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A Comprehensive HPLC–MS-MS Screening Method for 77 New Psychoactive Substances, 24 Classic Drugs and 18 Related Metabolites in Blood, Urine and Oral Fluid

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Abstract

To date, more than 800 molecules are classified as New Psychoactive Substances (NPS), and it is reported that this number increases every year. Whereas several cases of polydrug consumption which led to acute intoxication and death are reported, a lack of effective analytical screening method to detect NPS and classical drug of abuse in human matrices affects the prompt identification of the probable cause of intoxication in emergency department of hospitals. In this concern, a fast, simple and comprehensive high-performance chromatography–tandem mass spectrometry (HPLC–MS-MS) screening method to detect and quantify 77 NPS, 24 classic drugs and 18 related metabolites has been successfully developed and validated in blood, urine and oral fluid. A small volume (100 µL) of whole blood samples spiked with internal standard deuterated mixture was added to 70 µL of M3® buffer and after precipitation of blood proteins, the supernatant was evaporated to dryness and reconstituted in 1 mL of mobile phase. Same volume (100 µL) of urine and oral fluid samples spiked with internal standard deuterated mix were only diluted with with 500 µL of M3 ® reagent. One microliter samples of each matrix was injected into HPLC–MS-MS equipment. The run time lasted 10 min with a gradient mobile phase. Mass spectrometric analysis was performed in positive ion MRM mode. The method was linear for all analytes under investigation with a determination coefficient always better than 0.99. The calibration range for blood and oral fluid was from limits of quantification (LOQs) to 200 ng/mL, whereas that for urine was LOQs to 1000 ng/mL. Recovery and matrix effect were always higher than 80%, whereas intra-assay and inter-assay precision was always better than 19% and accuracy was always within 19% of target in every matrix. Applicability of the method was verified by analysis of samples from real cases.

Keywords: New Psychoactive Substances, HPLC–MS-MS, biological fluids

Introduction

A comprehensive definition of “New Psychoactive Substances” (NPS) includes all those substances of abuse not controlled by the 1961 Single Convention on Narcotic Drugs and/or the 1971 Convention on Psychotropic Substances (1). Although the number of new NPS is recently decreasing, every year dozens of new molecules appear for the first time on the illicit market and little or nothing is known about their toxicological profile. In 2018, more than 800 molecules from different chemical classes were reported as NPS to the United Nation Office On Drugs and Crime (UNODC) Early Warning Advisory (EWA) on NPS, 55 of which have been detected for the first time in Europe during the same year (1, 2). The main chemical classes of substances illicitly commercialized are aminoidanes, synthetic cannabinoids, synthetic cathinones, phencyclidine type substances, piperazines, and tryptamines (1, 3–5). Nevertheless, fentanyl analogues represent an increasing alarm for the public health due to the number of fatalities reported and the increasing rate of overdose cases related to their abuse (2, 6, 7). Therefore, consumers are not aware of the most threatening consequences of NPS and often it leads to acute intoxications and fatalities due to the self-experimentation of the abusers (8–10). At the same time, classic drugs of abuse consumption still represents a major public health issue beside to NPS abuse, being cannabis the most abused drug worldwide followed by cocaine, and heroin the first cause of overdose related deaths (1, 2, 9, 11). Moreover, several intoxications due to combinations of NPS from different classes and common drug of abuse have been reported in literature (12–14).

In this concern, the prompt analytical identification of newly introduced NPS is affected by the unavailability of rapid screening tests to detect them in emergency departments and by the paucity of feasible confirmatory analytical procedures and certified reference materials. Since immunochemical or colorimetric assays are not feasible for the high number and different chemistry of NPS, hyphenated techniques are recommended (15). To date, several gas chromatographic–mass spectrometric methods and liquid chromatography–mass spectrometric methods (16–24) have been developed and validated to detect a wide range of NPS in blood, urine or oral fluid in a single run to be applied as screening methods. Since the phenomenon of NPS is constantly changing, and new substances come up to the illicit market every year, more comprehensive and updated screening methods allowing also to assess the poly-drug consumption of both classic psychotropic drugs and emerging new psychoactive substances are required.

In this study, we developed and validated a fast and simple screening method by high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS-MS) to detect and quantify 77 among the most abused NPS (36 synthetic cannabinoids, 12 fentanyl analogues, 16 synthetic cathinones, 7 tryptamines, and 6 phenethylamine) (1-5), 10 available metabolites, 24 classic illicit drugs and 8 metabolites in a single chromatographic run, in whole blood, urine and

oral fluid. Real samples from antemortem and postmortem cases have been analyzed to confirm the suitability of this method.

Experimental

Chemicals and reagents

Working standards of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, 3,4-dimethylmethcathinone, 4-acetoxy-N,N-diisopropyltryptamine, 4-fluoromethcathinone, 4-hydroxy-N,N-diehtyltryptamine, 4-methylethcathinone, 5-APB, 6-APB, 5-chloro-AB PINACA, 5-EAPB, 5-fluoro-ADB, 5-fluoro-AKB48, 5-fluoro-NNEI-2, 5-methoxy-α-methyltryptamine, 5-methoxy-dipropytryptamine, 5-methoxy-N,N-monoisopropyltryptamine, 6-MAPB, 6-O-monoacetylmorphine (6-MAM), AB CHMINACA, AB FUBINACA, acetoxy-dimehtyltryptamine, acetylnorfentanyl, alfentanyl, AM-2201, AM-2233, AM-694, JWH-302, amphetamine, APP FUBINACA, benzoylecgonine, buphedrone, buprenorphine, buthylone, butyrylfentanyl, butyrylfentanyl-carboxy metabolite, butyrylnorfentanyl, carfentanyl, CB-13, cis-3-metylnorfentanyl, clobazam, cocaethylene, cocaine, codeine, CP47,497-CB, CUMYL-5-fluoro- PINACA, CUMYL-PEGACLONE, cyclopropylfentanyl, cyclopropylnorfentanyl, despropionyl-p-fluorofentanyl, diethylcathinone, dihydrocodeine, dimthylcathinone, ethcathinone, ethylone, fentanyl, flunitrazepam, furanylethylfentanyl, furanylnorfentanyl, JWH 203, JWH 251, JWH-007, JWH-016, JWH-019, JWH-081, JWH-098, JWH-122, JWH-147, JWH-210, JWH-398, ketamine, lorazepam, 3,4-methylendioxyamphetamine (MDA), 3,4-methylendioxy-N-ethylamphetamine (MDEA), 3,4-methylenedioxymethamphetamine (MDMA), mephedrone, metamphetamine, methadone, methcathinone, methoxyacetylfentanyl, methoxyacetylnorfentanyl, methylenedioxypyrovalerone, methylone, MMB 2201, morphine, N,N-diallyl-5-methoxy-tryptamine, naphyrone, norbuprenorfine, nordiazepam, norfentanyl, norketamine, norsufentanyl, N-phenetyl-4-piperidinone,oxycodone, penthedrone, penthylone, phenazepam, phenylacetyl fentanyl, pravadoline, PX-1, PX-2, ADB FUBINACA, RCS-4, RCS-8, JWH-018, sufentanyl, temazepam, THJ-018, tramadol, trans-3-metyl-norfentanyl, UR 144, valeryl-fentanyl carboxy metabolite, zolpidem, Δ^9-tetrahydrocannabinol, β-hydroxyfentanyl, β-hydroxythiofentanyl, and deuterated internal standards (IS; 6-MAM-d_3, amphetamine-d_6, benzoylecgonine-d_3, buprenorphine-d_4, cocaethylene-d_3, cocaine-d_3, codeine-d_3, fentanyl-d_5, ketamine-d_4, MDA-d_5, MDEA-d_5, MDMA-d_5, metamphetamine-d_5, methadone-d_3, morphine-d_3, norbuprenorphine-d_3, Δ^9-tetrahydrocannabinol-d_3) were purchased from Cayman Chemical (Ann Arbor, MI, USA) and stored at –20°C until use. Working standards of 4'-methyl-α-pyrrolidinohexiophenone (MPHP), acetylfentanyl, furanylfentanyl, and ritalinic acid were purchased from LGC, (Queens Road, Teddington, Middlesex, UK) and stored at –20°C until use.

