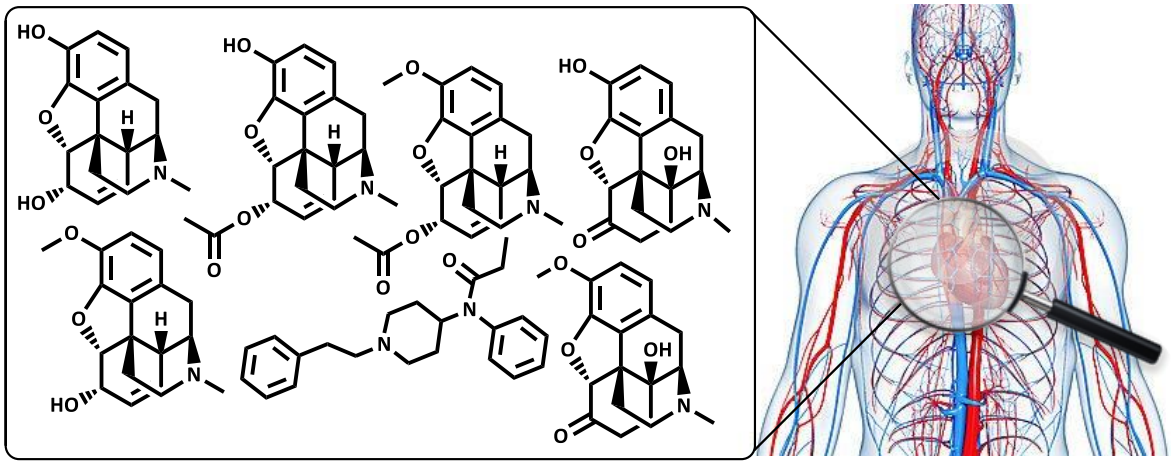
Elisa Gabriela Monteiro Ferreira

A Validated Methodology for the Forensic Toxicological Analysis of Opioids in Biological Fluids Using Solid Phase Extraction, Microwave Derivatization and Gas Chromatography-Mass Spectrometry



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Resumo

O desenvolvimento do presente estudo tornou-se pertinente devido ao consumo indevido de opióides e ao número de mortes por *overdoses* associadas ao seu consumo, tanto em Portugal como no resto do mundo. Por outro lado, sendo o recurso a matrizes biológicas *postmortem* alternativas ao sangue, uma área de crescente interesse em toxicologia forense estudámos a viabilidade do uso do líquido pericárdico na determinação das substâncias selecionadas.

Assim, o objetivo deste trabalho foi o desenvolvimento, otimização e validação de uma metodologia analítica para a determinação qualitativa e quantitativa de alguns opióides em sangue e líquido pericárdico.

Os opióides estudados foram: morfina, codeína, 6-acetilmorfina, 6-acetilcodeína, oxicodona, oximorfona e o fentanil.

O estudo incluiu a otimização do procedimento analítico e do método cromatográfico. Otimizámos a extração em fase sólida (SPE), a derivatização com e sem recurso a hidroxilamina aquosa a 1% e o tempo de derivatização induzida por micro-ondas com o reagente químico MSTFA (n-metil-n-(trimetilsilil) trifluoroacetamida)+5% TMCS (trimetilclorosilano).

O método mais eficiente e seletivo correspondeu ao seguinte procedimento: precipitação com acetonitrilo de volumes de 250 µL de amostras de sangue e de líquido pericárdico, derivatização das substâncias de interesse usando 1% hidroxilamina aquosa em PBS (1:2, v/v) promovida por irradiação de micro-ondas, durante 30 segundos com uma potência de 900 W a 50%. Procedeu-se à extração dos analitos de interesse por SPE. Após evaporação dos eluatos (sob corrente de azoto a 40 °C) os extratos foram derivatizados com MSTFA+5% TMCS sob ação de micro-ondas durante 100 segundos com uma potência de 900 W a 100%. Seguidamente os extratos derivatizados foram injetados (2 µL, *splitless*) diretamente no sistema de cromatografia de gases associado à espectrometria de massas (GC-MS-EI) com monitorização dos iões selecionados (modo SIM) e com o forno à temperatura inicial de 50 °C.

Após a otimização, o método foi validado seguindo as normas da *Scientific Working Group for Forensic Toxicology* (SWGTOX) de forma a garantir que o método é adequado para os fins a que se destina e assim atestar a sua fiabilidade na interpretação dos resultados analíticos. O método apresentou linearidade no intervalo 5-1000 ng/mL com coeficientes de determinação superiores a 0.99 para todos os analitos. Os limites de deteção (LOD) variaram entre 3 e 4 ng/mL, dependendo da substância e/ou da matriz analisada e os limites de quantificação (LOQ) foram de 5 ng/mL para todas as substâncias. Em relação às precisões (intra-dia e intermédia) todos os níveis de concentração apresentaram valores de CV <20% e a exatidão situou-se dentro do intervalo ±20%.

Verificou-se ainda que as substâncias apresentaram estabilidade sob as seguintes condições: nos extratos deixados no amostrador em condições ambientais por pelo menos 24 h; nas amostras de sangue e líquido pericárdico deixadas na bancada de trabalho durante 4 h e nas amostras de líquido pericárdico durante 3 ciclos de congelação e descongelação ao longo de pelo menos 4 semanas.

Por fim, a metodologia analítica foi aplicada a amostras reais disponibilizadas pelo Serviço de Química e Toxicologia Forenses da Delegação do Centro do Instituto Nacional de Medicina Legal e Ciências Forenses, I.P..

De acordo com a revisão bibliográfica efetuada, este foi o primeiro método desenvolvido para a deteção e quantificação simultânea deste grupo de substâncias em sangue e líquido pericárdico com recurso à derivatização promovida por micro-ondas com os reagentes químicos hidroxilamina e MSTFA+5% TMCS.

Palavras-Chave

Opióides, sangue, líquido pericárdico, derivatização por micro-ondas, CG-EM

Abstract

The development of the present study became pertinent due to the misuse of opioids and the number of overdose deaths associated with its use, both in Portugal and in the rest of the world. On the other hand, being the use of alternative biological matrices to *postmortem* blood, an area of growing interest in forensic toxicology, we studied the feasibility of using pericardial fluid in the determination of the selected substances.

Thus, the objective of this work was the development, optimization and validation of an analytical methodology for the qualitative and quantitative determination of some opioids in blood and pericardial fluid.

The opioids studied were: morphine, codeine, 6-acetylmorphine, 6-acetylcodeine, oxycodone, oxymorphone and fentanyl.

The study included the optimization of the analytical procedure and the chromatographic method. We have optimized solid phase extraction (SPE), derivatization with and without 1% aqueous hydroxylamine and microwave derivatization time with the chemical reagent MSTFA (n-methyl-n-(trimethylsilyl) trifluoroacetamide)+5% TMCS (trimethylchlorosilane).

The most efficient and selective method was as follows: precipitation with acetonitrile of 250 µL volumes of blood and pericardial fluid samples, derivatization of the substances of interest using 1% aqueous hydroxylamine in PBS (1:2, v/v) with microwave action for 30 seconds with a power of 900 W at 50%. The samples were cooled and then the analytes of interest were extracted by SPE. After evaporation of the eluates (under nitrogen stream at 40 °C) the extracts were derivatized with MSTFA+5% TMCS under microwave action for 100 seconds with a power of 900 W at 100%. Then the derivatized extracts were injected (2 µL, *splitless*) directly into a gas chromatography mass spectrometry system (GC-MS-EI) with selective ion monitoring mode (SIM mode) and with the oven at the initial temperature of 50 °C.

After optimization, the method was validated following the standards of the Scientific Working Group for Forensic Toxicology (SWGTOX) to ensure that the method is suitable for its intended purpose and thus attest to its reliability in interpreting the analytical results. The method presented linearity in the range 5-1000 ng/mL with coefficients of determination above 0.99 for all analytes. The limits of detection (LOD) ranged from 3 to 4 ng/mL, depending on the substance and/or matrix analysed and the limits of quantitation (LOQ) were 5 ng/mL for all substances. Regarding the precision (intra-day and intermediate) all concentration levels presented CV values <20% and the bias was within ±20%. It was also verified that the substances presented stability under the following conditions: in the extracts left in the autosampler under environmental

conditions for at least 24 h; blood and pericardial fluid samples on the workbench for 4 h; pericardial fluid samples for 3 freeze-thaw cycles for at least 4 weeks.

Finally, the analytical methodology was applied to real samples provided by the Serviço de Química e Toxicologia Forenses da Delegação do Centro do Instituto Nacional de Medicina Legal e Ciências Forenses, I.P. (Forensic Chemistry and Toxicology department of the Centre Branch of the National Institute of Legal Medicine and Forensic Sciences, I.P.).

According to the literature review, this was the first method developed for the simultaneous detection and quantification of this group of substances in blood and pericardial fluid using microwave induced derivatization with the chemical reagent's hydroxylamine and MSTFA+5% TMCS.

Keywords

Opioids, whole-blood, pericardial fluid, microwave derivatization, GC-MS

Thesis Overview

This thesis is organized in four chapters: The first chapter begins with a brief overview of opioids, including a historical review, highlighting the situation in Portugal, Europe, and the world as well as the characterization of each substance.

The second chapter introduces an overview of the bioanalytical procedures, with a special focus on the aspects involved in this study. Then, a compilation of previous studies, including at least one of the substances is introduced (up to 10 years, with 6-acetylcodeine, oxycodone and oxymorphone as exception, and regarding to pericardial fluid as matrix, being up to 20 years).

The third chapter introduces the results achieved during the optimization of the current methodology together with a discussion. The validation of the methodology is presented in an article format (submitted but awaiting acceptance) with a final overall discussion.

Finally, in the fourth chapter are introduced the conclusions of the study.

It should be noted that throughout this work, the term opioid will include all substances under study.

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List of Abbreviations

6-ACCOD: 6-Acetylcodeine

6-MAM: 6-Acetylmorphine

AMPA receptor: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

BL: Blood

BSA: N,O-bis (trimethylsilyl) acetamide

BSTFA: N,O-bis-(trimethylsilyl) trifluoroacetamide

CE: Common/current Era

CGRP: Calcitonin gene-related peptide

CV: Coefficient of Variation

DBS: Dried blood spot

DMF: Dimethylformamide

DMSO: Dimethyl sulfoxide

DUS: Dried urine spot

EI: Electron impact

FDA: Food and Drugs Administration

GABA: γ-aminobutryric acid

GC-MS: Gas chromatography – mass spectrometry

HMDS: Hexamethyldisilazane

HPLC-MS: High performance liquid chromatography - mass spectrometry

HPLC-MS/MS: High performance liquid chromatography - tandem mass spectrometry

HPLC-PDA: High performance liquid chromatography - photometric diode array

IM: Intramuscularly

IN: Intranasal

INMLCF, I.P.: National Institute of Legal Medicine and Forensic Sciences- Instituto Nacional de Medicina Legal e Ciências Forenses, I.P.

IS: Internal standard

IT: Intrathecal

IV: Intravenous

LC-qTOF-MS: Liquid chromatography quadrupole Time-of-Flight - mass spectrometry

LC-MS: Liquid chromatography- mass spectrometry

LC-MS/MS: Liquid chromatography - tandem mass spectrometry

LLE: Liquid- Liquid extraction

LOD: Limit of detection

LOQ: Limit of quantification

MSTFA: N-methyl-n-(trimethylsilyl) trifluoroacetamide

MTBSTFA: N-(tert-butyldimethylsilyl-N-methyltrifluoroacetamide)

M3G: Morphine-3-glucuronide

M6G: Morphine-6-glucuronide

NK1R: Neurokinin 1 receptor

NMDA receptor: N-methyl-D-aspartate receptor

PF: Pericardial fluid

PP: Protein precipitation

SC: Subcutaneously

SICAD: Serviço de Intervenção nos Comportamentos Aditivos e nas Dependências - Intervention service in additive behaviors and dependencies

SIM: Selected Ion Monitoring

SP: Substance P

SPE: Solid-Phase Extraction

SQTF-C: Serviço de Química e Toxicologia Forenses da Delegação do Centro - Laboratory of Forensic Chemistry and Toxicology department of the centre branch

SWGTOX: Scientific Working Group for Forensic Toxicology

TD: Transdermal

THF: Tetrahydrofuran

TIC: Full scanning mode (total ion chromatogram)

TM: Transmucosal

TMS: Tetramethylsilane

TMCS: Trimethylchlorosilane

UHPLC-MS/MS: Ultrahigh-performance liquid chromatography - tandem mass spectrometry

UHPLC-orbitrap: Ultrahigh-performance liquid chromatography – orbitrap

UNODC: United Nations Office on Drugs and Crime

UPLC-MS/MS: Ultra-performance liquid chromatography - tandem mass spectrometry

VAMS: Volumetric absorptive microsampling

Vd: Volume distribution

WHO: World Health Organization

Chapter 1. Introduction

The opium designation has Greek origin, being extracted from *Papaver somniferum* (poppy). Opium is composed of at least 25 alkaloids, which are classified into two groups: phenanthrenes, such as morphine, codeine and thebaine; and the benzyl isoquinoline derivatives such as papaverine.[1–3] Its derivatives, however, have already undergone changes in their designation over the years, having already been designated narcotics, narcoanalgesics, hypnoanalgesics and opiates, initially in a generic way comprising natural and some semi-synthetic derivatives of opium.[1,4] Natural derivatives refer to substances extracted from opium, such as morphine, codeine and thebaine. Semi-synthetic derivatives correspond to substances that have undergone partial modification, such as diacetylmorphine (heroin), oxycodone, oxymorphone, among others.[4] The term opioid, comprises drugs structurally distinct from morphine but with similar action i.e. synthetic derivatives (e.g. methadone and fentanyl). However, the designation of opioid is often use to include all substances regardless of being, natural, semi-synthetic or synthetic.[1,4,5]

1.1. History

The beginning of consumption and cultivation of opium is undoubtedly difficult to establish, yet the earliest and most consistent records of cultivation are related to the Sumerians at 5000 years (who lived where today is Iraq). They called the opium "gil", which means joy, and the poppy "hul gil" meaning plant of joy. Throughout history there are records of opium cultivation, consumption and even some archaeological pieces with poppy images.[1,4,6]

Hippocrates (the father of medicine) prescribes what is thought to be the juice of the poppy (meconium) as purgative and narcotic and for leucorrhoea (vaginal discharge). However, it is believed that the analgesic properties were not yet known having only been recognized by the Romans.[1,4] In the 1st century CE (Common/Current Era), a Roman physician recommended opium for pain relief. In the 2nd century CE Galen, a physician of the Emperor Antonino (believed to have been dependent on the drug) understood the risks associated with the exaggerated use of opium. Opium was recognized by Avicenna (an Arabic physician) as the most powerful analgesic used in otalgias and arthralgias (such as gout) and was also indicated in the treatment of diarrhea and eye disease. Some authors claim that Avicenna had an abusive use of this substance and eventually have died due to its overdosage.[1,7] In the 9th century, appeared references to a mixture used to promote anesthesia for surgeries, which consisted of opium, mandrake, hemlock and hyoscythus.[1,4] Opium was reintroduced in Western Europe by Paracelsus (1493-1541). Several individuals gave

rise to several distinct preparations containing opium. One of these formulations was included in the London Pharmacopoeia of 1721. From 1700 onwards, the adverse effects of opium began to be recognized, but its use was maintained. In 1800, the Chinese Government banned the importation of opium, and a deposit of the East India Company was destroyed, unleashing the "opium war", between England and China.[1] In the 19th century, morphine was discovered by Friedrich Sertürner, initially titled *principium somniferum*, and then *morphium*, honoring the Greek god of sleep (Morpheus).[3,6,8] In 1816, Sertürner published the details of his chemical and pharmacological investigation, being designated by morphine due to the proposal of Gay Lussac. Sertürner continued to investigate morphine, performing a self-experience, observing adverse effects.[1,3] In 1832 codeine (methyl-morphine) was isolated by Robiquet, and in 1835 thebaine (dimethyl-morphine) by Pelletier and Thibouméry. After the determination of the chemical structure of the natural derivatives, the creation of semi-synthetic derivatives was started. Only in 1860, opium became a medical and social problem, due to the statistical data of the mortality associated with its consumption.[1] From the 19th century to the 20th century, the replacement of morphine by heroin was proclaimed as it relieved the symptoms of withdrawal syndrome caused by morphine.[1,6] Only 12 years later, they concluded that heroin promoted addiction even more quickly. However, only in the 20th century, the concepts of tolerance, physical and psychic dependence, and addiction began to be discussed.[1] In 1916 Martin Freund and Edmund Speyer synthesized and introduced oxycodone into the pharmaceutical market.[9] Oxymorphone was available in parenteral formulation in 1959.[10] Finally, fentanyl in 1960 became available and between 1974 and 1976 several of its analogues were developed.[1]

In this chapter we only mentioned some of the synthesized substances, those of interest in this study, however there are many others, continuing to appear daily new derivatives of opium.

1.2. Portuguese Legislation

Nowadays, Decree-Law no. 15/93 of 22 January (Legal regime of trafficking and consumption of narcotic drugs and psychotropic drugs) reinforces and complements the measures conjected in the 1961 Narcotic Drugs Convention (already modified). In Chapter I, Article 15 (Medical prescription) it is indicated that the substances and preparations included in Tables I to II can only be provided for treatment upon presentation of a special medical prescription. When these substances are consumed without this medical prescription it constitutes a misdemeanour as indicated in Law no. 30/2000, of November 29. In Chapter III, Article 21 of the quoted Decree-Law,

it is presented all illegal activities (e.g. cultivate, produce, sell) related to the plants, substances or preparations listed in Tables I to III.

Thus, the consumption of these substances can either come from a legal act (with special medical prescription) or an illegal act, according to the legislation. In this study, all the substances of interest are included in Table I-A (Annex 1), and thus covered by the legislation presented.[11]

1.3. Current Consumption

According to the World Drug Report (2018), about 275 million people (15-64 years) used at least one drug in 2016, 31 million of whom are drug addicts. Worldwide the number of opioid users is about 34 million and opiates of 19 million. In this report they also mention that opium production in that year was the highest estimate they had collected since they began monitoring (early 21st century).[12] At European level, a high-risk opioid (15-64 years) consumption of 0.4% (1.3 million consumers) is estimated in 2016, while at national level (Portugal) a high-risk consumption of opioids (15-64 years) is estimated to be between less than 1 and more than 8 cases per 1000 inhabitants.[13] According to the annual report published by SICAD (Serviço de Intervenção nos Comportamentos Aditivos e nas Dependências-Intervention service in additive behaviors and dependencies), heroin consumption continues to be relevant although it has declined relative to other drugs. In the population aged 15-74 years the prevalence of their lifetime consumption is 0.5%, recent consumption 0.1% and current consumption of 0.0%, while in the population aged 15-34 years it is 0.3%, 0.0% and 0.0%, respectively. It should be noted that in the last year, 60% of heroin users consumed every day, while 20% consumed one time per month and the remaining 20% rarely. In the population aged 15-64 years the prevalence of high-risk opioid use at a rate of 1000 inhabitants, 5.2% indicated to be recent consumers. Consumers aged 18 years indicated opioid use in the last year, 34% between 1 to 5 times, 31% in 20 or more times and 23% a daily/almost daily consumption.[14]

Non-medical/abusive use of pharmaceutical opioids has increased, being diverted from the pharmaceutical industry or produced illegally, thus becoming a concern both at law enforcement authorities and public health professionals. This abusive use differs across regions, in North America, there are numerous overdoses due to the use of fentanyl with heroin or other drugs. In Europe the main opioid is heroin, but methadone, buprenorphine and fentanyl have also been registered.[12,13] West and North Africa and the Near and Middle East is the use of tramadol.[12]

Analysing the 3 reports, it is verified that opioids despite not being the most consumed are the most implicated substances in overdose deaths (76% worldwide and 84% at European level).[12–14] There has been an increase in the number of overdoses with opioids especially with the presence of methadone in Portugal.[14] The improper consumption of prescription drugs, usually opioids and benzodiazepines were involved in 21% of the cases.[13] According to INMLCF, I.P. (Instituto Nacional de Medicina Legal e Ciências Forenses - National Institute of Legal Medicine and Forensic Sciences) data, in 2017 opioids were detected in 42% of overdoses, most in association with other licit and/or illicit substances. They were 45 years old or older in about 38% of cases and 31% under 35 years old. About 94% of these opioid deaths were male. In the deaths with other causes (accident, natural, homicide and suicide) 27% had the presence of opioids and 13% of methadone and again the majority of the cases have association with other illicit and/or licit substances.[14] According to the World Drug Report (2018) fatal overdoses with opioids are often in association with benzodiazepines.[12] According to the European Drug Report, in 2016, the main reason to start treatment for drug addiction in Europe was the consumption of opioids (177000 users or 37%). Patients who started treatment reported abuse of methadone, buprenorphine, fentanyl, codeine, morphine, tramadol and oxycodone. The most widely prescribed opioid replacement drug is methadone at both European (63%) and national level (around 70%). Buprenorphine, morphine, and slow release heroin have also been prescribed.[13]

Although there has been a decline in opioid users at treatment centers in Europe, it was observed an increase in those over 40 years of age, such as overdose deaths. This may be due to the aging of users who would start injecting heroin into heroin "epidemics" in the 1980s and 1990s.[12,13] To emphasize that it is not only a concern the abusive use of heroin and other opioids, but also the unsafe practices of the injections due to its use, thus leading to a 5 to 10-fold increase in the risk of dying, mostly related to overdose cases but also causes of death due to infections, accidents, violence and suicide.[12–14]

The concomitant use of opioids and other drugs were observed in cases of deaths, but also by indication of the consumers who started specialized treatment. In 2016, the concomitant use of opioids with cocaine was reported by more than 53000 consumers.[13] However, a mixture of fentanyl and its analogues with heroin and other drugs such as cocaine, MDMA or ecstasy has been reported, leading users to be unaware of the true content of what they consume resulting in a higher number of fatal overdoses.[12]

According to World Drug Report (2018) the use of non-medical opioids is similar between men and women (may be higher in women). However, men are more likely to use cannabis, cocaine and opiates. In relation to age, opioids are used by adolescents as well as adults and older

adulthood, whereas heroin is more used in adults.[12] At the European level heroin users are also mostly male (80%) and the average age at the start of their consumption is 23 years old. In Portugal, the male sex users continue to predominate, although in the lifetime consumption this predominance tend to reduce. In 2017, 18 years old reported life-long opioid use at a rate of 2% throughout life, 1% in the last 12 months and 1% in the last 30 days. This consumption is higher in boys, with a recent and current consumption of 2%, in relation to 0.6% and 0.4% of girls.[14]

A concern for SICAD is the results of the perception of young people in Portugal (15-24 years old) of access to heroin (higher than the average for young Europeans), with about 24% considering it easy to acquire if desired in 24 h, compared to 13% of the European average. Considering consumers throughout life (15-74 years old) it was found that 70% indicated how easy or very easy to access heroin in a 24-hour period.[14]

1.4. Structure and Classification

The substances under study, except for fentanyl (synthetic), have a chemical structure very similar to that of morphine. In figure 1 (A) the core structure of morphine, codeine, heroin (diacetylmorphine), 6-acetylmorphine, 6-acetylcodeine are represented with the substitution pattern outlined in the table 1; (B) the core structure of oxycodone and oxymorphone are represented with the corresponding substituents presented in table 1. In the following sub-chapters, additional details of the features of each substance will be addressed. It should be noted that morphine is classified as a prototypic, serving the standard drug, i.e. the remaining opioids will be compared to morphine relatively to analgesic potency and chemical structure.[7,8,15,16]

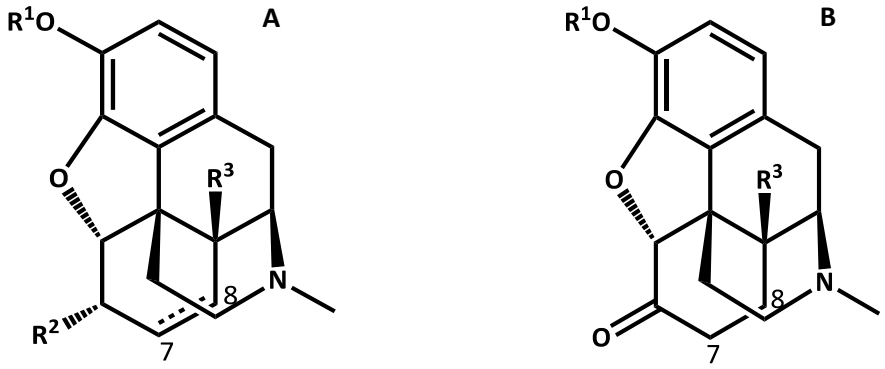

Figure 1. Natural and semi-synthetic opioids structure. (A) structure relative to morphine, codeine, diacetylmorphine, 6-acetylmorphine and 6-acetylcodeine. (B) structure relative to oxycodone and oxymorphone.

Table 1. Functional groups differences of the structures presented in figure 1.

Substance	R^1	R^2	R^3	Δ7-8
Morphine	*H*	*OH*	*H*	*Yes*
Codeine	*CH_3*	*OH*	*H*	*Yes*
Heroin	*CH_3CO*	*$OOCH_3$*	*H*	*Yes*
6-Acetylmorphine	*H*	*$OOCH_3$*	*H*	*Yes*
6-Acetylcodeine	*CH_3*	*$OOCH_3$*	*H*	*Yes*
Oxycodone	*CH_3*		*OH*	*No*
Oxymorphone	*H*		*OH*	*No*

1.4.1. Morphine

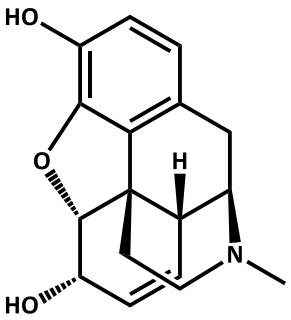

Figure 2. Morphine structure.

Molecular formula: $C_{17}H_{19}NO_3$

Molecular weight: 285.343 g/mol

IUPAC name: (4*R*,4a*R*,7*S*,7a*R*,12b*S*)-3-methyl-2,4,4a,7,7a,13 hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinoline-7,9-diol.[17]

pKa: 8.2

Log P: 0.9 [18]

Morphine is a natural derivative of opium and a strong opioid agonist of the μ (mu) opioid receptor, also having a lower action on the κ (kappa) receptor and even lower on the δ (delta) and minimum on σ (sigma). Its mechanism of action is classified as a full opioid agonist.[5,17,19–21] Since it has effects in the central nervous system and on smooth muscle, it is used for chronic pain and as anesthetics.[17,19–21] It could be taken orally, subcutaneously (SC), intramuscularly (IM), intravenous (IV), epidural, intrathecal (IT) and rectal.[5,16,19] Plasma half-life is 2-3.5 h, and its excretion is 85% renally.[16,21] In 72 h, 87% of the dose is excreted: 75% as morphine-3-glucuronide (M3G), 10% as morphine and the remaining as morphine-6-glucuronide (M6G), morphine-3-sulfate, normorphine and conjugates.[5] The volume distribution (Vd) is between 1-6 L/kg and protein binding 30 to 40%.[18] Morphine is metabolised by cytochrome CYP2D6 and then by glucuronoconjugation (at positions 3 and 6): M3G (about 50% of the metabolites) has no analgesic action, while M6G (10%) has a much higher action than morphine and normorphine (5%) and it is

an active but less potent metabolite.[5,16,21,22] Morphine metabolism is presented in figure 3. There are studies that indicate the metabolism of morphine in hydromorphone (Annex 2).[23]

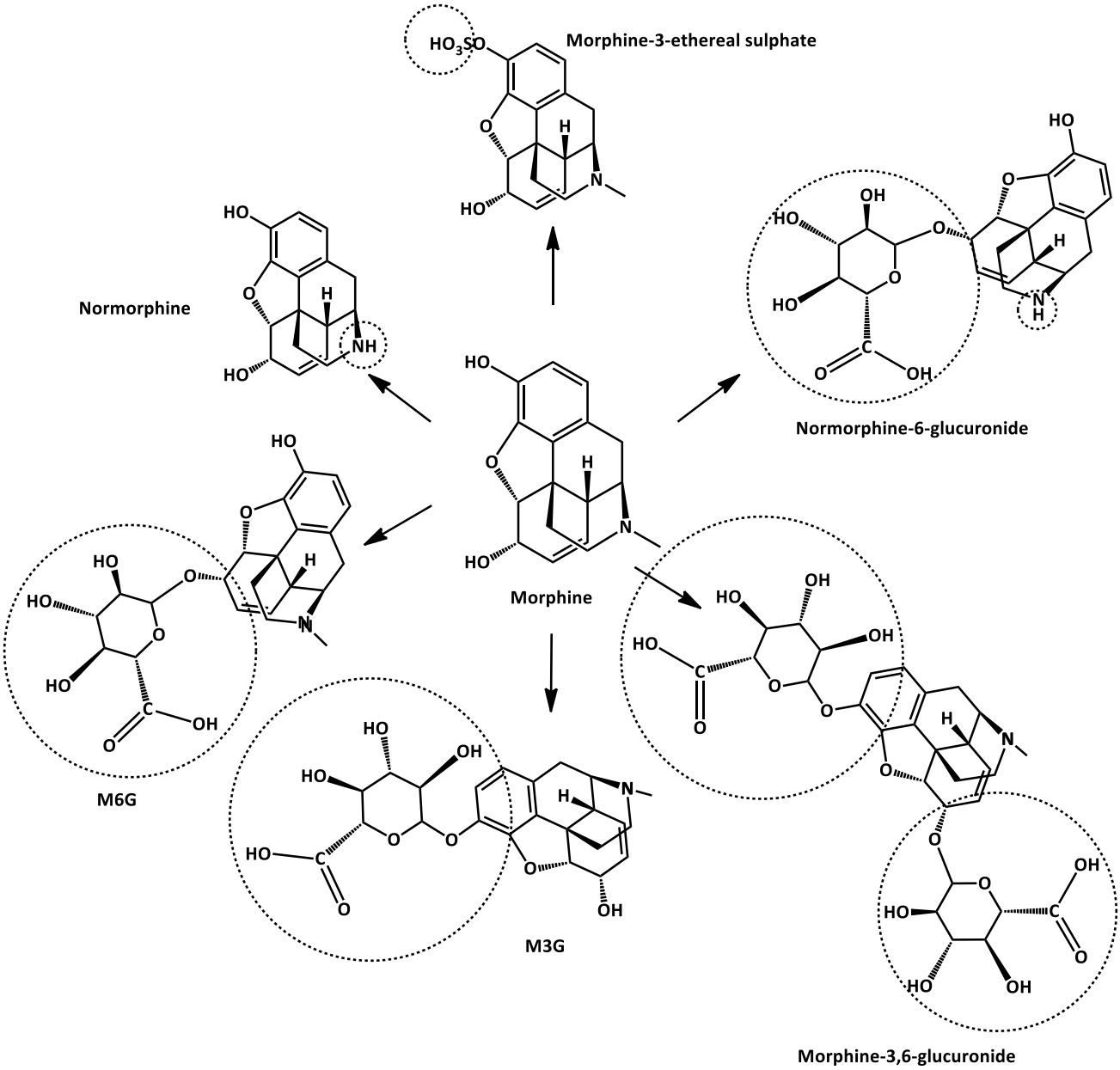

Figure 3. Morphine metabolism, with chemical alterations identified. Adapted from Shrabani et al.[24]

1.4.2. Codeine

Molecular formula: $C_{18}H_{21}NO_3$

Molecular weight: 299.37 g/mol

IUPAC name: (4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-methoxy-3-methyl-2,4,4a,7,7a,13-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinoline-7-ol [17]

pKa: 9.2

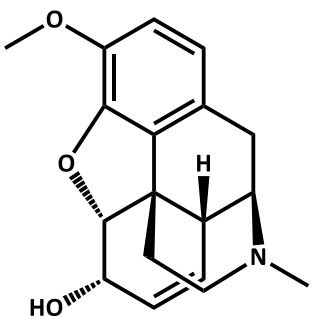

Figure 4. Codeine structure.

Log P: 1.3 [18]

Codeine is a weak opioid agonist of opioid receptors, a natural opiate derivative, and its mechanism of action is classified as a full opioid agonist.[17,20,21] It is also classified as a pro-drug, since it requires metabolization into morphine.[5,16,21,25] Its analgesic action is about 1/6 compared to morphine.[21] Due to the fact that it is a weak opioid, it is also classified as a drug of step 2 of the pain, it is prescribed in the treatment of moderate pain[20] and as antitussive.[17,25,26] The administration can be made orally, IM and SC.[16] Codeine plasma half-life is between 2-4 h and is excreted (metabolites as well) 5-15% by renal route.[16] The protein binding is 7 to 25% and the Vd is between 2.5-3.5 L/kg.[18] In figure 5, codeine metabolism is represented, wherein most of the dose is glucuronidated, although glucuronide derivative being inactive as an analgesic.[16] In this context, the detection of morphine in the blood may indicate both the consumption of morphine itself but also of codeine, since it is one of its metabolites. There are indications that codeine is also metabolized in hydrocodone.[23]

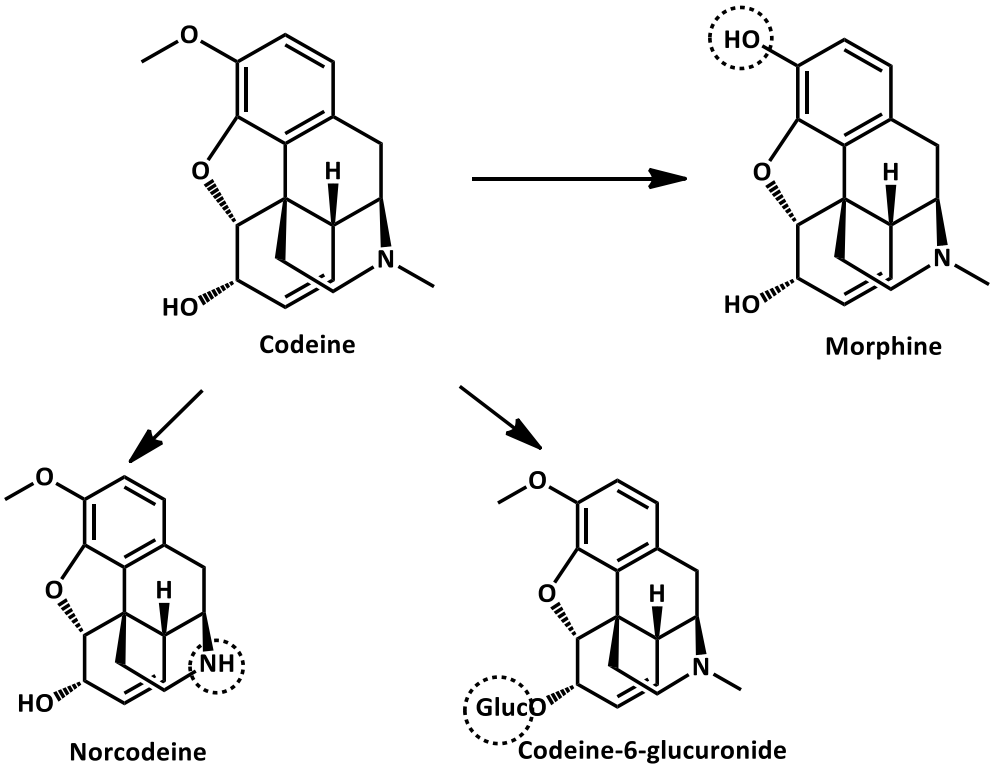

Figure 5. Codeine metabolism with chemical alterations identified. Adapted from Frost et al.[27]

1.4.3. Heroin

Heroin also referred as diacetylmorphine, is a semi-synthetic opiate derivative, and its mechanism of action is classified as an opioid μ receptor agonist and as a prodrug, since it becomes

active with its metabolites 6-acetylmorphine (6-MAM) and morphine.[5,16,17,28] Heroin is used as a recreational drug and can be administrated by IM, IV, Intranasal (IN) and smoking routes.[5,16,17,29] Plasma half-life is very short (1.3 minutes to 14 minutes) and is rapidly converted to 6-MAM and morphine (figure 6 and Annex 2). Thus, its detection in blood and urine is difficult. [15,16,23,28,30] Once again, it should be highlighted that the detection of morphine may not only indicate the consumption of morphine or codeine but also of heroin.

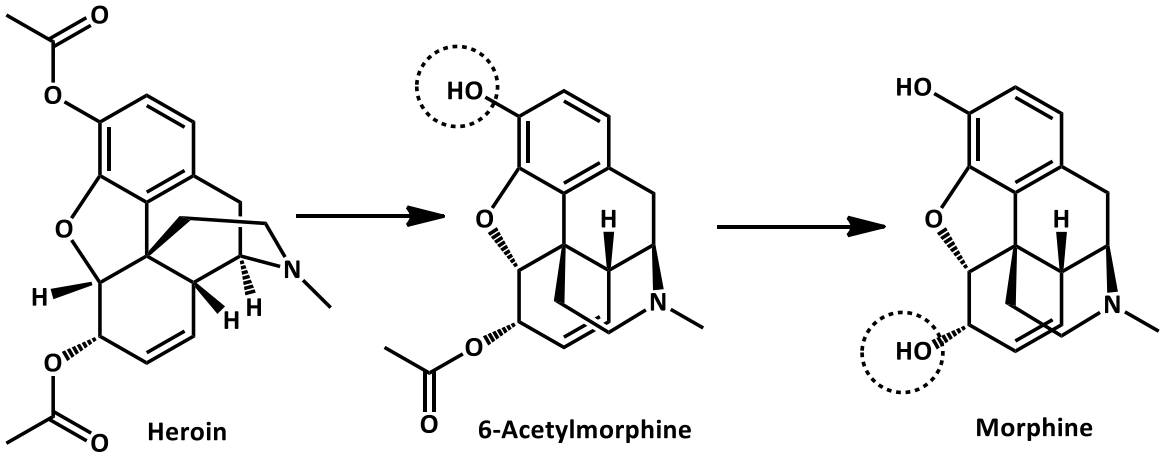

Figure 6. Heroin metabolism with chemical alterations identified. Adapted from Telepchak et al.[31]

1.4.4. 6-acetylmorphine

Molecular formula: $C_{19}N_{21}NO_4$

Molecular weight: 327.38 g/mol

IUPAC name: [(4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-hydroxy-3-methyl-2,4,4a,7,7a,13-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-yl] acetate [17]

pKa: 9.08

Log P: 1.31-1.9 [32]

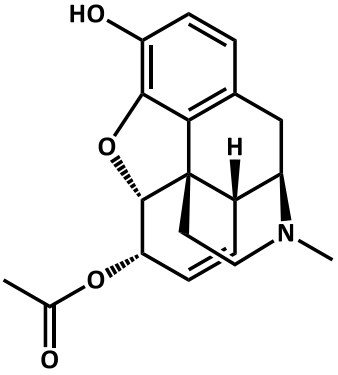

Figure 7. 6-Acetylmorphine structure.

As stated in the previous chapter the difficulty in detecting heroin in the blood (due to rapid metabolism) has been reported, so 6-acetylmorphine has become a specific marker for the illicit use of heroin.[15,23,33,34] This substance has an estimated plasma half-life of 3 to 52 min and only a small amount is excreted via urine.[23]

1.4.5. 6-acetylcodeine

Molecular formula: $C_{20}H_{23}NO_4$

Molecular weight: 341.407 g/mol

IUPAC name: [(4*R*,4a*R*,7*S*,7a*R*,12b*S*)-9-methoxy-3-methyl-2,4,4a,7,7a,13-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-yl] acetate [17]

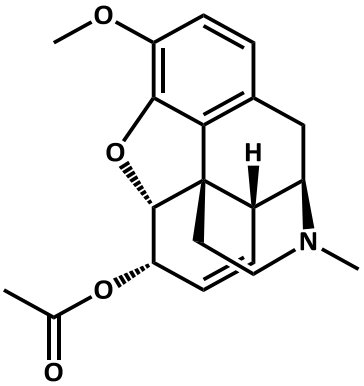

Figure 8. 6-Acetylcodeine structure.

Such as noscapine and papaverine, 6-acetylcodeine is an impurity of street heroin. In this way, it can be used as an indicator of heroin use. However, it should be taken in account that the impurities present will depend on the method of production and the primary composition of the opium used.[15,23] This substance is metabolized into codeine (figure 9), however 0.4% of 6-acetylcodeine is excreted unchanged via urine.[15,23] It has an approximate plasma half-life of 4 h.[15] The detection of codeine is also an example that may indicate both its own consumption and that of heroin, because it is a metabolite of 6-acetylcodeine.

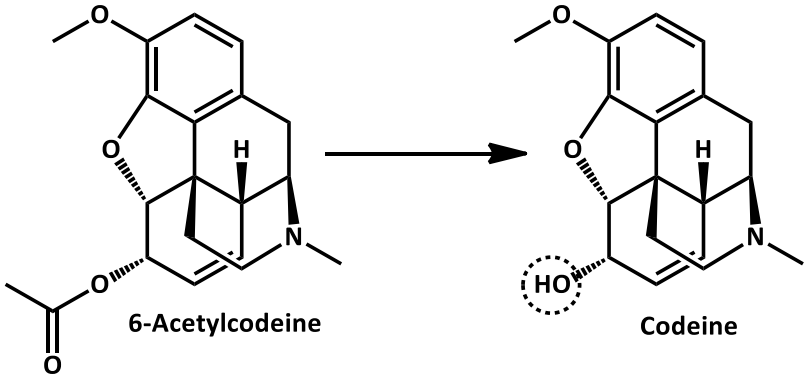

Figure 9. 6-Acetylcodeine metabolism adapted from Maas et al.[23]

1.4.6. Oxycodone

Molecular formula: $C_{18}H_{21}NO_4$

Molecular weight: 315.369 g/moL

IUPAC name: (4*R*,4a*S*,7a*R*,12b*S*)-4a-hydroxy-9-methoxy-3-methyl-2,4,5,6,7a,13-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-one [17]

pKa: 8.2

Log P: 1.0 [18]

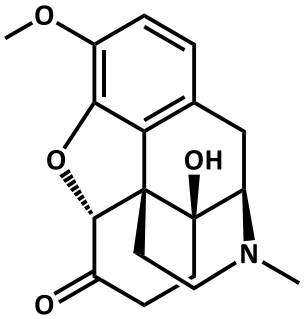

Figure 10. Oxycodone structure.

