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A new LC-MS/MS confirmation method for the determination of 17 drugs of abuse in oral fluid and its application to real samples

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ABSTRACT

A new liquid chromatography–tandem mass spectrometry (LC–MS/MS) confirmation method for the direct analysis of 17 drugs starting from 200 μL of diluted oral fluid (OF), in a single chromatographic run, was developed and validated. Cocaine, benzoylecgonine (BEG), cocaethylene, Δ-9-tetrahydrocannabinol (Δ-9-THC), buprenorphine, 6-acetylmorphine (6AM), morphine, codeine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), 3,4-Methylenedioxy-N-ethylamphetamine (MDE), ketamine, N-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine (MBDB) were determined in a chromatographic run of 12 min only with no sample pre-treatment, after the addition of 15 different internal standards (ISs). The method met all requirements in terms of linearity, accuracy (precision and trueness), recovery, and stability requested by FDA guidelines. Carry-over and interferences were negligible, as well as the matrix effects. LLOQs are below the limits defined by European guidelines and Italian national laws. The original oral fluid collections are stable at least six months at −20 °C and one week at +4 °C.

1. Introduction

Drug of abuse represent a worldwide problem. Illicit drugs use may lead to sub-optimal or erroneous driving behavior resulting in accidents and sometimes in death of the involved subjects. In such cases or in routine road checks carried out by the police consider the collection of a salivary sample, which is analyzed with first level screening methods. These semi-quantitative results must be confirmed with confirmatory techniques, such as liquid chromatography combined with mass spectrometry. Oral fluids (OF) has been introduced recently as a biological matrix suitable for *on-road* illicit drug determination because time course of drug in oral fluid may resemble that in plasma. Moreover OF can be considered a valid alternative specimen for confirmation testing as in OF drugs are excreted mainly as parent compound [1–4]. In fact police officers, without medical supervision, cannot use invasive methods while are authorized to collect OF samples. In new Italian traffic laws, OF has been considered as an alternative biological specimen for the determination of drug of abuse [5]. A very comprehensive review about analysis of DOA in OF is the paper published by Reinstadler et al. [6].

A number of studies have been published that highlight how both the sample treatment process [7] and the use of hyphenated instrumentation [8,9] are important in order to obtain analytical performances that satisfy the current regulations in terms of sensitivity, selectivity and fast confirmatory analysis.

The aim of this study is the development and validation of a quantitative (and confirmatory) method for the analysis of the major substances of abuse founded nowadays during checks by law enforcement, on a salivary sample with LC–MS/MS systems, with a minimal sample pre-treatment, in a single chromatographic run, and with analytical performances able to respect the actual laws.

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To meet these needs, the "*dilute-and-shot*" approach is applied in this work. The method considers only the sample dilution and the subsequent analysis in LC–MS/MS through the acquisition in SRM mode with two different transitions (one for qualitative purposes and one for quantitative purposes).

2. Materials and methods

2.1. Chemicals and reagents

The chemicals (cocaine, BEG, cocaethylene, Δ-9-THC, buprenorphine, 6AM, morphine, codeine, methadone, EDDP, amphetamine, methamphetamine, MDMA, MDA, MDE, ketamine, MBDB) and the deuterated internal standards (IS, cocaine d3, benzoylecgonine d8, cocaethylene d3, THC d3, buprenorphine d4, 6-acetylmorphine d6, morphine d3, codeine d6, methadone d9, EDDP d3, amphetamine d11, methamphetamine d11, MDMA d5, MDA d5, MDE d5), all with purity ≥99%, were purchased from Sigma-Aldrich (St. Louis, MO).

Water, acetonitrile (AcN), formic acid, ammonium formiate and methanol (all LC/MS-grade) were purchased from Carlo Erba Reagents (Milan, Italy), and used without further purification.

