Research Article

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Simultaneous Quantification of the New Psychoactive Substances 3-FMC, 3-FPM, 4-CEC, and 4-BMC in Human Blood using GC-MS

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Abstract: A gas chromatography-mass spectrometry (GC-MS) method for simultaneous quantification of 3-fluoromethcathinone (3-FMC), ($\pm$)-3-fluorophenmetrazine (3-FPM), 4-chloroethcathinone (4-CEC) and 4-Bromomethcathinone (4-BMC) in human blood with ($\pm$)-methcathinone-D3 as internal standard has been developed and validated. Whole blood samples were treated with 10% trichloroacetic acid for protein precipitation before solid phase extraction. The method was selective, the calibration curves showed linearity for all substances with R^2 ranging from 0.991 to 0.998 in the range 5-1.000 ng/mL. Analysis of blank samples showed no-sign of carryover. Precision and accuracy were acceptable with values less than 20% (RSD) and $\pm$ 20% (Bias). The limit of quantification (LOQ) for all substances was 5ng/mL. Intra-day and inter-day precision were 2.1-11.7% and 1.3-10.2% respectively and accuracy biases were between -10.6-19.6% (intra-day) and 11-12.1% (inter-day). The extraction efficiencies were 85.4, 82.8, 79.1 and 74.9% for 3-FMC, 3-FPM, 4-CEC and 4-BMC respectively.

A robust and reliable simultaneous quantification method using gas chromatography-mass spectrometry in selected ion monitoring mode (GC-MS-SIM) is reported.

Keywords: Forensic Science; Cathinones; Validation; Chromatography; Human Blood.

1 Introduction

New psychoactive substances (NPS) are becoming increasingly widespread. They are frequently sold online

as research chemicals or bath salts. In order to detect them comprehensive bioanalytical methods are needed [1]. Important examples of these substances are synthetic derivatives of cathinone [2].

Khat shrub (*Catha edulis*) leaves contain the psychostimulant cathinone which is a natural amphetamine. Various synthetic derivatives of cathinone are being used as 'legal highs'. As a consequence, some countries have introduced legislation restricting them [3,4]. Drugs of abuse have been detected in both conventional biological matrices (urine and blood) and non-conventional matrices (e.g. saliva, hair, nails and sweat) [4-6]. Compared to blood, urine carries higher concentrations of compounds and has a longer detection period [1, 7, 8]. Urine is therefore a primary source used in forensic screening analysis.

The symptoms of acute drug toxicity correlate, however, with the concentration of substances in the blood and not in urine. Therefore blood remains the most important specimen in the interpretation of the results of drugs of abuse detection [9,10].

Synthetic cathinones can be much more potent and hazardous than natural cathinones, or even lethal in some cases [11]. The metabolism of 3-Bromomethcathinone (3-BMC) and 3-fluoromethcathinone (3-FMC) in rat urine and human liver microsomes was studied by Meyer et al. [12] using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). Based on the results, their metabolism is understood to involve N-demethylation, reduction of the keto group to the corresponding alcohol, hydroxylation of the aromatic system and combinations of these steps. Mardal et al. [13] studied the metabolites of 3-fluorophenmetrazine (3-FPM) using GC-MS and LC-MS. They found that it remains mainly unchanged and is excreted as N-oxide in human urine with microbial biotransformation, and as aryl hydroxylated metabolites in rat urine.

Immunoassay techniques (IA) are rapid and simple for handling samples and are therefore used extensively

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for screening whole blood and serum in cases of drugs of abuse. However, these techniques have serious limitations relating to sensitivity and selectivity and can produce false positive as well as negative results [8, 9, 14, 15]. IA can also be used as a screening technique for the detection of synthetic cathinones in biological samples [16, 17].

Chromatographic techniques have been used to increase reliability and accuracy [1, 9, 14], and hyphenated chromatographic techniques have further improved reliability, particularly GC-MS [18, 19] and LC-MS [20, 21]. GC-MS enables the detection of a larger range of substances in a single analysis and is more cost efficient than LC-MS. It is therefore the primary analysis method used in forensics. However, there are limitations of GC-MS for blood analysis: they are not sensitive enough to identify the full range of compounds that are significant on the total ion chromatogram (TIC) [22], even in full scanning mode and with the use of specific search algorithms and mass spectral libraries. Selected-ion monitoring (SIM) mode has been used to improve sensitivity, but this, in turn, affects the detection spectrum.

