



Simultaneous determination of 20 drugs of abuse in oral fluid using ultrasound-assisted dispersive liquid–liquid microextraction

P. Fernández¹ · M. Regenjo¹ · A. Ares² · A. M. Fernández³ · R. A. Lorenzo² · A. M. Carro²

Received: 26 July 2018 / Revised: 9 September 2018 / Accepted: 10 October 2018 / Published online: 25 October 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Drugs of abuse and new psychoactive substances (NPS) for recreational purposes are in constant evolution, and their consumption constitutes a significant risk to public health and road safety. The development of an analytical methodology to confirm the intake of illicit drugs in biological fluids is required for an effective control of these substances. An ultra-performance liquid chromatography–tandem mass spectrometry method (UPLC-MS/MS) was developed for simultaneous determination of 10 synthetic cathinones and 10 illicit drugs in oral fluid easily sampled through non-invasive maneuvers. The UPLC-MS/MS method was coupled to an ultrasound-assisted dispersive liquid–liquid microextraction (US-DLLME), which is a miniaturized and inexpensive technique that uses reduced volumes of solvents and samples. The US-DLLME was optimized by using a $2^1 3^4 4^1 // 18$ asymmetric screening design and a Doehlert design. Sample volume, dispersion and extraction solvent volumes, pH, US time, and amount of sodium chloride were evaluated. The US-DLLME-UPLC-MS/MS method was validated according to international guidelines. Limits of quantitation (LOQs) ranged from 0.25 to 5 ng mL⁻¹, and the linear range spanned from LOQ to 500 ng mL⁻¹ with R^2 higher than 0.9907, for most of the target drugs. Precision ranged from 1.7 to 14.8 %RSD. Accuracy, i.e., extraction recovery, ranged from 74 to 129%. The proposed method was successfully applied to the analysis of 15 samples from patients on a drug detoxification program.

Keywords Drugs of abuse · Oral fluid · Scopolamine · Synthetic cathinones · UPLC-MS/MS · Ultrasound-assisted dispersive liquid–liquid microextraction

Introduction

According to a report issued in 2016 by the European Monitoring Center for Drugs and Drug Addiction

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00216-018-1428-5>) contains supplementary material, which is available to authorized users.

✉ R. A. Lorenzo
rosaantonia.lorenzo@usc.es

✉ A. M. Carro
tuchi.carro@usc.es

¹ Forensic Toxicology Service, Faculty of Medicine, Institute of Legal Medicine, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

² Department of Analytical Chemistry, Faculty of Chemistry and Health Research Institute of Santiago de Compostela (IDIS), University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

³ Drug Addiction Assistance Unit, Monforte de Lemos, 27400 Lugo, Spain

(EMCDDA), the drug market has increased in complexity due to the availability of new psychoactive substances (NPS) via the internet, their more potent stimulant effects compared to already consumed drugs, the new polydrug use patterns, and lack of regulation. The scarce information on NPS and the high-risk drug use has become a threat to public health and driving safety and justifies the need for fast and reliable control policies and methodologies [1, 2]. Synthetic cathinones are the second largest group of NPS monitored by EMCDDA. Since they were first detected in Europe in 2004, 103 new cathinones have been identified: 31 in 2014 and 26 in 2015 [1]. They are generally sold as “legal” substitutes for stimulant drugs such as amphetamine, MDMA, and cocaine [1, 3].

Recently, a non-recreational drug, scopolamine, is gaining popularity because of its hallucinogenic effects from misuse to sedate and manipulate the victims of robberies or rapes [4].

Oral fluid (OF) screening test is ideal for detecting recent intake of drugs in situations such as traffic offenses, accidents, or sexual assault. The OF test requires a small sample that is collected under direct supervision, reducing possible adulteration and reflecting recent drug use. The drugs most

commonly screened are THC (marijuana), opiates, cocaine, amphetamines, methamphetamine, ecstasy, and PCP (phencyclidine) [5]. Furthermore, the different OF collection devices are intended to screen for the presence of drugs and drug metabolites in saliva with predetermined concentration thresholds, but a more exhaustive quantitative analysis has to be carried out in the laboratory. Screening methods are limited tools for identifying positive samples in the analysis for NPS on biological fluid, and more selective and sensitive techniques, such as chromatographic methods, are required for a confirmatory purpose [6–8]. Gas chromatography–mass spectrometry (GC-MS) is the technique used to separate and determine drugs of abuse such as methamphetamine cannabinoids in OF. To avoid the derivatization step before analysis, some designer stimulants from OF are analyzed in liquid chromatography–tandem mass spectrometry (LC-MS/MS), including some cathinones and cannabinoids [8–10], amphetamines [11], and amphetamines and synthetic opioids [12, 13]. The extraction of NPS from OF is generally carried out using solid phase extraction (SPE) [8, 14] and liquid–liquid extraction (LLE) [7, 13, 15]. However, an important consideration in the use of OF is the limited sample availability and the possibility to find drug concentrations in trace levels. These challenges have motivated the need for a deepening in the development of miniaturized sample preparation strategies. The increasing use of NPS and the adverse effects on human health have led to the development of microextraction methods for the sensitive determination of these emerging drugs in alternative biological matrices of toxicological interest. Dispersive liquid–liquid microextraction (DLLME) was developed by Assadi et al. in 2006 and is based on a ternary system of solvents in which the extraction and dispersion solvents are rapidly injected into the aqueous sample with a syringe. After gentle stirring of the mixture, a turbid solution is formed, and the analytes are extracted into a droplet obtained by centrifugation [16]. Different classes of substances such as narcotics, drugs of abuse, hallucinogens, cannabinoids, benzodiazepines, and pollutants have been determined using DLLME in forensic control applications [17, 18]. Nevertheless, to the best of our knowledge, there is no information concerning the optimization of ultrasound-assisted US-DLLME and UPLC-MS/MS method for the determination of the drug of abuse and NPS selected in this study on OF.

The primary aim of this work is the development and validation of an analytical methodology for multianalyte determination of 20 drugs on oral fluid, including NPS, such as 10 cathinones, scopolamine, and drugs used in substitution therapy programs by US-DLLME and UPLC-MS/MS. For the first time, important variables influencing the US-DLLME procedure, such as sample volume, dispersion solvent volume, extraction solvent volume, ultrasound time, amount of NaCl, and pH, were tested and optimized by experimental designs.

Materials and methods

Reagents and standards

All the standards of 20 drugs (1 mg mL^{-1}), morphine (MOR), naloxone (NAL), methylone (MET), ethylcathinone ephedrine (ETCATEP), ethylcathinone (ETCAT), scopolamine (SCO), ethylone (ETH), 6-monoacetylmorphine (6-MAM), methylephedrine (METEP), butylone (BUT), mephedrone (MEP), pentadone (PENT), benzoylcegonine (BEG), cocaine (COC), methylenedioxypyrovalerone (MDPV), cocaethylene (COCET), pyrovalerone (PYR), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), buprenorphine (BUP), and methadone (METHA); and the 15 internal standards (IS) ($100 \text{ } \mu\text{g mL}^{-1}$) MOR d6, NAL d5, ETCATEP d5, ETH d5, 6-MAM d3, METEP d3, BUT d3, MEP d3, BEG d8, COC d3, COCET d8, MDPV d8, EDDP d3, BUP d4, and METHA d9 were purchased from Cerilliant (Round Rock, TX, USA). LC-MS–grade solvents of acetonitrile (ACN), methanol (MeOH), acetone, isopropanol, chloroform, carbon tetrachloride, ethylene tetrachloride, chlorobenzene, dichloromethane, dichloroethane, and other chemicals of analytical-reagent grade (potassium hydroxide, sodium hydroxide, potassium dihydrogen phosphate, ammonium hydroxide, formic acid, and acetic acid) were obtained from Merck (Darmstadt, Germany). Milli-Q water used in the study was obtained from Millipore water system (Bedford, MA, USA).

