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SIMULTANEOUS QUANTIFICATION OF MORPHINE AND COCAINE IN HAIR SAMPLES BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Master Thesis

Carla Alexandra Pereira Gouveia

Master Degree in Forensic Sciences

Dezembro de 2011

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Dissertação de candidatura ao grau de Mestre em Ciências Forenses apresentada à Faculdade de Medicina da Universidade do Porto

Dissertation thesis for the degree of Master of Philosophy in Forensic Sciences submitted to the Faculty of Medicine of Porto University

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"Not everything that counts can be counted and not everything that can be counted counts."

Albert Einstein

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ABSTRACT

Generally, toxicological analyses are usually based on blood and urine. Liver and kidney are also commonly used since they represent the major metabolic and eliminator organs, respectively. Nevertheless, these specimens only reflect administrations of several hours or days. Therefore, it is imperative the use of an alternative biological matrix such hair, that could provide relevant and important retrospective information about the use of drugs, namely cocaine and morphine which are commonly abused.

The present work, a qualitative and quantitative method for the simultaneous determination of cocaine and morphine in human hair was developed and validated. Hair samples (20 mg), after decontamination with dichloromethane, water and methanol, were incubated with a mixture of methanol/hydrochloric acid (2:1) at 65ºC ON (~16h) in order to extract the xenobiotics of the matrix. The purification of standards was performed in mixed-mode extraction cartridges. After their derivatization with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), they were analyzed by gas chromatography by electron impact ionization, equipped with a mass spectrometer, GC-EI-MS. The method validation was performed by evaluating the following parameters: selectivity, linearity, specificity, precision, accuracy, detection limit, quantification limit and the percentage of recovery.

The Part I of this thesis a brief review about the state of the art of drugs of abuse classification as well as considerations about pharmacokinetic and pharmacodynamic of cocaine and morphine, was performed. To complete this part, a review was made on analytical methods to quantify cocaine and morphine in hair.

The part II is reserved for the general and specific objectives of the thesis, which is the development of an analytical method properly validated, that enables the quantification of cocaine and morphine in hair using gas chromatography with electron impact ionization coupled with mass spectrometer, GC-EI-MS, with a previous treatment of the hair samples.

Part III is divided in three chapters that enclose the description of the experimental work in order to achieve the general and specific objectives of the thesis, the method validation and its application to real hair samples. It also presents the obtained results and discussion, ending with the conclusions which resulted of experimental work.

In part V is presented all the references consulted in this thesis work.

The developed method proved to be specific, accurate and precise across the calibration range (0.25 – 10 ng/mg), where good linearity was observed for both the

analytes with correlation coefficients ranging 0.9989 and 0.9991. The coefficients of variation oscillated between 0.83% and 14.6%. The limits of detection (LOD) were 0.01 and 0.02 ng/mg, and the limits of quantification (LOQ), 0.03 and 0.06 ng/mg for cocaine and morphine, respectively. Accordingly the Society of Hair Testing rules, these results are acceptable.

The proposed GC–EI-MS method was then successfully applied in the screening and quantification of these compounds xenobiotics in real samples, and therefore the method is suitable for application in Forensic Toxicology.

Keywords: Hair drug analysis; Cocaine; Morphine; GC-EI-MS.

RESUMO

Geralmente, os métodos utilizados em análises toxicológicas baseiam-se essencialmente no uso de sangue e urina. O fígado e os rins são também comummente utilizados visto que representam os órgãos principais de metabolização e excreção, respetivamente. No entanto, estas matrizes apenas refletem a exposição num curto período de tempo após administração, que vai desde algumas horas a alguns dias. Nesse sentido, é imperativo a utilização de uma matriz biológica alternativa que permita fornecer informações retrospetivas relevantes e importantes acerca do uso de drogas de abuso, nomeadamente cocaína e morfina.

O presente estudo teve como objetivo principal o desenvolvimento e a validação de um método qualitativo e quantitativo para a determinação simultânea de cocaína e morfina em cabelo. Após a descontaminação com diclorometano, água e metanol, as amostras de cabelo (20 mg) foram incubadas com uma mistura de metanol / ácido clorídrico (2:1) a 65ºC durante a noite (16h~), a fim de extrair os xenobióticos da matriz. A purificação dos padrões foi realizada utilizando extração em fase sólida. Após derivatização com *N*-metil-*N*-(trimetilsilil) trifluoroacetamida (MSTFA), procederam-se as análises através de cromatografia gasosa por impacto eletrónico, equipado com um espectrómetro de massa, GC-EI-MS. A validação do método foi realizada através da avaliação dos seguintes parâmetros: seletividade, linearidade, especificidade, precisão, exatidão, limite de deteção, limite de quantificação e a percentagem de recuperação.

Na Parte I da presente dissertação, abordam-se conceitos relativos às drogas de abuso, assim como o estado da arte relativo à fármacocinética e fármacodinâmica da cocaína e morfina. Por fim apresenta-se uma revisão de literatura sobre os métodos analíticos para a quantificação de cocaína e morfina em cabelo

Na Parte II, encontram-se definidos os objectivos gerais e específicos deste trabalho, ou seja, o desenvolvimento de um método analítico devidamente validado que permita proceder à quantificação de cocaína e morfina em cabelo, através de cromatografia gasosa por impacto eletrónico acoplada a um espectrómetro de massa, GC-EI-MS, com um pré-tratamento da amostra.

A Parte III divide-se em três capítulos, faz uma breve descrição de todo o trabalho experimental realizado no âmbito dos objetivos propostos, a validação do método desenvolvido e a sua aplicação a casos reais, permitindo, desta forma a implementação desta técnica analítica acima descrita. Por fim, após a discussão dos resultados, apresentam-se as reflexões finais de toda a investigação.

Na Parte IV estão descritas todas as referências bibliográficas consultadas para a realização deste trabalho.

O método desenvolvido provou ser preciso, específico e exato no intervalo de calibração considerado (0,25 - 10 ng/mg), onde se observou uma boa linearidade para ambos os analitos, com coeficientes de correlação variando 0,9989 e 0,9991. Os coeficientes de variação oscilaram entre 0,83% e 14,6%. Já os limites de deteção (LOD) foram 0,01 e 0,02 ng/mg e os limites de quantificação (LOQ) foram 0,03 e 0,06 ng/mg de cocaína e morfina, respetivamente, valores que, de acordo com a Sociedade de Análise de Drogas em cabelo, são considerados aceitáveis. O método GC-EI-MS proposto foi de seguida aplicado com sucesso no rastreio e quantificação destes xenobióticos em amostras reais, sendo como tal apropriado para aplicação em Toxicologia Forense.

Palavras-chave: Análise de drogas em cabelo; Cocaína; Morfina; GC-EI-MS

CONGRESS PRESENTATIONS

The results described in this dissertation will be presented in two scientific meetings. The list of the submitted abstracts is presented:

Carla Alexandra Gouveia, Ana Oliveira, Sandra Pinho, Roxana Falcão Moreira, Ricardo Jorge Dinis-Oliveira; Validation of an analytical technique by gas chromatography mass spectrometry for the quantification of cocaine and morphine in hair samples; 7th National Meeting on Chromatography; Porto; Portugal; 9 – 11 January; 2012.

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ABREVIATION LIST

6-MAM	6-monoacetylmorphin
A	Amphetamine
AEME	Anhydroecgonine Methyl Ester
B.C.	Before Christ
BE	Benzoylecgonine
BSTFA	Bis (trimethylsilyl) Trifluoroacetamide
cAMP	cyclic Adenosine Monophosphate
CAT	Centro de Atendimento a Toxicodependentes
CE	Cocaethylene
CNS	Central Nervous System
COC	Cocaine
CV	Coefficient of Variation
CZE	Capillary Zone Electrophoresis
CZE-MS	Capillary Zone Electrophoresis – Mass Spectrometry
DTE	dithioerythritol
DTT	Dithiothreitol
EI	Electron Impact ionization
EME	Ecgonine Methyl Ester
EMIT	Enzyme multiplied immunoassay technique
ESI - MS/MS	Electrospray Ionization – Tandem Mass Spectrometry
EtOH	Ethanol
GC	Gas Cromatography
GC - ion trap – MS	Gas Chromatography – ion trap – Mass Spectrometry
GC – MS/MS	Chromatography - Tandem Mass Spectrometry
GC –EI - MS	Chromatography - Electron Impact ionization – Mass Spectrometry
GCxGC/TOF-MS	Gas Chromatography x Gas Chromatography /Time-of-Flight Mass Spectrometry
GC-PCI-MS	Gas Chromatography - Positive Chemical Ionization - Mass Spectrometry
GC-NCI-MS	Gas Chromatography - Negative Chemical Ionization- Tandem Mass Spectrometry
GHB	gamma-Hydroxybutyric Acid

GTFCh	*Gesellschaft für Toxikologische und Forensische Chemie*
hCE-1	Carboxylesterase-1
hCE-2	Carboxylesterase-2
HFBA	Heptafluorobutyric anhydride
HFIP	Hexafluoroisopropanol
HFPOH	Heptafluoropropanol
HPLC	High Pressure Liquid Chromatography
HPLC - DAD	High Pressure Liquid Chromatography – Diod – Array Detector
HPLC - MS	High Pressure Liquid Chromatography - Mass Spectrometry
HS-SPME	Headspace – Solid Phase Microextraction
LC	Liquid Chromatography
LC-APCI-MS/MS	Liquid Chromatography - Atmospheric Pressure Chemical Ionization– Tandem Mass Spectrometry
LC-ESI-MS/MS	Liquid Chromatography - Electrospray Ionization- Tandem 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
LSD	Lysergic Acid Diethylamide
M3G	Morphine-3-Glucuronide
M6G	Morphine-6-Glucuronide
m/z	Mass-to-charge ratio
MA	Methamphetamine
MAE	Microwave-Assisted Extraction
MBTFA	*N*-methyl-bis(trifluoroacetamide)
MBHFBA	*N*-methyl-bis(heptafluorobutyramide
MCX	Mixed-mode cation – exchange cartridge
MDA	3,4-methylenedioxyamphetamine
MDE	3,4-methylenedioxy-N-ethylamphetamine
MDMA	3,4-methylenedioxymethampthetamine
MeOH	Methanol
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry

MSHFBA	Methyl-trimethylsilylheptafluorobutyramid
TMSIM	Trimethylsilylimidazole
MSPD	Matrix Solid-Phase Dispersion
MSTFA	*N*-methyl-*N*-(trimethylsilyl) Trifluoroacetamide
MSTFA:NH_4I:DTE	*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide:ammonium iodide: dithioerythritol
MTBSTFA	*N*-methyl-*N*-(t-butyldimethylsilyl)trifluoroacetamide
NCOC	Norcocaine
ON	Overnight
PAA	Propionic Acid Anhydride
PChE	Pseudocholinesterase
PFPA	Pentafluoropropionic Anhydride
PFPOH	Pentafluoropropanol
PTFE	Polytetrafluoroethylene
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SFE	Supercritical Fluid Extraction
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
SPME	Solid - Phase Microextraction
TFA	Trifluoroacetic acid
TFAA	Trifluoroacetic Anhydride
THC	Δ9-tetrahidrocanabinol
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TMSIM	Trimethylsilylimidazole
t_R	Retention Time
UDP	Uridine Diphosphate
UGP	Uridine Diphosphate Glucuronosyl Transferase
UPLC-TOF-MS	Ultra-Performance Liquid Chromatography - Time-of-Flight – Mass Spectrometry
WHO	World Health Organization

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PART I: General Introduction

Chapter I

Drugs of Abuse: Concepts and Classification

Drug abuse has been part of our society for centuries. The history of drugs has always been an essential part of our culture, religious rituals and relationships, being continuously reported through the history of mankind. They have been abused for millennia (Gwinnell and Adamec, 2008). Since antiquity man turns to drugs in an attempt to alter the level of consciousness and emotional state. These types of habits were part of the social acquaintanceship and helped to integrate individuals into the community through religious or cultural ceremonies for therapeutic purposes or simply recreational, this consumption was always under the control of community. In most cases, drugs that were once considered acceptable by society later became unacceptable or even illegal. An example of these drugs are opiates and cocaine that were generally considered acceptable substances in the 19th Century and were commonly used by many people for medical and nonmedical reasons; however, the nonmedical use of these drugs became illegal in the 20th century and medical uses were sharply curtailed (Karch, 1998).

1. Drugs of abuse: concepts and classification

According with World Health Organization (World Health Organization. (November) a drug is “any substance that, when taken into the living organism may modify one or more functions” (WHO, 1969). The term “abuse” is typically used to describe the “non-medical self-administration of a substance” aiming psychoactive effects, intoxication or body image alteration, and usually despite knowledge of the risks involved (Wills, 2005).

“Drugs of abuse” are considered “psychoactive substances and their abuse can lead to dependence syndrome - a cluster of behavioral, cognitive, and physiological phenomena that develop after repeated substance use and that typically include a strong desire to take the drug, difficulties in controlling its use, persisting in its use despite harmful consequences, a higher priority given to drug use than to other activities and obligations, increased tolerance, and sometimes a physical withdrawal state” (World Health Organization. (November, 2011).

Drugs can be classified as licit drugs and illicit drugs, of natural, semi-synthetic or synthetic origin, which in turn can be divided into stimulants, Central Nervous System (CNS) depressant or hallucinogenic (Jickells and Negrusz, 2008; World Health Organization. (November, 2011). The natural drugs are those obtained directly from nature, such as cannabinoids. The semi-synthetic drugs are obtained by modifying the

structure of substances of natural origin, such as heroin. Synthetic drugs are those obtained by laboratory synthesis, such as methadone (Eaton and Gallagher, 2010).

Although all drugs interfere within the CNS, they are divided into different categories, according to the effects produced on the individual. Thus, separated into depressants that produce a sedative effect, generally depress brain activity (e.g., alcohol); narcotics, causing a numbing effect on sensory experiences (e.g., opiates); stimulants, which exert a stimulating effect contributing to the overall increase in brain activity (e.g. cocaine) and hallucinogens, which have a distorting effect on sensory experiences, acting on the brain perception of reality distorted by alteration of the senses (e.g., LSD, cannabinoids) (Miller, 2002; World Health Organization. (November, 2011). **Table 1** resumes the classification of drugs according to their mechanism of action and effects on the CNS.

Table 1 - Classification of drugs according to their mechanism of action and effects on the CNS (adapted from Miller, 2002; da Costa, 2010).

Type of drug	Mechanism of action	Effects on the CNS	Examples
Depressants	Depress center stimulation	Sedation	Alcohol Barbiturates Benzodiazepines
Narcotics	Reduce neural transmission	Numbness of senses and pain relief	Morphine Heroin Methadone
Stimulants	Activate the neural transmission	Stimulation	Cocaine Caffeine, Nicotine amphetamines
Hallucinogenic	Depends on the drug	Sensory and perceptual distortion	Cannabis Ecstasy; LSD

The definitions of “drugs of abuse” suggested by several authors are limited, and generally because it excludes those pharmaceutical products that may be misused in the sense that they could lead to accidental or deliberate overdose (e.g. paracetamol and aspirin) or that could contribute to vehicle accidents (e.g. antihistamines) or are banned by sporting organizations (e.g. doping). Alcohol, tobacco, and caffeine are also excluded because the use of those substances is considered socially acceptable in

many countries. It also excludes the types of substances involved in glue sniffing as they are typically not controlled substances (Jickells and Negrusz, 2008).

Dependence is a concept defined as “an inappropriate compulsion to take a substance regularly, which may cause physical, mental and/or behavioral impairment” (Wills, 2005). The drug is taken to make the user feel good or more usually to avoid withdrawal, but in either case it is clear that the user has lost control over their behavior and gives the drug greater priority over other behaviors that previously were more important (Wills, 2005). The biological adaptation of the body to long-term use of a drug reflects in a physical dependence. When exposed to a drug for the first time, the body may have a strong reaction, such as intoxication. After chronic consumption of a drug, physical adjustments of body are visible; it often results in stopping the production of natural neurochemicals that are similar to the drug abuse. In other words, the body begins to expect the presence of a chronically used drug. In addition, the body becomes tolerant to the effects of the drug (Landry, 1994).

“Tolerance” is a term used to describe the physical process during which the same amount of drug begins to have less pronounced effect. As tolerance develops, increasing amounts of the drug must be consumed to compensate the lack of effects that were produced in the presence of smaller doses. Once physical dependence and tolerance have been developed, withdrawal will occur if the drug is abruptly stopped. The stimulants and depressants are well known for the development of tolerance and will tend to increase drug use (Wills, 2005) .

Drug withdrawal is the physical process that occurs as the consequence to the absence of a drug on which it is physically dependent. Because withdrawal is physically and psychologically uncomfortable, the chronic drug user is compelled to use more drugs to avoid the withdrawal effects. Many drugs promote two independent but sometimes overlapping types of withdrawal: acute and prolonged (Landry, 1994). To understand dependence, tolerance and withdrawal the type of substance used, routes of administration, use of other substances, individual variability are taken in consideration (Jickells and Negrusz, 2008).

1.1. Cocaine

Cocaine is a naturally occurring alkaloid found in a plant of the genus *Erythroxylum* (Erythroxylaceae family), more precisely in coca (*Erythroxylum coca*). Coca leaves, the source of cocaine, have been chewed and ingested for thousands of years, and the purified bioactive compound, cocaine hydrochloride, has been an abused substance for more than 100 years (Karch, 1998). The stimulating qualities of

the coca leaf were well known and described by the ancient Peruvians and other pre-Columbian Andean societies. Nowadays, coca cultivation is distributed throughout the central and northern Andean Ridge, with approximately 60% in Peru, 30% in Bolivia and the remainder in Columbia, Ecuador, Venezuela, Brazil, Argentina and Panama (Gwinnell and Adamec, 2008; Jickells and Negrusz, 2008; Eaton and Gallagher, 2010).

Cocaine is known as powerfully addictive stimulant that directly affects the brain. It is one of the oldest known psychoactive substances and one of the most used drugs (Instituto da Droga e da Toxicodependência, 2010).

1.1.1. Physical and chemical properties

Cocaine IUPAC name is [1R, 2R, 3S, 5S]-3-(benzoyloxy)-8-methyl-8-azabicyclo-octane-2-carboxylic acid methyl ester, or the methyl ester of benzoylecgonine (**Fig. 1**). It is a white, crystal-like powder, and when in the form of “crack” (further discussed), cocaine base usually occurs as small (100 – 200 mg) rocks (Freye, 2009).

