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A Novel Bioanalytical Method for the Determination of Opioids in Blood and Pericardial Fluid

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Abstract

Opioids are the drugs most commonly detected in overdose deaths and the second most consumed worldwide. An analytical methodology has been optimized and fully validated for the determination of codeine, morphine, 6-acetylmorphine, 6-acetylcodeine, oxycodone, oxymorphone and fentanyl in whole blood and pericardial fluid. The internal standards used were codeine-d₃, morphine-d₃, 6-acetylmorphine-d₃ and fentanyl-d₅. Before solid-phase extraction, volumes of 250 µL of blood and pericardial fluid were subjected to a protein precipitation (with 750 µL of ice-cold acetonitrile) and a microwave-induced oximation was performed using a solution of 1% aqueous hydroxylamine hydrochloride in phosphate-buffered saline (1:2, v/v). Finally, the dried extracts were further derivatized with a solution of *n*-methyl-*n*-(trimethylsilyl) trifluoroacetamide + 5% trimethylchlorosilane under microwave irradiation. The chromatographic analysis was carried out using gas chromatography–mass spectrometry operating in electron impact and selected ion monitoring mode. For all analytes, the method was linear between 5 and 1,000 ng/mL with determination coefficients (r^2) >0.99. Depending on the analyte and matrix, the limit of detection varies between 3 and 4 ng/mL. Intra- and intermediate precision (<20%) and bias (±20%) were acceptable for all analytes in both matrices. The stability of the substances in the studied matrices was guaranteed, at least, 24 h in the autosampler, 4 h at room temperature and 30 days after three freeze/thaw cycles. This methodology was applied to real samples from the Laboratory of Chemistry and Forensic Toxicology, Centre Branch, of the National Institute of Legal Medicine and Forensic Sciences, Portugal.

Introduction

Opioids are classified as natural (e.g., morphine, codeine), semisynthetic (e.g., oxycodone, heroin, oxymorphone) and synthetic (e.g., fentanyl) compounds (1–4). They can also be classified according to their affinity for different opioid receptors μ (mu), κ (kappa), δ (delta) and σ (sigma), as pure agonist (morphine, fentanyl and oxycodone), partial agonist/antagonist and as agonist/antagonist (3,

5–8). These receptors have inhibitory effect and are distributed in the brain, spinal cord and peripheral nervous system (7, 9). Several effects are associated with its consumption, such as analgesia, respiratory depression, nausea and vomiting, euphoria, sedation, miosis, dry mouth, antitussive effect, hypotension and bradycardia, constipation, contraction of the sphincter of Oddi and vesical, cognitive changes (hallucinations, delusions) and tolerance (5–7, 10). In spite of

this, opioids are prescribed and used mainly for pain management, although they are sometimes overused by patients and also used as recreational drugs (4).

Overdoses deaths are caused mainly to induced coma, respiratory depression, which will trigger apnea and consequently death (5, 7, 11). The effects of these substances begin to appear between <25 min and 1 h and they have distribution volumes of 1–25 L/kg. Elimination ratio varies from substance to substance (11, 12).

Opioids are the second most widely used drug in the world and the most detected in deaths related to overdoses. According to annual reports, opioids were detected in 76, 84 and 42% of fatal overdoses in the world, Europe and Portugal, respectively (13–15). Seizure of opioids other than heroin has increased in 2016 and 2017, including methadone, tramadol, fentanyl derivatives, morphine, opium, codeine and oxycodone. Also, treatment entrants indicated misused of methadone, buprenorphine, fentanyl, codeine, morphine, tramadol and oxycodone (14, 16). Due the synthetic opioids crisis of fentanyl, its analogs and tramadol, affecting mainly North America and parts of Africa, Asia and the Middle East, respectively, the United Nations Office on Drugs and Crime (UNODC) launched in June 2018 an integrated strategy to support countries facing this problem (17). In this context, it is important to develop analytical methodologies that identify and quantify these substances in several biological matrices. To perform the analysis, blood (BL) is one of the most important matrices, but collection is not always feasible (e.g., severe exsanguination, advanced putrefaction). Therefore, it is important to have an alternative or even a complementary biological matrix, such as pericardial fluid (PF), which can provide relevant information. In forensic toxicology, the use of PF has several advantages such as the volume available to be collected (between 5 and 35 mL), it is easily obtained from the pericardial cavity, is well protected from contamination by pathogens and relatively stable during the postmortem period (18–22). However, if a huge quantity of a drug is present in the stomach, PF can be contaminated by postmortem diffusion (21). Despite the scarce existing studies, they suggest a good or moderate correlation between PF and peripheral BL, and recommend its inclusion to the routine autopsy specimens (19, 20, 23).

Concerning to the studied opioids in PF, the published studies are scarce, comprising only codeine (18, 23), morphine (18, 20, 24) and 6-acetylmorphine (6-MAM) (24).

In this study, solid-phase extraction (SPE) was implemented due to its several advantages, namely, high reproducibility, efficiency, quickness, use of a smaller sample volume and production of less solvent waste. Liquid–liquid extraction, in turn, requires larger amounts of sample and solvents (some flammable and/or toxic) and can lead to the formation of emulsions with mutual solubility of the analytes in both phases (5, 25, 26).

In opioid determination, the sample pretreatment using deproteinization with acetonitrile prior to the SPE and chromatographic analysis by gas chromatography coupled with mass spectrometry (GC–MS) offers the great advantage of eliminating the matrix effect [a problem associated with analysis by liquid chromatography coupled to mass spectrometry (LC–MS)] and simultaneously allows the quantification of very low concentrations of substances in small volumes of biological samples (27). However, it requires a derivatization step to improve opioids detectability and stability (28, 29). Silylation or fluoroacetylation are the preferred methods for opioids derivatization. Oxycodone and oxymorphone, being keto-opioids, may originate multiple derivatives (via tautomerization) as their carbonyl group may be in an enol or keto form, which can compromise

the achievement of an accurate quantification of these substances (28, 30–34). One way to avoid the formation of these derivatives and improve the chromatographic resolution of the compounds is to perform the reaction with hydroxylamine to form the corresponding oximes, prior to other derivatizations (namely the addition of trimethylsilyl or propionyl groups). With the inclusion of this crucial step (oximation) in the preparation of the samples, the formation of oximes derivatives and multiple derivatization of these compounds is avoided. This multiple derivatization is due to the tautomerization that will permit the formation of products from the enol isomer and from the unreacted keto isomer. It must be highlighted that the addition of hydroxylamine does not affect the other opioids [codeine, morphine, 6-MAM, 6-acetylcodeine (6-ACCOD) and fentanyl] since their structures do not have a functional ketone group (30, 31, 33, 35–42). There are also studies that used methoxylamine (30, 34, 35, 43–47) as derivatization reagent.

