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A novel method for the simultaneous determination of drugs of abuse, ethyl glucuronide and synthetic opioids in human hair through a single digestion, purification and analysis in LC-MS/MS

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ABSTRACT

Polydrug use is a serious health and social problem worldwide. Over the past several years, there has been an increasing tendency to combine narcotics, alcohol, sedatives, and/or stimulants. To the traditional drugs of abuse and alcohol, an increase of new abuse drugs such as synthetic opioids has been added. In the current study, the development and validation of an innovative and fast analytical procedure has been presented to determine drugs of abuse, ethyl glucuronide and synthetics opioids in 30 mg of human hair through a single digestion, purification and analysis in LC-MS/MS. A combine simple preparation of hair sample followed to a single chromatographic run of 10 min has been proposed. A full validation for 54 target analytes for the parameters of selectivity, linearity, limit of detection, limit of quantification, accuracy, precision, matrix effects, recovery, and dilution integrity was successful completed. The method was linear in different ranges with r values of at least 0.990; the value to the validated LLOQ values were in the range 0.1–100 pg/mg. The method offered satisfactory precisions (CV<15 % and accuracy ± 20 %). In conclusion, a significant reduction in the overall times of the analytical procedure and the reduction of consumables costs make this method extremely advantageous and undoubtedly useful in routine laboratory workflow analyses and open the way to the prospect of a further implementation which also includes other classes of xenobiotics.

1. Introduction

Drug use patterns have changed radically over recent years and polydrug use is the rule rather than the exception worldwide, causing a serious public health problem [1]. This concerns the consumption of multiple drugs by a subject, either as simultaneous use (use of different drugs at the same time) or as sequential use (separate use of different drugs) [1]. Moreover, mixing of alcohol with other drugs of abuse and medications has become an emerging trend, exacerbating the public health concerns [2–5]. Mixing of alcohol with other drugs may additively or synergistically augment the seriousness of the adverse effects such as the withdrawal symptoms, cardiovascular disorders, liver damage, reproductive abnormalities, and behavioural abnormalities [6].

To the traditional drugs of abuse and alcohol, in recent years, the availability and the consequent consumption of new psychoactive substances have proliferated at an unprecedented rate, such as synthetic cannabinoids, synthetic cathinones, and more recently, the new synthetic opioids [7,8]. In a recent study, Ramirez Fernandez et al. [9] presented the development and validation of an ultra-performance liquid chromatography-tandem mass spectrometry method for the analysis of 16 synthetic opioids in segmental hair. The method was applied to 17 authentic hair samples: the synthetic opioids hair concentrations and the poly-drug history of fentanyl users was evaluated. In lights of the results, the authors recommended the analysis of synthetic opioids in heroin, but even in tramadol, methadone, buprenorphine, cocaine and amphetamine-like drugs users.

In order to characterize consumption patters of drugs, hair analysis is the gold matrix [8]. In forensic toxicology community, hair analysis has become increasingly widespread as it provides undoubted advantages compared to traditional matrices represented by blood and urine. As is known the keratin matrix allows a much wider window of detectability, less invasiveness than other traditional matrices such as blood, better stability over time also considering the unnecessary refrigerated storage

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[10]. The fields of application of these investigations have been implemented over time and in addition of the traditional medical-legal investigations regarding issuance of driving licenses, drug-related death cases, drug-facilitated crime and long-term therapeutic monitoring/follow-up of patients receiving substitution therapy, the practice has also become commonly used in workplace drug testing, firearms licensing, child custody, drug consumption behaviour and finally, in a clinical context, it is used for screening of prenatal/infant exposure control, and follow-up of liver transplantation patients [11,12].

Many analytical procedures have been developed and validated for the determination of xenobiotics in hair by following the solvent extraction phase with a purification phase using a solid-phase extraction (SPE) or a liquid–liquid extraction (LLE) to improve the cleanliness of the extract, the sensitivity and the signal-to-noise ratio [13]. When multiclass determination includes substances with very different chemical characteristics, this purification step is often excessively selective, favoring only a portion of compounds but sacrificing others. For this reason, several analytical methods are focused on class specific groups of compounds or multi-class drugs with similar chemical characteristics. However, if different procedures are used, the result is an increase in analysis times, costs and finally the quantity of sample required but often, in hair analysis, only a small quantity of matrix is available, while instead it is necessary the determination of multiple substances, even structurally very different and with different concentration ranges. [14]. Usually, the patients are not very cooperative as they are forced to undergo such investigations by the judicial authority, by the counterparty in judicial proceedings or by driving license commissions. Since it is necessary to be able to have an adequate quantity of sample available that allows the multiclass evaluation of substances, often in segmented samples, preferably in duplicate, and possibly sufficient to repeat the analyses, having a method that halves the necessary quantity of sample, halving costs and working times is a great advantage [15,16].

In light of the above, in routine laboratory workflow analyses, the development of new methods is necessary with the view to improve and combine sample preparation methods and to lower the time and costs analyses. In current study the development and validation of a novel multi analytical procedure for the simultaneous identification and quantification of DoA, synthetics opioids and EtG with a single extraction, a single SPE purification, a single chromatographic column and a single chromatographic run in LC-MS/MS has been presented.

