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Broad Screening and Identification of Novel Psychoactive Substances in Plasma by High-Performance Liquid Chromatography–High-Resolution Mass Spectrometry and Post-run Library Matching

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Abstract

Drug abuse is today a growing global problem. Often the consumers are not aware about the type of substances they are using and the correlated risks. In recent years, new psychoactive substances (NPS) appeared in the illicit market. The presence of NPS, such as synthetic cathinones, cannabinoids and phenethylamines, which are known to be pharmacologically and toxicologically hazardous, has been frequently reported. The aim of this study was the development of a liquid chromatography–high-resolution mass spectrometry (LC–HRMS) method for a broad screening of NPS in plasma. Data acquisition was in MS/MS and full-scan modes and the method was validated for 25 NPS belonging to different chemical classes. Quantitative results have been obtained for these analytes with limits of quantification ranging from 0.03 to 0.4 ng/mL. The method was proven to be suitable for the screening of additional substances; to this aim, a post-run library matching was conducted for every sample with an in-house database containing over 300 NPS and known metabolites. The library may be constantly expanded with new drugs, in order to obtain a broad screening of NPS in biological matrices.

Introduction

Drug abuse is today a growing global problem that affects every society and people of all ages. Often the consumers are not aware of the type of substances they are using and the correlated risks. In recent years, new psychoactive substances (NPS) often sold as "legal-highs" (1) (psychoactive compounds not included in the list of controlled substances) appeared in the illicit market. These substances are new molecules, natural or synthetic, which are sold in smart shops as incense, bath salts or standard not for human use. The United Nations and the European Union have repeatedly reported the presence of NPS, such as synthetic cathinones, cannabinoids and phenethylamines, which has been shown to be pharmacologically and toxicologically hazardous (2). The number of NPS, >450 according to the last EMCDDA report (3), has already exceeded the total number of substances under international control.

Screening of drugs of abuse in biological matrices has been traditionally performed with immunological methods that allow rapid and cheap analysis. However, these methods may not be appropriate

for the detection of the new molecules continuously appearing on the illicit market (4). Alternative screening methods are based on chromatography coupled with mass spectrometry (MS); these techniques are the most used in forensic medicine and toxicology for confirmation purposes (5–7). Gas chromatography coupled with MS (GCMS) has been the most used technique for general unknown screening or for systematic toxicological analysis both in clinical and forensic toxicology (8); however, the identification is based on library spectra or reference standard and information on NPS is not always readily available. Today, liquid chromatography coupled with MS (LC–MS) is frequently used for the analysis of NPS and several methods may be found in the literature (9); however, they are often not efficient because the standards are not always available. Using triple quadrupoles, ion traps or hybrids analyzers, several qualitative LC–MS-MS strategies can be employed, that is, full-scan (10–12), information-dependent acquisition (13–15) and targeted multi-reaction monitoring (16, 17). High-resolution mass spectrometry (HRMS) exceed the limits of low resolution analyzers through the determination of psychoactive substances and their metabolites with high mass accuracy (5, 9). Based on the exact masses of a molecule and the isotopic pattern, the chemical formula of a compound can be confirmed or even ascertained, with a low rate of false positives. The study of the fragments may be an additional tool for identification of unknown substances, especially using data-independent acquisition strategies (18). Several authors have developed methods for the screening of multiple NPS (19, 20) mainly on biological matrices such as blood (21–25), urine (19, 20, 26–32) and hair (33) but in most cases, the screening is limited to groups of related substances. A comprehensive method for screening and quantification is still required for NPS.

In this study, we developed a sensitive and quantitative method for the determination of NPS in plasma including 16 cathinones and 9 synthetic cannabinoids by LC–Q-Orbitrap HRMS in targeted-MS/MS acquisition mode. Sample preparation is based on a simple protein precipitation and the method was validated according to SWGTOX guidelines. The method was then proven to be suitable for the screening of substances not included within the 25 initially considered; to this aim, a parallel full-scan acquisition is conducted for every sample, allowing a post-run processing. The mass spectra obtained are then compared with an in-house library built with TraceFinder Software containing over 300 NPS and known metabolites; the library may be constantly expanded with new drugs found, in order to obtain a broad screening of NPS in biological matrices.

Materials and methods

Chemicals and materials

The available standards were separated into two groups; Set one (SET1): methcathinone; methylone; ethylcathinone; 4-fluoromethcathinone (4-FMC); butylone; dimethylcathinone, 3,4-dimethylmetcathinone (3,4 DMMC), 4-methoxymethcathinone (methedrone); buphedrone; ethylone; 4-methylethcathinone (4-MEC); pentylone; pentedrone; MDPV; 1-naphyrone; naphyrone; AM 694; JWH 251; JWH 203; JWH 016; JWH 007; JWH 081; JWH 122; JWH 019; JWH 210 (1 mg/mL).