Oxycodone is a moderately potent semi-synthetic opioid agonist of μ, and κ opioid receptors, and its mechanism of action is classified as a full opioid agonist.[17,21,35] It is recommended in the treatment of moderate to severe pain, been prescribed in both step 2 and 3 of pain.[16,35] It has higher analgesic properties and similar side effects than morphine.[16,21,35] Although it is considered a good alternative to codeine (lesser dosage causes equal analgesia with minor side effects), it shows an abuse liability similar to heroin.[16,35] The administration can be made orally, IM, IV, rectally and as an epidural.[16,21,35] Oxycodone is metabolized to noroxycodone (main metabolite without considerable analgesic activity) and oxymorphone (much higher analgesic activity than morphine). Both of these metabolites will be metabolized into noroxymorphone (figure 11). Oxycodol, oxymorphol, and noroxycodol are formed by reduction of oxycodone, oxymorphone and noroxymorphone, respectively, and oxycodone-6-glucuronide, oxymorphone-3-glucuronide and noroxycodone-glucuronide are formed by glucuronidation of oxycodone, oxymorphone and noroxycodone, respectively.[21,35]. Plasma half-life is around 2 to 3 h and it is especially excreted renally.[16] The protein binding is 45% and the Vd is 2.6 L/kg.[18]

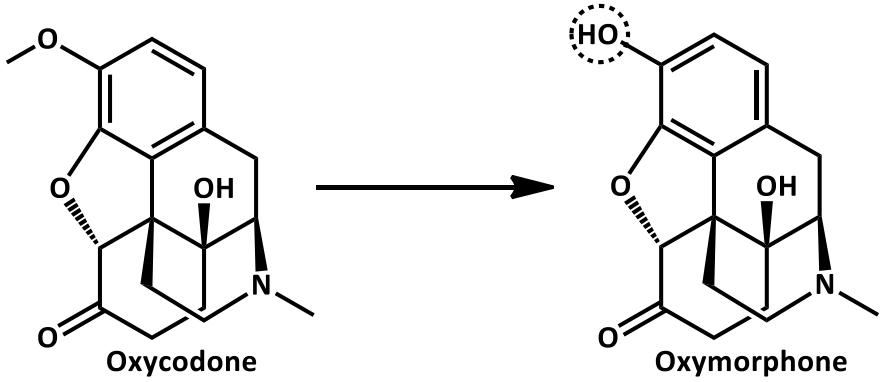

Figure 11. Oxycodone metabolism adapted from McKinley et al.[36]

1.4.7. Oxymorphone

Molecular formula: $C_{17}H_{19}NO_4$

Molecular weight: 301.342 g/mol

IUPAC name: (4*R*,4a*S*,7a*R*,12b*S*)-4a,9-dihydroxy-3-methyl-2,4,5,6,7a,13-hexahydro-1*H*-4,12-methanobenzofuro[3,2-*e*]isoquinolin-7-one [17]

pKa: 10.9

Log P: 0.8 [18]

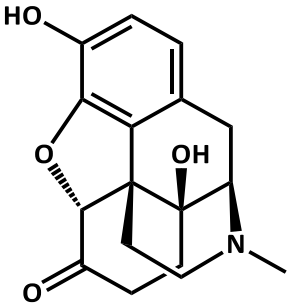

Figure 12. Oxymorphone structure.

Oxymorphone is a metabolite of oxycodone, however, is also classified as a semi-synthetic opioid agonist of μ and δ opioid receptors. Its mechanism of action is classified as a full opioid

agonist. It is prescribed in the treatment of moderate to severe pain and as an adjunct to anesthesia.[17,37–40] It has 6-10 higher analgesic properties than morphine, similar side effects with those appearing with other opioids and it shows an abuse liability similar to morphine.[5,17,37–40] Co-administration with alcohol increased the possibility of an overdose.[37] Although in Portugal it is not commercialized, it is available in other countries in injectable, suppository and oral formulations.[26,37,38] Oxymorphone-3-glucuronide and 6-OH-oxymorphone (has almost the same analgesic potency as oxymorphone) are the two major metabolites of oxymorphone.[38,39] When oxymorphone is detected it may occur due to consumption of the oxymorphone itself but also due to consumption of oxycodone since it is an oxycodone metabolite (figure 11). Its Vd is 3 L/kg and protein binding 10 to 12%.[18]

1.4.8. Fentanyl

Molecular Formula: $C_{22}H_{28}N_2O$

Molecular Weight: 336.479 g/mol

IUPAC Name: N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl]propanamide [17]

pKa: 8.8

Log P: 3.8 [18]

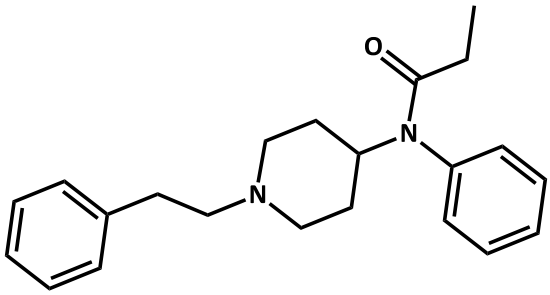

Figure 13. Fentanyl structure.

Fentanyl is a potent synthetic opioid analgesic and a full opioid agonist of μ opioid receptors, although it also has activity on the δ and κ receptors .[5,16,19,21,41,42] Belongs to the piperidine derivatives and chemically is related to pethidine.[17]It is prescribed in the treatment of severe pain (being approximately 50 to 100-fold more potent than morphine) and is also used as an anesthesic and sedative and is an aid in the treatment of epilepsy.[5,16,19,21,42] It has similar analgesic properties than morphine and similar side effects, however, detoxification symptoms are less severe, onset withdrawal symptoms is slower and the development is more prolonged.[5,16,17] The administration can be made orally, IM, IV, IN, epidural, IT, via transmucosal (TM) and transdermal (TD).[5,16,21,26,42] Norfentanyl is an inactive metabolite of fentanyl, but there are others in smaller quantity (hydroxyfentanyl, hydroxynorfentanyl, and despropionylfentanyl). Fentanyl has a plasma half-life of 3-12 h [5,8,16,19,42] a Vd between 3-8 L/kg and protein binding about 80 to 85%.[18] According to Barratt *et al.,* fentanyl has been sold as heroin, without the knowledge of their

consumers, leading to many cases of overdose (fentanyl has 30-50 times higher potency than heroin).[43]

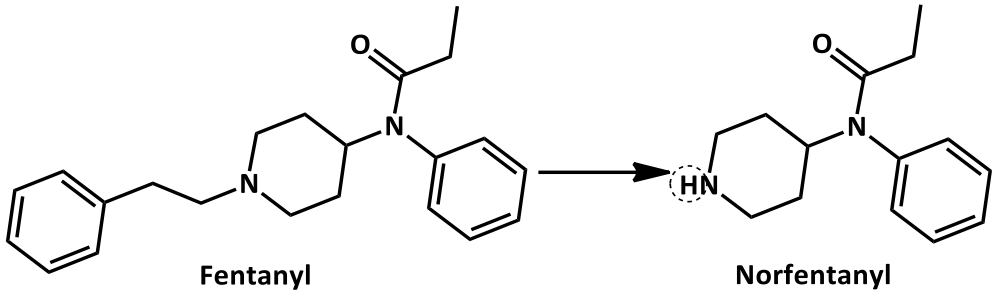

Figure 14. Fentanyl metabolism adapted from Jannetto et al.[41]

1.5. Pharmacology

Opioids are primarily used in the treatment of pain, thus according to World Health Organization (WHO), pain has 3 degrees (The WHO Pain Ladder- three step), mild pain, moderate pain and severe pain. Depending on the degree of pain, different dosage is appropriate. Opioids are advised from step 2 as is the case of codeine, however, opioids classified as strong (morphine, fentanyl, oxycodone and others) are only indicated in step 3.[20,44] Opioids can be classified according to their affinity for different opioid receptors: a pure agonist exerts effect on all receptors, a partial agonist/antagonist exerts a partial agonist effect on a receptor and antagonist effect on one or more receptors, and an agonist/antagonist exerts a pure agonist effect on a receptor and antagonistic effect on one or more receptors.[16,20] However, they are also used as a recreational way, because of their euphoric effects.[23,38] The main effects for each opioid receptor are compiled in table 2. The κ receptors analgesia occurs especially in the spinal cord, compared to μ respiratory depression and miosis (action on the oculomotor nucleas) is of lower intensity.[5]

Table 2. Principal opioids receptors and their effects. [5,7,20]

Receptors	**μ (mu)**	**κ (kappa)**	**δ (delta)**
Effect	Analgesia [5,7,20]	Analgesia [5,7,20]	Analgesia [7,20]
	Respiratory depression [5,7,20]	Respiratory depression [5,20]	Respiratory depression [20]
	Euphoria [5,7,20]	Dysphoria [20]	
	Constipation [5,7,20]	Sedation [7,20]	
	Miosis [5,7,20]	Miosis [5,7,20]	

1.5.1. Pharmacokinetics

Pharmacokinetics is the study of the quantitative dose relationship administered with plasma/blood concentrations. It is divided into absorption, distribution, metabolism or biotransformation and excretion. It is important to know the metabolism of the substances as a support for the interpretation of the obtained results. Another important factor to consider is the half-life of the substances, i.e., the time that the drug's plasma concentrations decrease by 50%.[5] The pharmacokinetic behaviour between pure agonists (morphine, fentanyl, methadone and oxycodone) is similar and their half-life is approximately 3-4 hours.[5,20] However, there are several pharmacokinetic studies that have demonstrated significant interindividual variations. Some factors to take into account are age, genetic constitution, disease and concomitant drug therapy that may affect dose-concentration relationship.[16] Opioids are mostly basic drugs (pKa ranging from 7.5 to 10.9), and in this group of drugs fentanyl is the most lipophilic (Log P: 3.8) which permits rapid diffusion through membranes. The relation between the quantity of drug in the body and the concentration of drug measured in blood/plasma (Vd) will be smaller if drugs are highly bound to plasma proteins. These characteristics indicate the potentiality of the drugs to undergo *postmortem redistribution* (lipophilicity (log P), basicity (pKa), Vd >3 L/Kg, and protein binding).[18]

1.5.2. Toxicokinetic

Toxicokinetic, is the study of the passage of hazardous substances or its metabolites through the body of an individual. In certain cases, the pharmacokinetics may change with high dose administration, e.g., morphine delays gastric emptying which will lead to greater absorption.[5]

1.5.3. Signals and Symptoms

Opioids cause very similar effects among themselves, the differences are essentially in relation to the potency that each one has in relation to certain receptors, therefore in the triggering of certain effects. The central effects are: analgesia, respiratory depression, nausea and vomiting (stimulation of the trigger zone in the marrow, in relation to the neurotransmitter dopamine and reduction of peristalsis), euphoria, sedation, miosis, antitussive effect (in the brainstem nuclei in the cough reflex pathway), hypotension and bradycardia. The peripheral effects are constipation (reduced peristalsis of the gastrointestinal tract caused by a maintained contraction of the smooth

muscle of the gut), contraction of the sphincter of Oddi and vesical, and analgesia in inflamed tissues.[5,16,20,29] The most common undesirable effects are nausea and vomiting, constipation, dry mouth, sedation and tolerance. Less frequently, respiratory depression, cognitive changes (hallucinations, delusions), hyperalgesia, myoclonus, dyspepsia and pruritus.[20] However, although they are described as secondary effects, opioids may be used as cough suppressants, and antidiarrheal agents. Its abuse comes from the effects on mood-altering effects, tolerance, and physical and psychological dependence.[5] The most common cause of death due to an overdose of opioids is the decrease in the sensitivity of the respiratory centers to *p*CO2, decreasing the automatic drive to respiration, which will cause the individual to fall asleep and die.[29] In this way it is described that in severe intoxication this can lead to coma, respiratory depression, which will trigger apnea and finally death.[5] It is thought that due the accumulation of morphine metabolites in patients it will lead to toxicity, while in the case of oxycodone this is not the case since there are no morphine metabolites.[35]

1.6. Mechanisms of Action

The human body has at least four classes of opioid receptors, μ, κ, δ and σ, and three endogenous opioid peptides the enkephalins (large affinity with μ and δ), endorphins (large affinity with μ and δ) and dynorphins (high affinity with κ).[29,45] These receptors have inhibitory effect, since they reduce activity, transmitter release and electrical firing.[29] They are distributed in the brain, spinal cord and peripheral nervous system and are predominantly in presynaptic locations but also in postsynaptic sites.[29,45] The stimulation of these receptors can control the release of several neurotransmitters as glutamate, substance P (SP), γ-aminobutyric acid (GABA) and acetylcholine. The central receptors lead to analgesic effects and side effects while peripheral receptors to side effects. In order to better understand the mechanism underlying the analgesic effect of opioids, it is necessary to explain slightly how information is transmitted in the human body. In the presynaptic, transmitters chemicals such as glutamate (principal pain neurotransmitters) and SP are released accordingly with the pain intensity by the primary afferents. Figures 15,16 and 17 illustrates the mechanisms subjacent in pain. Glutamate acts on NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, increasing calcium inflow (figure 15). Figure 16 illustrates SP acting on nerokinin-1 receptors (NK1R) increasing also intracellular calcium by removing magnesium-ion-block that is present in the NMDA receptor by a signal transduction. In figure 17, CGRP (calcitonin gene related peptide) release and action on the

CGRP receptor is illustrated.[46] Finally, figure 18 illustrates opioids action and their endogenous neurotransmitters, so basically, they bind to protein G inhibitory receptors that will close voltage channels (Ca^{2+}) decreasing neurotransmitters transmission (glutamate, SP and CGRP). It will also stimulate the opening of the potassium channels permitting the efflux of potassium and leading to a hyperpolarization, decreasing neuronal excitability.[7,29,45,46]

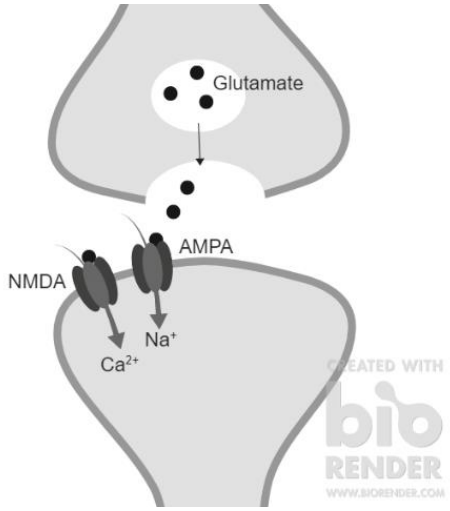

Figure 15. Example of one pathway of the pain transmission (glutamate), created with biorender.

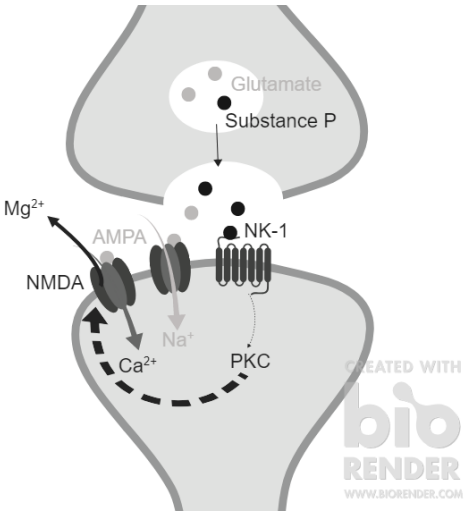

Figure 16. Example of one pathway of the pain transmission (Substance P), created with biorender.

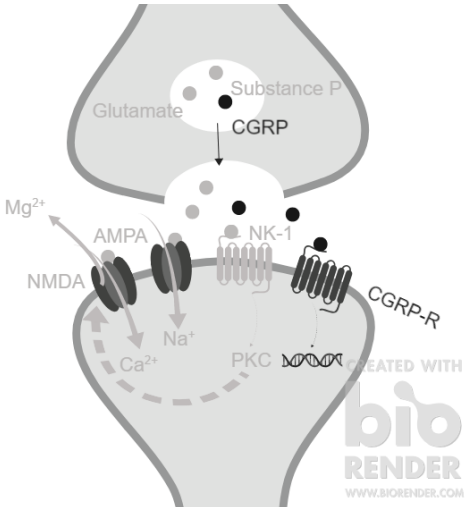

Figure 17. Example of one pathway of the pain transmission (CGRP), created with biorender

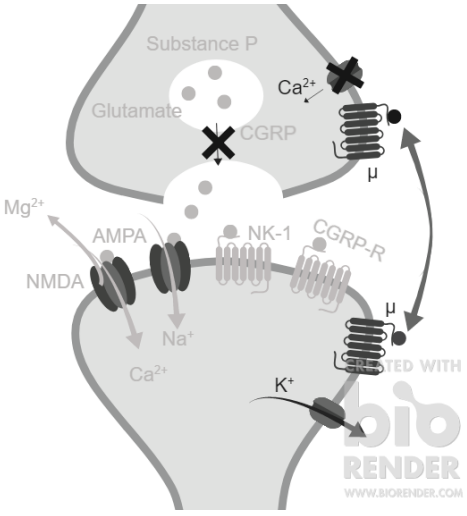

Figure 18. Example of opioids and endogenous opioids action on opioid receptor μ (analgesic effect), created with biorender

1.7. Biological Matrices

Toxicological analyses in most forensic cases are performed using blood (BL), with peripheral blood being less affected by *postmortem* redistribution in relation to cardiac blood. The reference concentrations of the substances are mostly in the peripheral blood.[47] However, BL may not be available in situations such as: carbonization, advanced decomposition, and extensive bleeding.[47–50] Thus, it is necessary to perform the toxicological analyses on an alternative BL matrix, such as pericardial fluid (PF), vitreous humor, skeletal muscle, among others.[47,48] The PF is composed of phospholipids and corresponds to an ultrafiltered plasma due to the electrolytes and small molecules (urea, uric acid, glucose, creatinine) present.[51,52] In terms of proteins their concentrations are smaller than those of BL and in different proportions. Its function is to lubricate the heart.[51] The main advantages of PF are: available volume (between 5-35 mL); easy collection; well protected from contamination and *postmortem* changes. However, it should be noted that it may be contaminated by *postmortem* diffusion if there are high amounts of a substance in the stomach.[48] In this way, due to its various advantages, it becomes a good alternative matrix as well as complementary (not enough blood to do the confirmation).[48,49,52] Despite the advantages of using this matrix, the studies carried with it are very scarce, making it important to develop methods for the detection and quantification of more substances.[47,49,50,52]

Chapter 2. Bioanalytical Procedures for Opioids Determination in Biological Specimens

2.1. Analytical Chemistry

Analytical chemistry is a subdivision of chemistry that allows identification (qualitative) or quantification (quantitative) of species or chemical elements. The methodologies used in analytical chemistry include classical methods of separation (precipitation, extraction and distillation) as well as chromatographic techniques and quantification methods such as volumetry, gravimetry, spectroscopy/spectrometry techniques, electroanalytical and miscellaneous techniques.[53] According to the IUPAC "Analytical chemistry is a scientific discipline that develops and applies methods, instruments, and strategies to obtain information on the composition and nature of matter in space and time, as well as on the value of these measurements, i.e., their uncertainty, validation, and/or traceability to fundamental standards". (https://iupac.org/who-we-are/divisions/division-details/?body_code=500, accessed 28 of September 2019)

2.1.1. Preparation of the Sample

The steps to be performed in the preparation of a sample depends on its type, i.e. for solid particles can be carried out with the use of filters and centrifugation, whereas for biological matrices components precipitation, liquid-liquid extraction (LLE) and/or solid phase extraction can be used.[54] Extraction is a separation/purification technique where its purpose is to separate the analytes from within a mixture, whether solid (leaching or solid/liquid), semi-solid or liquid (liquid/liquid or solid phase).[54] Since the analytes in this study are in biological matrices, the sample preparation is even more critical for the analytical method.[55] For example, BL contains proteins at a relatively high concentration which may interfere with the analysis of the analytes. In this way, the problem can be solved with a deprotonation/precipitation of the proteins.[56]

Solid-phase extraction (SPE) extract analytes from gas or liquid samples by their retention in the solid phase and then, by elution the analytes are recovered. The main goals of SPE are trace enrichment (concentration), sample clean-up and medium exchange.[57] The advantages of SPE is their highly reproducibility, efficiency (helps extend life time of gas chromatography (GC) and liquid chromatography (LC) columns), quickness, the use of less amount of sample and producing less waste solvents. However, the disadvantage is that it usually requires expensive columns.[5,31,55,56] There is a great diversity of samples and analytes, so, there are several types of columns or disks and solvents suitable for use in each situation. Some examples of adsorbents that the columns have are silica gel, alumina, graphitic carbon, etc.[56,57] The type of column used in this study are the mixed-

mode columns (sorbents) containing co-bonded ion-exchange and alkyl groups. This type of columns is widely used in pharmaceutical and clinical laboratories in the extraction of drugs from within biological fluids.[57]

The SPE involves four steps: column conditioning, sample addition, column washing (with solvents to remove interfering substance), dryness and analyte elution (figure 19). Each step depends on the analyte of interest and the type of extraction column.[5,31]

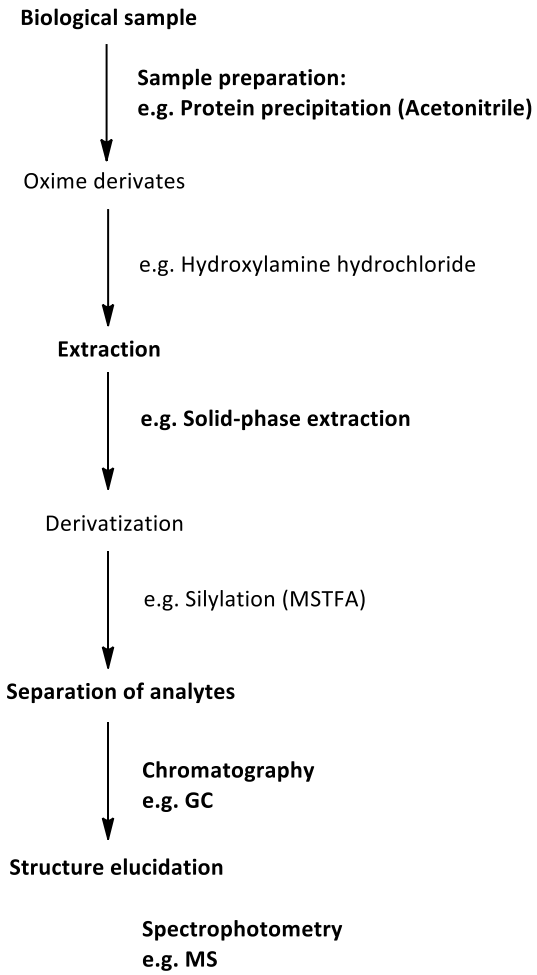

Figure 19. Example for the isolation and characterization of analytes (at bold), with the addition of the reagents and techniques used in this study. Adapted from Nnane et al. [56]

2.1.2. Gas Chromatography

Chromatography is an analytical technique for separating substances from a given mixture.[2] There are several types of chromatography techniques, however they all have certain common characteristics, such as: the use of a mobile phase and a stationary phase; samples are transported

through the mobile phase along the stationary phase; the separation of the different compounds from the mixture depends on the affinity of the various substances with the mobile and/or stationary phase.[2,58,59]

Gas chromatography (GC) is an instrumental analytical method. This technology uses an inert gas (helium, argon, hydrogen, or nitrogen) as the mobile phase, i.e. it does not interact with the analytes, and as stationary phase a capillary column consisting of fused silica coated with a liquid (most common are methyl silicones with 1.5 or 50% phenyl side chains) consisting of a polymer.[2,5,59] The columns may vary according to the chemical nature of the stationary phase, thickness (0.1 to 5 µm), length (10 to 60 meters) and diameter (0.20 to 0.53 mm). It can separate the components of a mixture according to the different boiling points/vapour pressure and polarity. In this case, the mobile phase has the transport function (drag gas) while the stationary phase separates the different components. The analysis begins with the introduction of the sample in the injector, via a handheld syringe (manually or by an automated device named autosampler). The analysis when performed with an autosampler is more precise and allows the instrument to run continuously, through an injection port into the injector (inlet).[5] The way of injection can be in *split* (only part of the sample is inserted in the column) or in *splitless* (almost total introduction of the sample in the column).[2] The mobile phase passes through the injector and transports the sample to the column (stationary phase). After the separation of the substances along the column these enter into the detector. There is a wide range of detectors, varying in detection capacity (specific or "universal") and detection limit. The detector to be used may be specific:

- electron capture detectors (ECD), sensitive only to- Halides, nitrates, nitriles, peroxides, anhydrides, organometallics;[2,5,58,59]
- nitrogen phosphorous detector (NPD) - N, P;[5,59]
- flame photometric detector (FPD) - S, P, Sn, B, As, Ge, Se, Cr;
- photo-ionization detector (PID) - Aromatics, ketones, esters, aldehydes, amines, heterocyclics, organosulphures, some organometallics.[59]

Or universal detectors such as:

- flame ionization detector (FID) - Most organic compounds;[2,5,58,59]
- thermal conductivity detector (TCD);
- mass spectrometer (MS).

The MS detector is considered an universal detector when used with full scanning mode (TIC), or a sensitive and selective detector when used in selected ion monitoring (SIM) mode.[2,59] The plot (chromatogram) has on the x axis the retention time (depends on the different boiling points/vapour pressure and on the affinity of the substances to the stationary phase, i.e. the time

the substance takes from the injection to the detection), this parameter is not specific for each substance, since there may be other substances with the same retention time, its depend on other factors such as operating conditions. The y-axis shows the peak abundance, which is a relative measure of the amount of substance present (peak height or peak area may be considered). For quantification, a calibration curve (analysis of known concentrations of the substances) will be necessary, and in order to guarantee precision, the addition of an internal standard (IS) is also necessary.[59]

2.1.3. Mass Spectrometry

It is a qualitative and quantitative technique that provides information on the elemental composition, molecular structure, and isotope ratios of the atoms in the sample.[2] In figure 20 the operation of an MS is shown: 1-Sample introduced by the interface in the ionization chamber; 2-substances ionized and fragmented in various ions; 3-the ions are accelerated according to the mass-charge ratio (m/z) throughout the analyser, which is made up of four electrical poles with a direct current voltage and radiofrequency voltage, vary these parameters only ions with certain m/z will cross and arrive at the detector (when the mass analyser is the quadrupole). The results are presented in a mass spectrum, in which the x-axis shows the m/z ratio and de y-axis the abundance of the ions.[2,5,59] There are several types of mass analysers, such as quadrupole, magnetic sector, flight time (TOF), ion entrapment.[2,59]

The ionization method will depend on the nature of the analyte, the sources of choice in GC-MS are: electron impact (EI) and chemical ionization (CI). The remaining sources of ionization are electrospray (ESI), atmospheric chemical ionization (APCI), atmospheric pressure photolonisation (APPI), fast atom bombardment (FAB) and matrix assisted laser desorption ionisation (MALDI).

One of the most widely used is the source of EI ions, where each organic molecule has a unique EI spectrum. This method is based on the following: a molecule is bombarded by an electron beam (70eV), and energy is transferred to the molecule and an electron is ejected, forming a species with an odd number of electrons and positive charge, the molecular ion. The molecular ion indicates the molecular weight of the molecule under study. Due to the excess of energy transferred, the molecular ion undergoes fragmentation into several ions of different m/z.[2,5]

Figure 20. MS components adapted from Skoog et al.[53]

2.1.4. Gas Chromatography-Mass Spectrometry

The hyphenated techniques take advantage of both techniques, in which the components of a mixture will be separated by the chromatographic method and later analysed by the MS (figure 21).[5,53]

GC-MS is recognized as one of the most efficient techniques used in toxicological laboratories for analysis of controlled and unknown substances, holding one of the highest degrees of specificity. The sample does not need to be pure and the various components in the mixture can be identified individually.[5]

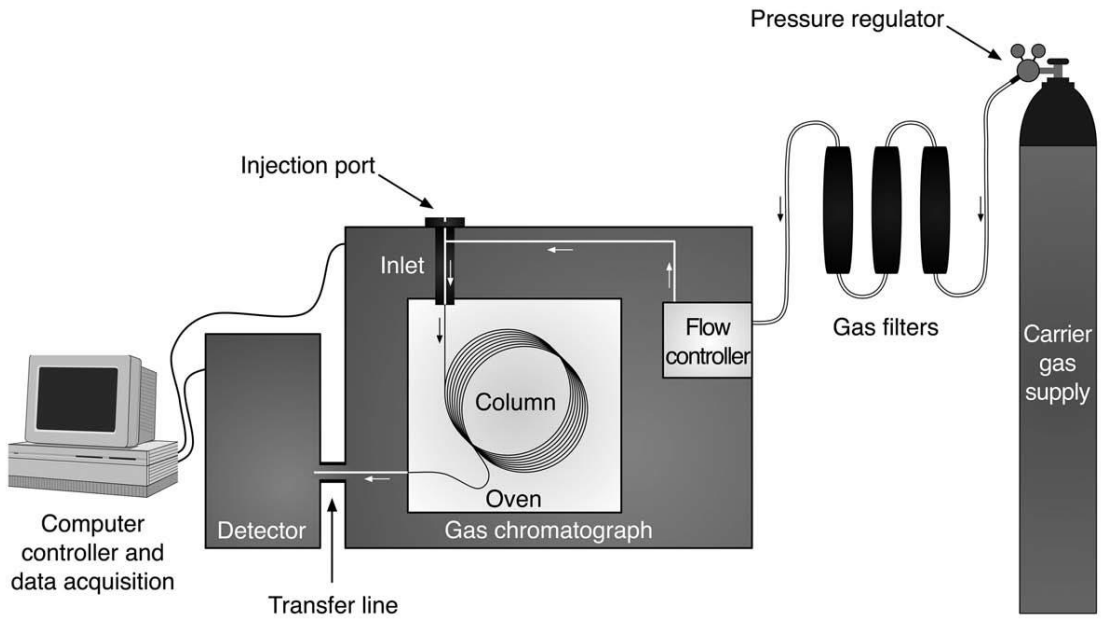

Figure 21. GC-MS. From Stauffer et al. [59]

2.1.5. Derivatization

The GC technique requires that the substances have certain chemical characteristics to be chromatographed, i.e. they need to be minimally volatile (the limit is ±300 °C) and thermally stable. In situations where these characteristics are not present, the problem can be solved with a chemical

derivatization aiming an adequate chromatographic performance.[2,60,61] Derivatization can be applied to make substances possible to be analysed and to improve chromatographic efficiency and detectability.[31,60,61] The functional groups that usually need derivatization are: hydroxyl, carboxylic and amines. The three main types of derivatization are silylation, alkylation, and acylation. Silylation is one of the most used. The reaction basically is the introduction of a silyl group (trimethylsilyl/Si(CH_3)$_3$/TMS) into the molecule by a nucleophilic attack on Si (Knapp), usually to substitute hydrogen in N-H, O-H and S-H groups, reducing the polarity of the compound and increasing the volatility (figure 22).[61] It is possible to use solvents such as acetonitrile, DMF (dimethylformamide), DMSO (dimethyl sulfoxide), pyridine, and THF (tetrahydrofuran), depending on the substances. Examples of silylation reagents are: N O-bis (trimethylsilyl) trifluoroacetamide (BSTFA), Trimethylchlorosilane (TMCS) usually added to increase strength of silyl donation, N O-bis (trimethylsilyl) acetamide (BSA), N- methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), Hexamethyldisilazane (HMDS) and N- (tert-butyldimethylsilyl-N-methyltrifluoroacetamide) (MTBSTFA).[31,61] Alternatively, alkylation and acylation of N-H, O-H and S-H groups can also be used to increase the volatility of a substance.[31,60,61] However, some mishaps may occur as poor chromatography, instability, incomplete derivatization, unsuitable ions and abundances, interferences or coelution, among others.[62]

Oxycodone and oxymorphone are characterized by keto-opioids and they are usually hard to analyse by GC-MS. The derivatization of these two compounds, with MSTFA+5% TMCS, was not enough to obtain a good chromatographic resolution. It was necessary to introduce a first step of oximation with hydroxylamine to increase the chromatographic efficiency and overcome this difficulty.[63]

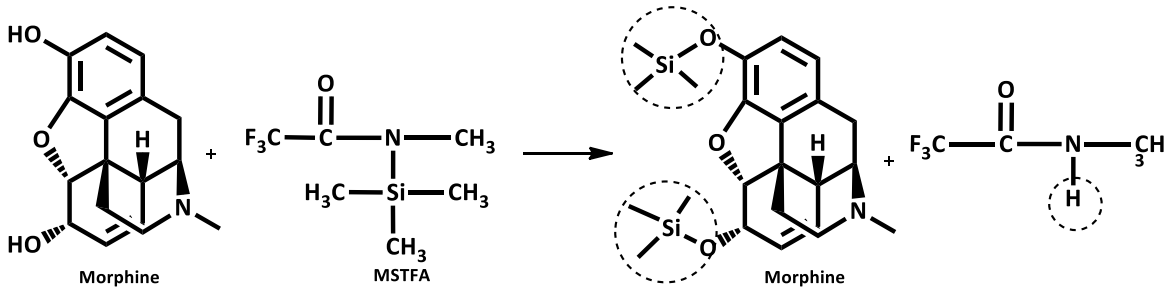

Figure 22. Silylation example of morphine with MSTFA

2.1.6. Hydroxylamine

Hydroxylamine (NH_2OH) is an inorganic compound used in organic synthesis and as a reducing agent. It is a white crystalline solid powder, soluble in water, ammonia and methanol.[17]

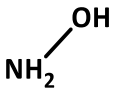

Figure 23. Hydroxylamine structure.

As already mentioned, during the derivatization by silylation of the keto-opioids oxycodone, oxymorphone, hydrocodone and hydromorphone several problems can occur, because they may interfere with codeine and morphine and also may lead to a poor chromatographic resolution. In this study, a step prior to the derivatization of the hydroxyl group by silylation, based on a pre-treatment/oximation with hydroxylamine was optimized and implemented (figure 24).[38,62,64–70] With the inclusion of this crucial step (oximation) in the preparation of the samples containing the substances of interest (as seen in the reaction of figure 24), formation of oximes derivatives occurs and multiple derivatization of these compounds is avoided. This multiple derivatization is due to the tautamerization which will permit the formation of products from the enol isomer and from the unreacted keto isomer.[63,65,69,71,72]

It must be highlighted that the addition of hydroxylamine will not affect other opioids such as codeine, morphine, 6-acetylmorphine, 6-acetylcodeine and fentanyl since their structure do not incorporate a ketone functional group.[62,63,73]

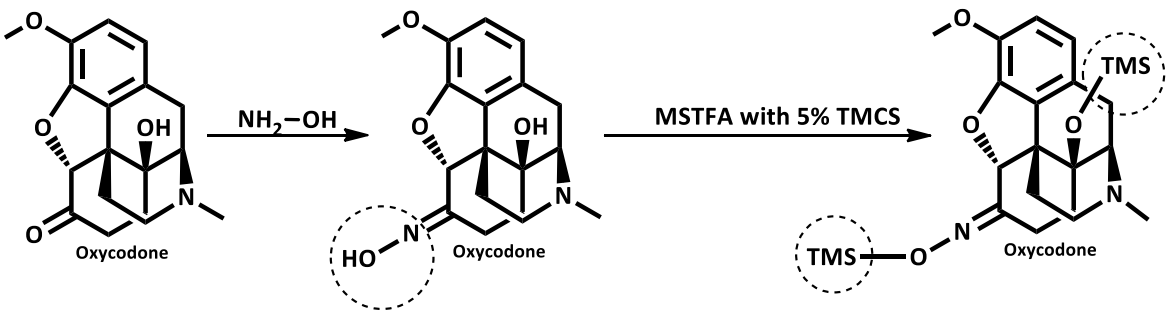

Figure 24. Example of oxycodone oximation using hydoxylamine and further a Silylation with MSTFA+5% TMCS.

2.1.7. Validation of a Method

The validation allows to estimate the efficacy and reliability of a new or modified (previously validated) analytical method, demonstrating evidence of the ability to successfully perform in the established parameters, and to verify the method's limitations. Thus, there are several guidelines such as: Standard Practices for Method Validation in Forensic Toxicology by SGWTOX (Scientific Working Group for Forensic Toxicology) and Guidance for Industry Bioanalytical

Method Validation by FDA (Food and Drug Administration). The parameters that will be evaluated in a quantitative analysis are:

- Bias – Required for all quantitative methods. It is the measurement of at least three samples per concentration (low, medium and high) over five different runs. The true value maximum acceptable is ±20%;

$$Bias\ (\%) at\ concentration_x = \left[\frac{Grand\ Mean\ of\ calculated\ concentration_x - Nominal\ concentrartion_x}{Nominal\ concentrartion_x}\right] \times 100$$

- Precision – Required for all quantitative methods. It is expressed as the coefficient of variation (%CV) and is acceptable when <20% CV for all concentrations;

$$\%CV = \frac{standard\ deviation}{mean\ response}$$

- Calibration model - Required for all quantitative methods. Determines the working range (concentrations range over which the method will be used) with at least six different non-zero concentrations and a minimum of five replicates per concentration. It is required determination coefficient r^2>0.99, acceptable bias and precision. It can also be evaluated using standardized residual plots;
- Carryover- After the analysis of positive samples (high concentrations) the appearance of unwanted signals in subsequent blank matrices must be investigated;
- Interference studies- Analyses of non-targeted compound which may affect the ability to detect, identify or quantitate a targeted compound. It should be included the most common drugs/metabolites detected in the laboratory;
- Limit of detection (LOD) - Lowest concentration of an analyte that can be identified. Reproducible instrument response greater or equal than three times the noise level of the background signal (≥ 3 S/N);
- Limit of quantification (LOQ)- Lowest concentration that achieves acceptable detection, identification, bias and precision criteria.

Additionally, it can be evaluated dilution integrity (assurance that bias and precision are not significantly affected), stability (analyte's resistance under specific conditions must be verified if they fall out bias criteria, e.g. by study the freeze/thaw multiple cycles during certain days) and recovery (comparison of the analytical results for extracted samples (spiked before extraction with analyte standards and after extraction is added IS) with biological samples spiked after extraction with analyte standards and IS (represents 100% recovery)).[74,75]

2.2. Previous Studies

A bibliographic search was carried out in order to verify the studies already done related to these substances, biological matrices and analytical instrumentation. In order to limit the search, we only analysed the studies in a period of 10 years (from 2009 to date) referring to the majority of substances except oxycodone, oxymorphone and 6-acetylcodeine being 20 years (from 1999 to date). However, studies within this period were reporting the remaining substances as well were also considered. The biological matrix pericardial fluid was also from 1999. This was done due to the scarce number of studies concerning the referred 3 substances and biological matrix.

Table 3 compiles all the studies found regarding the use of GC-MS and a biological fluid. It can be verified the scarcity of studies concerning the use of PF (only morphine and codeine, without data regarding the LOD and LOQ), and of 6-acetylcodeine (within these parameters); the shortage of studies with fentanyl in blood, among others. In the annex 3 there is a table with the compilation of all the studies found for the determination of opioids in several biological matrices and different analytical instrumentation as HPLC (High performance liquid chromatography)-MS[76], HPLC-MS/MS[77,78], HPLC-PDA[79], LC-MS [27], LC-MS/MS[80–105], LC-qTOF-MS[89,106,107], UHPLC (Ultrahigh-performance liquid chromatography)-orbitrap[108], UHPLC-MS/MS[47,109,110] and UPLC (Ultra-performance liquid chromatography)-MS/MS[22,111–115]. It is also worth noting that distinct studies were performed by using other analytical techniques such as DBS (dried blood spot),[94,101,116,117] DUS (dried urine spot),[94] VAMS (volumetric absorptive microsampling)[94] and other matrices such as expired air,[118,119] muscle,[27,47] fat,[27] brain,[27,109,120] liver,[121] gastric contents,[84] kidneys,[120,121] nails,[122,123] meconium,[82,124] bone,[125] teeth[121,126] and hair[63,86,121,123,127–134].

Table 3. Compilation of studies for the determination of our study group substances in biological liquid matrices by GC-MS.