All the additional information about chemico-physical properties of reported standards are listed in the Supplementary Table 1.

LC–MS-grade water, acetonitrile, methanol and LC-grade acetone were obtained from Sigma-Aldrich® (Milano, Italy). Ammonium formate buffer was prepared with ≥ 99% purity ammonium formate salt (Sigma-Aldrich®) dissolved in pure water at a concentration of 12.5 mM and brought to pH 9.5 with NH_4OH at 25% v/v (Sigma-Aldrich®). M3® buffer solution was purchased from Comedical® s.r.l. (Trento, Italy).

Human samples

Blank human blood, urine and oral fluid were obtained from the laboratory storehouse of blank biological samples. Pools of blank samples were prepared using 58 different postmortem blood, urine or antemortem oral fluid samples from the Section of Legal Medicine (Università Politecnica delle Marche, Ancona, Italy), pre-screened for the presence of any drug of abuse and pharmaceuticals. Postmortem blood and urine specimens from authentic cases were donated as discarded material by the Institute of Forensic Medicine of Strasbourg (France), and the Department of Medical and Health Sciences, Division of Drug Research of Linköping University (Sweden). Antemortem oral fluid specimens from authentic cases collected by spitting were donated as discarded material by the Polytechnique University of Marche. In addition, biological samples of in-house cases were also analyzed. No information about demographics, detection of other drugs and cause of death were available.

Calibrators and quality control solutions

Methanolic stock standard solution of each analyte at 1 mg/mL and working solutions containing all 119 non deuterated standard at 1 µg/mL, were prepared and stored at −20°C
Internal standards (ISs) stock solutions and ISs working solution with benzoylecgonine-d_3, morphine-d_3, amphetamine-d_6, MDA-d_5, codeine-d_3, metamphetamine-d_5, MDMA-d_5, MDEA-d_5, 6-O-monoacetylmorphine-d_3, cocaine-d_3, ketamine-d_4, cocaethylene-d_3, norbuprenorphine-d_3, methadone-d_3, fentanyl-d_5, buprenorphine-d_4, Δ^9-tetrahydrocannabinol-d_3 were prepared in methanol at 10 and 1 µg/mL.
Calibration samples daily prepared by spiking a pool of blank specimens with proper volumes of stock standard or working solutions and ISs working solution to obtain the concentrations reported in Supplementary Table 2. The first calibration samples and the low QC samples were prepared by spiking a pool of blank specimens with a proper volume of stock standard solutions of each analyte separately to obtain the concentration reported in Supplementary Table 2.

Sample treatment

Blood samples were pretreated according to the following procedure: 70 µL M3® and 500 µL acetone:acetonitrile (8:2, v/v) were added to 100 µL whole blood spiked with 1 µL ISs working solution. After vortexing and centrifuging, supernatant was evaporated to dryness under nitrogen at 45°C. Samples were reconstituted with 1 mL mobile phase, centrifuged and 1 µL supernatant was injected into the chromatographic system. The dilution ratio (1:10, v/v) between blood sample and reconstituted sample extract allowed to minimize the interferences due to endogenous substances in the matrix, without compromising the sensitivity.

Oral fluid and urine samples were pretreated according to the following procedure: 100 µL sample spiked with 1 µL and 5 µL ISs working solution, respectively, were added to 500 µL of M3® buffer solution, and 1 µL was injected into the chromatographic system.

Instrumentation

Chromatographic separation was carried out with an UPLC Waters Acquity I Class (Waters, Milford, MA, USA) instrument coupled with a Waters XEVO TQ-S Micro (Waters) tandem quadrupole MS. The reversed-phase column used for analytes separation was a Waters Oasis HLB (5 µm 4.6 x 20 mm), set at the temperature of 50°C.

A run time of 10 min with a gradient mobile phase composed by ammonium formate solution pH 9.5 (mobile phase A) and acetonitrile (mobile phase B) at the flow rate 1 mL/min was selected. Initial conditions were 75:25 (A:B). Phase A was gradually ramped down from 75 to 0% and phase B gradually ramped up from 25 to 100%. The injection volume was 1 µL for all the matrices.

MS analysis was performed in positive ion multiple reaction monitoring (ES+ MRM) mode, with two transitions for each analyte, and at least one transition for deuterated standards. The best MRM transitions of each analyte were selected by direct infusion of 1 µL stock standard solutions in the MS during the early stage of the method set up. Then, transitions were confirmed in spiked samples. Selected transitions of the analytes and relative cone voltage and collision energy are reported in Table I.

Method validation

The method was fully validated in whole blood, urine and oral fluid following a five-day validation protocol, in accordance to the accepted criteria for method validation in analytical toxicology (25, 26). Limits of detection (LODs) and quantification (LOQs) were estimated by analyzing pool of blank matrix samples with decreasing concentrations of the spiked analyte and thereafter calculating the signal-to-noise ratio (26). LOD was defined as the lowest concentration with good chromatography that yielded a signal-to-noise ratio higher than 3 and LOQ the lowest concentration with a signal-to-noise ratio higher than 10. The calibration range for blood and oral fluid was from their LOQs to 200 ng/mL, while that for urine was from LOQs to 1000 ng/mL. LOQs for each substance in every matrix are reported in Supplementary Table 3. Accuracy, precision,

selectivity, linearity, sensitivity, and carryover were calculated injecting five different daily replicates of calibration points and five replicates of QC samples along three subsequent working days. Precision and accuracy were considered acceptable whenever lower than 20%. Carryover was assessed by injecting drug-free samples of each matrix after the highest point of the calibration curve. Over-the-curve samples (drug free samples spiked with concentration of drugs five or 10 times higher than the highest calibration point) were tested for calibration curve fitting, precision and accuracy within 20%, once they were properly diluted.

Analytical recovery and matrix effect were determined using the experimental design proposed by Matuszewski et al. (27): set 1 was composed of 5 replicates of analytes diluted in the mobile phase (low, medium, and high QC concentrations); sets 2 and 3 were composed of 5 different lots of blank samples fortified with analytes after and before extraction, respectively (low, medium, and high QC concentrations). Matrix effect was calculated by dividing mean peak areas of set 2 by set 1, and analytical recovery was calculated by dividing mean peak areas of set 3 by set 2.

Results

As shown in Table I, the developed method determined all the analytes under investigation in a 10 min run time after a 10 min sample treatment of a quite small sample volume (Figures 1–3). Furthermore, the validation parameters obtained for different biological matrices satisfied the established international criteria (Supplementary Table 3) (25). No additional peaks due to endogenous substances and carryover interfering with analytes and ISs were detected. The method was linear for all analytes under investigation with a determination coefficient (r^2) always better than 0.99. Linearity of the curves was statistically confirmed by performing residual plots test, through Minitab® software (Minitab LLC, State College, PA, USA), that satisfied the Scientific Working Group of Forensic Toxicology (28). The linear range included the most commonly detected quantity of NPS in all the matrices investigated, and LODs ranged from 0.03 to 0.35 ng/mL in blood and from 0.03 to 0.25 ng/mL oral fluid, while they ranged from 0.02 to 0.25 ng/mL in urine. LOQs ranged between 0.08 and 1 ng/mL in blood, 0.07 and 0.8 ng/mL in oral fluid and between 0.06 and 0.5 ng/mL in urine. Recovery of analytes under investigation and matrix effect were always higher than 85%, whereas intra-assay and inter-assay precision and accuracy were always better than 19% in every matrix.