2. Material and methods

2.1. Chemicals and reagents

Standards of the DoA (as free bases or salts) of 6-acetylmorphine (6-MAM), amphetamine (AMP), benzoylecgonine (BEG), buprenorphine (BUP), dihydrocodeine (DHC), phencycline (PHC), hydrocodone (HC), hydromophone (HM), methadone (MET), N-desmethyltapentadolo (NTAP), Norbuprenophine (NBUP), Norcodeine (NCOD), Noroxycodone (NOC), oxycodone (OC), Oxymorphone (OM), Tapentadol (TAP), tramadol (TRAM), N-desmethyltramadol (NTRAM), O-desmethyltramadol (OTRAM), Tropicamide (TROP), cocaine (COC), cocaethylene (CE), codeine (COD), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) ketamine (KET), methamphetamine (METAM), methadone (MTD), methylendioxyamphetamine (MDA), methylendioxyethylamphetamine (MDEA), methylendioxymethamphetamine (MDMA), morphine (MOR), norketamine (NKET) were supplied as pure substance or methanolic solution (1.0 or 0.1 mg/mL) by Cerilliant® Corporation (Merck, Milan, Italy). Standards of the synthetic opioids (as free bases or salts) of (±)-*cis*-3-methyl norfentanyl ((±)-*cis*-3MeNFEN), (±)-*trans*-3-methyl norfentanyl ((±)-*trans*-3MeNFEN), Acetyl fentanyl (ACFEN), Acetyl norfentanyl (ACNFEN), Alfentanyl (ALFEN), Butyryl fentanyl (BUFEN), Butyryl fentanyl carboxy metabolite (BUFEN carboxy metabolite), Butyryl norfentanyl (BUNFEN), Carfentanyl (CARFEN), Cyclopropyl fentanyl (CyclopropylFEN), Fentanyl (FEN) Isobutyryl fentanyl (ISOBUFEN), Methoxyacetylfentanyl (MeACFEN), Methoxyacetyl norfentanyl (MeACNFEN), Norfentanyl (NFEN), Ocfentanyl (OCFEN), Sufentanyl (SUFEN), Valeryl fentanyl carboxy metabolite (VAFEN carboxy metabolite), β-hydroxy fentanyl (βOHFEN), β-hydroxy thiofentanyl (βOHTHIOFEN), 2-fluoro-*ortho*-fluoro-3-methylfentanyl (2F-o-F3MeFEN) were donated in the SNAP project (Sistema Nazionale di Allerta Precoce) from Istituto Superiore di Sanità ISS (Rome, Italy) and sent by Comedical (Trento, Italy) at a concentration of 10 μg/ml. EtG was purchased from Cerilliant® (Merck, Milan, Italy) at a concentration of 1 mg/ml. Deuterated internal standards (ISs) were purchased as methanolic or acetonitrile solution (1 or 0.1 mg/mL) from by Cerilliant Corporation (Merck, Milan, Italy): Cocaine D_3 (COC-D_3); Morphine D_3 (MOR-D_3); Benzoylecgonine D_3 (BEG-D_3); Buprenorphine D_4 (BUP-D_4); Norbuprenorphine D_3 (NBUP-D_3); Amphetamine D_6 (AMP-D_6); 6-acetylmorphine D_3 (6-MAM-D_3); 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine D_3 (EDDP-D_3); 3,4-methylenedioxymethamphetamine D_5 (MDMA-D_5); Methamphetamine D_5 (METAM-D_5); Methadone D_3 (MTD-D_3); Ethylglucuronide D_5 (EtG-D_5), Fentanyl D_5 (FEN-D_5). Water, Methanol, Formic Acid were of LC–MS purity grade (Baker-VWR, Milan, Italy). Ammonium hydroxide, Acetone, n-hexane used for specimen preparation were of analytical grade (Carlo Erba, Milan, Italy). Clean Screen® EtG extraction Columns (200 mg/3 mL) were supplied from UCT (Bristol. PA, USA).

2.2. Hair samples

Drug-free hair specimens for the preparation of quality control samples were collected from known volunteers abstinent from any drugs and ethanol. To assess method performance, two groups of hair samples were included in the study:

- For DoA and EtG molecules, a total of 18 ground human hair samples of Arvecon GmbH-proficiency tests (PT) were collected during three years (six round per year).
- For synthetics opioids, a total of 2 authentic human hair samples of ISS-Italy PT were collected during two years (one round per year).

2.3. Preparation of working solutions

A mixture of DoA analytes at 5000 ng/mL was obtained though the dilution of stock solutions (1.0 or 0.1 mg/mL in methanol or acetonitrile) of the analytes. Additional working solutions at 500 ng/mL, at 100 ng/mL and 10 ng/mL were prepared. A mixture of EtG at 50 ng/mL was obtained though the dilution of stock solution (1.0 mg/mL in methanol). Two different mixtures of synthetics opioids at 1000 ng/mL were obtained though the dilution of single stock solutions (10 μg/mL in methanol) of the analytes. Additional working solutions at 100 ng/mL, at 10 ng/mL and 1 ng/mL were prepared.

An internal standard working solution at 500 ng/mL was also prepared by diluting a stock solution (1.0 or 0.1 mg/mL in methanol or acetonitrile) of EDDP-D_3 and MTD-D_3 with methanol, at 250 ng/mL of BEG-D_3, BUP-D_4, NBUP D_4, AMP-D_6, MDMA-D_5, METAM-D_5, COC-D_3, at 1 ng/mL of MOR-D_3 and 6-MAM-D_3 and at 50 ng/mL of EtG-D_5 and FEN-D_5. All solutions were stored at − 20 °C.

2.4. LC–MS/MS conditions

LC analyses were performed on an Agilent 1290 Infinity II LC system consisting of a binary pump, an autosampler, an on-line degasser and a thermostatted column compartment (Agilent, Waldbronn, Germany). Samples were analysed on a Force Biphenyl (50 x 3.0 mm, 3.0 μm – Restek®, Milan, Italy) preceded by an UltraShield UHPLC precolumn filter (0.2 μm frit) (Restek®, Milan, Italy). The mobile phase was composed of (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in methanol using the following gradient program: 0.0–7.0 min, linear

gradient from 4 % to 100 % (B); 7.0–8.0 min, isocratic at 100 % (B), 8.0–8.01 min, linear gradient from 100 % to 4 % (B). A pre-equilibration period of 1.99 min was used between each run. The flow-rate was 0.8 mL/min and the column temperature was 40 °C. The injection volume was 10 µL. The autosampler was maintained at 10 °C and the injector needle was washed with methanol/0.05 % formic acid in water (1:9 v/v) prior to any injection. The chromatographic conditions were optimized by analysing the standard solutions and also extracts of blank hair spiked with the target analytes. Tandem mass spectrometry was performed using a SCIEX API 6500 QTRAP mass analyser equipped with a Turbo Ion Spray source (SCIEX Toronto, Canada) operating in ESI negative mode (Experimental 1: from 0.00 to 1.40 min) and in positive mode (Experimental 2: from 1.40 to 10.00 min). Detection and quantitation of all analytes were accomplished using multiple reaction monitoring mode (MRM) in scheduled mode due to the achieved high selectivity and sensitivity. The ESI source settings were: ion-spray voltage: − 4000 V (experimental 1) and 5500 V (experimental 2), source temperature: 450 °C, nebulization and heating gas (air): 40 psi and 40 psi, respectively. Multiple reaction monitoring (MRM) was optimized using nitrogen as collision gas, produced by a gas generation system (Nitrogen Generator model 75–72, Whatman Inc., MA, USA). MS/MS parameters were optimized by direct infusion of each individual analyte at 100 ng/mL in the initial LC mobile phase at a flow of 10 µL/min. MRM transitions and optimized parameters are presented in Table 1. The mass spectrometer was calibrated to < 2.0 mDa mass error prior to each batch analysis. The Analyst® Software (version 1.7.3, AB SCIEX, Foster City, CA, USA) was used for instrument control, data acquisition and qualitative data analyses. Quantitative data analyses were performed by the SCIEX OS software (version 2.0.1).