Analyte	Matrix	Sample volume	Sample Preparation	Derivatization	LOD/LOQ	Ref.
Morfine	Blood	-	SPE	BSTFA	LOD/LOQ: 20 ng/mL	[34]
		950 µL	SPE	BSTFA+1% TMCS	LOD: 1.50 ng/mL LOQ: 5.00 ng/mL	[135]
		1 mL	SPE	MSTFA	-	[136]
		1 mL	PP (acetonitrile); LLE	2%methoxyamine in pyridine; propionic anhydride	LOD: 2 ng/mL; LOQ: 10 ng/mL	[73]
		1 mL	PP (10% Trichloroacetic acid); SPE	BSTFA+ 1% TMCS	LOD: 1 ng/mL; LOQ: 2.5 ng/mL	[137] *
		2 mL	SPE	Hydroxylamine 1% in pyridine; MSTFA+1% TMCS	-	[39]
		2 mL	SPE	2% Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA+1% TMCS	LOD: 10 ng/mL	[84]

		2 mL	PP (methanol); SPE	10% hydroxylamine; BSTFA+1% TMCS	LOD: 2.5 ng/mL	[65]
		3 mL	PP (acetonitrile); SPE	10% hydroxylamine; ethyl acetate and BSTFA+1% TMCS	LOD: 3.12 ng/mL; LOQ: 12.5 ng/mL	[69]
	Pericar-dial Fluid	1 mL	Hydrolyzed, SPE	MSTFA, MBTFA	-	[52]
		1 mL	PP (10% TCA and concentrated HCl), LLE	Trifluoroacetic anhydride	-	[52]
	Serum	600 µL	PP (acetonitrile + isopropanol); SPE	Isooctane/pyridine/MSTFA (14/5/1, v/v/v)	LOD: 1.2 ng/mL; LOQ: 4.9 ng/mL	[120]
	Urine	-	SPE	BSTFA	LOD/LOQ: 20 ng/mL	[34]
		1 mL	Liquid nitrogen	Pyridine + 2% methoxyamine HCl, propionic Anhydride, MSTFA	LOD/LOQ: 50/200 ng/mL	[138]
		1 mL	LLE	2% Methoxyamine HCl; propionic anhydride	LOD: 10 ng/mL; LOQ: 25 ng/mL	[72]
		1 mL	SPE	MSTFA	-	[136]
		1 mL	Hydrolysis, LLE	MSTFA/ethanethiol/NH_4I (500:4:2)	LOD: 10 ng/mL	[139]
		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA+1% TMCS	LOD: 10 ng/mL	[84]
		2 mL	Hydrolysis; SPE	BSTFA+1% TMCS	LOD: 60 ng/mL; LOQ: 100 ng/mL	[140]
		2 mL	Hydrolysis, LLE	MSTFA+1% TMCS	LOD: 25 ng/mL	[141]
		3 mL	Hydrolysis, LLE	MSTFA/NH_4I/DTE (1000:2:4, v/w/w)	LOD: 30 ng/mL	[142]
		3 mL	Hydrolysis; SPE	10% hydroxylamine; BSTFA+1% TMCS	LOD: 25 ng/mL; LOQ: 50 ng/mL	[66]
		3 mL	SPE	Methoxyamine HCl in pyridine; BSTFA+pyridine	LOQ: 150 ng/mL	[143]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA	LOD: 50 ng/mL	[144]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH_4I/ethanethiol (640:1:2, v/w/v)	LOD: 100 ng/mL	[145]
	Oral Fluid	100 µL	PP (HCL, CH_3CN); SPE (DPX)	MSTFA	LOD: 500 ng/mL; LOQ: 1520 ng/mL	[146]
		1 mL	LLE	BuAc:ACN(1:1) + MSTFA (1:3)	LOQ: 5 ng/mL	[147]
Codeine	Blood	-	SPE	BSTFA	LOD/LOQ: 20 ng/mL	[34]
		950 µL	SPE	BSTFA+1% TMCS	LOD: 1.50 ng/mL LOQ: 5.00 ng/mL	[135]
		1 mL	PP (10% TCA); SPE	BSTFA+ 1% TMCS	LOD: 1 ng/mL; LOQ: 2.5 ng/mL	[137]
		1 mL	PP (acetonitrile); LLE	2% methoxyamine in pyridine; propionic anhydride	LOD: 2 ng/mL; LOQ: 10 ng/mL	[73]
		2 mL	SPE	Hydroxylamine 1% in pyridine; MSTFA+1% TMCS	-	[39]
		2 mL	PP (methanol); SPE	10% hydroxylamine; BSTFA+1% TMCS	LOD: 3.5 ng/mL	[65]
		2 mL	SPE	2% Mehtoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA+1% TMCS	-	[84]
		2 mL	LLE/ SPE	-	LOD: 100/50 ng/mL	[148]
		3 mL	PP (acetonitrile); SPE	10% hydroxylamine; ethyl acetate and BSTFA+1% TMCS	LOD:1.56 ng/mL; LOQ: 6.25 ng/mL	[69]
	Pericar-dial Fluid	1 mL	Hydrolyzed, SPE	MSTFA, MBTFA	-	[52]
		1 mL	SPE	-	-	[50]
	Plasma	1 mL	SPE	-	LOD: 0.10 ng/mL; LOQ: 0.20 ng/mL	[149]
	Serum	600 µL	PP (acetonitrile + isopropanol); SPE	Isooctane/pyridine/MSTFA (14/5/1, v/v/v)	LOD: 0.4 ng/mL; LOQ: 2.6 ng/mL	[120]

	Urine	-	SPE	BSTFA	LOD/LOQ: 20 ng/mL	[34]
		1 mL	Liquid nitrogen	Pyridine+2% methoxyamine HCl, propionic Anhydride, MSTFA	LOD/LOQ: 50/100 ng/mL	[138]
		1 mL	Hydrolysis, LLE	MSTFA/ethanethiol/NH_4I (500:4:2)	LOD: 10 ng/mL	[139]
		1 mL	LLE	2% Methoxyamine HCl; propionic anhydride	LOD: 10 ng/mL; LOQ: 25 ng/mL	[72]
		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA+1% TMCS	-	[84]
		2 mL	Hydrolysis; SPE	BSTFA+1% TMCS	LOD: 300 ng/mL; LOQ: 500 ng/mL	[140]
		2 mL	Hydrolysis, LLE	MSTFA+1% TMCS	LOD: 25 ng/mL	[141]
		3 mL	SPE	Methoxyamine HCl in pyridine; BSTFA+pyridine	LOQ: 150 ng/mL	[143]
		3 mL	Hydrolysis; SPE	10% hydroxylamine; BSTFA+1% TMCS	LOD: 25 ng/mL; LOQ: 50 ng/mL	[66]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA	LOD: 20 ng/mL	[144]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH_4I/ethanethiol (640:1:2, v/w/v)	LOD: 100 ng/mL	[145]
		10 mL	SPE	-	LOD: 0.10 ng/mL; LOQ: 0.20 ng/mL	[149]
	Oral Fluid	1 mL	LLE	BuAc:ACN(1:1) + MSTFA (1:3)	LOQ: 5 ng/mL	[147]
	Vitreous Humor	100 µL	PP (HCL, CH_3CN); SPE (DPX)	MSTFA	LOD: 160 ng/mL; LOQ: 490 ng/mL	[146]
6-Acetylmor-phine	Blood	-	SPE	BSTFA	LOD/LOQ: 4 ng/mL	[34]
		950 µL	SPE	BSTFA+1% TMCS	LOD: 1.50 ng/mL; LOQ: 5.00 ng/mL	[135]
		1 mL	SPE	MSTFA	-	[136]
		1 mL	PP (acetonitrile); LLE	2% methoxyamine in pyridine; propionic anhydride	LOD: 2 ng/mL; LOQ: 10 ng/mL	[73]
		1 mL	PP (10% TCA); SPE	BSTFA+1% TMCS	LOD: 1 ng/mL; LOQ: 2.5 ng/mL	[137] *
		2 mL	SPE	Hydroxylamine 1% in pyridine; MSTFA+1% TMCS	-	[39]
		2 mL	SPE	2% Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA+1% TMCS	LOD: 5 ng/mL	[84]
		2 mL	PP (methanol); SPE	10% hydroxylamine; BSTFA+1% TMCS	LOD: 0.70 ng/mL; LOQ: 5 ng/mL	[65]
		3 mL	PP (acetonitrile); SPE	10% hydroxylamine; ethyl acetate and BSTFA+1% TMCS	LOD: 0.78 ng/mL; LOQ: 1.56 ng/mL	[69]
	Serum	600 µL	PP (acetonitrile + isopropanol); SPE	Isooctane/pyridine/MSTFA (14/5/1, v/v/v)	LOD: 0.3 ng/mL; LOQ: 0.8 ng/mL	[120]
	Urine	-	SPE	BSTFA	LOD/LOQ: 20 ng/mL	[34]
		1 mL	SPE	MSTFA	-	[136]
		1 mL	LLE	2% Methoxyamine HCl; propionic anhydride	LOD: 10 ng/mL; LOQ: 25 ng/mL	[72]
		1 mL	Hydrolysis, LLE	MSTFA/ethanethiol/NH_4I (500:4:2)	LOD: 20 ng/mL	[139]
		1 mL	Liquid nitrogen	Pyridine+2% methoxyamine HCl, propionic Anhydride, MSTFA	LOD/LOQ: 30 ng/mL	[138]
		2 mL	SPE	2% Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA+1% TMCS	LOD: 5 ng/mL	[84]
		2 mL	Hydrolysis; SPE	BSTFA+1% TMCS	LOD: 400 ng/mL; LOQ: 600 ng/mL	[140]
		2 mL	Hydrolysis, LLE	MSTFA+1% TMCS	LOD: 25 ng/mL	[141]
		3 mL	SPE	Methoxyamine HCl in pyridine; BSTFA+pyridine	LOQ: 150 ng/mL	[143]

		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH_4I/ethanethiol (640:1:2, v/w/v)	LOD: 100 ng/mL	[145]
	Oral Fluid	1 mL	LLE	BuAc:ACN(1:1) + MSTFA (1:3)	LOQ: 1 ng/mL	[147]
		200 µL	SPE	-	LOD: 0.4 ng/mL; LOQ: 1 ng/mL	[90]
	Vitreous Humor	100 µL	PP (HCL, CH_3CN); SPE (DPX)	MSTFA	LOD: 1250 ng/mL; LOQ: 3790 ng/mL	[146]
6-Acetylcode-ine	Plasma	1 mL	SPE	-	LOD: 0.10 ng/mL LOQ: 0.20 ng/mL	[149]
	Urine	10 mL	SPE	-	LOD: 0.10 ng/mL LOQ: 0.20 ng/mL	[149]
Oxycodone	Blood	-	SPE	BSTFA	LOD/LOQ: 20 ng/mL	[34]
		1 mL	Methanol, SPE	BSTFA+1% TMCS and ethyl acetate	LOD: 50 ng/mL; LOQ: 50 ng/mL	[150]
		1 mL	PP (acetonitrile); LLE	2% methoxyamine in pyridine; propionic anhydride	LOD: 2 ng/mL; LOQ: 10 ng/mL	[73]
		2 mL	SPE	2% Mehtoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA+1% TMCS	-	[84]
		2 mL	SPE	Hydroxylamine 1% in pyridine; MSTFA+1% TMCS	-	[39]
		2 mL	PP (methanol); SPE	10% hydroxylamine; BSTFA+1% TMCS	LOD: 2.5 ng/mL; LOQ: 20 ng/mL	[65]
		2 mL	PP (acetonitrile); SPE	10% Hydroxylamine; BSTFA+1% TMCS	LOQ:50 ng/mL	[64]
		2 mL	LLE/ SPE	-	LOD: 25/250 ng/mL	[148]
		2 mL	LLE	MSTFA (TMS)	LOQ: 100 ng/mL	[151]
		3 mL	PP (acetonitrile); SPE	10% hydroxylamine; ethyl acetate and BSTFA+1% TMCS	LOD: 6.25 ng/mL; LOQ: 12.5 ng/mL	[69]
		3 mL	LLE	2% methoxyamine in pyridine; MSTFA+1% TMCS and dimethylformamide	LOD: 0.5 ng/mL; LOQ: 1.0 ng/mL	[152]
	Urine	-	SPE	BSTFA	LOD/LOQ: 20 ng/mL	[34]
		1 mL	Liquid nitrogen	Pyridine+2% methoxyamine HCl, propionic Anhydride, MSTFA	LOD/LOQ: 50 ng/mL	[138]
		1 mL	LLE	2% Methoxyamine HCl; propionic anhydride	LOD: 10 ng/mL; LOQ: 25 ng/mL	[72]
		1 mL	Hydrolysis, LLE	MSTFA/ethanethiol/NH_4I (500:4:2)	LOD: 200 ng/mL	[139]
		2 mL	SPE	2%Mehtoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA+1% TMCS	-	[84]
		2 mL	Hydrolysis, SPE	Methoxylamine 10%; acetonitrile and acetic anhydride	LOD: 40 ng/mL; LOQ: 40 ng/mL	[36]
		2 mL	Hydrolysis; SPE	Acetate buffer/ 10% hydroxylamine; BSTFA	LOQ: 100 ng/mL	[70]
		2 mL	Hydrolysis; SPE	BSTFA+1% TMCS	LOD: 400 ng/mL; LOQ: 600 ng/mL	[140]
		2 mL	Hydrolysis, LLE	MSTFA+1% TMCS	LOD: 25 ng/mL	[141]
		3 mL	SPE	Methoxyamine HCl in pyridine; BSTFA+pyridine	LOQ: 150 ng/mL	[143]
		3 mL	Hydrolysis; SPE	10% hydroxylamine; BSTFA+1% TMCS	LOD: 25 ng/mL; LOQ: 50 ng/mL	[66]
		3 mL	Hydrolysis, LLE	MSTFA/NH_4I/DTE (1000:2:4, v/w/w)	LOD: 100 ng/mL	[142]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA	LOD: 20 ng/mL	[144]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH4I/ethanethiol (640:1:2, v/w/v)	LOD: 100 ng/mL	[145]
	Oral Fluid	-[4]	SPE	Methoxyamine 10% aq; BTSFA+1% TMCS	LOD: 3 ng/mL; LOQ: 2 ng/mg	[63]
		1 mL	SPE	1% hydroxylamine in pyridine; MSTFA+ 1% TMCS	LOQ: 10 ng/mL	[67]

	Vitreous Humor	1 mL	SPE	1% hydroxylamine; MSTFA+1% TMCS	LOD: 10 ng/mL; LOQ: 50 ng/mL	[68]
Oxymor-phone	Blood	-	SPE	BSTFA	LOD/LOQ: 20 ng/mL	[34]
		1 mL	PP (acetonitrile); LLE	2%methoxyamine in pyridine; propionic anhydride	LOD: 2 ng/mL; LOQ: 10 ng/mL	[73]
		1 mL	PP (acetonitrile); SPE	10% Hydroxylamine; BSTFA+1% TMCS	LOD:15 ng/mL; LOQ: 25 ng/mL	[38]
		2 mL	SPE	2% Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA+1% TMCS	-	[84]
		2 mL	SPE	Hydroxylamine 1% in pyridine; MSTFA+1% TMCS	-	[39]
		2 mL	PP (methanol); SPE	10% hydroxylamine; BSTFA+1% TMCS	LOD: 3.5 ng/mL	[65]
		2 mL	PP (acetonitrile); SPE	10% hydroxylamine; BSTFA+1% TMCS	LOQ: 50 ng/mL	[64]
		3 mL	LLE	2% methoxyamine in pyridine; MSTFA+1% TMCS and DMF	LOD: 0.5 ng/mL; LOQ: 1.0 ng/mL	[152]
	Urine	-	SPE	BSTFA	LOD/LOQ: 20 ng/mL	[34]
		1 mL	Liquid nitrogen	Pyridine+2% methoxyamine HCl, propionic Anhydride, MSTFA	LOD/LOQ: 30 ng/mL	[138]
		1 mL	LLE	2% Methoxyamine HCl; propionic anhydride	LOD: 10 ng/mL; LOQ: 25 ng/mL	[72]
		1 mL	PP (acetonitrile); SPE	10% Hydroxylamine; BSTFA+1%TMCS	LOD:15 ng/mL; LOQ: 25 ng/mL	[38]
		1 mL	Hydrolysis, LLE	MSTFA/ethanethiol/NH_4I (500:4:2)	LOD: 40 ng/mL	[139]
		2 mL	SPE	2% Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA+1%TMCS	-	[84]
		2 mL	Hydrolysis, SPE	Methoxylamine 10%; acetonitrile and acetic anhydride	LOD: 20 ng/mL; LOQ: 40 ng/mL	[36]
		2 mL	Hydrolysis; SPE	Acetate buffer/ 10% hydroxylamine; BSTFA	LOQ: 100 ng/mL	[70]
		2 mL	Hydrolysis; SPE	BSTFA+1%TMCS	LOD: 7000 ng/mL; LOQ: 12000 ng/mL	[140]
		2 mL	Hydrolysis, LLE	MSTFA+1%TMCS	LOD: 25 ng/mL	[141]
		3 mL	SPE	Methoxyamine HCl in pyridine; BSTFA+pyridine	LOQ: 150 ng/mL	[143]
		3 mL	Hydrolysis; SPE	10% hydroxylamine; BSTFA+1%TMCS	LOD: 25 ng/mL; LOQ: 50 ng/mL	[66]
		3 mL	Hydrolysis, LLE	MSTFA/NH_4I/DTE (1000:2:4, v/w/w)	LOD: 100 ng/mL	[142]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA	LOD: 200 ng/mL	[144]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH_4I/ethanethiol (640:1:2, v/w/v)	LOD: 100 ng/mL	[145]
Fentanyl	Blood	1 mL	SPE	MSTFA	LOD/LOQ: 0.10 ng/mL	[153]
		2 mL	LLE/ SPE	-	LOD: 50/50 ng/mL	[148]
	Urine	2 mL	Hydrolysis; SPE	BSTFA+1%TMCS	LOD: 200 ng/mL; LOQ: 300 ng/mL	[140]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA	LOD: 20 ng/mL	[144]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH_4I/ethanethiol (640:1:2, v/w/v)	LOD: 100 ng/mL	[145]

*GC-MS/MS; PP (protein precipitation), LLE (liquid-liquid extraction), TCA (trichloroacetic acid), HCL (hydrochloric acid)

2.3. Aims of this Study

According to the existing information about the consumption of the studied substances, the lack of studies carried out for its simultaneous detection and quantification by GC-MS and the greater analysis difficulty with oxycodone and oxymorphone (detection and identification) it has become important the development of an efficient analytical method for the determination of these substances in routine analyses of the SQTF-C. In this way, two objectives were outlined: the first one was the optimization of the procedure in order to obtain a faster, selective and specific method; and the second was the validation of the analytical methodology for the qualitative and quantitative determination of morphine, codeine, 6-acetylmorphine, 6-acetylcodeine, oxycodone, oxymorphone and fentanyl in BL and PF, able to be used in the routine analyses of the SQTF-C of the INMLCF.

Chapter 3. Results and Discussion

3.1. Optimization

This work started with the derivatization with MSTFA+5% TMCS of each analyte during 90 seconds under microwave irradiation (method already implemented in the SQTF-C for the determination of morphine, codeine and 6-acetylmorphine) followed by GC-MS analysis in SCAN mode in order to identify the retention times of each analyte and the most characterizing ions. Initially, the method started at 90 °C with a ramp of 20 °C/min up to 300 °C (Annex 4). After the selection of the qualitative and quantitative ions, based on the results obtained and in agreement with previous studies, the substances were injected and analysed in SIM mode. The selected ions were as follows:

- 371,178 and 196 for codeine;[145]
- 429, 236 and 414 for morphine;[144,145]
- 399,340 and 287 for 6-acetylmorphine;[65,139,141,143]
- 341, 229 and 282 for 6-acetylcodeine;[33,154]
- 245,189 and 146 for fentanyl[139,144,153].

However, oxycodone and oxymorphone exhibited two relatively abundant chromatographic peaks (figure 25). It was concluded after analysis and comparison of the mass spectra with the data available in the literature regarding the possible fragmentations, that the first chromatographic peak corresponded to bis-TMS and tris-TMS for oxycodone and oxymorphone, respectively and the second peak corresponded to oxycodone-TMS and oxymorphone-bis-TMS. Therefore, the following ions were considered: 459[139,141,142,145,151], 444[141,142,145,151], 312[139,141,151], 368[139,151] for the first peak (12.77 min) and 387[140,143,144,151], 372[143,144,151], 229 for the second peak (13.15 min) for oxycodone; in the case of oxymorphone, 517,502,355,412[145] were considered for the first peak (12.94 min) and 445, 430, 287[144] for the second peak (13.28 min). Most of the previously reported studies did not consider the existence of two chromatographic peaks and their decision criteria were not disclosed. Thus, we considered the chromatographic peak corresponding to the ions of higher molecular mass, i.e., the two first chromatographic peaks. Once the mixture of all the studied substances was analysed in SIM mode with the method already described (90 °C) and with another method based on Lerch *et al.*[120], that started at 140 °C (Annex 4). After the retention times and the selected ions have been defined and stabilized, the solid phase extraction was optimized by testing with different reagents and two different SPE columns (MCX® and MCX® Prime from Waters Corp.).

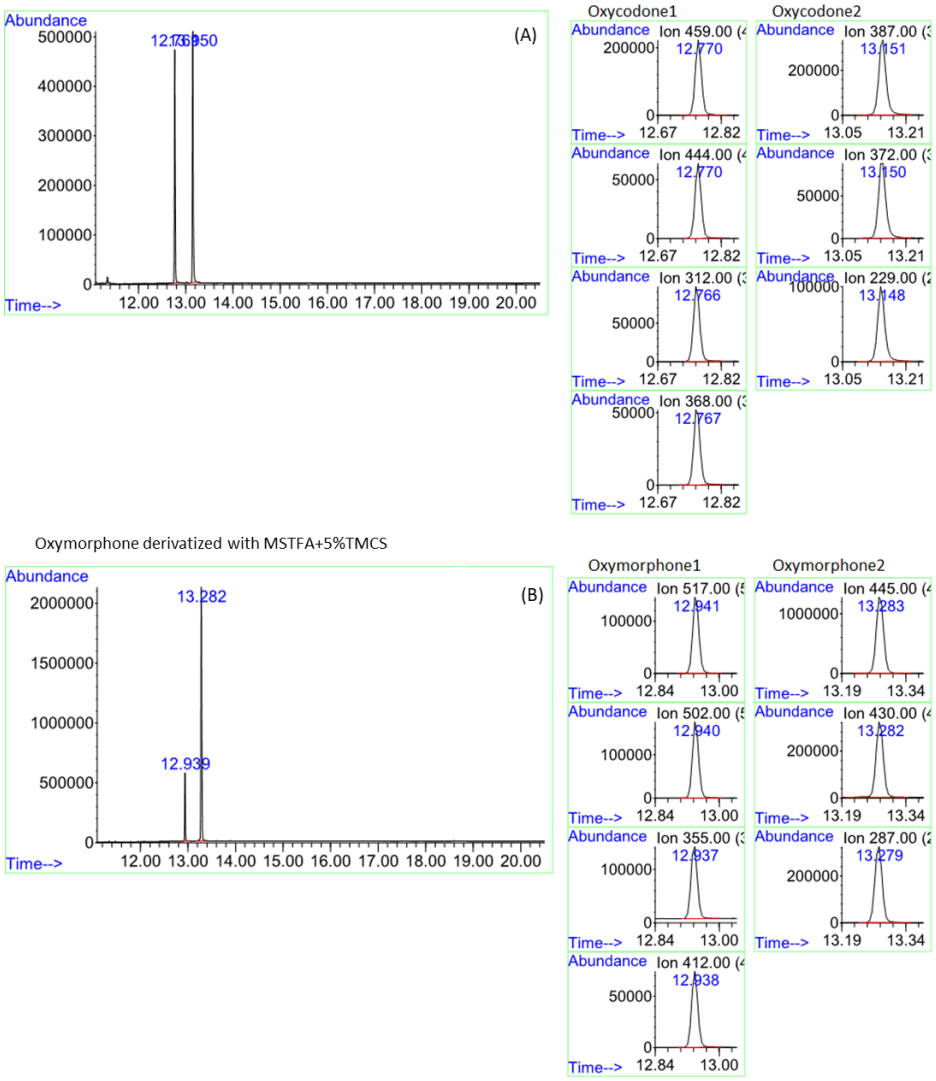

Figure 25. Ion chromatograms of standard solutions (2 µg/mL) of oxycodone (A) and oxymorphone (B) derivatized with MSTFA+5% TMCS (microwaves 90 s at 900 W) by GC-MS method with initial oven temperature at 90 °C.

3.1.1. Extraction Reagents and Procedure

Initially, we compared the extraction A method (Annex 5), already implemented in the SQTF-C, with two similar methods: substitution of the carbonate buffer 0.15 M by 2% formic acid (B) (Annex 6) and substitution of dichloromethane:2-propanol:ammonia by 5% ammonium hydroxide in Methanol (C) (Annex 7). These two reagents were indicated by the SPE columns company (Waters Corp.). Then the substances were analysed with both methods (90 °C and 140 °C). Method C was discarded because undissolved suspended particles were formed (figure 26). Comparing all the tested methods, it was concluded that the method used in the SQTF-C (A) was more efficient than method (B) and the chromatographic method at 140 °C was more efficient than 90 °C (Annex 4 and figure 27).

Figure 26. Residue after dryness under nitrogen stream at 40 °C (Extraction C - Annex 7).

Then, another method used in the SQTF-C for another group of drugs (D) (Annex 8) was also analysed and modified with the same reagents already described (method E-substitution of 1 N hydrochloric acid with 2% formic acid (Annex 9) and method F- substitution of dichloromethane:2-propanol:ammonia by 5% ammonia hydroxide in methanol (Annex 10)). It was observed that method E was more effective than the method D (figure 28). Once again, the method that uses 5% ammonia hydroxide/MeOH was discarded because undissolved suspended particles were formed (figure 29).

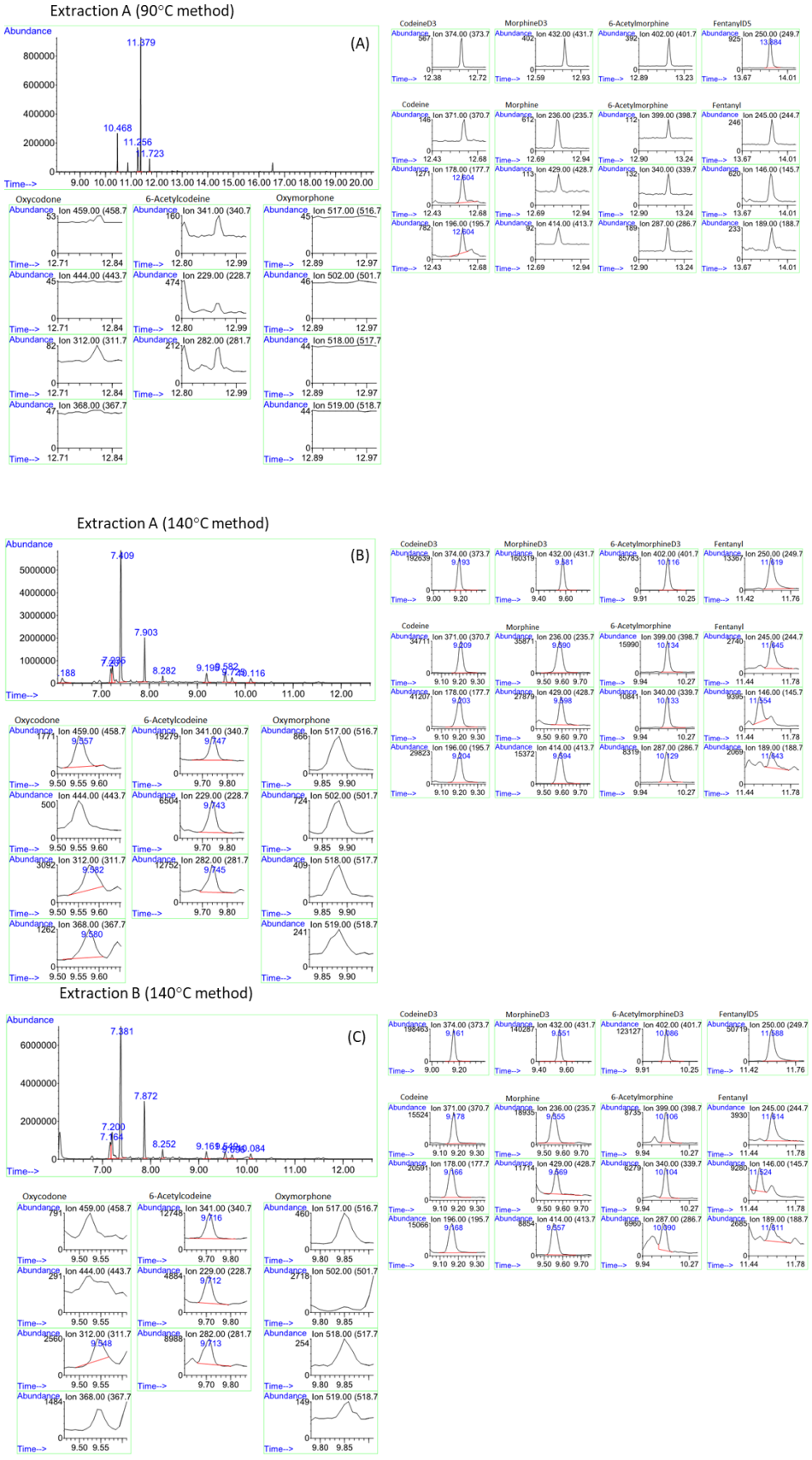

Figure 27. Ion chromatograms of extracted samples at 50 ng/mL by extraction A methodology (A), (B) (Annex 5), with two GC-MS methods: initial oven temperature at 90 °C (A) and 140 °C (B); Extraction B methodology (Annex 6) with initial oven temperature at 140 °C (C).

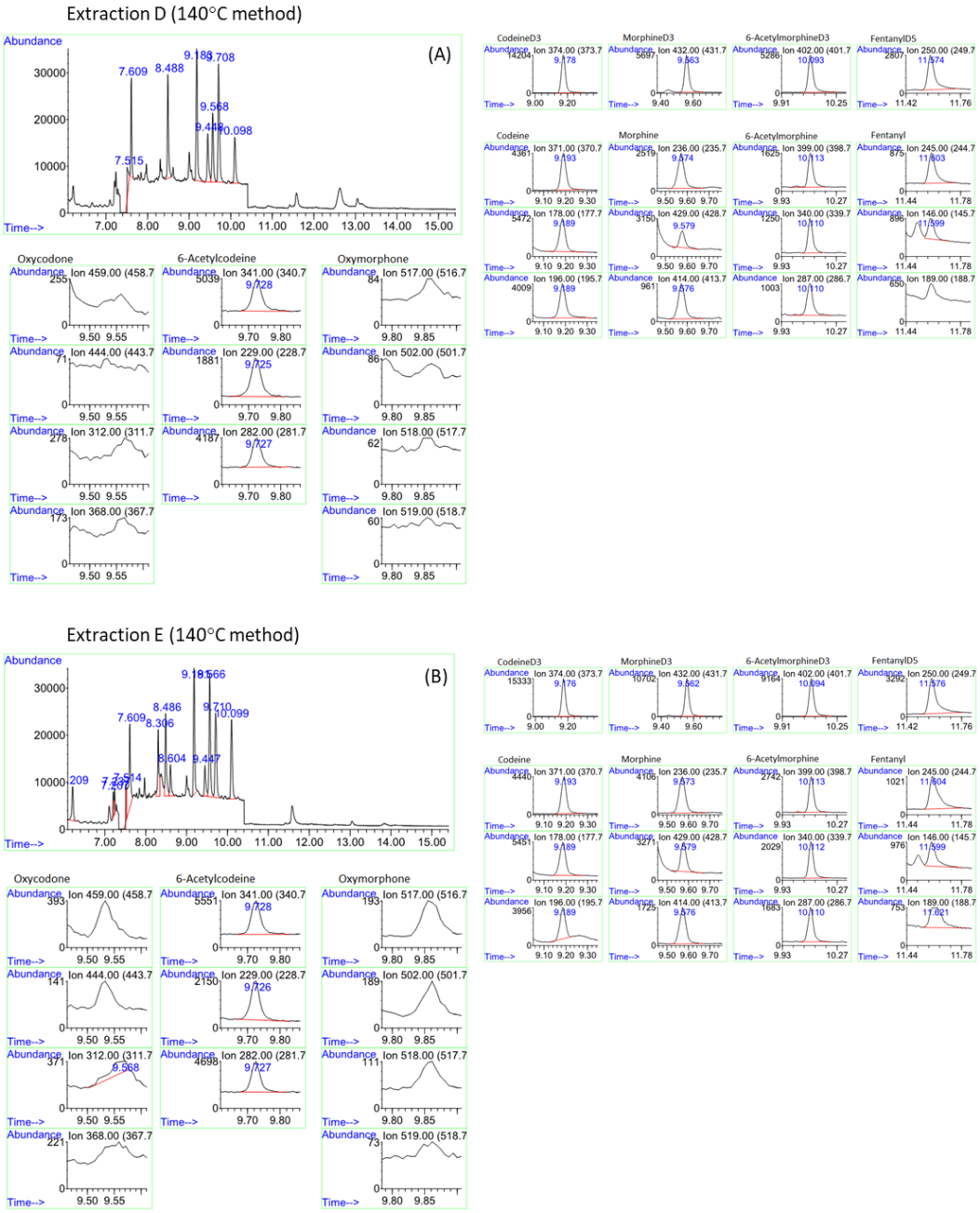

Figure 28. Ion chromatograms of extracted samples at 25 ng/mL by Extraction D methodology (A) (Annex 8) with 140 °C GC-MS method (Annex 4) and extraction E methodology (Annex 9) with initial oven temperature at 140 °C (B).

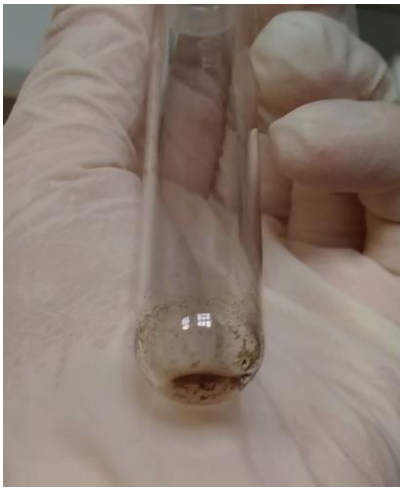

Figure 29. Undissolved suspended formed after dryness under nitrogen stream at 40 °C - Extraction F (Annex 10) and addition of MSTFA+5% TMCS.

3.1.2. Protein Precipitation and Oximes Derivatives

After the first extraction it was found that it would not be feasible to maintain the sample pre-treatment used in the SQTF-C for opiates determination, considering that oxycodone and oxymorphone were not efficiently detected at 50 ng/mL (figure 27) and 25 ng/mL (figure 28). The hydroxylamine step was implemented, to form oxime derivatives and therefore avoid the formation of multiple derivatives due to the existing ketonic group. The ions previously chosen would have to be changed since oxycodone and oxymorphone undergo a structural change with the addition of the oxime group in their structure as mentioned. Thus, again the mass spectrum was analysed in considering the molecular structure and the corresponding fragmentations supported by studies previously published, the following ions were selected: 474[39,65,66,69], 475[66], 459[39,65,69] for oxycodone and 532, 533, 517[39,66] for oxymorphone (Annex 11).

Proteins precipitation with ice-cold acetonitrile was also included in order to increase the extractive efficiency and to make feasible the implementation of the second step, the derivatization with hydroxylamine prior SPE. The method was based on the procedure of Knittel *et al.*[68]. However, we made some changes such as the use of a solution of 1% aqueous hydroxylamine in PBS (1:2, v/v), instead of the use of each one in separate, and microwave assisted derivatization (30 seconds, at 50% power, 900 W) instead of the traditional method (by heating for 1 h at 65 °C using a thermal block), with and without the addition of the buffer solution at the end of the procedure.

Since oxycodone and oxymorphone were the only analytes (from our group of substances) to undergo changes with the addition of hydroxylamine, an initial test was performed. The 1%

aqueous hydroxylamine was added to the standards of these substances (1 h in thermal block at 65 °C), evaporated to dryness and then derivatized with a solution of MSTFA+5% TMCS to be injected in SCAN mode. The retention times and characteristic ions of the substances were verified. A substantial increase in abundance was observed, such as the appearance of a single abundant peak (figure 30).

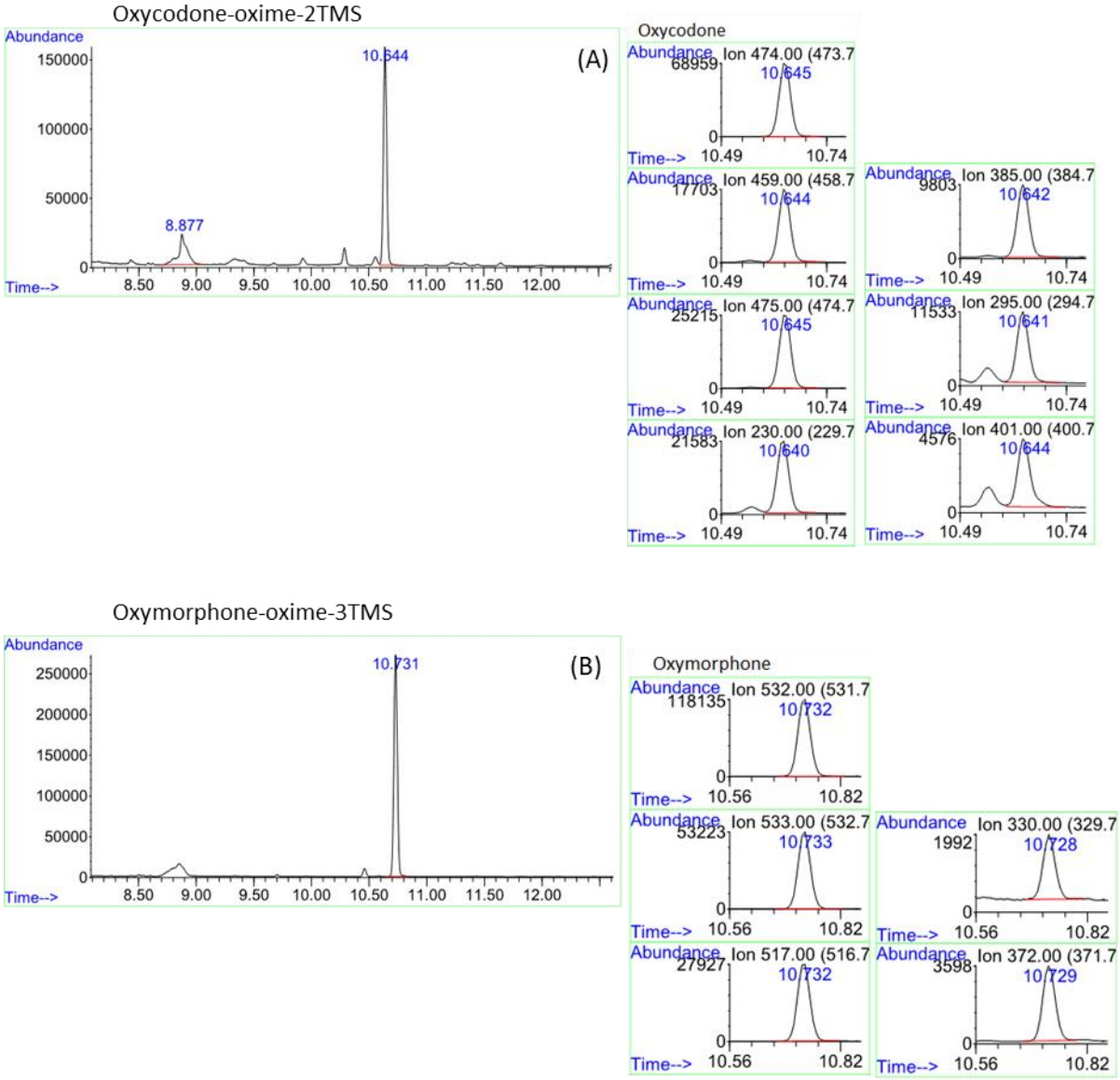

Figure 30. Ion chromatograms of standard solutions (100 ng/mL) of oxycodone (A) and oxymorphone (B) derivatized with 1% of aqueous hydroxylamine (1 h bath at 65 °C) and MSTFA+5% TMCS (microwaves 90 s at 900 W). GC-MS method with initial oven temperature at 140 °C.

Since microwave assisted derivatization with hydroxylamine was not published until now we began to study and compare the use of microwave irradiation (900 W) for 30 s with 80% potency with the thermal block (implemented in the procedure of Knittel *et al.*[68]) for 1 h at 65 °C using the extraction method E (Annex 12, 13 14 and 15). It was compared the use of a single solution (mixing

the 1% aqueous hydroxylamine with PBS (1:2, v/v)) with the use of them separately according to Knittel *et al.*[68]. We concluded that the use of a single solution was more advantageous when comparing with the addition of the separated solutions, however there were no significant differences between conventional heating and derivatization induced by microwave (figure 31 and 32).