The individual standard stock solutions of drugs are commercially available methanolic solutions (1.0 mg mL^{-1}) and $100 \text{ } \mu\text{g mL}^{-1}$ for the ISs (see Electronic Supplementary Material (ESM) Table S1). Mixed working standard solutions were freshly prepared by appropriate dilution of the stock mixed standard solutions in mobile phase (A, 0.1% formic acid in water; and B, 0.1% formic acid in acetonitrile, 90:10, v:v) for the concentrations required. Calibration standards and quality control (QC) samples were prepared freshly using drug-free matrix blank fortified OF. The spiking solutions for calibration standards were prepared to obtain working solutions ($0.5\text{--}100 \text{ ng mL}^{-1}$). The deuterated standards used as IS were prepared (25 ng mL^{-1}) by proper dilution of the standard solutions. QC samples were prepared at three concentration levels (low, middle, and high) by adding the calculated amount of the corresponding stock or working solution. All standard solutions were stored at $4 \text{ } ^\circ\text{C}$ before analysis.

OF samples

During the development and validation of the procedure, analysis of drug-free blank samples of OF was performed. OF samples were collected from healthy volunteers using Salivette® devices (Sarstedt, Germany). The device contains a cotton swab which is introduced directly into the mouth. The swab should not be touched with the fingers and it should be

gently chewed and rolled around in the sample donor mouth for 2 min. The swab is transferred to a tube for centrifugation and stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

All studies were performed in accordance with the Autonomic Committee of Research Ethics of Galicia (Spain) and the principles of the Helsinki Declaration [19].

Instrumentation

An Acquity UPLC H-Class system coupled with the Xevo® TQD Triple Quadrupole Mass Spectrometry from Waters® (Milford, MA, USA) was used for chromatographic analysis. Data acquisition was performed using MassLynx V4.1 software (Waters). The UPLC was equipped with Waters autosampler thermostated at $10\text{ }^{\circ}\text{C}$. The analytical column Acquity UPLC™ BEH Shield RP 18 chromatographic column ($100\text{ mm} \times 2.1\text{ mm id}$, $1.7\text{ }\mu\text{m}$) operated at $55\text{ }^{\circ}\text{C}$ was used for chromatographic separation. The mobile phase (A) consisted of 0.1% formic acid in water, and mobile phase (B) consisted of 0.1% formic acid in acetonitrile, filtered through a $0.2\text{-}\mu\text{m}$ filter (Phenomenex, CA, USA). Chromatographic separation was performed at 0.4 mL min^{-1} flow rate in gradient mode and the program was as follows: 0–0.2 min, 90% solvent A; 3.5 min, 30% solvent A; and 4–5 min, 90% solvent A. The injection volume was $5\text{ }\mu\text{L}$ and the complete run time was 3 min. The mass spectrometer was operated in electrospray ionization (ESI) in positive ionization mode. Nitrogen was used as a nebulizer gas (50 L h^{-1}) and drying gas (1100 L h^{-1}). The source temperature was $120\text{ }^{\circ}\text{C}$, capillary voltage was set at 0.6 kV, and compound MS/MS parameters were optimized before the extraction of all individual drugs and ISs. The retention times (R_t), specific multiple reaction monitoring mode (MRM) transitions, cone voltage, collision energies for quantification and confirmation transitions, and IS are summarized in ESM Table S1. These parameters were determined by injecting the individual methanolic solutions ($1\text{ }\mu\text{g mL}^{-1}$) directly and in combined position using mobile phase, at a flow of $20\text{ }\mu\text{L min}^{-1}$ before developing the analytical method.

Oral fluid US-DLLME

In DLLME, $500\text{ }\mu\text{L}$ of OF samples was diluted with deionized water at a ratio of 1:4, v:v. Then, 1.4 mL of MeOH (protein precipitant and dispersant solvent) was added, mixed, and centrifuged for 5 min at 1640 rcf (3500 rpm). The supernatant was placed in a conical bottom glass tube, and 1.5 mL of water (pH 8.0) containing 0.1 g NaCl was added. Finally, $200\text{ }\mu\text{L}$ of CHCl_3 (extraction solvent) was rapidly injected into the previous solution by using a Hamilton syringe. A cloudy solution of droplets of CHCl_3 dispersed into the sample solution was formed. The tube was placed in an ultrasound bath for 5 min and centrifuged at 3500 rpm for 5 min. After centrifugation,

extraction solvent droplet was deposited in the bottom of the conical tube and collected with a syringe and transferred to a glass vial where it was evaporated to dryness under stream nitrogen at $40\text{ }^{\circ}\text{C}$. The sediment was reconstituted with $50\text{ }\mu\text{L}$ of the mobile phase, and $5\text{ }\mu\text{L}$ was injected into the UPLC-MS/MS.

Experimental designs

When only one factor at a time is varied and optimized, the classical univariate procedure one-variable at-a-time (OVAT) approach is applied. Alternatively, experimental design is an experimental strategy to simultaneously assess several factors at two or more levels in a reduced number of experiences performed. NemrodW® statistical software was used for the experimental design generation, the data evaluation, and to plot effects and response surfaces [20].

First, an asymmetrical or mixed-level factorial design ($2^1 3^4 4^1 // 18$) was used to screen six variables at different numbers of levels. The full factorial design consists of all possible combinations of the factors levels; that is $2 \times 3 \times 3 \times 3 \times 3 \times 4 = 648$ experiments runs. In this asymmetrical screening design, the 18-experiment design was a part of the complete design [21]. One factor was studied at two levels, four factors at three levels, and one factor at four levels. The studied variables were as follows: b_1 , volume of sample (0.25 and 0.50 mL); b_2 , dispersant volume (0.5, 1, and 2 mL); b_3 , extractant volume (50, 100, and 200 μL); b_4 , US time (1, 3, and 5 min); b_5 , NaCl (0, 0.1, and 0.2 g); and b_6 , pH (7, 8, 9, and 10).

The responses of the design, as $y =$ peak area for each drug, were obtained from UPLC-MS/MS analysis of the mixture of 20 drugs. The response y is assumed to be related with the five factors according to the following model:

$$y = b_0 + b_1 / BA^{x_{BA}} \sum_{i=2}^5 b_i / BA^{x_{BA}} + b_i / CA^{x_{CA}} + b_i / CB^{x_{CB}} \\ + b_6 / BA^{x_{BA}} + b_6 / CA^{x_{CA}} + b_6 / DA^{x_{DA}} + b_6 / CB^{x_{CB}} \\ + b_6 / DB^{x_{DB}} + b_6 / DC^{x_{DC}}$$

Where, $A = 1$; $B = 2$; $C = 3$; $D = 4$ (factor levels)

The model describes the effects on the DLLME when replacing the level of a factor with respect to another. The terms x_{BA} are presence-absence variables. The coefficient $b_{1/21}$ defines the differential effect on the DLLME of the drugs of replacing 0.5 mL by 0.25 mL volume of sample. The constant term b_0 is the mean response for all the experimental runs. The experimental conditions of the design are provided as ESM (Table S2).