The analytical techniques of first choice for the detection of synthetic cathinones are GC and LC coupled with different spectroscopic instruments. GC-MS is applicable to cathinone compounds producing a very simple mass spectrum [23-26]. LC-MS is employed in the analysis of cathinones more frequently than GC-MS, mostly in the electrospray ionization mass spectrometric (ESI-MSn) mode, allowing the characterization of the respective protonated molecular ions using the product ion formation patterns [25, 27-30].

Most of the methods reported for whole blood analysis focused on only a single analyte and their metabolites or on small analyte groups [31-33]. As the range of different analytes is frequently unknown before testing and samples are often of a limited size, simultaneous quantification of analytes of interest is preferable in forensic analyses as it is faster and more effective. Due to this multi-analyte methods are now more widely used than in previous years [34-36].

In this work, a simultaneous quantification method for the determination of 3-FMC, 3-FPM, 4-CEC, and 4-BMC in human blood was developed using GC-MS-SIM. The internal standard used was ($\pm$)-methcathinone-D3. The analytical procedure reported was validated in accordance with the guidelines laid down by the Scientific Working Group on Forensic Toxicology (SWGTOX) on validation of bioanalytical methods [37].

2 Materials and Methods

2.1 Chemicals and Reagents

The drug standards were obtained as methanolic solutions, (1.0 mg/mL) of ($\pm$)-3-fluorophenmetrazine hydrochloride (3-FPM), 3-fluoromethcathinone hydrochloride (3-FMC), and 4-chloroethcathinone hydrochloride (4-CEC) purchased from Cerilliant (USA). A 1.0 mg standard 4-bromomethcathinone hydrochloride (4-BMC) (also known as Buphedrone) was obtained from Cayman Chemical (USA). ($\pm$)-Methcathinone-D3 hydrochloride solution 100 μ g/mL in acetonitrile and Trifluoroacetic anhydride (TFAA), Hydrogen chloride ReagentPlus[®] $\geq 99\%$ and HPLC water were obtained from Sigma Aldrich (USA).

The clean screen extraction column (UCT, size 200mg/3ml) was purchased from United Chemical Technologies (USA) consisting of octyl (C8) with benzyl sulfonic acid. Acetic acid 99.7%, ammonium hydroxide 28 - 30%, ethyl acetate, HPLC grade methanol, and HPLC grade acetone were acquired from Fisher Scientific (UK). GC grade dichloromethane and GC grade Pyridine were acquired from Riedel-DeHaën (Germany). Trichloroacetic Acid (TCA) was obtained from Biochemicals (USA). UHPLC grade Acetonitrile was acquired from Applichem Panreac (Germany) and Isopropyl alcohol (2-propanol) was acquired from Chem-Lab (Belgium).

2.2 Samples

Blank human blood samples were obtained from ten different volunteers and collected in 5 mL Vacutainer tubes containing sodium heparin. The samples tested negative for synthetic cathinones (general GC-MS full scan test). 1 mL aliquots from each blank pool were analyzed in full scan mode to confirm the absence of potential interference prior to use, as a calibration matrix and quality control (QC) procedure. Before analysis, specimens were stored at -20°C .

2.3 Preparation of Calibrators and QC Samples

3-FMC, 3-FPM, 4-BMC and 4-CEC (1.0 mg base/mL) stock solutions were prepared in methanol. A mixed calibration stock solution of 50,000 ng/mL was made by combining and diluting the methanol standards of the four analytes (1.0 mg/mL). Concentrations of 50, 250, 500, 1,000, 5,000 and 10,000 ng/mL were used for working solutions. To yield final concentrations of 5 – 1,000 ng/g, 100 μ L aliquots

Table 1: Retention times and m/z values chosen for the GC-MS-SIM analysis of 3-FMC, 3-FPM, 4-CEC, 4-BMC and the IS Methcathinone-D3 (three time windows).