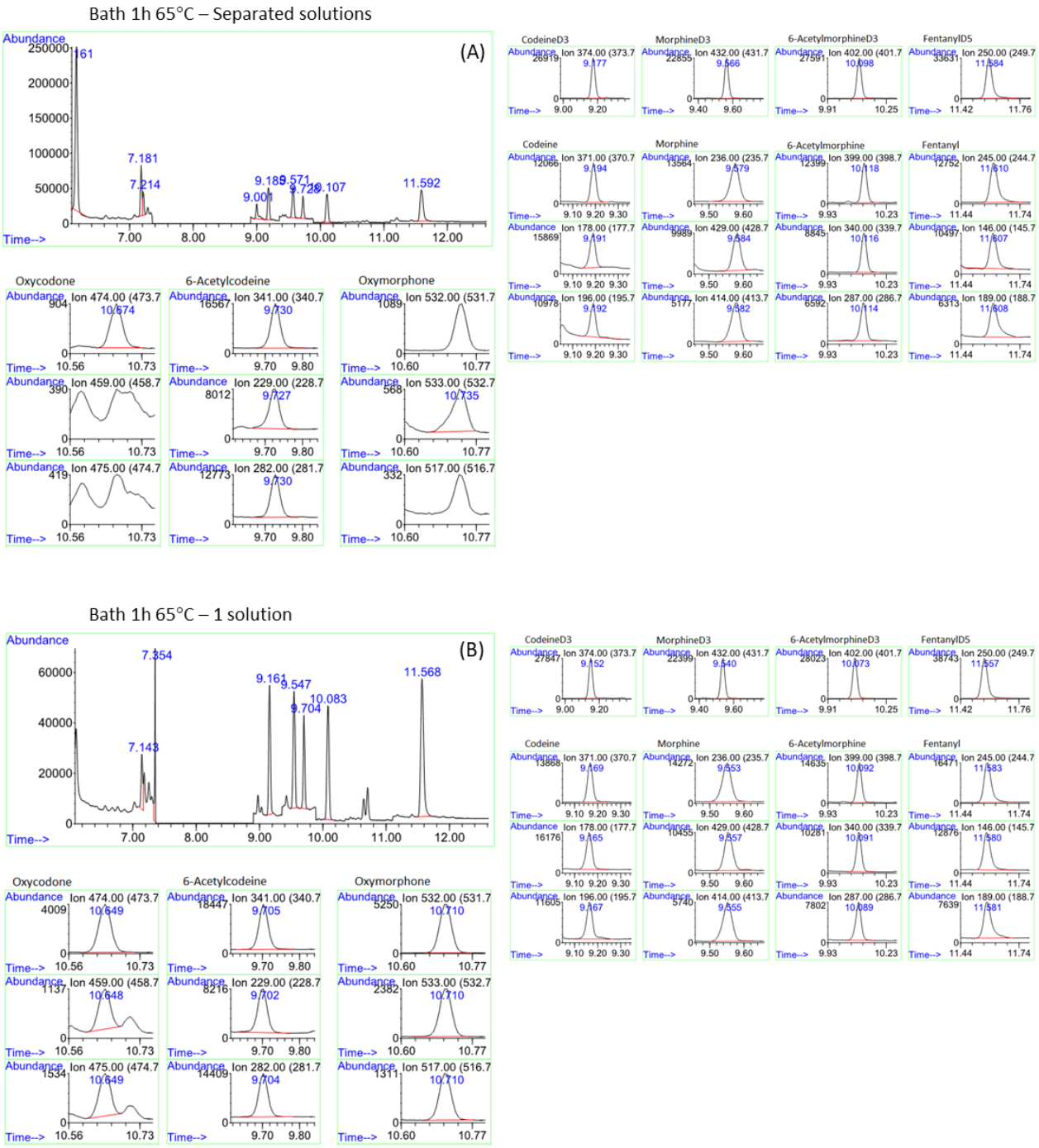

Figure 31. Ion chromatograms of extracted samples at 25 ng/mL (method E) subject to conventional heating during 1 h at 65 °C (A) and (B). Separate addition of 1% hydroxylamine and PBS (A) (Annex 12) and one solution of 1% aqueous hydroxylamine in PBS (1:2, v/v) was used (B) (Annex 13). GC-MS method with initial oven temperature at 140 °C.

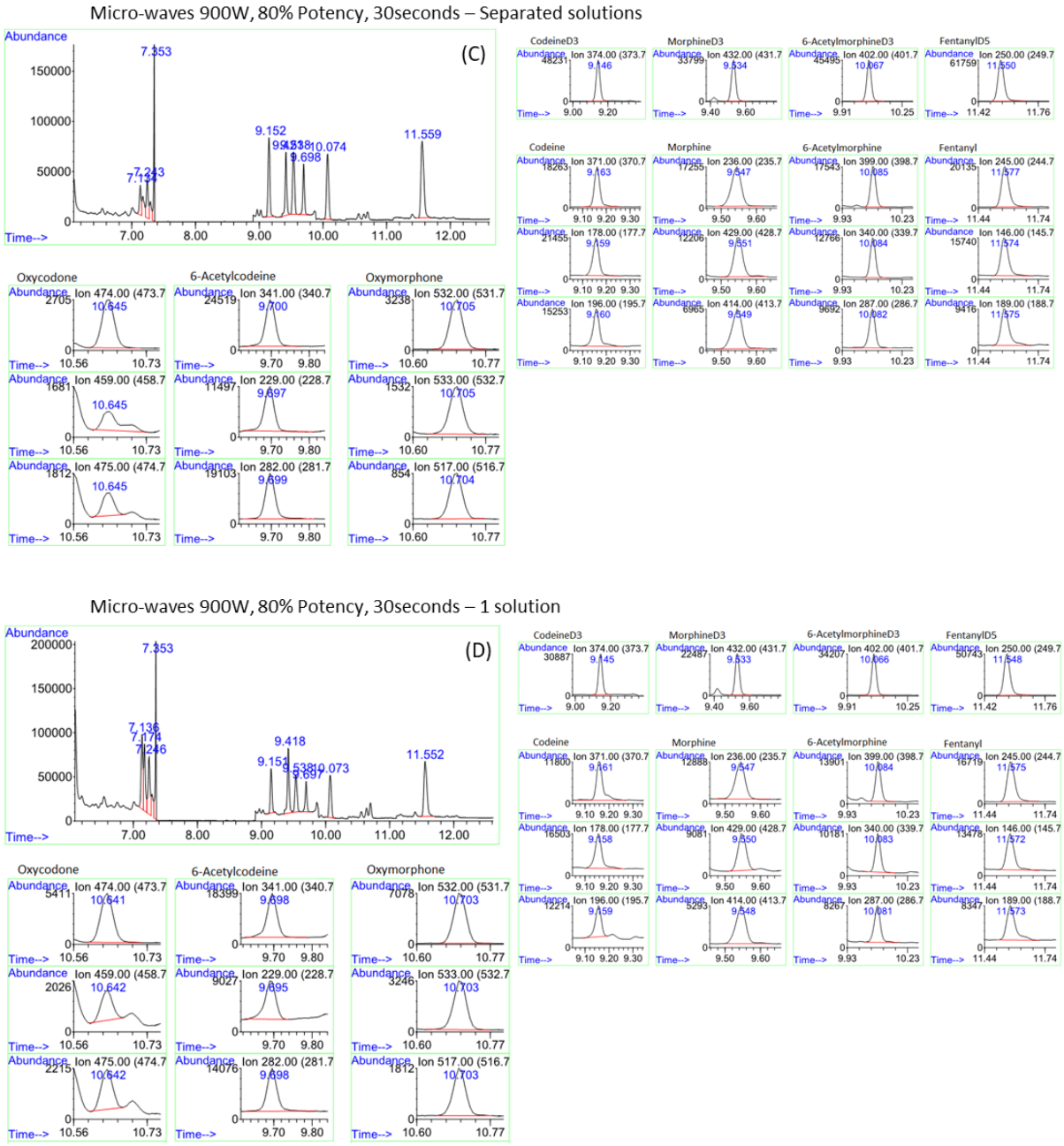

Figure 32. Ion chromatograms of extracted samples (method E) (25 ng/mL) subject by microwave irradiation (900 W) 30 s at 80% potency (C) and (D). Separate addition of 1% hydroxylamine and PBS (C) (Annex 14) and one solution of 1% aqueous hydroxylamine in PBS (1:2, v/v) was used (D) (Annex 15) GC-MS method with initial oven temperature at 140 °C.

With the implementation of these two new steps, a substantial increase of the chromatographic resolution was observed, making it possible to carry out the analyse at 25 ng/mL (figure 31 and 32).

With respect to the chromatographic method, as the analytes were dissolved in MSTFA+5% TMCS and its boiling point is 70 °C for MSTFA [155] and 57 °C for TMCS [156] a new method was tested with a lower initial oven temperature at 50 °C with a ramp of 20 °C/min and a flow rate of 1 mL/min.

A lower microwave potency at 50% potency was also experimented, due to pressure build-up of in the reaction vessel at 80% potency (the slight lifting of the tube caps was observed) (figure 33 and 34).

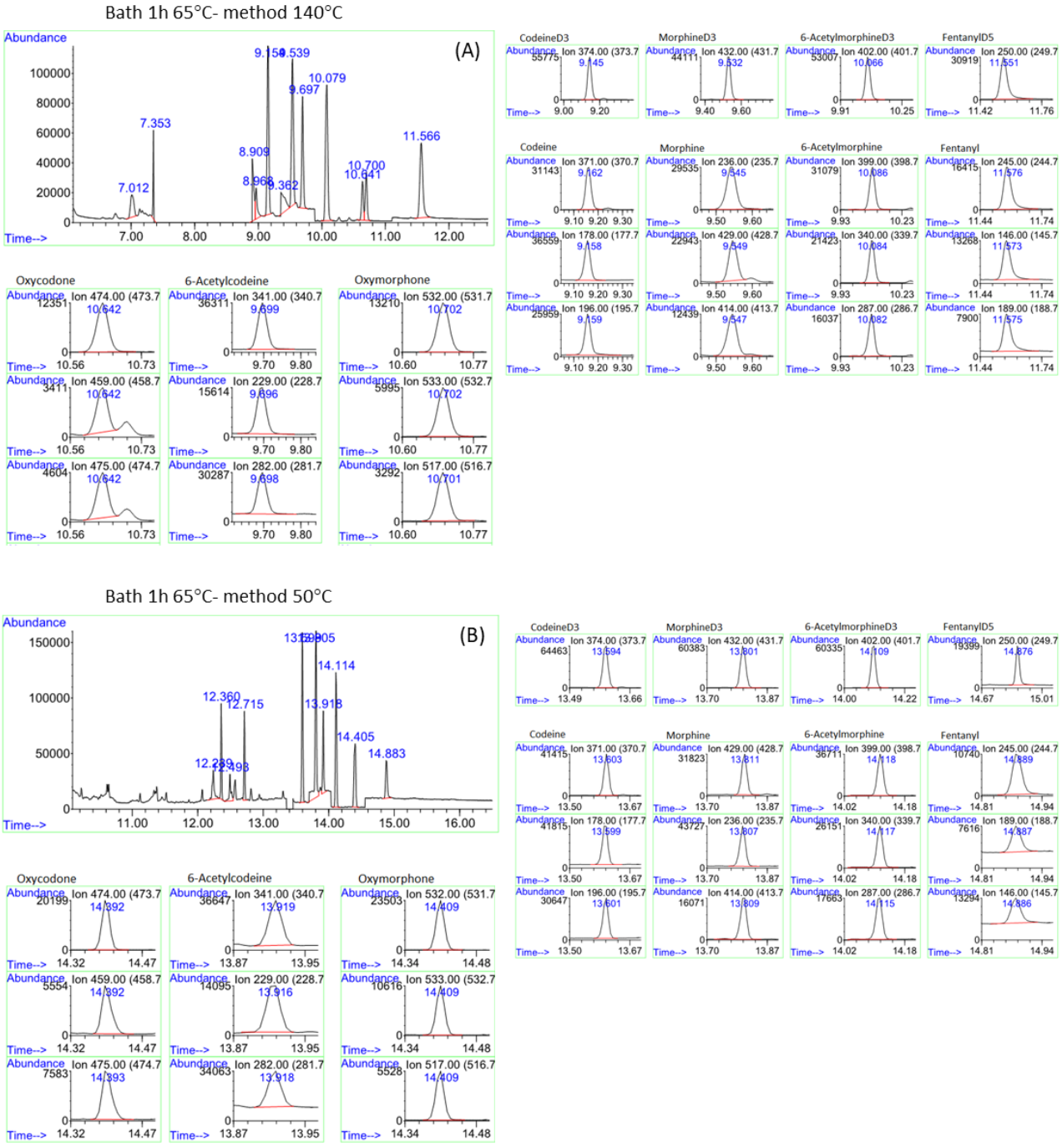

Figure 33. Ion chromatograms of standard mixture (25 ng/mL), using a mixture solution of 1% aqueous hydroxylamine in PBS (1:2, v/v) and subjected to thermal block during 1 h at 65 °C (A) and (B). GC-MS oven program initiated at 140 °C (A) and with initial oven temperature at 50 °C (B) (Annex 4).

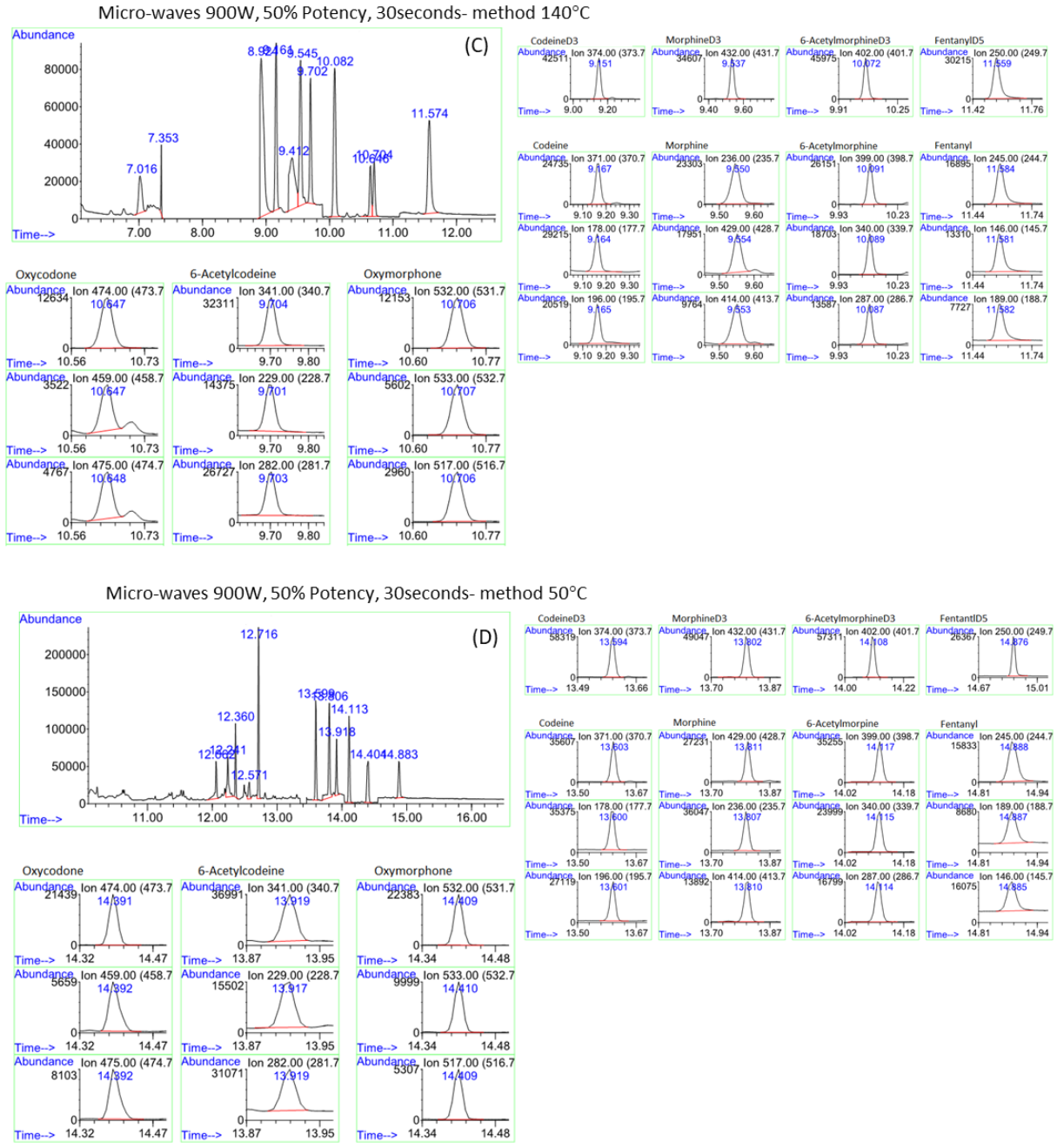

Figure 34. Ion chromatograms of standard mixture (25 ng/mL), using a mixture solution of 1% aqueous hydroxylamine in PBS (1:2, v/v) and subject to microwave irradiation (900 W) for 30 s at 50% potency (C), (D). GC-MS method with initial oven temperature at 140 °C (C) and initial oven temperature at 50 °C (D) (Annex 4).

The two most advantageous extractive methods (A and E) were compared (Annex 16, 17 and 18) with the implementation of the initial oven temperature at 50 °C (figure 35,36 and 37). It was concluded that the method at 50 °C had a better chromatographic resolution.

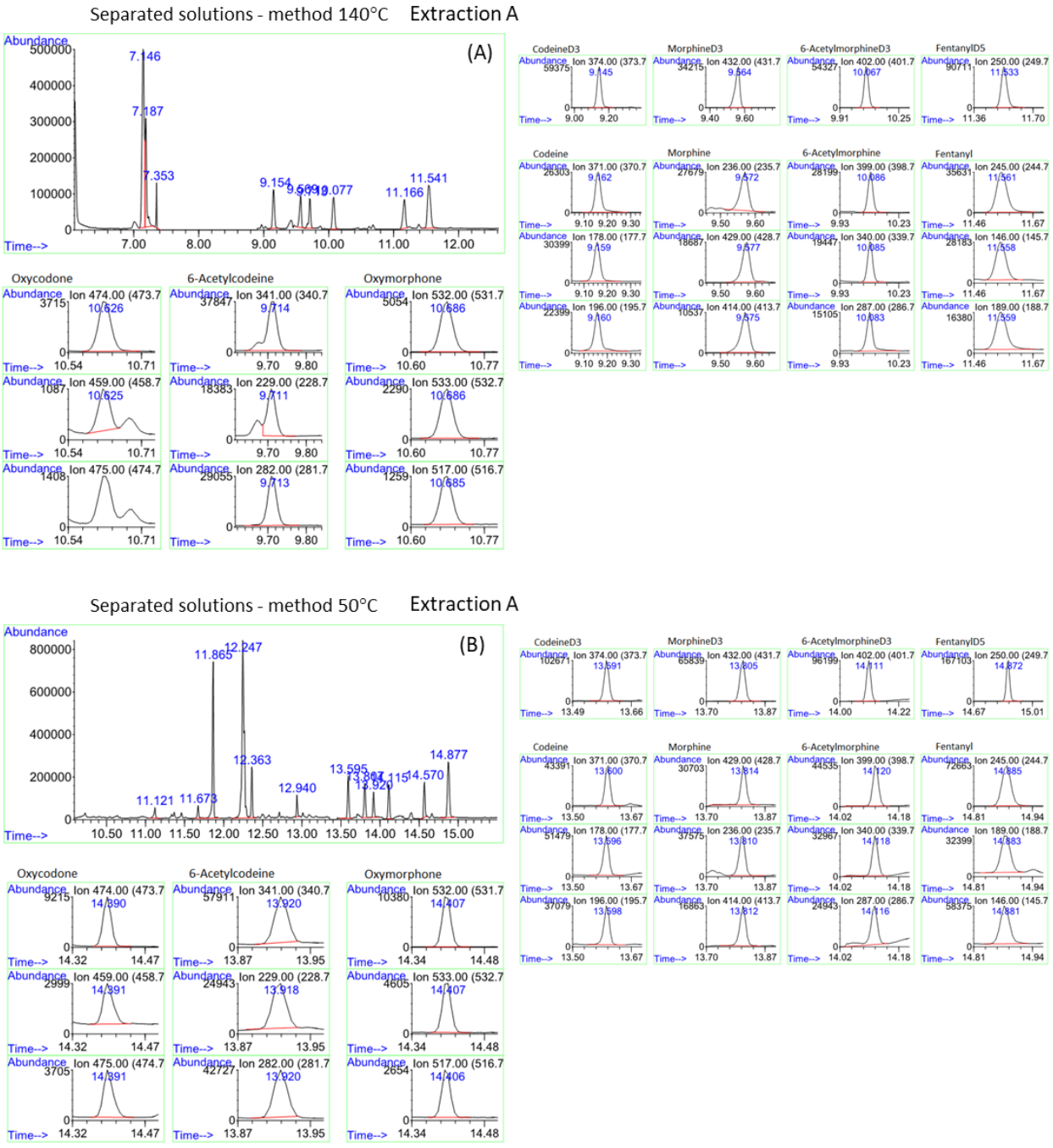

Figure 35. Ion chromatograms of extracted samples at 25 ng/mL (method A) subject to microwave irradiation (900 W) for 30 s at 50% potency and separate addition of 1% hydroxylamine and PBS (A,B) (Annex 16) GC-MS method with initial oven temperature at 140 °C (A) and initial oven temperature at 50 °C (B) (Annex 4).

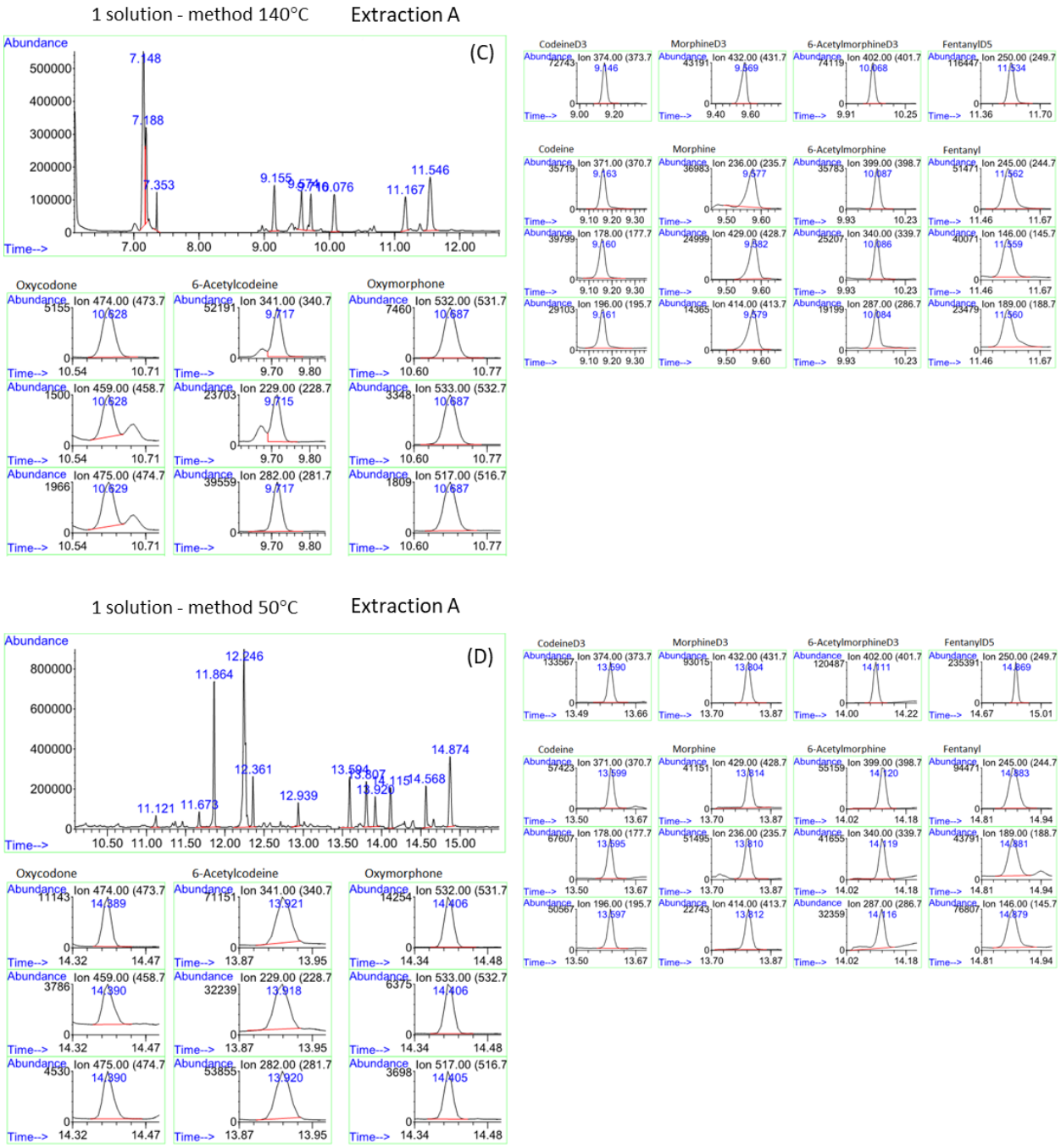

Figure 36. Ion chromatograms of extracted samples at 25 ng/mL (method A) using a mixture solution of 1% aqueous hydroxylamine in PBS (1:2, v/v) and subject to microwave irradiation (900 W) for 30 s at 50% potency (Annex 17). GC-MS method with initial oven temperature at 140 °C (C) and with initial oven temperature at 50 °C (D) (Annex 4).

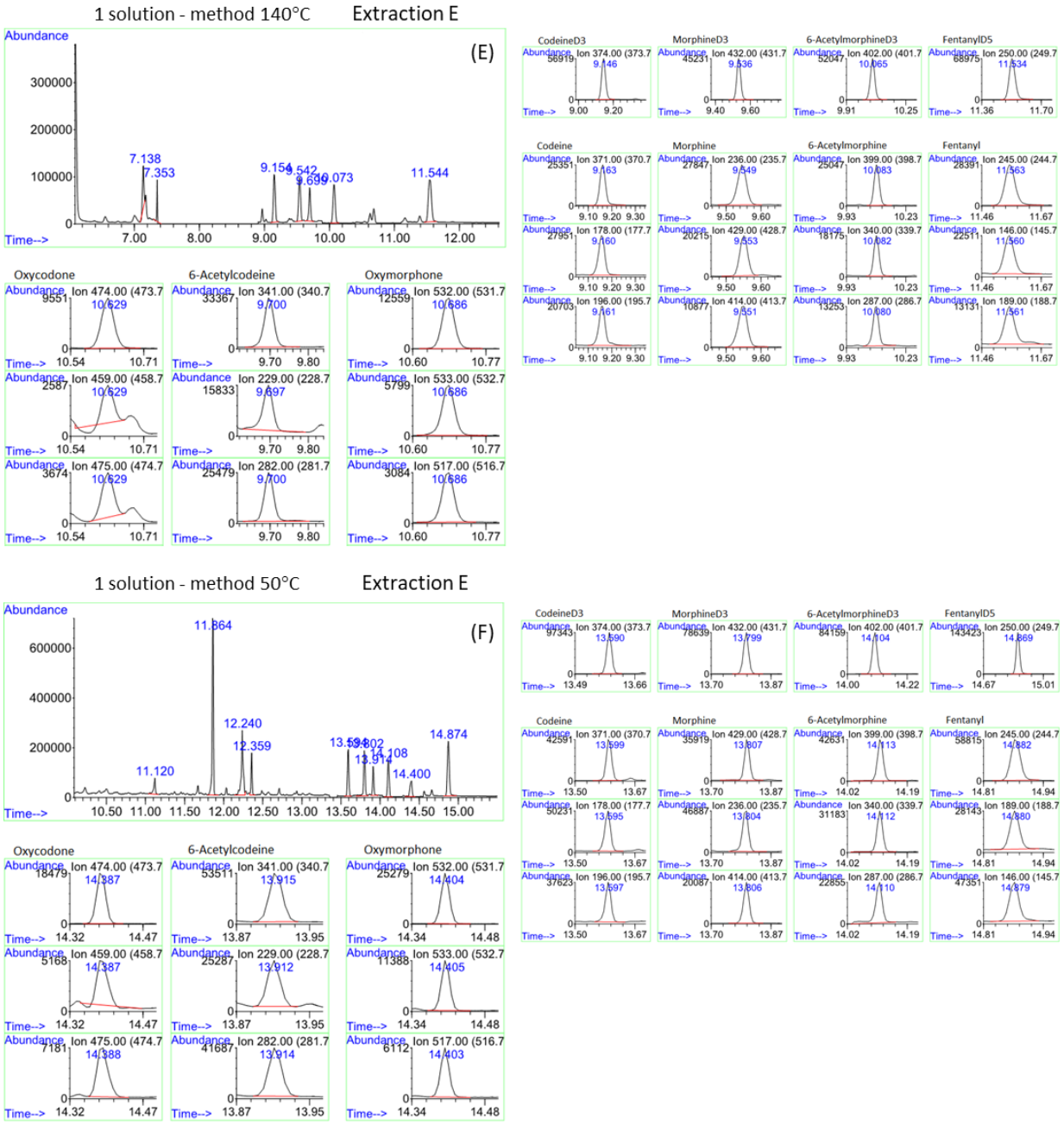

Figure 37. Ion chromatograms of extracted samples 25 ng/mL (method E) using one mixture solution of 1% aqueous hydroxylamine in PBS (1:2, v/v) and subject to microwave irradiation (900 W) for 30 s at 50% potency (Annex 18). GC-MS program with initial oven temperature at 140 °C (C) and with initial oven temperature at 50 °C (D) (Annex 4).

We also tried to use other extractive columns, the Prime MCX® (Waters Corp.) The same procedure was used and compared with method A (Annex 17). We concluded that there was no benefit in the use of these columns (figure 38).

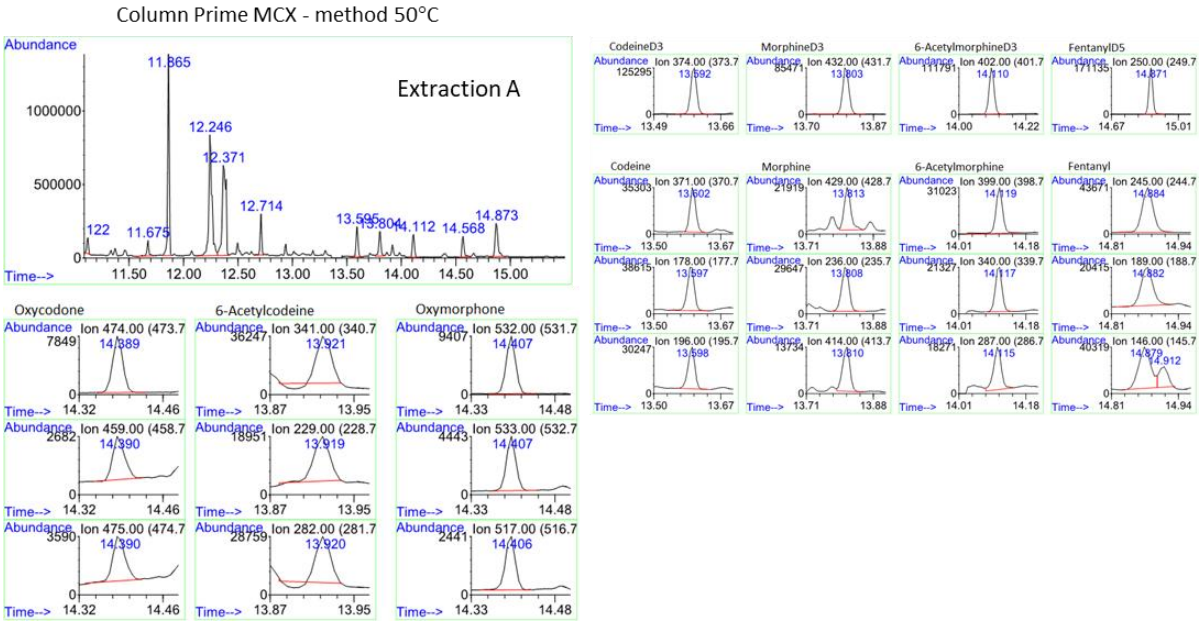

Figure 38. Ion chromatograms of extracted samples at 25 ng/mL (method A) using a mixture solution of 1% aqueous hydroxylamine in PBS (1:2, v/v) and subject to microwave irradiation (900 W) for 30 s at 50% potency (Annex 17). GC-MS method with initial oven temperature at 50 °C (D) (Annex 4).

To summarize, seven extractive methodologies were compared, six differing in the solvents used and one employing a different SPE column. Analysing the chromatograms, it was concluded that the extractive methods (A) and (E) were the most advantageous, demonstrating a better chromatographic resolution (better defined peaks without interferences and higher ion abundances). As extraction (A) was similar to that used in the SQTF-C for the opiated extraction and there were no significant differences between them (A and E) it was decided to adopt the (A) extraction.

Finally, in order to increase the derivatization efficiency under microwave irradiation, the use of 100 seconds (Annex 19) instead of 90 seconds (Annex 17) with MSTFA+5% TMCS was tested (figure 39). It was also compared the use of a fresh mixture of 1% aqueous hydroxylamine in PBS (1:2, v/v) (Annex 19) with the preparation of a solution by dissolving the powder of hydroxylamine hydrochloride 99% (purity) in PBS (Annex 20, figure 40). Considering the final derivatization with the solution of MSTFA+5% TMCS, the use of the microwave irradiation during 100 seconds (100% power, 900 W), demonstrated an improvement for oxycodone and oxymorphone. Relative to the use of the solution of hydroxylamine hydrochloride 99% in PBS did not demonstrate advantages.

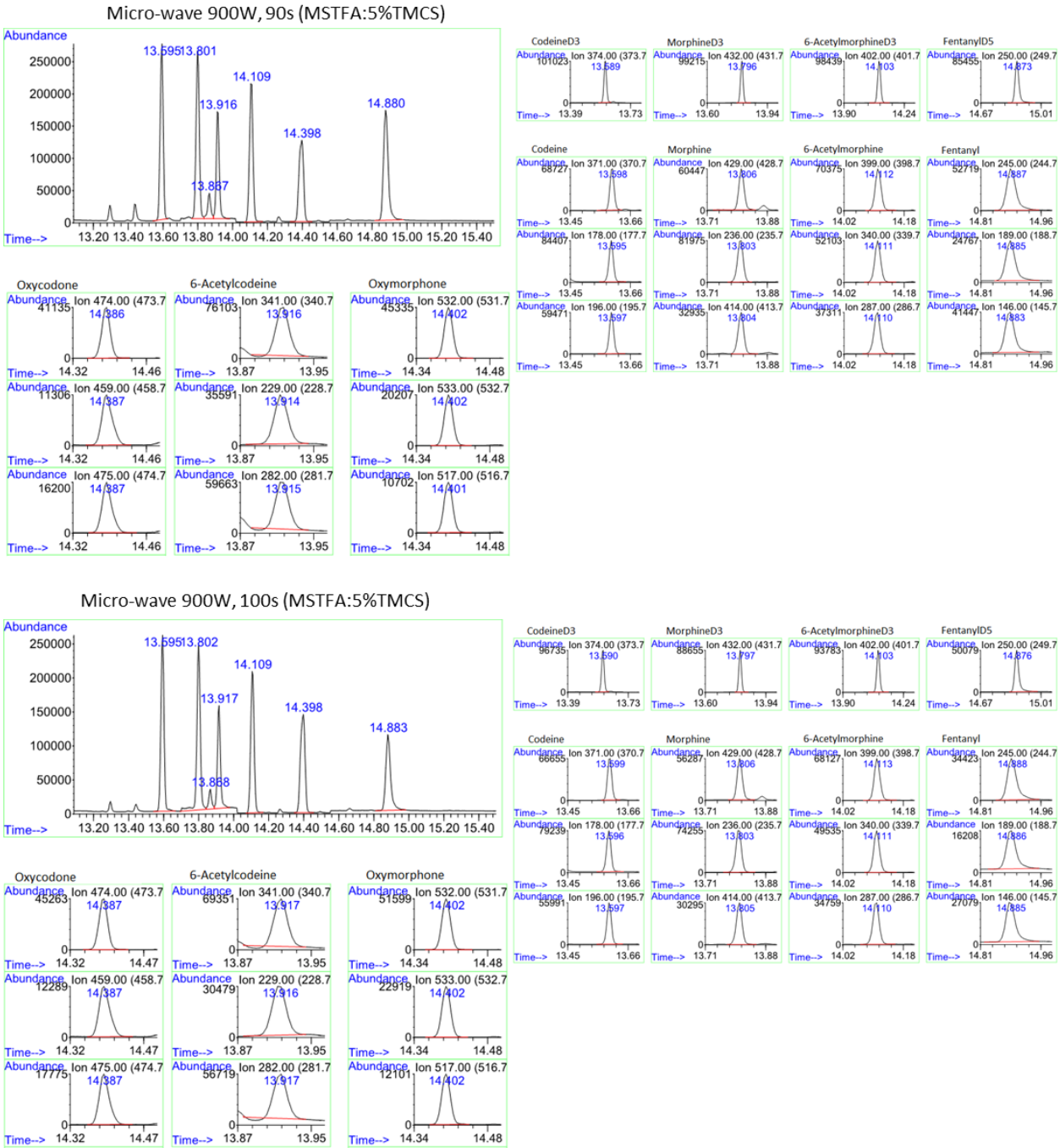

Figure 39. Ion chromatograms of extracted samples at 25 ng/mL (method A) using a mixture solution of 1% aqueous hydroxylamine in PBS (1:2, v/v) and subject to microwave irradiation (900 W) for 30 s at 50% potency (Procedure 12 and 14). GC-MS method with initial oven temperature at 50 °C (D).

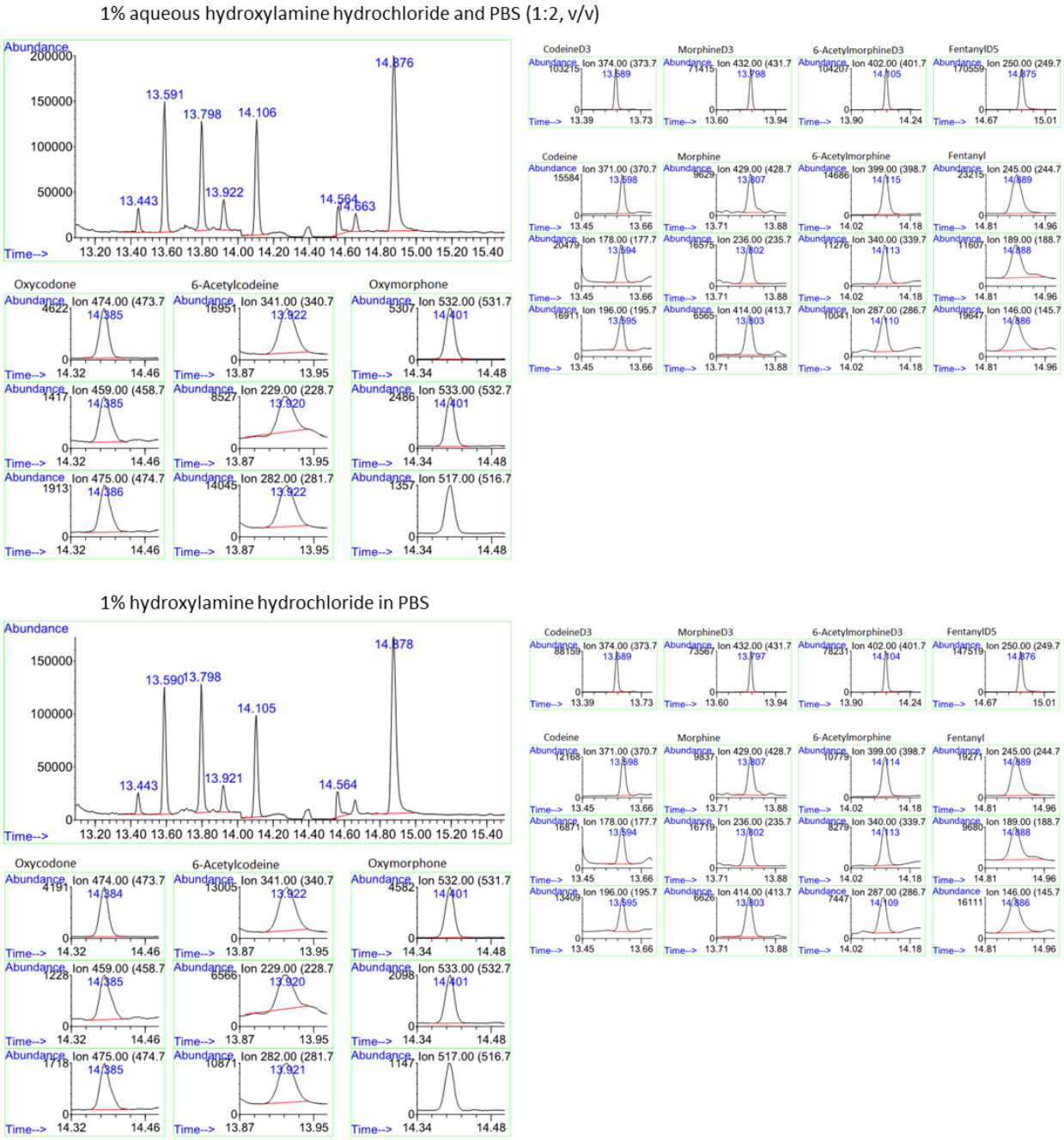

Figure 40. Ion chromatograms of extracted samples at 20 ng/mL (method A) using a fresh mixture solution of 1% aqueous hydroxylamine in PBS (1:2, v/v) (A) and one direct mixture of hydroxylamine hydrochloride in PBS (B) (Annex 19 and 20, respectively). GC-MS method with initial oven temperature at 50 °C.

3.2. Method Validation

Paper I-

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, morphined3, 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 PBS (1:2, v/v). Finally, the dried extracts were further derivatized with a solution of MSTFA+5% TMCS 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 5-1000 ng/mL with determination coefficients (r^2) higher than 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.

Keywords: Opioids, Microwave-induced oxime derivatization, Gas Chromatography–Mass Spectrometry

1. Introduction

Opioids are classified as natural (e.g. morphine, codeine), semi-synthetic (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 used also as recreational drugs [4].

Overdoses deaths are caused manly to induced coma, respiratory depression, which will trigger apnea and consequently death [5,7,11]. The effects of this substances begin to appear between less than 25 min to 1 hour 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 fatal overdoses 76%, 84% and 42% at worldwide, European and Portugal level, respectively [13–15]. Seizure of opioids other than heroin has increased between 2016-2017, 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 analogues 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 identifies and quantifies these substances in several biological matrices. To perform the analysis, blood (BL) is one of the most important matrices but not always feasible to be collected (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 5-35 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 blood and recommend its inclusion to the routine autopsy specimens [19,20,23].

