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RESEARCH ARTICLE

GC-MS Method for the Analysis of Thirteen Opioids, Cocaine and Cocaethylene in Whole Blood Based on a Modified Quechers Extraction

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Abstract: ***Background:*** QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) is a methodology previously developed to extract pesticides from vegetables and fruits and has been fully applied for different analytical approaches.

Objective: In the present study, a rapid and less laborious modified QuEChERS extraction method for the quantification of 13 opioids [codeine, morphine, heroin, 6-acetylmorphine (6-AM), desomorphine, ethylmorphine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP), papaverine, tramadol, *O*-desmetyltramadol (M1) and, tapentadol], cocaine and cocaethylene in whole blood was developed and validated by Gas Chromatography-Mass Spectrometry.

Method: The modification of QuEChERS method consisted in the pretreatment of the whole blood samples using ultrasonication, the use of ethyl acetate as extraction solvent and a previous step of sample alcalinization. The use of dispersive separation steps such as Dispersive Solid-Phase Extraction (dSPE) or sorbents such as Primary Secondary Amine (PSA) was suppressed to minimize the errors and, to improve the velocity of the analysis.

Results: The method proved to be selective and the regression analysis for the analytes was linear in the range of 31.2-2000 ng/mL with correlation coefficients > 0.98. The coefficients of variation did not exceed 15%. The lowest limit of detection and quantification for all the analytes were below the therapeutic range of the drugs. The recoveries of the analytes ranged from 52.4 to 95.0%.

Conclusion: The developed method can provide a rapid, effective and "greener" process for the analysis of a wide range of opioids drugs in whole blood samples and can be applied to clinical and forensic *antemortem* and *postmortem* cases.

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INTRODUCTION

Clinical and Forensic Toxicology are strongly based in Analytical Chemistry. Whole blood, urine and solid tissues as well as alternative samples such as oral fluid, hair and meconium are commonly used for toxicological analysis [1-2]. The use of such biological matrices demands an extraction/purification pre-treatment before its chromatographic analysis. Solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are common techniques used for this purpose. LLE is a classical and simple technique that uses solvents to selectively extract target compounds [3, 4]. However, LLE has a number of drawbacks that limit its use, namely reduced selectivity, large solvent consumption and difficulty of automation. Moreover, formation of emulsions can interfere with the phase-separation process due to the difficulty to separate emulsified organic phases, and errors

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can occur when extracting compounds of interest, consequently questioning the obtained quantitative values [5]. SPE has high selectivity and has been used for many biological matrices [6-7]. Although some automation exists, SPE is a complex, laborious, high cost and time-consuming methodology in an extensive multi-steps process [8].

In 2003, Anastassiades *et al.* [9] developed a new extraction method named QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) to analyze pesticides in vegetables and fruits, removing sugars, lipids, organic acids, steroids, proteins, pigments and water excess, all in one step. Its advantages are the quicker and easier handling, the use of low solvent volumes and low cost when compared with other extraction methods. This process involves a sample extraction using acetonitrile, followed by addition of anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) to decrease solubility of organic drugs in aqueous phase, and to reduce the amount of water in the organic phase [9-10]. Only few studies have applied QuEChERS in human samples. Some successful reports, included the extraction of pesticides [11] and drugs of abuse in human whole blood [10, 12] and muscle samples [13-15]. Most studies applying QuEChERS to complex biological samples such as whole blood, describes the use of a pre-treatment step with Primary Secondary Amine (PSA) [8, 12]. PSA showed to be an effective sorbent for removal of various matrices and significantly reducing matrix-enhancement effect. The surface of PSA contains many primary secondary amino groups, which could selectively adsorb fatty acids an important interference in whole blood samples [16].

Due the large number of cases that every year reaches clinical and forensic laboratories, it is imperative to use fast extraction, detection and quantitative analytical methods to analyze toxicological substances in biological specimens. Opioids have a great role in fatal intoxications in Europe, as reported in The European Report about Drugs. About 3.5% of all deaths of Europeans 15-39 years old were drug overdoses, opioids were found in about three-quarters of fatal overdoses [17].

Therefore, the goal of our work was the development and validation of a modified QuEChERS method for the simultaneous extraction of 13 opioids from whole blood samples and to analyze them by GC-MS.

MATERIAL AND METHODS

Reagents and Standards

Ethyl acetate and sodium sulfate were purchased from Carlo Erba (Milan, Italy), N-methyl-N-(trimethylsily) trifluoroacetamide (MSTFA) was purchased from Sigma-Aldrich (St Louis, MO, USA). Magnesium sulfate and sodium chloride in powder form and analytical grade were purchased from Merck (Darmstadt, Germany). Codeine, morphine, heroin, 6-AM, desomorphine, ethylmorphine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP), papaverine, tramadol, tapentadol, cocaine and cocaethylene certified standards were purchased from Lipomed AG (Arlesheim, Switzerland). *O*-desmetyltramadol (M1) was a generous gift from Grünenthal (Amadora, Portugal). Phenacetin (internal standard, IS) was obtained from LGC Standards. The purity of all standards were higher than 98.5%. Helium C-60 (99.99%) was ordered from Gasin (Portugal). Nitrogen was supplied by AirLiquid (Algés, Portugal). All the reagents used were from the highest available grade.

Sample Collection

Blank whole blood samples for method validation were obtained from healthy volunteers who gave informed consent, and collected in tubes containing EDTA. Any preservative such as sodium fluoride was added.

Preparation of Stock, Quality Control and Working Solutions

Separate commercially available 1 mg/mL methanolic solutions for each analyte and IS were used as stock solutions. They were prepared and stored at -20ºC until use. The heroin solutions were prepared in chloroform due to their instability when stored in methanolic solutions [9]. Working solutions (2000 ng/mL) were prepared by dilution from each stock solution in 1 mL of mL of blank whole blood. For calibration standards and quality control (QC) samples, the working solutions were diluted in blank blood by serial dilutions. For the calibration standards serial dilutions were made to originate the 2000, 1000, 500, 250, 125, 62.5 and 31.2 ng/mL concentrations. For QC spiking solutions (low, 31.2; medium, 250; high, 2000 ng/mL), serial dilutions from the work solutions were also used. No dilution integrity was performed as part of the method validation since selected standard concentrations cover most concentration found in clinical and forensic settings as previously demonstrated [10].