Compound	Time window (min)	Retention time (min)	Values of m/z in SIM mode	
			Quantitation Ion	Qualification Ions
3-FMC	8.00 – 10.00	8.97	154	110, 123
Methcathinone-D3	8.00 – 10.00	9.14	105	113, 157
3-FPM	10.00 – 10.70	10.32	167	70, 98, 290
4-CEC	10.70 – 12.50	11.11	168	111, 140
4-BMC	10.70 – 12.50	11.27	154	110, 183, 185

of working solutions were added to 900 μ L of blank blood. Final calibration concentrations for the method were as follows: 5, 25, 50, 100, 500, 1,000 ng/mL for each analyte.

Various ampules of 1.0 mg / mL methanol standards were used to prepare the QC working solutions used to make the calibration solutions; where available a different number of people were used to make these solutions. 15, 150, and 750 ng/mL working QC solutions were used to fortify blank whole blood samples. This yielded low, medium and high QC samples for each analyte.

100,000 ng/mL internal standard stock solution was prepared by diluting 1.0 mg/mL methanolic solution of the internal standard ($\pm$)-methcathinone-D3 in methanol. 25 μ L of a 4,000 ng/mL working solution of the internal standard prepared in methanol was added to each calibration and quality control sample. This yielded 100 ng/mL internal standard concentrations. All stock solutions were stored at -80°C before use and work solutions were prepared daily.

2.4 Instrumentation

GC analyses were performed by an Agilent 7890 GC coupled to an Agilent 5975A GC-MS Single Quad mass spectrometer (Agilent, Santa Clara, CA, USA), an ALS 7693 and a MassHunter GC/MS Acquisition (version B.07.04.2260, 28 October 2015) using an 5% phenyl methyl siloxane column (HP-5 MS UI; 30 m $\times$ 0.25 mm i.d.; 250 nm film thickness).

The GC conditions were as follows: splitless injection mode; injection port was set at 280°C ; carrier gas, helium; flow rate, 1 mL/min; The GC oven temperature was initially increased from 80°C (hold 2 minutes) to 280°C at a rate of $15^{\circ}\text{C}/\text{min}$, giving a total run time of 15.333 minutes; The temperatures of interface, ion source and the quadrupole were 250, 230 and 110°C respectively. Ion monitoring (SIM) mode (70 eV, electron impact mode) was used for the detection.

The retention times and m/z values selected for the detection in the selected ion mode (SIM, three time windows) are shown in Table 1. The mass spectrum of targeted cathinone derivatives is shown in Figure 1.

2.5 Samples Preparation

25 mL of the 4,000 ng / mL internal standard working solution and a defined volume of the corresponding working calibration or QC fortifying solution (if applicable) were added to 1.0 mL of whole blood. 1 mL fortified blood samples of calibration and QC samples were treated with 4 mL of 10% trichloroacetic acid (TCAA) for protein precipitation before being introduced to Solid phase extraction (SPE) column cartridge (UCT, size 200mg/3ml). Samples were mixed, vortexed for 5 minutes and centrifuged at 6000 rpm for 10 minutes at 5°C . The supernatant was transferred to clean test tubes for SPE. Sequential addition of 2.5 mL water and 2.5 mL methanol were used to condition the SPE cartridges. Samples were allowed to flow under gravity after being loaded on the columns. They were then washed and rinsed by sequential addition of 2.5 mL 0.1M HCl, 2.5 mL water, 2.5 mL methanol and 2.5 mL water. They were then dried for 10 minutes under full vacuum. Using 4 mL of freshly prepared dichloromethane/isopropyl alcohol/ammonium hydroxide [80:20:2, v/v/v], the analytes were eluted under gravity and collected in glass tubes. A gentle stream of nitrogen at $\leq 40^{\circ}\text{C}$ was used to evaporate the extracts to dryness. The residues were derivatized in capped tubes in 50 μ L of ethyl acetate and 50 μ L of TFAA by incubation for 30 minutes at 70°C in a heater block. Samples were then cooled to room temperature, evaporated to dryness under nitrogen at 50°C and reconstituted with 50 μ L of ethyl acetate. For GC-MS / SIM analysis, 2 μ L were injected.

Ethical approval: The conducted research is not related to either human or animal use.

Figure 1: EI Mass spectra, proposed structures and predominant fragmentation patterns of TFA derivatives of the target cathinones. 3-FPM indicates of (±)-3-Fluorophenmetrazine; 4-CEC, 4-Chloroethcathinone; 4-BMC, 4-Bromomethcathinone; 3-FMC, 3-Fluoromethcathinone using GC-MS, full scan mode.