Regarding 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].

The use of gas chromatography–mass spectrometry (GC-MS) to analyze opioids has several advantages. However, it requires a derivatization step to improve opioids detectability and stability [25,26]. When opioids are derivatized, silylation or fluoroacetylation are the preferred methods, although oxycodone and oxymorphone, being keto-opioids, may originate multiple derivatives (via tautomerization) as their carbonyl group may be in an enol or keto form [25,27– 31]. One way to avoid this multiple derivatization of these compounds is to carry out the reaction with hydroxylamine to form corresponding oximes prior to other derivatizations namely the addition of trimethylsilyl (TMS) or propionyl groups [28,30,32–38]. There are also studies that used methoxylamine [27,31,39–44] as derivatization reagent.

The use of microwave-assisted derivatization for the determination of drugs of abuse by GC–MS has already been published [26,45–50]. However, we didn't find any study, regarding the studied substances, that use the microwave-induced oxime derivatizations. Thus, we present a fast microwave-assisted derivatization procedure using hydroxylamine (30 seconds) instead of the reported 15 min [31,33,35,36,39,42], 20 min [44], 30 min [32,41,43] 1 h [27,28,30,34,38,40], or 2 h [37] procedures. A final fast microwave-assisted derivatization using MSTFA (n-methyl-n(trimethylsilyl) trifluoroacetamide)+ 5% TMCS (trimethylchlorosilane), requiring only 100 seconds instead of the 15-20 min [28,30,32,35–38,42,44], 30 min [27,33,41] or 1 h [34] spent in other known studies, is also described.

The aim of this work was the optimization and validation of an analytical methodology for qualification and quantification of codeine, morphine, 6-MAM, 6-acetylcodeine (6-ACCOD), oxycodone, oxymorphone and fentanyl using only 250 µL of BL and PF. Despite the need for a two derivatization steps, the implementation of microwave-assisted derivatization reduced enormously the time spent for the analysis. To the best of our knowledge, this is the first validated methodology that permits the simultaneous determination of these analytes in 250 µL of BL and PF by GC-MS using two step microwave-assisted derivatization.

2. Materials and methods

2.1. 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-d3, 6-MAM-d3, fentanyl-d5 and morphine-d3 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), dichloromethane, 2-propanol, ammonium, n-hexane, TMCS (trimethylchlorosilane) and hydroxylamine hydrochloride 99% were acquired from Merck Co. (Darmstadt, Germany), MSTFA (n-methyl-n-(trimethylsilyl) trifluoroacetamide) from Macherey–Nagel GmbH & Co. (Düren, Germany), phosphate-buffered saline (PBS, pH=7) and purified water from VWR (Radnor, Pennsylvania, EUA). Oasis® MCX extraction cartridges (3 mL, 60 mg) were purchased from Waters Corp. (Milford, MA, USA).

2.2. Biological samples

Blank BL samples were acquired from a local BL bank and were 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.

2.3. Instrumentation

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, Waldbronn, Germany).

2.4. Preparation of standard solutions, calibrators and controls

Stock solutions of each substance (50 µg/mL) were prepared in methanol with exception of 6ACCOD which was in acetonitrile. Working solutions of quality control (QC) and calibrators at 5 µg/mL were prepared with methanol and then by proper dilution were prepared at 0.5 µg/mL and 0.05 µg/mL. An internal working standard solution (mixture of codeine-d3, morphine-d3, 6MAM-d3 and fentanyl-d5) at 2 µg/mL was prepared in methanol. Working solutions and stock solutions were stored at a temperature of 5 °C and -20 °C, respectively, and protected from light.

2.5. Sample preparation and extraction

After the addition of 25 µL of the deuterated IS solution to volumes of 250 µL of PF and BL, the samples were precipitated by the addition of 750 µL of ice-cold acetonitrile and centrifuged at 3000 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 deionised water. Then, the cartridges were washed sequentially with 1 mL carbonate buffer (0.15 M), 1 mL of deionised 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 during 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 injected 2 µL into the GC-MS system.

2.6. GC-MS conditions

A capillary column HP-5 MS with 5% phenylmethylsiloxane (30 m × 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 °C/min and held for 2 min. As a carrier gas, was used a highly purified helium at a constant flow rate of 1 mL/min and it was used *splitless* injection mode (2 µL). The 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 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 (SIM) mode. The IS used to quantitate each substance were the following: codeine-d3 for codeine and 6-ACCOD, morphine-d3 for morphine, oxymorphone and oxycodone, 6-MAM-d3 for 6-MAM and fentanyl-d5 for fentanyl since these were available and the most similar to the substances. The ions monitored for each substance are presented in Table 1 in ascending order of their retention times.

2.7. Method validation

The described procedure was validated by evaluating interferences, linearity, limits of detection (LOD) and quantification (LOQ), carryover, precision (intra-day and intermediate), bias, stability (autosampler, bench-top and freeze/thaw cycles), dilution integrity and recovery based on the recommendations of Scientific Working Group for Forensic Toxicology (SWGTOX) [51].

2.7.1. 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 find in routine analyses of the SQTF-C (medicines, drugs of abuse and pesticides) at 5 ng/mL 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; duloxatine; 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; zisprasidone; 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; chlorfenvinphos; 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.

2.7.2. Linearity, limits of detection and quantification and carryover

Linearity was determined by plotting the peak area ratio between the analyte of interest and his IS against theoretical concentrations. The acceptance criteria were a $r^2 \geq 0.99$ and the calibrators

quantified within ±20%. Simultaneously, 4 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 (±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 retention times that will affect the capability to confirm them.

2.7.3. Precision and Bias

Precision was determined by calculating the coefficient of variation (CV, %) and acceptable values should be <20%. Bias was calculated on each group of QC samples: [(mean of measured concentrations - theoretical concentration)/ theoretical concentration x 100] and the limit of the acceptable variability was ±20%. Intra-day precision was determined by analyzing 4 QC samples levels in both matrices at the same day. The intermediate precision and bias were determined by the analysis of QC samples at 3 concentration levels (low, medium and high) in both matrices on 5 different days. Precision was acceptable when CV <20% and bias was acceptable when the value measured was ±20% of the spiked concentration, for all QC samples.

2.7.4. Stability

The stability on the autosampler was examined at 3 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). Bench-top stability and the three freeze/thaw cycles (after 1, 2, 3 and 4 weeks) were performed with 2 different QC levels (low and medium). Bench-top 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 2 levels QC samples frozen at -15 °C and thawed after the referred periods. Stability was considered acceptable if percentage of loss was within ± 20% of the freshly prepared QC samples.

2.7.5. 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 ±20% of the concentration 1150 ng/mL after the mentioned dilution

2.7.6. Recovery

The extraction efficiency was estimated by analysis of 2 sets of 3 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 (set1, n=6), with average blank samples fortified after the extraction (set2, n=6): set1/set2*100%.

3. 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 their retention times. All the studied substances were successfully identified in the fortified matrices, suggesting that the added compounds do not interfere with analysis of the analytes of interest. *(Annex 21)*

Linearity was verified in BL and PF samples from 5 to 1000 ng/mL with eight different points for each analyte, with 1/x weighted factor. *(Annex 22)* The 1/x weighting linear regression model was suitable to all calibration curves obtaining $r^2 \geq 0.99$ and calibrators quantitated within ±20%. The LODs achieved in BL were 4 ng/mL for all analytes except for oxymorphone and fentanyl (3 ng/mL). 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 2. The LOQs achieved in both matrices were 5 ng/mL for all substances. Related to PF, previous studies neither present values of LOD and LOQ using GC-MS, for codeine and morphine, nor the other compounds were studied in this matrix, being impossible to compare [18,20,23]. Although in some previous studies lower values were achieved for LODs and LOQs much higher volumes of BL sample were used, between 1 to 3 mL [28,30,56,57,33,38,39,44,52–55]. Previous published studies of 6-acetylcodeine in BL by GC-MS were not found. Carryover was not observed in both matrices analyzed for each substance.

The values obtained in BL for intra-day 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 intra-day 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 table 3 and 4.

The results obtained for the stability on the autosampler are summarized in table 5. All extracted analytes 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 6 are presented both the benchtop stability and three freeze/thaw cycles (after 1, 2, 3 and 4 weeks). Regarding bench-top 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 above 20%, (out of the limits established by the SWGTOX) having noticed a progressive increase of this difference over the different periods. Blank samples were fortified with the analytes in methanol which may have affected the stability of fentanyl and which may have led to a behavior different 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 ±20%.

The results obtained for the extraction efficiency of the developed procedure in BL, for all concentration levels (20 ng/mL, 200 ng/ mL and 900 ng/mL) ranged between 32.7-87.6% 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 were sufficiently low in volumes of 250 µL of BL.

It was not possible to study this parameter in PF, since an emulsion was formed when the 1% aqueous hydroxylamine was added to the sample after the fortification of the eluates. It was also observed the formation of insoluble material when the dried extracts were treated with the MSTFA+ 5% TMCS solution, which made the GC-MS analysis impossible. One reason for this could be the possibility of the formation of a salt of the neutralized acid during oximation [58].

4. 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 one single positive result in PF: codeine (50.2 ng/mL), morphine (540.4 ng/mL) and 6MAM (5.3 ng/mL). *(Annex 23)*

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 more than 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.

5. 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 µL of BL and PF. This new methodology brings several advantages like the small sample volumes required and the low limits (LOD and LOQ) obtained for all the substances. The optimization of two microwave-assisted derivatizations (30 seconds and 100 seconds) contributed to the reduction of the total time of the assay. To our knowledge this is the first procedure developed for the simultaneous determination of codeine, morphine, 6-acetylmorphine, 6-acetylcodeine, oxycodone, oxymorphone and fentanyl in whole blood and pericardial fluid with microwave-assisted derivatization with 1% aqueous hydroxylamine.

Furthermore, the results indicate the methodology suitability for the application in forensic toxicology laboratories in the routine analysis of these compounds.

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Tables

Table 1

Retention times (RT) and monitored ions of each substance

Analyte	Ions (m/z)	RT (min)
Codeine-D3	374*	13.58
Codeine	371*, 178, 196	13.59
Morphine-D3	432*	13.79
Morphine	429*, 236, 414	13.80
6-Acetylcodeine	341*, 282, 229	13.92
6-Aceylmorphine-D3	402*	14.10
6-Acetylmorphine	399*, 287, 340	14.11
Oxycodone	474*, 459, 475	14.38
Oxymorphone	532*, 517, 533	14.40
Fentanyl- D5	250*	14.87
Fentanyl	245*, 189, 146	14.89

**quantification ions.*

Table 2

Linearity results for all analytes studied in blood (BL) and pericardial fluid (PF).

Analytes	Matrix	Linear range (ng/mL)	Linearity			LOD**
			Slope(*)	Intercept(*)	r^2(*)	
Codeine	BL	5-1000	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	5-1000	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	5-1000	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	5-1000	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	5-1000	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	5-1000	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	5-1000	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

(*) mean values ± standard deviation (n=5). (**) n=6

Table 3

Intra-day precision and bias (n=6).

Analyte	**Matrix**	**Spiked Concentration (ng/mL)**											
		5			25			200			900		
		Concentration Found (ng/mL)	CV (%)	Bias (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)	Concentration Found (ng/mL)	CV (%)	Bias (%)
Codeine	BL	5.7	3.4	14.1	29.5	0.5	17.9	194.5	5.0	-2.7	854.7	3.0	-5.0
	PF	5.5	6.8	9.3	29.2	2.0	16.8	181.4	0.7	-9.3	826.8	0.7	-8.1
Morphine	BL	5.5	3.8	9.2	29.3	3.0	17.2	199.2	4.8	-0.4	825.9	2.8	-8.2
	PF	4.3	3.7	-14.9	28.3	5.1	13.1	186.5	0.7	-6.8	956.0	1.5	6.2
6-MAM	BL	5.5	1.5	9.9	28.6	1.2	14.6	198.1	4.7	-0.9	886.6	3.0	-1.5
	PF	4.8	8.5	-3.0	29.6	2.5	18.4	196.0	0.8	-2.0	992.8	1.5	10.3
6-ACCOD	BL	5.8	3.8	15.8	27.3	1.6	9.1	187.1	4.1	-6.5	847.3	2.9	-5.9
	PF	5.1	6.5	2.3	29.0	2.7	16.0	189.4	1.0	-5.3	996.9	3.8	10.8
Oxycodone	BL	5.7	6.1	13.6	27.3	7.8	8.2	208.8	6.6	4.4	895.0	9.9	-0.6
	PF	5.4	7.0	7.4	27.2	4.7	8.8	192.0	8.9	-4.0	977.8	1.3	8.6
Oxymorphone	BL	5.6	8.1	11.7	28.6	3.4	14.3	228.2	3.3	14.1	894.2	7.6	-0.6
	PF	4.9	9.4	-1.4	27.6	8.0	10.4	203.4	9.5	1.7	995.9	1.2	10.7
Fentanyl	BL	5.7	2.1	14.6	28.6	1.3	11.1	193.4	5.3	-3.3	895.9	3.7	-0.5
	PF	4.6	10.7	-8.8	29.0	1.3	15.8	199.2	4.7	-0.4	960.4	3.2	6.7

Table 4

Intermediate precision and bias (n=15).

Analyte	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 5

Stability data (%difference) after 24 h, 48 h and 7 days on the autosampler (n=3)

Analyte	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

Table 6

Stability data (%difference) after bench-top for 4 h at room temperature and after 3 freeze-thaw cycles (***(-15 ºC)***. (n=3)

Analyte	Matrix	Bench-top 4 h (Room temp)		Freeze-thaw 8 days (3 cycles)		Freeze-thaw 15 days (3 cycles)		Freeze-thaw 21 days (3 cycles)		Freeze-thaw 30 days (3 cycles)	
		20	200	20	200	20	200	20	200	20	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

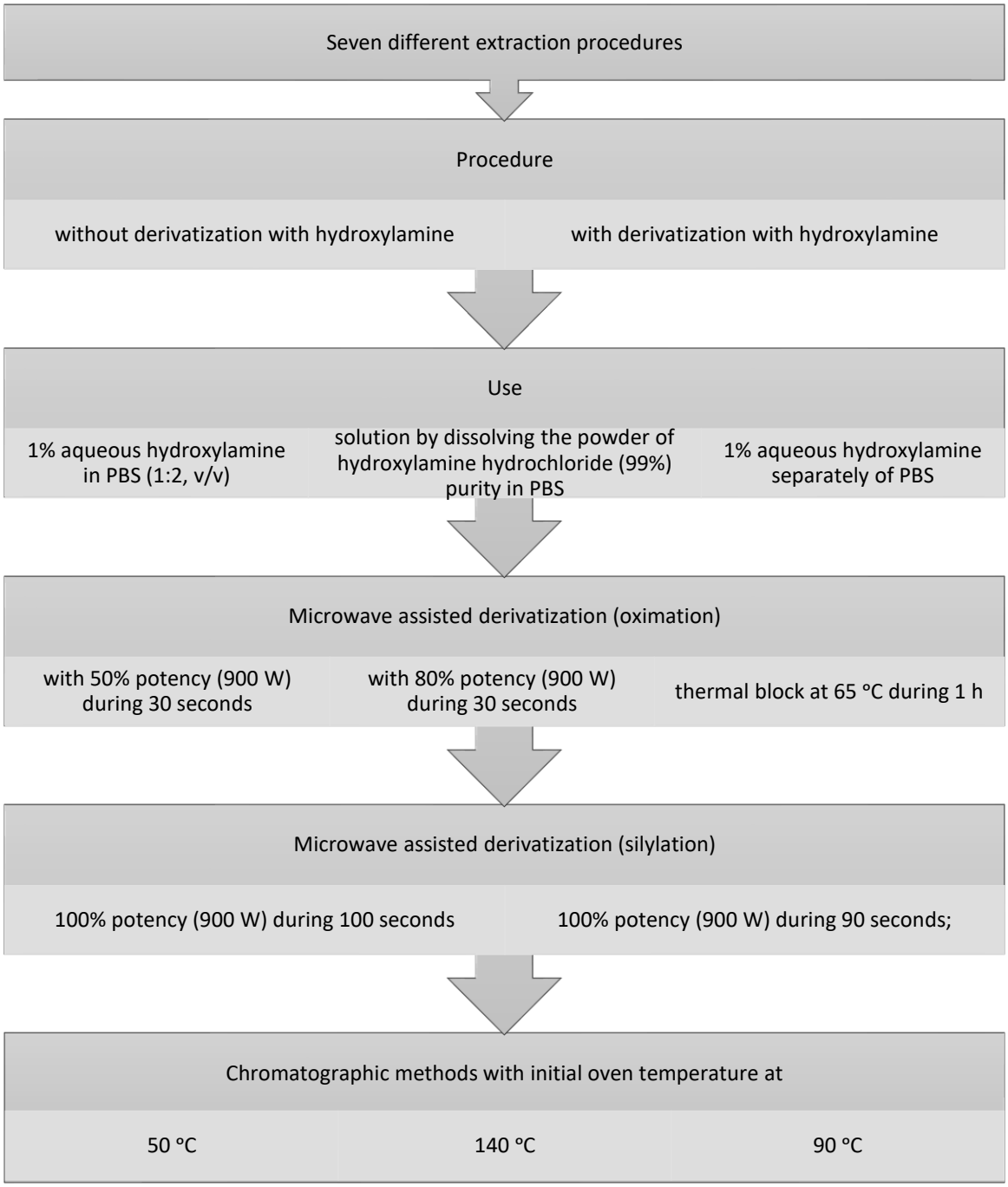
3.3. Overall Discussion

According to the previously referenced published reports, a seriously problem was identified, all over the world, related with the use of studied substances. The situation regarding synthetic opioids such as fentanyl, tramadol and its analogues has become so extreme that the United Nations Office on Drugs and Crime (UNODC) launched, in June 2018, an integrated strategy supporting the affected countries.[157] As consequence, a research was developed based on the widespread use of codeine for recreational purposes, especially among young people (Annex 24), who called purple drink/drank. This research has raised awareness for the possibility of illegally driving under the influence of codeine or other opioids (except for morphine and heroin) without consequences due to an absence of these substances in the list of analysed drugs in the Portuguese Highway Code.[158] Another reason for this situation was the lack of an analytical methodology that comprises this group of opioids in the SQTF-C. Therefore, it is important to have one that encloses a greater number of opioids in order to avoid the possibility of not identifying the cause of intoxication or even death by the illicit consumption of these substances.

It is important to maintain present that the detection of certain analytes may indicate their consumption and/or consumption of other substances: codeine, morphine, 6-acetylmorphine (heroin consumption indicator), 6-acetylcodeine (heroin consumption) and oxymorphone are examples of such.

It is also of high relevance that this study covered the use of an alternative matrix, since the BL (most commonly used matrix to confirm and quantitate the detected substances) may not be available to be collected during forensic autopsies. When considering our group of substances, we conclude that published analytical methodologies for the determination/quantification in PF are scarce, comprising only codeine[50,52], morphine[47,49,52] and 6-acetylmorphine[47,49,52].

Therefore, these were analysed and the following aspects were compared:



It was concluded that the best methodology was the Procedure 14 (Annex 19) as following:

Procedure 14	Use of a single solution (1% of aqueous hydroxylamine in PBS (1:2, v/v);
	First microwave- assisted derivatization at 50% power (900 W) for 30 seconds,
	Extractive methodology A;
	Second microwave- assisted derivatization at 100% power (900 W) for 100 seconds.

Regarding that same group of substances, especially when using hydroxylamine, no study demonstrating the use of a microwave-assisted derivation was found. Thus, we analysed the possibility of using the fast microwave-assisted derivatization with hydroxylamine in order to decrease the reaction time and even increased derivatization efficiency. Moreover we succeeded using only 30 seconds microwave irradiation time instead of 15 min[62,64,66,72,73,138], 20 min[152], 30 min[67,143], 1 h[38,63,65,68,69,133], or 2 h[70] used by other classic methodologies. Furthermore, the final microwave-assisted derivatization with MSTFA+5% TMCS was faster only requiring 100 seconds instead of 15-20 min [38,62,65–67,69,70,138,152], 30 min[63,64,143] or 1 h[68] reported in other studies.

Once the method was optimized, it was validated according to the international standards of SWGTOX. As it was seen, the present method achieved relatively low LOD (3 or 4 ng/mL) and LOQ (5 ng/mL) in small volume of sample (250 µL), especially when compared to previously published studies. Regarding whole blood, studies demonstrated LOD and LOQ values varying between 0.5-250 ng/mL and 0.10-100 ng/mL, although some had values significantly lower, all of these used sample amounts between 1 and 3 mL[34,38,64,65,69,73,84,148,150–153] and, in our case, 250 µL only. Regarding 6-acetylcodeine we didn't find any published work for its determination in BL using GC-MS. Concerning the PF, it was not possible to make a comparison since the previous studies didn't indicate the LOD and LOQ values.

Although extraction efficiency gave relatively low values for oxycodone and oxymorphone, this did not become relevant because LOD and LOQ were low. The extraction efficiency was still observed in terms of liner clean up. The chromatograms results were better with a clean liner.

The present study used GC-MS since that was the instrumentation available and adequate in the SQTF-C for the analysis of drugs of abuse. Even with the difficulties in the analysis of oxycodone and oxymorphone, low LOD and LOQ were obtained for all substances. Since GC-MS is the most accessible, adequate and economical instrument in the most Forensic Toxicological Laboratories, it was relevant to develop and implement this procedure in the SQTF-C for the routine analyses of these compounds.

Chapter 4. Conclusions

This study describes the optimization and development of an analytical methodology for the determination of morphine, codeine, 6-acetylmorphine, 6-acetylcodeine, oxycodone, oxymorphone and fentanyl in blood and pericardial fluid using solid-phase extraction, followed by gas chromatography-mass spectrometry analysis. It was full validated with two prior microwave-induced derivatizations.

Throughout this work the main accomplishments and conclusions obtained are the following:

- It was developed and fully validated a simple, faster and sensitive GC-MS method for the determination of opioids in BL and PF, using volume samples of 250 µL. This is very advantageous, since frequently a small amount of sample is available to do all the toxicological analyses usually required.
- The analytical methodology showed to be linear in the studied range (5-1000 ng/mL) with determination coefficients higher than 0.99, intra-day and intermediate precision (<11.9%) and bias (below ±19.4%) were according the followed guidelines. Extraction efficiencies ranged from 32.7 to 87.6%. Even though low limits of detection (3 or 4 ng/mL) and quantitation (5 ng/mL) were achieved for all analytes in the studied matrices and dilution integrity was assured. The stability of processed samples on the autosampler was guaranteed for at least 24 h at room temperature, for 4 h on workbench in both matrices and after the 3 freeze/thaw cycles for at least 30 days in PF.
- The optimized procedure is simple and faster than the previous ones, since it contains two very fast microwave-assisted derivatizations steps.

We used a single quadrupole mass spectrometer, nevertheless we achieved low limits for all the analytes using low volumes of samples, concluding that this validated methodology is sensitive and specific for the analysis of these study group of substances.

This is the first study in PF for the analysis of 6-acetylcodeine, oxycodone, oxymorphone and fentanyl. It is important to study the substances in alternative/complementary matrices since they could provide more information about their toxicokinetics and can also be useful in situations where BL is not available for analysis. However, it is important to study the correlations between BL concentrations and PF for better understanding of the results.

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Annex 1. Table I-A - Decree- Law nº 15/93, 22 January (Portuguese)

DL n.º 15/93, de 22 de Janeiro (versão actualizada)

LEGISLAÇÃO DE COMBATE À DROGA

Contém as alterações introduzidas pelos seguintes diplomas:

- Rectif. n.º 20/93, de 20 de Fevereiro

Versões anteriores deste artigo:

- 1ª versão: DL n.º 15/93, de 22 de Janeiro

TABELA I-A

Acetil-alfa-metilfentanil - N-(1-α metilfenetil-4-piperidil) acetanilida.

Acetildiidrocodeína - 3-metoxi-4,5-epoxi-6-acetoxi-17-metilmorfinano.

Acetilfentanilo - (N-fenil-N-[1-(2-feniletil) piperidina-4-il]acetamida).

Acetilmetadol - 3-acetoxi-6-dimetilamino-4,4-difenil-heptano.

Acetorfina - 3-0-acetiltetra-hidro-7α-(1-hidro-1-metilbutil)-6,14-endoetano-oripavina.

Alfacetilmetadol - alfa-3-acetoxi-6-dimetilamino-4,4-difenil-heptano.

Alfameprodina - alfa-3-etil-1-metil-4-fenil-4-propionoxipiperidina.

Alfametadol - alfa-6-dimetilamino-4,4-difenil-3-heptanol.

Alfa-metilfentanil - N-[1-(α metilfenetil)-4-piperidil] propionanilida.

Alfa-metiltiofentanil - N-[1-metil-2-(2-tienil) etil]-4-piperidil propionanilida.

Alfentanil - monocloridrato de N-{1[2-(4-etil-4,5-di-hidro-5-oxo-1H-tetrazol-1 il) etil]-4-(metoximetil)-4-piperidinil}-N-fenilpropanamida.

Alfaprodina - α-1,3-dimetil-4-fenil-4-propionoxipiperidina.

Alilprodina - 3-alil-1-metil-4-fenil-4-propionoxipiperidina.

Anileridina - éster etílico do ácido 1-para-aminofenetil-4-fenilpiperidino-4-carboxílico.

ANPP (4-anilino-N-fenetilpiperidina).

Benzilmorfina - 3-benziloxi-4,5-epoxi-N-metil-7-morfineno-6-ol; 3-benzilmorfina.

Benzetidina - éster etílico do ácido 1-(2-benziloxietil)-4-fenilpepiridino-4- carboxílico.

Betacetilmetadol - beta-3-acetoxi-6-dimetilamino-4,4-difenil-heptano.

Beta-hidroxifentanil - N-[1-(β-hidroxifenetil)-4-piperidil] propionanilida.

Beta-hidroxi-3-metilfentanil - N-[1-β-hidroxifenetil)-3-metil-4-piperidil] propionanilida.

Betameprodina - beta-3-etil-1-metil-4-fenil-4-propionoxipiperidina.

Betametadol - beta-6-dimetilamino-4,4-difenil-3-heptanol.

Betaprodina - beta-1,3-dimetil-4-fenil-4-propionoxipiperidina.

Bezitramida - 1-(3-ciano-3,3-difenilpropil)-4-(2-oxo-3-propionil-1-benzimidazolinil)-piperidina.

Butirato de dioxafetilo - etil-4-morfolino-2,2-difenilbutirato.

Butirfentanilo - (N-fenil-N-[1-(2-feniletil)-4-iperidinil]butanamida).

Carfentanilo - (1-(2-feniletil)-4-[fenil(propanoil)amino]piperidina-4-carboxilato de metilo).

Cetobemidona - 4-meta-hidroxifenil-1-metil-4-propionilpiperidina.

Clonitazeno - 2-para-clorobenzil-1-dietilaminoetil-5-nitrobenzimidazol.

Codeína - 3-metoxi-4,5-epoxi-6-hidroxi-17-metil-7-morfineno; 3-metil-morfina.

Codeína N-óxido - 3-metoxi-4,5-epoxi-6-hidroxi-17-metil-7-morfineno-17-oxi-ol.

Codoxina - di-hidrocodeinona-6-carboximetiloxina.

Concentrado de palha de papoila - matéria obtida por tratamento da palha de papoila em ordem a obter a concentração dos seus alcalóides, logo que esta matéria é colocada no comércio.

Desomorfina - 3-hidroxi-4,5-epoxi-17-metilmorfinano; di-hidrodoximorfina.

Dextromoramida - (+)-4-[2-metil-4-oxo-3,3-difenil-4 (1-pirrolidinil)-butil]-morfolina.

Dextropropoxifeno - (+)-4-dimetilamino-3-metil-1,2-difenil-2-butanol propionato.

Diampromida - N-[(2-metilfenetilamino)-propil]-propionanilida.

Dietiltiambuteno - 3-dietilamino-1,1-di-(2'-tienil)-1-buteno.

Difenoxilato - éster etílico do ácido 1-(3-ciano-3,3-difenilpropil)-4- fenilpiperidino-4-carboxílico.

Difenoxina - ácido-1-(3-ciano-3,3-difenilpropil)-4-fenilisonipecótico.

Diidrocodeína - 6-hidroxi-3-metoxi-17-metil-4,5-epoximorfinano.

Diidroetorfina-7,8-diidro-7-α-[1-(R)-hidroxi-1- metilbutil]-6,14-enab- etanotetraidrooripavina.

Di-hidromorfina - 3,6-di-hidroxi-4,5-epoxi-17-metilmorfinano.

Dimefeptanol - 6-dimetilamino-4,4-difenil-3-heptanol.

Dimenoxadol - 2-dimetilaminoetilo-1-etoxi-1,1-difenilacetato.

Dimetiltiambuteno - 3-dimetilamino-1,1-di-(2'-tienil)-1-buteno.

Dipipanona - 4,4-difenil-6-piperidina-3-heptanona.

Drotebanol - 3,4-dimetoxi-17-metilmorfinano-6-beta, 14-diol.

Etilmetiltiambuteno - 3-etilmetilamino-1,1-di-(2'-tienil)-1-buteno.

Etilmorfina - 3-etoxi-4,5-epoxi-6-hidroxi-17-metil-7-morfineno; 3-etilmorfina.

Etonitazeno - 1-dietilaminoetil-2-para-etoxibenzil-5-nitrobenzimidazol.

Etorfina - tetra-hidro-7-(1-hidroxi-1-metilbutil)-6,14-endoetanooripavina.

Etoxeridina - éster etílico do ácido-1-[2-(2-hidroxietoxi)-etil]-4-fenilpiperidino-4-carboxílico.

Fenadoxona - 6-morfolino-4,4-difenil-3-heptanona.

Fenanpromida - N-(1-metil-2-piperidinoetil)-propionalida.

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Fenazocina - 2'-hidroxi-5,9-dimetil-2-fenetil-6,7-benzomorfano.

Fenomorfano - 3-hidroxi-N-fenetilmorfinano.

Fenopiridina - éster etílico do ácido 1-(3-hidroxi-3-fenilpropil)-fenilpiperidino-4- carboxílico.

Fentanil - 1-fenetil-4-N-propionilanilinopiperidina.

4-fluoroisobutirilfentanilo (4F-iBF, 4-FIBF, pFIBF, N-(4-fluorofenil)-N-(1-fenetilpiperidin-4-il)isobutiramida).

Folcodina - 3-(2-morfolino-etoxi)-6-hidroxi-4,5-epoxi-17-metil-7-morfineno; morfoliniletilmorfina.

Furanilfentanilo - (Fu-F; N-fenil-N-[1-(2-feniletil) piperidin-4-il)]furano-2-carboxamida).

Furetidina - éster etílico do ácido 1-(2-tetra-hidrofurfuriloxietil)-4-fenilpiperidino-4-carboxílico.

Heroína - 3,6-diacetoxi-4,5-epoxi-17-metil-7-morfineno; diacetilmorfina.

Hidrocodona - 3-metoxi-4,5-epoxi-6-oxo-17-metilmorfina; di-hidrocodeina.

Hidromorfinol - 3,6,14-triidroxi-4,5-epoxi-17-metilmorfinano; 14-hidroxidiidromorfina.

Hidromorfona - 3-hidroxi-4,5-epoxi-6-oxo-17-metilmorfinano; diidromorfinona.

Hidroxipetidina - éster etílico do ácido 4-meta-hidroxifenil-1-metilpiperidino-4-carboxílico.

Isometadona - 6-dimetilamino-5-metil-4,4-difenil-3-hexanona.

Levofenacilmorfano - (-)-3-hidroxi-N-fenacilmorfinano.

Levometorfano - (-)-3-metoxi-N-metilmorfinano (*).

Levomoramida - (-)-4-[2-metil-4-oxo-3,3-difenil-4-(1-pirrolidinil)-butil] morfina.

Levorfanol - (-)-3-hidroxi-N-metilmorfinano (*).

Metadona - 6-dimetilamino-4,4-difenil-3-heptanona.

Metadona, intermediário de - 4-ciano-2-dimetilamino-4,4-difenilbutano.

Metazocina - 2'-hidroxi-2,5,9-trimetil-6,7-benzomorfano.

Metildesorfina - 6-metil-delta-6-desoximorfina; 3-hidroxi-4,5-epoxi-6,17-dimetil-6-morfineno.

Metildiidromorfina - 6-metil-diidromorfina; 3,6-diidroxi-4,5-epoxi-6,17-dimetilmorfinano.

3-metilfentanil - N-(3-metil-1-fenetil-4-piperidil) propionanilida (e os seus dois isómeros cis e trans).

Metopão - 5-metil di-hidromorfinona; 3-hidroxi-4,5-epoxi-6-oxo-5,17 dimetilmorfinona.

Mirofina - miristilbenzilmorfina; tetradecanoato de 3-benziloxi-4,5-epoxi-17-metil-7-morfineno-6-ilo.

Morferidina – éster etílicodo ácido 1-(2-morfolinoetil)-4-fenilpiperidino-4-carboxílico.

Moramida, intermediário de - ácido 2-metil-3-morfolino-1,1-difenilpropano carboxílico.

Morfina - 3,6-diidroxi-4,5-epoxi-17-metil-7-morfineno.

Morfina, bromometilato e outros derivados da morfina com nitrogénio pentavalente.

Morfina-N-óxido - 3,6-diidroxi-4,5-epoxi-17-metil-7-morfineno-N-óxido.

MPPP - propionato de 1-metil-4-fenil-4-piperidinol.

Nicocodina - éster codeínico do ácido 3-piridinocarboxílico; 6-nicotinilcodeína.

Nicodicodina - éster diidrocodeínico do ácido 3-piridinocarboxílico; 6-nicotinildiidrocodeína.

Nicomorfina - 3,6-dinicotilmorfina.

NPP (N-fenetil-4-piperidona).

Noracimetadol - ±-α-3-acetoxi-6-metilamino-4,4-difenil-heptano.

Norcodeína - 3-metoxi-4,5-epoxi-6-hidroxi-7-morfineno; N-desmetilcodeína.

Norlevorfanol - (-)-3-hidroximorfinano.

Normetadona - 6-dimetilamino-4,4-difenil-3-hexanona.

Normorfina - 3,6-di-hidroxi-4,5-epoxi-7-morfineno; desmetilmorfina.

Norpipanona - 4,4-difenil-6-peperidino-3-hexanona.

Ocfentanilo (N-(2-fluorofenil) -2-metoxi-N-[1-(2-fenetil)piperidin-4-il]acetamida).

Ópio - o suco coagulado espontaneamente obtido da cápsula da *Papaver somniferum L.* e que não tenha sofrido mais do que as manipulações necessárias para o seu empacotamento e transporte, qualquer que seja o seu teor em morfina.

Ópio - mistura de alcalóides sob a forma de cloridratos e brometos.

Oripavina - (3-O-desmetiltebaína, o 6,7,8,14-tetradeshidro-4,5-α-epoxi-6-metoxi-17-metilmorfinan-3-ol)

Oxicodona - 3-metoxi-4,5-epoxi-6-oxo-14-hidroxi-17-metilmorfinano; 14- hidroxidiidrocodeínona.

Oximorfona - 3,14-diidroxi-4,5-epoxi-6-oxo-17-metilmorfinano; 14-hidroxidiidromorfinona.

Para-fluorofentanil-(4'-fluoro-N-(1-fenetil-4-piperidil) propionanilida.

PEPAP - acetato de 1-fenetil-4-fenil-4-piperidinol.

Petidina - éster etílico do ácido 1-metil-4-fenilpiperidino-4-carboxílico.

Petidina, intermediário A da - 4-ciano-1-metil-4-fenilpiperidina.

Petidina, intermediário B da - éster etílico do ácido-4-fenilpiperidino-4-carboxílico.

Petidina, intermediário C da - ácido 1-metil-4-fenilpiperidino-4-carboxílico.

Piminodina - éster etílico do ácido 4-fenil-1-[3-(fenilamino)-propilpiperidino]-4-carboxílico.

Piritramida - amida do ácido 1-(3-ciano-3,3-difenilpropil)-4-(1-piperidino)- piperidino-4-carboxílico.

Pro-heptazina - 1,3-dimetil-4-fenil-4-propionoxiazaciclo-heptano.

Properidina - éster isopropílico do ácido 1-metil-4-fenilpiperi-dino-4-carboxílico.

Propirano - N-(1-metil-2-piperidinoetil)-N-2-piridilpropionamida.

Racemétorfano - ±-3-metoxi-N-metilmorfinano.

Racemoramida - ±-4-[2-metil-4-oxo-3,3-difenil-4-(1-pirrolidinil)-butil]-morfolina.

Racemorfano - ±-3-hidroxi-N-metilmorfinano.

Remifentanilo-1-(2-metoxicarboniletil)-4-(fenilpropionilamino) piperidina-4-carboxilato de metilo

Sufentanil - N-{4-metoximetil-1-[2-(2 (tienil) etil]-4-piperidil} propionanilida.

Tabecão - 3-metoxi-4,5-epoxi-6-acetoxi-17-metilmorfinano; acetidil-hidrocodeínona.

Tapentadol - {3-[(1R,2R)-3-(dimetilamino)-1-etil-2-metilpropil]fenol}

Tebaína - (3,6-dimetoxi-4,5-epoxi-17-metil-6,8-morfinadieno).

Tetra-hidrofuranilfentanilo (THF-F; N-fenil-N-[1-(2-feniletil)piperidin-4-il] tetra-hidrofurano-2-carboxamida).

Tilidina – (±)-etil-trans-2-(dimetilamino)-1-fenil-3-ciclo-hexeno-1-carboxilato.

Tiofentanil - N-{1-[2-(2-tienil) etil]-4-piperidil} propionanilida.

Trimeperidina - 1,2,5-trimetil-4-fenil-4-propionoxipiperidina.

U47700 (3,4-dicloro-N-(2-dimetilaminociclo-hexil)-N-metilbenzamida).

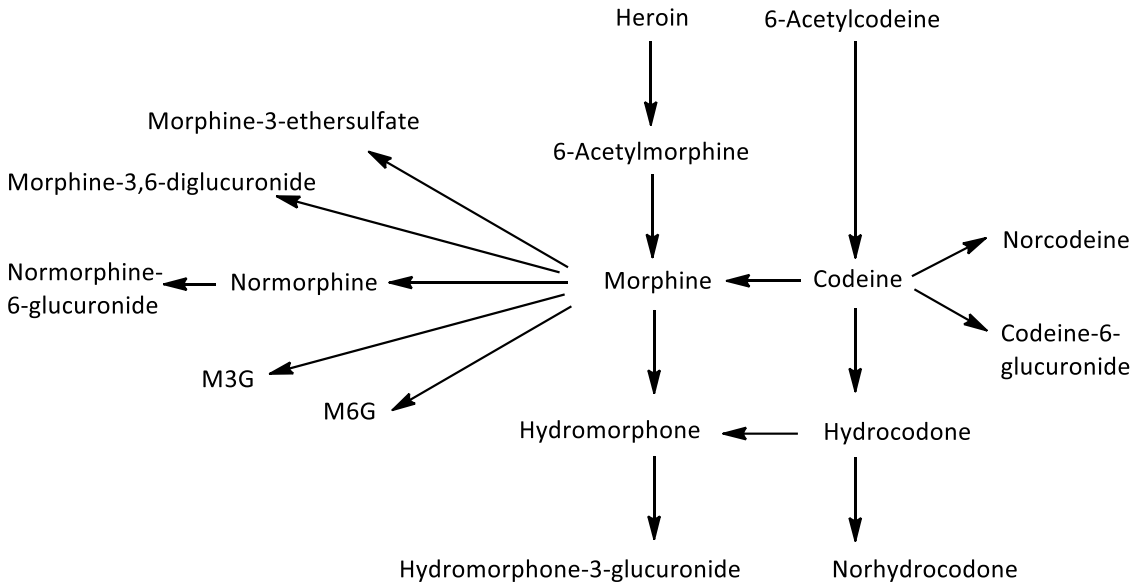
Os isómeros das substâncias inscritas nesta tabela em todos os casos em que estes isómeros possam existir com designação química específica, salvo se forem expressamente excluídos.

Os ésteres e os éteres das substâncias inscritas na presente tabela em todas as formas em que estes ésteres e éteres possam existir, salvo se figurarem noutra tabela.

Os sais das substâncias inscritas na presente tabela, incluindo os sais dos ésteres e éteres e isómeros mencionados anteriormente sempre que as formas desses sais sejam possíveis.

(*) O dextrometorfano (+)-3-metoxi-N-metilmorfinano e o dextrorfano (+)-3-hidroxi-N-metilmorfineno estão especificamente excluídos desta tabela.