According to the results of the screening design, a response surface design, named Doehlert (uniform shell) design, was applied to evaluate two factors (dispersant volume and pH) in 9 experiments [21]. This experimental design is suitable for

the exploration of the quadratic response surface and the construction of a second-order polynomial model:

$$y = b_0 + \sum_{i=1}^2 b_i x_i + \sum_{i=1}^2 b_{ii} x_{ii}^2 + \sum_{i=1}^2 \sum_{j=1}^2 b_{ij} x_{ij}$$

where (y) is the calculated response function (the peak area obtained from UPLC-MS/MS analysis for each drug), x_i represents the factors, and b_0 ; b_i ; b_{ii} ; b_{ij} are the estimated coefficients. The constant term b_0 is the mean response for all the experimental runs. This design for two factors consists of the six vertices of a hexagon with three center points, requiring nine runs. Unlike other response surface designs, the factors were varied at different numbers of levels (one at three and one at five levels in the two-factor design). Dispersant volumes between 0.5 and 2.5 mL and pH values between 6 and 10 were evaluated. The experimental conditions of the Doehlert design are provided as ESM (Table S3). Desirability functions, based on partial Derringer functions [21], were included in the experimental design methodology to find the experimental optimal conditions of compromise so that each one of the responses is within an acceptable range. Each response was transformed to a dimensionless partial desirability function, d_i , which varies from 0 (undesirable response) to 1 (optimal response), without additional experiments. The overall objective function D representing the global desirability function is defined as the weighted geometric average of n individual desirability functions.

Method validation

Method validation was performed following FDA guidelines of the US Food and Drug Administration (FDA) [22, 23], the International Conference on Harmonization (ICH) [24], and Eurachem [25] in terms of selectivity, linearity, limits of detection (LOD) and limit of quantification (LOQ), intraday and interday precisions, accuracy, and matrix effect (ME). The selectivity was evaluated by comparison of chromatograms of six drug-free OF samples and the corresponding OF samples spiked with standards of the target drugs and IS. Linearity was assessed by constructing a calibration curve using fortified OF samples at concentration ranges between LOQ to 500 ng mL⁻¹ (seven concentration levels) of all analytes and internal standards. Linearity range for morphine was 50–1000 ng mL⁻¹. The individual drugs at each calibration level were injected in triplicate and plotting the ratio of the quantitative ion peak area for each compound to that for the IS against the corresponding drug concentration (ng mL⁻¹). The LOD and LOQ were calculated at a signal-to-noise ratio of 3 ($S/N=3$) and 10 ($S/N=10$), respectively.

Known levels of drugs were added to a blank OF to determine accuracy and precision. Precision was established through the evaluation of relative standard deviation (%)

RSD). Aliquots of the blank OF were fortified with three QC samples at, low level (5 ng mL⁻¹), medium level (25 ng mL⁻¹), and high level (100 ng mL⁻¹), within the linear range of the calibration curve of the analytes. Precision analyses were carried out using six replicates of each QC sample on the same day (intraday precision), and 16 replicates on 4 different days (interday precision). The concentration levels of QC for morphine were low level (50 ng mL⁻¹), medium level (100 ng mL⁻¹), and high level (500 ng mL⁻¹). Accuracy was evaluated as average trueness of the quantified standard mix concentration added to blank OF samples and as an absolute recovery that estimates the process efficiency (PE), which involves the matrix effect and recovery of the extraction by DLLME [26]. It was also performed at the same low-, medium-, and high-concentration QC samples and calculated according to the following formula:

$$R(\%) = C/A \times 100$$

where “C” represents the compound concentration measured experimentally in spiked OF samples before extraction and was used to calculate the ratio of the peak area for each target analyte to that for IS spiked before extraction; “A” represents the concentration of the analyte in the mobile phase and was used to calculate the ratio of the peak area for each compound to that for IS in the mobile phase.

The presence of ME was evaluated, and matrix-matched calibration curves were constructed in a blank OF extract. The matrix effects on analyte ionization were evaluated by the quotient of the slope of the matrix-matched curve and the slope of the standard calibration curve.

Results and discussion

US-DLLME optimization

The variables dispersant and extractant were initially optimized by OVAT procedure in triplicate. In all experiments, OF samples were spiked with 100 ng mL⁻¹ of drug standards and IS. Selection of disperser solvent was based on the miscibility of the disperser solvent with the aqueous phase and extraction solvent. Acetonitrile (ACN), acetone, isopropanol, methanol (MeOH), 0.1% formic acid in methanol, and 5% ammonium hydroxide in methanol (NH₃) were tested. As shown in ESM Fig. S1, the normalized chromatographic responses of each solvent demonstrated that extraction ability of methanol is suitable for the extraction of 14 of the analytes considered although for five cathinones and scopolamine, the best dispersant is methanol slightly alkalized, so the pH should be considered a factor to be studied, as explored below.

The selection of an appropriate extractant solvent is essential in US-DLLME. Extractant solvent should be denser than water, high extraction capability of target compounds, low water solubility, and low boiling point [26]. Based on these characteristics, chloroform (CHCl₃), dichloroethane

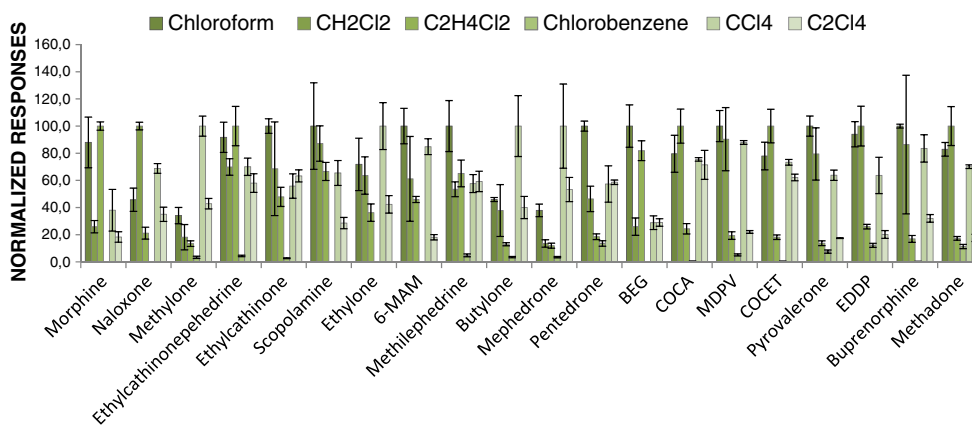


Fig. 1 Effect of different extractant solvents on the US-DLLME procedure ($n = 3$). Conditions: OF, 0.5 mL; without salt addition; pH, 7; dispersant solvent (MeOH) volume, 2 mL; ultrasound time, 5.0 min; centrifugation 1640 ref for 5.0 min