2.5. Hair extraction

All samples were decontaminated as recommended by the Society of Hair Testing guidelines [15] with acetone (2 x 10 mL) and n-hexane (2 x 10 mL) and then dried at room temperature. The washed hair samples were pulverized by Precellys® 24 (Bertin Technologies-Alphatech SpA, Genova, Italy) at a rotation frequency of 6000 rpm for 30 s; this treatment provided 1–2 mm length segments and the temperature inside the device did not exceed 40 °C, thus avoiding any overheating of the samples.

30 mg aliquots of pulverized hair were added with 50 µL of the internal standard solution and 1.5 mL of 0.5 % formic acid in water, then sonicated at 40 kHz for 90 min then at 45 °C overnight. The extracts were purified by SPE procedure on clean Screen® EtG extraction Columns (200 mg/3 mL) pre-conditioned with 3 mL of 1 % formic acid in methanol and 3 mL of 1 % formic acid in water. The samples were loaded at a flow-rate of 1 mL/min; then the SPE cartridges were washed with 1 mL of methanol and dried under full vacuum for 3 min. Finally, the analytes were eluted with 2 mL of 1 % formic acid in methanol and successively with 2 mL of 2 % ammonium hydroxide in methanol. The obtained eluates were evaporated to dryness under nitrogen flow, reconstituted in 100 µL of the initial LC mobile phase and a 10.0 µL aliquot was subjected to LC–MS/MS analysis

2.6. Validation

The present study was validated accomplished according to the Standard practices for method validation in forensic toxicology, ANSI/ASB Standard 036, First Edition 2019 [17].

The following parameters were evaluated: selectivity, calibration model, limit of detection (LOD), lower limit of quantitation (LLOQ), precision, accuracy, carry-over, matrix effects, extraction recovery and dilution integrity.

2.6.1. Calibration and quality control samples

Calibration samples were prepared at concentration of 5, 10, 25, 50, 200, 500, 1000, 2500, 5000 pg/mg hair for DoA, 0.1, 0.15, 0.25, 0.5, 2.5, 10, 50, 100, 300, 500 pg/mg hair for synthetics opioids and 2.5, 5, 10, 20, 30, 50, 100, 200 pg/mg hair for EtG. Quality control samples (QC) were prepared to three concentrations levels: low level at 5 pg/mg for EtG, 0.5 pg/mg for synthetics opioids except for ISOBUFEN, MeACFEN, MeACNFEN, NFEN, OCFEN, OHTHIOFEN, VAFEN carboxy metabolite, 2F-o-F3MeFEN, (±)-*cis*-3MeNFEN, (±)-*trans*-3MeNFEN, ACNFEN, BUNFEN, CARFEN, CyclopropylFEN at 2.5 pg/mg and 0.005 ng/mg for DOA except for AMP, DHC, PHC, HC, HM and NBUP at 0.025 ng/mg and for 6-MAM, EDDP, MDA, NOC, OM at 0.01 ng/mg; medium level at 30 pg/mg for EtG, 50 pg/mg for synthetics opioids and 0.05 ng/mg for DoA except for AMP, DHC, PHC, HC, HM and NBUP at 0.5 ng/mg and for 6-MAM, EDDP, MDA, NOC, OM at 0.2 ng/mg; high level at 100 pg/mg for EtG, 300 pg/mg for synthetics opioids and 1 ng/mg for DoA except for AMP, DHC, PHC, HC, HM and NBUP at 2.5 ng/mg (n = 6 for each level).

2.6.2. Selectivity

Aliquots (30 mg) of pulverized hair samples, obtained from 10 different volunteers abstinent from ethanol and drugs, were added with 100 µL of methanol and processed as described above. These blank samples were individually assessed for the presence of any interference across the retention window of each analyte and the ISs [17].

2.6.3. Calibration model

The processed calibration samples were analysed in triplicate. Calibration curves were generated from the peak-area ratio of each analyte quantifier transition to the assigned IS. The ratio was then plotted on the y-axis against the nominal analyte concentration to generate the standard curves by the method of least squares using a weighed (1/x) linear regression model.

2.6.4. LOD and LLOQ

The sensitivity of the developed analytical procedure was evaluated by determining the limit of detection (LOD) and the lower limit of quantitation (LLOQ) for each analyte. Sets of blank hair were fortified at 2.5, 1, 0.5 pg/mg hair for EtG, 1, 0.25, 0.1, 0.05 pg/mg hair for synthetics opioids and 0.01, 0.05, 0.001 ng/mg hair for DoA and subjected to the described sample processing. The LLOQ value represented the lowest concentration of the analyte that was capable of reproducibility providing symmetrical peaks and the minimum mass spectral identification ratios, while maintaining a bias of ± 20 % and % CV<20 %. The LOD value for each analyte was estimated from the standard deviation of the y-intercept (s_y) and the average slope (Avg_m) as: $LOD=3.3\ s_y/Avg_m$ [17].

2.6.5. Precision and accuracy

Method precision and accuracy were determined by replicate analyses of the QC samples spiked at low, medium and high levels (n = 6, each). The QC samples were analysed in triplicate over the course of five different days. The acceptable bias range was within ± 20 % of each QC level. Precision was evaluated through analysis of variance (ANOVA) approach for the coefficient of variance (%CV) of within-run and between-run values, which should not exceed 20 % for each QC level. [17].