Annex 2 – Heroin and 6-Acetylcodeine metabolism. Adapted from Maas *et al*.[23]



Annex 3- Literature review regarding opioids determination between the years 1999-2019

Analyte	Matrix	Sample volume	Sample Preparation	Derivatization	Analytical Procedure	LOD/LOQ	References
Morfine	Blood	-	SPE	BSTFA	GC-MS	LOD/LOQ: 20 ng/mL	[34]
		50 µL	Automated sample preparation system	-	LC-MS-MS	LLOQ: 5 ng/mL	[23]
		100 µL	Ice-cold acetonitrile:methanol (85:15); SPE	-	UHPLC-MS-MS	LOQ: 8.6 ng/mL	[47]
		100 µL	PP (acetonitrile)	-	LC-MS/MS	LOD: 0.39 ng/mL-46 ng/mL	[95]
		100 µL	LLE	-	UPLC-MS/MS	LOD: 0.26 ng/mL; LLOQ: 2.5 ng/mL	[114]
		300 µL	SLE (suported liquid extraction)	-	UPLC-orbitrap®	LOD: 3 ng/mL or better	[108]
		500 µL	SPE	-	LC-MS/MS	LOQ: 2.5 ng/mL	[85]
		1 mL	SPE	MSTFA	GC-MS	-	[136]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LOQ: 5 ng/mL	[100]
		1 mL	PP (10% Trichloroacetic acid); SPE	BSTFA+ 1%TMCS	GC-MS/MS	LOD: 1 ng/mL; LOQ: 2.5 ng/mL	[137]
		1 mL	PP (acetonitrile); LLE	2%methoxyamine in pyridine; propionic anhydride	GC-MS	LOD: 2 ng/mL; LOQ: 10 ng/mL	[73]
		1 mL	SPE	-	LC-MS/MS	LOD: 0.2 ng/mL LOQ: 0.7 ng/mL	[80]
		1 mL	SPE	-	LC-MS	LOD: 0.75 ng/mL; LOQ: 1.5 ng/mL	[27]
		1 mL	LLE	-	LC-MS/MS	LOD: 7 ng/mL; LOQ:8 ng/mL	[97]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA-1%TMCS	GC-MS	LOD: 10 ng/mL	[84]
		2 mL	SPE	Hydroxylamine 1% in pyridine; MSTFA+1%TMCS	GC-MS	-	[39]
		2 mL	PP (methanol); SPE	10% hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD: 2.5 ng/mL	[65]
		3 mL	PP (acetonitrile); SPE	10% hydroxylamine; ethyl acetate and BSTFA+1%TMCS	GC-MS	LOD:3.12 ng/mL; LOQ: 12.5 ng/mL	[69]
	Pericardial Fluid	100 µL	Ice-cold acetonitrile:methanol (85:15); SPE	-	UHPLC-MS-MS	LOQ: 8.6 ng/mL	[47]
		1 mL	Hydrolyzed, SPE	MSTFA, MBTFA	GC-MS	-	[52]
		1 mL	PP (10% TCA and concentrated HCl), LLE	-	GC/MS; GC-FTD; GC-FID	-	[49]
	Plasma	50 µL	Automated sample preparation system	-	LC-MS-MS	LLOQ: 5 ng/mL	[23]
		100 µL	PP (acetonitrile)	-	UPLC-MS/MS	LLOQ: 0.5 ng/mL	[22]

		200 µL	PP (acetonitrile+ acetone or only acetone), LLE	-	UPLC-MS/MS	LLOQ: 1.00 ng/mL	[113]
	Serum	50 µL	PP, filtration	-	HPLC-MS/MS	LOD: 1.3 ng/mL; LOQ: 9.1 ng/mL	[77]
		100 µL	PP (acetonitrile)	-	LC-MS/MS	LOD: 0.23 ng/mL- 0.55 ng/mL	[95]
		100 µL	PP (acetonitrile)	-	LC-MS/MS	LLOD: 0.1 ng/mL; LLOQ: 0.25 ng/mL	[101]
		100 µL (serum)	PP (methanol: zinc sulphate 0.1 M (4:1, v/v)) Online extraction	-	LC-MS/MS	LOD: 0.5 ng/mL; LOQ: 1.5 ng/mL	[98]
		200 µL	SPE	-	LC-MS/MS	LOD: 0.60 ng/mL; LOQ: 1.0 ng/mL	[104]
		600 µL	PP (acetonitrile + isopropanol); SPE	Isooctane/pyridine/MSTFA (14/5/1, v/v/v)	GC-MS	LOD: 1.2 ng/mL; LOQ: 4.9 ng/mL	[120]
	Serum/plasma	1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
	Urine	-	-	-	LC-TIMS-qTOF MS / LC-qTOF-MS	LOD: 27.9 ng/mL; LOQ: 138.6 ng/mL / LOD: 31.9 ng/mL; LOQ: 159.4 ng/mL	[107]
		-	SPE	BSTFA	GC-MS	LOD/LOQ: 20 ng/mL	[34]
		70 µL	Hydrolysis	-	LC-MS/MS	LLOQ: 20 ng/mL	[91]
		100 µL	Ice-cold acetonitrile:methanol (85:15); SPE	-	UHPLC-MS-MS	LOQ: 8.6 ng/mL	[47]
		100 µL	PP (acetonitrile)	-	UPLC-MS/MS	LLOQ: 2 ng/mL	[22]
		100 µL	PP (acetonitrile)	-	LC-MS/MS	LOD: 1.7 ng/mL- 4.2 ng/mL	[95]
		100 µL	Hydrolyzed	-	LC-MS/MS; LC-qTOF-MS	LOD ≈ 2/3 ng/mL; LOQ ≈ 7,5 /10 ng/mL	[89]
		300 µL	SLE (supported liquid extraction)	-	UPLC-orbitrap®	LOD: 3 ng/mL or better	[108]
		1 mL	SPE	MSTFA	GC-MS	-	[136]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LOQ: 5 ng/mL	[100]
		1 mL	LLE (2 different methodology)	-	LC-MS/MS	LOD:5 ng/mL-10 ng/mL	[105]
		1 mL	LLE	2% Methoxyamine HCl; propionic anhydride	GC-MS	LOD: 10 ng/mL; LOQ: 25 ng/mL	[72]
		1 mL	Hydrolysis, LLE	MSTFA/ethanethiol/NH_4I (500:4:2)	GC-MS	LOD: 10 ng/mL	[139]
		1 mL	Hydrolysis, LLE	-	LC-MS/MS	LOD: 4 ng/mL; LOQ: 8 ng/mL	[97]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
		2 mL	DLLME	-	HPLC-PDA	LOD:25 ng/mL	[79]

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		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA-1%TMCS	GC-MS	LOD: 10 ng/mL	[84]
		2 mL	Hydrolysis; SPE	BSTFA+1%TMCS	GC-MS	LOD: 60 ng/mL; LOQ: 100 ng/mL	[140]
		2 mL	SPE	-	LC-MS/MS	LOD: 0.6 ng/mL; LOQ: 1.2 ng/mL	[96]
		2 mL	Hydrolysis, LLE	MSTFA+1%TMCS	GC-MS	LOD: 25 ng/mL	[141]
		3 mL	SPE	Methoxyamine HCl in pyridine; BSTFA+pyridine	GC-MS	LOQ: 150 ng/mL	[143]
		3 mL	SPE	-	HPLC-MS	LOD:65 ng/mL; LLOQ: 100 ng/mL	[76]
		3 mL	Hydrolysis; SPE	10% hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD: 25 ng/mL; LOQ: 50 ng/mL	[66]
		3 mL	Hydrolysis, LLE	MSTFA/NH_4I/DTE (1000:2:4, v/w/w)	GC-MS	LOD: 30 ng/mL	[142]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA	GC-MS	LOD: 50 ng/mL	[144]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH_4I/ethanethiol (640:1:2, v/w/v)	GC-MS	LOD: 100 ng/mL	[145]
	Oral Fluid	-	SPE	-	UPLC-MS/MS	LLOQ: 0.5ng/mg	[112]
		200 µL	SPE	-	LC-MS/MS	LOD: 2 ng/mL; LOQ: 4 ng/mL	[90]
		750 µL	SPE	-	LC-MS-MS	LOD:0.02 ng/mL; LLOQ: 1.5 ng/mL	[87]
		1 mL	LLE	BuAc:ACN(1:1) + MSTFA (1:3)	GC-MS	LOQ: 5 ng/mL	[147]
	Vitreous Humor	100 µL	Ice-cold acetonitrile:methanol (85:15); SPE	-	UHPLC-MS-MS	LOQ: 8.6 ng/mL	[47]
		100 µL	PP (HCL and CH3CN); SPE (DPX)	MSTFA	GC	LOD: 500 ng/mL; LOQ: 1520 ng/mL	[146]
		1 mL	SPE	-	LC-MS	LOD: 0.30 ng/mL; LOQ: 1.5 ng/mL	[27]
Codeine	Blood	-	SPE	BSTFA	GC-MS	LOD/LOQ: 20 ng/mL	[34]
		50 µL	Automated sample preparation system	-	LC-MS-MS	LLOQ: 1 ng/mL	[23]
		100 µL	PP (acetonitrile)	-	LC-MS/MS	LOD: 0.09 ng/mL- 0.44 ng/mL	[95]
		100 µL	LLE	-	UPLC-MS/MS	LOD: 0.64 ng/mL; LLOQ: 5 ng/mL	[114]
		300 µL	SLE (supported liquid extraction)	-	UPLC-orbitrap®	LOD: 3 ng/mL or better	[108]
		500 µL	SPE	-	LC-MS/MS	LOQ: 2.5 ng/mL	[85]
		1 mL	LLE	-	LC-MS/MS	LLOQ: 50 ng/mL	[88]

		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LOQ: 5 ng/mL	[100]
		1 mL	PP (10% TCA); SPE	BSTFA+ 1%TMCS	GC-MS/MS	LOD: 1 ng/mL; LOQ: 2.5 ng/mL	[137]
		1 mL	PP (acetonitrile); LLE	2%methoxyamine in pyridine; propionic anhydride	GC-MS	LOD: 2 ng/mL; LOQ: 10 ng/mL	[73]
		1 mL	SPE	-	LC-MS	LOD: 0.30 ng/mL; LOQ: 0.75 ng/mL	[27]
		1 mL	LLE	-	LC-MS/MS	LOD: 3 ng/mL; LOQ:3 ng/mL	[97]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
		1 mL	SPE	-	LC-MS/MS	LOD: 0.32 ng/mL LOQ: 1.08 ng/mL	[80]
		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA-1%TMCS	GC-MS	LOD-	[84]
		2 mL	SPE	Hydroxylamine 1% in pyridine; MSTFA+1%TMCS	GC-MS	-	[39]
		2 mL	PP (methanol); SPE	10% hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD: 3.5 ng/mL	[65]
		2 mL	LLE/ SPE	-	GC-MS	LOD: 100/50 ng/mL	[148]
		3 mL	PP (acetonitrile); SPE	10% hydroxylamine; ethyl acetate and BSTFA+1%TMCS	GC-MS	LOD:1.56 ng/mL; LOQ: 6.25 ng/mL	[69]
	Pericardial Fluid	1 mL	Hydrolyzed, SPE	MSTFA, MBTFA	GC-MS	-	[52]
		1 mL	SPE	-	GC-MS	-	[50]
	Plasma	50 µL	Automated sample preparation system	-	LC-MS-MS	LLOQ: 1 ng/mL	[23]
		450 µL	Online SPE	-	LC-MS/MS	LOQ: 5 ng/mL	[102]
	Serum	50 µL	PP, filtration	-	HPLC-MS/MS	LOD: 3.8 ng/mL; LOQ: 6.4 ng/mL	[77]
		100 µL	PP (acetonitrile)	-	LC-MS/MS	LOD: 0.26 ng/mL- 0.11 ng/mL	[95]
		100 µL	PP (methanol: zinc sulphate 0.1 M (4:1, v/v)) Online extraction	-	LC-MS/MS	LOD: 0.1 ng/mL; LOQ: 1.5 ng/mL	[98]
		200 µL	LLE	-	LC-qTOF-MS	LOD: 7 ng/mL	[106]
		200 µL	SPE	-	LC-MS/MS	LOD: 0.20 ng/mL; LOQ: 0.5 ng/mL	[104]
		600 µL	PP (acetonitrile + isopropanol); SPE	Isooctane/pyridine/MSTFA (14/5/1, v/v/v)	GC-MS	LOD: 0.4 ng/mL; LOQ: 2.6 ng/mL	[120]

d

	Serum/plasma	1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
	Urine	-	-	-	LC-TIMS-qTOF MS / LC-qTOF-MS	LOD: 9.9 ng/mL; LOQ:49.6 ng/mL / LOD: 3.0 ng/mL; LOQ: 15.0 ng/mL	[107]
		-	SPE	BSTFA	GC-MS	LOD/LOQ: 20 ng/mL	[34]
		70 µL	Hydrolysis	-	LC-MS/MS	LLOQ: 20 ng/mL	[91]
		100 µL	PP (acetonitrile)	-	LC-MS/MS	LOD: 0.66 ng/mL- 1.1 ng/mL	[95]
		100 µL	Hydrolyzed	-	LC-MS/MS; LC-qTOF-MS	LOD ≈ 3/ 1,5 ng/mL; LOQ ≈ 10 / 5 ng/mL	[89]
		300 µL	SLE (suported liquid extraction)	-	UPLC-orbitrap®	LOD: 3 ng/mL or better	[108]
		1 mL	Liquid nitrogen	Pyridine + 2% methoxyamine HCl, propionic Anhydride, MSTFA	GC-MS	LOD/LOQ: 50/100 ng/mL	[138]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LOQ: 5 ng/mL	[100]
		1 mL	LLE (2 different methodology)	-	LC-MS/MS	LOD:2 ng/mL-2 ng/mL	[105]
		1 mL	LLE	2% Methoxyamine HCl; propionic anhydride	GC-MS	LOD: 10 ng/mL; LOQ: 25 ng/mL	[72]
		1 mL	Hydrolysis, LLE	MSTFA/ethanethiol/NH_4I (500:4:2)	GC-MS	LOD: 10 ng/mL	[139]
		1 mL	Hydrolysis, LLE	-	LC-MS/MS	LOD: 3 ng/mL; LOQ: 3 ng/mL	[97]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
		2 mL	DLLME	-	HPLC-PDA	LOD:9 ng/mL	[79]
		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA-1%TMCS	GC-MS	-	[84]
		2 mL	Hydrolysis; SPE	BSTFA+1%TMCS	GC-MS	LOD: 300 ng/mL; LOQ: 500 ng/mL	[140]
		2 mL	SPE	-	LC-MS/MS	LOD: 0.6 ng/mL; LOQ: 1.2 ng/mL	[96]
		2 mL	Hydrolysis, LLE	MSTFA+1%TMCS	GC-MS	LOD: 25 ng/mL	[141]
		3 mL	SPE	Methoxyamine HCl in pyridine; BSTFA+pyridine	GC-MS	LOQ: 150 ng/mL	[143]
		3 mL	SPE	-	HPLC-MS	LOD:6 ng/mL; LLOQ: 100 ng/mL	[76]
		3 mL	Hydrolysis; SPE	10% hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD: 25 ng/mL; LOQ: 50 ng/mL	[66]

e

		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA	GC-MS	LOD: 20 ng/mL	[144]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH4I/ethanethiol (640:1:2, v/w/v)	GC-MS	LOD: 100 ng/mL	[145]
	Oral Fluid	-	SPE	-	UPLC-MS/MS	LLOQ: 0.5 ng/mg	[112]
		200 µL	SPE	-	LC-MS/MS	LOD: 2 ng/mL; LOQ: 4 ng/mL	[90]
		750 µL	SPE	-	LC-MS-MS	LOD:0.04 ng/mL; LLOQ: 1.5 ng/mL	[87]
		1 mL	LLE	BuAc:ACN(1:1) + MSTFA (1:3)	GC-MS	LOQ: 5 ng/mL	[147]
	Vitreous Humor	100 µL	PP(HCL, CH_3CN); SPE (DPX)	MSTFA	GC	LOD: 160 ng/mL; LOQ: 490 ng/mL	[146]
		1 mL	SPE	-	LC-MS	LOD: 0.15 ng/mL; LOQ: 1.5 ng/mL	[27]
6-Acetylmorphine	Blood		SPE	BSTFA	GC-MS	LOD/LOQ: 4 ng/mL	[34]
		50 µL	Automated sample preparation system	-	LC-MS-MS	LLOQ: 5 ng/mL	[23]
		100 µL	Ice-cold acetonitrile:methanol (85:15); SPE	-	UHPLC-MS-MS	LOQ: 3.3 ng/mL	[47]
		100 µL	LLE	-	UPLC-MS/MS	LOD: 0.05 ng/mL; LLOQ: 5 ng/mL	[114]
		300 µL	SLE	-	UPLC-orbitrap®	LOD: 3 ng/mL or better	[108]
		500 µL	SPE	-	LC-MS/MS	LOQ: 2.5 ng/mL	[85]
		1 mL	SPE	MSTFA	GC-MS	-	[136]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LOQ: 5 ng/mL	[100]
		1 mL	PP (10% TCA); SPE	BSTFA+ 1%TMCS	GC-MS/MS	LOD: 1 ng/mL; LOQ: 2.5 ng/mL	[137]
		1 mL	PP (acetonitrile); LLE	2%methoxyamine in pyridine; propionic anhydride	GC-MS	LOD: 2 ng/mL; LOQ: 10 ng/mL	[73]
		1 mL	LLE	-	LC-MS/MS	LOD: 2 ng/mL; LOQ:3 ng/mL	[97]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
		1 mL	SPE	-	LC-MS/MS	LOD: 0.16 ng/mL LOQ: 0.5 ng/mL	[80]
		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA-1%TMCS	GC-MS	LOD: 5 ng/mL	[84]
		2 mL	SPE	Hydroxylamine 1% in pyridine; MSTFA+1%TMCS	GC-MS	-	[39]
		2 mL	PP (methanol); SPE	10% hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD: 0.70 ng/mL; LOQ: 5 ng/mL	[65]

f

		3 mL	PP (acetonitrile); SPE	10% hydroxylamine; ethyl acetate and BSTFA+1%TMCS	GC-MS	LOD:0.78 ng/mL; LOQ: 1.56 ng/mL	[69]
	Pericardial Fluid	100 µL	Ice-cold acetonitrile:methanol (85:15); SPE	-	UHPLC-MS-MS	LOQ: 3.3 ng/mL	[47]
	Plasma	50 µL	Automated sample preparation system	-	LC-MS-MS	LLOQ: 5 ng/mL	[83]
	Serum	50 µL	PP, filtration	-	HPLC-MS/MS	LOD: 3.8 ng/mL; LOQ: 30.7 ng/mL	[77]
		100 µL	PP (methanol: zinc sulphate 0.1M (4:1, v/v)) Online extraction	-	LC-MS/MS	LOD: 0.2 ng/mL; LOQ: 1.3 ng/mL	[98]
		200 µL	SPE	-	LC-MS/MS	LOD: 0.10 ng/mL; LOQ: 1.0 ng/mL	[104]
		600 µL	PP (acetonitrile + isopropanol); SPE	Isooctane/pyridine/MSTFA (14/5/1, v/v/v)	GC-MS	LOD: 0.3 ng/mL; LOQ: 0.8 ng/mL	[120]
	Serum/plasma	1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
	Urine	-	SPE	BSTFA	GC-MS	LOD/LOQ: 20 ng/mL	[34]
		70 µL	Hydrolysis	-	LC-MS/MS	LLOQ: 10 ng/mL	[91]
		100 µL	Ice-cold acetonitrile:methanol (85:15); SPE	-	UHPLC-MS-MS	LOQ: 3.3 ng/mL	[47]
		300 µL	SLE	-	UPLC-orbitrap®	LOD: 3 ng/mL or better	[108]
		1 mL	SPE	MSTFA	GC-MS	-	[136]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LOQ: 5 ng/mL	[100]
		1 mL	LLE	2% Methoxyamine HCl; propionic anhydride	GC-MS	LOD: 10 ng/mL; LOQ: 25 ng/mL	[72]
		1 mL	Hydrolysis, LLE	MSTFA/ethanethiol/NH_4I (500:4:2)	GC-MS	LOD: 20 ng/mL	[139]
		1 mL	Hydrolysis, LLE	-	LC-MS/MS	LOD: 2 ng/mL; LOQ: 5 ng/mL	[97]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
		1 mL	Liquid nitrogen	Pyridine + 2% methoxyamine HCl, propionic Anhydride, MSTFA	GC-MS	LOD/LOQ: 30 ng/mL	[138]
		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA-1%TMCS	GC-MS	LOD: 5 ng/mL	[84]
		2 mL	Hydrolysis; SPE	BSTFA+1%TMCS	GC-MS	LOD: 400 ng/mL; LOQ: 600 ng/mL	[140]
		2 mL	SPE	-	LC-MS/MS	LOD: 0.02 ng/mL; LOQ: 0.05 ng/mL	[96]

		2 mL	Hydrolysis, LLE	MSTFA+1%TMCS	GC-MS	LOD: 25 ng/mL	[141]
		3 mL	SPE	Methoxyamine HCl in pyridine; BSTFA+pyridine	GC-MS	LOQ: 150 ng/mL	[143]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH_4I/ethanethiol (640:1:2, v/w/v)	GC-MS	LOD: 100 ng/mL	[145]
	Oral Fluid	-	SPE	-	UPLC-MS/MS	LLOQ: 0.5ng/mg	[112]
		200 µL	SPE	-	LC-MS/MS	LOD: 0.4 ng/mL; LOQ: 1 ng/mL	[90]
		750 µL	SPE	-	LC-MS-MS	LOD:0.04 ng/mL; LLOQ: 0.4 ng/mL	[87]
		1 mL	LLE	BuAc:ACN(1:1) + MSTFA (1:3)	GC-MS	LOQ: 1 ng/mL	[147]
	Vitreous Humor	100 µL	Ice-cold acetonitrile:methanol (85:15); SPE	-	UHPLC-MS-MS	LOQ: 3.3 ng/mL	[47]
		100 µL	PP (HCL and CH_3CN); SPE (DPX)	MSTFA	GC	LOD: 1250 ng/mL; LOQ: 3790 ng/mL	[146]
6-Acetylcodeine	Blood	1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LOQ: 5 ng/mL	[100]
		1 mL	SPE	-	LC-MS/MS	LOD: 0.26 ng/mL LOQ: 0.88 ng/mL	[80]
	Urine	1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LOQ: 5 ng/mL	[100]
	Oral fluid	200 µL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LOQ: 2 ng/mL	[90]
Oxycodone	Blood	-	SPE	BSTFA	GC-MS	LOD/LOQ: 20 ng/mL	[34]
		50 µL	Automated sample preparation system	-	LC-MS-MS	LLOQ: 1 ng/mL	[23]
		100 µL-500 µL	SPE	-	LC-MS/MS	LOD:0.2 ng/mL LLOQ: 1 ng/mL	[81]
		300 µL	SLE	-	UPLC-orbitrap®	LOD: 3 ng/mL or better	[108]
		500 µL	SPE	-	LC-MS/MS	LOQ: 2.5 ng/mL	[85]
		1 mL	LLE	-	LC-MS/MS	LLOQ: 10 ng/mL	[88]
		1 mL	SPE	BSTFA+1%TMCS and ethyl acetate	GC-MS	LOD: 50 ng/mL; LOQ: 50 ng/mL	[150]
		1 mL	PP (acetonitrile); LLE	2%methoxyamine in pyridine; propionic anhydride	GC-MS	LOD: 2 ng/mL; LOQ: 10 ng/mL	[73]
		1 mL	LLE	-	LC-MS/MS	LOD: 0.2 ng/mL; LOQ:0.2 ng/mL	[97]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
		1 mL	SPE	-	LC-MS/MS	LOD: 0.58 ng/mL LOQ: 1.93 ng/mL	[80]

h

		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA-1%TMCS	GC-MS	-	[84]
		2 mL	SPE	Hydroxylamine 1% in pyridine; MSTFA+1%TMCS	GC-MS	-	[39]
		2 mL	PP (methanol); SPE	10% hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD: 2.5 ng/mL; LOQ: 20 ng/mL	[65]
		2 mL	PP (acetonitrile); SPE	10% Hydroxylamine; BSTFA+1%TMCS	GC-MS	LOQ:50 ng/mL	[64]
		2 mL	LLE/ SPE	-	GC-MS	LOD: 25/250 ng/mL	[148]
		2 mL	LLE	MSTFA (TMS)	GC-MS	LOQ: 100 ng/mL	[151]
		3 mL	PP (acetonitrile); SPE	10% hydroxylamine; ethyl acetate and BSTFA+1%TMCS	GC-MS	LOD:6.25 ng/mL; LOQ: 12.5 ng/mL	[69]
		3 mL	LLE	2% methoxyamine in pyridine; MSTFA+1%TMCS and dimethylformamide	GC-MS	LOD: 0.5 ng/mL; LOQ: 1.0 ng/mL	[152]
	Plasma	50 µL	Automated sample preparation system	-	LC-MS-MS	LLOQ: 1 ng/mL	[23]
		50 µL	PP (acetonitrile)	-	LC-MS/MS	LLOQ: 0.200 ng/mL	[99]
		100 µL	Methanol	-	LC-MS/MS	LOD: 0,03 ng/mL; LOQ: 0,10 ng/mL	[94]
		500 µL	LLE	-	UHPLC-MS/MS	LOD:0.06 ng/mL; LOQ:0.2 ng/mL	[110]
		1 mL	LLE	-	HPLC-MS/MS	LLOQ: 0.2 ng/mL	[78]
	Serum	200 µL	SPE	-	LC-MS/MS	LOD: 0.30 ng/mL; LOQ: 1.0 ng/mL	[104]
	Serum/plasma	1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
	Urine	-	SPE	BSTFA	GC-MS	LOD/LOQ: 20 ng/mL	[34]
		70 µL	Hydrolysis	-	LC-MS/MS	LLOQ: 20 ng/mL	[91]
		100 µL-500 µL	SPE	-	LC-MS/MS	LOD:0.2 ng/mL LLOQ: 1 ng/mL	[81]
		200 µL	LLE	-	LC-MS/MS	LOD: 0,01 ng/mL; LOQ: 0,02 ng/mL	[94]
		200 µL	LLE	-	HPLC-MS/MS	LLOQ: 10 ng/mL	[78]
		300 µL	SLE	-	UPLC-orbitrap®	LOD: 3 ng/mL or better	[108]
		500 µL	LLE	-	UHPLC-MS/MS	LOD:0.06 ng/mL; LOQ:0.2 ng/mL	[110]

		1 mL	Liquid nitrogen	Pyridine + 2% methoxyamine HCl, propionic Anhydride, MSTFA	GC-MS	LOD/LOQ: 50 ng/mL	[138]
		1 mL	LLE (2 different methodology)	-	LC-MS/MS	LOD:1 ng/mL-1 ng/mL	[105]
		1 mL	LLE	2% Methoxyamine HCl; propionic anhydride	GC-MS	LOD: 10 ng/mL; LOQ: 25 ng/mL	[72]
		1 mL	Hydrolysis, LLE	MSTFA/ethanethiol/NH_4I (500:4:2)	GC-MS	LOD: 200 ng/mL	[139]
		1 mL	Hydrolysis, LLE	-	LC-MS/MS	LOD: 0.2 ng/mL; LOQ: 0.2 ng/mL	[97]
		1 mL	SPE	-	LC-MS/MS	LOD: 1 ng/mL; LLOQ: 2 ng/mL	[82]
		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA-1%TMCS	GC-MS	-	[84]
		2 mL	Hydrolysis, SPE	Methoxylamine 10%; acetonitrile and acetic anhydride	GC-MS	LOD: 40 ng/mL; LOQ: 40 ng/mL	[36]
		2 mL	Hydrolysis; SPE	Acetate buffer/ 10% hydroxylamine; BSTFA	GC-MS	LOQ: 100 ng/mL	[70]
		2 mL	Hydrolysis; SPE	BSTFA+1%TMCS	GC-MS	LOD: 400 ng/mL; LOQ: 600 ng/mL	[140]
		2 mL	Hydrolysis, LLE	MSTFA+1%TMCS	GC-MS	LOD: 25 ng/mL	[141]
		3 mL	SPE	Methoxyamine HCl in pyridine; BSTFA+pyridine	GC-MS	LOQ: 150 ng/mL	[143]
		3 mL	Hydrolysis; SPE	10% hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD: 25 ng/mL; LOQ: 50 ng/mL	[66]
		3 mL	Hydrolysis, LLE	MSTFA/NH_4I/DTE (1000:2:4 v/w/w)	GC-MS	LOD: 100 ng/mL	[142]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA	GC-MS	LOD: 20 ng/mL	[144]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH_4I/ethanethiol (640:1:2, v/w/v)	GC-MS	LOD: 100 ng/mL	[145]
	Oral Fluid		SPE	Methoxyamine 10% aq; BTSFA+1%TMCS	GC-MS	LOD: 3 ng/mL; LOQ: 2ng/mg	[63]
		200 µL	SPE	-	LC-MS/MS	LOD: 2 ng/mL; LOQ: 4 ng/mL	[90]
		750 µL	SPE	-	LC-MS-MS	LOD:0.02 ng/mL; LLOQ: 1.5 ng/mL	[87]
		1 mL	SPE	1% hydroxylamine in pyridine; MSTFA+1%TMCS	GC-MS	LOQ: 10 ng/mL	[67]

	Vitreous Humor	1 mL	SPE	1% hydroxylamine; MSTFA+ 1%TMCS	GC-MS	LOD: 10 ng/mL; LOQ: 50 ng/mL	[68]
Oxymorphone	Blood	-	SPE	BSTFA	GC-MS	LOD/LOQ: 20 ng/mL	[34]
		100 µL-500 µL	SPE	-	LC-MS/MS	LOD:0.3 ng/mL LLOQ: 1 ng/mL	[81]
		500 µL	SPE	-	LC-MS/MS	LOQ: 2.5 ng/mL	[85]
		1 mL	PP (acetonitrile); LLE	2%methoxyamine in pyridine; propionic anhydride	GC-MS	LOD: 2 ng/mL; LOQ: 10 ng/mL	[73]
		1 mL	PP (acetonitrile); SPE	10% Hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD:15 ng/mL; LOQ: 25 ng/mL	[38]
		1 mL	SPE	-	LC-MS/MS	LOD: 0.295 ng/mL LOQ: 0.98 ng/mL	[80]
		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA-1%TMCS	GC-MS	-	[84]
		2 mL	SPE	Hydroxylamine 1% in pyridine; MSTFA+1%TMCS	GC-MS	-	[39]
		2 mL	PP (methanol); SPE	10% hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD: 3.5 ng/mL	[65]
		2 mL	PP (acetonitrile); SPE	10% hydroxylamine; BSTFA+1%TMCS	GC-MS	LOQ: 50 ng/mL	[64]
		3 mL	LLE	2% methoxyamine in pyridine; MSTFA+1%TMCS and dimethylformamide	GC-MS	LOD: 0.5 ng/mL; LOQ: 1.0 ng/mL	[152]
	Plasma	100 µL	Methanol	-	LC-MS/MS	LOD: 0,03 ng/mL; LOQ: 0,10 ng/mL	[94]
		500 µL	LLE	-	UHPLC-MS/MS	LOD:0.06 ng/mL; LOQ:0.2 ng/mL	[110]
		1 mL	LLE	-	HPLC-MS/MS	LLOQ: 0.2 ng/mL	[78]
	Serum	200 µL	SPE	-	LC-MS/MS	LOD: 0.06 ng/mL; LOQ: 0.1 ng/mL	[104]
	Urine	-	SPE	BSTFA	GC-MS	LOD/LOQ: 20 ng/mL	[34]
		70 µL	Hydrolysis	-	LC-MS/MS	LLOQ: 20 ng/mL	[91]
		100 µL-500 µL	SPE	-	LC-MS/MS	LOD:0.3 ng/mL LLOQ: 1 ng/mL	[81]
		200 µL	LLE	-	LC-MS/MS	LOD: 0.01 ng/mL; LOQ: 0.02 ng/mL	[94]
		200 µL	LLE	-	HPLC-MS/MS	LLOQ: 10 ng/mL	[78]

		500 µL	LLE	-	UHPLC-MS/MS	LOD:0.06 ng/mL; LOQ:0.2 ng/mL	[110]
		1 mL	Liquid nitrogen	Pyridine + 2% methoxyamine HCl, propionic Anhydride, MSTFA	GC-MS	LOD/LOQ: 30 ng/mL	[138]
		1 mL	LLE (2 different methodology)	-	LC-MS/MS	LOD:1 ng/mL-1 ng/mL	[105]
		1 mL	LLE	2% Methoxyamine HCl; propionic anhydride	GC-MS	LOD: 10 ng/mL; LOQ: 25 ng/mL	[72]
		1 mL	PP (acetonitrile); SPE	10% Hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD:15 ng/mL; LOQ: 25 ng/mL	[38]
		1 mL	Hydrolysis, LLE	MSTFA/ethanethiol/NH_4I (500:4:2)	GC-MS	LOD: 40 ng/mL	[139]
		2 mL	SPE	2%Methoxyamine in pyridine; 1:1 (ethyl acetate and MSTFA-1%TMCS	GC-MS	-	[84]
		2 mL	Hydrolysis, SPE	Methoxylamine 10%; acetonitrile and acetic anhydride	GC-MS	LOD: 20 ng/mL; LOQ: 40 ng/mL	[36]
		2 mL	Hydrolysis; SPE	Acetate buffer/ 10% hydroxylamine; BSTFA	GC-MS	LOQ: 100 ng/mL	[70]
		2 mL	Hydrolysis; SPE	BSTFA+1%TMCS	GC-MS	LOD: 7000 ng/mL; LOQ: 12000 ng/mL	[140]
		2 mL	Hydrolysis, LLE	MSTFA+1%TMCS	GC-MS	LOD: 25 ng/mL	[141]
		3 mL	SPE	Methoxyamine HCl in pyridine; BSTFA+pyridine	GC-MS	LOQ: 150 ng/mL	[143]
		3 mL	Hydrolysis; SPE	10% hydroxylamine; BSTFA+1%TMCS	GC-MS	LOD: 25 ng/mL; LOQ: 50 ng/mL	[66]
		3 mL	Hydrolysis, LLE	MSTFA/NH_4I/DTE (1000:2:4, v/w/w)	GC-MS	LOD: 100 ng/mL	[142]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA	GC-MS	LOD: 200 ng/mL	[144]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH_4I/ethanethiol (640:1:2, v/w/v)	GC-MS	LOD: 100 ng/mL	[145]
	Oral Fluid	200 µL	SPE	-	LC-MS/MS	LOD: 2 ng/mL; LOQ: 4 ng/mL	[90]
		750 µL	SPE	-	LC-MS-MS	LOD: 0.04 ng/mL; LLOQ: 1.5 ng/mL	[87]
Fentanyl	Blood	200 µL	SPE	-	UHPLC-MS/MS	0.004 mg/Kg	[109]
		500 µL	SPE	-	LC-MS/MS	LLOQ: 0.1 ng/mL	[93]
		1 mL	SPE	-	UPLC-MS/MS	LOQ:0.5 ng/mL	[111]

		1 mL	SPE	MSTFA	GC-MS	LOD and LOQ: 0.10 ng/mL	[153]
		1 mL	SPE	-	LC-MS/MS	LOD: 0,5 ng/mL	[84]
		1 mL	LLE	-	LC-MS/MS	LOD: 0.08 ng/mL; LOQ:0.1 ng/mL	[97]
		2 mL	Acetonitrile; SPE	-	LC-MS/MS	LOQ:1.0 ng/mL	[103]
		2 mL	LLE/ SPE	-	GC-MS	LOD: 50/50 ng/mL	[148]
	Plasma	50 µL	PP (acetonitrile)	-	UPLC-MS/MS	LLOQ: 0.10 ng/mL	[115]
		200 µL	PP (acetonitrile+ acetone), LLE	-	UPLC-MS/MS	LLOQ: 0.100 ng/mL	[113]
		250 µL	LLE	-	LC-MS/MS	LOD: 0.01 ng/mL LOQ: 0.05 ng/mL	[92]
	Serum	50 µL	PP, filtration	-	HPLC-MS/MS	LOD: 1.2 ng/mL; LOQ: 7.4 ng/mL	[77]
		200 µL	SPE	-	LC-MS/MS	LOD: 0.02 ng/mL; LOQ: 1.0 ng/mL	[104]
	Urine	250 µL	LLE	-	LC-MS/MS	LOD: 0.03 ng/mL LOQ: 0.09 ng/mL	[92]
		1 mL	LLE (2 different methodology)	-	LC-MS/MS	LOD:0.5 ng/mL-1 ng/mL	[105]
		1 mL	Hydrolysis, LLE	-	LC-MS/MS	LOD: 0.1 ng/mL; LOQ: 0.1 ng/mL	[97]
		2 mL	Hydrolysis; SPE	BSTFA+1%TMCS	GC-MS	LOD: 200 ng/mL; LOQ: 300 ng/mL	[140]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA	GC-MS	LOD: 20 ng/mL	[144]
		4 mL (3+1 mL)	Hydrolysis, LLE	MSTFA/NH_4I/ethanethiol (640:1:2, v/w/v)	GC-MS	LOD: 100 ng/mL	[145]

TCA (Trichloroacetic acid), HCL (hydrochloric acid), SLE (supported liquid extraction), DLLME (dispersive liquid-liquid microextraction)

m

Annex 4. GC-MS conditions for all the tested methodologies

Injection volume and mode	2 µL, *Splitless*
Initial Flux	1.0 mL/min
Injector temperature	250 °C
Oven initial temperature/Initial time	90 °C/2 min
Ramp	20 °C/min
Final oven temperature/final time	300 °C/8 min
Ionization mode/acquisition	EI/SIM
Detector temperature	280 °C

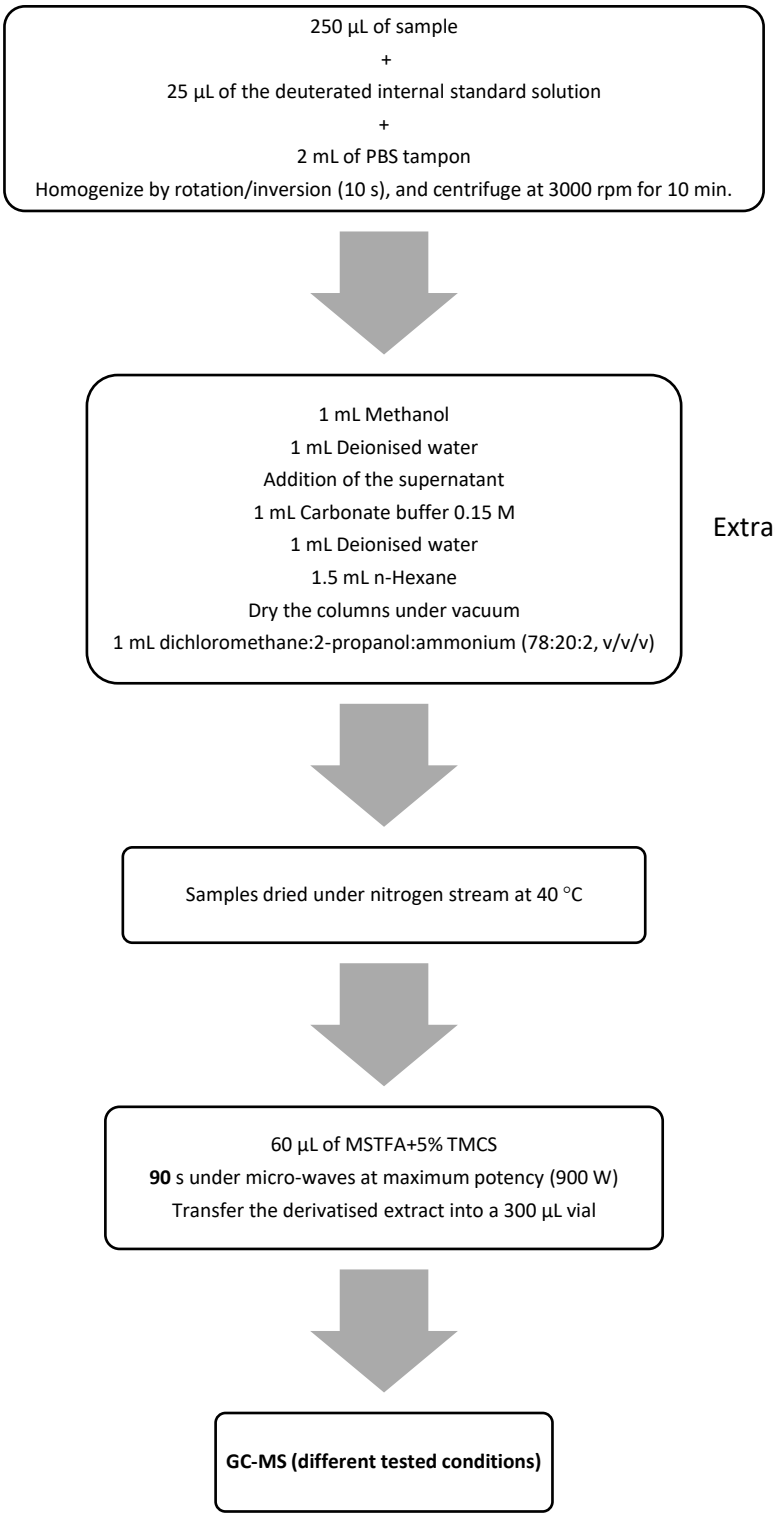
Injection volume and mode	2 µL, *Splitless*
Initial Flux	1.2 mL/min
Injector temperature	250 °C
Oven initial temperature/Initial time	140 °C/1 min
Ramp 1	120 °C/min until 225 °C (hold: 5.29);
Ramp 2	120 °C/min until 275 °C (hold: 5.20);
Final oven temperature/final time	300 °C/2.50 min
Ionization mode/acquisition	EI/SIM
Detector temperature	280 °C