Analysis of real samples

Authentic human samples of blood, urine and oral fluid specimens (n = 56) from different subjects have been analyzed and the concentrations of target compounds are reported in the Tables II–IV, respectively. In case of target analytes concentration higher than the highest point of the

calibration curve, the analysis was repeated performing a dilution of the sample. Blood samples from 17 real cases were tested positive for fentanyl analogues, three of which were positive also for at least one classic illicit drug. Cyclopropylfentanyl and its metabolite cyclopropylnorfentanyl were detected at an average concentration ± standard deviation (SD) of 7.7 ± 7.0 ng/mL and 29 ± 18 ng/mL (n = 8), respectively. Methoxyacetylfentanyl and its metabolite methoxyacetylnorfentanyl were detected at an average concentration ± SD of 36 ± 38 ng/mL and 4.1 ± 2.3 ng/mL (n = 4), respectively. Acetylfentanyl and its metabolite acetylnorfentanyl were detected at an average concentration ± SD of 41 ± 40 ng/mL and 45 ± 22 ng/mL (n = 3), respectively. 4-ANPP was detected at an average concentration ± SD of 3.5 ± 2.3 ng/mL (n = 9). Moreover, furanylfentanyl and its metabolite furanylnorfentanyl, sufentanyl, morphine and codeine, and oxycodone were detected each in one sample.

Urine samples from 23 real cases were tested positive for classic illicit drugs and fentanyl analogues.

Cyclopropylfentanyl and its metabolite cyclopropylnorfentanyl were detected at an average concentration ± SD of 47 ± 39 ng/mL and 420 ± 300 ng/mL (n = 11), respectively. Methoxyacetylfentanyl and its metabolite methoxyacetylnorfentanyl were detected at an average concentration ± SD of 790 ± 850 ng/mL and 750 ± 910 ng/mL (n = 4), respectively. Acetylfentanyl and its metabolite acetylnorfentanyl were detected at an average concentration ± SD of 1900 ± 1700 ng/mL and 6600 ± 3200 ng/mL (n = 5), respectively. 4-ANPP was detected at an average concentration ± SD of 51 ± 52 ng/mL (n = 10). Fentanyl and its metabolite norfentanyl were detected at an average concentration ± SD of 180 ± 350 ng/mL and 430 ± 840 ng/mL (n = 4), respectively. Nordiazepam was detected at an average concentration ± SD of 580 ± 310 ng/mL (n = 4). Morphine was detected at an average concentration ± SD of 180 ± 170 ng/mL (n = 3). Codeine was detected at an average concentration ± SD of 85 ± 65 ng/mL (n = 4). 6-MAM was detected at an average concentration ± SD of 67 ± 74 ng/mL (n = 2). In addition, one sample was positive to furanylfentanyl and its metabolite furanylnorfentanyl, one other to ritalinic acid and a third one to noroxycodone.

Classic drugs of abuse, fentanyl analogues and several NPS were quantified in 14 oral fluids samples. Cocaine was detected in four samples at an average concentration ± SD of 28 ± 54 ng/mL, and its metabolite was quantified in only two samples at an average concentration of 8.6 ± 12 ng/mL. 6-MAM was quantified at an average concentration ± SD of 0.47 ± 0.45 ng/mL, but morphine was also quantified as a metabolite in one case. Thus, morphine was present in two samples at an average concentration ± SD of 0.5 ± 0.6 ng/mL. Aco-DMT was quantified at an average concentration ± SD of 60 ± 84 ng/mL (n = 2). The average concentration ± SD of THJ 018 detected in four samples was 25 ± 48 ng/mL. Butylone was founded in three samples at an average concentration ± SD of 40 ± 69 ng/mL, and carfentanyl was detected in only one oral fluid sample.

Discussion and Conclusions

The detection of newly spread NPS represents a challenging issue in forensic and clinical toxicology since their structures are closely correlated to controlled drugs. The development of screening analytical methods to detect and quantify a broad range of NPS and classic illicit drugs in different biological matrices represent an important tool, due to the frequent association of NPS to other more common drugs of abuse by consumers. As recently reported by Graziano et al. (15), hyphenated techniques have to be preferred to immunochemical or colorimetric assays as screening tool to identify NPS in biological matrices due to the extensive cross-reactivity of antibodies to structurally related substances. However, immunochemical methods are easier to include in clinical routine and forensic analysis since they often require a simple pretreatment of little quantity of specimens. Recently, several chromatography–mass spectrometry methods to detect a wide range of NPS in a single run have been developed and validated with the purpose to be applied as screening methods. In 2015, Odoardi et al. (22) proposed an UHPLC–MS-MS screening method to detect 78 NPS of different classes in whole blood. A volume of 0.5 mL of whole blood was pre-treated by dispersive liquid-liquid microextraction (DLLME) after deproteinization. Due to the structural differences between analytes classes, the author developed a chromatographic method for the separation of cannabinoids and another for the separation of cathinones and other stimulants. The first one separated cannabinoids in a 12-min gradient mobile phase run, and the second method applied a different gradient to a run that lasted 11 min. The detection of all the substances was performed through multiple reaction monitoring (MRM). Although both the methods showed a certain sensitivity (LODs ranging between 0.2 and 2 ng/mL), the quantitative validation of the methods was not performed, thus only the qualitative detection of substances was allowed. Furthermore, a large volume of sample was required and fentanyl analogues were not considered. Vaiano et al. (21) developed and fully validated an HPLC–MS-MS assay to detect and quantify 74 substances among NPS and psychostimulants drugs in blood. After a deproteinization of 0.2 mL of specimen, the samples were reconstituted in methanol and injected in the chromatograph. The analytes were separated through a gradient elution in a 15 min run and the detection performed in MRM mode. The method showed good sensitivity and specificity but the lack of validation in other matrices represented an eventual limit in complex toxicological cases. A qualitative LC–MS-MS screening method to identify 80 designer stimulants in whole blood has been validated by Adamowicz and Tokarczyk (29). A volume of 0.2 mL of blood was precipitated with acetonitrile, centrifuged, dried under nitrogen at 30°C and finally dissolved in 100 µL 0.1% formic acid in acetonitrile. The chromatographic run conducted in gradient mobile phase of 0.1% formic acid in acetonitrile and 0.1% formic acid in water, lasted 14 min. The detection of the target compound was performed in multiple reaction monitoring. The simultaneous quantification of classic drug and NPS in oral fluid was investigated by Malaca et al. (30), and a simple and fast UHPLC–MS-MS method, which requires a simple dilution of the sample, to detect

13 molecules in oral fluid was developed and validated. The chromatographic separation through a C18 column (2.1 mm × 75 mm, 1.7 μm) was achieved in 6 min working in reversed phase with 0.1% formic acid in water and 0.1% formic acid in acetonitrile as mobile phases. The target compounds were injected by positive electrospray ionization in the triple quadruple working in MRM. The method showed good linearity between LOQs (range: 0.5–5 ng/mL) and 250 ng/mL and good recovery and matrix effect, always higher than 90%, while precision and accuracy were always better than 15%.

To the best of our knowledge, for the first time a unique HPLC–MS-MS method for detecting and quantifying 119 among substances of abuse and their metabolites in three different matrices has been developed and fully validated. Unfortunately, the unavailability of several metabolites reference standards, such as those of synthetic cannabinoids did not allow a clear and complete toxicokinetic evaluation in some cases.

The HPLC system was equipped with the HLB Oasis column, usually marketed as an on-column SPE. Nevertheless, being a hydrophilic-lipophilic reverse phase column, stable at extreme pH and for a wide spectrum of solvents, the HLB Oasis was experimentally tested for the current chromatographic separation without the aid of a further reverse phase column in the circuit. It has been proven to perfectly fit to the analysis of substances with different polarity, such as the molecules evaluated in our work. Whereas peaks efficiency was not exceptional, given the reduced length of the column, the employed column resulted satisfactory for the versatility in separating compounds with different polarity as in the present case. Furthermore, the use of M3® buffer at pH 3.5 during the preparation of samples determined the ionization on analytes at pH 9.5.