3 Results

3.1 Method validation

The international guidelines on bioanalytical method validation [SWGFT] were the basis for performing the described analytical procedure. Stability (autosampler, bench-top and short-term freeze/thaw), precision (intra-day and intermediate), selectivity, limits of detection (LOD) and quantitation (LOQ), carryover, linear range, bias, extraction and efficiency were studied.

3.2 Selectivity

10 pools from different sources of blank samples of blood were analyzed to study selectivity. Ions were monitored for each significant analyte and respective internal standard (IS). The pools were monitored and investigated for interferences at the relevant retention times. From each pool, two sets of samples ($n = 10$) were spiked with the same concentration (150 ng/mL) of all analytes of interest (Methcathinone-D33-FMC, 3-FPM, 4-BMC and 4-CEC). Ten negative and ten positive samples were submitted to the above methodologies. All analytes were identified successfully in all the spiked matrices. No interference of

the matrices was observed at the retention times and at the m/z values of the monitored ions. This was shown by the analysis of the negative blank pools. Based on these results, the procedure described was considered selective for identifying the selected substances in human blood matrix. Total ion chromatograms for the target cathinone TFA derivatives are shown in Figure 2.

3.3 Linearity

The peak area ratio of each analyte and the IS were used to construct the calibration curves ($n=6$) by linear regression. In the whole blood, the linear range was 5 to 1,000 ng/mL (5, 25, 50, 100, 500, 1,000 ng/mL) for each substance. A blank sample with IS was added, and three separate concentration levels (15, 150, and 750 ng/mL) of QC samples ($n=3$) were then analysed. After the highest point of the calibration curve, a blank sample was analyzed and showed no peaks for the target analytes that the method is free of carryover. The LOQ was measured in SIM mode using blank blood fortified with all analytes at concentrations of 1, 2.5, 5, and 10 ng/mL. The S/N ratio was calculated from triplicate measurements. The lowest concentration at which the S/N ratio was equal or greater than three was considered as the LOD.

Figure 2: Total Ion Chromatograms of the given ions at 70, 98, 105, 110, 111, 113, 123, 140, 154, 157, 167, 168, 183, 185 and 290 indicating TFA derivatives of the target cathinones. 3-FPM indicates of (±)-3-Fluorophenmetrazine; 4-CEC, 4-Chloroethcathinone; 4-BMC, 4-Bromomethcathinone; 3-FMC, 3-Fluoromethcathinone, obtained after protein precipitation and SPE extraction (SPE column UCT, size 200mg/3ml) of Whole Blood using GC-MS (GC column HP-5ms, SIM mode).

Table 2: Linearity results for 3-FMC, 3-FPM, 4-CEC, 4-BMC in whole blood.

Analytes	Linear Rang (ng/mL)	Linearity			LOD (ng/mL)	LOQ (ng/mL)
		Slope	Intercept	r^2		
3-FMC	5-1,000	0.00224±0.00002	-0.00513±0.00114	0.997	2.5	5
3-FPM	5-1,000	0.00135±0.00008	-0.00207±0.00386	0.998	2.5	5
4-CEC	5-1,000	0.00288±0.00007	-0.03654±0.00340	0.991	2.5	5
4-BMC	5-1,000	0.00174±0.00001	-0.01013±0.00564	0.998	2.5	5

To evaluate the LOQ, the minimum concentration of the analytes measured with adequate precision ($CV \leq 20\%$) and bias ($\pm 20\%$) was used. The acceptance criteria for calibration data have been accomplished (Table 2). Selected ion monitoring chromatograms for the analysis of whole blood blank and fortified LOQ samples are shown for all analytes in Figure 3.

3.4 Precision and accuracy

Coefficient of variation (CV, %) was used to express precision. The intra-day precision and bias were determined by analyzing batches of five QC samples on the same day at three different concentration levels (15, 150 and 750 ng/mL) in whole blood. The intermediate precision and bias were evaluated by the analysis of five QC samples (15, 150 and 750 ng/mL) in whole blood over three consecutive days ($n=27$). The bias was calculated as $[(\text{mean of measured concentrations} - \text{theoretical$

concentration)]/theoretical concentration $\times 100$] for each group of concentrations of QC samples. Acceptable values for $CV \pm 20\%$ and a bias variability of $\pm 20\%$ have been established. The obtained values were acceptable at all concentrations for intra-day precision (%RSD; 2.1-11.7%) and bias (-10.6-19.6%), as well as to the intermediate precision (%RSD; 3.7-9.6%) and bias (-11-12.1%) in the studied matrices (Tables 3 and 4).