The use of microwave-assisted derivatization for the determination of drugs of abuse by GC–MS has already been published (29, 48–53). However, we did not find any studies on the substance in the current study that used microwave-induced oxime derivatizations. Thus, we present a fast microwave-assisted derivatization procedure using hydroxylamine with a reaction time of just 30 s, much shorter than found in other publications: 15 min (34, 35, 37, 39, 40, 45), 20 min (47), 30 min (36, 44, 46), 60 min (30, 31, 33, 38, 42, 43) or 120 min (41). A final fast microwave-assisted derivatization using *n*-methyl-*n*-(trimethylsilyl) trifluoroacetamide (MSTFA) + 5% trimethylchlorosilane (TMCS), requiring only 100 s instead of the 15–20 min (31, 33, 36, 39–42, 45, 47), 30 min (30, 37, 44) or 60 min (38) spent in other published studies, was also described.

The aim of this work was the development, optimization and validation of an analytical methodology for confirmation and quantification of codeine, morphine, 6-MAM, 6-ACCOD, oxycodone, oxymorphone and fentanyl using only 250 μ L of BL and PF with application of two steps of fast microwave-assisted derivatization, which reduced the total analysis time remarkably. To the best of our knowledge, this is the first developed methodology for the simultaneous determination of these seven opioids in postmortem matrices, under the studied conditions.

Experimental

Chemicals and reagents

The analytical standards of codeine and morphine were purchased from Lipomed AG (Arlesheim, Switzerland). The substances, 6-MAM, 6-ACCOD, oxycodone, oxymorphone, fentanyl and the internal standards (IS), codeine- d_3 , 6-MAM- d_3 , fentanyl- d_5 and morphine- d_3 were supplied from Cerilliant (Round Rock, TX, USA). Acetonitrile and deionized water were purchased from Riedel-de Haën (Seelze, Germany). Carbonate buffer (0.15 M at pH = 9.5), dichloromethane, 2-propanol, ammonium, *n*-hexane, TMCS and hydroxylamine hydrochloride 99% were acquired from Merck Co. (Darmstadt, Germany), MSTFA from Macherey–Nagel GmbH & Co. (Düren, Germany), phosphate-buffered saline (PBS, 0.1 M at pH = 7) and purified water from VWR (Radnor, PA, USA). Oasis[®] MCX extraction cartridges (3 mL, 60 mg) were purchased from Waters Corp. (Milford, MA, USA).

Biological samples

Blank BL samples were acquired from a local BL bank and used for calibration purposes and validation experiments. The blank

PF was collected during autopsies performed at the Medico-Legal Offices of the National Institute of Legal Medicine and Forensic Sciences, Centre Branch, Portugal and were also used for calibration purposes and validation experiments. All samples were stored at -15°C and screened for drugs of abuse before being used.

Apparatus

A Turbo Vap[®] LV (Caliper Life Science) with nitrogen gas was used for the solvents evaporation. The chromatographic analyses were achieved using an Agilent Technologies 7890B GC system (Hewlett-Packard, Waldbronn, Germany), coupled with a 5977A, mass-selective detector (Hewlett-Packard).

Methods

Preparation of standard solutions, calibrators and controls

Stock solutions of each substance (50 $\mu\text{L}/\text{mL}$) were prepared in methanol with exception of 6-ACCOD, which was prepared in acetonitrile. Working solutions of quality control (QC) and calibrators at 5 $\mu\text{g}/\text{mL}$ were prepared in the corresponding solvents (acetonitrile for ACCOD and methanol for the remaining compounds) and then by proper dilution were prepared at 0.5 and 0.05 $\mu\text{g}/\text{mL}$. An internal working standard solution (mixture of codeine- d_3 , morphine- d_3 , 6-MAM- d_3 and fentanyl- d_5) at 2 $\mu\text{g}/\text{mL}$ was prepared in methanol. Working solutions and stock solutions were stored at a temperature of 5 and -20°C , respectively, and protected from light.

Sample preparation and extraction

After the addition of 25 μL of the deuterated IS solution to volumes of 250 μL of PF and BL, a protein precipitation of the samples was performed by the addition of 750 μL of ice-cold acetonitrile and centrifuged at 3,000 rpm for 10 min. The supernatant was decanted into clean glass tubes, and 375 μL of the 1% aqueous hydroxylamine hydrochloride in PBS (1:2, v/v) solution (prepared freshly) was added, agitated and derivatized in a microwave reactor of 900 W at 50% power during 30 s. After cooling down to room temperature, the samples were added to the extraction cartridges, beforehand conditioned with 1 mL methanol and 1 mL of deionized water. Then, the cartridges were washed sequentially with 1 mL carbonate buffer (0.15 M), 1 mL of deionized water and 1.5 mL of *n*-hexane. After the columns were dried under full vacuum, the analytes were eluted with 1 mL of a mixture of dichloromethane:2-propanol:ammonium (78:20:2, v/v/v). The extracts were evaporated at 40°C under a gentle nitrogen stream (until dryness). Lastly, derivatization was performed by addition of 60 μL of MSTFA + 5% TMCS over 100 s at 100% power in a microwave reactor of 900 W. After cooling down to room temperature, the solutions were transferred to the GC autosampler vials and 2 μL injected into the GC-MS system.

GC-MS conditions

A capillary column HP-5 MS with 5% phenylmethylsiloxane (30 m \times 0.25 mm I.D., 0.25-mm film thickness) supplied by J&W Scientific (Folsom, CA, USA) was used.

The oven temperature program was as follows: 50°C held for 1 min, increased to 300°C at $20^{\circ}\text{C}/\text{min}$ and held for 2 min. Highly purified helium was the carrier gas at a constant flow rate of 1 mL/min, and it was used in splitless injection mode (2 μL). The

Table I. RTs and Monitored Ions of Each Substance

Analytes	Ions (m/z)	RT (min)
Codeine- d_3	374 ^a	13.58
Codeine	371 ^a , 178, 196	13.59
Morphine- d_3	432 ^a	13.79
Morphine	429 ^a , 236, 414	13.80
6-ACCOD	341 ^a , 282, 229	13.92
6-Aceylmorphine- d_3	402 ^a	14.10
6-MAM	399 ^a , 287, 340	14.11
Oxycodone	474 ^a , 459, 475	14.38
Oxymorphone	532 ^a , 517, 533	14.40
Fentanyl- d_5	250 ^a	14.87
Fentanyl	245 ^a , 189, 146	14.89

^aQuantification ions.

mass spectrometer worked with an emission current of 300 μA in the electron ionization mode with an electron energy of 70 eV. The temperatures of the injection port and detector were set at 250 and 280°C , respectively. Initially, the retention times (RTs) and characteristics ions of the substances were identified with a full scan mode (scan range 50–550 m/z), and then the analyses were performed with a selected ion monitoring mode. The IS used to quantitate each substance were the following: codeine- d_3 for codeine and 6-ACCOD, morphine- d_3 for morphine, oxymorphone and oxycodone, 6-MAM- d_3 for 6-MAM and fentanyl- d_5 for fentanyl, since these were available and the most similar to the substances. The ions monitored for each substance are presented in Table I in ascending order of their RTs.