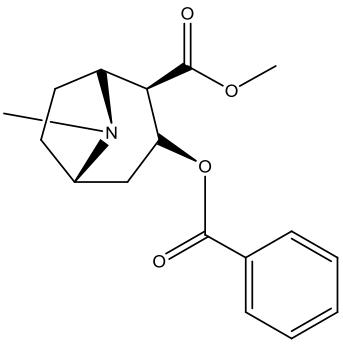

Fig. 1 – Chemical structure of cocaine

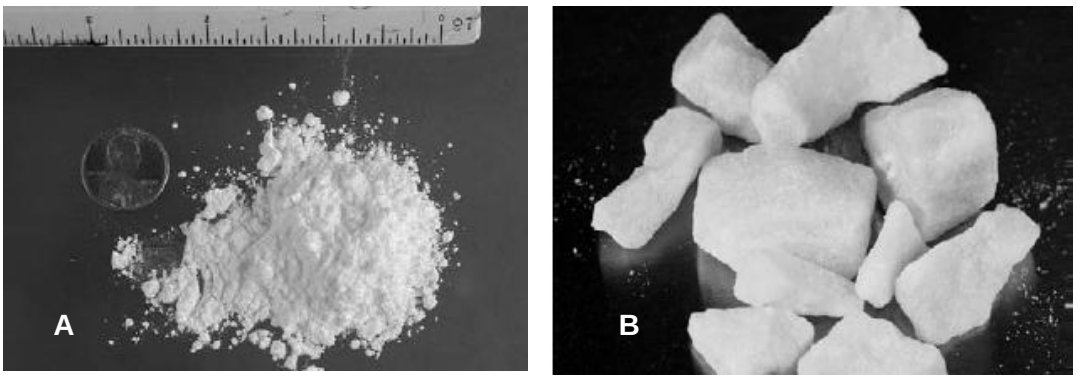

Fig. 2 – Cocaine forms: **A** - Cocaine hydrochloride **B** - Crack cocaine. (Reproduced from Jickells and Negrusz, 2008)

Cocaine is available in two forms: the hydrochloride salt and the “freebase”. Cocaine hydrochloride (**Fig. 2A**) is prepared by dissolving the alkaloid in hydrochloric acid to form a water-soluble, crystalline powder that decomposes when heated. “Crack” (**Fig. 2B**) is the street name given to cocaine processed from cocaine hydrochloride. It is prepared by adding baking soda to aqueous cocaine hydrochloride and heating it until the freebase cocaine precipitates into small pellets. The mixture is cooled and filtered and the “rocks” are smoked in a crack pipe (Jickells and Negrusz, 2008).

Street cocaine used by addicts can present a number of impurities, these include amphetamines (A), anti-histamines, benzocaine, lactose, lidocaine, opioids, caffeine and even flour or talc. The estimated minimum lethal dose is 1.2 g but it depends on the user susceptibility (Jickells and Negrusz, 2008; IPCS - CCOHS. (October, 2011). Toxic effects have been noted with blood concentrations in the range 0.25 to 5 mg/L and fatalities have occurred with concentrations of 1 mg/L or more (Moffat *et al.*, 2004). **Table 2** summarizes physical and chemical properties of cocaine.

1.1.2. Pharmacokinetics

Cocaine is a CNS stimulant and an appétit suppressant and induces what has been described as a “euphoric sense of happiness and increased energy” (White and Lambe, 2003).

The peaks of absorption vary depending on the routes of administration. Intranasal insufflations of cocaine remain the most common method of self-administration; nevertheless there are several alternative forms of administration that can be used. After oral administration, cocaine appears in blood after about 30 minutes, the maximum concentration is reached in 50 to 90 minutes. In acid medium, cocaine is ionized and fails to cross into cells, in alkaline conditions, the absorption increases rapidly and there is less ionization (Lizasoain *et al.*; Blaho *et al.*, 2000). When administrated by the nasal route, clinical effects are evident 3 minutes after administration, and last 30 to 60 minutes, the peak plasma concentration being around 15 minutes. Oral or intra-nasal route, 60 to 80% of cocaine is absorbed, if inhaled the absorption can vary from 20 to 60%, the variability being related to secondary vasoconstriction. Intravenous route leads to high peaks in blood concentrations within a few minutes (IPCS - CCOHS. (October, 2011).

The main *in vivo* metabolism of cocaine is hydrolysis by hepatic and plasma esterases (carboxylesterase). Carboxylesterase-1 (hCE-1) is responsible for the hydrolysis of cocaine to benzoylecgonine (BE). Cocaine may also be hydrolyzed to ecgonine methyl ester (F. Musshoff *et al.*) by liver carboxylesterase-2 (hCE-2) and

plasma cholinesterase (PChE). Only a minor part is *N*-demethylated by CYP3A in humans to norcocaine (NCOC). During crack smoking, cocaine undergoes thermal breakdown to anhydroecgonine methyl ester (AEME). In the presence of ethanol (EtOH), hCE-1 catalyzes the ethyl transesterification of cocaine to cocaethylene (CE) (Jatlow, 1988; Valente *et al.*, 2010; IPCS - CCOHS. (October, 2011).

The different cocaine metabolites present unique pharmacokinetic and toxicological profiles (Valente *et al.*, 2010). Cocaine can be detected in blood or urine for only several hours after its use. In contrast, its metabolites are detectable in blood or urine for 24 – 36 h after ingestion, thereby providing a useful indicator of recent drug ingestion (Eaton and Gallagher, 2010). **Table 2** summarizes pharmacokinetics and properties of cocaine.

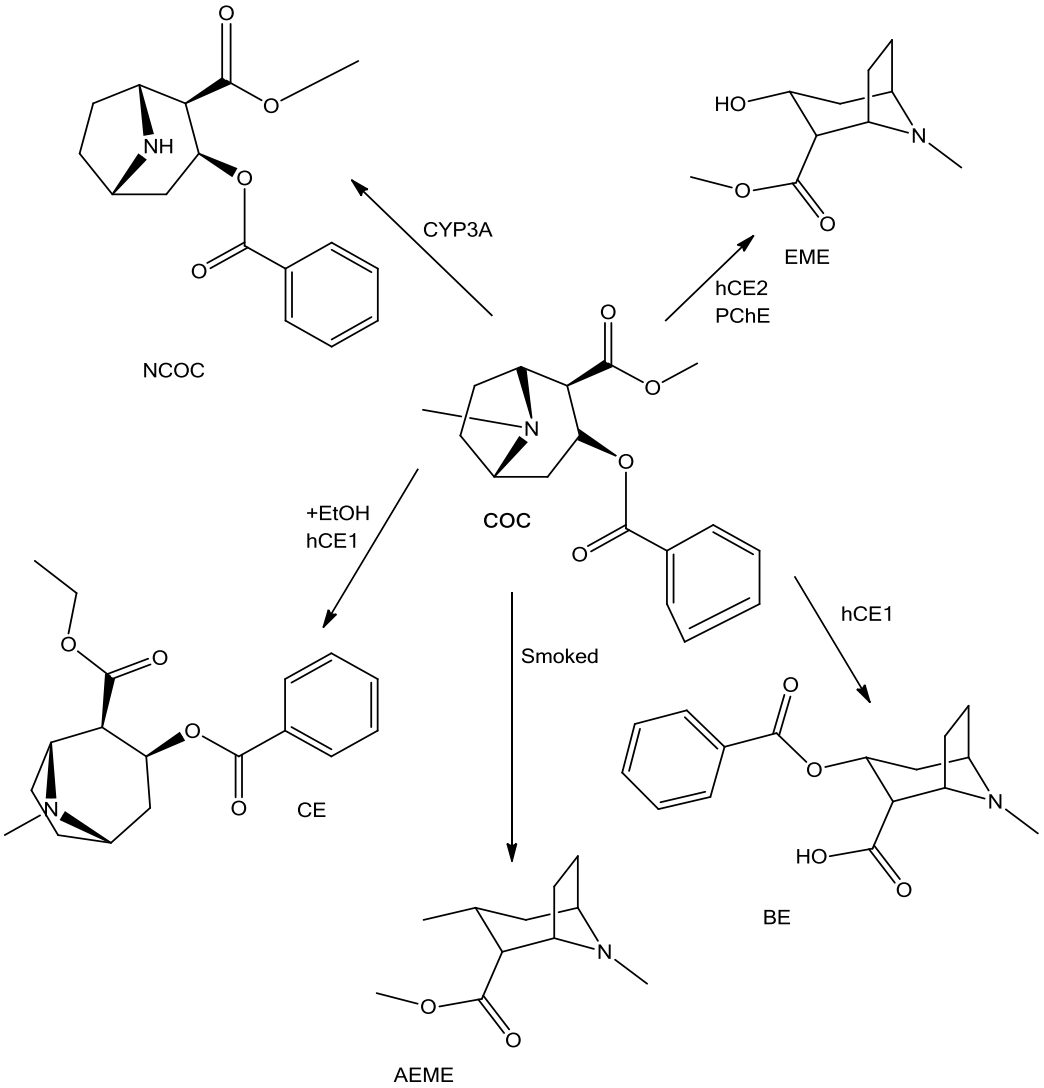

Fig. 3 - Schematic representation of major metabolites pathways of cocaine. The main *in vivo* metabolism of cocaine is its hydrolysis to benzoylecgonine (BE) mainly by liver carboxylesterase-1 (hCE-1). Cocaine may also be hydrolyzed to ecgonine methyl ester (F. Musshoff *et al.*) by liver carboxylesterase-2 (hCE-2) and plasma cholinesterase (PChE). Only a

minor part is N-demethylated by CYP3A in humans to norcocaine (NCOC). During crack smoking, cocaine undergoes thermal breakdown to anhydroecgonine methyl ester (AEME). In the presence of ethanol (EtOH), hCE-1 catalyzes the ethyl transesterification of cocaine to cocaethylene (CE).

1.1.3. Toxicodynamics

The main target organs are the cardiovascular system and the CNS (Lizasoain *et al.*; Gwinnell and Adamec, 2008; Freye, 2009). The effects depend on the dose, route of administration, other substances taken and obviously individual susceptibility. The main effects of cocaine are the result of its sympathetic action: cocaine prevents the re-uptake of dopamine and noradrenaline, induces accumulation and stimulation of neuronal receptors, the release of serotonin, a ”sedative” neurotransmitter, is inhibited (Lizasoain *et al.*; Jatlow, 1988; Freye, 2009).