Target compounds included in this study belong to the most representative classes of substances taking into consideration also recent trends of abuse, published scientific literature and commercial availability of standards (2, 31). The range of linearity for every analyte includes the concentration expected to be detected in each matrices in acute intoxication or fatal cases, according to values reported in literature (32–34). Fentanyl analogues and related metabolites were the most prevalent detected compounds in blood, being 4-ANPP quantified in the larger number of samples (n = 9). The opiates morphine and codeine were detected in combination of cyclopropylfentanyl and cyclopropylnorfentanyl in one case, and oxycodone was coassumed with cycloproprylfentanyl in another case. As was expected, the highest concentrations of target compounds were reached in urine specimens, in which 11 different fentanyl analogues, 1 benzodiazepine, 1 phenetylamine and 4 different opiates were quantified. Fentanyl compounds were the most prevalent molecules detected also in urine samples. Polyconsumption of drugs belonging to different chemical classes was revealed in 14 urine samples, while cyclopropylfentanyl and its metabolite and acetylfentanyl and its N-dealkylated metabolite acetylnorfentanyl were found alone. Finally, fentanyl analogues, opiates, a tryptamine and a synthetic cannabinoid were revealed in oral fluid samples with polydrug detection occurred in three cases.

The method here reported allows not only to screen a broad range of compounds belonging to different chemical classes in a single run of 10 min, but also to reach a good analytical sensitivity, which makes it eligible application to cases of acute intoxication and fatalities. Clearly, the positive findings need always to be confirmed in a real context by a confirmatory analytical method. The best advantage of this method is represented by the very simple and fast pretreatment, which allows to include this method in hospital routine. The easy manageable samples pretreatment and the of the analytes make this method easy to be extended to the inclusion of eventual new NPS requiring determination in biological fluids of consumers. The current method employing a simple pretreatment of small amount (100 µL) of biological samples and substance characterization via direct infusion into the MS can be easily expanded to include a greater number of NPS. Moreover, due to its brief run time (10 min), it can be used in high throughput laboratories saving time and economic resources and providing effective toxicological information.

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Figure 1. HPLC–MS-MS chromatogram of whole blood spiked with all the target analytes at a concentration of 5 ng/mL (II calibration point) (ES+TIC: 1-7e6). Deuterated standard peaks are not shown. 1: BZG; 2: Methoxyacetyl norfentanyl; 3: Acetyl Norfentanyl; 4: Ritalinic Acid; 5: Butyryl Fentanyl Carboxy metabolite, 6: Morphine; 7: 4-fluoromethcathinone; 8: Valeryl Fentanyl Carboxy metabolite; 9: Norfentanyl; 10: Methcathinone; 11: Amphetamine;12: Dihydrocodeine;13: Methoxyacetylfentanyl;14: Furanyl Norfentanyl; 15: Cis-3-Metyl Norfentanyl; 16: Trans-3-Metyl Norfentanyl; 17: Methylone; 18: Codeine; 19: MDA; 20: Metamphetamine; 21: Butyryl Norfentanyl; 22: MDMA; 23: 5-MeO-AMT; 24: AcO DMT; 25: Cyclopropyl Norfentanyl; 26: Mephedrone; 27: Buphedrone; 28: 6-MAM; 29: MDEA; 30: 4 OH DET; 31: Buthylone; 32: Ethylone; 33: 5-APB; 34: 6-APB; 35: 5-MeO-MIPT; 36: Dimethylcathinone; 37: 6-MAPB; 38: Diethylcathinone; 39: 3,4-dimethylmethcathinone; 40: 4-methylethcathinone; 41: Norketamine; 42: Ethcathinone; 43: 4-AcO-DIPT; 44: Oxycodone; 45: Tramadol; 46: Norsufentanyl; 47: Penthedrone; 48: 5-EAPB; 49: Zolpidem; 50: Penthylone; 51: Lorazepam; 52: EDDP; 53: β-hydroxyfentanyl; 54: Cocaine; 55: Alfentanyl; 56: Ketamine; 57: PX-1; 58: AB FUBINACA; 59: Cocaethylene; 60: 5-MeO-DPT; 61: 5 CL AB PINACA; 62: Norbuprenorfine; 63: Nordiazepam; 64: PX-2; 65: Flunitrazepam; 66: Temazepam; 67: Clobazam; 68: Cyclopropylfentanyl; 69: Phenazepam; 70: β-hydroxythiofentanyl; 71: ADB Fubinaca; 72: Methadone; 73: Furanylethyl Fentanyl; 74: Acetyl fentanyl; 75: MDPV; 76: AB CHMINACA; 77: Furanyl Fentanyl; 78: MMB 2201; 79: Pravadoline; 80: 5-MeO-DALT;81: Fentanyl; 82: APP FUBINACA; 83: Carfentanyl; 84: 5-F ADB; 85: MPHP; 86: Butyryl Fentanyl; 87: Sufentanyl; 88: Despropionyl-para-fluorofentanyl; 89 4-ANPP; 90: AM-2233; 91: CUMYL 5F PINACA; 92: Naphyrone; 93: Phenyl Acetyl Fentanyl; 94: AM-694; 95: JWH 302; 96: CUMYL PEGACLONE; 97: RCS-4; 98: JWH 251; 99: AM-2201; 100: Buprenorphine; 101: UR 144; 102: JWH 203; 103: 5f NNEI-2; 104: 5F-AKB48; 105: RCS-8; 106: THC; 107: JWH 018; 108: CP47, 497-C8; 109: JWH 016; 110: JWH 098; 111: THJ 018; 112: JWH 081; 113: JWH 122; 114: JWH 019; 115: JWH 007; 116: JWH 210; 117: JWH 147; 118: JWH 398; 119: CB-13

Figure 2. HPLC–MS-MS chromatogram of urine spiked with all the target analytes at a concentration of 50 ng/mL (II calibration point) (ES+TIC: 2-5e7). Deuterated standard peaks are not shown. 1: BZG; 2: Methoxyacetyl norfentanyl; 3: Acetyl Norfentanyl; 4: Ritalinic Acid; 5: Butyryl Fentanyl Carboxy metabolite, 6: Morphine; 7: 4-fluoromethcathinone; 8: Valeryl Fentanyl Carboxy metabolite; 9: Norfentanyl; 10: Methcathinone; 11: Amphetamine;12: Dihydrocodeine;13: Methoxyacetylfentanyl;14: Furanyl Norfentanyl; 15: Cis-3-Metyl Norfentanyl; 16: Trans-3-Metyl Norfentanyl; 17: Methylone; 18: Codeine; 19: MDA; 20: Metamphetamine; 21: Butyryl Norfentanyl; 22: MDMA; 23: 5-MeO-AMT; 24: AcO DMT; 25: Cyclopropyl Norfentanyl; 26: Mephedrone; 27: Buphedrone; 28: 6-MAM; 29: MDEA; 30: 4 OH DET; 31: Buthylone; 32: Ethylone; 33: 5-APB; 34: 6-APB; 35: 5-MeO-MIPT; 36: Dimethylcathinone; 37: 6-MAPB; 38: Diethylcathinone; 39: 3,4-dimethylmethcathinone; 40: 4-methylethcathinone; 41: Norketamine; 42: Ethcathinone; 43: 4-AcO-DIPT; 44: Oxycodone; 45: Tramadol; 46: Norsufentanyl; 47: Penthedrone; 48: 5-EAPB; 49: Zolpidem; 50: Penthylone; 51: Lorazepam; 52: EDDP; 53: β-hydroxyfentanyl; 54: Cocaine; 55: Alfentanyl; 56: Ketamine; 57: PX-1; 58: AB FUBINACA; 59: Cocaethylene; 60: 5-MeO-DPT; 61: 5 CL AB PINACA; 62: Norbuprenorfine; 63: Nordiazepam; 64: PX-2; 65: Flunitrazepam; 66: Temazepam; 67: Clobazam; 68: Cyclopropylfentanyl; 69: Phenazepam; 70: β-hydroxythiofentanyl; 71: ADB Fubinaca; 72: Methadone; 73: Furanylethyl Fentanyl; 74: Acetyl fentanyl; 75: MDPV; 76: AB CHMINACA; 77: Furanyl Fentanyl; 78: MMB 2201; 79: Pravadoline; 80: 5-MeO-DALT;81: Fentanyl; 82: APP FUBINACA; 83: Carfentanyl; 84: 5-F ADB; 85: MPHP; 86: Butyryl Fentanyl; 87: Sufentanyl; 88: Despropionyl-para-fluorofentanyl; 89 4-ANPP; 90: AM-2233; 91: CUMYL 5F PINACA; 92: Naphyrone; 93: Phenyl Acetyl Fentanyl; 94: AM-694; 95: JWH 302; 96: CUMYL PEGACLONE; 97: RCS-4; 98: JWH 251; 99: AM-2201; 100: Buprenorphine; 101: UR 144; 102: JWH 203; 103: 5f NNEI-2; 104: 5F-AKB48; 105: RCS-8; 106: THC; 107: JWH 018; 108: CP47, 497-C8; 109: JWH 016; 110: JWH 098; 111: THJ 018; 112: JWH 081; 113: JWH 122; 114: JWH 019; 115: JWH 007; 116: JWH 210; 117: JWH 147; 118: JWH 398; 119: CB-13