2.2. Samples collection and treatment

Six drug-free samples were obtained from healthy voluntary laboratory staff by mean of Quantisal® oral fluid collector from Alere (Scorzé, Italy). These devices are actually used and approved by Italian police officers during *on-site* patrols and samples are either send to authorized laboratory for the analysis or directly analyzed *on-site* with screening methods. Immediately after the oral fluid sample collection by means of the collection pad, it is completely introduced in the test tube, finds itself immersed in the 3 mL of buffer, and remains there until the analysis. In addition, in this case, however, the collection pad always remains immersed in the solution.

The analytical procedure (based on the "*dilute-and-shot*" approach) consists of a simple dilution of 200 μL of diluted oral fluid sample (1:4, *v:v*), in a 1.5 mL polypropylene conical tube, with 200 μL of Internal Standards containing 15 different drug of abuse labeled with stable isotopes. After the centrifugation for 10 min at 14,000 × *g*, the supernatant is transferred in glass vials and then 2 μL were directly injected in LC–MS/MS system. All reported values are referred to the final diluted sample injected into the LC–MS/MS instrumentation. Specifically, the elaborated instrumental signal is referred to the diluted sample, while the concentration levels used for the validation are referred to the original oral fluid collection concentrations, as commonly is performed in bioanalytical chemistry during quantitative analysis.

2.3. Preparation of spiked OF samples and calibration curves

Methanolic solutions containing 1 mg/mL of each drug standards were used. Calibration curves and internal quality controls (QCs) were prepared by adding the appropriate amount of each standards to 1 mL of blank OF, to obtain the concentrations listed in Table 1.

2.4. LC–MS/MS equipment and method

LC–MS/MS assays were performed on a Sciex 4500 triple quadrupole mass spectrometers coupled with UHPLC module Nexera composed by a binary pump, degasser, column oven, autosampler from Sciex, part of Danaher (Washington, D.C., USA). The analyses were performed on a Hypersil PFP Gold column (50 × 2.1 mm, 1.9 μm particle size) purchased from Thermo Fisher Scientific (Waltham, Massachusetts,). Column temperature was set at 40 °C (±1 °C) and the injection volume was 2 μL. Mobile phase M1 and M2 were Millipore water (Millipore, Sigma, Milano, Italy) with 0.1% formic acid and 50:50 methanol/AcN with 0.1% formic acid, respectively. The starting conditions for the gradient elution were with a 0.4 mL/min flow rate. The total run time was 12 min. The gradient elution program was as follows: 0–8 min, linear decrease from 95 to 25% A; 8–8.1 min, 0% A; 8.1–10 min, 0% A, 10–10.1 min, 95% A, 10.1–12 min, 95% A.

MS analysis was performed using an electrospray source ionization (ESI source) operating in positive mode. Analytes and deuterated IS were detected using multiple reaction monitoring (MRM) of the specific transitions shown in Table 2. Data were acquired in a selective reaction monitoring (SRM) mode, cycle time 10 ms. Ion spray voltage was set at 5.4 kV for positive polarity. The vaporizer temperature was 450 °C. For collision-induced dissociation (CID), high-purity nitrogen was used. Data processing was performed using the Analyst software version 1.6.2 obtained from Sciex, part of Danaher (Washington, D.C., USA).

Table 1
Calibration curves, QC levels, LLOQ, ULOQ.

	R^2	6 points calibration curve ng/mL	L-QC ng/mL	H-QC ng/mL	LLOQ ng/mL	Recommended maximum cut-off values for confirmation tests in oral fluid (ng/mL) [ref. 13]	ULOQ ng/mL
Cocaine	0.995	0.5	10	30	0.5	8	50
6 AM	0.997	5				2	
Buprenorphine	0.997	15				1	
Δ9-THC	0.995	25				2	
BEG	0.995	40				8	
		50					
Amphetamine	0.994	5	75	180	**1**	15	250
MDA	0.998	50				15	
MDE	0.998	100				15	
MDMA	0.993	150				15	
Methadone	0.993	200				20	
Methamphetamine	0.994	250				15	
Morphine	0.993					15	
MBDB	0.993					15	
Ketamine	0.993					no cut-off available	
Codeine	0.994					15	
Cocaethylene	0.991					no cut-off available	
EDDP	0.996					no cut-off available	

Table 2
Analytes and associated deuterated internal standards (IS), MRM transitions, collision energies, and retention times.