Samples Extraction Using QuEChERS

For extraction, 300 µL of spiked whole blood with work concentrations of each analyte was alkalinized using one drop of NaOH 0.1M, to obtain a pH around 10.0. Samples were vortexed and then centrifuged at 10,000g for 5 minutes. The resulting supernatants were separated and, then sonicated for 5 min in an ultrasonic bath (Fig. **1A**). 300 µL of the supernatant was pipetted into a tube containing a previously pulverized mixture of 50mg of NaCl and 100mg of $MgSO_4$ (1:2), 500 µL of ethyl acetate and two metal spheres. The samples were vortexed for 10 seconds and then centrifuged at 7,300g for 2 minutes. The organic phase was carefully transferred to a dry and clean glass tube. This extraction process was repeated to maximize extraction efficiency. All samples were evaporated to dryness using a nitrogen flow at room temperature (Fig. **1B**). The time effort was approximately 30 minutes for fourteen different samples and a daily calibration curve. 30 µL of N-methyl-N-(trimethylsily) trifluoroacetamide (MSTFA) were added and samples heated at 80ºC for 30 min to accomplish silylation. An aliquot of 1 µL of the derivatized extract was injected into the GC-IT/MS system (Fig. **1C**).

GC-MS Conditions

Quantitative and qualitative GC-MS analyses were performed on a Trace GC 2000 Series ThermoQuest gas chromatograph equipped with ion-trap GCQ Plus ThermoQuest Finnigan mass detector (Austin, Texas, USA). Chroma-

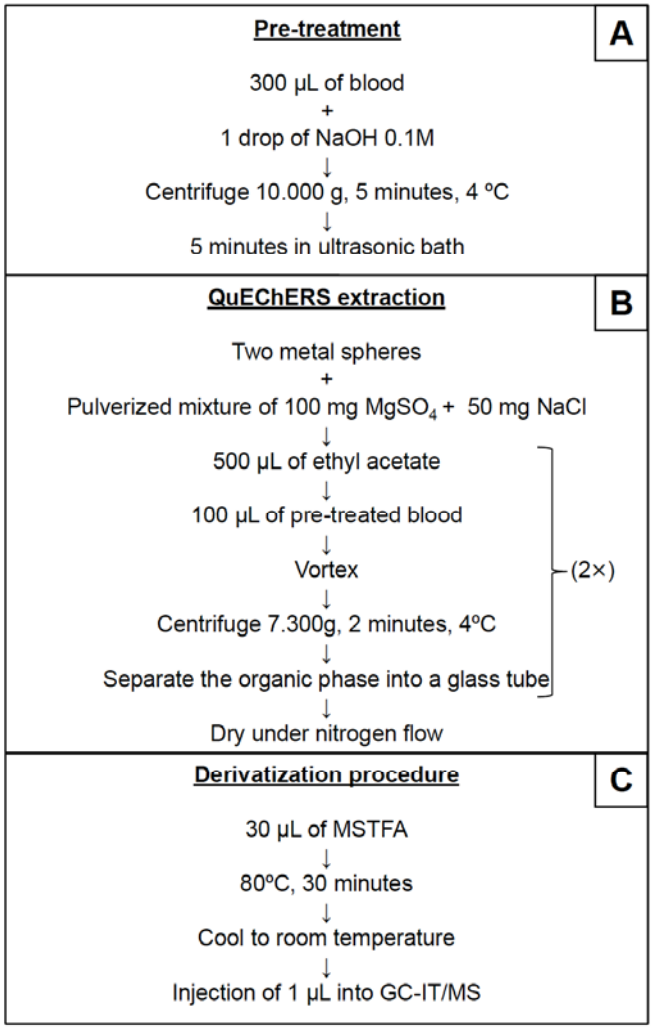

Fig. (1). Sample preparation procedure. **A** – Pre-treatment of blood samples. **B** - Extraction of analytes by modified QuEChERS method. **C** – Derivatization procedure.

tographic separation was achieved using a capillary columm (30 m × 0.25 mm × 0.25 µm, cross-linked 5% diphenyl and 95% dimethyl polysiloxane) from Restek® (Pennsylvania, USA) and high-purity helium C-60 as carrier gas. An initial temperature of 80ºC was maintained for 1 min, increased to 300ºC at 10ºC/min rate, and held for 5 min giving a total run time of 28 min. The flow of the carrier gas was maintained at 1.0 mL/min. The injector port was set at 280ºC. Quantification was performed in selected ion monitoring mode (SIM) with splitless injection. The most abundant ions with higher *m/z* were selected for quantification and, second and third more abundant *m/z* ions were used for qualification, considering the mass spectra of the standards previously analyzed and the retention times of each analyte [11-13]. Elucidation of their structures was not performed. The designated *m/z* for the each analyte is present in Table **1**. For qualitative analysis all analytes were analyzed simultaneously.

Phenacetin was chosen as IS since it proved to be effective for opioids analysis as described previously [14]. Although phenacetin has been reported to be used to "cut" heroin and cocaine samples, we performed a thorough retrospective analysis of forensic intoxication involving heroin and cocaine in Portugal between 2001 and 2013; these cases did not register positivity for phenacetin. Typical recommendations on sample preparation of biological specimens for systematic toxicological analysis suggests that for any systematic toxicological assay procedure, active drugs should only be used as internal standards when no alternative is available and after proofing their absence in the sample, which corresponds to the present case [15].

Table 1. The designated *m/z* for the opioids analyzed.

Analyte	*m/z*
Phenacetin-tms	162, 236, 251
Desomorphine-tms	148, 271, 286
Codeine	146, 234, 371
Morphine-tms	234, 268, 429
6-AM-tms	203, 268, 399
Heroin-tms	327, 369, 310
Methadone	72, 233, 294
EDDP	232, 262, 277
EMDP	115, 130, 193
Tramadol	58, 264, 336
M1	58, 322, 394
Ethylmorphine-tms	192, 234, 385
Papaverine	293, 324, 338
Tapentadol	58, 133, 221
Cocaine	82, 182, 303
Cocaethylene	82, 196, 317

Method Validation

The validation of the method was performed accordingly to European Medicines Agency [16]. The evaluated parameters were selectivity, limit of detection (LOD), lower limit of quantification (LLOQ), precision, accuracy, recovery, matrix effect and linearity of the method. Calibration curves were prepared by serial dilutions starting from the working solutions in blank whole blood prepared with standard solutions of codeine, morphine, heroin, 6-AM, desomorphine, ethylmorphine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP), papaverine, tramadol, *O*-desmetyltramadol (M1), tapentadol, cocaine and cocaethylene. The QC samples were also prepared from working solutions starting from the high concentration with serial dilutions for medium and low concentrations.