The frequent use of these substances leads to symptoms of arousal, irritability and self-confidence, going through several episodes of agitation, aggression, psychosis, cocaine and cardiac syncope, as consequences of an overdose. Its use leads to chronic ulceration of the nasal septum, acute psychotic reaction anxiety, irritability, depression, paranoid feelings, tactile hallucinations and insomnia (da Costa, 2010). Euphoria, confusion, agitation, and hallucination result from an increase in the action of dopamine in the limbic system (White and Lambe, 2003)

It was demonstrated that cocaine metabolism in the liver into NCOC plays an important role in its hepatotoxic effects, while BE and EME are pharmacologically inactive and nontoxic metabolites (Eaton and Gallagher, 2010; Valente *et al.*, 2010).

Table 2 – Overview of physical and chemical properties, pharmacokinetics and toxicodynamics of cocaine (adapted from Moffat *et al.*, 2004; Freye, 2009).

Appearance	**Color and odorless, bitter taste, crystal powder**
Molecular weight	303.4 g/mol
Chemical formula of the base	$C_{17}H_{21}NO_4$
pKa	8.6
Melting point	Cocaine hydrochloride: 197ºC
	Cocaine base: 98ºC
Solubility	*Cocaine hydrochloride:* Fair solubility in alcohol, chloroform; insoluble in ether
	Cocaine base: good solubility in ether

Metabolism	Pseudocholinestarese of blood plasma, liver enzymes
Mean distribution half time mean time till max. onset of action, nasal	15 - 20 minutes
Mean plasma half time mean duration of action, nasal	1 – 2.5h
Bioavailability	Oral : 33% Nasal: 19% Note: cocaine can also be administered rectally, vaginally and urethrally
Excretion	Renal as BE and EME
Main target organs	Cardiovascular system CNS

1.2. Opiates: morphine

The medicinal value of opiate alkaloids extracted from the opium poppy plant (*Papaver somniferum*) has been documented in the ancient literature as early as 1500 B.C. (Skarke and Lötsch, 2002). The term “opioids” includes natural alkaloids (usually denoted “opiates”) such as morphine and codeine; semisynthetic substances as diacetylmorphine or as commonly called, heroin; and synthetic alkaloid derivatives either prepared from opium or synthesized possessing a morphine-like activity, methadone, oxycodone, fentanyl and tramadol, between others. (Karch, 1998; Miller, 2002; dos Santos Baptista, 2005).

Morphine was isolated from opium (10 to 17% morphine) in 1804 by Adam Sertürner and classified as a narcotic drug of the opioid group, forming the basis of the natural opiates and semi-synthetic (Eaton and Gallagher, 2010). Its name is related to Morpheus, the Greek god of dreams, it has analgesic, sedative and anxiolytic properties (Skarke and Lötsch, 2002) and is the most widely used as potent opioid analgesic for chronic pain (Karch, 1998).

1.2.1. Physical and chemical properties

Chemically, morphine is (5*α*,6*α*)-7,8-Didehydro–4,5–epoxy–17–methylmorphinan–3,6–diol (**Fig. 4**). This compound is the principal alkaloid obtained from opium and when isolated is obtained as a white crystalline powder or colorless or white acicular crystals (Moffat *et al.*, 2004).

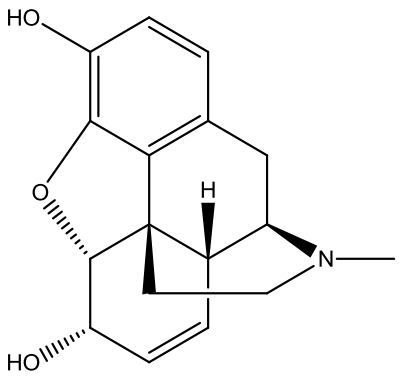

Fig. 4 - Chemical structure of morphine

The estimated minimum oral lethal dose for adults is 200 mg but addicts can present higher tolerance, up to 10 times as much. Morphine is initially eliminated from the blood relatively fast, therefore blood concentrations are difficult to interpret, especially as toxic effects depend on the degree of tolerance of each individual (Moffat *et al.*, 2004). Physical and chemical properties of morphine are presented in **Table 3**.

1.2.2. Pharmacokinetics

Pharmacologically is an opioid agonist. It is administered in the form of powder, liquid or bar, orally or injected, which quickly spreads through the bloodstream, rapidly coming to the CNS, for which presents great tropism (da Costa, 2010). The main route of biotransformation of morphine corresponds to its esterification with glucuronic acid *via* the hydroxyl groups and the main route of elimination in bile and urine. In urine, all metabolites can be detected as well as small amounts of morphine (Eaton and Gallagher, 2010), with only 2 to 12% excreted as the parent molecule, while 60 to 80% is excreted in the urine as the conjugated glucuronide. About 56% of the morphine molecules are metabolized to morphine-3-glucuronide (M3G), and about 10% to

morphine-6-glucuronide (M6G) (Skarke and Lötsch, 2002; Mercadante and Arcuri, 2004).

Morphine can be originated as a metabolite of other xenobiotics (**Fig. 5**). For example, considering heroin metabolism, this drug is rapidly biotransformed first to 6-monoacetylmorphine (6–MAM) and then to morphine. Both heroin and 6-MAM disappear rapidly from the blood ($t½$ = 3 min, 5 to 10 min, respectively). Thus, morphine levels rise slowly, persist longer, and decline slowly. Codeine is extensively metabolized, primarily to the 6-glucuronide conjugate. About 10 to 15% of a dose is demethylated to form morphine and norcodeine conjugates. Therefore, morphine in free and conjugated form can appear in the urine after codeine and heroin consumption (Stuart-harris *et al.*, 2000; Barile, 2004). Overview of pharmacokinetics profile of morphine is presented in **Table 3**.

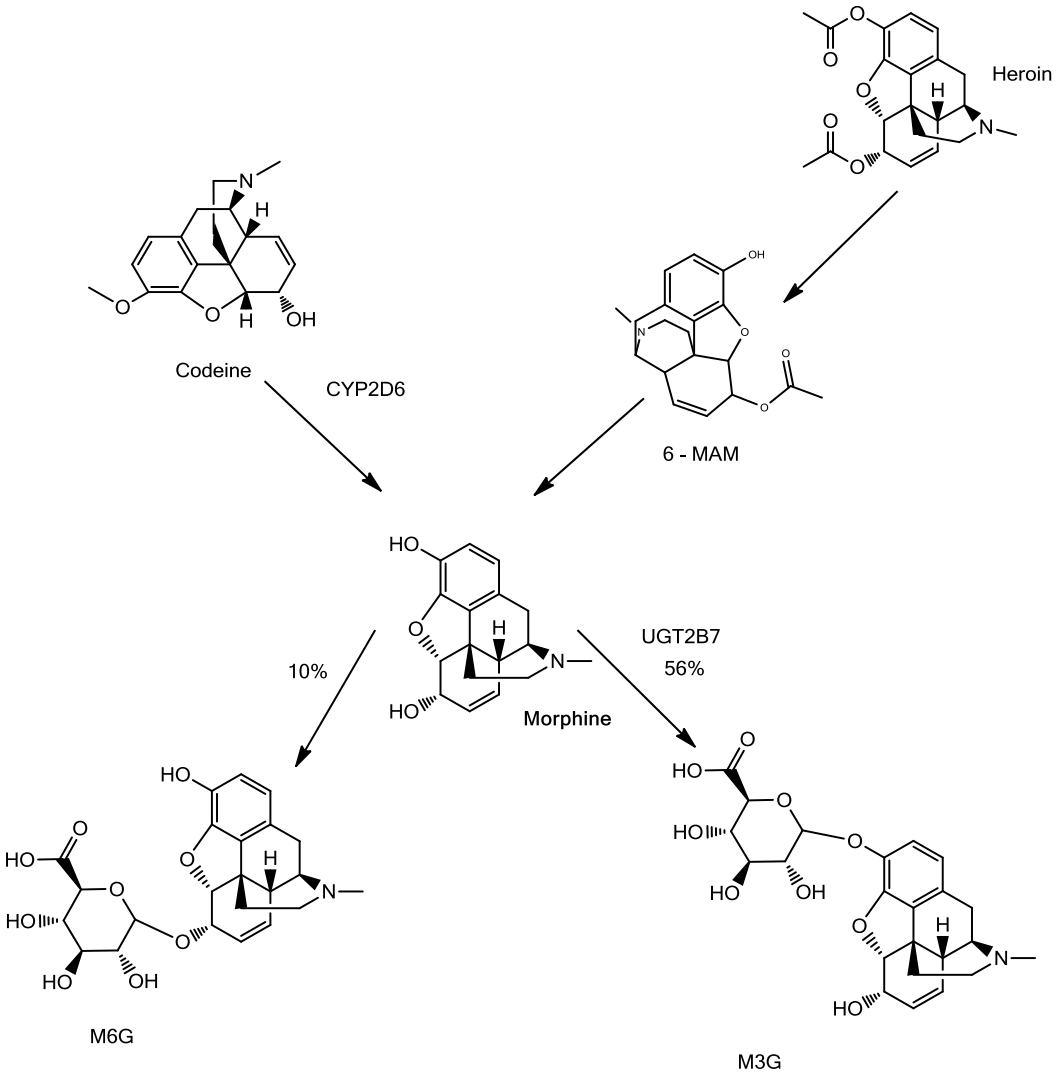

Fig. 5 - Schematic representation of major metabolites pathways of morphine in humans with the enzymes involved. The major metabolite of morphine is, morphine–3–glucuronide (M3G)

originated by glucuronidation mainly mediated by the uridine diphosphate (UDP) glucuronosyl transferase (UGT) 2B7. (Lötsch, 2005).