Drug/IS	Associated IS	Parent Ion m/z	Product Quantifier Ion m/z	Product Qualifier Ion m/z	Collision energy Volts	Retention time min
Cocaine	Cocaine d3	304.2	182.2	105.2	27/34	6.0
6 AM	6 AM d6	328.0	165.0	211.2	53/38	3.9
Buprenorphine	Buprenorphine d4	468.2	396.1	414.3	53/48	7.8
Δ9-THC	THC d3	315.2	193.2	123.2	33/45	8.6
BEG	BEG d8	290.1	168.1	105.0	25/44	4.2
Amphetamine	Amphetamine d11	136.1	91.0	119.0	27/13	3.7
MDA	MDA d5	180.2	133.1	135.1	25/27	4.1
MDE	MDE d5	208.2	163.1	135.1	29/25	5.1
MDMA	MDMA d5	194.2	163.0	105.0	18/34	4.5
Methadone	Methadone d9	310.2	265.1	223.1	22/30	8.9
Methamphetamine	Methamphetamine d11	150.1	119.1	91.0	16/29	4.2
Morphine	Morphine d6	286.1	165.0	181.0	60/48	1.8
MBDB	MDE d5	208.2	135.2	177.0	31/15	5.4
Ketamine	MDE d5	238.0	125.0	179.1	38/25	5.3
Codeine	Codeine d6	300.1	165.1	153.1	64/62	3.4
Cocaethylene	Cocaethylene d3	318.1	197.1	151.0	28/35	6.8
EDDP	EDDP d9	278.1	234.0	249.1	42/33	8.0
Cocaine d3	–	307.1	185.0	–	28	6.0
6 AM d6	–	334.2	211.0	–	40	3.9
Buprenorphine d4	–	472.2	400.2	–	55	7.8
THC d3	–	318.2	196.2	–	32	8.6
BEG d8	–	298.0	171.0	–	27	4.2
Amphetamine d11	–	147.0	98.0	–	29	3.7
MDA d5	–	185.0	138.0	–	26	4.1
MDE d5	–	213.0	163.0	–	20	5.1
MDMA d5	–	199.0	165.0	–	20	4.5
Methadone d9	–	319.3	268.0	–	22	8.9
Metamphetamine d11	–	161.1	127.2	–	16	4.2
Morphine d6	–	292.0	181.0	–	50	1.8
Codeine d6	–	306.0	165.2	–	60	3.4
Cocaethylene d3	–	321.2	199.0	–	28	6.8
EDDP d9	–	281.2	234.0	–	42	8.0

2.5. Method validation

The method was validated in accordance with the FDA guidelines for bioanalytical method validation [10,11]. The validation testing of selectivity, carry over, calibration curve, accuracy (precision and trueness), stability, dilution integrity was performed. Selectivity and intra-matrix variability were evaluated by analyzing oral fluid from six healthy volunteers who do not use drugs of abuse. For the analytes identification, the following criteria should be met: Consistency of retention time and the presence of at least two transitions (one qualifier ion and one quantifier ion) (see Table 2) in MRM mode and their relative intensities. For the identification of an analyte, retention times should not vary more than ±2.5%, while the relative ion intensities should not vary more than ±20%.

Carry over was assessed by injecting six blank samples after repeated injections of the highest calibrator sample (C5). Dilution integrity was determined by diluting (1:2, *v:v*) a sample, above the highest calibrator (C5), which was almost twice the C4 calibrator. Accuracy and imprecisions were calculated based on six diluted samples per analyte. Within- and between-run accuracy (precision and trueness) were evaluated by analyzing five replicates of QC samples, low QC (L-QC) and high QC (H-QC), in a single run and from five runs analyzed on five different days, respectively. Precision and trueness were calculated as the mean measured concentration *versus* the nominal concentration (%) and relative bias. Matrix effects (ME) and recoveries (RE), were evaluated according to Matuszewski et al. [12], based on blank samples collected from 6 different individuals. ME was calculated as follow:

$$\frac{B}{A} \cdot 100\%$$

where B corresponds to the peak areas for each compound spiked to a blank matrix extract (spiking after extraction) and A the peak areas for analytes in standard solution. RE was calculated as follow:

$$\frac{C}{B} \cdot 100\%$$

where C represents the peak areas of analytes in the spiked sample extract (spiking before extraction).