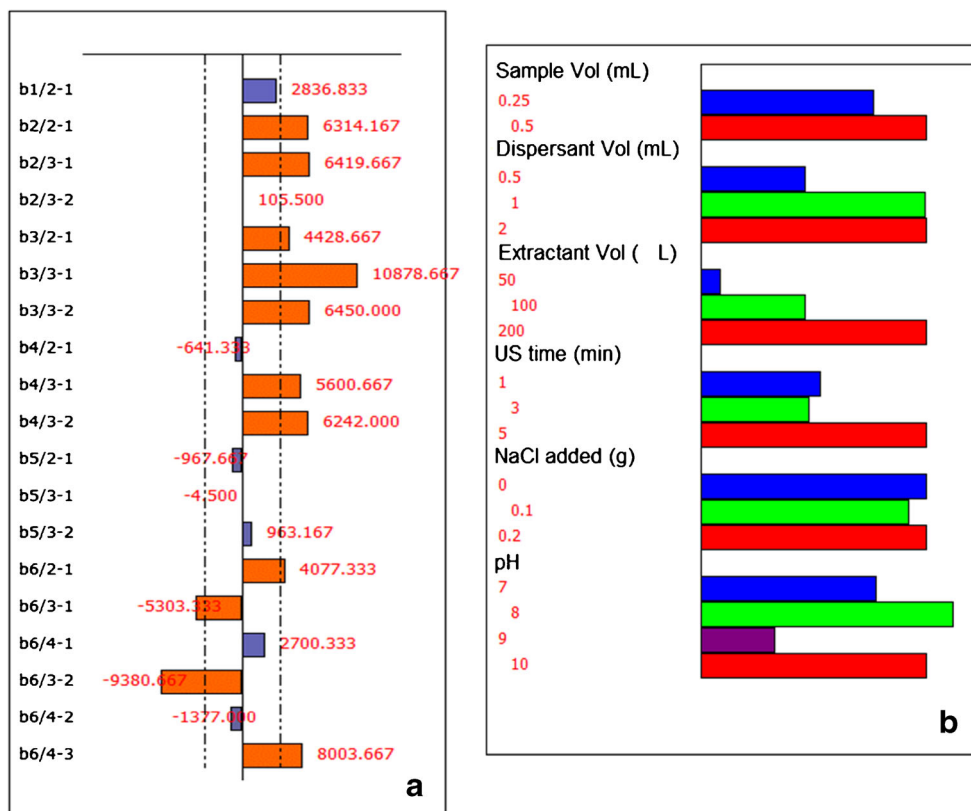
(C₂H₄Cl₂), dichloromethane (CH₂Cl₂), chlorobenzene (C₆H₅Cl), ethylene tetrachloride (C₂Cl₄), and carbon tetrachloride (CCl₄) were tested as extractant solvent. As shown in Fig. 1, chloroform proved the best results, except for some drugs such as naloxone, methylone, butylone, and mephedrone. The extraction of these analytes improved when optimizing other factors, such as the pH or the volume of the dispersant.

Optimization of multivariate parameters affecting US-DLLME process such as the dispersant and extractant volumes, sample volume, pH, ionic strength (g NaCl added), and US time was studied using experimental designs [21]. This

chemometric approach simultaneously assesses several factors at two or more levels in a reduced number of experiments.

First, an asymmetric screening ($2^1 3^4 4^1$) was applied to find the critical factors among the considered six. Analysis of variance (ANOVA) was evaluated, and the factors showed statistical significance (95% confidence level, $p < 0.5$) in US-DLLME for three drugs (pentedrone, cocaine, and cocaethylene). The effect of five variables, dispersant volume (b_2), extractant volume (b_3), US time (b_4), NaCl addition (b_5), and pH (b_6), was statistically significant for some of the drugs studied. By way of example, the significant factors for

Fig. 2 Delta weights (a) and total effects (b) plots for pentadrene



pentedrone are shown in delta weights (Fig. 2A) and total effects (Fig. 2B) [21]. The vertical dotted line on the delta weights plot specifies the most significant factors which affect the efficiency of the US-DLLME process. The length of each bar on total effects plot is proportional to the effect of that level of a factor on the extraction. As can be seen, dispersant and extractant volumes, US time, and pH have significant effects on US-DLLME, but sample volume (b_1) and NaCl addition do not have any significant influences. An extractant volume of 200 μL and 5 min of US were chosen for subsequent experiments because most analytes showed this same trend. A sample volume of 0.5 mL is also recommended for most of the compounds. Although the salt addition has no significant effect on US-DLLME, the influence of ionic strength (0.1 g of NaCl) is advantageous, especially, for morphine, methylene, ethylcathinone, EDDP, and methadone; and this value was kept constant for the next experiments. The results of the screening design did not clarify the most convenient values for dispersant volume and pH. For some drugs such as morphine and buprenorphine, 1 mL of the dispersant was better. Similarly, a pH of 9 was suitable to extract EDDP and 6-MAM, but for BEG, pH 7 was better. For these reasons, the optimal values of

dispersant volume and pH were evaluated by response surface methodology (Doehlert design) [21]. The experimental results were evaluated by ANOVA and regression was statistically significant, assuming a 95% confidence, for pH (b_1), dispersant volume (b_2), the effect of interaction between both factors (b_{12}), and the effect of quadratic terms pH^2 (b_{11}) and dispersant volume² (b_{22}). The response surface plots in Fig. 3 indicate that the effect of the two factors on US-DLLME is different for the drugs studied. As an example, three different trends are shown: both high pH and dispersant volume are required to extract morphine (Fig. 3A); medium–high pH values and low dispersant volume provide the microextraction of methylene (Fig. 3B); medium pH value and medium–low dispersant volume provided better results for ethylcathinone (Fig. 3C). The most probable explanation for these results is the different characteristics and properties of the compounds studied.

These results required a multicriteria decision analysis, based on desirability functions to predict the optimal conditions [21] suitable for the simultaneous extraction of all compounds (Fig. 3D). The best values obtained for the factors tested are pH 8 and 1.4 mL of dispersant solvent. The extraction efficiency improves when the acid–base equilibrium of

Fig. 3 Estimated response surfaces for morphine (a), methylene (b), and ethylcathinone (c). Global desirability function for all selected drugs (d)