Figure 3. HPLC–MS-MS chromatogram of oral fluid spiked with all the target analytes at a concentration of 5 ng/mL (II calibration point) (ES+TIC: 1-8e6). Deuterated standard peaks are not shown. 1: BZG; 2: Methoxyacetyl norfentanyl; 3: Acetyl Norfentanyl; 4: Ritalinic Acid; 5: Butyryl Fentanyl Carboxy metabolite, 6: Morphine; 7: 4-fluoromethcathinone; 8: Valeryl Fentanyl Carboxy metabolite; 9: Norfentanyl; 10: Methcathinone; 11: Amphetamine;12: Dihydrocodeine;13: Methoxyacetylfentanyl;14: Furanyl Norfentanyl; 15: Cis-3-Methyl Norfentanyl; 16: Trans-3-Methyl Norfentanyl; 17: Methylone; 18: Codeine; 19: MDA; 20: Metamphetamine; 21: Butyryl Norfentanyl; 22: MDMA; 23: 5-MeO-AMT; 24: AcO DMT; 25: Cyclopropyl Norfentanyl; 26: Mephedrone; 27: Buphedrone; 28: 6-MAM; 29: MDEA; 30: 4 OH DET; 31: Buthylone; 32: Ethylone; 33: 5-APB; 34: 6-APB; 35: 5-MeO-MIPT; 36: Dimethylcathinone; 37: 6-MAPB; 38: Diethylcathinone; 39: 3,4-dimethylmethcathinone; 40: 4-methylethcathinone; 41: Norketamine; 42: Ethcathinone; 43: 4-AcO-DIPT; 44: Oxycodone; 45: Tramadol; 46: Norsufentanyl; 47: Penthedrone; 48: 5-EAPB; 49: Zolpidem; 50: Penthylone; 51: Lorazepam; 52: EDDP; 53: β-hydroxyfentanyl; 54: Cocaine; 55: Alfentanyl; 56: Ketamine; 57: PX-1; 58: AB FUBINACA; 59: Cocaethylene; 60: 5-MeO-DPT; 61: 5 CL AB PINACA; 62: Norbuprenorfine; 63: Nordiazepam; 64: PX-2; 65: Flunitrazepam; 66: Temazepam; 67: Clobazam; 68: Cyclopropylfentanyl; 69: Phenazepam; 70: β-hydroxythiofentanyl; 71: ADB Fubinaca; 72: Methadone; 73: Furanylethyl Fentanyl; 74: Acetyl fentanyl; 75: MDPV; 76: AB CHMINACA; 77: Furanyl Fentanyl; 78: MMB 2201; 79: Pravadoline; 80: 5-MeO-DALT;81: Fentanyl; 82: APP FUBINACA; 83: Carfentanyl; 84: 5-F ADB; 85: MPHP; 86: Butyryl Fentanyl; 87: Sufentanyl; 88: Despropionyl-para-fluorofentanyl; 89 4-ANPP; 90: AM-2233; 91: CUMYL 5F PINACA; 92: Naphyrone; 93: Phenyl Acetyl Fentanyl; 94: AM-694; 95: JWH 302; 96: CUMYL PEGACLONE; 97: RCS-4; 98: JWH 251; 99: AM-2201; 100: Buprenorphine; 101: UR 144; 102: JWH 203; 103: 5f NNEI-2; 104: 5F-AKB48; 105: RCS-8; 106: THC; 107: JWH 018; 108: CP47, 497-C8; 109: JWH 016; 110: JWH 098; 111: THJ 018; 112: JWH 081; 113: JWH 122; 114: JWH 019; 115: JWH 007; 116: JWH 210; 117: JWH 147; 118: JWH 398; 119: CB-13.

Table I. MS Parameters for Analytes and Internal Standards

n.	Analyte	Retention time (min)	Cone Voltage (V)	Quantifier MRM transitions (m/z)	Collision energy (eV)	Qualifier MRM transition (m/z)	Collision energy (eV)
classic drugs							
1	**Benzoylecgonine-d_3**	0.38	30.00	293.10 > 171.10	20.00	-	-
2	**Benzoylecgonine**	0.38	30.00	290.10 > 168.10	20.00	290.1 > 105.1	33.00
3	**Morphine-d_3**	0.75	35.00	289.00 > 61.00	28.00	-	-
4	**Morphine**	0.76	35.00	286.00 > 165.10	40.00	286 > 153	40.00
5	**Norfentanyl**	0.93	25.00	233.10 > 84.20	20.00	233.10 > 55.30	34.00
6	**Amphetamine-d_6**	1.01	10.00	142.20 > 93.10	16.00	-	-
7	**Amphetamine**	1.01	15.00	136.00 > 119.10	8.00	136.00 > 91.10	15.00
8	**Dihydrocodeine**	1.03	35.00	302.10 > 199.10	34.00	302.10 > 201.10	30.00
9	**Codeine**	1.18	30.00	300.10 > 215.10	25.00	300.10 > 199.20	27.00
10	**3,4-Methylenedioxyamphetamine (MDA)**	1.20	20.00	180.00 > 133.10	18.00	180.00 > 163.10	10.00
11	**Methamphetamine**	1.21	20.00	150.10 > 91.10	12.00	150.10 > 119.10	10.00
12	**MDA-d_5**	1.23	20.00	184.71 > 137.20	18.00	184.71 > 109.70	10.00
13	**Codeine-d_3**	1.26	40.00	303.00 >215.10	25.00	303.00 > 199.10	30.00
14	**Methamphetamine-d_5**	1.29	20.00	154.80 > 91.80	12.00	154.80 > 119.10	10.00
15	**3,4-Methylenedioxymetamphetamine-d_5 (MDMA-d_5)**	1.29	20.00	199.10 > 116.51	12.00	199.10 > 135.25	20.00
16	**3,4-Methylenedioxymetamphetamine (MDMA)**	1.29	20.00	194.10 > 163.00	14.00	194.10 > 133.10	20.00
17	**6-O-monoacetylmorphine (6-MAM)**	1.46	30.00	328.10 > 165.10	40.00	328.10 > 181.20	40.00
18	**3,4-methylenedioxy-N-ethylamphetamine- d_5 (MDEA-d_5)**	1.51	20.00	213.10 > 163.10	14.00	213.10 > 105.10	26.00
19	**3,4-methylenedioxy-N-ethylamphetamine (MDEA)**	1.55	20.00	208.10 > 163.10	14.00	208.10 > 135.10	14.00
20	**Norketamine**	1.90	20.00	224.10 > 207.10	10.00	224.10 > 125.00	25.00
21	**Oxycodone**	1.99	25.00	316.10 > 241.20	30.00	316.10 > 256.10	30.00
22	**Tramadol**	2.02	25.00	264.10 > 58.10	15.00	-	-
23	**Norsufentanyl**	2.02	25.00	277.00 > 128.10	15.00	277.00 > 96.00	25.00
24	**6-O-monoacetylmorphine- d_3 (6-MAM-d_3)**	2.09	30.00	331.00 > 61.10	30.00	-	-
25	**Zolpidem**	2.13	45.00	308.10 > 235.20	34.00	208.10 > 263.10	28.00
26	**Lorazepam**	2.21	30.00	312.10 > 229.00	30.00	321.00 > 275.00	20.00
27	**2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)**	2.28	45.00	278.20 > 234.20	26.00	278.20 > 186.20	35.00
28	**Cocaine**	2.57	30.00	304.20 > 182.26	20.00	304.20 > 82.30	30.00
29	**Cocaine-d_3**	2.59	30.00	307.00 > 184.70	20.00	307.00 > 84.80	30.00
30	**Ketamine-d_4**	2.61	20.00	242.20 > 129.10	25.00	242.20 > 211.10	15.00
31	**Ketamine**	2.62	20.00	238.20 > 125.10	25.00	238.20 > 220.20	15.00
32	**Cocaethylene**	2.89	30.00	318.10 > 196.10	20.00	318.10 > 82.10	30.00
33	**Cocaethylene-d_3**	2.93	30.00	321.10 > 199.10	20.00	321.10 > 85.00	30.00
34	**Norbuprenorphine-d_3**	2.95	55.00	417.20 > 100.80	40.00	417.20 > 56.80	40.00
35	**Norbuprenorphine**	2.95	55.00	414.20 > 101.30	40.00	414.20 > 83.20	55.00
36	**Nordiazepam**	2.97	40.00	271.10 > 140.00	27.00	271.10 > 165.10	27.00
37	**Flunitrazepam**	3.02	52.00	314.10 > 239.10	36.00	314.10 > 268.10	26.00
38	**Temazepam**	3.04	34.00	301.00 >177.00	20.00	301.00 > 255.00	38.00
39	**Clobazam**	3.04	38.00	301.10 > 224.00	36.00	301.10 > 259.00	22.00
40	**Phenazepam**	3.08	25.00	350.70 > 104.70	45.00	350.70 > 206.00	35.00
41	**Methadone**	3.27	30.00	310.30 > 265.20	14.00	310.30 > 105.10	32.00
42	**Methadone-d_3**	3.30	38.00	313.20 > 105.10	28.00	-	-
43	**Fentanyl**	3.87	35.00	337.20 > 105.20	38.00	337.20 > 188.20	30.00
44	**Fentanyl-d_5**	3.89	35.00	342.20 > 105.20	38.00	342.20 > 188.20	30.00
45	**Sufentanyl**	4.41	16.00	387.20 > 238.10	38.00	387.20 > 111.00	18.00
46	**Buprenorphine-d_4**	5.65	55.00	472.20 > 59.20	80.00	-	-