Proof of Applicability

Whole blood samples were collected from volunteers undergoing methadone administration as a replacement therapy of heroin dependence and from rats exposed to desomor-

phine, tramadol and tapentadol. The human samples were obtained under the consent of the volunteers.

The animal study was performed using adult male Wistar rats obtained from Harlan (Udine, Italy), with mean weight of 250±10g. Animals were kept under standard laboratory conditions (12h/12h light/darkness, 22±2ºC room temperature, 50-60% humidity) for at least 1 week (quarantine) before starting the experiments. Animals were allowed access to tap water and rat chow ad libitum during the quarantine period. Animals' experiments were licensed by the Portuguese General Directorate of Veterinary Medicine and approved by the Ethical Committee of Faculty of Pharmacy of the University of Porto (protocol number 0269/26/EA). Housing and experimental treatment of animals were in accordance with the guidelines defined by the European Council Directive (2010/63/EN). Six male Wistar rats were daily exposed to desomorphine (1 mg/Kg) during five consecutive days. After the last day, animals were euthanized and whole blood was collected. For tapentadol and tramadol analysis sixteen male Wistar rats were divided in two groups. At the first group, tramadol was administered in two doses (therapeutic - 10 or toxic - 25 mg/Kg) in four animals *per dose*. The second group had the same procedure but tapentadol was used. Both group animals were euthanized 24 h after the injections to collect whole blood. For both substances experiments, blood was obtained by exsanguination using a hypodermic heparinized needle. Blood samples were centrifuged (3000g, 4ºC, 10 min) and plasma was aliquoted and stored (-80ºC) for further analysis.

RESULTS AND DISCUSSION

Several authors have applied QuEChERS to clean up biological samples for subsequent detection of drugs [8, 17-21]. Occasionally, sorbents were used after QuEChERS to improve removal of proteins and lipids, which are the most common interferents in biological samples [17-19]. To develop a fast and reliable method for clinical and forensic applications, we aimed to reduce sample preparation steps in order to minimize errors and cost.

Modified QuEChERS method

Sample clean-up: Sample clean-up is a very important step in toxicological analysis aiming to isolate target substances from tissue interfering components such as proteins and lipids, and to concentrate analytes present in the specimen. This method adds a rapid pre-treatment step using centrifugation and sonication to release the compounds linked to blood proteins, especially albumin, followed by ethyl acetate as the extraction solvent replacing the acetonitrile of the classic QuEChERS methodology. These adaptations were shown to be suitable for the detection and quantification of the tested drugs of abuse in whole blood samples (Fig. **2A**).

Choice of solvent: The drug analytes tested are more soluble in ethyl acetate, it is easier and safer to handle, and is considered a "solvent of choice" among a wide variety of multiresidue tests available for different kinds of analytes [22]. Previously, basified acetonitrile was tested as extraction solvent but recoveries lower than 15% were obtained. Raising the ethyl acetate extraction pH to 10 takes into account the chemical nature of all compounds which in an acidic environment should be ionized and consequently not soluble in organic solvents. In our work, the use of an alkaline pH during the extraction process was an important modification to compensate the absence of the Primary Secondary Amine (PSA) step.

Salting out steps: The "salting out" process follows the original method using $MgSO_4$ and NaCl salts, being only miniaturized for adaptation to whole blood samples [23]. Anhydrous $MgSO_4$ is an effective drying agent with an exothermic process of hydration, which increases the extraction efficiency [22, 24] and NaCl decreases the solubility of organic drugs in their aqueous phase, increasing their concentration in the organic solvent.

Sample preservation: Fluoride preservation with a final concentration of 1-5% sodium (or potassium) fluoride by weight is usually recommended for whole blood samples and has great role avoiding the enzymatic loss of esters [1]. The whole blood sample collection was performed using only EDTA once the samples were not stored and all the analysis were performed on the same day after the collection.

Additional drugs: Besides opioids, in this method validation we also included cocaine and cocaethylene. The presence of these compounds relies on the fact that many polydrug users self-administer combinations of heroin with cocaine (*i.e.*, "speedball") and ethanol [25]. Benzoylecgonine is the major cocaine metabolite in plasma by all routes of administration [26-28]. Although benzoylecgonine is considered pharmacologically inactive, it has a longer elimination plasma half-life (0.5-1.5h *versus* 4-7h) [32-35] and, therefore, it is the most commonly monitored metabolite for the determination of cocaine abuse. Although previously considered for validation purposes, benzoilecgonine co-eluted with other analytes and presented low recoveries even after extraction and pH optimization and therefore our QuEChERS methodology proved not be effective for this analyte.

Method Validation

The analytical figures of merit of the developed method are discussed in the following topics.

Selectivity. Selectivity is "the ability of an analytical method to differentiate and quantify the analyte in the presence of other endogenous components in the sample" [16]. Six blank matrix samples were analyzed to evaluate chromatographic interferences. No interference peaks were detected, either in the retention times of all the analytes or in the phenacetin (IS) retention time (Fig. **2A**; Fig. **2B**). Therefore, the analytical method is capable to differentiate our 15 analytes and IS from endogenous components present in the sample.

Carry-over. During the validation process, injections of calibration standards containing 20 μg/mL of each analyte (10 times the concentration of the higher curve calibration point) were followed by six blank sample injections of ethyl acetate, to ensure that there was no carry-over from one injection to the next one. The obtained carry-over results were less than 20% of the LLOQ and less than 5% for the IS, which are within the proposed acceptance limits for this parameter [16].