The LOD was defined as the concentration value giving a signal to noise ratio > 3 for all the transitions considered for each substance. This parameter was determined by mean of scalar dilutions of the substances of interest in OF in five samples, analyzed in five different days. The linearity of the method for each compound was studied in the range from the LOD of each substance to 50 or 250 ng/mL, depending on the analyte (see Table 1), performing five analyses for each concentration level. Calibration curves were built by linear regression of the peak area ratio of each substance with respective IS *versus* the concentration of analyte. Stability was evaluated as percentage difference from expected values using L-QC and H-QC samples in triplicates, analyzed against a fresh calibration curve after one week at 4 °C and six month at −20 °C. Extracted L-QC and H-QC stored in autosampler were also analyzed after 3 days to assess the stability until injection. Freeze-thaw stability of calibration curve and QC samples in oral fluid was also evaluated over three freeze-thaw cycles at −20 °C up to six months period.

3. Results and discussion

An LC–MS/MS method for the determination of 17 different drugs of abuse, in oral fluid samples, was successfully developed and validated according to FDA guidelines for bioanalytical method validation.

The method was considered to be selective: Analyzing six blank samples there were no unexpected endogenous interferences >20% of LLOQ for all drugs, nor >5% of the IS signals. Peak

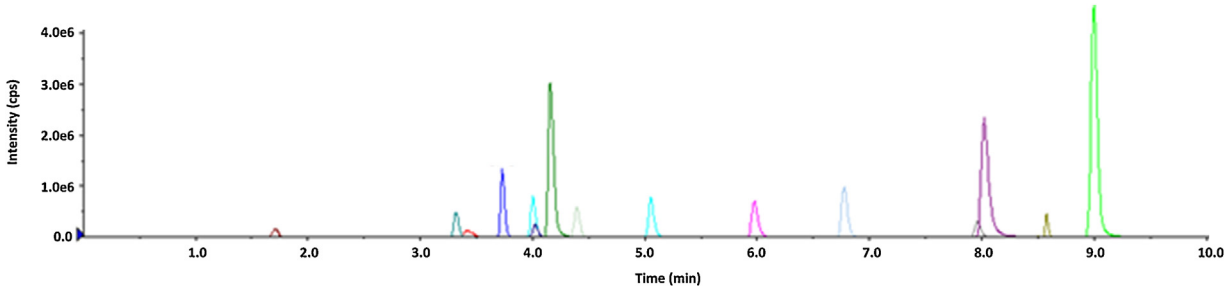

Fig. 1. Transition chromatograms of a blank OF with internal standards.