3.5 Recovery

The efficiency of extraction was measured by comparing two sets of six different QC samples at each concentration level (15, 150 and 750 ng / mL) in which the IS was added after extraction. Blank samples were fortified in set 1 with all analytes, while analytes were added to the QC samples after the extraction procedure (100 percent recovery) in set 2. The average peak range ratio was compared as follows: $\text{Set1/Set2} \times 100\%$. The extraction efficiencies for

Figure 3: Total ion chromatograms of TFA derivatives of cathinones at (A) limit of quantification (5 ng/mL) and (B) blank whole blood. 3-FPM indicates of (±)-3-Fluorophenmetrazine; 4-CEC, 4-Chloroethcathinone; 4-BMC, 4-Bromomethcathinone; 3-FMC, 3-Fluoromethcathinone after protein precipitation and SPE extraction (SPE column UCT, size 200mg/3ml) of the Whole Blood GC-MS (GC column HP-5ms, SIM mode).

Table 3: Intra-day precision and accuracy (n=5).

Substance	Spiked Concentrations (ng/mL)								
	15			150			750		
	Concentration Found (ng/mL) Mean $\pm$ SD	RSD (%)	Bias (%)	Concentration Found (ng/mL) Mean $\pm$ SD	RSD (%)	Bias (%)	Concentration Found (ng/mL) Mean $\pm$ SD	RSD (%)	Bias (%)
3-FMC	17.9 $\pm$ 0.5	2.7	19.6	161.5 $\pm$ 9.3	5.7	-5.6	753 $\pm$ 53.7	7.1	-7.3
3-FPM	16.3 $\pm$ 1.3	7.9	-8.7	142.4 $\pm$ 7.7	5.4	5.0	787.8 $\pm$ 57.4	7.6	-5.0
4-CEC	16.8 $\pm$ 1.9	11.7	-12	143.4 $\pm$ 7.7	5.3	4.4	729.6 $\pm$ 15.2	2.1	2.7
4-BMC	16.6 $\pm$ 1.5	9.0	-10.6	163.6 $\pm$ 3.1	2.2	-9.1	769.8 $\pm$ 30.6	3.9	-2.6

Table 4: Inter-day precision and accuracy (n=15).

Substance	Spiked Concentrations (ng/mL)								
	15			150			750		
	Concentration Found (ng/mL) Mean $\pm$ SD	RSD (%)	Bias (%)	Concentration Found (ng/mL) Mean $\pm$ SD	RSD (%)	Bias (%)	Concentration Found (ng/mL) Mean $\pm$ SD	RSD (%)	Bias (%)
3-FMC	16.2 $\pm$ 1.3	8.0	6.1	146.9 $\pm$ 7.3	5.0	3.7	768.1 $\pm$ 42.6	5.5	6.9
3-FPM	15.7 $\pm$ 1.6	10.2	-3.2	153.3 $\pm$ 5.6	3.7	-4.2	738.3 $\pm$ 26.4	3.6	3.7
4-CEC	16.8 $\pm$ 1.1	6.5	-11	160.5 $\pm$ 2.1	1.3	6.5	763.6 $\pm$ 22.9	3.0	-10.8
4-BMC	15.2 $\pm$ 0.9	5.9	9.6	156.8 $\pm$ 6.2	4.0	7.1	759.7 $\pm$ 32.4	4.3	12.1

Table 5: Extraction efficiency for 3-FMC, 3-FPM, 4-CEC and 4-BMC.