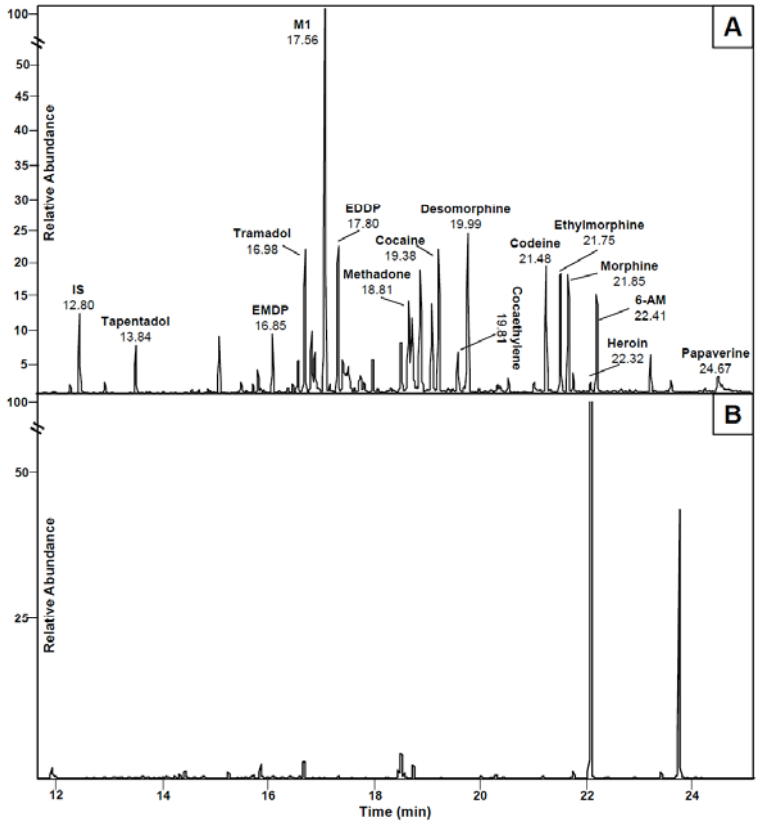

Fig. (2) A- Chromatogram of analytes extracted by modified QuEChERS method at concentrations of 2 μg/mL (1 –IS; 2 – Tapentadol; 3 – EMDP; 4 – Tramadol; 5 – M1; 6 – EDDP; 7 – Methadone; 8 – Cocaine; 9 – Cocaethylene; 10 – Desomorphine; 11 – Codeine; 12 – Ethylmorphine; 13 – Morphine; 14 – 6-AM; 15 – Papaverine; 16 - Heroine. **B** - Chromatogram of a blank sample. M1 – *O*-desmethyltramadol; 6-AM – 6-acethylmorphine; EDDP - 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EMDP - 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline; IS – Internal Standard.

Matrix Effect. For the matrix effect evaluation six calibration curves were performed. Three curves were prepared using neat standard solutions diluted in pure solvent in a range of 31.2-2000 ng/mL and, three curves were prepared using spiked blank whole blood samples on the same concentration range. The matrix effect factor was calculated considering the response of the analytes with matrix and in the absence of matrix. The values for all analytes were between 80-120%. For the method tested the %CV obtained were less than 15%. Matrix-induced signal enhancement occurs in GC when active surfaces in the system (*i.e.*, injector, column and detector) cause retention and/or degradation of analytes. In standard solutions without sample matrix, only the analytes fill the active sites, which reduce the percentage of injected molecules eventually detected. In complex injected extracts, the active sites are filled predominantly by matrix components, thereby increasing efficiency of analyte transfer through the GC system to the detector [29]. The ionic suppression or enhancement at the interface affects accuracy because standards in pure solvent do not undergo this process [30]. The exact mechanism of ion suppression is not known; it seems that it may be caused by non-volatile material 31 or by compounds of high surface activity [32]. The chemical nature of the analyte plays an important role too. Taylor [33] observed that matrix effects of polar compounds are more relevant than those of less polar compounds. Some instrumental parameters such as the ionization source [31], ionization mode [34], and flow rate [35] have been found to influence the extent of the matrix effect. There are different approaches to avoid matrix effect in GC analysis such as adding masking agents or analyte protectants to standards and samples but an efficient clean-up process is the best strategy especially for complex matrices [36].

Linearity. Peak-area ratios of analytes of interest to the internal standard were calculated. Calibration curves were obtained by plotting peak-area ratios against concentration. The determination coefficient (r^2) was obtained for each linear regression of three different curves of each analyte obtained from independent working solutions. The results obtained are showed in Table **2**. The r^2 were >0.98 over the concentration range, showing good linearity for all analytes.

Limit of Detection and Limit of Quantification. LOD and LLOQ were determined based on signal-to-noise ratio, by comparing measured noise signals from samples with known standard concentrations and establishing the minimum concentration level at which analyte can be detected and quantified. A signal-to-noise ratio of 3:1 and 10:1 was considered acceptable for LOD and LLOQ, respectively. The values of LOD and LLOQ are listed on Table **2**. Although LLOQ values obtained were higher than most forensic data in literature, we found them acceptable considering chromatographic system limitations, the time consuming analysis and the cost.

Table 2. A - Parameters of the analytical curves of thirteen opioids, cocaethylene and cocaine solutions (31.2 – 2000 ng/mL) obtained by the least squares method in three different days. B - Precision, accuracy and recovery (%) for desomorphine and codeine evaluation at 3 different spiked concentrations. LOD, limit of detection; LLOQ, limit of quantification.