1.2.3. Pharmacodynamics

Several types of opioid receptors have been identified in the body. The receptor responsible for the cardiovascular effects of opioids is the mu (μ) receptor, originally named after its identification as the morphine-binding site. This receptor mostly acts at the spinal level and its stimulation results in respiratory depression, bradycardia, hypotension, and peripheral vasodilatation (Kieffer, 1999). Respiratory depression, miosis, reduced gastrointestinal activity, and euphoria are direct consequences produced by the binding with μ-receptors produces (Karch, 1998). In addition, drugs that bind selectively to kappa (κ) receptors also produce analgesia but act principally in the spinal cord, though it results in miosis or respiratory depression when compared with μ agonists but with less intensity. The κ agonists produce analgesia (spinal level), sedation, and slight miosis whereas sigma (σ) agonists produce dysphoric psychomimetic effects (Brownstein, 1993; Karch, 1998).

The effects of opioids are mediated through either a cyclic adenosine monophosphate (cAMP) pathway or modulation of the calcium and potassium channels (Eaton and Gallagher, 2010). Morphine when associated to the receptor closes Ca^{2+} channels on presynaptic nerve terminals, thereby reducing neurotransmitter release. It also opens K^{+} channels inhibiting postsynaptic neurons (Brownstein, 1993; Jickells and Negrusz, 2008).

The respiratory depression is due to a direct effect of the drug on the respiratory centers of the brainstem by reducing the responsiveness to carbon dioxide. The constriction of the pupil is caused by an excitatory action on the parasympathetic nerve innervating the pupil. It also causes nausea and vomiting consequence of direct stimulation of the chemoreceptor trigger zone in the medulla oblongata (Karch, 1998).

Among the cited adverse reactions, the potential for induction of physical and psychic dependence, which is high for heroin, morphine and opium, and moderate for codeine (dos Santos Baptista, 2005).

Table 3 - Overview of physical and chemical properties, pharmacokinetics and pharmacodynamics of morphine (adapted from Moffat *et al.*, 2004)

Appearance	**White crystalline powder or colorless or white acicular crystals**
Molecular weight	285.4 g/mol
Chemical formula of the base	$C_{17}H_{19}NO_3$
pKa	7.9
Melting point	254 ºC to 256 ºC, with decomposition
Solubility	Good solubility in water, alcohol, chloroform. Insoluble in ether
Metabolism	Glucuronidation
Mean plasma half time mean duration of action	About 2 to 3 h
Bioavailability	20 to 30% (Oral) 100% (Intravenous)
Excretion	Bile and urine
Main target organs	Respiratory system (depression)

Chapter II

Hair in Forensic Sciences

2. Drug testing in hair

The use of hair as a biological matrix in forensic toxicology was introduced in the late 70s by Baumgartner, where this sample proved to be suitable for retrospective analysis of drugs of abuse in specific for opiates (Staub, 1995; Hadidi *et al.*, 2003; Gottardo *et al.*, 2007). Since then, hair analysis has gained great importance, it provides a great number of information about consumption over a long period, less invasive to collect and in forensic toxicology it is specially used as a supplementary tool to disclose or confirm previous drug abuse (Dinis-Oliveira *et al.*, 2010).

Hair analysis was normally used to evaluate exposure to toxic heavy metals, such as arsenic, lead or mercury. This kind of analysis was achieved using atomic absorption spectroscopy that allowed detection in the nanogram range. At that time, 60s and early 70s, examination of hair for organic substances, especially drugs, was not possible since the analytical methods were not sensitive enough to analyze that kind of substances (Tobin, 2005)

Due to the difficulties and constraints of biological samples, blood and urine, preferably used in drug screening, detection and quantification, including the constitution of these matrices (proteins, salts, acids, alkalis and organic compounds), the detection windows of each drug, as well as the dose and time elapsed since the intake of the same to the collection of samples, becomes substantial the recourse to another biological sample - hair - who provides, with a larger window of detection, data about the presence or absence of certain substances. Today, the hair has been recognized as the third leading biological sample (Gentili *et al.*, 2004; Kronstrand *et al.*, 2004; Gottardo *et al.*, 2007; Pragst and Balikova, 2008; Nielsen *et al.*, 2010). In some *post-mortem* cases, it is difficult or its not available to collect specimens for toxicological analysis, and hair, due to his resistance to decay, could be the only sample available for testing (Dinis-Oliveira *et al.*, 2010).

The development of analytical techniques, specially the progression of separation techniques, increased selectivity and sensitivity of detection, enables the detection and quantification of illicit drugs consumed in a voluntary or involuntary, in the order of µmol/ng hair (Nakahara, 1999; Clauwaert *et al.*, 2000; Musshoff and Madea, 2007a).

Recognizing the seriousness of problems related to drug abuse and dependence, the rising consumption and easy access to drugs of abuse in society, it becomes imperative a more accurate analytical means and type of sample used for the detection of drugs of abuse in each individual. Due to its importance, the scientific and legal validity of drug evidence analyses must be defensible to be effective. Therefore, this requires application of the proper legal statutes, knowledge of the relevant

pharmacological properties, and selection of the optimal analytical approach. In addition, chain of custody dictates that evidence integrity be protected by acceptable collection, packaging, documentation, and storage (Kintz, 1996; Musshoff and Madea, 2007a).

2.1. Drug incorporation into hair

Hair is a complex tissue originated in a hair follicule, it grows in a cycle composed of the anagen (active growing, 4 - 6 years), catagen (transition, few weeks) and telogen (resting, 4 - 6 months) stages (Wennig, 2000). It is constituted by proteins, mostly keratin (65 - 95%), water (15 - 35%), lipids (1 - 9%) and minerals (0.25 - 0.95%) (Dinis-Oliveira *et al.*, 2010).

Drugs penetrate the hair shaft with nutrients from the blood during the growth phase, and remain in the hair matrix throughout the life of the structure (months to years). The hair grows from 0.6 - 1.4 cm with an average of 0.6 cm/month for scalp and pubic hair (Dinis-Oliveira *et al.*, 2010), and every inch of hair, keeps track of each individual corresponding to one month of exposure or consumption drug (Kintz, 1996; Pragst and Balikova, 2008).

In general, the incorporation of drugs in hair can occur by three processes: absorption of the environment, that results from passive exposure to the drugs from an external source (smoke, dirty hands, and secondary to dissolution of the drugs into drug-free sweat) (Tobin, 2005; Dinis-Oliveira *et al.*, 2010). Passive diffusion from blood capillaries, the incorporation into the growing hair shaft takes place through the blood capillaries that feed the hair follicle (Moffat *et al.*, 2004; Pragst and Balikova, 2008). However, the exact mechanism of drug incorporation in hair is still undefined, so it is still the subject of considerable.

The incorporation of drugs can be influenced by several factors, namely the amount of melanin present in the hair, the lipophilicity and basicity of the substance itself, which are the most relevant, among others. The merger made through the blood is controlled, usually by pharmacological principles of drug distribution (Pragst and Balikova, 2008). The lipophilic organic molecules can easily penetrate the membrane and diffuse according to the concentration gradient of the cell matrix. However, for hydrophilic molecules and ions of organic molecular mass, the membrane forms an impermeable barrier. As for acidic or basic drugs, which are ionized to a higher pH, they can penetrate the membrane, but only when they suffer deprotonation or protonation state until they are neutral (Kintz, 2007; Pragst and Balikova, 2008; Dinis-Oliveira *et al.*, 2010).

Nevertheless, it is of extreme importance to take into account that there is only a limited correlation between the frequency of drug use or ingested dose and the drug concentration found in hair, this is based on interindividual differences in the rate of metabolism, on differing drug incorporation rates, hair pigmentation and the physical state of the hair (shampooing, bleaching, dyeing, permanent wave) (Musshoff and Madea, 2007a).

Chapter III

Literature Review

3. Review of the analytical methods to quantify morphine and cocaine in hair

Nowadays, hair analysis has gained a great relevance as a tool for detection of drug abuse in forensic science, occupational and traffic medicine as well as in clinical toxicology. The widespread use of this type of analysis is due to the recent advances in analytical techniques (Kintz, 1996; Sachs and Kintz, 1998b; Romolo *et al.*, 2003; Cognard *et al.*, 2005; Kintz, 2007; Musshoff and Madea, 2007a; Barroso *et al.*, 2010; Guthery *et al.*, 2010).

Hair analysis procedures requires scientific demands that have been discussed in several studies published in 2004 by Society of Hair Testing (Nakahara *et al.*, 1994; Nakahara, 1999; Wennig, 2000; Jurado and Sachs, 2003; Society of Hair Testing, 2004; Musshoff and Madea, 2007a). These analyses usually involve several measures to ensure the validation and reliability of the results.

A classic hair testing includes several steps with a variety of possible sources of error, this includes decontamination or washing steps to remove non-specific endogenous and exogenous substances in order to eliminate possible interferences in the next steps and for the results interpretation (Tagliaro *et al.*, 1997; Wennig, 2000; Jurado and Sachs, 2003; Kintz, 2007). The cleaning of the hair is important to remove external impurities as completely as possible, but efforts should be made to avoid the extraction of the drugs from the hair matrix (Pragst and Balikova, 2008). Usually, dichloromethane (CH_2Cl_2) and/or a detergent or organic solvent are used (Kintz and Mangin, 1995; Clauwaert *et al.*, 2000; Girod and Staub, 2000; Toledo *et al.*, 2003; Cognard *et al.*, 2005; Cordero and Paterson, 2007; Kintz, 2007; Musshoff and Madea, 2007a; Barroso *et al.*, 2008; Bucelli *et al.*, 2009; Huang *et al.*, 2009; Rossi *et al.*, 2009; Barroso *et al.*, 2010).