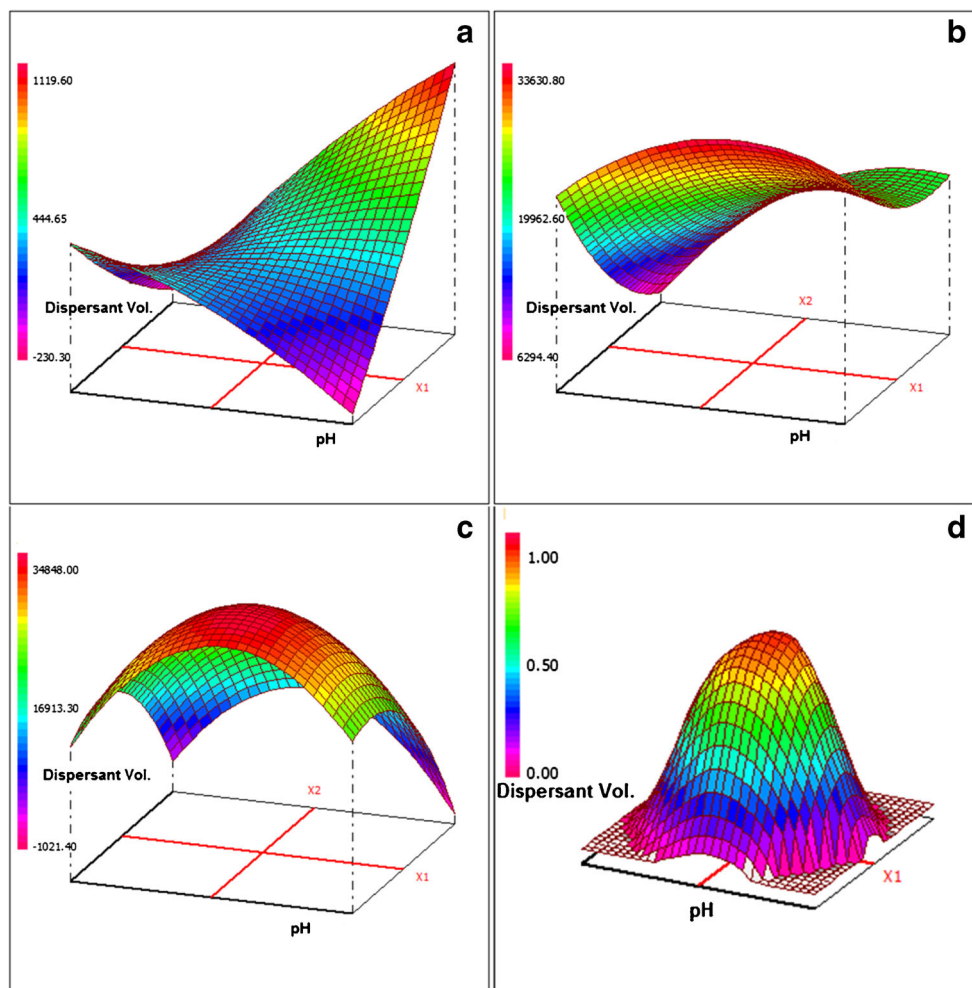


Table 1 Method performance obtained for the target drugs in terms of linearity range, limits, and precision in OF

Compound	Linear range (ng mL ⁻¹)	R ²	LOD (ng mL ⁻¹)	LOQ (ng mL ⁻¹)	%ME	Intraday precision %RSD (n = 6)		Interday precision %RSD (n = 16)			
						5 ng mL ⁻¹	25 ng mL ⁻¹	5 ng mL ⁻¹	25 ng mL ⁻¹	5 ng mL ⁻¹	25 ng mL ⁻¹
Morphine*	50–1000	0.9907	25	50	66.7	12.4	10.1	12.3	11.3	10.1	13.5
Naloxone	1–500	0.9916	0.5	1	95.6	14.5	9.8	6.3	14.5	12.4	14.1
Methylone	2.5–500	0.9985	1	2.5	63.1	9.3	6.6	9.3	9.7	13.6	13.7
Ethylethinone	1–500	0.9968	0.5	1	75.6	14.3	8.1	7.9	11.1	13.3	10.9
Ethylethinone	5–500	0.9967	0.25	5	65.1	13.6	9.0	8.6	13.5	14.7	13.4
Scopolamine	1–500	0.9901	0.5	1	77.0	7.7	6.2	7.5	14.5	12.5	10.9
Ethylone	0.5–500	0.9975	0.25	0.5	77.4	9.2	9.2	5.7	12.9	13.7	5.5
6-MIAM	2.5–500	0.9972	1	2.5	85.4	10.1	4.8	10.8	12.3	14.1	12.8
Methylephedrine	5–500	0.9981	2.5	5	67.6	8.7	14.3	2.9	14.0	14.2	11.5
Butylone	1–500	0.9938	0.5	1	85.7	5.4	14.1	14.6	10.3	13.3	13.0
Mephedrone	2.5–500	0.9960	1	2.5	85.3	7.0	8.7	9.2	13.2	12.9	10.8
Pentadrone	2.5–500	0.9950	1	2.5	84.9	8.5	8.1	6.8	13.5	14.8	11.0
BEG	0.5–500	0.9907	0.25	0.5	87.0	12.7	10.1	14.8	13.2	13.2	7.9
Cocaine	0.5–500	0.9982	0.25	0.5	91.6	4.5	2.7	1.7	4.0	2.4	2.4
MDPV	0.5–500	0.9977	0.1	0.25	87.5	2.4	3.2	3	12.9	5.5	9.4
COCET	0.5–500	0.9995	0.25	0.5	82.4	3.7	4.9	3.4	13.4	6.3	12.6
Pyrovalerone	0.5–500	0.9968	0.1	0.25	105.0	11.8	6.1	8.3	12.5	9.8	7.5
EDDP	1–500	0.9965	0.5	1	82.4	8.9	7.3	5.3	13.0	14.8	10.2
Buprenorphine	2.5–500	0.9926	1	2.5	83.4	10.0	4.7	7.5	12.8	9.5	12.8
Methadone	0.5–500	0.9976	0.1	0.25	76.1	2.5	4.2	3.2	10.2	8.3	7.2

*QC levels for morphine: 50, 100, and 500 ng mL⁻¹

most of the compounds is shifted to the dissociated basic form at high pH values (pK_a values for the drugs ranged from 3.4 to 9.5). Also, medium MeOH volume ensures the precipitation of OF proteins and the dispersant solvent utility [27].

Performance parameters and validation

Some analytical parameters as described in the “Method validation” section were determined under the optimal conditions to evaluate the proposed US-DLLME-UPLC-MS/MS method. ESM Fig. S2 shows the chromatograms corresponding to drug-free OF and OF spiked with standards of them with the absence of interfering peaks, demonstrating the selectivity provided by the MS/MS technique. The linearity was studied by spiking drug-free blank samples of OF with target analytes, using IS, in the ranges expanded from 0.5 to 500 ng mL⁻¹ (33% of the analyzed samples), 1.0 to 500 ng mL⁻¹ (24% of the analyzed samples), and 2.5 to 500 ng mL⁻¹ (24% of the analyzed samples). Shorter linearity ranges from 5 to 500 ng mL⁻¹ (14% of the analyzed samples) and 50 to 1000 ng mL⁻¹ for morphine were obtained (Table 1). Determination coefficients (R^2) ranging from 0.9901 to 0.9995 were obtained with a minimum of seven

concentration levels in triplicate. The LODs ranged from 0.1 to 2.5 ng mL⁻¹, except for morphine (25 ng mL⁻¹). LOQs ranged from 0.25 to 5 ng mL⁻¹, except for morphine (50 ng mL⁻¹). As can be seen in Table 1, nine compounds show a matrix effect from 63.1 to 105%. A value < 100% indicates signal suppression, whereas a value > 100% shows signal enhancement. The closer the value is to 100%, the less affected is the determination of the compound by ME. Some drugs showed moderate ME as it is shown in Table 1. Morphine, methylone, ethylcathinone, ephedrine, ethylcathinone, scopolamine, ethylone, methylephedrine, and methadone presented ME between 63 and 77%; and therefore, their mass spectrometry analysis can be considered affected by the matrix interferences. All remaining drugs were not affected by matrix effects, with values higher than 80%; however, matrix-matched calibrations were used in combination with IS in the simultaneous quantification of the target drugs.

Method precision and recoveries were calculated, as previously indicated, in the QC samples at three concentration levels (5, 25, and 100 ng mL⁻¹) except for morphine (50, 100, and 500 ng mL⁻¹). The intraday precision ($n = 6$) ranged from 1.7–14.8 %RSD and interday precision ($n = 16$) ranged from 2.4–14.8 %RSD (see Table 1).