Method validation

The described procedure was validated by evaluating interferences, linearity, limits of detection (LOD) and limits of quantification (LOQ), carryover, precision (intraday and intermediate), bias, stability (autosampler, benchtop and freeze/thaw cycles), dilution integrity and recovery based on the recommendations of the Scientific Working Group for Forensic Toxicology (SWGTOX) (54).

Acceptance criteria for compounds identification

The criteria for identification of compounds were established according to the recommendations of the World Anti-Doping Agency (55). For chromatography, the relative RT of the substance must fall within a 1% window, or ± 0.1 min in absolute terms, from that of the same substance in a QC sample prepared and analyzed contemporaneously. Mass spectrometric identification must include at least three diagnostic ions, and their relative intensities should not differ by more than a tolerated amount from those generated by the same substance in a QC sample prepared and analyzed contemporaneously (if the relative intensity of the ion is within a 25–50% interval of the base peak in the control sample, a maximum relative tolerance of 20% will be allowed for the same ion in the sample; if this intensity is $<25\%$ or $>50\%$ in the control sample, then absolute tolerances of 5 and 10%, respectively, will be allowed for the ion in the sample). A signal-to-noise ratio greater than three to one (3:1) of all diagnostic ions is also required (55).

Interference study

Interferences were studied by analyzing one group of 10 different blank samples of both matrices (BL and PF) spiked with the substances most commonly found in routine analyses of the SQTFC (medicines, drugs of abuse and pesticides) at 5 and 100 ng/mL and one group of 10 different blank samples of both matrices only fortified with the IS. The interferences used were amisulpride; amitriptyline; amlodipine; aripiprazole; atenolol; bisoprolol; buprenorphine; bupropion; buspirone; carbamazepine; carvedilol; ciamemazine; citalopram; clomipramine; clonazepam; chlorpromazine; clozapine; diltiazem; dosulepine; doxylamine; duloxetine; esmolol; phenytoin; phenobarbital; felbamate; fluphenazine; fluoxetine; fluvoxamine; gabapentin; haloperidol; hydrochlorothiazide; imipramine; indapamide; ketamine; lamotrigine; lercanidipine; levetiracetam; lidocaine; maprotiline; melperone; methadone; metoprolol; mianserin; mirtazapine; nifedipine; nimesulide; nortriptyline; olanzapine; oxcarbazepine; paliperidone; paracetamol; paroxetine; pentobarbital; pethidine (meperidine); primidone; promethazine; propafenone; propranolol; quetiapine; risperidone; sertraline; sildenafil; tadalafil; telmisartan; tapentadol; tiapride; tiopental; tramadol; trazodone; trimipramine; vardenafil; warfarin; venlafaxine; zispraside; zolpidem; THC; THCOH; THCCOOH; cocaine; benzoylecgonine; ecgoninamethylester; d,l-amphetamine; d,l-methamphetamine; d,l-MDA; d,l-MDMA; d,l-MDEA; d,l-MBDB; mephedrone; methedrone; d,l-methcathinone; d-cathine; 1s,2r(+)-ephedrine; d,l-DCB; d,l-PMA; 2C-B; 2C-H; 2C-I; 2C-T-2; 2C-T-4; 2C-T-7; MDPV; α -pVP; pentylone, r(-)-Bromo DragonFLy; d,l-NNDMA; methylone; d,l-4-MTA; 2C-P; ethylone; buphedrone; flephedrone; r(+)-cathinone; azinfos ethyl; azinfos methyl; chlorfeniphos; chlorpyrifos; chlorpyrifos methyl; demeton-s-methyl sulfone; diazinon; dimethoate; etoprophos; fenamiphos; fenthion; phosalone; foxime; malathion; methamidophos; methidathion; mevinphos; ometoate; paraoxon; parathion ethyl; parathion methyl; quinalfos; sulfotep and terbufos. This parameter was studied to investigate the presence of matrix interferences at the RT of monitored ions for each compound and respective IS, and also the method's ability to identify the substances of interest in the presence of other compounds routinely analyzed in the laboratory.

Linearity, LOD and LOQ and carryover

Linearity was determined by plotting the peak area ratio between the analyte of interest and its IS against theoretical concentrations. The acceptance criteria were $r^2 \geq 0.99$ and the calibrators quantified within $\pm 20\%$. Simultaneously, four different QC samples at 5, 25, 200 and 900 ng/mL and blank samples of BL and PF with IS added were also analyzed.

The LODs were determined as the lowest concentrations with a signal/noise ratio ≥ 3 . The LOQs were defined as the minimum concentration of each substance that could be quantified with adequate precision (coefficient of variation, CV < 20%) and bias ($\pm 20\%$). Carryover was analyzed by injecting blank samples after the highest concentration of the calibration curve and verifying if it was present ionic signals of the substances of interest at their RTs that will affect the capability to confirm them.

Precision and bias

Precision was determined by calculating the CV (%) and acceptable values should be < 20%. Bias was calculated on each group of QC samples: [(mean of measured concentrations – theoretical

concentration)/theoretical concentration $\times 100$] and the limit of the acceptable variability was $\pm 20\%$. Intraday precision was determined by analyzing four QC samples levels in both matrices at the same day. The intermediate precision and bias were determined by the analysis of QC samples at three concentration levels (low, medium and high) in both matrices on five different days. Precision was acceptable when CV < 20% and bias was acceptable when the value measured was $\pm 20\%$ of the spiked concentration, for all QC samples.

Stability

The stability on the autosampler was examined at three QC levels (low, medium and high) by reinjecting the extracts after 24 h, 48 h, and 7 days, under the conditions of the GC-MS laboratory. This parameter was evaluated by comparing the mean concentration obtained in each of the days ($n = 3$), with the mean concentration of the freshly extracts obtained on the first day ($n = 3$). Benchtop stability and the three freeze/thaw cycles (after 1, 2, 3 and 4 weeks) were performed with two different QC levels (low and medium). Benchtop evaluation was done analyzing the QC samples leaved at room temperature for 4 h and compared with freshly spiked QC samples at the same concentrations. The evaluation of freeze/thaw cycles was done with the two levels QC samples frozen at -15°C and thawed after the referred periods. Stability was considered acceptable if percentage of loss was within $\pm 20\%$ of the freshly prepared QC samples.

Dilution integrity

This parameter was evaluated by diluting fortified QC samples (BL and PF) at 1150 ng/mL samples to achieve 1:2 dilution; prior protein precipitation IS was added to the samples and the methodology previously described was applied. Dilution integrity is accepted if substances quantify within $\pm 20\%$ of the concentration 1,150 ng/mL after the mentioned dilution.

Recovery

The extraction efficiency was estimated by analysis of two sets of three different concentration levels (low, medium and high) in which the IS was added after the extraction. This parameter was determined comparing average blank samples fortified before extraction (set 1, $n = 6$), with average blank samples fortified after the extraction (set 2, $n = 6$): set 1/set 2 $\times 100\%$.