Set two (SET2) 2-fluoromethcathionone (2-FMC); 2,5-dimethoxy-4-bromophenethylamine (2C-B); 2,5-dimethoxyphenethylamine (2C-H); 2,5-dimethoxy-4-isopropylthiophenethylamine (2C-T-4); 2,5-dimethoxy-4-propylthiophenetylamine (2C-T-7); 2-methoxymethcathinone (2MeOMC); para-fluorophenylpiperazine (4-FPP); 1-(4-methoxyphenyl)-piperazine (4-MeOPP); α-Pyrrolidinopentiophenone (α-PVP); diethylcathinone; mephedrone (4MMC); Methoxetamine; AM 1220; JWH 018; JWH 018 2-hydroxyindole metabolite; JWH 018 N-(5-hydroxypentyl) metabolite; JWH 018 N-pentanoic acid metabolite; JWH 073; JWH 081; JWH 081 N-(5-hydroxypentyl) metabolite JWH 200; JWH 250; MAM 2201; MAM 2201 N-pentanoic acid metabolite; (±)-JWH 018 N-(4-hydroxypentyl) metabolite; UR-144; UR-144 N-(5-hydroxypentyl)metabolite; WIN 55; XLR 11; XLR 11 N-(4-hydroxypentyl) metabolite. Both SET1 and SET2 were purchased from Cayman Chemical (Ann Arbor, MI, USA) and LGC standard (Sesto San Giovanni, Milan, Italy) at a concentration of 1 or 0.1 mg/mL based on availability. Formic acid, methanol, acetonitrile and water were acquired from Fisher Scientific (Fair Lawn, NJ, USA). All solvents employed in the extraction were ultra-performance liquid chromatography (UPLC) grade, and LC–MS grade in the chromatographic system.

Extraction procedure

In a 2-mL Eppendorf tube, 250 µL of human plasma was mixed with 750 µL of ACN/MeOH (80/20) and maintained at −20°C for 5 min. The sample is then centrifuged at 4,000 × *g*, 4°C for 15 min. The supernatant is transferred into a second Eppendorf tube and centrifuged at 12,000 × *g*, 4°C for 15 min. Finally, 100 µL of the supernatant are mixed with 100 µL of water and the mixture is transferred into screw top autosampler vials prior to injection.

Preparation of standard stock solution

Stock solutions were obtained by diluting each standard in the proper amount of methanol in order to obtain individual stock solutions at 0.01 and 0.001 mg/mL. All the stock solutions were stored at −20°C.

Instrumentation

A Thermo Scientific Ultimate 3000 RSLC system coupled with a Thermo Scientific Q Exactive Mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used for the analysis. The Ultimate 3000 RSLC system consisted of a degasser, a tertiary loading pump, a binary eluting pump, a column oven and an RS autosampler.

Liquid chromatography

Chromatographic separation was achieved with a BetaBasic18 column, 150 × 2.1 mm (Thermo Scientific, Bremen, Germany) held at a temperature of 40°C and a flow rate of 0.6 mL/min. Mobile phases were 0.1% formic acid in water (Phase A) and 0.1% formic acid in acetonitrile (Phase B). The gradient elution was as follows: the initial composition (5% B) was increased from 5% to 50% B over 4.5 min, from 50% to 100% over 0.5 min, held at 100% for 2 min and returned to initial conditions over 1 min. A 2-min equilibration followed, yielding a total run time of 10 min.

Mass spectrometry

Detection was performed using a Q Exactive mass spectrometer equipped with a heated electrospray ionization source (HESI-II); ionization was operated in positive mode. Source conditions were as follows: spray voltage 4 kV, heater temperature 425°C, capillary temperature 400°C, S-lens RF level 50, sheath gas flow rate 30 and auxiliary gas flow rate 15. The gas used for spray stabilization, collision-induced dissociation experiments in the higher energy

collision dissociation (HCD) cell, and as damping gas in the C-trap was nitrogen. The instrument was calibrated both in positive and negative modes everyday. The mass spectrometer acquired a targeted MS/MS (the inclusion list consists of SET1 analytes) and a full scan at a resolution of 70,000 (full width at half maximum at *m/z* 450); scan range 50–550 *m/z*. Automatic gain control (AGC) was 2e5 and maximum injection time 100 ms. In targeted MS/MS, precursor ions are selected in the quadrupole with a 0.4 *m/z* window and subsequently fragmented in the HCD cell (normalized collision energy (NCE) was set to 35%). The mass spectrometer performs a scan of all the fragment ions at a resolution of 70,000 as well; two specific product ions are used for data analysis with a mass tolerance of 5 ppm. The selected precursor masses and fragments are reported in Table I.

TraceFinder™ software was used for method development and routine analysis during validation.

Method validation

Linearity, limits of detection (LODs), limits of quantification (LOQs), selectivity, matrix effect, precision and accuracy were evaluated according to SWGTOX guidelines (34) for the analytes included in SET1.

Linearity, LODs and LOQs

Calibration standards were prepared in water/methanol (50:50). They were prepared at nine concentration levels (each point was analyzed in five separate runs), from LOQ to 50 ng/mL (in detail, calibrator samples concentration were 0.02–0.05–0.1–0.5–1–5–10–25–50). LOD was estimated for each compound in blank plasma samples as the smallest concentration that gave a signal to noise ratio (*S/N*) of 3.

Table I. LC–HRMS parameters and retention times (Rt) of the analytes acquired in target-MS/MS mode