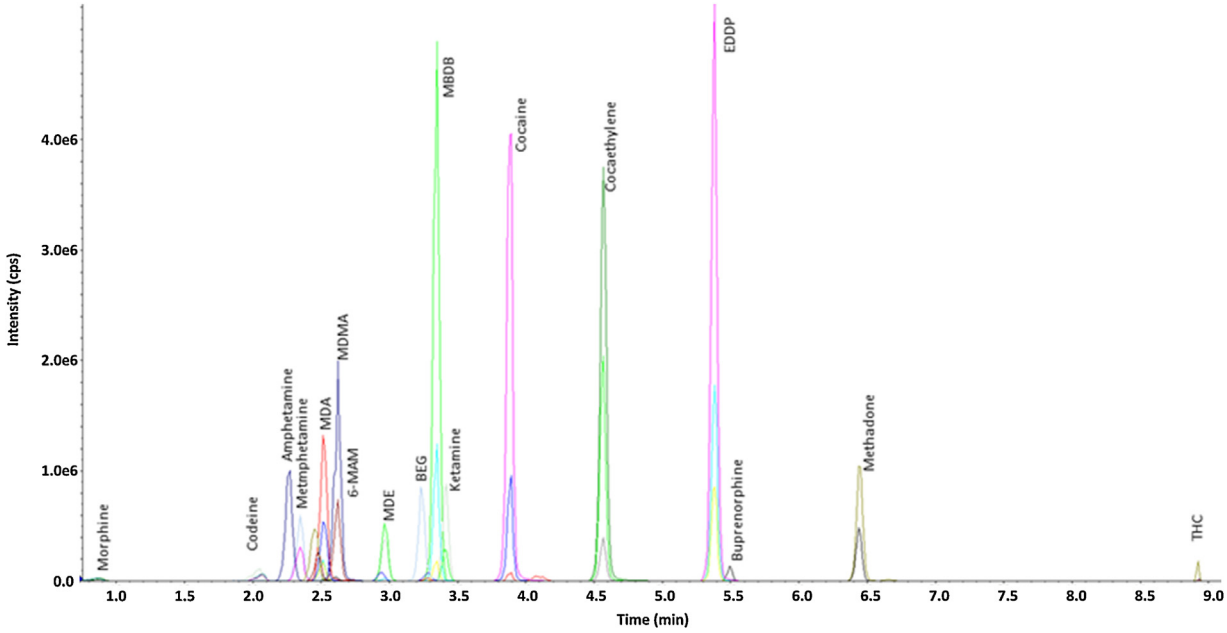

Fig. 2. Spiked sample with all the substances investigated at a concentration of level high concentration.

identification was consistent in all the runs according to the parameters described in paragraph 2.5 (Method validation). Two representative chromatograms are shown in Fig. 1 (blank sample with IS) and in Fig. 2 (spiked sample).

Recoveries from fortified samples as it relates to ion suppression and matrix effects in the LC–MS/MS system were comprised between 76.6–115.3% and 76.6–112.6% respectively. No matrix effects were found (Table 3). These values show that there are no significant matrix effects or ion suppression in the procedure shown here, and that the values obtained comply with the limits set out in the International Guidelines.

The method was linear for all drugs with $R^2 \geq 0.99$ in all cases (Table 1). Intra-assay (CVs variable from 1.04 to 8.3%) and inter-assay (CVs variable from 3.37 to 10.14%) reproducibility analyses demonstrated trueness and precision within the acceptance criteria (Table 4).

Carry-over and interferences were negligible. Lower limits of quantitation are below the limits defined by international guidelines [13] and Italian national laws [5] (see Table 1). Samples were stable at least six months at −20 °C and one week at +4 °C as all concentration values varied less than 15%.

The method was applied to real OF samples collected during random traffic controls. In all these cases, the identification criteria were fulfilled. Fig. 3 shows a real OF samples containing three of the herein considered drugs of abuse, specifically: cocaine (upper trace), benzoylecgonine (middle trace) and cocaethylene (bottom trace).

Table 3
Matrix effect (ME%) and recovery (RE%).

	ME (Cv%)		RE %	
	L-QC	H-QC	L-QC	H-QC
Cocaine	108.9	111.6	105.4	115.3
6 AM	94.7	97.8	103.7	91.3
Amphetamine	109.7	106.7	81.4	86.4
Buprenorphine	91.8	100.3	96.9	98.7
MDA	92.3	90.5	89.1	91.4
MDE	102.1	103.4	88.3	90.1
MDMA	105.9	101.4	95.9	88.8
Methadone	97.2	95.4	96.0	94.1
Methamphetamine	112.6	99.2	85.3	92.4
Morphine	98.3	99.6	76.6	82.2
MBDB	104.6	103.4	86.9	84.8
Ketamine	111.4	102.4	86.0	84.3
Δ9-THC	76.6	78.6	96.3	87.9
BEG	93.7	111.0	87.4	92.4
Codeine	88.8	89.5	97.2	98.6
Cocaethylene	100.6	99.8	91.2	89.8
EDDP	90.1	94.1	93.9	90.4

Although a number of similar methods has been published, their main drawback is related to the use of SPE purification methods (or other preconcentration procedures) to achieve the desired sensitivity or the use of particular LC–MS instruments, which are not available in most laboratories or with very different sampling strategies [14–18].