2.6.6. Carry-over

Carry-over effect was evaluated by injecting extracts of blank hair samples after analyses of calibration samples spiked at the upper limit of the calibration range. For acceptance, the peak areas in the blank sample should be above the method's LOD areas. This carryover concentration for each analyte shall be confirmed using triplicate analyses [17].

2.6.7. Matrix effect and recovery

According to Matuszewski et al. [18], the matrix effect (ME) and extraction recovery were evaluated with a set of three different samples

Table 1
LC-MS/MS parameters for all target analytes.

Analyte	MRM transitions	CE (Hz)	Tr (min)
Drugs of Abuse			
AMP	136.1 → **119.2**, 91.1	**14**, 24	2.22
BEG	290.3 → **105.0**, 168.1, 150.0	**44**, 29, 34	3.72
BUP	468.5 → **396.4**, 101.2, 267.3	**54**, 60, 65	4.62
CE	318.2 → **82.0**, 196.2, 150.3	**45**, 29, 37	4.24
COC	304.1 → **150.1**, 105.0, 82.0	**35**, 46, 47	3.90
COD	300.2 → **153.2**, 165.2, 183.0	**65**, 60, 38	2.71
DHC	303.6 → **200.3**, 172.0	**44**, 53	2.66
EDDP	278.1 → **234.4**, 249.4, 186.0	**20**, 26, 46	5.06
PHC	245.0 → **160.2**, 86.0	**20**, 17	4.75
HC	300.1 → **199.0**, 128.0, 183.0	**50**, 70, 37	2.94
HM	286.0 → **185.2**, 227.1, 199.3, 157.1	**40**, 37, 40, 56	2.06
KET	238.2 → **207.1**, 220.1, 179.1	**22**, 23, 26	3.69
6-MAM	328.3 → **165.2**, 191.1, 211.2	**51**, 45, 37	2.72
MDA	180.1 → **163.1**, 133.1	**15**, 27	2.59
MDE	208.2 → **133.0**, 163.1	**30**, 21	3.17
MDMA	194.2 → **105.2**, 163.0	**29**, 20	2.89
MTD	310.4 → **105.1**, 265.4	**38**, 23	5.31
METAM	150.2 → **119.1**, 90.9	**17**, 26	2.56
MOR	286.2 → **165.0**, 181.0, 153.2	**54**, 48, 50	1.83
NTAP	208.1 → **107.0**, 121.0	**38**, 30	3.28
NBUP	414.3 → **83.0**, 101.3, 187.1	**70**, 60, 51	4.12
NCOD	286.1 → **165.0**, 152.0	**58**, 75	2.36
NKET	224.2 → **178.9**, 207.1, 124.8	**23**, 18, 37	3.41
NOC	302.1 → **187.1**, 284.1, 198.1	**27**, 21, 61	2.61
OC	316.3 → **240.9**, 298.2, 256.1	**40**, 27, 36	2.84
OM	302.0 → **227.1**, 284.0	**40**, 27	1.89
TAP	222.3 → **1** → **21.1**, 107.2, 135.0	**30**, 38, 27	3.43
TRAM	264.1 → **58.1**, 42.2	**43**, 103	3.55
NTRAM	250.2 → **44.0**, 42.0	**97**, 97	3.65
OTRAM	250.1 → **42.0**, 58.1	**97**, 97	2.69
TROP	285.0 → **255.0**, 134.0, 107.1	**29**, 35, 49	3.47
Synthetic opioids			
(±)-*cis*-3MeNFEN	247.2 → **97.9**, 68.9	**48**, 54	5.02
(±)-*trans*-3MeNFEN	247.2 → **97.9**, 68.9	**48**, 54	4.93
4-ANPP	281.2 → **105.0**, 77.2	**50,** 30	5.74
ACFEN	323.1 → **105.1**, 188.1	**50,** 31	5.63
ACNFEN	219.2 → **84.1**, 55.2	**37,** 47	4.05
ALFEN	417.1 → **197.1**, 268.1	**30,** 17	5.88
BUFEN	351.2 → **105.0**, 188.1	**28,** 34	6.22
BUFEN carboxy metabolite	381.3 → **188.0**, 105.0	**33,** 32	5.61
BUNFEN	247.4 → **83.9**, 177.0, 164.2	**28,** 23, 20	5.18
CARFEN	395.2 → **113.0**, 335.0	**36,** 18	6.08
CyclopropylFEN	349.2 → **105.0**, 188.1	**36,** 16	6.22
FEN	337.3 → **188.2**, 216.2, 105.2	**33**, 30, 34	5.97
ISOBUFEN	351.2 → **105.0**, 188.1	**61,** 23	6.15
MeACFEN	353.2 → **188.0**, 105.0	**22,** 27	5.52
MeACNFEN	249.1 → **84**, 55.1	**19,** 57	3.90
NFEN	233.2 → **83.7,** 177.3, 150.1	**28**, 23, 23	3.42
OCFEN	371.0 → **188.0**, 105.0	**22,** 19	5.56
SUFEN	387.2 → **238.1**, 111.0	**24,** 32	6.34
VAFEN carboxy metabolite	395.2 → **188.2**, 105.0	**34,** 54	5.36
βOHFEN	353.2 → **204.0**, 335.0	**20,** 36	5.73
βOHTHIOFEN	359.2 → **192.1**, 111.0	**35,** 60	5.62
2F-o-F3MeFEN	387.2 → **123.1**, 103.1	**50,** 60	6.31
Ethylglucuronide			
EtG	221.0 → **85.1**, 75.0, 112,8	**–23,** −21, −20	0.75
Internal standards			
AMP-D_6	142.1 → **125.3**, 94.0	**14**, 30	2.19
BEG-D_3	293.3 → **171.2**, 104.9, 293.3	**30**, 45, 34	3.69
BUP-D_4	472.4 → **400.0**	**55**	4.60
COC-D_3	307.4 → **185.1**, 153.1, 85.0	**28**, 37, 45	3.87
6-MAM-D_3	331.0 → **165.4**	**50**	2.70
MDMA-D_5	199.5 → **165.1**, 135.2, 107.0	**17**, 28, 34	2.86
MTD-D_3	313.3 → **268.4**	**22**	5.30
METAM-D_5	155.1 → **121.0**, 92.0	**20**, 26	2.54
MOR-D_3	289.3 → **165.1**, 181.1, 201.2	**54**, 50, 36	1.80
NBUP-D_3	417.0 → **100.8**	**54**	4.11
EDDP-D_3	281.0 → **234.0**, 249.1	**30**, 25	5.02
FEN-D_5	342.2 → **188.1**	**36**	4.60
EtG-D_5	225.5 → **85.0**, 75.0	**–22,** −24	0.78

at low (LQC) and high (HQC) concentration with ten different authentic samples: the neat standard (set 1), blank matrix spiked with target analytes after extraction (set 2) and blank matrix spiked before extraction (set 3) (n = 6 for each set). ME was evaluated comparing the average peak areas (X) of set 2 to those of set 1 as follows: ME (%) = [(X area of Set 2 /X area of set 1) −1] x 100. The ME% should be within ± 25 %. Extraction recovery was estimated by comparison of the average peak areas set 2 to those of set 3, expressed as percentages.