Substance	Extraction efficiency % (mean values $\pm$ standard deviation) (n=6)		
	15 (ng/mL)	150 (ng/mL)	750 (ng/mL)
3-FMC	82.2 $\pm$ 5.7	85.3 $\pm$ 3.8	88.6 $\pm$ 2.1
3-FPM	86.6 $\pm$ 3.5	82.4 $\pm$ 4.5	79.3 $\pm$ 0.9
4-CEC	79.1 $\pm$ 6.3	81.6 $\pm$ 0.5	76.5 $\pm$ 3.2
4-BMC	76.7 $\pm$ 4.2	79.3 $\pm$ 1.5	74.9 $\pm$ 6.7

3-FMC, 3-FPM, 4-CEC and 4-BMC were 85.4, 82.8, 79.1 and 74.9 respectively, as shown in Table 5.

4 Discussion

The study aimed at developing a GS-MS-SIM procedure for simultaneous analysis as well as for precise and accurate quantification of four synthetic derivatives of cathinone in whole human blood. Mass spectral profiles of cathinones are very simple in positive mode GC-MS. In the mass spectra obtained in the EI-MS mode each main fragment ion detected for 4-CEC, 3-FMC, 4-BMC and 3-FPM was at m/z 168, 154, 154 and 167, respectively. Other less intense fragments present in the spectra were at m/z 140 and 111 for 4-CEC, m/z 110 and 123 for 4-FMC, m/z 182 and

110 for 4-BMC, and m/z 98 and 123 for 3-FPM. Possible structures of the fragmentation products derived from the parent structures of the four compounds are presented in Figure 1. The obtained spectra showed the presence of the oxonium and iminium ions and were in accordance with reported fragmentation pathways of other cathinone derivatives [38].

Analysis of cathinones in biological materials (human blood, urine and cellular fraction) which has been carried out using SPE, liquid-liquid extraction LLE and solid-phase microextraction headspace (HS-SPME) followed by GC-MS methods has been widely reported [39-50]. LOD values using liquid-liquid extraction (LLE) were between 2-50 ng/mL [40-41, 43]. In another study LODs of eighteen synthetic cathinones (fledrone, methcathinone, ethcathinone, buphedrone, mephedrone, penthedrone, 4-MEC, 4-EMC, methedrone, methylone, ethylone, α -PVP, butylone, MPBP, pentylone, pyrovalerone, MDPBP, MDPV and naphyrone) ranged between 25-100 ng after mixed mode SPE extraction of the blood samples and GC-MS detection ion full scan [51]. Also the analytical recoveries for blood ranged between 47-73%. LOQ values were 2.5 ng/ml for some cathinones [4]. Linear range values were between 2-2000 ng/mL for LLE [40, 42-43], 10-2000 ng/mL for SPE [46], and 1-200 ng/mL for SPME [44].

By comparing the results of the previous studies using GC-MS SIM mode with the current study the

method developed has acceptable selectivity (it is free of interferences), linearity (5-1,000) (plus free from carryover), LOD (2.5 ng/mL), precision and accuracy, and a very good recovery (75-88%). The method gave very low LOQ values (5 ng/ml) for the four analytes. These values are in good agreement with the previous studies. This method meets or exceeds the cut-off requirements set out in SWGTOX 's Standard Practices for Validation of Methods in Forensic Toxicology [37].

The various cathinone administration modes used by abusers cause the concentration of cathinones in blood samples to vary. In fatal cases, the concentrations of the cathinones in blood were 400 ng/ml [46], 560–3,300 [43], 272 [52], and 60–1,120 ng/ml [53] for methylone; 1.2–22 [54], 5.1 [55], and 5.5 lg/ ml [56] for mephedrone; 55.2 ng/ml for a-PBP [57]; 486 [44] and 654 ng/ml [58] for a-PVP; 180 ng/ml [59] for PV9; and 170 [41], 82 [60], 1,200 [74], 440 [46], 17–38 [61], and 700 ng/ml [62] for MDPV. In non-fatal cases, concentrations ranged from 250-910 ng/mL for bk-MBDB [63]. The linearity range (5-1,000 ng/mL) as well as LOD and LOQ values in the recent study are in good agreement with the previous studies and can cover the possible blood concentrations in both fatal and non-fatal cases associated with studied cathinones. The results above provide a definite indication that the use of mixed mode SPE and GC-MS SIM mode could lead to reliable results for genuine samples with the studied analytes.

5 Conclusion

A robust and reliable simultaneous quantification method of four synthetic derivatives of cathinone in whole human blood using GC-MS-SIM technique is reported.

Conflict of interest: The author reports no conflicts of interest relevant to this article.

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