Results and Discussion

The procedure described above was full validated in BL and PF obtaining appropriate results using only 250 μL of samples. All compounds were well separated chromatographically in 15 min. In both blank matrices (BL and PF), no significant interferences (endogenous and exogenous) were observed at m/z of the monitored ions and at their RTs. All the studied substances were successfully identified in the fortified matrices, suggesting that the added compounds do not interfere with the analysis of the analytes of interest.

Linearity was verified in BL and PF samples from 5 to 1,000 ng/mL, using eight different points for each compound and 1/x weighted factor. The 1/x weighting linear regression model was suitable to all calibration curves obtaining $r^2 \geq 0.99$ and calibrators quantitated within $\pm 20\%$. The LODs achieved in BL were 4 ng/mL for all analytes except for oxycodone and fentanyl (3 ng/mL).

Table II. Linearity Results for All Analytes Studied in BL and PF

Analytes	Matrix	Linear range (ng/mL)	Linearity			LOD ^b
			Slope ^(a)	Intercept ^(a)	r^2 ^(a)	
Codeine	BL	5–1,000	5.8E–03 ± 3.9E–04	1.5E–02 ± 1.3E–03	0.999 ± 1.2E–03	4
	PF		8.9E–03 ± 2.6E–03	1.2E–02 ± 5.4E–03	0.999 ± 1.2E–04	3
Morphine	BL	5–1,000	5.39E–03 ± 1.56E–04	2.73E–02 ± 1.47E–02	0.998 ± 1.41E–03	4
	PF		7.49E–03 ± 1.91E–03	6.37E–02 ± 1.89E–02	0.997 ± 2.33E–03	3
6-MAM	BL	5–1,000	5.8E–03 ± 5.5E–04	1.3E–02 ± 5.0E–04	0.998 ± 1.2E–03	4
	PF		9.3E–03 ± 3.0E–03	1.5E–02 ± 7.3E–03	0.999 ± 9.1E–05	4
6-ACCOD	BL	5–1,000	7.3E–03 ± 6.9E–04	2.3E–02 ± 2.2E–02	0.999 ± 1.1E–03	4
	PF		1.3E–02 ± 4.5E–03	3.1E–01 ± 5.3E–01	0.999 ± 7.5E–05	4
Oxycodone	BL	5–1,000	4.9E–03 ± 1.4E–03	–4.4E–03 ± 5.3E–03	0.998 ± 2.4E–03	4
	PF		5.1E–03 ± 9.1E–04	–7.2E–03 ± 4.8E–03	0.995 ± 1.4E–03	3
Oxymorphone	BL	5–1,000	4.5E–03 ± 1.2E–03	–1.7E–03 ± 6.8E–03	0.998 ± 2.1E–03	3
	PF		5.5E–03 ± 1.1E–03	–3.1E–03 ± 8.9E–03	0.995 ± 2.8E–03	3
Fentanyl	BL	5–1,000	5.6E–03 ± 7.1E–04	1.0E–02 ± 5.7E–03	0.999 ± 8.3E–04	3
	PF		9.0E–03 ± 2.7E–03	2.1E–02 ± 3.4E–02	0.999 ± 5.4E–04	3

^aMean values ± standard deviation ($n = 5$). ^b $n = 6$.

For PF, all compounds reached LODs of 3 ng/mL with exception of 6-MAM and 6-ACCOD (4 ng/mL) as can be seen in Table II. The LOQs achieved in both matrices were 5 ng/mL for all substances (Figure 1A and B). Although in some previous studies lower values were achieved for LODs and LOQs much higher volumes of BL sample were used, between 1 and 3 mL (31, 33, 35, 37, 42, 47, 56–61). Related to PF, previous studies neither present values of LOD and LOQ using GC–MS, for codeine and morphine, nor the other compounds studied in this matrix, being impossible to make comparisons (18, 20, 23). According to the few review articles published on the determination of opiates in biological matrices, we present some comparisons of results obtained for both LOD and LOQ taking in account the sample volume (V) used and the chromatographic technology coupled with the mass spectrometry that was applied (GC–MS–MS and LC–MS–MS technologies) (27, 28). When LC–MS–MS was used, the LODs (LOQs) obtained in BL for morphine, codeine, 6-MAM, 6-ACCOD, oxycodone, oxymorphone and fentanyl are between 0.2 (0.7) and 7 (8) ng/mL ($V = 1$ mL) (62, 63); 0.09 (0.44) ($V = 100$ μ L) and 3 (5) ng/mL ($V = 1$ mL) (63–65); 0.05 (5) ($V = 100$ μ L) and 2 (3) ng/mL ($V = 1$ mL) (63, 66); 0.26 (0.88) and 1 (5) ng/mL ($V = 1$ mL) (62, 64); 0.2 (0.2) and 1 (2) ng/mL ($V = 1$ mL) (63, 67); 0.29 (0.98) ($V = 1$ mL) and 0.3 (1) ng/mL ($V = 300$ – 500 μ L) (68, 69); 0.08 (0.1) ng/mL ($V = 1$ mL) (63), respectively. With GC–MS–MS techniques, the LODs (LOQs) obtained in BL for the same compounds were: 1 (2.5) ($V = 1$ mL)–20 (20) ng/mL ($V =$ unknown) (59, 70); 1 (2.5)–2 (10) ng/mL ($V = 1$ mL) (35, 70); 0.78 (1.56) ($V = 3$ mL)–2 (10) ng/mL ($V = 1$ mL) (31, 35); no data available for 6-ACCOD; 0.5 (1) ($V = 3$ mL)–25 (250) ng/mL ($V = 2$ mL) (47, 58); 0.5 (1) ($V = 3$ mL)–15 (25) ng/mL ($V = 1$ mL) (42, 47); 0.1 (0.1) ($V = 1$ mL)–50 (50) ng/mL ($V = 2$ mL) (57, 58), respectively. Taking into account the technology (GC–MS) and the sample volume used (250 μ L), our results were quite satisfactory for all compounds in both matrices, when compared with those published.

Carryover was not observed in both matrices analyzed for each substance.

The values obtained in BL for intraday precision ($CV < 10.0\%$) and bias (0.0–17.9%), such as intermediate precision ($CV < 8.3$) and bias (0.0–19.4%), were adequate. We also obtained acceptable results

in PF for intraday precision ($CV < 8.4\%$) and bias (0.0–18.4%) and for intermediate precision ($CV < 11.9$) and bias (0.0–13.8%). These results are presented in Tables III and IV.

The results obtained for the stability on the autosampler are summarized in Table V. All extracted substances were stable during 24 h in both biological matrices with % differences between –19.0 and 3.7% for BL and between –17.1 and 5.0% for PF. At Table VI are presented both the benchtop stability and three freeze/thaw cycles (after 1, 2, 3 and 4 weeks). Regarding benchtop stability, all analytes were stable in BL and PF, with differences between –19.1 and 13.4 (BL) and between –16.0 and 13.9% (PF). However, after three freeze/thaw cycles, there was only stability for all substances in PF with differences between –16.6 and 19.9%. The results obtained to fentanyl during 7 days led to the need to reduce the analysis period to a shorter period of 5 days. Nevertheless, the results were similar, with the difference $>20\%$, (out of the limits established by the SWGTOX) having noticed a progressive increase of this difference over the different periods. The study of this parameter with fortified samples may have affected the stability of fentanyl and led to a slightly different behavior from a real biological sample.