2.6.8. Dilution integrity

During authentic sample analysis, excessively high concentrations that are above the established calibration range may be encountered. To bring the analyte concentration within the validated concentration range, the sample may be diluted, providing that accuracy and precision of the method are not significantly impacted [17]. The dilution integrity was assessed as follows: blank specimens for all matrices were spiked at 5 times the highest validation sample and mixed with additional blank matrix to achieve a 5-fold (n = 6), 10-fold (n = 6), 15-fold (n = 6) dilution. All obtained samples were analysed against the calculated calibration curves to assess if the criteria performance was still met. QC samples after 24 and 48 h on the autosampler were reanalysed in triplicate to assess the stability parameters. The analyte is considered stable until the average concentration values, after 24 and 48 h, compared to the time zero average concentration values falls outside of the method's acceptable bias.

3. Results and discussion

3.1. LC-MS/MS detection

The development of the chromatographic conditions was aimed to short retention time run with preserving the chromatographic resolution of the all studied analytes. Different columns under different mobile phases were tested to aim this purpose. Best results were reached with Force Biphenyl (50 x 3.0 mm, 3,0 μm − Restek®, Milan, Italy) both in terms of resolution and peak shape. The repeatability of the retention times was satisfactory (RSD%= 0.3 % for all analytes and the IS). Multiple reaction monitoring (MRM) in scheduled mode was used to carry out the quantitative analyses with high sensibility and selectivity. Two/three ion transitions in scheduled MRM mode were set up for each target analytes as shown in Table 1. The representative chromatographic profiles of drug-free hair spiked at LLOQ concentrations are showed in Fig. 1 for 6-MAM, BEG, NCOD, TRAM, OTRAM, CE, COD, KET, METAM, MDMA, MOR, NKET, EtG, ACFEN, ALFEN and FEN.

3.2. Extraction procedure

Numerous papers suggested various procedures for removing xenobiotics from matrix hair: enzymatic digestion, digestion by strong acids or bases at different temperatures or solvent extraction [19]. However, the choice of the digestion conditions must take into account the stability of the different target analytes, protecting them from breakdown. Incubation in an organic solvent or a buffer has been extensively used [19]. In this study, we carried out the extraction step by sonication at 40 kHz for 90 min in slight acidic condition (0.5 % HCOOH in water) and

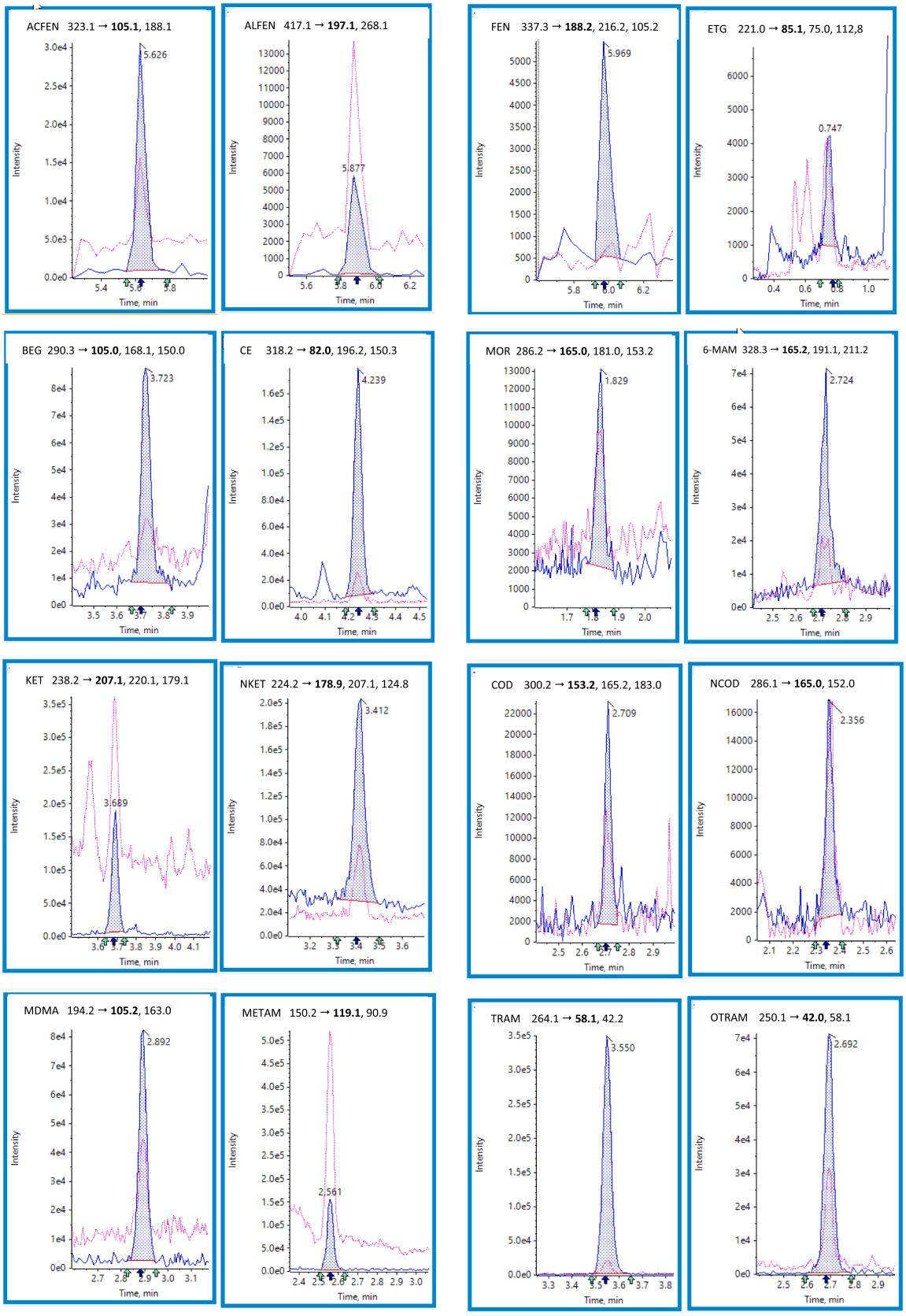

Fig. 1. The representative chromatographic profiles of drug-free hair spiked at LLOQ concentrations.