Table 4
Intra-assay and inter-assay precision and trueness.

	CV% intra-assay (n = 5)		CV% inter-assay (n = 5)		trueness intra-assay (n = 5), bias %		trueness inter-assay (n = 5), bias %	
	L-QC	H-QC	L-QC	H-QC	L-QC	H-QC	L-QC	H-QC
Cocaine	3.45	2.25	9.37	7.56	14.29	6.66	14.29	8.78
6 AM	8.3	2.91	8.14	4.33	8.57	5.01	12.60	6.18
Amphetamine	3.65	2.10	8.25	3.37	5.66	8.91	5.66	8.91
Buprenorphine	5.86	5.34	10.06	7.00	5.18	5.38	10.77	4.97
MDA	5.93	3.44	6.23	3.98	8.73	4.87	9.74	5.13
MDE	4.17	5.74	5.60	7.67	6.81	2.55	9.69	4.11
MDMA	3.16	3.49	9.72	9.06	9.06	5.02	11.44	8.14
Methadone	1.71	2.48	4.11	4.31	5.58	3.46	7.01	4.77
Methamphetamine	1.59	2.96	6.52	6.67	8.88	7.53	8.89	9.71
Morphine	5.93	5.15	9.34	8.21	12.68	5.48	12.64	9.62
MBDB	3.01	1.04	6.71	4.95	9.12	9.63	11.13	11.53
Ketamine	4.50	4.70	5.29	6.51	9.44	9.67	10.11	11.17
Δ9-THC	4.96	4.27	8.41	6.50	7.08	5.88	8.60	8.36
BEG	2.70	4.00	9.15	6.28	13.21	6.10	13.40	8.81
Codeine	1.93	1.32	7.03	6.04	1.94	1.05	8.79	7.32
Cocaethylene	4.80	3.91	10.14	6.34	6.11	2.37	10.76	6.67
EDDP	2.64	3.35	6.07	8.02	2.43	2.87	9.33	6.31