A							B				
Xenobiotic	**n=3**	**y = mx + b**	**Concentration range (ng/mL)**	r^2	**LOD (ng/mL)**	**LLOQ (ng/mL)**	**Quality control concentration (ng/mL)**	**Intra-day precision (%, n=3)**	**Inter-day precision (%, n=3)**	**Accuracy (%, n=3)**	**Recovery (%)**
Codeine	day1	y = 0.00006x – 0.0059	31.2 - 2000	0.9851	63.6	73.4	31.2	11.6	5.6	104.0	78.8
	day2	y = 0.00003x + 0.0028	31.2 - 2000	0.9842			250	13.5	15.1	112.7	89.7
	day3	y = 0.0001x – 0.0189	31.2 - 2000	0.9817			2000	5.3	14.6	111.0	85.5
Morphine	day1	y = 0.00002x – 0.0025	31.2 - 2000	0.9966	31.8	46.3	31.2	11.5	5.1	110.0	58.6
	day2	y = 0.00001x + 0.0028	31.2 - 2000	0.9951			250	6.9	5.3	106.0	72.4
	day3	y = 0.00001x + 0.0030	31.2 - 2000	0.9844			2000	9.4	8.8	102.4	71.4
Heroin	day1	y = 0.000004x + 0.0042	31.2 - 2000	0.9958	32.7	65.2	31.2	12.3	11.5	110.1	79.3
	day2	y = 0.000004x + 0.0063	31.2 - 2000	0.9969			250	13.7	8.8	107.3	82.1
	day3	y = 0.00005x + 0.0002	31.2 - 2000	0.9975			2000	14.2	13.1	102.8	78.3
6-acethylmorphine	day1	y = 0.00003x – 0.0042	31.2 - 2000	0.9815	81.7	123.4	31.2	11.8	11.7	88.23	59.8
	day2	y = 0.00002x + 0.0033	31.2 - 2000	0.9961			250	6.4	9.3	89.0	52.4
	day3	y = 0.00002x + 0.0053	31.2 - 2000	0.9830			2000	13.9	10.1	84.5	53.3
Desomorphine	day1	y = 0.0001x – 0.0057	31.2 - 2000	0.9873	30.8	102.6	31.2	11.0	3.7	102.4	82.5
	day2	y = 0.00005x + 0.0081	31.2 - 2000	0.9981			250	12	13.9	96.4	76.3
	day3	y = 0.00006x + 0.0091	31.2 - 2000	0.9946			2000	15.1	14.8	112.3	83.7
Ethylmorphine	day1	y = 0.00006x + 0.0002	31.2 - 2000	0.9808	16.2	54.1	31.2	8.5	11.6	81.6	87.8
	day2	y = 0.00005x + 0.0070	31.2 - 2000	0.9982			250	6.4	11.0	90.8	63.2
	day3	y = 0.00006x + 0.0075	31.2 - 2000	0.9978			2000	2.5	15.8	92.5	67.0
Methadone	day1	y = 0.000004x + 0.0041	31.2 - 2000	0.9805	21.2	70.7	31.2	14.7	16.8	114.5	78.9
	day2	y = 0.000007x + 0.0005	31.2 - 2000	0.9977			250	14.7	6.4	94.3	77.0
	day3	y = 0.00001x + 0.0006	31.2 - 2000	0.9838			2000	5.7	16.2	104.3	84.4
EDDP	day1	y = 0.00007x + 0.0003	31.2 - 2000	0.9985	7.1	33.6	31.2	2.2	1.7	99.9	93.9
	day2	y = 0.00009x + 0.0015	31.2 - 2000	0.9999			250	2.7	15.8	108.2	95.0
	day3	y = 0.00008x + 0.0010	31.2 - 2000	0.9985			2000	3.0	11.5	104.2	83.6
EMDP	day1	y = 0.0001x – 0.0015	31.2 - 2000	0.9980	6.4	32.3	31.2	3.2	3.8	120.8	88.2
	day2	y = 0.0001x – 0.0001	31.2 - 2000	0.9960			250	2.6	7.4	106.7	86.2
	day3	y = 0.0001x + 0.0001	31.2 - 2000	0.9998			2000	1.5	1.4	106.1	90.6
Papaverine	day1	y = 0.00007x – 0.0081	31.2 - 2000	0.9825	32.7	109.1	31.2	13.0	16.5	136.0	65.4
	day2	y = 0.00004x + 0.0026	31.2 - 2000	0.9849			250	7.8	14.5	94.6	67.3
	day3	y = 0.00003x – 0.0016	31.2 - 2000	0.9849			2000	9.3	0.6	99.1	72.9
Tramadol	day1	y = 0.00005x + 0.0030	31.2 - 2000	0.9894	7.4	31.2	31.2	11.1	13.4	114.0	89.7
	day2	y = 0.00004x + 0.0062	31.2 - 2000	0.9940			250	6.8	11.1	101.5	76.0

Table (2) contd….

A							B				
Xenobiotic	**n=3**	**y = mx + b**	**Concentration range (ng/mL)**	**r^2**	**LOD (ng/mL)**	**LLOQ (ng/mL)**	**Quality control concentration (ng/mL)**	**Intra-day precision (%, n=3)**	**Inter-day precision (%, n=3)**	**Accuracy (%, n=3)**	**Recovery (%)**
	day3	y = 0.00004x + 0.0059	31.2 - 2000	0.9952			2000	6.6	11.9	105.4	74.7
M1	day1	y = 0.00005x + 0.0014	31.2 - 2000	0.9958	12.4	49.2	31.2	17.5	8.4	104.0	87.2
	day2	y = 0.00004x + 0.0032	31.2 - 2000	0.9969			250	5.9	15.3	96.5	59.7
	day3	y = 0.00004x + 0.0002	31.2 - 2000	0.9923			2000	8.7	12.3	92.8	72.5
Tapentadol	day1	y = 0.00200x + 0.0027	31.2 - 2000	0.9999	32.1	31.6	31.2	2.4	8.9	110.9	85.0
	day2	y = 0.00200x + 0.0058	31.2 - 2000	0.9987			250	2.8	6.2	113.7	81.9
	day3	y = 0.00200x + 0.0099	31.2 - 2000	0.9986			2000	6.9	10.7	111.0	84.3
Cocaine	day1	y = 0.00007x – 0.0024	31.2 - 2000	0.9880	15	62.5	31.2	12.7	13.9	94.4	73.1
	day2	y = 0.00005x + 0.0069	31.2 - 2000	0.9989			250	11.9	13.9	92.8	79.4
	day3	y = 0.00008x + 0.0048	31.2 - 2000	0.9972			2000	5.5	11.1	95.6	74.7
Cocaethylene	day1	y = 0.00004x – 0.0022	31.2 - 2000	0.9873	62	125	31.2	15.1	10.4	104.0	68.5
	day2	y = 0.00002x + 0.0033	31.2 - 2000	0.9845			250	14.7	7.3	97.0	54.1
	day3	y = 0.00002x + 0.0049	31.2- 2000	0.9913			2000	8.8	9.3	103.5	70.6

Most literature, presents LLOQ data obtained by High Pressure Liquid Chromatography (HPLC) coupled with tandem mass spectrometry (MS) techniques [36-39] which is much more sensitive for drug analysis. Nevertheless, GC-MS is a useful technique for forensic and clinical analysis due its reproducibility. Lerch and coworkers [40] described a GC-MS technique for detection of opioids and cocaine obtaining slightly lower LLOQ using SPE as the extraction methodology. Even with limitations, our technique showed to be comparable with data described in literature. Moreover, the obtained LLOQ and LOD were derived from a multiresidual operation for simultaneous analysis of thirteen analytes. Improvement of LOD and LLOQ can be made case by case, by reducing the numbers of ions included in the SIM data collection process when a substance is suspected. Indeed, as previously suggested the suspicion is an extremely important pre-analytical step to vectorize which xenobiotics must be included in the toxicological analysis [41-44].