Validated and optimized GC-MS method

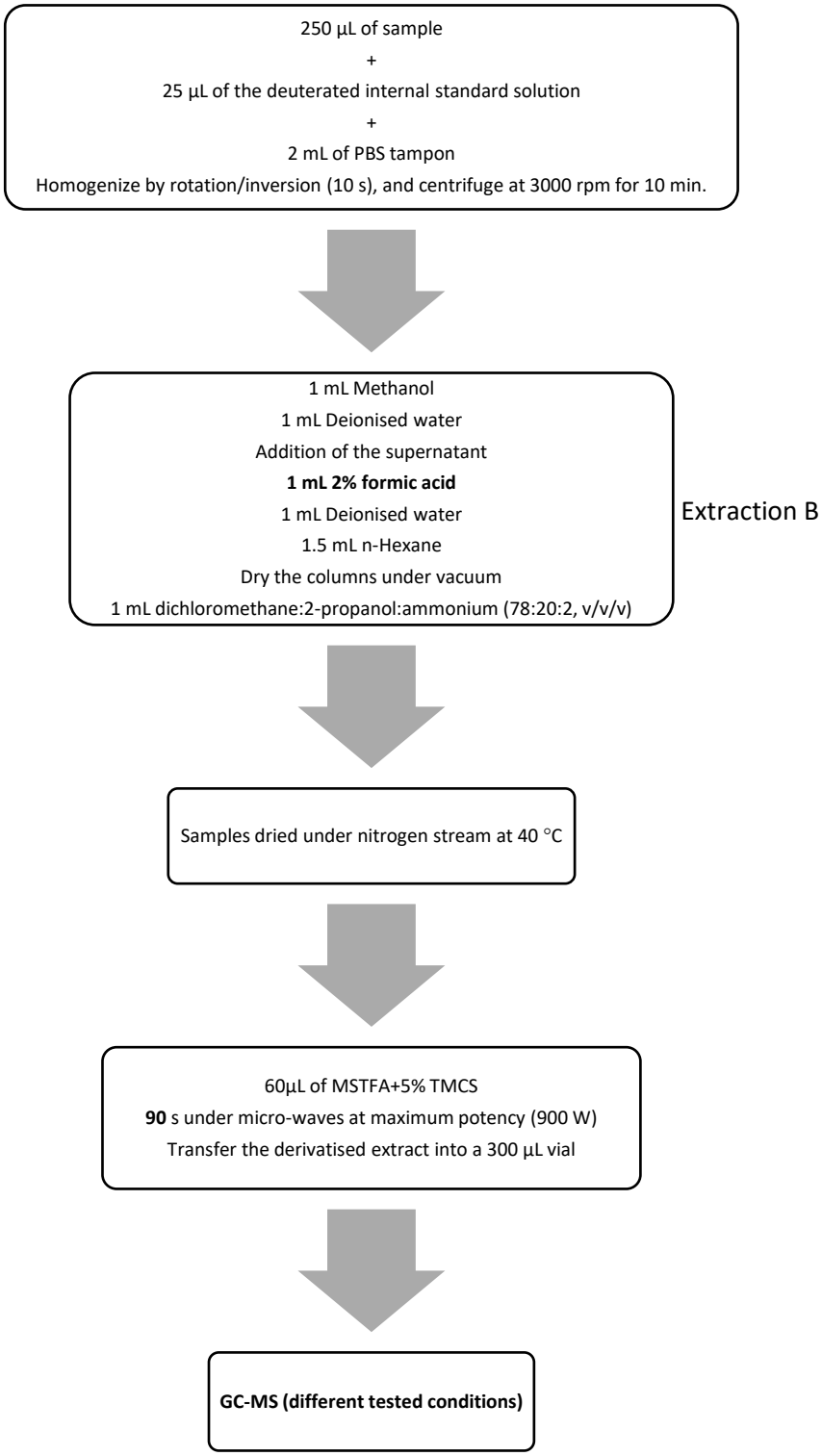
Injection volume and mode	2 µL, *Splitless*
Initial Flux	1 mL/min
Injector temperature	250 °C
Oven initial temperature/Initial time	50 °C /1 min
Ramp	20 °C/min
Final oven temperature/final time	300 °C /2 min
Ionization mode/acquisition	EI/SIM
Detector temperature	280 °C

n

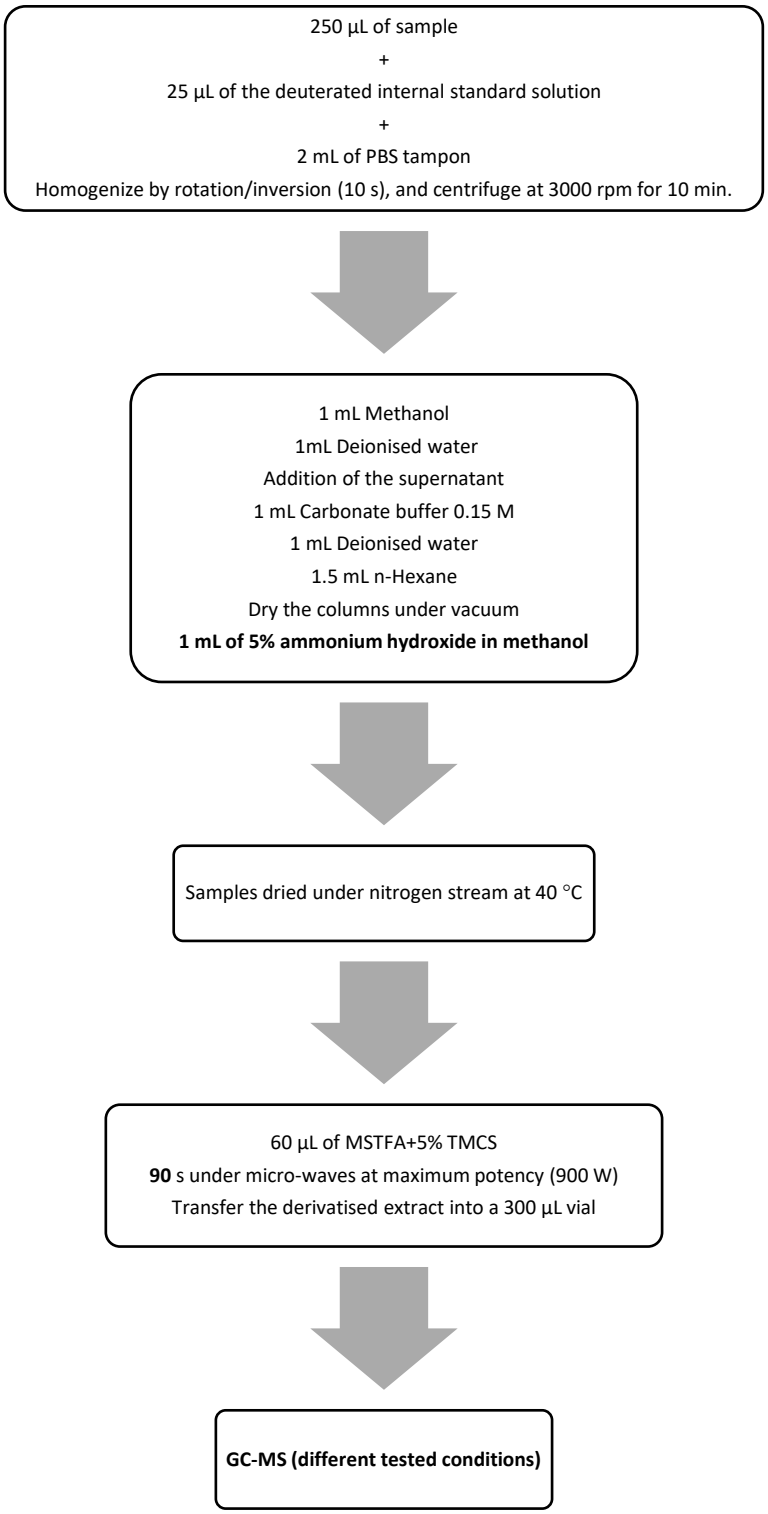
Annex 5. Procedure 1



Annex 6. Procedure 2

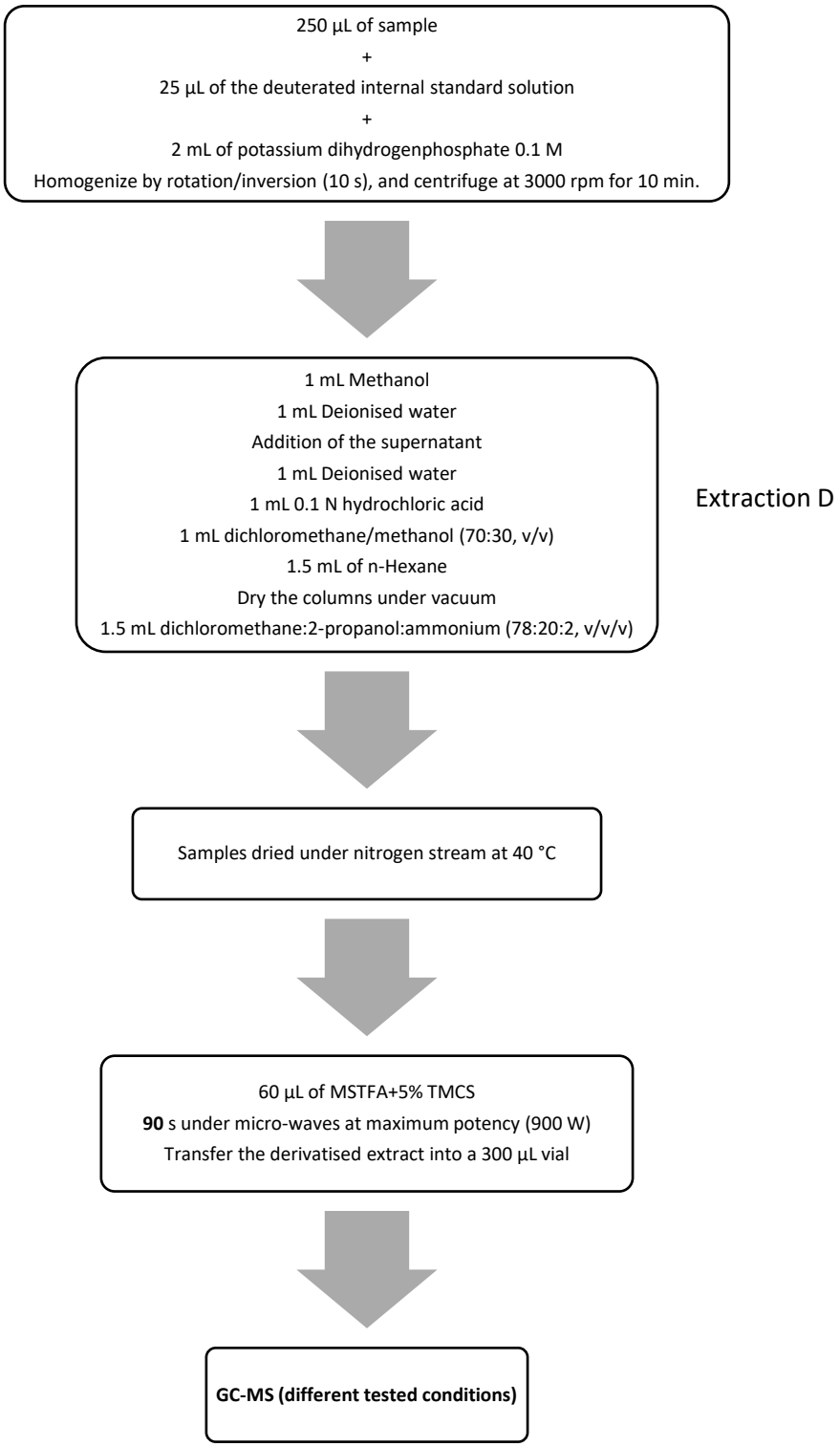


Annex.7. Procedure 3



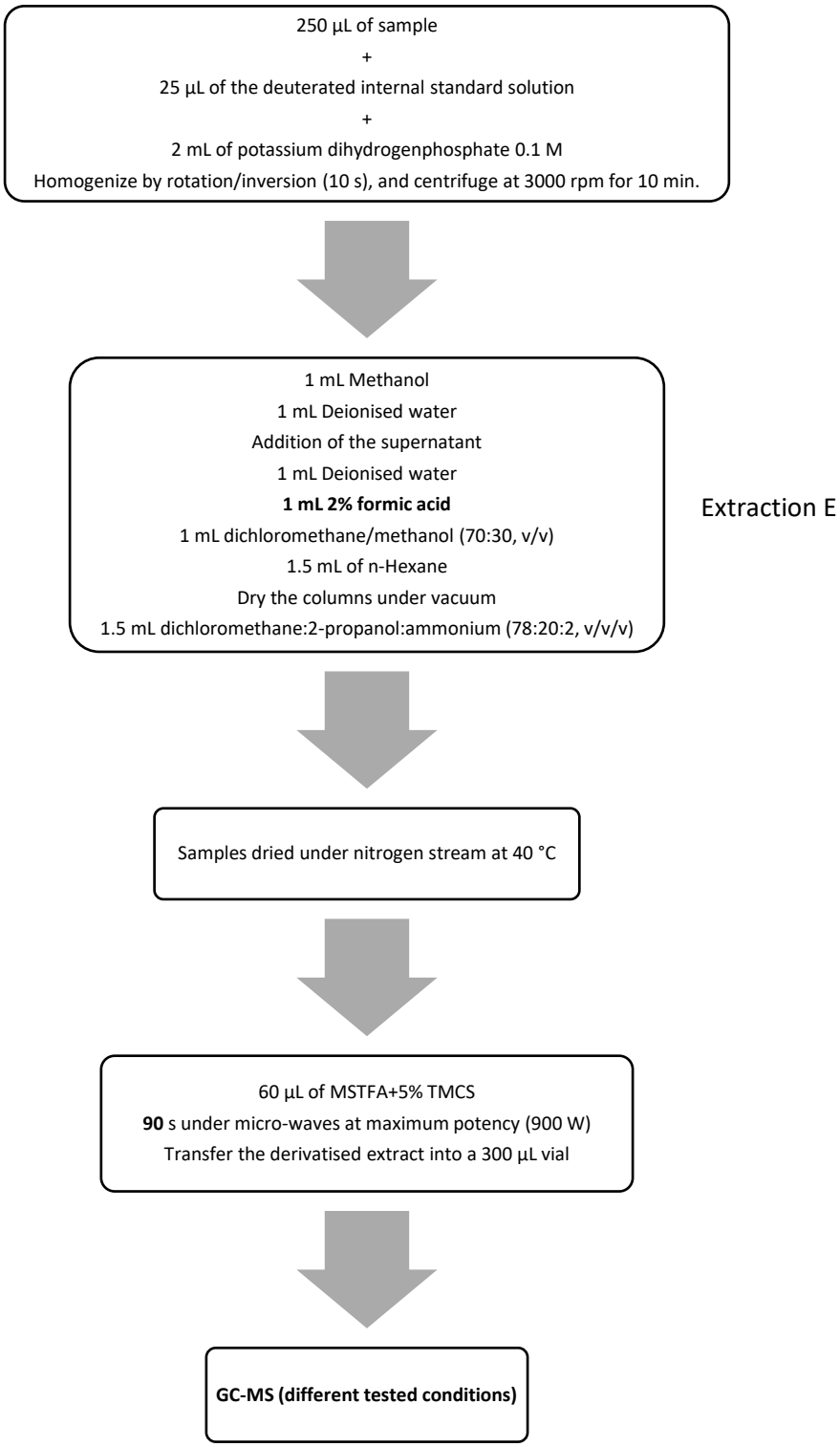
Extraction C

Annex 8. Procedure 4

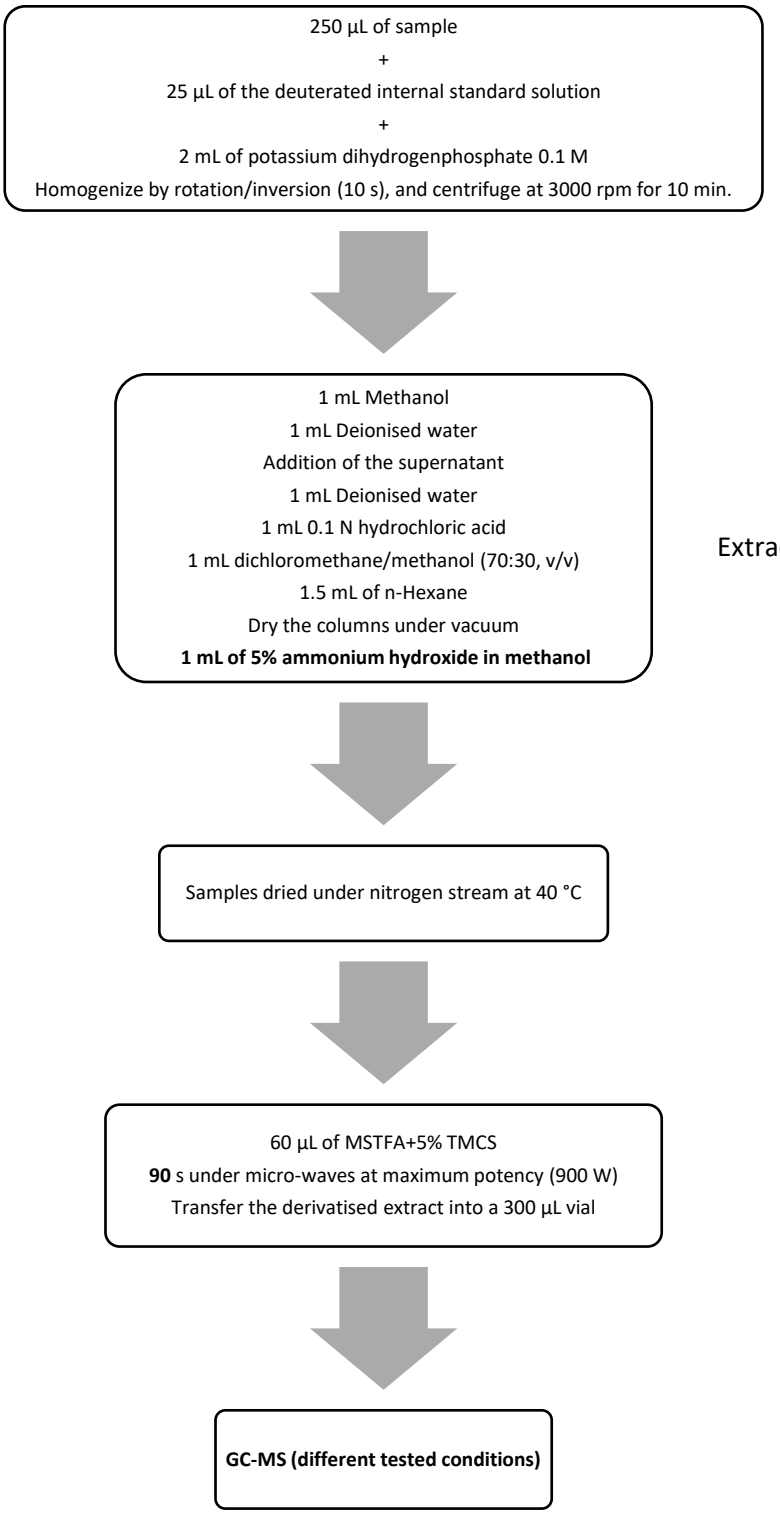


r

Annex 9. Procedure 5

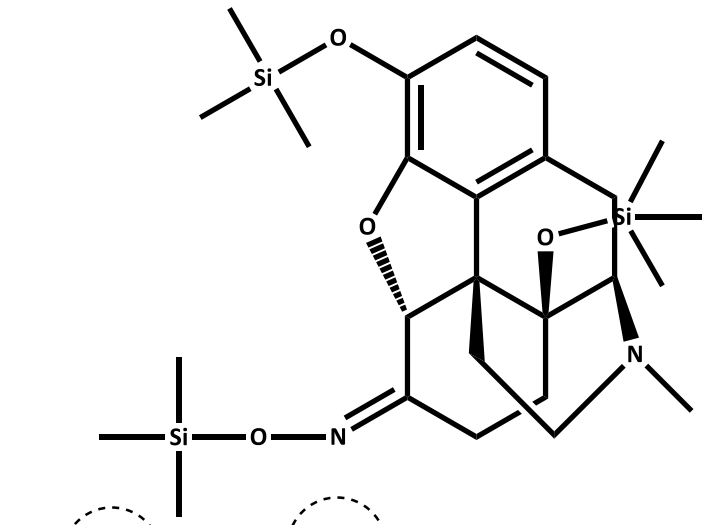


Annex 10. Procedure 6



t

Annex 11. *Oxymorphone -oxime-3TMS example fragments*



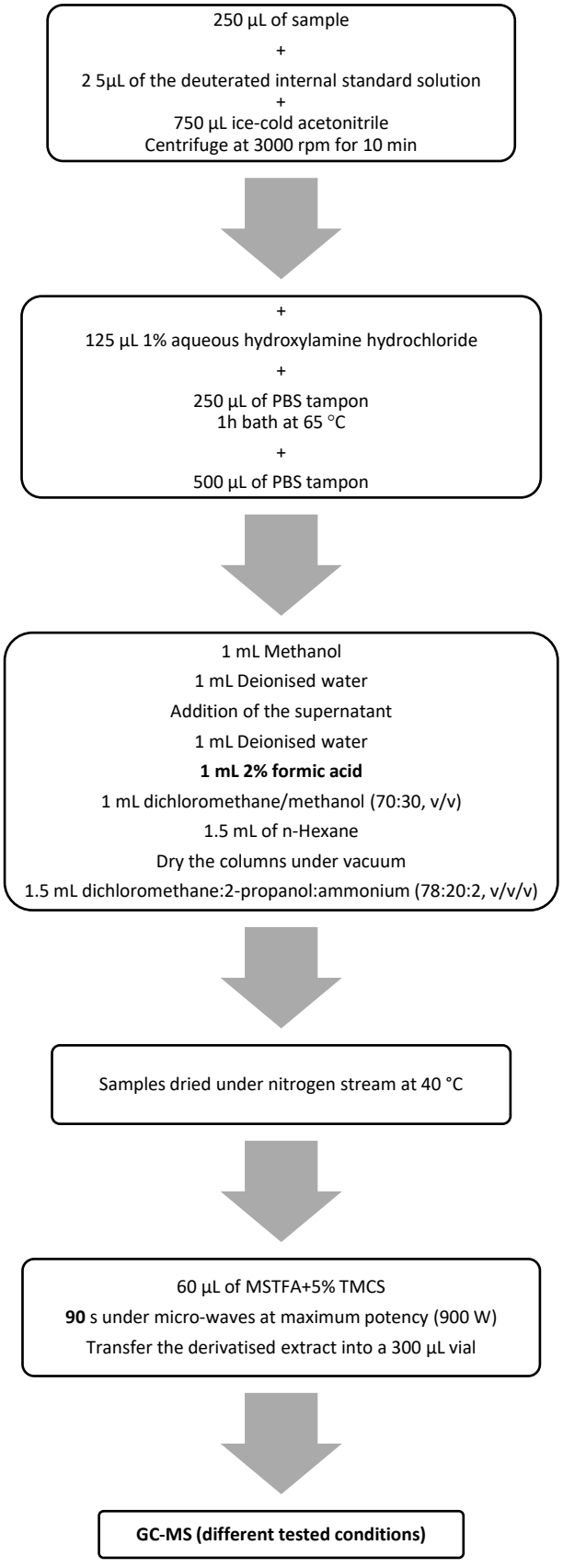
m/z: 532.26 (100.0%), 533.26 (44.1%), 534.26 (15.4%), 534.27 (4.9%), 535.26 (4.3%), 535.27 (1.2%)

$\cdot CH_3$

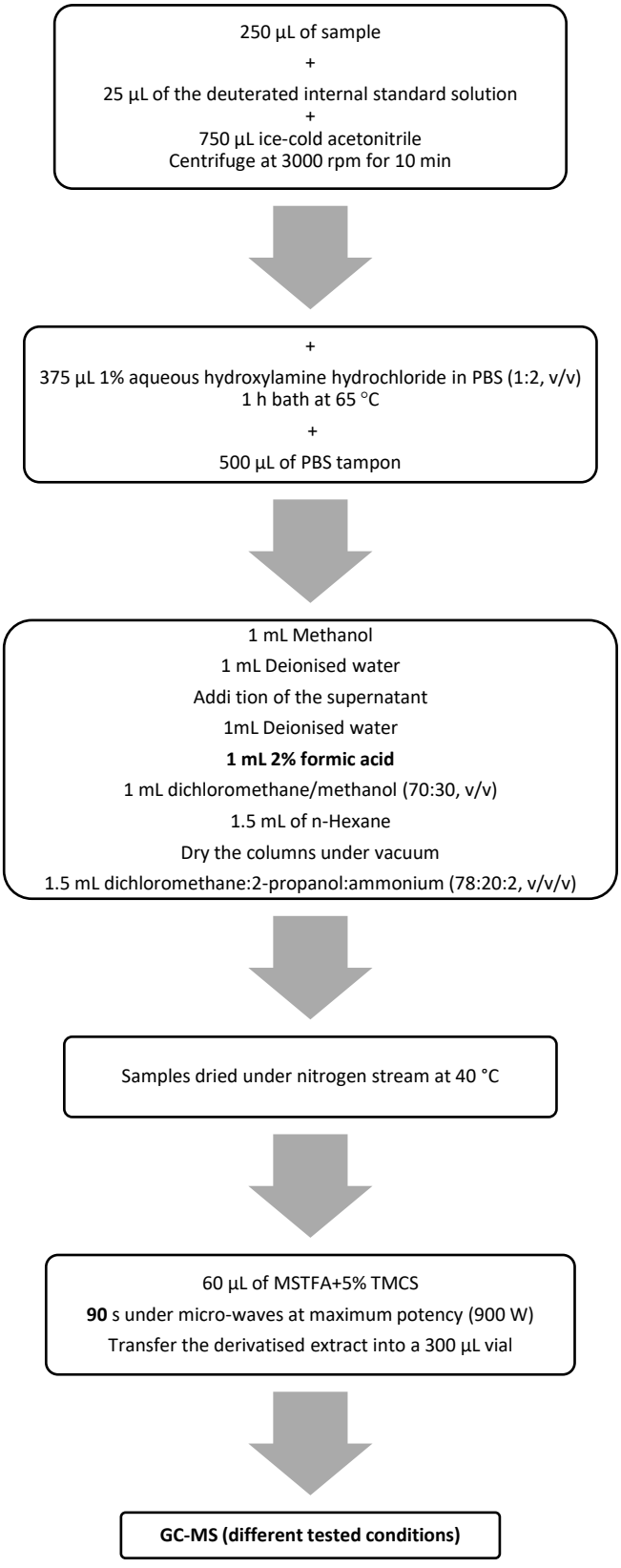
Chemical Formula: $CH_3^{\bullet}$
Exact Mass: 15.02