Table 2 Accuracy expressed as average trueness and absolute recoveries in OF

Compound	Low QC ^a		Medium QC ^b		High QC ^c	
	Concentration found $\pm S$ (ng mL ⁻¹)	Recovery \pm RSD (%)	Concentration found $\pm S$ (ng mL ⁻¹)	Recovery \pm RSD (%)	Concentration found $\pm S$ (ng mL ⁻¹)	Recovery \pm RSD (%)
Morphine	59.1 \pm 6.0	118.2 \pm 11.9	86.5 \pm 12.2	86.5 \pm 12.2	554.5 \pm 66.8	110.9 \pm 13.4
Naloxone	6.5 \pm 0.4	129.0 \pm 7.4	21.5 \pm 2.4	85.8 \pm 9.7	93.7 \pm 3.0	93.7 \pm 3.0
Methylone	5.1 \pm 0.3	101.5 \pm 5.8	25.9 \pm 3.2	103.8 \pm 13.0	116.8 \pm 16.0	116.8 \pm 16.0
Ethylcathinone ephedrine	3.7 \pm 0.5	74.6 \pm 11.4	23.0 \pm 3.0	91.9 \pm 12.0	100.0 \pm 11.4	100.0 \pm 11.4
Ethylcathinone	6.1 \pm 0.7	122.7 \pm 13.2	21.1 \pm 3.6	84.5 \pm 10.4	96.3 \pm 12.3	96.3 \pm 12.3
Scopolamine	3.8 \pm 0.5	77.1 \pm 9.8	22.9 \pm 5.8	91.7 \pm 23.1	92.2 \pm 5.8	92.2 \pm 5.8
Ethylone	5.5 \pm 1.2	110.6 \pm 23.4	20.6 \pm 5.4	82.5 \pm 10.6	105.2 \pm 5.6	105.2 \pm 5.6
6-MAM	5.8 \pm 0.8	115.4 \pm 13.9	20.2 \pm 2.3	80.9 \pm 9.4	111.7 \pm 13.8	111.7 \pm 13.8
Methylephedrine	3.7 \pm 0.6	73.8 \pm 12.2	28.3 \pm 2.3	113.1 \pm 9.0	96.0 \pm 9.6	96.0 \pm 9.6
Butylone	4.9 \pm 0.8	98.5 \pm 16.2	21.7 \pm 2.9	96.8 \pm 11.6	113.4 \pm 11.6	113.4 \pm 11.6
Mephedrone	3.8 \pm 0.5	76.6 \pm 11.3	21.8 \pm 3.0	87.1 \pm 12.2	98.3 \pm 10.8	98.3 \pm 10.8
Pentedrone	5.4 \pm 1.1	107.2 \pm 1.2	24.5 \pm 4.0	98.1 \pm 16.0	92.1 \pm 10.4	92.1 \pm 10.4
BEG	5.1 \pm 1.2	102.7 \pm 23.3	19.2 \pm 2.8	76.8 \pm 11.2	126.2 \pm 20.6	126.2 \pm 20.6
Cocaine	5.1 \pm 0.2	102.6 \pm 4.0	25.4 \pm 0.7	101.0 \pm 1.7	101.6 \pm 2.7	101.6 \pm 2.7
MDPV	5.3 \pm 0.5	105.5 \pm 10.5	22.7 \pm 2.9	96.8 \pm 11.6	110.2 \pm 10.2	110.2 \pm 10.2
COCET	5.0 \pm 0.6	100.7 \pm 11.8	26.6 \pm 1.8	106.5 \pm 7.3	118.2 \pm 13.0	118.2 \pm 13.0
Pyrovalerone	6.1 \pm 0.4	121.7 \pm 8.5	21.4 \pm 1.9	85.8 \pm 7.4	113.5 \pm 8.4	113.5 \pm 8.4
EDDP	6.3 \pm 0.5	125.3 \pm 9.6	23.5 \pm 3.1	93.9 \pm 12.4	109.5 \pm 10.9	109.5 \pm 10.9
Buprenorphine	4.6 \pm 0.6	92.3 \pm 30.7	22.0 \pm 2.1	87.8 \pm 8.8	108.9 \pm 14.6	108.9 \pm 14.6
Methadone	5.2 \pm 0.6	103.9 \pm 11.6	28.3 \pm 2.4	113.2 \pm 9.4	106.4 \pm 11.8	106.4 \pm 11.8

^a Low QC, 5 ng mL⁻¹ (50 ng mL⁻¹ for morphine)

^b Medium QC, 25 ng mL⁻¹ (100 ng mL⁻¹ for morphine)

^c High QC, 100 ng mL⁻¹ (500 ng mL⁻¹ for morphine)

Accuracy as average trueness of the quantified 100 ng mL^{-1} standard mix added to OF and standard deviation (S) of five replicate samples is shown in Table 2. The overall PE was evaluated by absolute recoveries (Table 2), and the obtained values are indicators of the good performance of the method. Most drug recoveries of the drugs using low and medium QC samples ($5\text{--}25 \text{ ng mL}^{-1}$ or 50 ng mL^{-1} for morphine) were in the range of $80\text{--}115\%$ and between 80 and 110% at high levels of QC samples (100 ng mL^{-1} or 500 ng mL^{-1} for morphine), which is the range marked by FDA “Guidelines for the Validation of Chemical Methods” [24]. Recoveries of only six analytes presented slight deviations, at the high levels of QC samples, compared to the guideline values (between 1.7% for 6-MAM and 19% for BEG). Small deviations at low and medium QC samples were found (6.7% for pyrovalerone 10.3% for EDDP and 14% for naloxone) which show that the proposed methodology is appropriately validated.

Application of the proposed method to OF

The proposed method was successfully applied for the analysis in duplicate of the target drugs in 15 OF samples from patients participating in methadone and buprenorphine/naloxone substitution therapy programs. It was necessary to dilute some positive samples that had concentrations higher than the linear range studied, for its correct quantification. Also, due to the small volume of OF collected in the Salivette® devices, the means were performed in duplicate, except those for which limited amount was available and only a single analysis was possible to carry out (see ESM Table S4). The results are presented in box plots (Fig. 4) and the MRM chromatograms for positive sample 9 (ESM Table S4) are shown in Fig. 5. The central mark represents the median value, the edges of the whiskers are the minimum and maximum values, and the box is limited by the first

Fig. 4 Box–whiskers plot of concentrations (ng mL^{-1}) determined for detection of positive cases for drugs of abuse: morphine, naloxone, 6-MAM, BEG, cocaine, cocaethylene, EDDP, buprenorphine, and methadone