47	**Buprenorphine**	5.65	55.00	468.20 > 55.20	80.00	468.20 > 84.20	40
48	**Δ^9-tetrahydrocannabinol-d_3 (Δ^9-THC-d_3)**	5.79	45.00	318.00 > 123.00	34.00	-	-
49	**Δ^9-tetrahydrocannabinol (Δ^9-THC)**	6.19	45.00	315.20 > 193.10	34.00	315.20 > 123.00	22.00
synthetic cannabinoids							
50	**PX-1**	2.80	36.00	396.30 > 144.00	44.00	379.10 > 134.80	24.00
51	**AB-FUBINACA**	2.83	36.00	369.30 > 109.00	40.00	369.30 > 253.00	24.00
52	**5-Cl-AB-PINACA**	2.94	36.00	366.00 > 249.00	24.00	366.00 > 145.00	44.00
53	**PX-2**	2.97	26.00	397.30 > 145.00	46.00	397.30 > 233.00	22.00
54	**ADB-Fubinaca**	3.15	25.00	383.20 > 109.00	42.00	383.20 > 253.00	25.00
55	**AB CHMINACA**	3.46	38	357.40 > 241.20	28.00	357.40 > 145.00	46.00
56	**MMB 2201**	3.52	34.00	363.30 > 231.90	12.00	363.30 > 143.90	38.00
57	**Pravadoline**	3.77	45.00	379.10 > 113.90	32.00	379.10 > 134.80	24.00
58	**APP-FUBINACA**	3.9	20.00	417.30 > 109.00	40.00	417.30 > 253.00	24.00
59	**5-F-ADB**	4.1	45.00	378.30 > 105.00	24.00	378.30 > 318.00	14.00
60	**AM-2233**	4.75	45.00	459.00 > 111.90	22.00	459.00 > 97.80	34.00
61	**Cumyl-5-F-PINACA**	4.77	32.00	368.30 > 250.00	10.00	368.30 > 233.00	18.00
62	**AM-694**	4.99	45.00	436.00 > 202.70	40.00	436.00 > 230.70	28.00
63	**JWH 302**	5.16	45.00	336.10 > 121.10	22.00	322.00 > 134.80	26.00
64	**Cumyl-PEGACLONE**	5.26	30.00	373.30 > 255.00	10.00	373.30 > 119.00	24.00
65	**RCS-4**	5.33	45.00	322.00 > 106.80	40.00	322.00 > 134.80	24.00
66	**JWH 251**	5.51	45.00	319.80 > 105.90	22.00	319.80 > 214.20	15.00
67	**AM-2201**	5.59	45.00	360.20 > 126.90	40.00	360.20 > 154.90	28.00
68	**UR 144**	5.68	18.00	312.20 > 55.00	36.00	312.20 > 125.00	22.00
69	**JWH 203**	5.69	45.00	340.40 > 124.80	28.00	340.40 > 187.80	20.00
70	**5-F-NNEI-2**	5.76	22.00	375.30 > 232.00	20.00	375.30 > 144.00	42.00
71	**5-F-AKB48**	5.93	35.00	384.00 > 106.90	45.00	384.0 > 134.90	25.00
72	**RCS-8**	6.13	45.00	376.10 > 90.85	40.00	376.10 > 120.83	26.00
73	**JWH 018**	6.27	45.00	342.10>127.00	25.00	342.10 >155.00	34.00
74	**CP47, 497-C8**	6.32	45.00	386.70 > 104.80	22.00	386.70 > 120.80	24.00
75	**JWH 016**	6.33	45.00	341.70 > 127.10	44.00	341.70 > 155.10	24.00
76	**JWH 098**	6.33	45.00	385.80 > 157.20	42.00	385.80 > 185.10	26.00
77	**THJ 018**	6.36	25.00	377.20 > 248.90	16.00	377.20 > 212.90	24.00
78	**JWH 081**	6.38	45.00	371.80 > 157.09	40.00	371.80 > 185.08	26.00
79	**JWH 122**	6.48	45.00	356.10 > 140.90	40.00	356.10 > 168.80	26.00
80	**JWH 019**	6.52	45.00	356.10 > 255.07	26.00	356.10 > 228.10	26.00
81	**JWH 007**	6.56	45.00	355.80 > 127.09	48.00	355.80 > 155.09	26.00
82	**JWH 210**	6.69	45.00	369.80 > 183.10	26.00	369.80 > 214.20	24.00
83	**JWH 147**	6.70	45.00	382.10 > 127.09	48.00	382.10 > 155.06	22.00
84	**JWH 398**	6.90	45.00	376.06 > 161.07	48.00	376.06 > 189.06	26.00
85	**CB-13**	7.71	45.00	369.20 > 155.08	26.00	369.20 > 170.80	28.00
fentanyl analogues							
86	**Methoxyacetyl norfentanyl**	0.50	15.00	249.10 > 84.10	14.00	246.10 > 55.00	38.00
87	**Acetyl norfentanyl-d_5**	0.58	25.00	224.20 > 84.00	18.00	-	-
88	**Acetyl norfentanyl**	0.59	25.00	219.20 > 84.05	18.00	219.20 > 55.20	36.00
89	**Butyryl fentanyl carboxy metabolite**	0.76	25.00	381.20 > 105.00	42.00	381.20 > 188.10	30.00
90	**Valeryl fentanyl carboxy metabolite**	0.87	40.00	395.30 > 105.25	44.00	395.30 > 188.15	26.00
91	**Methoxyacetyl fentanyl**	1.1	30.00	353.30 > 188.00	20.00	249.10 > 84.10	18.00
92	**Furanyl norfentanyl**	1.15	16.00	271.00 >84.20	18.00	271.00 > 55.10	38.00
93	**Cis-3-metyl norfentanyl**	1.15	25.00	247.00 > 98.00	16.00	247.00 > 69.20	30.00
94	**Trans-3-metyl norfentanyl**	1.15	25.00	247.00 > 98.10	16.00	247.00 > 69.10	30.00
95	**Butyryl norfentanyl**	1.26	25.00	247.10 > 84.15	20.00	247.10 > 55.30	36.00
96	**Cyclopropyl norfentanyl**	1.33	25.00	245.20 > 177.10	18.00	245.20 > 84.10	20.00
97	**β-hydroxyfentanyl**	2.5	25.00	389.20 > 238.00	16.00	389.20 > 111.00	38.00