The mean of triplicate fortified samples were quantified within –12.0 to 16.4% in BL and –9.8 to 11.4% in PF, using 1:2 dilution factor. All the substances have shown adequate bias within the acceptance criteria of $\pm 20\%$.

The results obtained for the extraction efficiency of the developed procedure in BL, for all concentration levels (20, 200 and 900 ng/mL) ranged between 32.7 and 87.6% for all the substances. Oxycodone and oxymorphone were the two substances with the lowest values ($<65.3\%$). These results are adequate since the obtained LOD and LOQ are sufficiently low in volumes of 250 μ L of BL. It was not possible to study this parameter in PF, because an unexpected formation of an emulsion occurred after adding the 1% aqueous hydroxylamine to the samples with unextracted standards (set 2). An insoluble material was also formed when the dried extracts were treated with the MSTFA + 5% TMCS solution. This side reaction may have occurred due to the formation of a salt of the neutralized acid during oximation, caused by an eventual pH change that prevented the complete reaction (71). However, the LOD and

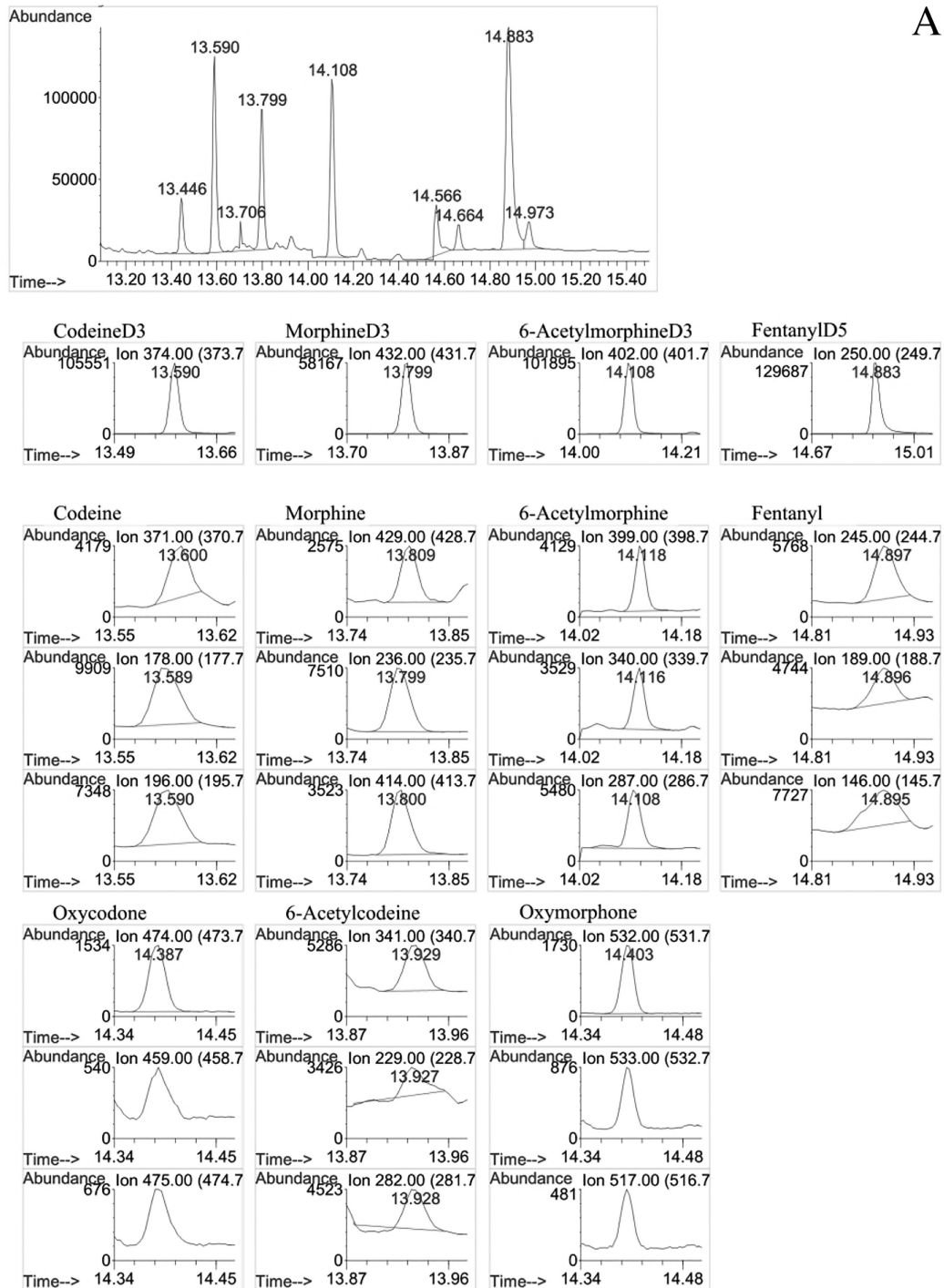


Figure 1. Ion chromatograms of BL (A) and PF (B) samples fortified at the LOQ of each compound.