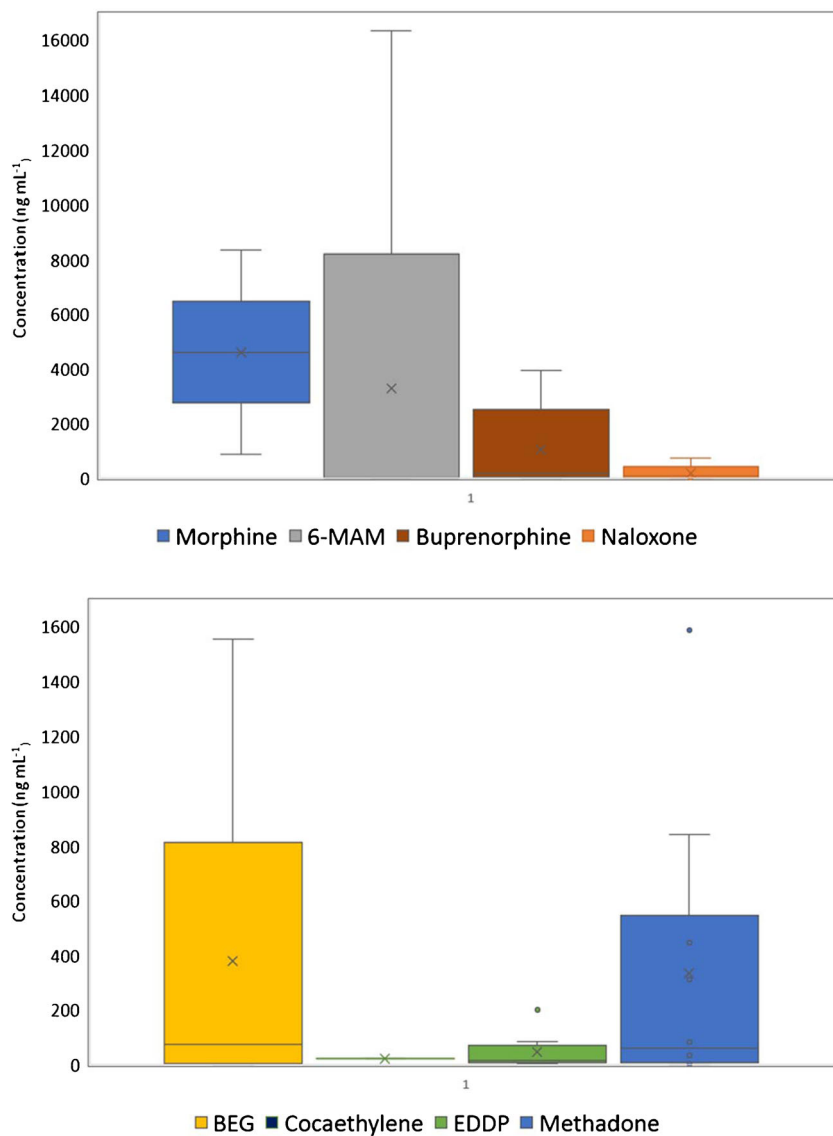
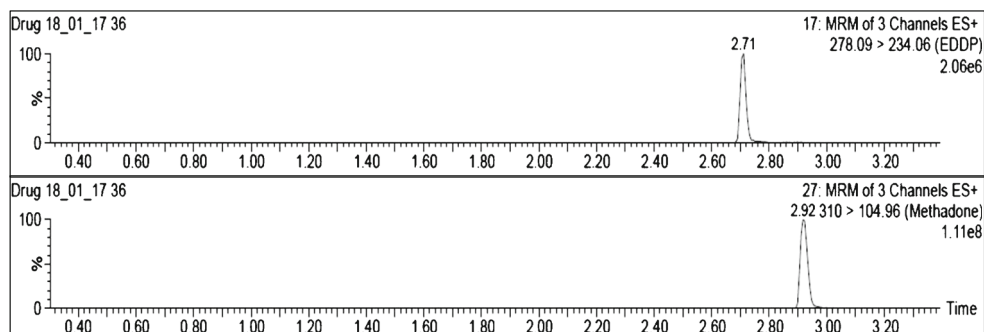


Fig. 5 UPLC-MS/MS chromatogram in MRM mode of two detected drugs (EDDP and methadone) in the real sample 9 from Table S4



quartile and third quartile values for eight drugs found in the OF samples. As expected, NPS drugs were not detected in the OF samples. Patients who follow methadone treatment also have concentrations of their EDDP metabolite, except in one case where the methadone concentration found is very low (0.6 ng mL^{-1}) and EDDP is not found in that sample. Positive cases in these real samples ranged from 875.8 to $8323.7 \text{ ng mL}^{-1}$ for morphine, 8.0 to $16,336.8 \text{ ng mL}^{-1}$ for 6-MAM, 2.0 to $12,529.3 \text{ ng mL}^{-1}$ for cocaine, 0.6 to $1554.6 \text{ ng mL}^{-1}$ for BEG, only a positive sample of 23.3 ng mL^{-1} for cocaethylene, 0.6 to 841.1 ng mL^{-1} for methadone, 4.4 to 84.0 ng mL^{-1} for EDDP, 1.9 to 730.8 ng mL^{-1} for naloxone, and 20.9 to $3938.3 \text{ ng mL}^{-1}$ for buprenorphine. Results from Fig. 4 show that 67% of the subjects used methadone and 33% used buprenorphine/naloxone therapy. However, 47% of these patients consumed cocaine and present concentrations of the metabolites BEG and cocaethylene (linked to cocaine co-consumption with alcohol) in 40% and 7% of cases, respectively. Some patients exhibited the presence of morphine (13% of cases) and 6-MAM (33% of cases) that were compatible with heroin consumption. Polydrug use of heroin and cocaine is compatible in four cases with the simultaneous determination of some of their metabolites.

Conclusions

A fast and robust method for the multianalyte qualification of 20 illegal drugs, including 10 synthetic cathinones, in OF samples, has been developed and adequately validated with satisfactory results. The method is based on US-DLLME, a miniaturized version of liquid–liquid extraction (LLE) and UPLC-MS/MS selective determination at low levels in nanogram per milliliter range. Matrix ionization effects were observed for eight drugs, but quantification was satisfactorily corrected using isotopically labeled surrogates. The proposed methodology is a good choice to eliminate false positives that may appear in drug screening tests. Application of the proposed method to a set of 15 OF samples showed the presence of naloxone/buprenorphine in five OF analyzed while methadone was detected in 10 OF, all of them from patients on a

drug substitution therapy program. Drug consumption is compatible with cocaine (seven cases) or heroin (five cases). Cathinones were not detected in any sample.

Acknowledgments The authors are grateful to Professor M.I. Loza (BioFarma Research Group–USC) for assistance with the instrumentation.

Funding information This work was funded by Ministerio del Interior-DGT (Project SPIP2015-01838) of Spain and the European Regional Development Fund (FEDER).

Compliance with ethical standards

This study has been carried out using oral fluid samples from healthy volunteers and patients participating in substitution therapy programs, authorized by the Comité Autonómico de Ética da Investigación de Galicia Consellería de Sanidade. Xunta de Galicia (SPAIN), with the reference 2015/488, according to Spanish Regulation Law 14/2007, of biomedical investigation and the Real Decreto 1716/2011, of November 18. All the samples have been provided by volunteers and patients who did not oppose to the use of these samples for the development of an analytical method and signed the corresponding informed consent.

Conflict of interest All authors declare that they have no conflict of interest.