98	**Alfentanyl**	2.6	24.00	417.10 > 197.05	26.00	417.10 > 268.10	16.00
99	**Cyclopropylfentanyl**	3.06	25.00	349.20 > 105.00	30.00	349.20 > 188.10	25.00
100	**β-hydroxythiofentanyl**	3.14	25.00	359.20 > 192.00	22.00	359.20 > 111.00	38.00
101	**Furanylethyl fentanyl**	3.31	25.00	327.20 > 95.10	34.00	327.20 > 178.10	16.00
102	**Acetyl fentanyl**	3.33	25.00	322.20 > 105.00	36.00	322.20 > 188.00	20.00
103	**Furanyl fentanyl**	3.5	30.00	375.10 > 188.00	20.00	375.10 > 105.00	25.00
104	**Carfentanyl**	3.93	22.00	395.20 > 113.00	32.00	395.20 > 335.00	16.00
105	**Butyryl fentanyl**	4.22	30.00	351.20 > 105.00	40.00	351.20 > 188.10	22.00
106	**Despropionyl-para-fluoro fentanyl**	4.52	15.00	299.10 > 105.10	16.00	299.10 > 188.10	38.00
107	**Despropionyl fentanyl (4-ANPP)**	4.55	22.00	281.20 > 105.00	22.00	281.20 > 188.00	14.00
108	**Phenyl acetyl fentanyl**	4.96	46.00	399.30 > 188.05	24.00	399.30 > 105.05	44.00
synthetic cathinones							
109	**4-fluoromethcathinone**	0.83	35.00	205.00 > 102.80	28.00	205.00 > 148.70	26.00
110	**Methcathinone**	0.97	30.00	163.90 > 104.80	22.00	163.9 > 130.7	20.00
111	**Methylone**	1.15	30.00	208.1 > 159.9	16.00	208.1 > 131.9	26.00
112	**Mephedrone**	1.44	30.00	178.01 > 145	18.00	178.01 > 119	22.00
113	**Buphedrone**	1.45	30.00	178 > 130.3	26.00	178 > 91	32.00
114	**Ethylone**	1.63	30.00	222 > 174	20.00	222 > 146	26.00
115	**Buthylone**	1.63	25.00	222 > 173.9	20.00	222 > 145.9	26.00
116	**Dimethylcathinone**	1.80	30.00	177.70 > 72.10	22.00	177.7 > 105.30	20.00
117	**Diethylcathinone**	1.84	25.00	206.30 > 100.00	22.00	206.30 > 105.00	20.00
118	**3,4-dimethyl methcathinone**	1.85	30.00	192.00 > 143.90	28.00	192.00 > 158.80	22.00
119	**4-methyl ethcathinone**	1.89	35.00	192.00 > 145.30	18.00	192.00 > 91.00	34.00
120	**Ethcathinone**	1.94	30.00	177.7 > 72	16.00	177.7 > 105.20	22.00
121	**Penthedrone**	2.09	35.00	192.10 > 90.90	20.00	192.10 > 131.70	20.00
122	**Penthylone**	2.15	35.00	236.10 > 174.90	22.00	236.10 > 187.80	18.00
123	**Methylenedioxypyrovalerone (MDPV)**	3.42	30.00	276.1 > 126	26.00	276.1 > 134.8	24.00
124	**Naphyrone**	4.93	45.00	282.10 > 126.2	36.00	282.10 > 140.9	26.00
tryptamines							
125	**Acetyl-O-dimethyltryptamine (AcO-DMT)**	1.33	28.00	247 > 58.1	24.00	247 > 160	14.00
126	**5-methoxy-α-methyltryptamine (5-MeO-AMT)**	1.33	22.00	205.1 > 147	20.00	205.1 > 173	22.00
127	**4-hydroxy-diethyltryptamine (4-OH-DET)**	1.6	16.00	233.1 > 86.1	18.00	233.1 > 160	14.00
128	**5-methoxy-N-methyl-N-isopropyltryptamine (5-MeO-MIPT)**	1.8	10.00	247.1 > 86	14.00	247.1 > 174	16.00
129	**4-acetoxy-diisopropyltryptamine (4-AcO-DIPT)**	1.99	15.00	303.1 > 114	18.00	303.1 > 160	28.00
130	**5-methoxy-dipropyltryptamine (5-MeO-DPT)**	2.92	14.00	275.2 > 174	16.00	275.2 > 114	14.00
131	**5-methoxy-diallyltryptamine (5-MeO-DALT)**	3.78	24.00	271.2 > 110	14.00	271.2 > 174	18.00
phenylethylamines							
132	**Ritalinic Acid**	0.63	20.00	220.1 > 84.1	20.00	220.1 > 56	46.00
133	**5-(2-aminopropyl)benzofuran (5-APB)**	1.74	26.0	176.2 > 91	28.00	176.2 > 77	38.00
134	**6-(2-aminopropyl)benzofuran (6-APB)**	1.75	22.00	176.2 > 91	26.00	176.2 > 77	40.00
135	**1-(benzofuran-6-yl)-N-methylpropan-2-amine (6-MAPB)**	1.83	22.00	190.15 > 159	10.00	190.15 > 131	18.00
136	**1-(benzofuran-5-yl)-N-ethylpropan-2-amine (5-EAPB)**	2.11	24.00	204.15 > 131	20.00	204.15 > 91	30.00
137	**4'-methyl-α-pyrrolidinohexiophenone (MPHP)**	4.20	10.00	260.2 > 105	22.00	260.2 > 189	16.00

Table II. Concentration (ng/mL) of Target Analytes in Real Whole Blood Samples

Sample name	Cyclopropyl fentanyl	Cyclopropyl norfentanyl	Methoxyacetyl fentanyl	Methoxyacetyl norfentanyl	Furanylfentanyl	Furanylnorfentanyl	Acetylfentanyl	Acetylnorfentanyl	4-ANPP	Sufentanyl	Morphine	Codeine	Oxycodone
B002	4.3	5.0	–	–	–	–	–	–	–	–	148	100	–
B004	3.0	54	–	–	–	–	–	–	–	–	–	–	–
B005	6.4	9.9	–	–	–	–	–	–	–	–	–	–	–
B006	21	42	–	–	–	–	–	–	–	–	–	–	–
B007	16	46	–	–	–	–	–	–	–	–	–	–	–
B008	0.8	22	–	–	–	–	–	–	–	–	–	–	–
B010	4.9	36	3.5	1.1	–	–	–	–	0.2	–	–	–	–
B011	–	–	–	–	3.6	0.9	–	–	7.9	–	–	–	–
B012	–	–	–	–	–	–	–	–	–	–	–	–	–
B013	–	–	26	4.4	–	–	–	–	3.8	–	–	–	–
B014	–	–	23	4.1	–	–	–	–	3.6	–	–	–	–
B016	–	–	91	6.7	–	–	–	–	4.6	–	–	–	–
B017	–	–	–	–	–	–	87	70	4.3	–	–	–	–
B018	–	–	–	–	–	–	16	26	1.2	–	–	–	–
B019	–	–	–	–	–	–	20	40	2.5	–	–	–	–
B020	5.1	21	–	–	–	–	–	–	–	–	–	–	241
B022	–	–	–	–	–	–	–	–	0.4	–	–	–	–
B025	–	–	–	–	–	–	–	–	–	0.7	–	–	–

4-ANPP: 4-anilino-N-phenethyl-piperidine.