Table 2
Linearity range, correlation coefficient, limit of detection (LOD) and lower limit of quantification (LLOQ) of all target analytes (pg/mg).

Analyte	LOD	LLOQ	Linearity	r
AMP	10	25	25–5000	0.999
BEG	1	5	5–5000	0.998
BUP	25	50	50–5000	0.998
CE	1	5	5–5000	0.998
COC	10	25	25–5000	0.999
COD	1	5	5–5000	0.999
DHC	10	25	25–5000	0.998
EDDP	5	10	10–5000	0.999
PHC	10	25	25–5000	0.996
HC	10	25	25–5000	0.996
HM	10	25	25–5000	0.996
KET	1	5	5–5000	0.996
6-MAM	5	10	10–5000	0.999
MDA	5	10	10–5000	0.998
MDE	1	5	5–5000	0.995
MDMA	1	5	5–5000	0.999
MTD	1	5	5–1000	0.997
METAM	1	5	5–2500	0.998
MOR	1	5	5–5000	0.999
NTAP	1	5	5–1000	0.998
NBUP	50	100	100–5000	0.995
NCOD	1	5	5–5000	0.998
NKET	1	5	5–5000	0.995
NOC	5	10	10–5000	0.996
OC	1	5	5–5000	0.998
OM	5	10	10–5000	0.999
TAP	1	5	5–1000	0.997
TRAM	1	5	5–1000	0.998
NTRAM	1	5	5–5000	0.998
OTRAM	1	5	5–1000	0.997
TROP	1	5	5–5000	0.993
EtG	1	2.5	2.5–200	0.999
(±)-*cis*-3MeNFEN	1	2.5	2.5–500	0.999
(±)-*trans*-3MeNFEN	1	2.5	2.5–500	0.999
4-ANPP	0.1	0.25	0.25–500	0.999
ACFEN	0.1	0.25	0.25–500	0.995
ACNFEN	1	2.5	2.5–500	0.995
ALFEN	0.5	1.5	1.5–500	0.999
BUFEN	0.1	0.25	0.25–500	0.997
BUFEN carboxy metabolite	0.5	1.5	1.5–500	0.996
BUNFEN	1	2.5	2.5–500	0.995
CARFEN	1	2.5	2.5–500	0.997
CyclopropylFEN	1	2.5	2.5–500	0.998
FEN	0.05	0.1	0.1–500	0.996
ISOBUFEN	1	2.5	2.5–500	0.998
MeACFEN	1	2.5	2.5–500	0.993
MeACNFEN	1	2.5	2.5–500	0.998
NFEN	1	2.5	2.5–500	0.997
OCFEN	1	2.5	2.5–500	0.994
SUFEN	0.1	0.25	0.25–500	0.999
VAFEN carboxy metabolite	1	2.5	2.5–500	0.994
βOHFEN	0.1	0.25	0.25–500	0.995
βOHTHIOFEN	1	2.5	2.5–500	0.995
2F-o-F3MeFEN	0.25	1	1–500	0.996

then at 45 °C overnight. In particular, we tested different digestion conditions applied at authentic hair samples (collected from proficiency tests) using water, 0.1 % HCOOH in water, 0.5 % HCOOH in water, 1 % HCOOH in water. The incubation test was studied at different times: 30 min, 60 min, 90 min and 120 min. Following different experimental assay relative to digestion conditions, the optimized settings satisfied the maximum extraction of analytes from matrix with the best quality/time ratio.

As mentioned above, in general the extracted solution must be purified and concentrated to improve the signal-to-noise ratio. Moreover, the clean-up of the biological matrix is fundamental in the case of analytes based on LC-MS because of the matrix effects (ion suppression and ion enhancement). To achieve the required LLOQ of target analytes, the obtained extracts were purified by SPE procedure on Clean Screen® EtG extraction Columns as described above. The dried cartridge was eluted using 1 mL of 1 % formic acid in methanol followed by 1 mL of 2 % ammonium hydroxide in methanol to carry out a complete recovery of acid, neutral and basic target analytes. Following different experimental assay relative to eluent composition and volume, the chosen eluent satisfied the conditions for maximum extraction from matrix, recovery and the shortest drying time. The main advantage of the combined procedure is the drastically reduction of hair sample amount and this is an important aspect especially in segmental analysis. Moreover, if both EtG and DoA are demanded on the same sample, the cost and the time of analysis can be considerably reduced.