Precision, Accuracy and Recovery. The results obtained are showed in Table **2**. Precision was evaluated considering the threshold of 15% for %CV and 20% for the concentration closer to the LLOQ. The precision of the analytical method describes the closeness of repeated individual measures of the analyte. Intra-day precision data was quantified by analyzing peak-area ratios of analytes of interest to the internal standard of three replicates of three different concentrations (low, 31.2; medium, 250; high, 2000 ng/mL) and calculating %CV. Peak-areas ratios of the same three concentrations, injected on three consecutive days, were used to calculate inter-day precision. For this analysis all the samples were freshly fortified and extracted each day of analysis.

The accuracy of an analytical method describes the closeness of the determined value obtained by the method to the nominal concentration of the analyte in percentage [16]. Accuracy percentage should be within ±20% of actual value for LLOQ and ±15% for other concentrations along the linearity range. The accuracy was evaluated by spiking blank whole blood samples with three different analytes concentrations (low, 31.2; medium, 250; high, 2000 ng/mL) and through the calculation of the deviation of the percentage between the calculated and the nominal value obtained from de QC samples concentrations (low, 31.2; medium, 250; high, 2000 ng/mL) [accuracy (%) = (experimental concentration/ QC concentration)×100].

The extraction recovery was calculated comparing the concentration of the analyte extracted from the matrix and the concentration of the analyte in a curve not extracted. It was evaluated by spiking blank whole blood samples with three different analytes concentrations (low, 31.2; medium, 250; high, 2000 ng/mL) and through the calculation of the deviation of the percentage between the extracted and the non-extracted value [Recovery (%) = (extracted [standard]/non-extracted [standard]) × 100]. Usually recovery of extraction methods should be between 80-120%. According to Anzillotti and colleagues [8], for QuEChERS it is acceptable to achieve an overall recovery value of 60–70% for non-, medium- and polar compounds. Therefore, the method showed good recovery values considering there was no dispersive solid-phase extraction (dSPE) step. Moreover, basic pH of extraction step proved to be important to extract basic drugs and may be used as an exchange to the addition of sorbents like primary secondary amine (PSA).

We also compared our validated modified QuEChERS methodology with classic solid-phase extraction (SPE). Considering the basic nature of analytes, Oasis MCX SPE Column was used. Although slightly higher recoveries were obtained with SPE (data not shown), the cost and the time consuming were significantly greater. Moreover, QuEChERS methodology proved to be effective in the proof of applicability step.

Stability. Stability studies were performed concurrently with precision and accuracy analysis. The stock solutions of the analytes did not show deviations (*i.e.*, acceptable %CV) during the 85 days that took the optimization and validation process.

PROOF OF APPLICABILITY

From all 34 human samples, 23 were positive for opioids (Table **S1**), namely methadone and its main metabolites, EDDP and EMDP. Methadone therapy is the most extensively evaluated and most used treatment for opioids addiction [45]. It occurs due to its pharmacological characteristics such as high oral bioavailability, long half-live and the availability of a specific antagonist, needed for overdose cases [46]. In Portugal, the therapy consist in a weekly dose of 40 mg of methadone chloride, which may be progressively increased until 200 mg, lasting 1 to 3 years. The variability of methadone concentrations observed in our samples may be due to its kinetic characteristics and the inter-individual variability of absorption and metabolism. Methadone taken orally suffers first-pass effect and is detectable in plasma about 30 min after its administration [46], occurring the plasmatic peak after 4 hours [47]. Its oral bioavailability varies from 41–95% [47]. Consequently, following the administration of equal doses, quite different whole blood concentrations are obtained in different subjects [46, 47]. Two samples presented low concentrations of tramadol and M1 and, one sample showed M1 under the LLOQ (data not showed). This may be explained by the fact that even in the most effective rehabilitation programs a non-negligible number of the patients still continue using opioids [45].

In both *in vivo* experiments, our developed method proved to be effective to detect and quantify therapeutic and toxic concentrations of tapentadol (Table **S2**), tramadol and *O*-desmethyltramadol (Table **S3**).

CONCLUSIONS

A sensitive, reproducible and relatively simple GC-MS method was developed and validated for qualitative and quantitative analysis of 15 drugs in whole blood samples. Since this method requires no special equipment, it is less laborious and consumes a minimal time, it shows a great potential as a useful tool for clinical and forensic routine analysis. Moreover, since original QuEChERS methodology is not adapted for highly complex matrix such as whole blood, dSPE with the addition of PSA are usually required. Our modified QuEChERS methodology was validated even without these steps. Whole blood was considered in this work since it is a frequent sample namely in *postmortem* toxicology [1, 2]. Other samples, such as serum, liver, kidney and lung, muscle and adipose tissue should be considered for further work to widen the applicability of the developed method in clinical and forensic toxicology [48, 49].

CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest, particularly no financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] Dinis-Oliveira, R.J.; Carvalho, F.; Duarte, J.A.; Remiao, F.; Marques, A.; Santos, A.; Magalhaes, T. Collection of biological samples in forensic toxicology. *Toxicol. Mech. Methods*, **2010**, *20* (7), 363-414.

[2] Dinis-Oliveira, R. J.; Magalhaes, T., Forensic toxicology in drug-facilitated sexual assault. *T Toxicol. Mech. Methods*, **2013**, *23* (7), 471-8.

[3] Jones, G.R., Postmortem Toxicology. In: *Clarke's Analycal Forensic Toxicology*, Jickells, S.; Negrusz, A., Eds. Pharmaceutical Press: London, UK, **2008**, 191-218.