Analytes	Rt	Precursor ion	Fragment-1	Fragment-2
Methcathinone	2.14	164.11	146.09625	131.07292
Methylone	2.25	208.10	190.08960	160.07550
Dimethylcathinone	2.26	178.12	133.06464	105.07040
4-FMC	2.35	182.10	164.08667	149.06319
Ethylcathinone	2.37	178.12	160.11169	132.08052
Ethylone	2.47	222.11	204.10153	174.09102
Methedrone	2.52	194.12	176.10669	161.08325
Buphedrone	2.58	178.12	160.11176	147.08012
Butylone	2.69	222.11	204.10153	191.06985
4-MEC	3.00	192.14	174.12746	159.10396
Pentedrone	3.11	192.14	174.12746	161.09575
Pentylone	3.18	236.13	218.11707	205.08548
3,4 DMMC	3.32	192.14	174.12746	159.10396
MDPV	3.48	276.16	205.08559	175.07507
1-Naphyrone	4.39	282.18	211.11140	169.06451
Naphyrone	4.54	282.18	211.11140	155.04886
AM 694	6.14	436.06	309.15189	230.92987
JWH 251	6.34	320.20	214.12234	188.14307
JWH 203	6.36	340.15	312.15051	214.12239
JWH 016	6.37	342.18	214.12215	155.04881
JWH 081	6.44	372.20	214.12218	185.05939
JWH 007	6.45	356.20	228.13777	155.04883
JWH 122	6.47	356.20	214.12238	169.06458
JWH 019	6.50	356.20	214.12238	155.04881
JWH 210	6.58	370.22	214.12242	183.08320

As recommended by the SWGTOX guidelines, LODs were derived experimentally on the fragment ion with lowest *S/N* analyzing blank plasma samples and blank plasma samples spiked with decreasing amount of standard solution at appropriate concentration. LOQs were determined similarly as the lowest concentration of a substance needed to give a *S/N* of 10. In addition, it was verified that relative standard deviation (RSD%) and bias were within ±20% at the LOQs level.

For positive identification of an analyte, the two selected fragments must be observed at the same retention time (Rt) as in the quality control (QC) samples (only deviations within ±0.02 min were acceptable) and the quantifier/qualifier ratio were required to be within 20% of those in QC samples.

Recovery and matrix effect

Six plasma samples were spiked with the proper amount of standard stock solution before and after the extraction to reach a concentration of LOQ and 50 ng/mL. Recovery was calculated comparing the average peak area of the samples spiked before (A) and after extraction (B). Accordingly, $R\ (\%) = A/B \times 100$. Matrix effect was estimated by comparison of the calibration curves obtained in blank plasma and in water/methanol (50:50) for each compound. Matrix effect was obtained by (b_m/b_s), where b_s represents the slope of the curve prepared in solvent and b_m is the slope of the curve in matrix.

Accuracy and precision

Accuracy and precision were estimated at three concentration levels (LOQ, 5 and 50 ng/mL) in fortified plasma samples; QC samples were prepared by adding 10 uL of a methanolic solution at a suitable concentration to 240 uL of blank plasma specimens; the concentrations of the compounds in the plasma samples were calculated from freshly prepared calibration curves. Precision was calculated as RSD (RSD% = SD/mean × 100). Precision was evaluated for each analyte in three different days from the areas of six plasma samples per day spiked before extraction. Accuracy was calculated from six plasma samples fortified before extraction step; the concentration corresponding the mean peak area (C_c) was calculated using the equation of the calibration curve and was compared with the theoretical concentration (C_t). Accordingly $A\% = C_c/C_t \times 100$.

Selectivity

The presence of matrix interferences at the Rt of the considered analytes was observed in 10 blank samples.

Post-run screening analysis

A post-run processing of the full-scan data is performed by means of TraceFinder software. The mass spectra are hence compared with an in-house library. An additional MS/MS targeted scan of the precursor ions found is performed on the positive samples in order to generate fragmentation spectra and confirm the identity of the compounds.

Creation of a NPS mass spectra library

A mass spectra library including over 300 NPS and metabolites was built and introduced into TraceFinder software for a post-run analysis of the acquired samples. For each analyte, the library contains the elemental composition (molecular formula) and the theoretical accurate mass as well as the mass of the fragments and the t_r whenever possible.

If a reference standard was available, the library information related to the analyte was collected by injecting standard solutions. Exact masses of precursor ions for which a standard was not available were calculated using Xcalibur™ software; when possible the theoretical exact masses of the most prominent fragments were also added in the database, based on a comprehensive review of literature data and on the known fragmentation route of analogous compounds.

The settings in TraceFinder program include a threshold override of 10,000, with *S/N* equal to 5 and a mass tolerance of 5 ppm for the molecular ion, while a threshold of 5,000 and a mass tolerance of 5 ppm for the fragments.

Performance evaluation of the established spectra library

In order to evaluate the performance of the in-house library, 25 different plasma samples were spiked with the analytes included in SET2 at different concentrations down to 0.5 ng/mL. The samples were processed following the presented method and analyzed. A post-run analysis was then conducted with TraceFinder software by library search.