3.3. Validation

Monitoring the MRM transitions shown in Table 1, no interfering peaks were observed in the extracts of 10 different blank hair samples for all analytes and its deuterated IS., demonstrating the specificity of the method. The linear weighted (1/x) model was chosen to evaluate the calibration curves and the calculated calibration parameters are shown in Table1. The correlation coefficients for all target analytes were ≥ 0.99 and the linearity range is listed in Table 2. The LOD and LLOQ values for each analyte were estimated as described above and are shown in Table 2. The obtained LODs and LLOQs are similar to other published methods [5,9,14], confirming the satisfactory sensitivity of the developed procedure and providing suitable sensitivity for the interpretation using the SoHT cut off values. The obtained data relative to within-run precision, between-run precision and accuracy fulfilled the acceptance criteria (CV% $<$ 20 %, accuracy $\pm$ 20 %) for each analyte at low, medium and high concentrations. The obtained data are show in Table 3. The carry over effects were negligible for all target compounds, being the peak areas in the blank sample $<$ 5 % of the peak areas found for the calibrator spiked at the lowest concentration. This result is within the proposed acceptance limits for this parameter. The recoveries were satisfactory for most of the analytes as showed in Table 4. The recovery values were higher than 60 % excepted for BEG, CE, COC, HC and HM, which had extraction recoveries of 37 %, 45 %, 53 %, 45 % and 54 %, respectively. Despite the low recovery values found for these analytes, it should be noted that the sensitivity achieved with these analytes met largely the interpretation criteria for the hair matrix [9]. In fact, the tested LLOQs at 0.005 ng/mg and 0.025 ng/mg for BEG and COC respectively are well below the cut off recommended by the SoHT for the DoA. Matrix effects showed generally acceptable results for most analytes and are presented in Table 4. Some analytes were outside of the acceptable criteria. A considerable matrix effect in terms of ionization enhancement of 125 % and 75 % at low and high level respectively was tested for EDDP, whereas a moderate ion enhancement was estimated for 6-MAM and amphetamine at 34 % and 26 % respectively. BEG, CE, MDE and NTRAM showed effects of ion suppression ranging from –32 % to −41 %. This evidence indicates the presence in the extracts of matrix components affecting the ionization process only for the cited analytes. To compensate efficiently these effects, the quantitative analyses were carried out by the use of isotopically labelled ISs, characterized by similar retention time and ionization behaviour. In this way, the matrix effect can be minimized and the variability (RSD%) of the matrix effect measured in different QC samples was less than 20 %. Concerning the dilution integrity studies, the obtained results fulfilled the suggested acceptance criteria.

3.4. Method application

The validated analytical procedure was applied to a total of 18 ground human pulverized hair samples (six rounds per year) collected from Arvecon GmbH proficiency tests (PT) during a period of three years for EtG and DoA and to a total of 2 (one ground per year) from ISS-Italy proficiency tests during a period of two years for fentanyl and analogues molecules. The analytes were tested at different hair concentration ranges: 7.1–76.0 pg/mg for EtG, 11.7–2457 pg/mg for DoA and

Table 3
Bias and precision data for all target analytes at low, medium and high concentrations.

Analyte	Bias %			CV %					
	Low	Medium	High	Low		Medium		High	
				wr[a]	br[b]	wr	br	wr	br
AMP	3.2	4.9	9.4	6.9	16.5	2.8	2.7	2.7	2.8
BEG	2.1	1.9	−3.1	8.2	8.2	2.7	3.8	2.1	2.7
BUP	−5.5	2.3	−5.5	14.6	12.0	4.9	5.5	2.9	2.8
CE	−0.8	9.5	1.9	7.1	6.0	8.9	7.2	1.4	0.9
COC	4.5	5.3	−1.4	3.7	4.7	2.5	2.5	2.9	1.9
COD	−9.3	0.1	0.8	4.2	5.2	2.8	4.7	2.7	3.8
DHC	−12.9	−5.7	−1.4	2.3	4.8	4.0	6.8	1.4	4.2
EDDP	5.7	14.4	1.0	3.0	2.6	4.3	4.2	1.0	1.9
PHC	−3.4	13.9	−0.1	6.2	7.1	3.1	2.9	5.7	5.3
HC	8.6	−10.6	−5.6	6.3	5.3	7.0	6.7	5.2	6.0
HM	9.5	−13.6	−8.5	2.7	2.8	6.8	17.3	1.3	2.6
KET	9.6	12.7	−4.6	4.1	6.0	2.3	3.3	2.1	4.8
6-MAM	4.5	5.3	2.5	2.1	1.8	4.2	5.2	2.0	2.35
MDA	−14.4	4.2	3.3	6.4	9.4	7.5	6.6	6.2	5.4
MDE	−9.4	9.2	−7.8	2.8	3.1	2.4	2.6	1.6	2.1
MDMA	3.3	13.1	4.3	4.2	5.6	0.6	1.9	1.0	2.5
MTD	−5.9	8.7	−3.2	1.8	1.6	2.1	2.2	3.0	7.2
METAM	3.5	8.6	−9.0	3.5	3.2	0.5	1.2	1.3	2.0
MOR	−0.7	4.7	−2.2	2.5	2.3	1.2	1.1	2.2	1.7
NTAP	14.6	13.3	10.3	3.5	3.6	5.1	4.2	5.0	3.5
NBUP	−3.1	−5.7	2.3	10.3	8.7	7.9	9.4	12.3	14.
NCOD	−11.4	4.7	0.1	5.9	5.0	1.2	1.8	3.6	3.0
NKET	7.9	11.0	1.5	7.6	7.2	5.6	5.5	3.7	3.4
NOC	13.5	1.2	−9.7	5.1	5.6	10.2	9.1	3.6	2.9
OC	−14.7	−3.3	−2.5	2.8	2.7	2.6	3.6	1.1	3.3
OM	−13.3	3.5	4.5	11.1	12.1	3.2	2.7	4.4	4.0
TAP	0.7	6.7	3.5	6.2	9.3	4.8	6.7	7.0	2.9
TRAM	−1.7	13.4	7.5	5.7	5.6	3.4	9.1	3.0	5.9
NTRAM	1.9	14.3	5.5	3.9	3.3	4.0	3.5	2.2	2.4
OTRAM	6.3	8.4	5.3	5.0	7.5	2.1	2.5	4.0	5.5
TROP	−4.0	13.2	12.3	5.7	6.6	3.7	3.8	3.3	3.7
EtG	4.1	3.1	0.5	5.5	4.8	3.8	3.6	2.6	3.0
(±)-*cis*-3MeNFEN	7.2	5.2	1.7	8.4	5.9	7.9	8.8	9.5	10.2
(±)-*trans*-3MeNFEN	4.4	6.2	3.3	5.7	3.4	8.1	10.1	6.5	8.2
4-ANPP	5.2	5.8	2.5	2.3	4.2	6.5	9.2	4.5	5.5
ACFEN	7.4	9.6	3.8	3.7	5.3	8.3	8.4	2.5	9.6
ACNFEN	−3.5	4.5	9.2	5.6	7.2	7.4	6.5	8.9	4.8
ALFEN	−9.1	7.5	7.2	6.0	2.4	6.5	7.6	3.3	7.2
BUFEN	3.10	4.6	1.0	5.7	7.2	2.8	4.4	1.3	2.1
BUFEN carboxy metabolite	8.2	5.8	1.6	7.8	5.0	6.9	4.9	1.3	4.4
BUNFEN	−9.2	7.5	8.2	8.9	5.3	9.2	10.3	4.2	5.2
CARFEN	2.9	4.5	3.5	2.2	6.8	8.3	7.2	3.5	5.0
CyclopropylFEN	12.7	7.5	5.8	3.4	−2.0	8.0	7.3	4.6	7.8
FEN	−0.9	5.0	−2.3	3.8	10.1	1.7	2.3	4.3	7.7
ISOBUFEN	−11.2	−5.8	−2.3	6.2	6.8	2.7	1.2	2.9	2.6
MeACFEN	5.6	−0.2	6.0	3.5	10.3	9.2	5.6	4.5	2.8
MeACNFEN	−0.3	4.5	5.3	5.3	7.8	8.8	5.5	6.3	6.5
NFEN	9.7	−2.1	0.5	2.8	4.0	5.6	5.8	7.3	3.9
OCFEN	4.5	−1.2	−0.5	4.5	12.1	5.2	1.3	2.9	3.9
SUFEN	7.8	4.5	7.2	5.2	10.3	7.3	4.5	3.6	6.5
VAFEN carboxy metabolite	12.5	2.3	−2.3	5.5	8.5	4.2	0.9	2.7	4.5
βOHFEN	10.2	5.2	−3.7	9.2	7.6	2.2	3.5	5.6	8.2
βOHTHIOFEN	5.7	3.6	0.5	9.9	5.2	2.9	6.2	2.5	7.2
2F-o-F3MeFEN	6.8	6.2	2.4	2.4	3.6	3.6	4.5	3.2	3.5