The washing step is generally followed by a further treatment, the extraction or digestion of hair matrix, this is considered to be the most sensible and complex step of hair testing procedures (Kronstrand *et al.*, 2004). As mentioned above, the drugs are firmly enclosed in the hair structure and partly bound to proteins, lipids or melanin of the cell membrane complex. The extraction can be achieved using different methodological approaches, including digestion of the hair matrix in alkaline or enzymatic conditions, incubation in organic solvents or acid media at elevated temperatures (e.g. 65ºC) or buffer extraction of the hair, as well as supercritical fluid extraction to elute the analytes from the matrix without complete destruction (Tagliaro *et al.*, 1997; Kronstrand *et al.*, 2004; Pragst and Balikova, 2008). It is important to take under consideration the type of drug to be extracted, since basic conditions degrade

substances such as heroin, cocaine and benzodiazepines and acid conditions can promote the hydrolysis of several substances, 6–MAM to morphine leading to false-positive results (Kronstrand *et al.*, 2004; Barroso *et al.*, 2010). Methanolic extraction (3 – 18 h) in an ultrasonic bath is universally applied (Nakahara *et al.*, 1994; Kauert and Röhrich, 1996; Uhl, 2000; Toledo *et al.*, 2003; Kronstrand *et al.*, 2004; Scheidweiler and Huestis, 2004; Musshoff and Madea, 2007a; Moller *et al.*, 2010; Favretto *et al.*, 2011). In 2009, Fernández *et al.* proposed a new approach to COC and opiates extraction from hair matrix, Microwave-Assisted Extraction (MAE). The MAE method uses microwave energy to heat the sample–solvent mixtures in sealed or open vessels. The extraction solvents used for MAE must absorb microwaves, although the use of solvent mixtures with and without dipole moments opens up a variety of potential solvent mixtures (Fernández *et al.*, 2009)

In general, after the drugs have been extracted from the hair matrix, the mixture obtained is not suitable for direct detection, thus, it has to be subsequently cleaned up and concentrated to improve the signal-to-noise ratio (Musshoff and Madea, 2007a). Liquid-Liquid Extraction (LLE) (Gottardo *et al.*, 2007; Favretto *et al.*, 2011) or Solid-Phase extraction (SPE), using different kind of columns, have been proposed by the literature as the essential clean-up procedure (Kintz, 2007; Musshoff and Madea, 2007a; Paterson *et al.*, 2009; Moller *et al.*, 2010).

Supercritical Fluid Extraction (SFE) with carbon dioxide has also been used for the cleanup. The advantages of this extraction technique include the high speed of the extraction (30 min) and the potential to be connected on-line with gas chromatography-mass spectrometry (GC-MS) (Kintz, 2007; Musshoff and Madea, 2007a).

Most of the procedures used for the detection of drugs in hair are based on gas chromatography coupled with mass spectrometry (GC–MS). Cappillary GC–MS with electron impact ionization source (GC–EI–MS) is the method most frequently used when testing for a wide variety of drugs and its metabolites as it presents adequate accuracy at very low concentrations (Kintz and Mangin, 1995; Kauert and Röhrich, 1996; Sachs and Kintz, 1998a; Girod and Staub, 2000; Kintz, 2007; Musshoff and Madea, 2007b; Musshoff and Madea, 2007a; Barroso *et al.*, 2008; Favretto *et al.*, 2011).

Prior to chromatographic analysis, derivatization can be required either because of limited thermal stability or insufficient volatility, that sometimes can be made amenable by means of derivatization, especially for drugs with free amino, hydroxyl or carboxyl groups, therefore drugs are transformed into derivatives, more volatiles, with a similar chemical structure. A general aim in this type of derivatization is the reduction of polarity of the analyte by chemical substitution of its active protons (Niessen, 2001). It

is frequently applied a mixed derivatization reaction that addresses different functional groups, e.g. using pentafluoropropionic anhydride/pentafluoropropionic anhydride-pentafluoropropanol (PFPA-PFPOH) (Musshoff and Madea, 2007a) or heptafluorobutyric anhydride-heptafluoropropanol (HFBA-HFPOH) (Girod and Staub, 2000). After the extraction procedure there are several options that can be made concerning the derivatization step, *N*-methyl-*N*-(t-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) (Musshoff and Madea, 2007a) and *N*-methyl-bis(trifluoroacetamide) (MBTFA) (Girod and Staub, 2000; Musshoff and Madea, 2007a; Guthery *et al.*, 2010) are used to get a tetrabutyldimethylsilyl derivative and a trifluoroacetic acid derivative, respectively.

The sensitivity and specificity is an important parameter in the analyses, and it can be enhanced by employing different analytical methods such us positive or negative chemical ionization (GC–PCI–MS or GC–NCI–MS) (Kintz, 2007; Musshoff and Madea, 2007a) used mainly for compounds such as benzodiazepines. Tandem mass spectrometry coupled with gas chromatography (GC–MS/MS) has proved to be highly sensitive and specific (Uhl, 2000; Bermejo *et al.*, 2006; Musshoff and Madea, 2007a). This technique increases the information content in a spectrum, thereby increasing the certainty of identification (Smith, 2005).

Even though not so commonly used, high-performance liquid chromatography (HPLC) or liquid chromatography (LC) methods have been reported in a few studies due to their ability to separate a large range of not derivatized substances. When comparing HPLC with GC techniques, the HPLC methods present a lower chromatographic resolution (Smith, 2005; Pragst and Balikova, 2008), therefore an efficient use of HPLC methods in hair analysis is only possible when coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS). This method has been describe by several articles for the determination of benzodiazepines and metabolites as well as cocaine and opiates (Kronstrand *et al.*, 2004; Scheidweiler and Huestis, 2004; Kintz, 2007; Musshoff and Madea, 2007a). Despite of its advantages, this analytical approach remains limited since it is very expensive (Musshoff and Madea, 2007b; Musshoff and Madea, 2007a; Pragst and Balikova, 2008).

Advanced and sophisticated methods have been recently described, e.g., ultra-performance liquid chromatography (UPLC) combined with mass spectrometry using a time-of-flight (TOF) analyzer (Musshoff and Madea, 2007a; Nielsen *et al.*, 2010). UPLC combines short runtimes with a higher resolution chromatographic separation. TOF-MS provides high informative and very accurate mass spectrometry data. Therefore, the combination of these techniques is suited for specific and fast multi-analytes analyses and detection. It represents a significant advantage in specificity, selectivity and speed

in the analytical procedure of several drugs of abuse and pharmaceuticals (Pragst and Balikova, 2008; Nielsen *et al.*, 2010)

Capillary zone electrophoresis (CZE) and electrospray ionization (Montagna *et al.*) (Montagna *et al.*) have been also described (Gottardo *et al.*, 2007; Míguez-Framil *et al.*, 2011). These techniques when compared with HPLC methods have many different modes of separation, which allows some flexibility for difficult analyses and reduces drastically solvent consumption (Smith, 2005).

The validation of procedures and quality control are two important parameters that are worthy of especial attention, since there are several guidelines and recommendations for quality management in analytical laboratories to be applied in hair analyses (Kintz, 1996; Kintz, 2007; Pragst and Balikova, 2008). Quality control in hair testing is more difficult than for other body fluids, since spiked control samples cannot substitute the real hair of the drug user (Musshoff and Madea, 2007b). However, for internal quality control spiked samples, if adequately prepared, they may substitute the hair from drug users and for the external quality control, only the authentic hair specimens should be used, because these samples are the ones that yield control over all steps of the all hair procedure (Musshoff and Madea, 2007b; Musshoff and Madea, 2007a; Pragst and Balikova, 2008).

There are several recommendations for the validation of an analytical procedure; the selectivity, used to exclude possible interferences, precision and accuracy, linearity, and analytical limits (Limit of Detection (LOD) and Limit of Quantification (LOQ)) are the principal parameters to take under consideration. This topic will be discussed in detail in the second part of this thesis.

The last item in the hair analysis procedure is not necessarily concerned with practical performance but refers to the interpretation of results, even though they are connected. This part represents one of the most thorough parts, since there are various aspects to take into account when interpreting results of a hair analysis. As mentioned above, hair is a complex matrix (Kintz, 1996; Tagliaro *et al.*, 1997; Nakahara, 1999; Society of Hair Testing, 2004; Smith, 2005; Kintz, 2007; Pragst and Balikova, 2008). Differences in hair growth rate depends on the anatomical region, that is the reason why the collection of the hair is made in the posterior vertex region of scalp; gender, age, ethnicity and interindividual variability, makes that interpretation of the concentrations of drugs found in hair is not straightforward (Wennig, 2000). In order to facilitate the interpretation of results, some recommendations about the specimen collection (collection site, collect as close as possible to the skin, wrapping of the hair in aluminium foil, storage at room temperature) should be followed in order to maintain integrity and avoid contamination, criteria for obtaining a positive result since the

evaluation of a possible contamination, identification of metabolites and assay values of decontamination washes (Wennig, 2000; Society of Hair Testing, 2004; F. Musshoff *et al.*, 2009).