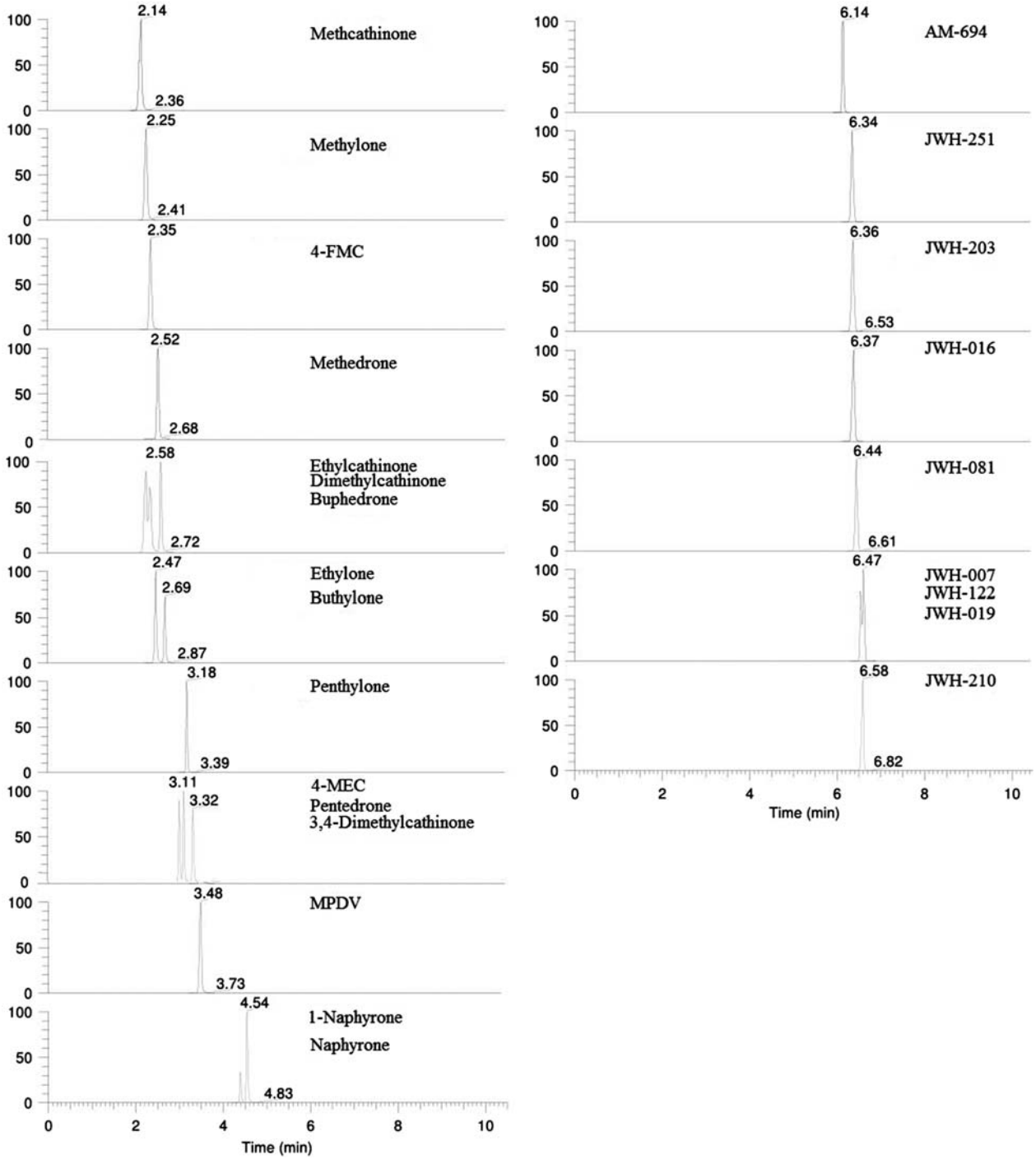

Figure 1. Extracted ion currents of SET1 analytes obtained by targeted-MS/MS scan from a spiked plasma sample.

Results and discussion

LC–MS/MS optimization

The targeted LC–HRMS method proposed for the determination of cathinones and synthetic cannabinoids includes a total of 25 analytes (SET1). The chromatographic conditions were optimized in order to obtain the best separation, particularly for the analytes with the same exact mass (Figure 1), that cannot be discriminated only by MS. Two columns, Thermo Scientific BetaBasic8 and Kinetex PFP of different length (15 and 10 cm) and different particle sizes (5 and 2.6 μm, respectively), were tested. The Betabasic column was chosen since it allowed to achieve a better separation. Both methanol and acetonitrile were tested as organic mobile phases but the latter was chosen because the noise within the entire run was significantly lower. The addition of formic acid resulted in a slight enhancement of the signal for all the analytes.