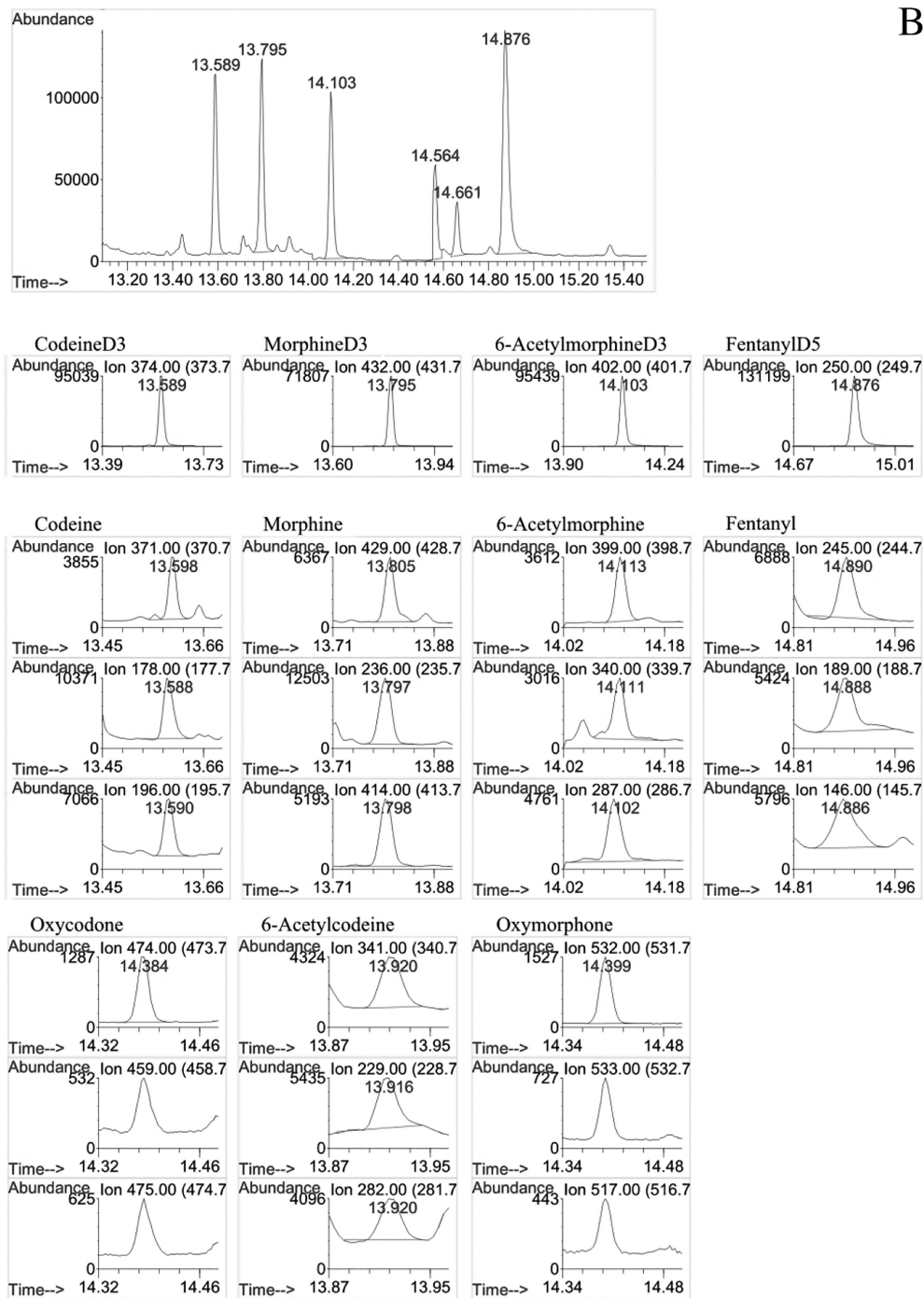


Figure 1. Ion chromatograms of BL (A) and PF (B) samples fortified at the LOQ of each compound.

Table III. Intraday Precision ($n = 6$)

Analytes	Matrix	Spiked concentration (ng/mL)											
		5			25			200			900		
		Concentration found (ng/mL)	Bias (%)	CV (%)	Concentration found (ng/mL)	Bias (%)	CV (%)	Concentration found (ng/mL)	Bias (%)	CV (%)	Concentration found (ng/mL)	Bias (%)	CV (%)
Codeine	BL	5.7	3.4	14.1	29.5	17.9	0.5	194.5	17.9	5.0	854.7	-2.7	3.0
	PF	5.5	6.8	9.3	29.2	16.8	2.0	181.4	16.8	0.7	826.8	-9.3	0.7
Morphine	BL	5.5	3.8	9.2	29.3	17.2	3.0	199.2	17.2	4.8	825.9	-0.4	2.8
	PF	4.3	3.7	-14.9	28.3	13.1	5.1	186.5	13.1	0.7	956.0	-6.8	1.5
6-MAM	BL	5.5	1.5	9.9	28.6	14.6	1.2	198.1	14.6	4.7	886.6	-0.9	3.0
	PF	4.8	8.5	-3.0	29.6	18.4	2.5	196.0	18.4	0.8	992.8	-2.0	1.5
6-ACCOD	BL	5.8	3.8	15.8	27.3	9.1	1.6	187.1	9.1	4.1	847.3	-6.5	2.9
	PF	5.1	6.5	2.3	29.0	16.0	2.7	189.4	16.0	1.0	996.9	-5.3	3.8
Oxycodone	BL	5.7	6.1	13.6	27.3	8.2	7.8	208.8	8.2	6.6	895.0	4.4	9.9
	PF	5.4	7.0	7.4	27.2	8.8	4.7	192.0	8.8	8.9	977.8	-4.0	1.3
Oxymorphone	BL	5.6	8.1	11.7	28.6	14.3	3.4	228.2	14.3	3.3	894.2	14.1	7.6
	PF	4.9	9.4	1.4	27.6	10.4	8.0	203.4	10.4	9.5	995.9	1.7	1.2
Fentanyl	BL	5.7	2.1	-14.6	28.6	11.1	1.3	193.4	11.1	5.3	895.9	-3.3	3.7
	PF	4.6	10.7	-8.8	29.0	15.8	1.3	199.2	15.8	4.7	960.4	-0.4	3.2

Table IV. Intermediate Precision ($n = 15$)

Analytes	Matrix	Spiked concentration (ng/mL)								
		25			200			900		
		Concentration found (ng/mL)	CV (%)	Bias (%)	Concentration found (ng/mL)	CV (%)	Bias (%)	Concentration found (ng/mL)	CV (%)	Bias (%)
Codeine	BL	29.7	1.4	18.8	218.8	5.2	9.4	918.7	3.1	2.1
	PF	28.5	4.2	13.8	205.0	5.0	2.5	887.3	6.6	-1.4
Morphine	BL	28.6	5.1	14.6	233.6	1.7	16.8	912.8	1.6	1.4
	PF	27.9	4.7	11.5	207.4	8.2	3.7	917.1	6.6	1.9
6-MAM	BL	29.0	3.3	16.0	225.1	4.1	12.6	918.9	1.4	2.1
	PF	28.2	6.4	12.8	203.7	3.4	1.8	911.3	5.7	1.3
6-ACCOD	BL	28.6	4.8	14.5	216.2	5.4	8.1	945.4	3.3	5.0
	PF	26.4	11.8	5.6	200.5	3.8	0.2	911.7	6.3	1.3
Oxycodone	BL	28.9	6.8	15.7	222.6	5.9	11.3	860.2	8.3	-4.4
	PF	25.9	9.8	3.7	204.2	8.8	2.1	917.1	10.4	1.9
Oxymorphone	BL	29.3	4.5	17.1	226.1	3.7	13.0	906.1	6.1	0.7
	PF	27.1	11.2	8.5	206.5	8.6	3.2	919.9	10.3	2.2
Fentanyl	BL	29.8	1.1	19.4	205.8	3.3	2.9	933.7	2.5	3.7
	PF	28.0	5.7	11.9	200.8	3.7	0.4	917.0	5.5	1.9

Table V. Stability Data (% Difference) after 24 h, 48 h and 7 days on the Autosampler ($n = 3$)