[a] within run;
[b] between run.

9.7–48.2 pg/mg for synthetic opioids. For all authentic hair samples, the PT results of our laboratory presented the acceptable zeta score values. In particular, for EtG the z-score value range was tested at −0.72–1.45, for DOA at −0.72–0.34 and for fentanyl and analogues at −0.78–0.40.

4. Conclusion

A new and single analytical procedure was developed and validated for the simultaneous extraction, identification and quantification of 54 target analytes including 31 DoA, EtG and 22 synthetic opioids in human hair. Usually in hair analysis, the sample amount for a complete analysis is limited. To face this limiting aspect, the presented method proposes the use of only one aliquot of 30 mg hair for the determination of different substances classes in a wide quantitative range. A combine sample preparation of hair sample followed to a single chromatographic run of 10 min has been proposed. All the validated parameters met the required criteria for clinical and forensic toxicology. The high method performance has been confirmed by passing international proficiency tests such as Arvecon and ISS. A significant reduction in the overall times of the analytical procedure and the reduction of consumables costs make this method extremely advantageous and open the way to the prospect of a further implementation which also includes other classes of xenobiotics such as Cannabinoids, NPS and pharmaceutical drugs.

Author statement

Table 4
Matrix effects and recoveries of the LC-MS/MS method for all target analytes.

Analyte	Matrix Effect (%)		Recovery (%)	
	Low	High	Low	High
AMP	26.0	23.0	75.3	79.2
BEG	−38.0	−16.0	37.0	38.2
BUP	83.0	31.0	78.0	80.3
CE	–32.0	−6.8	53.0	55.0
COC	−25.0	−14.0	45.0	48.0
COD	14.0	17.7	71.0	75.0
DHC	−10.0	9.8	74.0	84.2
EDDP	125	75	77.0	79.2
PHC	–23.0	13.5	91.0	92.3
HC	−14.0	−0.3	45.0	48.2
HM	7.7	2.5	54.0	55.0
KET	−4.0	−4.7	93.0	94.0
6-MAM	34.0	13.6	74.0	79.0
MDA	7.9	6.0	73.0	75.2
MDE	−39.0	−8.7	79.0	86.2
MDMA	−16.0	−3.8	99.0	98.1
MTD	13.0	−0.2	90.9	95.0
METAM	21.0	14.0	87.9	88.5
MOR	5.0	10.0	71.0	75.0
NTAP	−13.0	−5.5	95.9	98.0
NBUP	−2.0	−8.2	70.0	75.0
NCOD	9.6	−3.2	70.5	77.0
NKET	−6.0	−12.4	78.5	82.0
NOC	−12.0	−3.1	60.0	65.0
OC	−1.0	5.2	61.0	63.0
OM	24.0	8.1	66.0	70.0
TAP	−2.8	−2.9	91.0	95.0
TRAM	−24.0	0.1	73.0	76.0
NTRAM	−41.0	−13.2	83.0	88.0
OTRAM	–23.0	−15.5	79.0	85.0
TROP	−19.0	−4.2	61.0	63.0
EtG	−25.0	–23.5	78.0	80.2
(±)-*cis*-3MeNFEN	6.7	5.2	85.2	89.0
(±)-*trans*-3MeNFEN	7.2	8.2	87.3	88.6
4-ANPP	−9.3	7.2	88.2	89.2
ACFEN	−5.3	1.2	91.3	96.0
ACNFEN	−8.2	−2.2	88.2	87.0
ALFEN	−3.2	−7.2	89.7	91.0
BUFEN	3.2	−0.5	91.2	93.5
BUFEN carboxy metabolite	−5.2	3.2	90.2	92.5
BUNFEN	2.5	7.2	95.3	96.8
CARFEN	5.7	4.5	96.2	99.0
CyclopropylFEN	−9.2	6.5	89.9	93.0
FEN	−6.0	−1.3	99.0	99.3
ISOBUFEN	−7.2	−5.2	90.0	96.0
MeACFEN	−9.3	−11.2	90.2	93.0
MeACNFEN	−10.2	−9.0	91.3	95.0
NFEN	7.3	−1.9	89.0	87.0
OCFEN	6.5	6.2	87.3	89.3
SUFEN	8.6	9.2	88.4	92.4
VAFEN carboxy metabolite	8.8	5.6	91.2	95.0
βOHFEN	4.5	7.2	87.3	88.9
βOHTHIOFEN	−5.3	6.2	87.4	88.9
2F-o-F3MeFEN	−7.0	−2.1	88.4	95.2

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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