Cut-off values constitute a major controversial parameter for interpreting hair analyzes results, according to Kintz (Kintz, 2007) the incorporation of the different drugs into hair is different. The proposed cut-off values for COC and morphine are 0.5 and 0.2 ng/mg of hair, respectively (**Table 4**) (Wennig, 2000; Society of Hair Testing, 2004; Kintz, 2007).

Table 4 - Proposed Cut-off values for COC and morphine

Compound	Cut-off values (ng/mg)
COC	0.5
Morphine	0.2

During hair analysis, in case of COC the concentration of the parent drug is present predominantly, but the analysis of one or two metabolites are recommended namely the major metabolite, BE (Karch, 1998; Wennig, 2000; Society of Hair Testing, 2004; Smith, 2005; Kintz, 2007; Gwinnell and Adamec, 2008; Pragst and Balikova, 2008; Huang *et al.*, 2009; Míguez-Framil *et al.*, 2011). The use of metabolite-to-parent ratio is essential to obtain a positive result, there are defined cut-off values as well, namely to the consumption of COC: COC/BE > 0.05 ng/mg and for heroin: 6-MAM/morphine, >1.3 ng/mg (Society of HairSociety of Hair Testing, 2004)

A review of all findings concerning not only the analytical procedure of a hair analysis but all the parameters listed is needed to guarantee a valid interpretation of hair analysis.

3.1. Cocaine

The simultaneous determination of opiates and COC is possible true several procedures (Kintz and Mangin, 1995; Kronstrand *et al.*, 1998; Girod and Staub, 2000; Montagna *et al.*, 2000; Uhl, 2000; Romolo *et al.*, 2003; Kronstrand *et al.*, 2004; Scheidweiler and Huestis, 2004; Cordero and Paterson, 2007; Gottardo *et al.*, 2007;

Bucelli *et al.*, 2009; Fernández *et al.*, 2009; Huang *et al.*, 2009; Musshoff *et al.*, 2009; Guthery *et al.*, 2010; Nielsen *et al.*, 2010; Favretto *et al.*, 2011; Míguez-Framil *et al.*, 2011). As mentioned in Part I, COC abuse can be proved through the detection of the main analyte, COC, the major analyte founded in hair samples, and the metabolites resulting from its biotransformation (Musshoff and Madea, 2007a). One of the hair handicaps as biological matrix is the exposure to environmental contact and hence contamination. Consequently, is probable that certain drugs such as cocaine can contaminate a nonuser's hair (Kintz, 2007). This knowledge emphasizes the important role of decontamination procedure. There is no general consensus concerning the washing procedure, methanol (MeOH) followed by the non-protic solvent CH_2Cl_2 are the selected solvents in the majority of the studies reported in literature (Kintz and Mangin, 1995; Montagna *et al.*, 2000; Romolo *et al.*, 2003; Toledo *et al.*, 2003; Kintz, 2007; Musshoff and Madea, 2007a; Guthery *et al.*, 2010). However, the use of a single procedure is not the most recommended one, a multiple step wash procedure is the mostly mentioned (e.g., water, acetone, detergents (Tween or Sodium dodecyl sulfate (SDS)), petroleum ether, isopropanol, *n*-hexane, phosphate buffer) (Nakahara *et al.*, 1994; Kauert and Röhrich, 1996; Kronstrand *et al.*, 1998; Girod and Staub, 2000; Uhl, 2000; Kintz, 2007; Musshoff and Madea, 2007a; Barroso *et al.*, 2008; Fernández *et al.*, 2009; Huang *et al.*, 2009; Barroso *et al.*, 2010).

COC has the particularity of being easily hydrolyzed to BE and EME in alkaline conditions, thus, the extraction with various concentrations of acids has demonstrated to be useful, 0.1M HCl is the most used concentration (Kintz and Mangin, 1995; Montagna *et al.*, 2000; Cordero and Paterson, 2007; Gottardo *et al.*, 2007; Kintz, 2007; Musshoff and Madea, 2007a; Guthery *et al.*, 2010). Despite the fact that this is the most adopted procedure, Clauwaert *et al.* when carrying out HPLC analysis of COC and metabolites, faced with unsatisfying results, being them the appearance of large interfering peaks ((Clauwaert *et al.*, 2000). The use of other solvents as MeOH (Kauert and Röhrich, 1996; Kronstrand *et al.*, 1998; Uhl, 2000; Toledo *et al.*, 2003; Musshoff and Madea, 2007a; Huang *et al.*, 2009; Musshoff *et al.*, 2009) and buffer solutions (that create optimal conditions for COC extraction) (Romolo *et al.*, 2003; Kronstrand *et al.*, 2004; Bucelli *et al.*, 2009) is also referred. SFE were reviewed by Musshoff and Madea as an alternative method for the extraction COC (Musshoff and Madea, 2007a).

Concerning the clean-up, SPE is considered to be a "golden standard", and multiple procedures using this technique are described for the simultaneous quantification of morphine and COC (Kronstrand *et al.*, 1998; Clauwaert *et al.*, 2000; Girod and Staub, 2000; Montagna *et al.*, 2000; Uhl, 2000; Romolo *et al.*, 2003; Kintz, 2007; Musshoff and Madea, 2007a; Barroso *et al.*, 2008; Huang *et al.*, 2009; Musshoff

et al., 2009; Guthery *et al.*, 2010). A rapid screening procedure based on Headspace Solid-Phase Microextraction (HS-SPME) was developed for the analysis of COC, metabolites and other drugs. Neverthless, because no derivatization was performed, only cocaine was detected, but not the polar metabolites (Gentili *et al.*, 2004; Cognard *et al.*, 2005). A different clean-up and derivatization strategy was used by Toledo *et al.*, performing derivatization with butylchloroformate after Solid-phase microextraction (SPME) (Toledo *et al.*, 2003; Bermejo *et al.*, 2006).

GC-EI-MS (Kintz and Mangin, 1995; Girod and Staub, 2000; Montagna *et al.*, 2000; Romolo *et al.*, 2003; Toledo *et al.*, 2003; Gentili *et al.*, 2004; Kintz, 2007; Musshoff and Madea, 2007a; Barroso *et al.*, 2008; Rossi *et al.*, 2009; Favretto *et al.*, 2011) continuous to be the number one method followed by LC-MS (Clauwaert *et al.*, 2000; Kintz, 2007). One of the advantages LC methods is that the polar compounds can be analyzed directly, which eliminates the need for any previous derivatization procedure. MS/MS Spectrometry coupled with GC or LC and more recently, Electrospray Ionization (Montagna *et al.*; Míguez-Framil *et al.*, 2011), is used to enhance the sensitivity to metabolites at minor concentrations (Uhl, 2000; Kronstrand *et al.*, 2004; Scheidweiler and Huestis, 2004; Cognard *et al.*, 2005; Musshoff and Madea, 2007a; Huang *et al.*, 2009; Míguez-Framil *et al.*, 2011).

3.2. Morphine

Several developments of the methods used for opiates, cocaine, cannabinoids and A (Barroso *et al.*, 2008; Fernández *et al.*, 2009; Barroso *et al.*, 2010; Guthery *et al.*, 2010; Moller *et al.*, 2010; Nielsen *et al.*, 2010; Favretto *et al.*, 2011; Míguez-Framil *et al.*, 2011) have been reported, this developments include the different steps of the analytical procedures. Morphine can be detected as a result of absorption of heroin, morphine, codeine and 6-MAM.

The most popular decontamination procedures used for morphine analysis does not diverge of those mentioned for COC, MeOH and CH_2Cl_2, still being the elected solvents.

Extraction method, as referred before, is the most rigorous step. There are several studies concerning extraction methods and their effects on recovery and stability of the analytes (Barroso *et al.*, 2011). The hair sample can be pulverized in a ball-mill prior to testing, or cut into segments of about 1 mm, or the entire hair can be treated for extraction (Kintz, 2007). This step usually involves solvent hydrolysis or buffer extraction, MeOH (Kauert and Röhrich, 1996; Kronstrand *et al.*, 1998; Uhl, 2000; Scheidweiler and Huestis, 2004; Huang *et al.*, 2009; Musshoff *et al.*, 2009; Barroso *et*

al., 2010; Moller *et al.*, 2010) and acidic hydrolysis with HCl 0.1 M (Kintz and Mangin, 1995; Girod and Staub, 2000; Montagna *et al.*, 2000; Gentili *et al.*, 2004; Cognard *et al.*, 2005; Cordero and Paterson, 2007; Gottardo *et al.*, 2007; Kintz, 2007; Musshoff and Madea, 2007a; Guthery *et al.*, 2010). This seems to be commonly selected as method for the extraction of morphine and other opioids. Other methods are also reported, such as enzymatic digestion (e.g. glucuronidase arylsulfatase, protease) (Bermejo *et al.*, 2006; Musshoff and Madea, 2007a; Míguez-Framil *et al.*, 2011), a mixture trifluoroacetic acid (TFA)-MeOH (Nakahara *et al.*, 1994; Musshoff and Madea, 2007a; Favretto *et al.*, 2011) and MeOH-HCl (Barroso *et al.*, 2008; Barroso *et al.*, 2010; Barroso *et al.*, 2011). In general, the conditions of the extraction differ only on the temperature of incubation chosen because regarding the time of incubation, overnight (ON) is consensually chosen in the majority of the studies with the exception for the method used by Barroso *et al.*, that uses a 3h incubation with a good extraction yield (Barroso *et al.*, 2008).

In order to improve the signal-to-noise ratio, a sample cleanup procedure might be necessary; numerous methods have been suggested for the quantification of opioids in hair. The most important ones are described subsequently: SPE (Nakahara *et al.*, 1994; Kronstrand *et al.*, 1998; Girod and Staub, 2000; Montagna *et al.*, 2000; Uhl, 2000; Toledo *et al.*, 2003; Scheidweiler and Huestis, 2004; Cordero and Paterson, 2007; Musshoff and Madea, 2007a; Musshoff *et al.*, 2009; Barroso *et al.*, 2010; Míguez-Framil *et al.*, 2011) and LLE (Gottardo *et al.*, 2007; Favretto *et al.*, 2011).