[4] Namera, A.; Yashiki, M., Pretreatments of human specimens. In: *Drugs Poisons Humans: A Handbook Practical Analysis*, Suzuki, O.; Watanabe, K., Eds. Springer: New York, **2005**, 25-31.

[5] Cantwell, F.F.; Losier, M., Liquid-liquid extraction. In: *Comprehensive Analytical Chemistry*, Elsevier B.V.: Amsterdam, Netherlands, **2002**, 297-340.

[6] Al-Asmari, A.I.; Anderson, R.A., Method for quantification of opioids and their metabolites in autopsy blood by liquid chromatography-tandem mass spectrometry. *J. Anal. Toxicol.*, **2007**, *31*(7), 394-408.

[7] Carvalho, V. M.; Fukushima, A.R.; Fontes, L.R.; Fuzinato, D.V.; Florio, J.C.; Chasin, A.A. Cocaine postmortem distribution in three brain structures: a comparison with whole blood and vitreous humour. *J. Forensic. Leg. Med.*, **2013**, *20*(3), 143-5.

[8] Anzillotti, L.; Odoardi, S.; Strano-Rossi, S., Cleaning up blood samples using a modified "QuEChERS" procedure for the determination of drugs of abuse and benzodiazepines by UPLC-MSMS. *Forensic Sci. Int.*, **2014**, *243*, 99-106.

[9] Guillot, J.G.; Lefebvre, M.; Weber, J.P. Determination of heroin, 6-acetylmorphine, and morphine in biological fluids using their propionyl derivtives with ion trap GC-MS. *J. Anal. Toxicol.* **1997**, *21*, 127-133.

[10] Schulz, M.; Iwersen-Bergmann, S.; Andresen, H.; Schmoldt, A. Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. *Crit. Care*, **2012**, *16*(4), R136.

[11] Pinho, S.; Oliveira, A.; Costa, I.; Gouveia, C.A.; Carvalho, F.; Moreira, R.F.; Dinis-Oliveira, R.J. Simultaneous quantification of tramadol and O-desmethyltramadol in hair samples by gas chromatography-electron impact/mass spectrometry. *Biomed. Chrom.* **2013**, *27*(8), 1003-11.

[12] Barroso, M.; Gallardo, E.; Vieira, D.N.; Queiroz, J.A.; Lopez-Rivadulla, M. Bioanalytical procedures and recent developments in

the determination of opiates/opioids in human biological samples. *Anal. Bioanal. Chem.*, **2011**, *400*(6), 1665-90.

[13] Alves, E.A.; Soares, J.X.; Afonso, C.M.; Grund, J.P.; Agonia, A. S.; Cravo, S.M.; Netto, A.D.; Carvalho, F.; Dinis-Oliveira, R.J. The harmful chemistry behind "krokodil": Street-like synthesis and product analysis. *Forensic Sci. Int.*, **2015**, *257*, 76-82.

[14] Gan, S.H.; Ismail, R.; Wan Adnan, W.A.; Wan, Z. Method development and validation of a high-performance liquid chromatographic method for tramadol in human plasma using liquid-liquid extraction. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, **2002**, *772*(1), 123-9.

[15] Stimpfl, T.; Muller, K.; Gergov, M.; LeBeau, M.; Polettini, A.; Sporkert, F.; Weinmann, W. Recommendations on sample preparation of biological specimens for systematic toxicological analysis. *TIAFT-Bulletin XLI - Number 2*, **2011**, 30-34.

[16] European Medicines Agency, *Guidelines on bioanalytical method validation*. London, **2011**.

[17] Matsuta, S.; Nakanishi, K.; Miki, A.; Zaitsu, K.; Shima, N.; Kamata, T.; Nishioka, H.; Katagi, M.; Tatsuno, M.; Tsuboi, K.; Tsuchihashi, H.; Suzuki, K. Development of a simple one-pot extraction method for various drugs and metabolites of forensic interest in blood by modifying the QuEChERS method. *Forensic Sci. Int.*, **2013**, *232*(1-3), 40-5.

[18] Plossl, F.; Giera, M.; Bracher, F. Multiresidue analytical method using dispersive solid-phase extraction and gas chromatography/ion trap mass spectrometry to determine pharmaceuticals in whole blood. *J. Chrom. A.*, **2006**, *1135*(1), 19-26.

[19] Usui, K.; Hayashizaki, Y.; Hashiyada, M.; Funayama, M. Rapid drug extraction from human whole blood using a modified QuEChERS extraction method. *Leg. Med.* **2012**, *14*(6), 286-96.

[20] Cerqueira, M.B.; Guilherme, J.R.; Caldas, S.S.; Martins, M.L.; Zanella, R.; Primel, E.G. Evaluation of the QuEChERS method for the extraction of pharmaceuticals and personal care products from drinking-water treatment sludge with determination by UPLC-ESI-MS/MS. *Chemosphere*, **2014**, *107*, 74-82.

[21] Prestes, O.D.; Friggi, C.A.; Adaime, M.B.; Zanella, R. QuEChERS - Um método moderno de preparo de amostra para determinação multirresíduo de pesticidas em alimentos por métodos cromatográficos acoplados à espectromketria de massas. *Química. Nova.*, **2009**, *32*(6), 1620-1634.

[22] Diez, C.; Traag, W.A.; Zommer, P.; Marinero, P.; Atienza, J. Comparison of an acetonitrile extraction/partitioning and "dispersive solid-phase extraction" method with classical multi-residue methods for the extraction of herbicide residues in barley samples. *J. Chrom. A.*, **2006**, *1131*(1-2), 11-23.

[23] Anastassiades, M.; Lehotay, S.J.; Stajnbaher, D.; Schenck, F.J. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *J. AOAC Int.*, **2003**, *86*(2), 412-31.

[24] Albinet, A.; Tomaz, S.; Lestremau, F. A really quick easy cheap effective rugged and safe (QuEChERS) extraction procedure for the analysis of particle-bound PAHs in ambient air and emission samples. *Sci. Total Environ.*, **2013**, *450-451*, 31-8.

[25] Rowlett, J.K.; Platt, D.M.; Yao, W.D.; Spealman, R.D. Modulation of heroin and cocaine self-administration by dopamine D1- and D2-like receptor agonists in rhesus monkeys. *J. Pharm. Exp. Ther.*, **2007**, *321*(3), 1135-43.