References

1. European Monitoring Centre for Drugs and Drug Addiction, European Drug Report 2016: trends and developments, Publications Office of the European Union, Luxembourg, 2016 <http://www.emcdda.europa.eu/system/files/publications/2637/TDAT16001ENN.pdf>
2. Miller BL, Stogner JM, Miller JM, Fernandez MI. The arrest and synthetic novel psychoactive drug relationship: observations from a young adult population. *J Drug Issues*. 2017;47:91–103. <https://doi.org/10.1177/0022042616678611>.
3. Rech MA, Donahey E, Cappiello Dziedzic JM, Oh L, Greenhalgh E. New drugs of abuse. *Pharmacotherapy*. 2015;35:189–97. <https://doi.org/10.1002/phar.1522>.
4. Benítez G, March-Salas M, Villa-Kamel A, Cháves-Jiménez U, Hernández J, Montes-Osuna N, et al. The genus *Datura* L. (Solanaceae) in Mexico and Spain—ethnobotanical perspective at the interface of medical and illicit uses. *J Ethnopharmacol*. 2018;219:133–51. <https://doi.org/10.1016/j.jep.2018.03.007>.

5. Bosker WM, Huestis MA. Oral fluid testing for drugs of abuse. *Clinical Chem.* 2009;55:1910–31. <https://doi.org/10.1373/clinchem.2008.108670>.
6. Aldigan AA, Torrance HJ. Bioanalytical methods for the determination of synthetic cannabinoids and metabolites in biological specimens. *TRAC Trends Anal Chem.* 2016;80:444–57. <https://doi.org/10.1016/j.trac.2016.03.025>.
7. Mercolini L, Protti M. Biosampling strategies for emerging drugs of abuse: towards the future of toxicological and forensic analysis. *J Pharm Biomed Anal.* 2016;130:202–19. <https://doi.org/10.1016/j.jpba.2016.06.046>.
8. Amaratunga P, Lemberg BL, Lemberg D. Quantitative measurement of synthetic cathinones in oral fluid. *J Anal Toxicol.* 2013;37:622–8. <https://doi.org/10.1093/jat/bkt080>.
9. Di Rago M, Chu M, Rodda LN, Jenkins E, Kotsos A, Gerostamoulos D. Ultra-rapid targeted analysis of 40 drugs of abuse in oral fluid by LC-MS/MS using carbon-13 isotopes of methamphetamine and MDMA to reduce detector saturation. *Anal Bioanal Chem.* 2016;40:3737–49. <https://doi.org/10.1007/s00216-016-9458-3>.
10. Sergi M, Montesano C, Odoardi S, Rocca LM, Fabrizi G, Compagnone D, et al. Microextraction by packed sorbent coupled to liquid chromatography tandem mass spectrometry for the rapid and sensitive determination of cannabinoids in oral fluids. *J Chromatogr A.* 2013;1301:139–46. <https://doi.org/10.1016/j.chroma.2013.05.072>.
11. Newmeyer MN, Concheiro M, Huestis MA. Rapid quantitative chiral amphetamines liquid chromatography-tandem mass spectrometry: method in plasma and oral fluid with a cost-effective chiral derivatizing reagent. *J Chromatogr A.* 2014;1358:68–74. <https://doi.org/10.1016/j.chroma.2014.06.096>.
12. Montesano C, Simeoni MC, Curini R, Sergi M, Lo Sterzo C, Compagnone D. Determination of illicit drugs and metabolites in oral fluid by microextraction on packed sorbent coupled with LC-MS/MS. *Anal Bioanal Chem.* 2015;407:3647–58. <https://doi.org/10.1007/s00216-015-8583-8>.
13. Liu HC, Lee HT, Hsu YC, Huang MH, Liu RH, Chen TJ, et al. Direct injection LC-MS-MS analysis of opiates, methamphetamine, buprenorphine, methadone and their metabolites in oral fluid from substitution therapy patients. *J Anal Toxicol.* 2015;39:472–80. <https://doi.org/10.1093/jat/bkv041>.
14. de Castro A, Lendoiro E, Fernández-Vega H, Steinmeyer S, López-Rivadulla M, Cruz A. Liquid chromatography tandem mass spectrometry determination of selected synthetic cathinones and two piperazines in oral fluid. Cross reactivity study with an on-site immunoassay device. *J Chromatogr A.* 2014;1374:93–101. <https://doi.org/10.1016/j.chroma.2014.11.024>.
15. Mercolini L, Protti M, Catapano MC, Rudge J, Sberna AE. LC-MS/MS and volumetric absorptive microsampling for quantitative bioanalysis of cathinone analogs in dried urine, plasma and oral fluid samples. *J Pharm Biomed Anal.* 2016;123:186–94. <https://doi.org/10.1016/j.jpba.2016.02.015>.
16. Rezaee M, Assadi Y, MilaniHosseini MR, Aghaee E, Ahmadi F, Berijani S. Determination of organic compounds in water using dispersive liquid-liquid microextraction. *J Chromatogr A.* 2006;1116:1–9. <https://doi.org/10.1016/j.chroma.2006.03.007>.
17. Jain R, Singh R. Applications of dispersive liquid-liquid microextraction in forensic toxicology. *TRAC Trends Anal Chem.* 2016;75:227–37. <https://doi.org/10.1016/j.trac.2015.07.007>.
18. Fisichella M, Odoardi S, Strano-Rossi S. High-throughput dispersive liquid/liquid microextraction (DLLME) method for the rapid determination of drugs of abuse, benzodiazepines and other psychotropic medications in blood samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and application to forensic cases. *Microchem J.* 2015;123:33–41. <https://doi.org/10.1016/j.microc.2015.05.009>.
19. World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects. *JAMA J Am Med Assoc.* 2013;310:2191–4. <https://doi.org/10.1001/jama.2013.281053>.
20. Mathieu D, Nony J, Phan-Tan-Luu R. NemrodW®, Version 2011, LPRAI, Marseille
21. Lewis GA, Mathieu D, Phan-Tan-Luu R. *Pharmaceutical experimental design.* New York: Marcel Dekker; 1999. [https://doi.org/10.1016/S0378-5173\(99\)00384-1](https://doi.org/10.1016/S0378-5173(99)00384-1).
22. U.S. Department of Health and Human Services Food and Drug Administration, Guidance for industry: bioanalytical method validation, draft guidance, U.S. Department of Health and Human Services Food and Drug Administration, 2013. <https://www.fda.gov/downloads/Drugs/20GuidanceComplianceRegulatoryInformation/Guidances/UCM368107.pdf> (Accessed on June 2018).
23. U.S. Food and Drug Administration Foods and Veterinary Medicine Science and Research Steering Committee, Guidelines for the validation of chemical methods, 2015. <https://www.fda.gov/downloads/scienceresearch/fieldscience/ucm273418.pdf> (Accessed on June 2018).
24. ICH harmonized tripartite guideline: validation of analytical procedures: text and methodology Q2(R1), International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005. <https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm265700.htm> (Accessed on June 2018).
25. Magnusson B, Örnemark U. *Eurachem guide: the fitness for purpose of analytical methods—a laboratory guide to method validation and related topics.* 2nd ed. Teddington: Eurachem; 2014.
26. Krue A, Rebane R, Kipper K, Oldekop M-L, Evard H, Herodes K, et al. Tutorial review on validation of liquid chromatography–mass spectrometry methods: part II. *Anal Chim Acta.* 2015;870:8–28. <https://doi.org/10.1016/j.aca.2015.02.016>.
27. Mansour FR, Khairy MA. Pharmaceutical and biomedical applications of dispersive liquid-liquid microextraction. *J Chromatogr B.* 2017;1061–1062:382–91. <https://doi.org/10.1016/j.jchromb.2017.07.055>.