Analytes	Matrix	Autosampler 24 h			Autosampler 48 h			Autosampler 7 days		
		20	200	900	20	200	900	20	200	900
		(ng/mL)								
Codeine	BL	2.9	0.5	0.5	8.9	-0.4	-0.1	8.6	-6.1	-3.5
	PF	0.3	0.6	0.6	2.3	1.5	-0.4	0.7	-2.1	-3.3
Morphine	BL	1.8	0.3	0.2	-16.9	1.6	0.3	-12.3	3.9	4.3
	PF	1.6	0.1	0.3	7.4	3.2	3.0	13.8	1.6	2.9
6-MAM	BL	2.2	0.7	0.0	0.1	-0.9	-0.2	5.1	-6.1	-4.3
	PF	-0.4	-0.2	5.0	-0.1	2.7	0.2	2.7	-1.6	-1.0
6-ACCOD	BL	-1.7	-2.5	-0.6	-17.9	-8.4	0.5	5.3	-5.9	-0.3
	PF	-0.3	-1.1	-0.8	0.2	-1.9	-0.4	3.7	0.2	-1.8
Oxycodone	BL	-19.0	-14.9	-8.6	-15.3	-15.3	-13.6	-61.7	-57.1	-54.0
	PF	-11.3	-17.1	-7.5	-31.2	-22.2	-21.6	-18.8	-23.2	-22.3
Oxymorphone	BL	-11.9	-7.2	-4.0	-9.3	-10.4	-9.4	-33.6	-32.9	-31.0
	PF	-8.4	-11.7	-7.2	-19.9	-19.9	-13.5	-8.4	-13.1	-11.9
Fentanyl	BL	3.7	0.4	0.9	6.3	2.7	-0.5	9.4	-0.6	-1.3
	PF	1.4	-0.3	0.9	3.8	2.3	2.0	1.2	-0.3	-1.2

LOQ reached in 250 μ L of PF were low enough to demonstrate that the method is sensitive.

Method application

The applicability of the method was evaluated by their application to 44 and 31 BL and PF samples, respectively. In BL, we had 11 positive results for codeine (5.8–54.8 ng/mL), 33 for morphine (4.2–386.0 ng/mL), 4 for 6-MAM (4.6–13.2 ng/mL) and 3 for fentanyl (4.4–45.2 ng/mL). We had only one positive result in PF: codeine (50.2 ng/mL), morphine (540.4 ng/mL) and 6-MAM (5.3 ng/mL) (Figure 2A and B).

The restricted number of positive cases in PF and the absence of positive cases for some of the substances (6-ACCOD,

oxycodone, oxymorphone and fentanyl) is the main limitation of our methodology, considering the existence of records that they are consumed and the evolution on its consumption. However, the case samples were from >2 years ago, and according to the obtained stability results most of the substances degrades over time; this can be an explanation for the lack of positive results.

Conclusion

We were able to develop a sensitive and selective methodology combining the SPE extraction and the analyses by gas chromatography with a single quadrupole mass spectrometer for the determination of opioids and some metabolites in volumes of 250 μ L of BL and PF. Since often small amounts of samples are available to perform

Table VI. Stability Data (% Difference) after Benchtop for 4 h at Room Temperature and after Three Freeze/Thaw Cycles ($n = 3$)

Analytes	Matrix	Benchtop 4 h (room temp.)		Freeze/Thaw (-15°C) 8 days (3 cycles)		Freeze/Thaw (-15°C) 15 days (3 cycles)		Freeze/Thaw (-15°C) 21 days (3 cycles)		Freeze/Thaw (-15°C) 30 days (3 cycles)	
		20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL
Codeine	BL	-1.7	9.2	1.8	19.5	12.3	15.3	25.7	31.1	42.1	38.0
	PF	1.8	5.1	2.7	1.3	-11.6	-1.4	-11.2	-5.6	4.7	7.5
Morphine	BL	3.9	13.4	-3.0	13.8	-2.4	-3.2	-3.9	-1.3	6.3	5.2
	PF	7.8	7.8	9.1	2.5	-5.4	1.9	-4.0	-1.1	14.6	14.9
6-MAM	BL	-19.1	-16.1	-17.4	-15.8	-36.6	-43.1	-40.3	-51.8	-40.5	-47.7
	PF	-8.6	-7.3	-7.8	-8.5	-16.6	-5.6	-16.2	-8.7	-8.0	-4.4
6-ACCOD	BL	-15.6	-15.9	-14.7	-18.0	-43.2	-54.9	-49.9	-65.9	-51.7	-59.4
	PF	-5.6	2.3	-9.4	-3.5	-9.0	-3.2	-11.5	-5.3	1.9	2.7
Oxycodone	BL	-16.9	4.7	-15.7	18.7	9.8	-16.4	-32.5	-45.4	-41.8	-23.6
	PF	6.1	-16.0	9.8	6.6	-5.9	19.4	4.3	-11.7	19.9	16.2
Oxymorphone	BL	-17.5	2.9	-18.4	18.8	6.9	-17.6	-26.4	-43.1	-36.3	-17.3
	PF	13.9	-11.1	17.3	15.3	-12.5	12.9	1.4	-8.3	14.0	15.6
Fentanyl	BL	-17.5	-11.7	-53.7	55.9	-71.6	-75.7	-78.7	-78.4	-77.4	-80.8
	PF	1.0	0.5	-1.6	-2.3	-13.3	-2.1	-12.2	-5.2	3.3	4.9