[26] Jufer, R.A.; Walsh, S.L.; Cone, E.J. Cocaine and metabolite concentrations in plasma during repeated oral administration: development of a human laboratory model of chronic cocaine use. *J. Anal. Toxicol.*, **1998**, *22*(6), 435-44.

[27] Isenschmid, D.S.; Fischman, M.W.; Foltin, R.W.; Caplan, Y.H. Concentration of cocaine and metabolites in plasma of humans following intravenous administration and smoking of cocaine. *J. Anal. Toxicol.*, **1992**, *16*(5), 311-4.

[28] Cone, E.J. Pharmacokinetics and pharmacodynamics of cocaine. *J. Anal. Toxicol.*, **1995**, *19*(6), 459-78.

[29] Liu, Y.; Shi, X.W.; Liu, E.H.; Sheng, L.S.; Qi, L.W.; Li, P. More accurate matrix-matched quantification using standard superposition method for herbal medicines. *J. Chrom. A.*, **2012**, *1254*, 43-50.

[30] Lucini, L.; Molinari, G.P. Performance and matrix effect observed in QuEChERS extraction and tandem mass spectrometry analyses of pesticide residues in different target crops. *J. Chromatogr. Sci.*, **2011**, *49*(9), 709-14.

[31] King, R.; Bonfiglio, R.; Fernandez-Metzler, C.; Miller-Stein, C.; Olah, T. Mechanistic investigation of ionization suppression in electrospray ionization. *J. Am. Soc. Mass Spectrom.*, **2000**, *11*(11), 942-50.

[32] Cech, N.B.; Enke, C.G. Relating electrospray ionization response to nonpolar character of small peptides. *Anal. Chem.*, **2000**, *72*(13), 2717-23.

[33] Taylor, P. J. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. *CLB*, **2005**, *38*(4), 328-34.

[34] Thurman, E.M.; Ferrer, I.; Barcelo, D. Choosing between atmospheric pressure chemical ionization and electrospray ionization interfaces for the HPLC/MS analysis of pesticides. *Anal. Chem.*, **2001**, *73*(22), 5441-9.

[35] Kloepfer, A.; Quintana, J.B.; Reemtsma, T. Operational options to reduce matrix effects in liquid chromatography-electrospray ionization-mass spectrometry analysis of aqueous environmental samples. *J. Chromatogr. A.*, **2005**, *1067*(1-2), 153-60.

[36] Bjork, M.K.; Nielsen, M.K.; Markussen, L.O.; Klinke, H.B.; Linnet, K. Determination of 19 drugs of abuse and metabolites in whole blood by high-performance liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.*, **2010**, *396*(7), 2393-401.

[37] Bouzas, N.F.; Dresen, S.; Munz, B.; Weinmann, W. Determination of basic drugs of abuse in human serum by online extraction and LC-MS/MS. *Anal. Bioanal. Chem.*, **2009**, *395*(8), 2499-507.

[38] Jones, A.W.; Holmgren, A.; Kugelberg, F.C. Driving under the influence of opiates: concentration relationships between morphine, codeine, 6-acetyl morphine, and ethyl morphine in blood. *J. Anal. Toxicol.*, **2008**, *32*(4), 265-72.

[39] Namera, A.; Nagao, M.; Nakamoto, A.; Miyazaki, S.; Saito, T. Monolithic spin column: a new extraction device for analysis of drugs in urine and serum by GC/MS and HPLC/MS. *J. AOAC Int.*, **2011**, *94*(3), 765-74.

[40] Lerch, O.; Temme, O.; Daldrup, T. Comprehensive automation of the solid phase extraction gas chromatographic mass spectrometric analysis (SPE-GC/MS) of opioids, cocaine, and metabolites from serum and other matrices. *Anal. Bioanal. Chem.*, **2014**, *406*(18), 4443-51.

[41] Dinis-Oliveira, R.J.; Carvalho, F.; Duarte, J.A.; Proenca, J.B.; Santos, A.; Magalhaes, T. Clinical and forensic signs related to cocaine abuse. *Curr. Drug Abuse Rev.*, **2012**, *5*(1), 64-83.

[42] Dinis-Oliveira, R.J.; Carvalho, F.; Moreira, R.; Duarte, J.A.; Proenca, J.B.; Santos, A.; Magalhaes, T., Clinical and forensic signs related to opioids abuse. *Curr. Drug Abuse Rev.*, **2012**, *5*(4), 273-90.

[43] Dinis-Oliveira, R.J.; Carvalho, F.; Moreira, R.; Proenca, J.B.; Santos, A.; Duarte, J.A.; Bastos, M.D.; Magalhaes, T. Clinical and forensic signs related to chemical burns: A mechanistic approach. *Burns*, **2015**, 41(4), 658-79.

[44] Dinis-Oliveira, R.J.; Magalhaes, T.; Moreira, R.; Proenca, J.B.; Pontes, H.; Santos, A.; Duarte, J.A.; Carvalho, F. Clinical and forensic signs related to ethanol abuse: a mechanistic approach. *Toxicol. Mech. Meth.*, **2014**, *24*(2), 81-110.

[45] Graziani, M.; Nistico, R. Gender differences in pharmacokinetics and pharmacodynamics of methadone substitution therapy. *Front. Pharmacol.*, **2015**, *6*, 122.

[46] Ferrari, A.; Coccia, C.P.; Bertolini, A.; Sternieri, E. Methadone--metabolism, pharmacokinetics and interactions. *Pharmacol. Res.*, **2004**, *50*(6), 551-9.

[47] Dinis-Oliveira, R.J. Metabolomics of methadone: clinical and forensic toxicological implications and variability of dose response. *Drug Metab. Rev.*, **2016**, *48*(4), 568-76.

[48] Dinis-Oliveira, R. J., Vieira, D. N., Magalhães, T. Guidelines for collection of biological samples for clinical and forensic toxicological analysis. *Forensic Sci. Res.*, **2016**, *1*, 42-51.

[49] Launiainen, T.; Ojanpera, I., Drug concentrations in post-mortem femoral blood compared with therapeutic concentrations in plasma. *Drug Test. Anal.*, **2014**, *6*(4), 308-16.