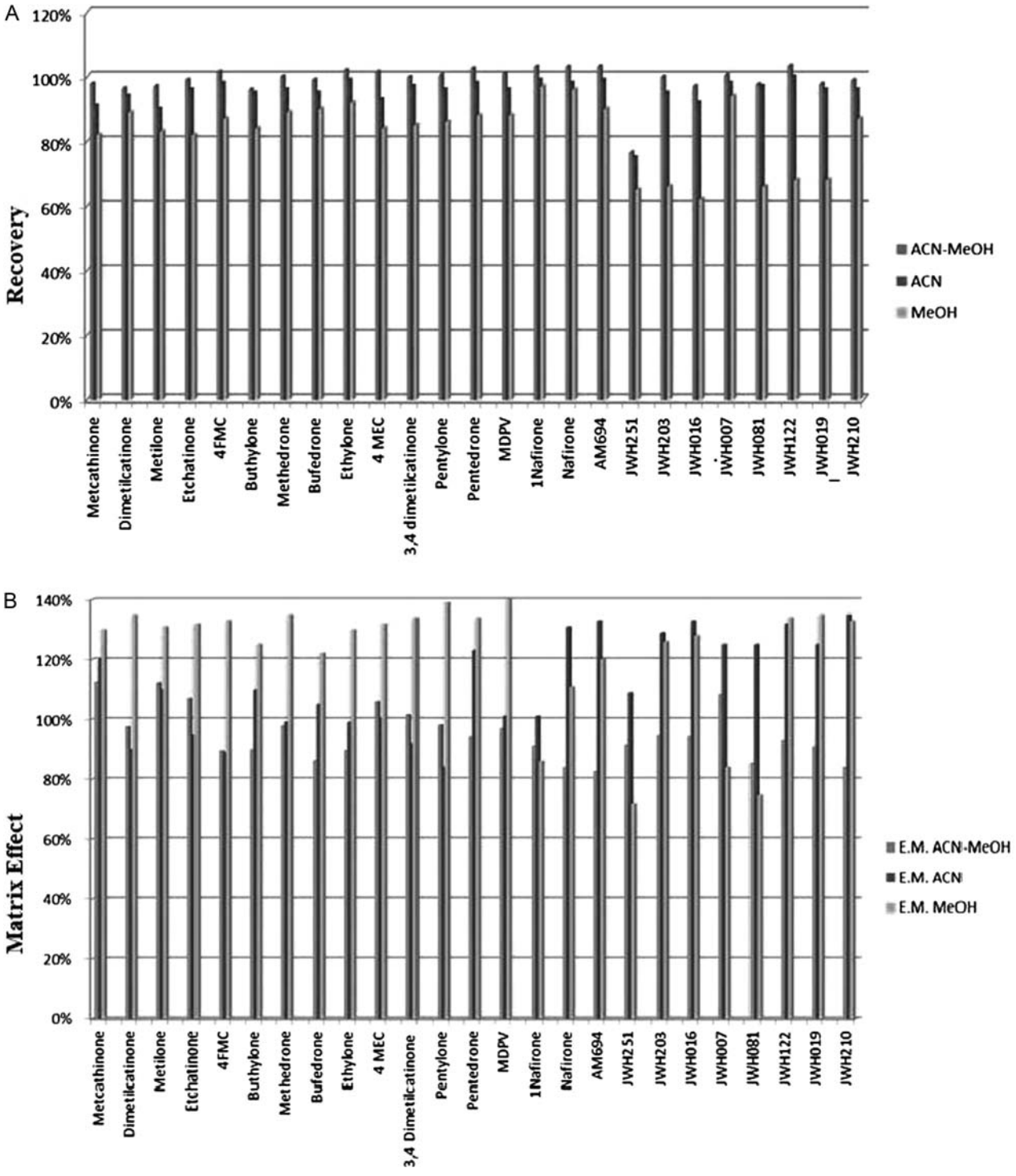

Figure 2. Recovery (A) and matrix effect (B) obtained in the different tested conditions.

Initially, the sample was injected directly after the extraction procedure, but this resulted in a split of the chromatographic peaks, especially for the most polar analytes such as cathinones. For this reason, the sample was diluted 1:1 with water before the injection. In these conditions, drugs belonging to different chemical classes are included in a single chromatographic run; while the methods found in the literature, except for the one proposed by Sundstrom *et al.* (28), generally consider a single class of analytes or perform two separate runs when cathinones and cannabinoids are investigated in the same method (24, 33).

Spray voltage, gas temperature and flows, which must be the same over the whole run, were properly tuned to the conditions given in Section 2.4 by injecting all the analytes, in direct flow injection analysis. In addition, the effect of AGC, resolution, maximum ion time, scan range, NCE, LC flow rate, on the sensitivity as well as the number of scans per chromatographic peak was thoroughly studied and optimized to obtain an optimal sensitivity. The Q Exactive spectrometer gave the opportunity to perform various acquisition modes: full-scan, target SIM and target MS/MS were tested in this study. We decided to use targeted-MS/MS mode, with an inclusion list of 25 analytes (SET1), coupled with a full-scan mode for a broader screening. Target SIM allowed to reach a sensitivity similar to target MS/MS; however, to achieve the minimum requirement of identification points according to EU Commission Decision 2002/657/EC (35), in addition to the fulfillment of Rt criteria, at least a precursor ion and a product ion must be included in the method.

Plasma extraction

Plasma is a complex matrix composed of 92% water and mineral salts and 8% proteins; protein precipitation was selected as sample preparation because it is not analyte specific and is then suitable for untargeted screening; additionally, it requires a reduced sample handling and is less expensive than other extraction techniques.

We firstly evaluated the influence of the amount of organic solvent used on the extent of protein precipitation. To obtain an almost complete precipitation, it would be appropriate to add a volume at least twice the volume of the sample to process (36); it was then decided to add the solvent in a ratio of 3:1.

Different extraction solvents or mixture were evaluated, that is, methanol, acetonitrile and acetonitrile:methanol. Based on the results shown in Figure 2A, the mixture 80:20 (v:v) was chosen. In fact, similar recoveries were obtained with the three tested solvents: however, using the solvent mixture, it was possible to obtain a lower matrix effect (calculated as the ratio between the peak areas of the reference samples in the matrix and the reference samples in solution model) for all the tested analytes (Figure 2B). Similar results were found by Vincenti *et al.* (37).

The developed extraction method allows to obtain good recoveries (>75%) for all the tested analytes despite the considerable differences of the chemical properties; cathinones, similar to amphetamines are relatively hydrophilic, while synthetic cannabinoids are extremely lipophilic. It is then likely that NPS not included within the tested analytes are properly extracted with this method. By selecting an appropriate mixture of solvent, it was additionally possible to obtain a reduced matrix effect despite the nonspecifity of the method.

Validation

Identification and linearity

Detection of analytes was performed in targeted MS/MS; identification is based on Rt, the exact mass of the precursor ion and two diagnostic fragments, fulfilling the EU Commission Decision 2002/657/EC confirmation criteria.

Calibration standards were prepared in water:methanol. QC samples were prepared with blank plasma specimens; according to SWGTOX guidelines (34), the use of matrix-matched calibrators is not essential if the validation parameters are accomplished.

