and optimal linear response, together with good repeatability and accuracy for quantitative determinations in the concentration range useful for confirmation analysis. The analytical results on real samples confirm the diffusion of cathinones in the geographical region of our laboratory. In particular, the present study highlights the occurrence of poly-abuse of traditional and new drugs in the investigated population, irrespective of the deliberate or unaware intake of the latter class. The range of different circumstances under which the positive detection of cathinones may be of utmost importance makes the present method profitably applicable to urine samples collected in all fields of intervention typical of a forensic toxicology laboratory.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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How to cite this article: Gerace E, Caneparo D, Borio F, Salomone A, Vincenti M. Determination of several synthetic cathinones and an amphetamine-like compound in urine by gas chromatography with mass spectrometry. Method validation and application to real cases. *J Sep Sci* 2019;42:1577–1584. https://doi.org/10.1002/jssc.201801249