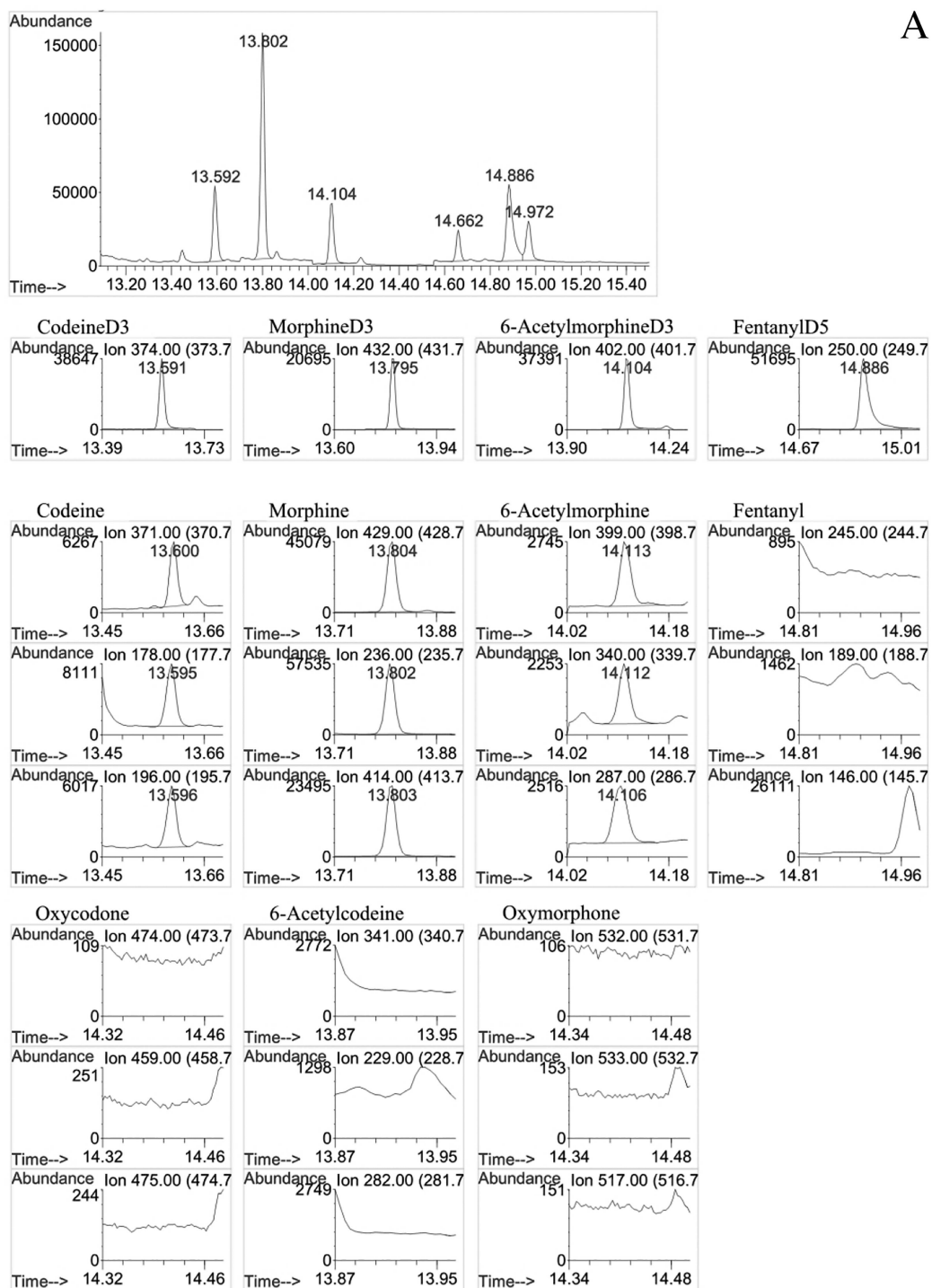


Figure 2. Ion chromatograms of authentic BL (A) and PF (B) samples.

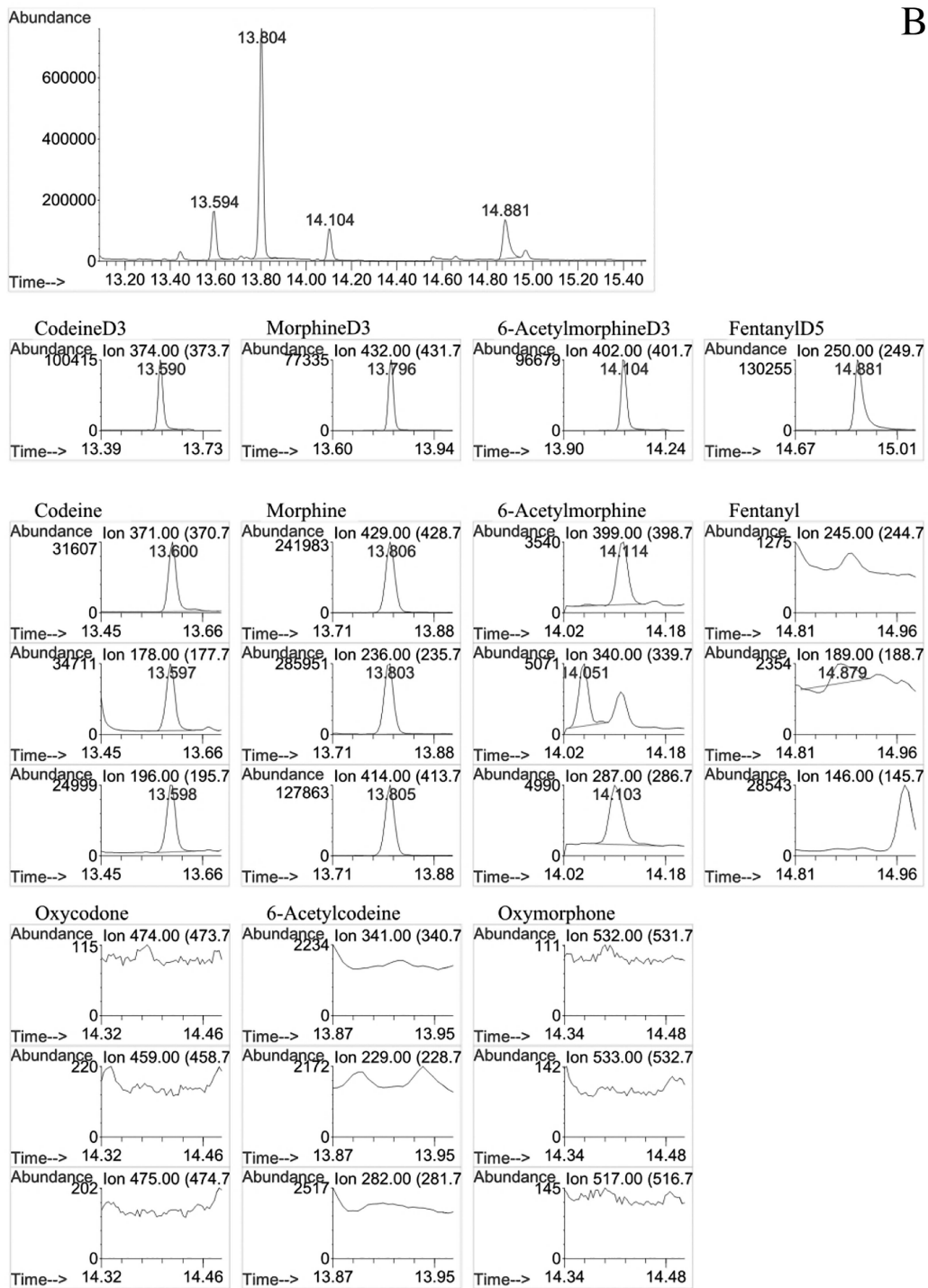


Figure 2. Ion chromatograms of authentic BL (A) and PF (B) samples.

all the required toxicological analyses, only 250 µL of required volume sample is very useful. This new methodology brings several advantages like the small sample and solvents volumes required and the low limits (LOD and LOQ) obtained for all the substances. The optimization of two microwave-assisted derivatizations (30 and 100 s) contributed to the reduction of the total time of the assay when compared to previous studies. Another advantage in the utilization of the GC-MS analytical instrumentation relates to its availability in most toxicology laboratories and because it offers robustness, sensitivity and is less expensive than other technologies.

To our knowledge, this is the first procedure developed for the simultaneous determination of codeine, morphine, 6-MAM, 6-ACCOD, oxycodone, oxymorphone and fentanyl in whole BL and PF with microwave-assisted derivatization with both 1% aqueous hydroxylamine and MSTFA + 5% TMCS.

Furthermore, the results indicate the methodology suitability for the application in forensic toxicology laboratories in the routine analysis of these compounds. Thus, the developed methodology prepares the forensic toxicology laboratory for future requests and for the unpredictable detection of these substances in the routine casework.

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