Chemical Formula: $C_{25}H_{41}N_2O_4Si_3^+$
Exact Mass: 517.24

Annex 12. Procedure 7



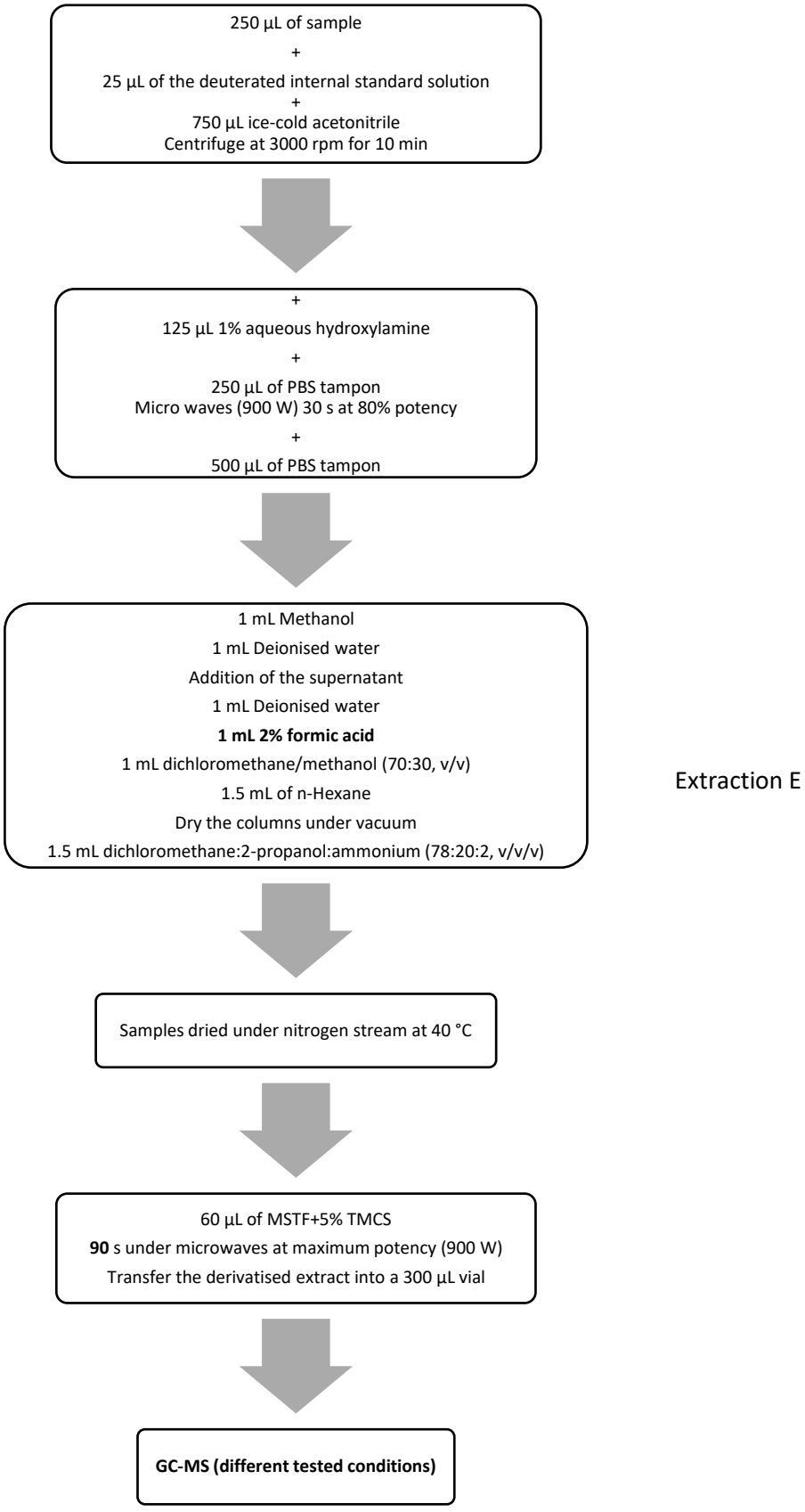
Annex 13. Procedure 8



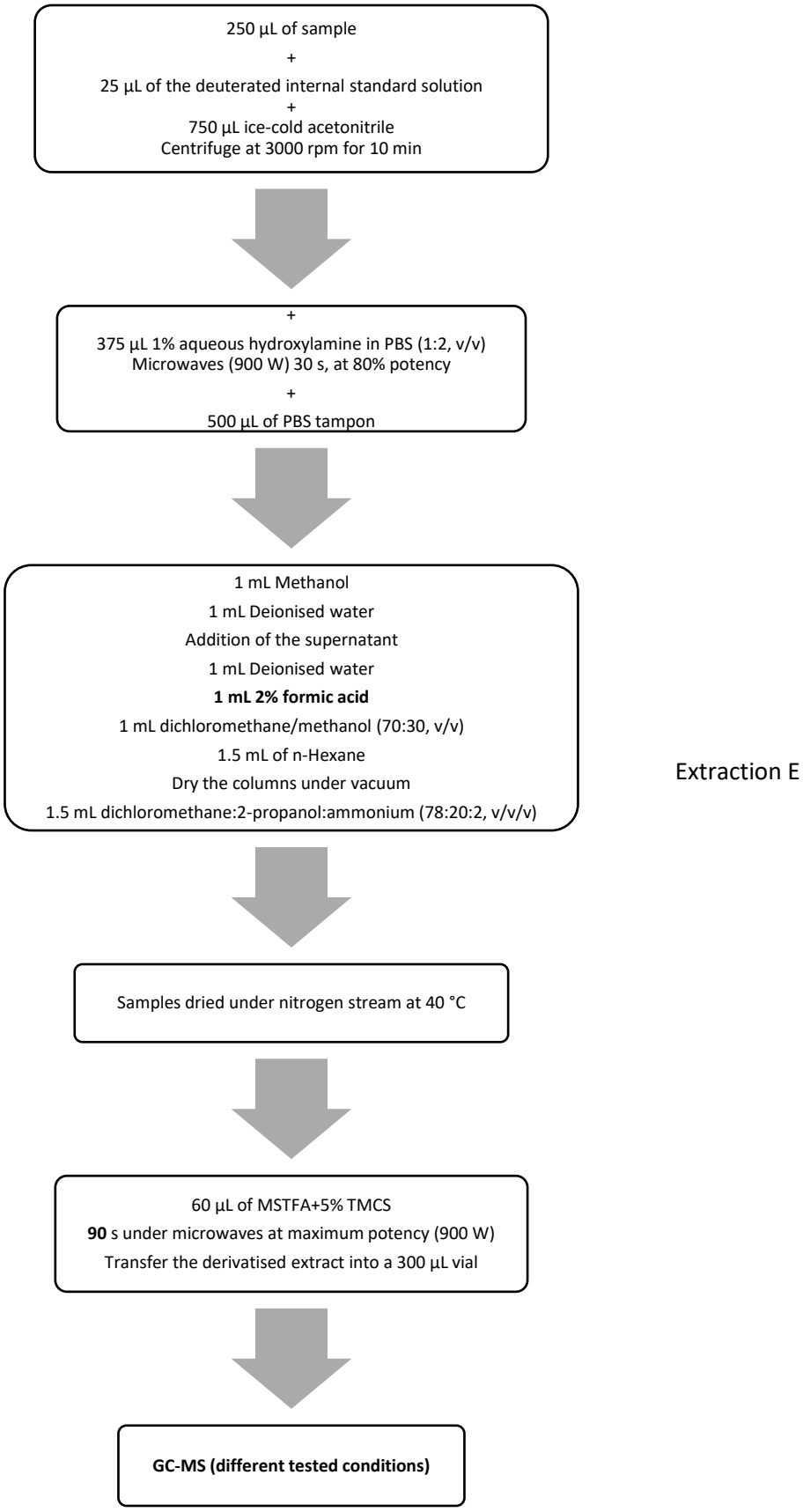
Extraction E

w

Annex 14. Procedure 9



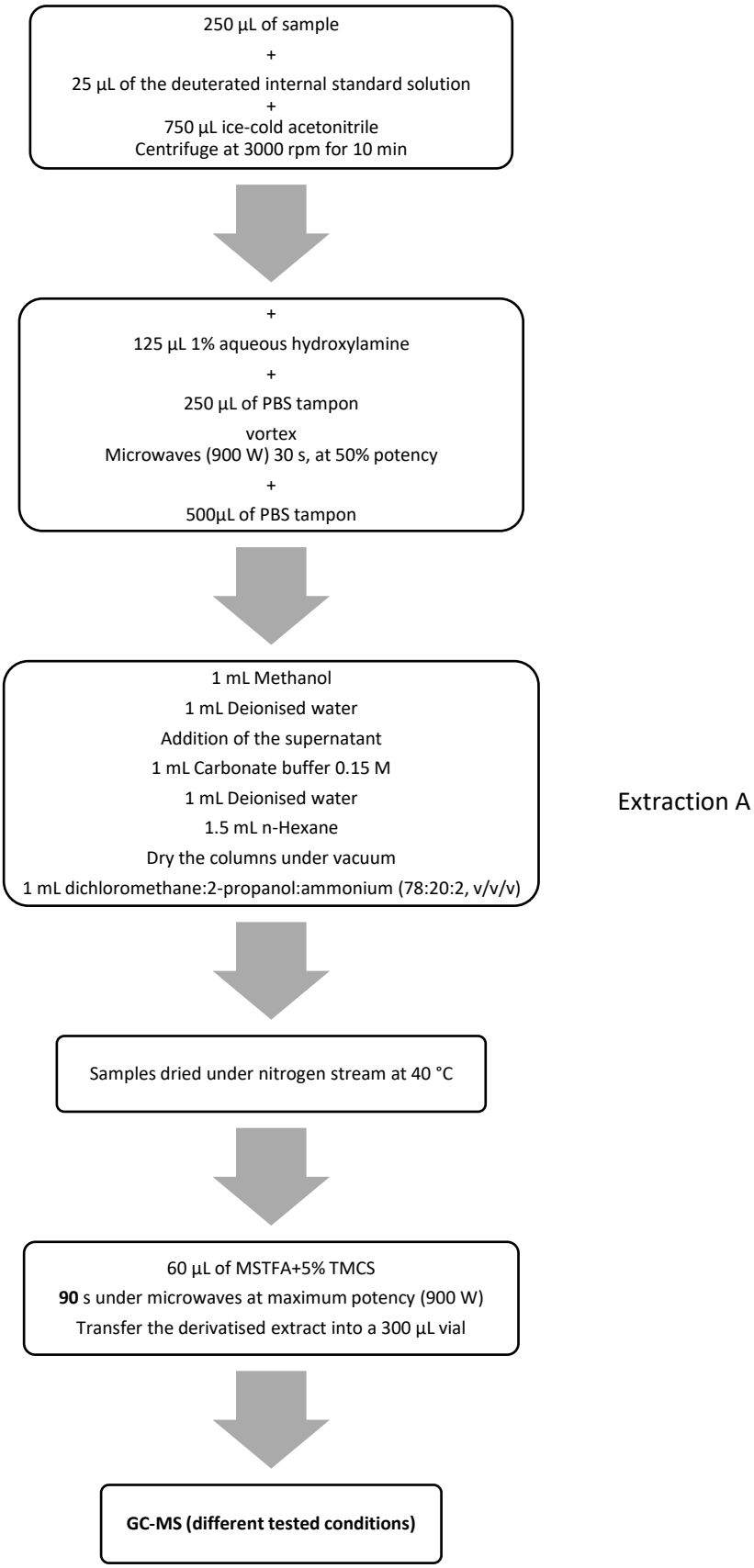
Annex 15. Procedure 10



Extraction E

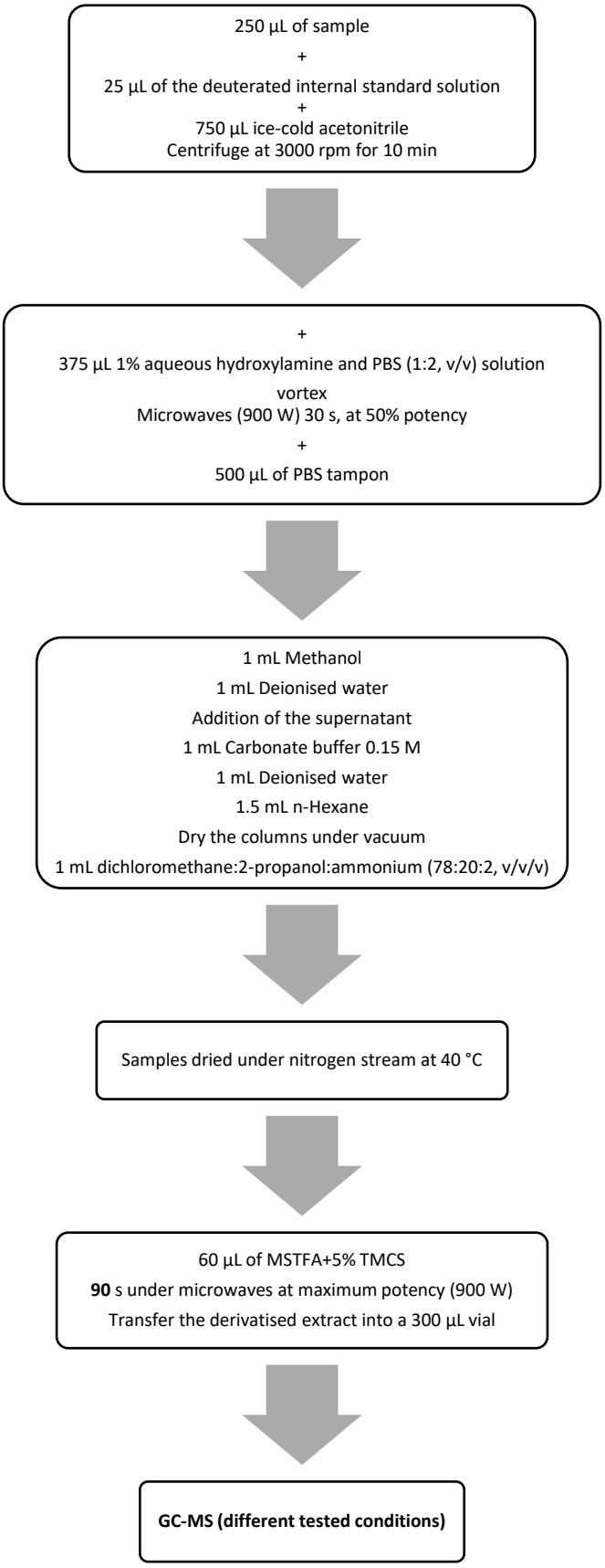
y

Annex 16. Procedure 11



z

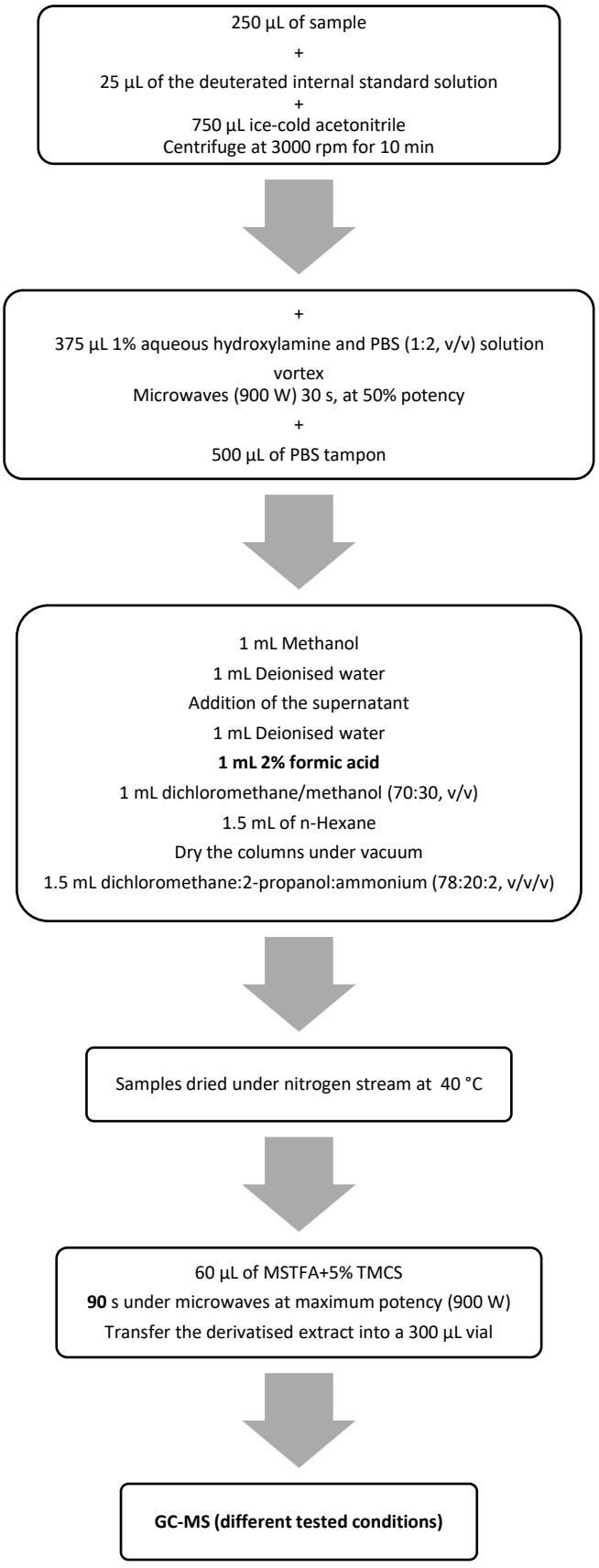
Annex 17. Procedure 12



Extraction A

aa

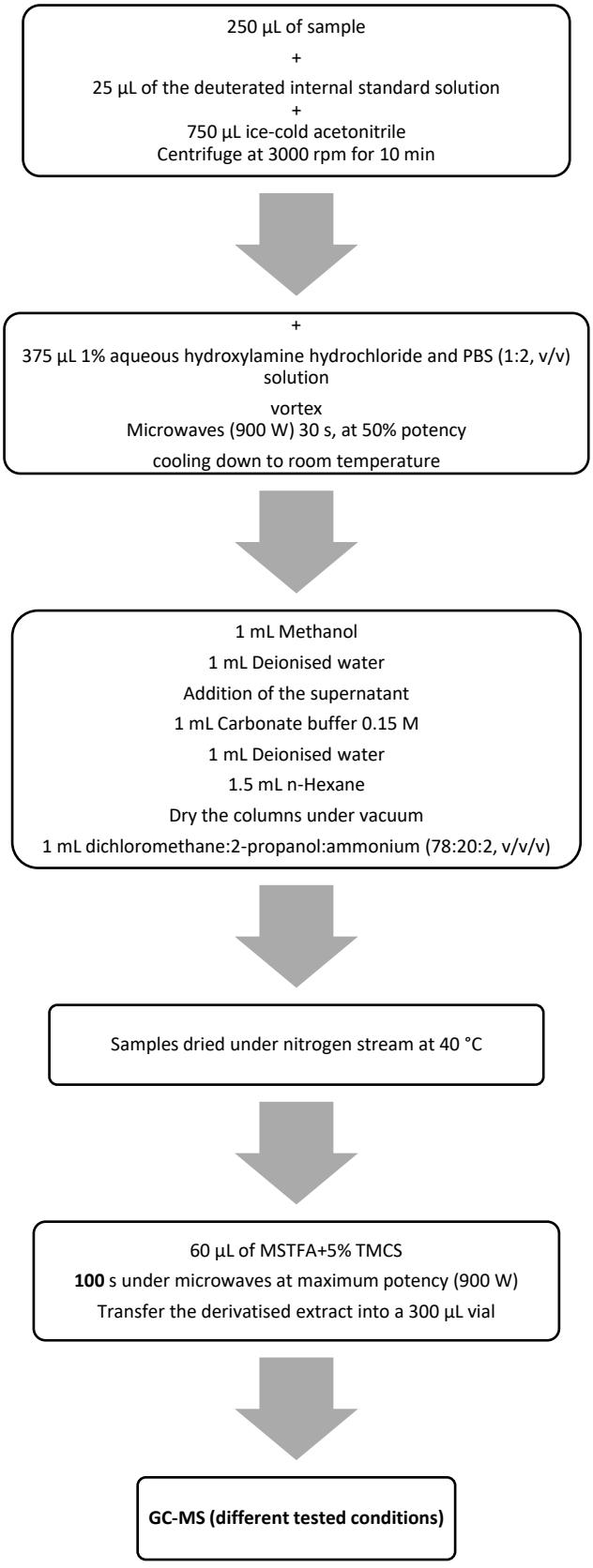
Annex 18. Procedure 13



Extraction E

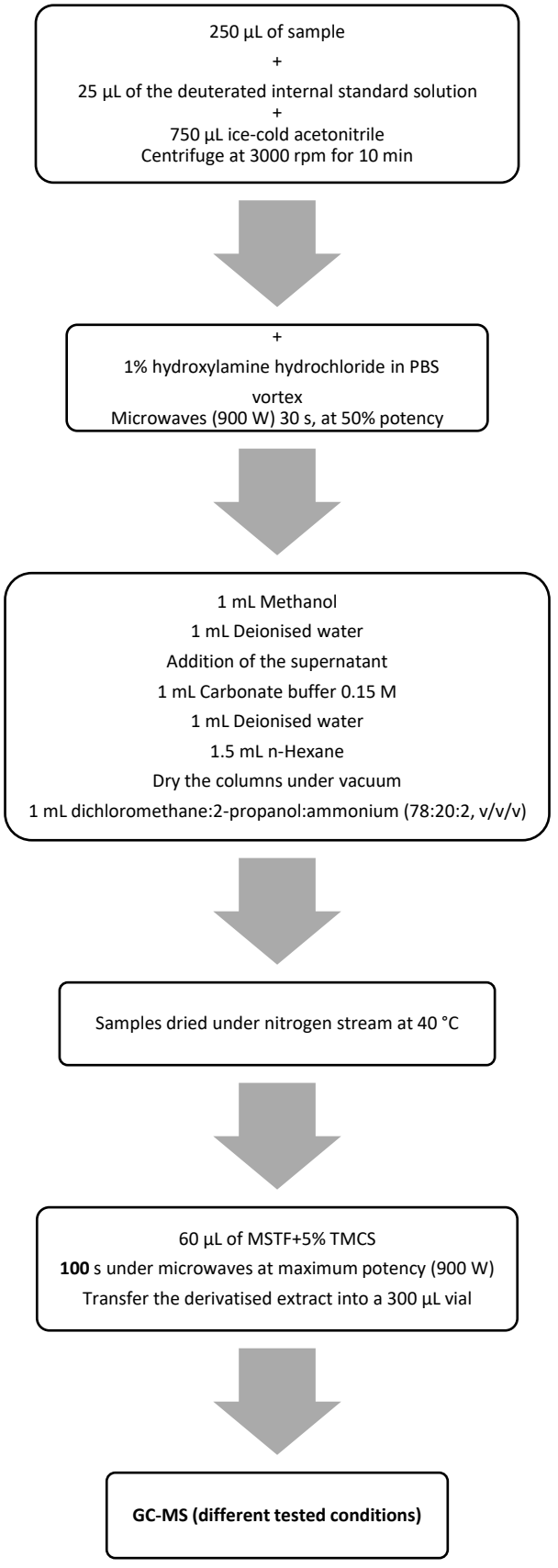
bb

Annex 19. Procedure 14



Extraction A

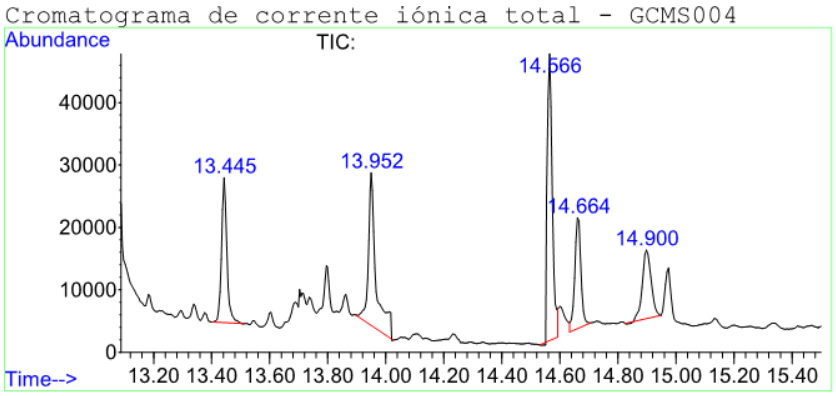
Annex 20- Procedure 15



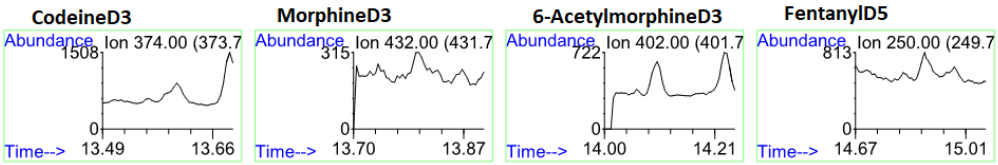
Extraction A

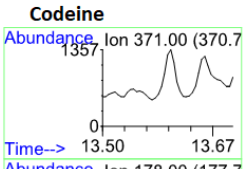
dd

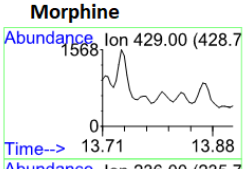
Annex 21. *Cromatograms*

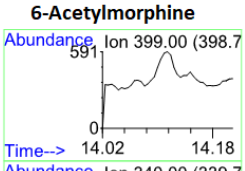


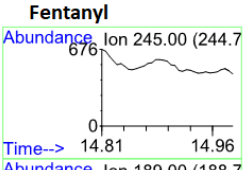
Interferences study

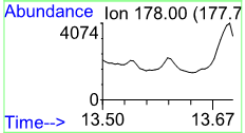


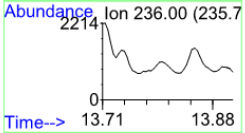


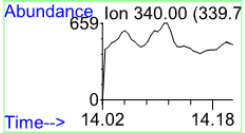


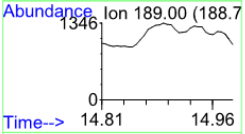


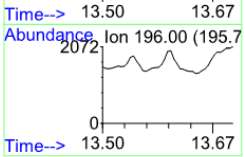


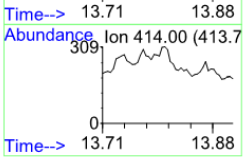


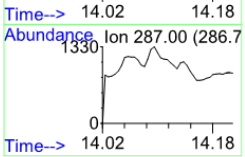


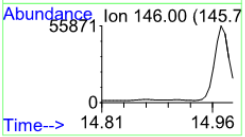


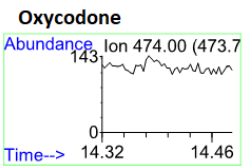


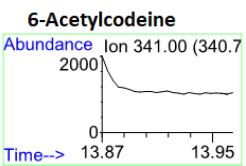


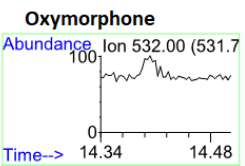


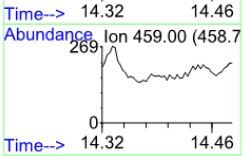


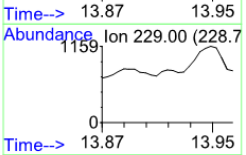


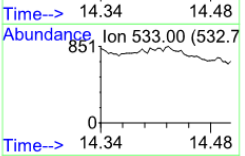


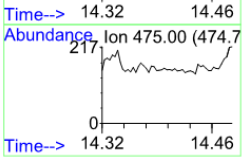


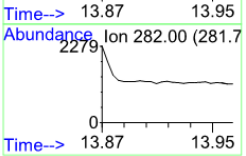


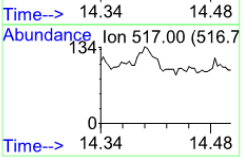






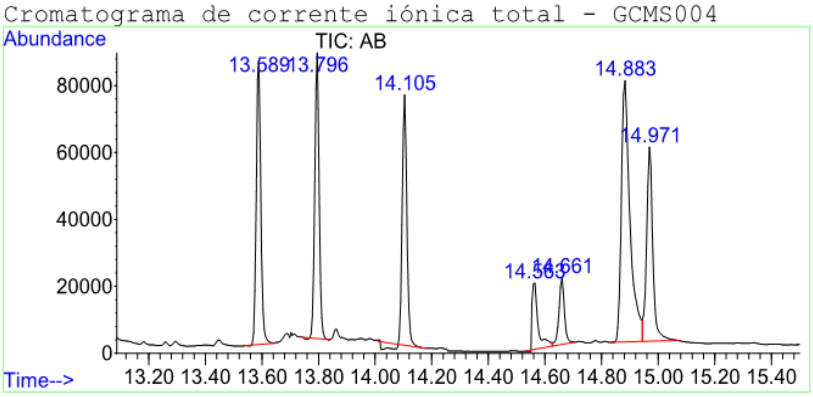






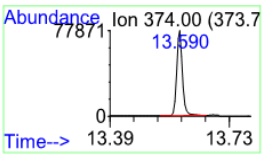
ee

Cromatograma de corrente iónica total - GCMS004

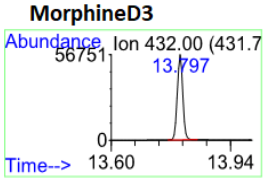


Blank Sample + IS (Blood)

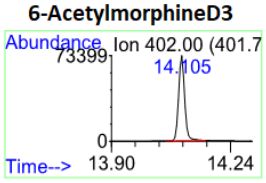
CodeineD3



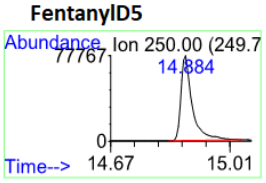
MorphineD3



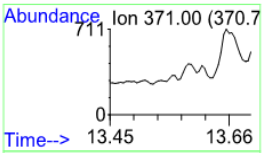
6-AcetylmorphineD3

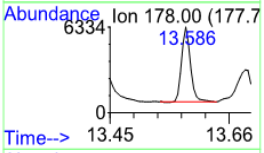


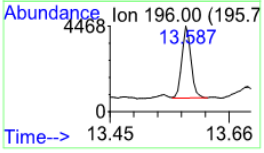
FentanylD5



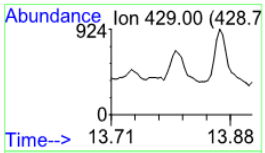
Codeine

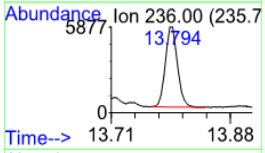


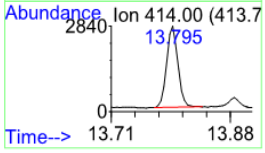




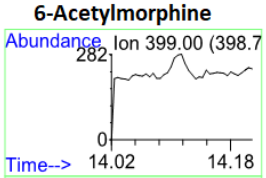
Morphine

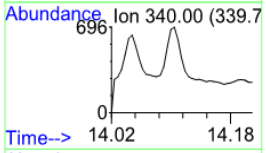


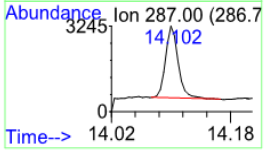




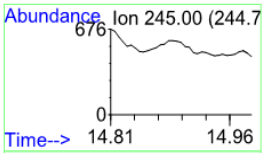
6-Acetylmorphine

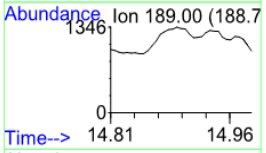


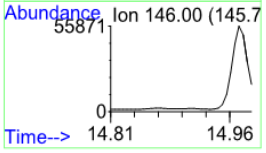




Fentanyl







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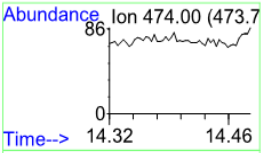
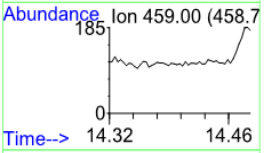
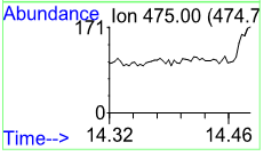
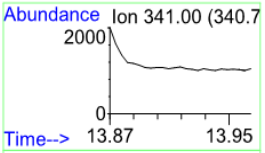
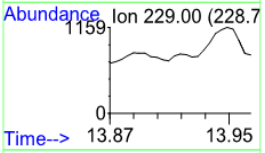


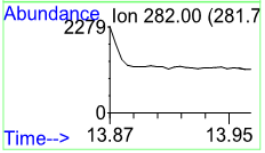




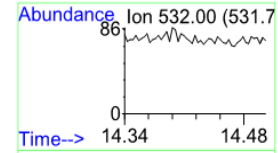
6-Acetylcodeine

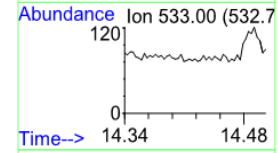


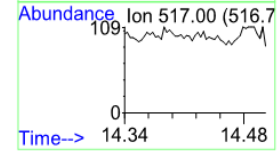




Oxymorphone







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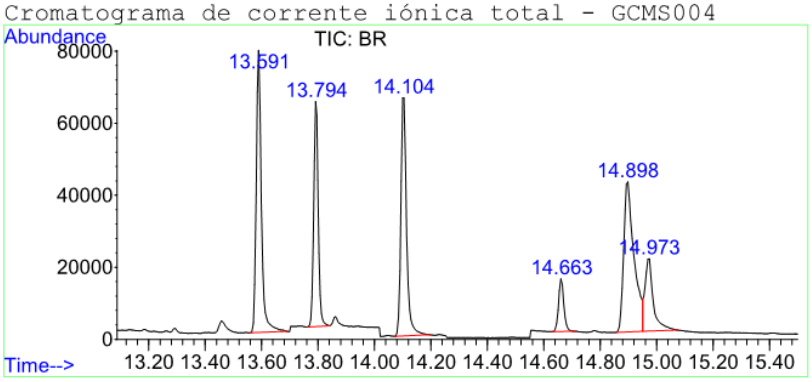
Cromatograma de corrente iónica total - GCMS004



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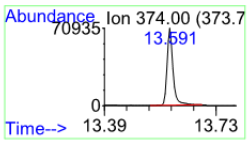
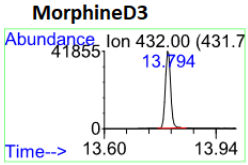
CodeineD3



MorphineD3



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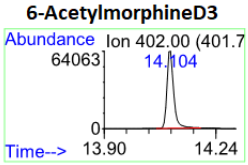

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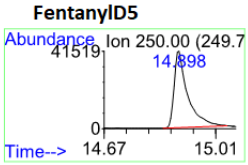
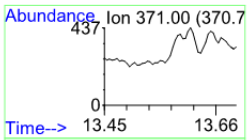
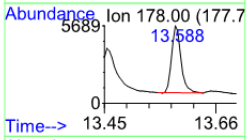
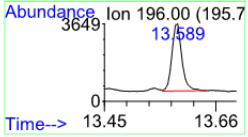


Codeine







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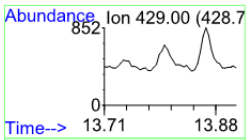

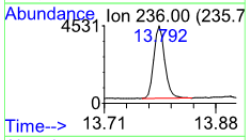

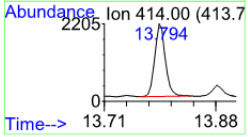

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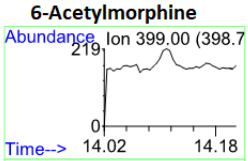
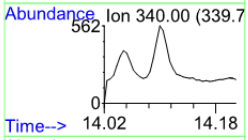
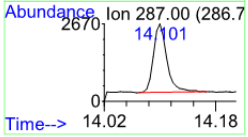
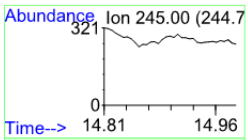
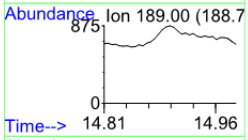


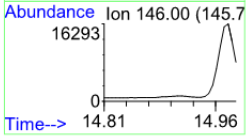




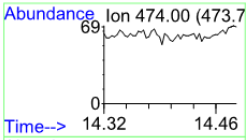
Fentanyl

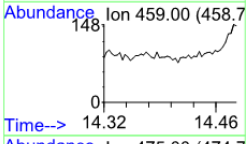


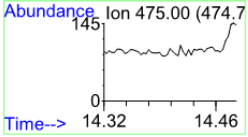




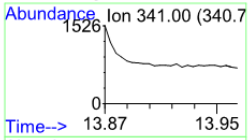
Oxycodone

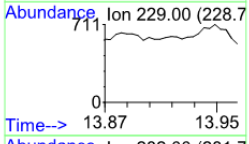


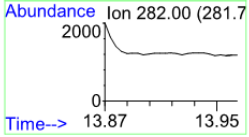




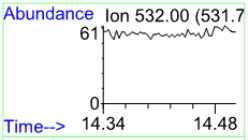
6-Acetylcodeine

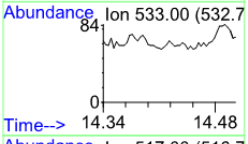


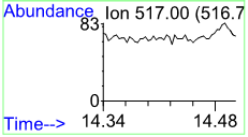




Oxymorphone







gg

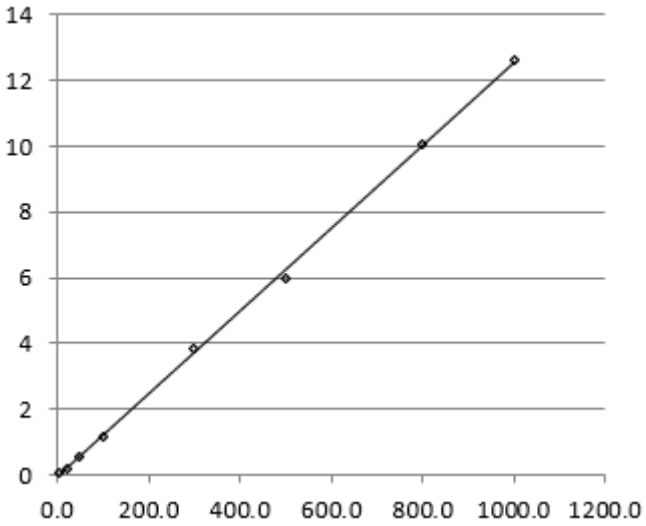
Annex 22. *Codeine linearity with different regression models*

Substância:	Codeína	Série Resultados "> r"	
Padrão Interno:	Codeína-d3	Concentrações Calculadas	650.22
Limite r:	20.00%		1053.35
Veracidade:	20.00%	Fator de ponderação (w) =	1 (não ponderada)
Unidades:	ng/mL	Declive (b) =	0.012597
Série:		Intercepção (a) =	-0.053780
Operador:	EF	r^2 =	0.999489
Data:	Rubrica	$S_{y/x}$ =	0.118065
Informação:	0	$CV_{P.L}$ =	9.74%
Observações:	Electrónica(PAUT058) PAUT022,	n =	8

Níveis CC	Conc. Teórica ng/mL	FD	Codeína Área	Codeína-d3 Área	Razão $A_{Subst.} / A_{PI}$	Conc. Calculada ng/mL	Resíduos	Desvio %
CL5-LP-A-190411	5.0	1.0	85912	1372566	0.062592254	3.237945438	4.238	84.8%
CL20-LP-A-190411	20.0	1.0	295278	1371929	0.215228339	21.35456231	1.355	6.8%
CL50-LP-A-190411	50.0	1.0	690122	1207146	0.57163721	49.65131244	-0.348	-0.7%
CL100-LP-A-190411	100.0	1.0	1465168	1264198	1.158970351	96.27105668	-3.729	-3.7%
CL300-LP-A-190411	300.0	1.0	4464212	1159397	3.850460136	309.9279472	9.928	3.3%
CL500-LP-A-190411	500.0	1.0	6541920	1089576	6.004037007	480.8887736	-19.111	-3.8%
CL800-LP-A-190411	800.0	1.0	13050736	1295851	10.07117022	803.7427625	3.743	0.5%
CL1000-LP-A-190411	1000.0	1.0	18471429	1466811	12.59231688	1003.325034	3.925	0.4%

Substância:	Codeína	Série Resultados "> r"	
Padrão Interno:	Codeína-d3	Concentrações Calculadas	650.22
Limite r:	20.00%		1053.35
Veracidade:	20.00%	Fator de ponderação (w) =	1/x
Unidades:	ng/mL	Declive (b) =	0.012477
Série:		Intercepção (a) =	-0.012132
Operador:	EF	r^2 =	0.999192
Data:	Rubrica	$S_{y/x}$ =	0.128121
Informação:	0	$CV_{P.L}$ =	9.74%
Observações:	Electrónica(PAUT058) PAUT022,	n =	8

Níveis CC	Conc. Teórica ng/mL	FD	Codeína Área	Codeína-d3 Área	Razão $A_{Subst.} / A_{PI}$	Conc. Calculada ng/mL	Resíduos	Desvio %
CL5-LP-A-190411	5.0	1.0	85912	1372566	0.062592254	5.988889892	0.989	19.8%
CL20-LP-A-190411	20.0	1.0	295278	1371929	0.215228339	18.22210405	-1.778	-8.9%
CL50-LP-A-190411	50.0	1.0	690122	1207146	0.57163721	46.79175741	-3.208	-6.4%
CL100-LP-A-190411	100.0	1.0	1465168	1264198	1.158970351	93.8595141	-6.140	-6.1%
CL300-LP-A-190411	300.0	1.0	4464212	1159397	3.850460136	309.5724086	9.572	3.2%
CL500-LP-A-190411	500.0	1.0	6541920	1089576	6.004037007	482.178384	-17.822	-3.6%
CL800-LP-A-190411	800.0	1.0	13050736	1295851	10.07117022	808.1391661	8.139	1.0%
CL1000-LP-A-190411	1000.0	1.0	18471429	1466811	12.59231688	1010.247776	10.248	1.0%



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Substância:	**Codeína**	Série Resultados "> r	
Padrão Interno:	Codeína-d3	Concentrações Calculadas	650.22 1053.35
Limite r:	20.00%		
Veracidade:	20.00%	Fator de ponderação (w) =	1/x^2
Unidades:	ng/mL	Declive (b) =	0.012021
Série:		Intercepção (a) =	0.000571
Operador:	EF	r^2 =	0.995846
Data:	Rubrica	$S_{y/x}$ =	0.315214
Informação:	0	$CV_{P.L}$ =	9.74%
Observações:	Electrónica(PAUT058) PAUT022,	n =	8

Níveis CC	Conc. Teórica ng/mL	FD	Codeína Área	Codeína-d3 Área	Razão $A_{Subst.} / A_{PI}$	Conc. Calculada ng/mL	Resíduos	Desvio %
CL5-LP-A-190411	5.0	1.0	85912	1372566	0.062592254	5.159614572	0.160	3.2%
CL20-LP-A-190411	20.0	1.0	295278	1371929	0.215228333	17.85756098	-2.142	-10.7%
CL50-LP-A-190411	50.0	1.0	690122	1207146	0.57163721	47.51255783	-2.487	-5.0%
CL100-LP-A-190411	100.0	1.0	1465168	1264198	1.158370351	96.36838955	-3.632	-3.6%
CL300-LP-A-190411	300.0	1.0	4464212	1159397	3.850460196	320.2760837	20.276	6.8%
CL500-LP-A-190411	500.0	1.0	6541920	1089576	6.004037007	499.4392536	-0.561	-0.1%
CL800-LP-A-190411	800.0	1.0	13050736	1295851	10.07117022	837.7830846	37.783	4.7%
CL1000-LP-A-190411	1000.0	1.0	18471429	1466811	12.59231688	1047.563676	47.570	4.8%

Substância:	**Codeína**	Série Resultados "> r	
Padrão Interno:	Codeína-d3	Concentrações Calculadas	650.22 1053.35
Limite r:	20.00%		
Veracidade:	20.00%	Fator de ponderação (w) =	1/x^(1/2)
Unidades:	ng/mL	Declive (b) =	0.012543
Série:		Intercepção (a) =	-0.027213
Operador:	EF	r^2 =	0.999497
Data:	Rubrica	$S_{y/x}$ =	0.120514
Informação:	0	$CV_{P.L}$ =	9.74%
Observações:	Electrónica(PAUT058) PAUT022,	n =	8

Níveis CC	Conc. Teórica ng/mL	FD	Codeína Área	Codeína-d3 Área	Razão $A_{Subst.} / A_{PI}$	Conc. Calculada ng/mL	Resíduos	Desvio %
CL5-LP-A-190411	5.0	1.0	85912	1372566	0.062592254	7.159758218	2.160	43.2%
CL20-LP-A-190411	20.0	1.0	295278	1371929	0.215228333	19.32867561	-0.671	-3.4%
CL50-LP-A-190411	50.0	1.0	690122	1207146	0.57163721	47.74816322	-2.252	-4.5%
CL100-LP-A-190411	100.0	1.0	1465168	1264198	1.158370351	94.56854167	-5.431	-5.4%
CL300-LP-A-190411	300.0	1.0	4464212	1159397	3.850460196	309.1476663	9.148	3.0%
CL500-LP-A-190411	500.0	1.0	6541920	1089576	6.004037007	480.8464336	-19.154	-3.8%
CL800-LP-A-190411	800.0	1.0	13050736	1295851	10.07117022	805.0933386	5.094	0.6%
CL1000-LP-A-190411	1000.0	1.0	18471429	1466811	12.59231688	1006.140342	6.140	0.6%

Substância:	**Codeína**	Série Resultados "> r	
Padrão Interno:	Codeína-d3	Concentrações Calculadas	650.22 1053.35
Limite r:	20.00%		
Veracidade:	20.00%	Fator de ponderação (w) =	1/y
Unidades:	ng/mL	Declive (b) =	0.012470
Série:		Intercepção (a) =	-0.013109
Operador:	EF	r^2 =	0.999158
Data:	Rubrica	$S_{y/x}$ =	0.129307
Informação:	0	$CV_{P.L}$ =	9.74%
Observações:	Electrónica(PAUT058) PAUT022,	n =	8

Níveis CC	Conc. Teórica ng/mL	FD	Codeína Área	Codeína-d3 Área	Razão $A_{Subst.} / A_{PI}$	Conc. Calculada ng/mL	Resíduos	Desvio %
CL5-LP-A-190411	5.0	1.0	85912	1372566	0.062592254	6.070502075	1.071	21.4%
CL20-LP-A-190411	20.0	1.0	295278	1371929	0.215228333	18.31043029	-1.690	-8.4%
CL50-LP-A-190411	50.0	1.0	690122	1207146	0.57163721	46.89576378	-3.104	-6.2%
CL100-LP-A-190411	100.0	1.0	1465168	1264198	1.158370351	93.38935309	-6.011	-6.0%
CL300-LP-A-190411	300.0	1.0	4464212	1159397	3.850460196	309.8206392	9.821	3.3%
CL500-LP-A-190411	500.0	1.0	6541920	1089576	6.004037007	482.5213474	-17.479	-3.5%
CL800-LP-A-190411	800.0	1.0	13050736	1295851	10.07117022	808.6610294	8.661	1.1%
CL1000-LP-A-190411	1000.0	1.0	18471429	1466811	12.59231688	1010.880564	10.881	1.1%

Substância:	**Codeína**	Série Resultados	
Padrão Interno:	Codeína-d3	Concentrações Calculadas	650.22
Limite r:	20.00%		1053.35
Veracidade:	20.00%	Fator de ponderação (w) =	1/y^2
Unidades:	ng/mL	Declive (b) =	0.011942
Série:		Intercepção (a) =	0.000538
Operador:	EF	r^2 =	0.995456
Data:	Rubrica	$S_{y/x}$ =	0.357420
Informação:	0	$CV_{P.I.}$ =	9.74%
Observações:	Electrónica(PAUT058) PAUT022,	n =	8

Níveis CC	Conc. Teórica ng/mL	FD	Codeína Área	Codeína-d3 Área	Razão $A_{Subst.} / A_{PI}$	Conc. Calculada ng/mL	Resíduos	Desvio %
CL5-LP-A-190411	5.0	1.0	85912	1372566	0.062592254	5.196475385	0.196	3.9%
CL20-LP-A-190411	20.0	1.0	295278	1371929	0.215228339	17.97844483	-2.022	-10.1%
CL50-LP-A-190411	50.0	1.0	690122	1207146	0.57163721	47.8236631	-2.170	-4.3%
CL100-LP-A-190411	100.0	1.0	1465168	1264198	1.158970351	97.00878036	-2.991	-3.0%
CL300-LP-A-190411	300.0	1.0	4464212	1159397	3.850460136	322.398074	22.398	7.5%
CL500-LP-A-190411	500.0	1.0	6541920	1089576	6.004037007	502.7467684	2.747	0.5%
CL800-LP-A-190411	800.0	1.0	13050736	1295851	10.07117022	843.329424	43.329	5.4%
CL1000-LP-A-190411	1000.0	1.0	18471429	1466811	12.59291688	1054.504175	54.504	5.5%

Substância:	**Codeína**	Série Resultados	
Padrão Interno:	Codeína-d3	Concentrações Calculadas	650.22
Limite r:	20.00%		1053.35
Veracidade:	20.00%	Fator de ponderação (w) =	1/y^(1/2)
Unidades:	ng/mL	Declive (b) =	0.012542
Série:		Intercepção (a) =	-0.027880
Operador:	EF	r^2 =	0.999491
Data:	Rubrica	$S_{y/x}$ =	0.120535
Informação:	0	$CV_{P.I.}$ =	9.74%
Observações:	Electrónica(PAUT058) PAUT022,	n =	8

Níveis CC	Conc. Teórica ng/mL	FD	Codeína Área	Codeína-d3 Área	Razão $A_{Subst.} / A_{PI}$	Conc. Calculada ng/mL	Resíduos	Desvio %
CL5-LP-A-190411	5.0	1.0	85912	1372566	0.062592254	7.213739355	2.214	44.3%
CL20-LP-A-190411	20.0	1.0	295278	1371929	0.215228339	19.38408034	-0.616	-3.1%
CL50-LP-A-190411	50.0	1.0	690122	1207146	0.57163721	47.80689325	-2.193	-4.4%
CL100-LP-A-190411	100.0	1.0	1465168	1264198	1.158970351	94.63274303	-5.367	-5.4%
CL300-LP-A-190411	300.0	1.0	4464212	1159397	3.850460136	309.2369771	9.237	3.1%
CL500-LP-A-190411	500.0	1.0	6541920	1089576	6.004037007	480.9558362	-19.044	-3.8%
CL800-LP-A-190411	800.0	1.0	13050736	1295851	10.07117022	805.2413277	5.241	0.7%
CL1000-LP-A-190411	1000.0	1.0	18471429	1466811	12.59291688	1006.311131	6.311	0.6%

jj

Annex 23. *Table of the analysed positive cases*

kk

Case	Matrix	Age	Gender	Codeine	Morphine	6-MAM	Fentanyl	Other substances
1	Blood	N/A	N/A	5.9 ng/mL	95.9 ng/mL	13.2 ng/mL	-	N/A
2	Blood	N/A	N/A	10.4 ng/mL	128.3 ng/mL	< 5 ng/mL	-	N/A
3	Blood	N/A	N/A	-	6.9 ng/mL	-	-	N/A
4	Blood	N/A	N/A	<5 ng/mL	5.4 ng/mL	-	-	N/A
5	Blood	N/A	N/A	54.8 ng/mL	22.6 ng/mL	-	-	N/A
6	Blood	N/A	N/A	-	75.9 ng/mL	-	-	N/A
7	Blood	N/A	N/A	<5 ng/mL	25.7 ng/mL	-	-	Cocaine; Benzoylecgonine; EME; THC-COOH; Diazepam; Temazepam; Oxazepam; Nordiazepam; Levomepromazine; Haloperidol
8	Blood	N/A	N/A	11.8 ng/mL	174.7 ng/mL	-	-	THC; 11-OH-THC; THC-COOH; Cyamemazine
9	Blood	N/A	N/A	11.9 ng/mL	142.8 ng/mL	-	-	N/A
10	Blood	N/A	N/A	-	15.8 ng/mL	-	-	N/A
11	Blood	N/A	N/A	-	56.5 ng/mL	-	-	N/A
12	Blood	N/A	N/A	-	26.7 ng/mL	-	20.3 ng/mL	N/A
13	Blood	N/A	N/A	-	<5 ng/mL	-	-	N/A
14	Blood	N/A	N/A	-	31.4 ng/mL	-	-	N/A
15	Blood	N/A	N/A	-	11.7 ng/mL	-	-	N/A
16	Blood	N/A	N/A	-	<5 ng/mL	-	-	N/A
17	Blood	N/A	N/A	-	12.8 ng/mL	-	-	N/A
18	Blood	N/A	N/A	-	20.4 ng/mL	-	45.3 ng/mL	N/A

19	*Blood*	*N/A*	*N/A*	*-*	*107.3 ng/mL*	*-*	*<5 ng/mL*	*N/A*
20	*Blood*	*N/A*	*N/A*	*-*	*16.4 ng/mL*	*-*	*-*	*N/A*
21	*Blood*	*N/A*	*N/A*	*5.8 ng/mL*	*131.1 ng/mL*	*-*	*-*	*N/A*
22	*Blood*	*N/A*	*N/A*	*7.7 ng/mL*	*171.3 ng/mL*	*<5 ng/mL*	*-*	*N/A*
23	*Blood*	*N/A*	*N/A*	*-*	*6.5 ng/mL*	*-*	*-*	*N/A*
24	*Blood*	*N/A*	*N/A*	*-*	*36.84 ng/mL*	*-*	*-*	*N/A*
25	*Blood*	*N/A*	*N/A*	*-*	*11.1 ng/mL*	*-*	*-*	*N/A*
26	*Blood*	*N/A*	*N/A*	*-*	*11.1 ng/mL*	*-*	*-*	*N/A*
27	*Blood*	*N/A*	*N/A*	*14.3 ng/mL*	*163.5 ng/mL*	*-*	*-*	*N/A*
28	*Blood*	*N/A*	*N/A*	*-*	*93.2 ng/mL*	*9.9 ng/mL*	*-*	*N/A*
29	*Blood*	*N/A*	*N/A*	*20.3 ng/mL*	*386.0 ng/mL*	*-*	*-*	*N/A*
29	*Pericardial Fluid*	*N/A*	*N/A*	*50.2 ng/mL*	*540.4 ng/mL*	*5.3 ng/mL*	*-*	*N/A*
30	*Blood*	*N/A*	*N/A*	*-*	*7.2 ng/mL*	*-*	*-*	*N/A*
31	*Blood*	*N/A*	*N/A*	*-*	*13.6 ng/mL*	*-*	*-*	*N/A*
32	*Blood*	*N/A*	*N/A*	*-*	*8.2 ng/mL*	*-*	*-*	*N/A*
33	*Blood*	*N/A*	*N/A*	*-*	*13.6 ng/mL*	*-*	*-*	*N/A*

N/A: Not available

Annex 24. Poster presentations

CONSUMO DE CODEÍNA COM OU SEM FINS TERAPÊUTICOS?

Elisa Ferreira, Alice Castanheira, João Franco, Cláudia Margalho

17º Congresso Nacional de Medicina Legal e Ciências Forenses

Coimbra, Portugal, 22-24 Novembro 2018

DESENVOLVIMENTO DE UMA METODOLOGIA ANALÍTICA PARA A ANÁLISE DE SUBSTÂNCIAS OPIÓIDES POR GC/MS-EI

Elisa Ferreira, Alice Castanheira, João Franco, Cláudia Margalho

17º Congresso Nacional de Medicina Legal e Ciências Forenses

Coimbra, Portugal, 22-24 Novembro 2018

DEVELOPMENT AND VALIDATION OF A GC-MS METHODOLOGY FOR THE DETERMINATION OF OPIOIDS IN WHOLE BLOOD AND PERICARDIAL FLUID: APPLICATION TO AUTHENTIC SPECIMENS

Elisa Ferreira, Alice Castanheira, João Franco, Francisco Corte Real, Cláudia Margalho

The 57th Annual Meeting of the International Association of Forensic Toxicologists

Birmingham, UK, 2-6 September 2019

DEATH BY DROWNING AFTER CONSUMPTION OF HIGH DOSE OF METHADONE: A CASE REPORT

Cláudia Margalho, Elisa Ferreira, Alice Castanheira, João Franco, Francisco Corte Real

The 57th Annual Meeting of the International Association of Forensic Toxicologists

Birmingham, UK, 2-6 September 2019

A CASE OF DEATH AFTER THE CONSUMPTION OF N-ETHYLPENTYLONE AND MPHP

Cláudia Margalho, Alice Castanheira, Fernando Castanheira, Elisa Ferreira, João Franco, Francisco Corte Real

The 57th Annual Meeting of the International Association of Forensic Toxicologists

Birmingham, UK, 2-6 September 2019