LODs and LOQs

LODs were derived experimentally, as recommended by the SWGTOX guidelines and are listed in Table II. To ensure a correct identification of the analytes even at LOD levels, the latter were determined on the fragment with the smallest *S/N*. LOQs were calculated similarly; for the lowest concentration of calibrator, it was verified that a RSD within 20% could be routinely achieved and that ion ratios between the quantifying and qualifier ion were within ±20% of that established by the calibration standards.

Matrix effect and selectivity

Accurate assessment of matrix effects is crucial for LC–MS based bioanalytical methods both in confirmatory analysis and for screening purposes. The calculated values for matrix effect are reported in Table III. The variability of matrix effect, evaluated on five different plasma samples, was <15% for all the analytes.

At the Rt of the analytes, no interfering signals were recorded by analyzing drug-free samples, showing that the developed method provided an optimal selectivity for all the tested analytes.

Precision and accuracy

Accuracy and precision were measured at three concentration values using fortified plasma samples and resulted always within the limits of 15%, as shown in Table III.

Table II. Regression data, LODs, LOQs for selected analytes (SET1)

Analyte	Equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
Methcathinone	Y = 3E+06X + 570,562	0.9956	0.07	0.2
Dimethylcathinone	Y = 4E+06X + 2E+06	0.9927	0.08	0.3
Methylone	Y = 3E+06X + 2E+06	0.9921	0.02	0.2
Ethylcathinone	Y = 2E+06X + 61,022	0.9974	0.08	0.4
4-FMC	Y = 1E+06X + 107,749	0.996	0.08	0.3
Butylone	Y = 5E+06X + 4E+06	0.9929	0.04	0.1
Methedrone	Y = 2E+06X + 1E+06	0.9929	0.07	0.2
Buphedrone	Y = 2E+06X + 1E+06	0.9962	0.07	0.3
Ethylone	Y = 4E+06X +1E+06	0.9958	0.04	0.1
4-MEC	Y = 3E+06X + 3E+06	0.9949	0.03	0.1
3,4 DMMC	Y = 4E+06X + 705,141	0.998	0.07	0.2
Pentylone	Y = 4E+06X + 2E+06	0.9958	0.02	0.1
Pentedrone	Y = 3E+06X + 1E+06	0.9967	0.07	0.2
MDPV	Y = 8E+06X + 4E+06	0.9947	0.008	0.05
1-Naphyrone	Y = 5E+06X + 2E+06	0.9964	0.01	0.05
Naphyrone	Y = 1E+07X + 1E+07	0.9924	0.005	0.02
AM 694	Y = 6E+06X – 1E+06	0.9981	0.06	0.1
JWH 251	Y = 2E+07X – 919,156	0.9975	0.008	0.03
JWH 203	Y = 4E+06X + 1E+06	0.9944	0.01	0.05
JWH 016	Y = 7E+06X + 2E+06	0.992	0.008	0.03
JWH 007	Y = 1E+07X + 7E+06	0.9928	0.007	0.03
JWH 081	Y = 9E+06X + 2E+06	0.9926	0.009	0.03
JWH 122	Y = 2E+07X + 1E+07	0.9962	0.01	0.04
JWH 210	Y = 1E+07X + 806,324	0.9969	0.03	0.1
JWH 019	Y = 2E+07X + 1E+07	0.9962	0.06	0.2

Table III. Precision (expressed as RSD%), accuracy and matrix effect data

Analytes	Precision (RSD%)			Accuracy (%)			Matrix effect (%)
	LOQ	5	50	LOQ	5	50	
Methcathinone	11	2	3	99	90	97	113
Dimethylcathinone	1	4	1	103	109	91	97
Methylone	6	2	4	94	105	97	112
Ethylcathinone	11	12	13	84	102	101	107
4-FMC	5	5	2	101	102	99	90
Butylone	2	1	4	94	105	97	90
Methedrone	2	1	1	94	104	102	98
Buphedrone	4	2	8	96	102	101	86
Ethylone	2	4	3	95	104	100	90
4-MEC	2	3	6	95	102	97	106
3,4 DMMC	13	4	7	104	109	98	101
Pentylone	2	1	3	101	105	99	98
Pentedrone	1	2	7	97	102	94	94
MDPV	3	1	4	89	104	100	97
1-Naphyrone	7	3	4	80	107	102	91
Naphyrone	2	2	3	94	102	104	84
AM 694	1	2	9	98	101	96	83
JWH 251	7	4	9	84	89	97	92
JWH 203	9	10	13	85	87	84	95
JWH 016	5	4	10	91	90	87	95
JWH 007	2	2	2	85	95	93	108
JWH 081	13	2	7	125	111	100	85
JWH 122	2	3	2	95	99	90	93
JWH 210	5	4	8	87	96	95	91
JWH 019	2	2	4	95	96	92	84

Precision and accuracy are listed at three concentration levels, LOQ, 5 and 50 ng/mL.

Post-run screening and library matching

The method, developed for the 25 NPS, was further extended in order to obtain a broad screening of NPS in plasma samples.

Together with the MS/MS acquisition, a full-scan survey is performed, allowing later data re-interrogation to screen for unexpected compounds. The obtained mass spectra may be compared with spectral libraries in order to identify NPS, potentially included in the sample. Initially, mass-spectral identification is based on the precursor (molecular) ion exact mass and isotopic pattern; the ions that match with those in the library are then introduced in an inclusion list and in a subsequent run, a targeted MS/MS scan is performed to confirm their identity by comparing the generated fragments to the ones included in the library.