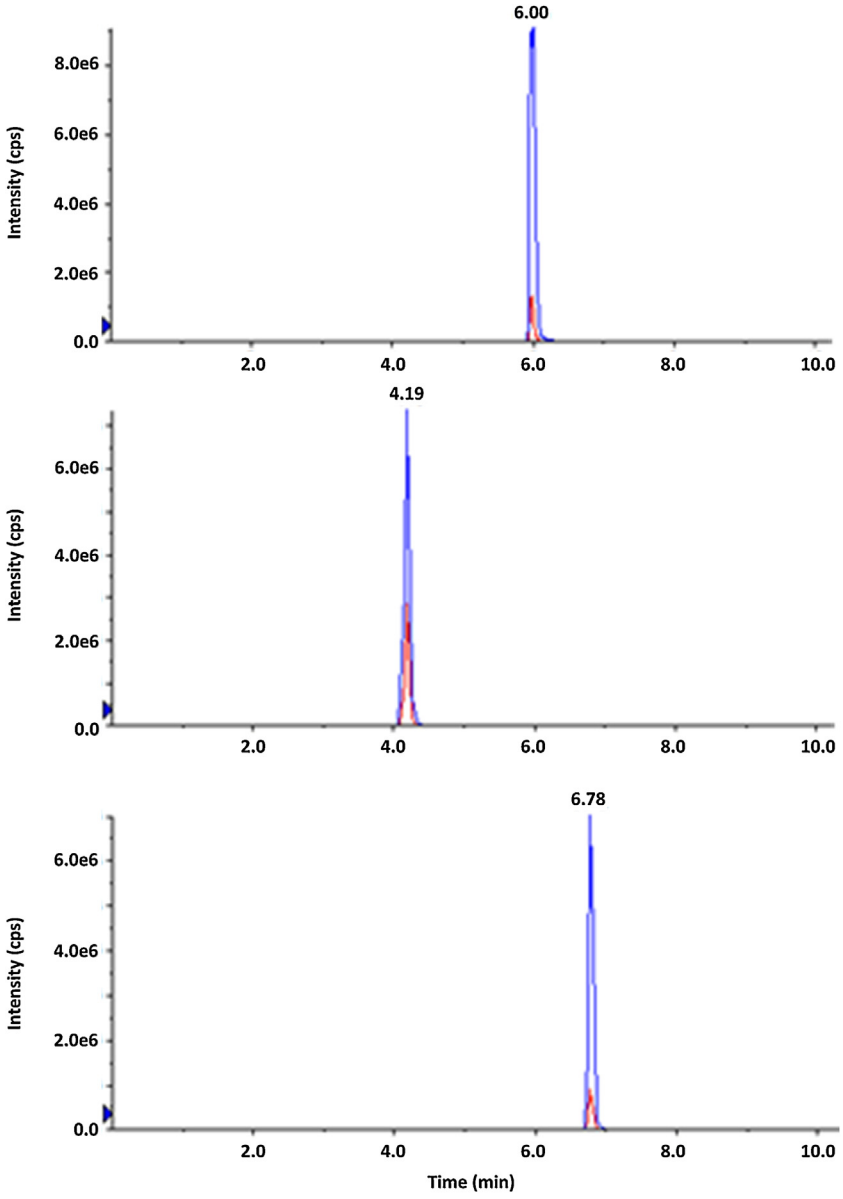

Fig. 3. Real OF samples showing the presence of cocaine (upper trace), benzoylecgonine (middle trace) and cocaethylene (bottom trace).

Moreover, the developed method is the more robust as it consider the use of 15 different IS compared to previous papers [14–18]. The use of 15 different IS allows to better control the whole analytical procedure, in addition to correct the analyte signal in case of anomalous fluctuations of both the instrumental response and the "*dilute and shoot*" procedure (which in itself reduces to minimum the possibility of sources of variability).

4. Conclusions

The proposed method allows the separation, identification, and quantification of 17 different drugs of abuse in oral fluid in a single chromatographic run with good sensitivity, linearity, and with a very short time of analysis. One of the main advantages of the herein validated method is that the sample preparation is minimal (reducing the possibility of sources of variability), hence the volume of oral fluids needed for the analysis is very small allowing, if necessary, the analysis of the same sample used for the screening test. Additionally, the herein reported procedure, based on the "*dilute-and-shot*" approach could be a valuable starting point to the development of other innovative procedures with minimal sample handling, reducing the analyte(s) loss and reducing the possible interferences/matrix effects.

The method can be a valid support for police officers, forensic doctors, and forensic toxicology laboratories in confirming the presence of classes of drugs of abuse in oral fluids after positive roadside screening tests, thanks to non-invasive (oral fluid), and reproducible sampling procedures that require low sample quantities.

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CRediT authorship contribution statement

Elisa Bassotti: Methodology, Validation, Supervision. **Giuseppe Maria Merone:** Data curation. **Annachiara D'Urso:** Data curation, Formal analysis. **Fabio Savini:** Conceptualization, Writing - original draft. **Marcello Locatelli:** Conceptualization, Writing - original draft. **Angela Tartaglia:** Data curation. **Paolo Dossetto:** Conceptualization, Writing - original draft. **Cristian D'Ovidio:** Conceptualization, Writing - original draft. **Ugo de Grazia:** Project administration, Formal analysis, Conceptualization, Writing - original draft, Investigation.

Declaration of Competing Interest

Elisa Bassotti is employee at Eureka Lab Division. Paolo Dossetto is employee at Sciex. The other authors declare that does not exist any economic interest or any conflict of interest.

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