Table III. Concentration (ng/mL) of Target Analytes in Real Urine Samples

Sample name	Cyclopropyl fentanyl	Cyclopropyl norfentanyl	Methoxyacetyl fentanyl	Methoxyacetyl norfentanyl	Furanylfentanyl	Furanylnorfentanyl	Acetylfentanyl	Acetyl norfentanyl	4-ANPP	Fentanyl	Norfentanyl	Ritalinic Acid	N
U000	–	–	–	–	–	–	–	–	–	–	–	62	–
U001	11	120	–	–	–	–	–	–	–	–	–	–	–
U002	108	240	–	–	–	–	–	–	–	–	–	–	–
U003	7.4	350	–	–	–	–	–	–	–	–	–	–	–
U004	20	380	–	–	–	–	–	–	–	–	–	–	–
U005	83	130	–	–	–	–	–	–	–	–	–	–	–
U006	100	910	–	–	–	–	–	–	–	–	–	–	–
U007	36	400	–	–	–	–	–	–	–	–	–	–	–
U008	28	830	–	–	–	–	–	–	–	–	–	–	–
U009	88	530	–	–	–	–	–	–	–	–	–	–	3
U010	41	720	180	170	–	–	–	–	5.6	–	–	–	–
U011	–	–	–	–	85	22	–	–	130	–	–	–	–
U012	1.5	16	–	–	–	–	–	–	–	–	–	–	6
U013	–	–	1900	840	–	–	–	–	39	–	–	–	–
U014	–	–	1000	2000	–	–	–	–	27	–	–	–	–
U016	–	–	70	4.2	–	–	–	–	1.6	–	–	–	–
U017	–	–	–	–	–	–	2800	8900	120	0.7	11	–	–
U018	–	–	–	–	–	–	2900	8400	68	0.9	12	–	–
U019	–	–	–	–	–	–	62	7800	9.4	–	–	–	–
U036	–	–	–	–	–	–	3600	7000	110	–	–	–	–
U037	–	–	–	–	–	–	7.1	910	–	–	–	–	–
U038	–	–	–	–	–	–	–	–	0.2	710	1700	–	3
U040	–	–	–	–	–	–	–	–	–	12	9.9	–	1

4-ANPP: 4-anilino-N-phenethyl-piperidine; 6-MAM: 6-O-Monoacetylmorphine

Table IV. Concentration (ng/mL) of Target Analytes in Real Oral Fluid Samples

Sample name	Butylone	Carfentanyl	AcO-DMT	BEG	Cocaine	Morphine	6-MAM	THJ 018
OF000	–	12	–	–	–	0.1	1.0	–
OF001	–	–	–	–	0.2	–	0.2	–
OF002	–	–	–	–	–	–	0.2	–
OF003	–	–	–	–	–	1.0	–	–
OF004	–	–	0.6	–	–	–	–	–
OF005	120	–	–	–	–	–	–	–
OF006	0.1	–	–	–	0.4	–	–	–
OF007	–	–	–	–	–	–	–	–
OF008	–	–	120	–	–	–	–	–
OF009	–	–	–	17	110	–	–	–
OF010	1.0	–	–	–		–	–	2.0
OF011	–	–	–	0.3	1	–	–	–
OF012	–	–	–	–	–	–	–	0.2
OF013	–	–	–	–	–	–	–	97
OF014	–	–	–	–	–	–	–	0.4

AcO DMT: Acetyl-o-dimethyltryptamine; BEG: Benzoylecgonine; 6-MAM: 6-O-Monoacetylmorphine

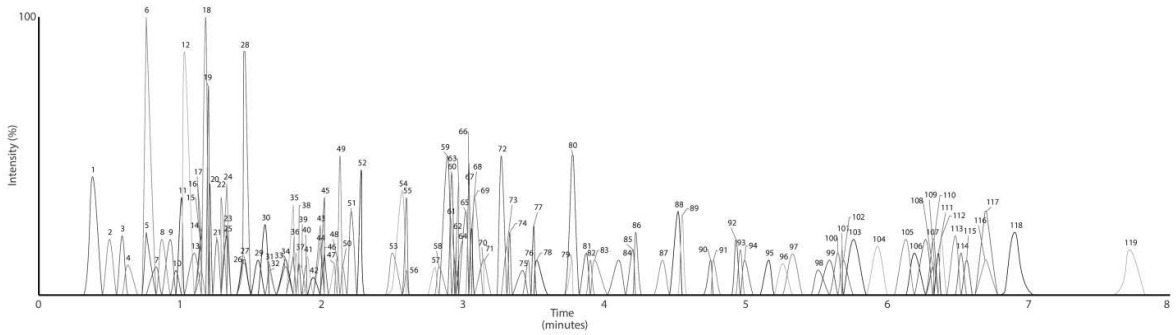

Fig 1

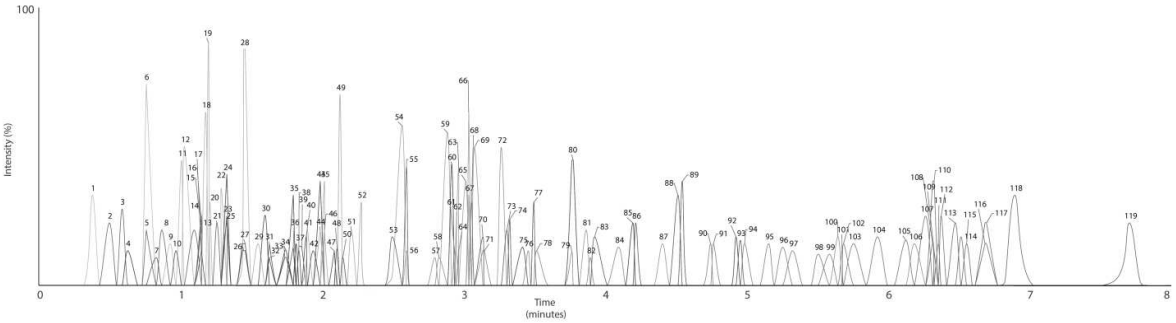

Fig 2

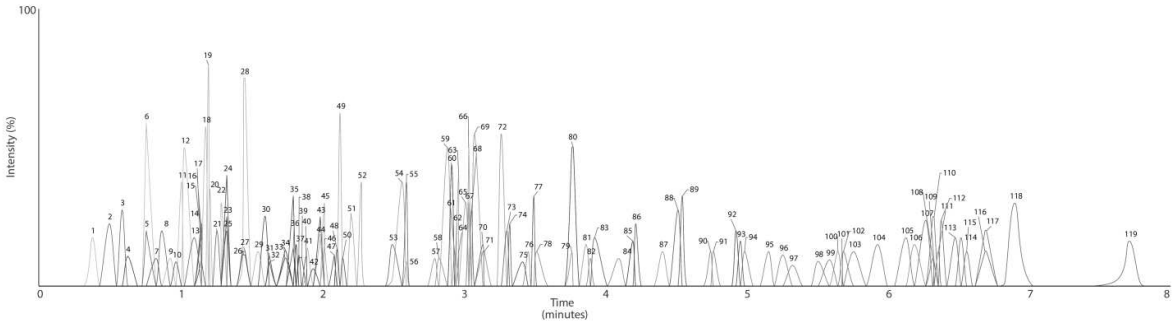

Fig 3