To this aim, an in-house library containing over 300 NPS and metabolites was built. The information included in the library are precursor ion accurate masses, Rts (when available) and MS/MS fragment ions. Rts were initially available only for the 25 substances included in the quantitative method; for the other compounds in the library, an intermediate Rt (4.5 min) with a ±310 s window was listed. In this way, the whole chromatographic run is covered and the software is able to identify substances even not knowing the exact Rt. In these conditions, Rt was not considered as a significant criterion in the library search strategy, anyhow it has been proved that reliable results are obtained.

The library search strategy was verified using 25 different samples of plasma obtained from volunteers. As proof of concept, the samples were separated into two aliquots, an aliquot was processed as is while the other was spiked with both the drugs included in SET1 and SET2; samples were prepared at five different concentrations (40, 10, 5, 1 and 0.5 ng/mL) in five replicates. These samples were then injected in the HPLC–HRMS system and acquired with the previously described method; a library matching was performed by using TraceFinder software.

Table IV. LODs values of the post-run screening for both SET1 and SET2 analytes

Analyte	LOD (ng/mL)
Methcathinone	1
Methylone	1
Dimethylcathinone	1
4-FMC	1
Ethylcathinone	1
Ethylone	1
Methedrone	1
Buphedrone	1
Butylone	5
Pentedrone	1
4-MEC	1
Pentylone	1
3,4 DMMC	1
MDPV	1
1-Naphyrone	1
Naphyrone	1
AM 694	0.5
JWH 251	0.5
JWH 203	0.5
JWH 016	1
JWH 081	0.5
JWH 007	0.5
JWH 122	0.5
JWH 019	0.5
JWH 210	0.5
2-FMC	5
2C-B	5
2C-H	5
2C-T-4	5
2C-T-7	5
2MeOMC	1
4-FPP	5
4-MeOPP	5
α-PVP	1
Diethylcathinone	1
Mephedrone	1
Methoxetamine	1
AM 1220	0.5
JWH 018	0.5
JWH 018 2-hydroxyindole metabolite	0.5
JWH 018 N-(5-hydroxypentyl) metabolite	0.5
JWH 018 N-pentanoic acid metabolite	0.5
JWH 073	0.5
JWH 081	0.5
JWH 081 N-(5-hydroxypentyl) metabolite	0.5
JWH 200	0.5
JWH 250	0.5
MAM 2201	0.5
MAM 2201 N-pentanoic acid metabolite	0.5
(±)-JWH 018 N-(4-hydroxypentyl) metabolite	0.5
UR-144	0.5
UR-144 N-(5-hydroxypentyl)metabolite	0.5
WIN 55	0.5
XLR 11	0.5
XLR 11 N-(4-hydroxypentyl) metabolite	0.5

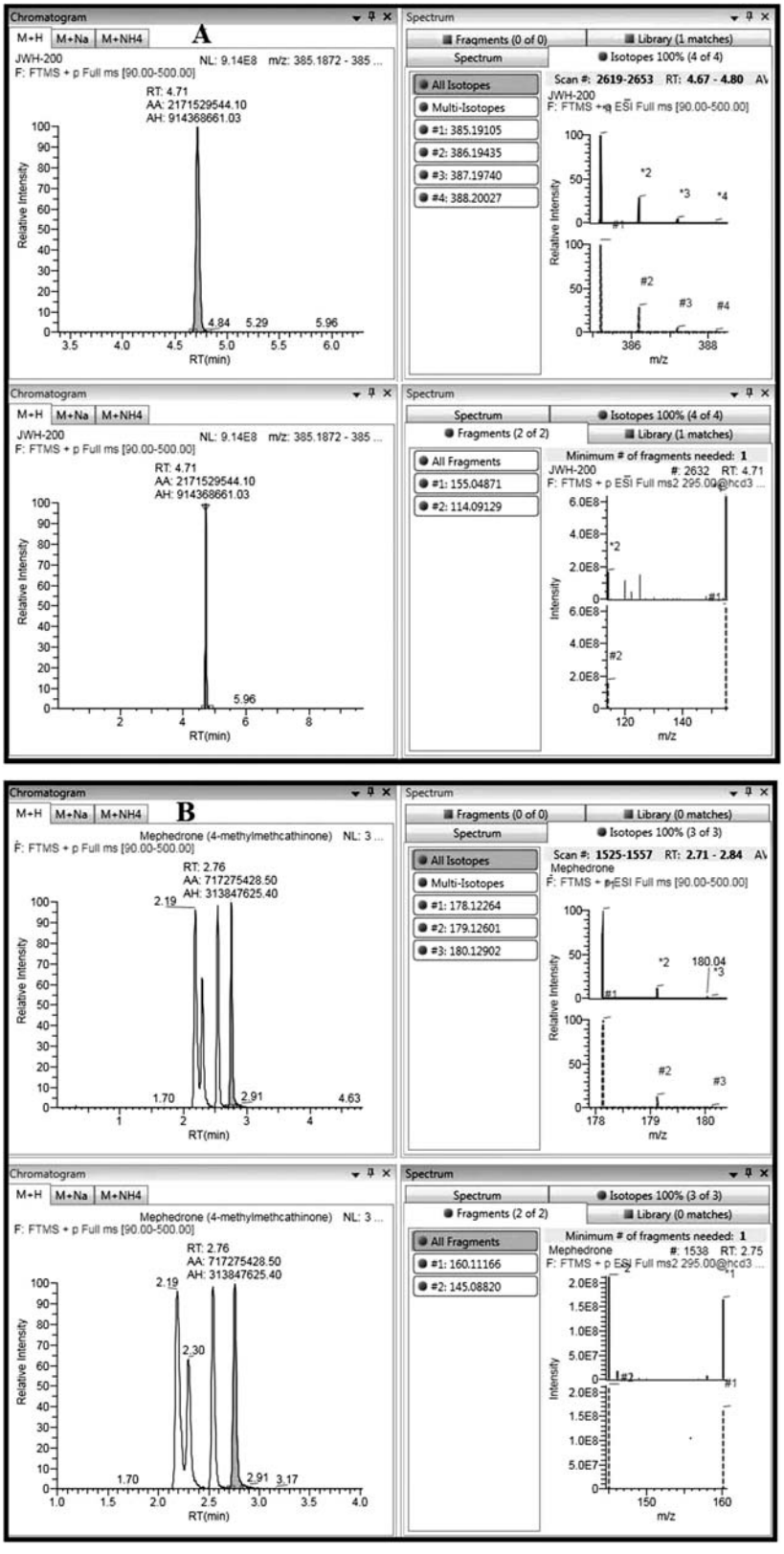

Figure 3. Examples of positive identifications with Tracefinder™ software: (A) JWH 200 (B) mephedrone.

The 25 drug-free aliquots were negative to all the substances included in the library, showing no false positives caused by endogenous plasma compounds. Instead, the other aliquots were positive to all the included drugs, at least for the most concentrated samples. In Table IV, we report the LODs values for the tested drugs; LOD was considered as the lowest concentration of analyte that yields a positive result on the library match.

An additional MS/MS targeted scan with an inclusion list of the precursor ions found was performed on the positive sample in order to generate fragmentation spectra and confirm the identity of the compounds; Rt was also confirmed and thence was included in the in-house library.

An example of a positive identification is given in Figure 3A, which represents the identification process of JWH 200. In the upper part of the figure, the first attempt of identification, based on the precursor ion exact mass and the isotopic pattern, is represented; at this first stage, the precursor ions masses recorded in full scan are compared against the library entries. In this example, a peak at Rt 4.71 with a mass of 385.1910 corresponds to JWH 200 entry; the exact mass matches with a mass tolerance of 5 ppm while the isotopic pattern give a score of 100%. To confirm the identity of the compound, the MS/MS fragments are compared to the ones included in the library; for this purpose, the sample was reanalyzed introducing the precursor exact mass identified in a targeted-MS/MS inclusion list. The results obtained are shown in the lower part of the figure; the product ion spectra of the ion 385.1910 include the fragments 155.0487 and 114.0913, which correspond to the fragments of JWH 200.

In Figure 3B, mephedrone identification is reported: in this example, four peaks corresponding to the exact mass 178.1226 are found. In fact, this mass corresponds to dimethylcathinone, ethylcathinone, buphedrone and mephedrone that are structural isomers. At the first stage of identification, based on exact mass and isotopic pattern, the four peaks could not be discerned; with the additional MS/MS experiment, the peak at 2.76 min could be unambiguously identified as mephedrone since the fragments 160.1117 and 145.0882 only matches with this entry.

Very recently, similar NPS screening approaches were described by Concheiro *et al.* (29) and Paul *et al.* (30). In the former study, the data were acquired in full-scan and data-dependent MS^2 mode with a Q-exactive analyzer; the method includes the determination of 40 NPS stimulants in urine but, being a data-dependent acquisition approach, additional NPS may easily be introduced in the mass-spectrometric method: however, no synthetic cannabinoids are included at this time. In the second study, the same approach was used with a quadrupole-time of flight analyzer, a data-dependent algorithm was combined with a preferred target list (not including synthetic cannabinoids) in order to obtain data-dependent MS^2 spectra for 49 target compounds and/or the most abundant untargeted precursor ions; this is a promising approach but the generation of a high number of MS/MS data causes a decreased strength of the signal that negatively influences LODs and LOQs.

In our study, we decided to perform a sensitive target MS/MS quantification of target compounds coupled with a full-scan screening, and if necessary, a targeted-MS/MS scan for confirmation of the positive findings. In this way, improved sensitivity is achieved, which is particularly important to the detection of synthetic cannabinoids, given that their median concentration in blood matrices (serum) is generally below 1 ng/mL (38). Another possible approach would have been a full scan coupled with all ion fragmentation, as reported by Sunstrom *et al.* (28); this strategy apart from giving a lower sensitivity may be susceptible to the interference effect of co-eluting matrix components that create problems in unambiguously tracing back an MS spectrum to its precursor ion.

Our approach combines a target method in MS/MS with a full-scan acquisition for broad screening; the MS/MS method, used for quantification may be expanded with every positive library match with minimal method validation steps. Screening for unexpected drugs and quantification of target compounds are then performed in as single run. Precursor ion accurate mass and isotopic pattern were previously shown to be powerful means of identification (39); however, to exclude false positives, the additional experiment in MS/MS is essential.

Conclusions

This work reports a LC–HRMS method for the determination of NPS in plasma, which is suitable both for a sensitive and quantitative analysis of cathinones and synthetic cannabinoids in targeted-MS/MS acquisition mode and for the screening of substances not included within the first set; to this aim, a parallel full-scan acquisition is conducted for every sample, allowing a reliable post-run processing.

Sample preparation is based on a simple protein precipitation and the method was validated according to SWGTOX guidelines. For the post-run screening, the mass spectra obtained is compared with an in-house library containing over 300 NPS and known metabolites.

The opportunity to perform screening and confirmation in one analytical run simplifies the workflow in forensic laboratories saving time, costs and specimen volume. The presented approach provides a very useful tool for the combined targeted analysis and broad screening of drugs of abuse in plasma.

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