The most frequently used chromatographic method is GC-EI-MS (Nakahara *et al.*, 1994; Kintz and Mangin, 1995; Kauert and Röhrich, 1996; Kronstrand *et al.*, 1998; Girod and Staub, 2000; Montagna *et al.*, 2000; Romolo *et al.*, 2003; Musshoff and Madea, 2007a; Musshoff *et al.*, 2009; Rossi *et al.*, 2009; Barroso *et al.*, 2010; Favretto *et al.*, 2011). In order to enhance the sensitivity of the method and also achieve characteristic mass fragments, there are a few derivatization procedures used by the reviewed literature. The most employed derivatizing agents for morphine analyzes are, bis(trimethylsilyl) trifluoroacetamide (BSTFA) sometimes together with 1% trimethylchlorosilane (TMCS) (Nakahara *et al.*, 1994; Kintz and Mangin, 1995; Kronstrand *et al.*, 1998; Musshoff and Madea, 2007a; Moller *et al.*, 2010), methyl-trimethylsilylheptafluorobutyramid, with trimethylsilylimidazole and *N*-methyl-bis(heptafluorobutyramide) (MSHFBA/TMSIM/MBHFBA) (Musshoff and Madea, 2007a) propionic acid anhydride (PAA) (Girod and Staub, 2000), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (Romolo *et al.*, 2003; Musshoff *et al.*, 2009; Barroso *et al.*, 2010; Favretto *et al.*, 2011), PFPA/PFPOH (Uhl, 2000; Musshoff and Madea, 2007a).

GC-MS/MS and LC-MS/MS has proved to be efficient and appropriate for the detection of drugs of abuse and equally sensitive and specific (Uhl, 2000; Kronstrand *et al.*, 2004; Scheidweiler and Huestis, 2004; Musshoff and Madea, 2007a; Barroso *et al.*, 2008; Huang *et al.*, 2009; Moller *et al.*, 2010). CZE has been also proposed for the quantitative determination of morphine in hair (Gottardo *et al.*, 2007). A more sophisticated UPLC-TOF-MS technique have been described by (Nielsen *et al.*, 2010).

Table 5 encloses some of the studies and progressions in hair analysis made since 1991 to 2011. There are enormous published articles, just a small sample of articles concerning the quantification of COC and morphine are presented below.

Table 5 - Literature review of analytical methodologies for the determination of COC and morphine in hair.

Analytes/Reference	Sample/ Sample amount	Decontamination	Extraction	Purification	Derivatization	Detection	LOD/LOQ ng/mg
Opiates (1991) (Musshoff and Madea, 2007a)	Hair/ 100 mg	MeOH	MeOH	Toluol/heptanes/ isoamyl alcohol from sodium bicarbonate buffer	MBTFA	GC-EI-MS	morphine: 0.1
COC, BE and EME (1991) (Musshoff and Madea, 2007a)	Hair/ 10 mg	H_2O, MeOH	Proteinase	SPE	MTBSTFA	GC-ion trap MS	COC: 0.1
Opiates (1993) (Musshoff and Madea, 2007a)	Hair/ 100 mg	MeOH	MeOH with MSTFA	-	-	GC-MS/MS	morphine: 0.25
Opiates, COC, BE and EME (1993) (Musshoff and Madea, 2007a)	Hair/ 10-30 mg	H_2O, Acetone	B-glucuronidase/arylsulfatase	SPE	PFPA/PFPOH	GC-EI-MS	COC: 0.1 morphine:0.04/ 0.16
6-acetylmorphine and morphine (Nakahara *et al.*, 1994)	Hair/ 4-8 mg	SDS, H_2O	MeOH/TFA (9:1)	SPE	BSTFA	GC-EI-MS	morphine: 0.2
Opiates, COC and major metabolites (Kintz and Mangin, 1995)	Hair/ ~30-50 mg	CH_2Cl_2	0.1M HCl	Chloroform/ isopropanol/*n*-heptane phosphate buffer	BSTFA/TMCS	GC-EI-MS	COC:0.05 morphine 0.10

THC, COC, morphine and 6-MAM (Kauert and Röhrich, 1996)	Hair/ 50-200 mg	H_2O, Acetone, Petroleum ether	MeOH	PAA	-	GC-EI-MS	COC: 0.1 morphine: 0.05
Opiates, A, and COC (Kronstrand *et al.*, 1998)	Hair/ 20-30 mg	Isopropanol, Deionized H_2O	MeOH	SPE	BSTFA/TMCS	GC-EI-MS	COC: 0.5 morphine: 0.25
morphine, COC, CE and EME (1998) (Musshoff and Madea, 2007a)	Hair/ 20 mg	-	Protease VIII/DTT	SPE	MSHFBA/TMSIM/ MBHFBA	GC-PCI-MS	COC: - morphine: 0.2 - 0.5
COC and metabolites (1998) (Kintz, 2007)	Hair/ -	CH_2Cl_2, H_2O	0.1M HCl	SPE	-	HPLC-FL	-
Opiates and COC (1999) (Musshoff and Madea, 2007a)	Hair/ 100 mg	CH_2Cl_2	MeOH	-	BSTFA/TMCS	GC-MS/MS	COC: 0.5 morphine: 0.1
COC and metabolites (1999) (Musshoff and Madea, 2007a)	Hair/ -	CH_2Cl_2	MeOH:TFA (9:1)	SPE	HBFA/HFPOH	GC-EI-MS	-
codeine, 6-MAM, morphine, COC, methadone, 3,4-methylenedioxyamphetamine (MDMA) and Eve (MDE) (Girod and Staub, 2000)	Hair / 50 mg	CH_2Cl_2, H_2O, MeOH	0.01 M HCl	SPE	PAA	GC-EI-MS	COC: 0.02 morphine: 0.05
Opiates, COC and metabolites (Montagna *et al.*, 2000)	Hair/ 50 mg	MeOH	0.1M HCl	SPE	MSTFA	GC-EI-MS	COC: 0.04 morphine: 0.04

COC and metabolites (2000) (Musshoff and Madea, 2007a)	Hair/ 20 mg	-	0.1M HCl	SPE	HFPOH/TFAA	GC-NCI-MS/MS	COC: 0.01
COC and metabolites (Clauwaert *et al.*, 2000)	Hair/ 100 mg	CH_2Cl_2, H_2O	0.1M HCl	SPE	-	LC-MS	0.025
COC and metabolites (2000) (Kintz, 2007)	Hair/ -	Tween, H_2O	0.1M HCl	SPE	-	HPLC-MS	-
COC and metabolites (2000) (Musshoff and Madea, 2007a)	Hair/ 25 mg	Isopropanol, phosphate buffer	Protease XI	SPE	BSTFA/TMCS	GC-EI-MS	COC: 0.1-0.3
A, methamphetamine (MA), MDA, MDMA (ecstasy), MDE, MBDB, methadone and metabolite, THC, COC, BE, CE, opiates (Uhl, 2000)	Hair/ 10 mg	*n*-hexane, Acetone	MeOH	SPE	PFPA/HFIP	GC-MS/MS	COC: - morphine: 0.05-0.1
COC and metabolites (2001) (Musshoff and Madea, 2007a)	-	CH_2Cl_2	-	MeOH	MTBSTFA	GC-EI-MS	COC: 0.12
COC and metabolites (2001) (Musshoff and Madea, 2007a)	Hair/ 30-70 mg	-	SFE	-	TMS/BSTFA or PFPA	GC-EI-MS	-
COC and metabolites (2001) (Musshoff and Madea, 2007a)	Hair/ 20 mg	4 washing procedures	0.1M HCl	SPE	-	LC-MS	-

COC and metabolites (2002) (Musshoff and Madea, 2007a)	Hair/ -	Isopropanol, phosphate buffer	Proteinase K, DTT, detergent	SPE	-	LC-MS/MS	-
Opiates, COC and BE (Romolo *et al.*, 2003)	Hair/ 20 mg	MeOH	Phosphate Buffer	SPE	MSTFA/TMS	GC-EI-MS	COC:0.05 morphine: 0.05
COC, BE and CE (Toledo *et al.*, 2003)	Hair/ -	CH_2Cl_2	MeOH	SPME	Butychloroformate	GC-EI-MS	COC: 0.1
Opiates, COC and Metabolites (Scheidweiler and Huestis, 2004)	Hair/ 20 mg	-	MeOH	SPE		LC-APCI-MS/MS	COC: 0.0085-0.017 morphine: 0.0415-0.083
COC and metabolites (2004) (Kintz, 2007)	Hair/ -	CH_2Cl_2	HCl 37%	SPE	PAA	GC-EI-MS	-
nicotine, cotinine, morphine, codeine, 6-acetylmorphine, ethylmorphine, A, MA, MDA, MDMA, BE, COC, 7-aminoflunitrazepam and diazepam (Kronstrand *et al.*, 2004)	Hair/ 10-50 mg	-	acetonitrile/ MeOH/ 20 mM formate buffer (10:10:80)	-	-	LC-MS/MS	COC: 0.01 morphine: 0.03-0.05
COC, MDMA, MDA, MA, A, MDE, Ketamine and methadone (Gentili *et al.*, 2004)	Hair/ 20 mg	H_2O, Acetone	1M HCl	HS-SPME	-	GC-EI-MS	COC: 0.35-1.05

Opiates (2005) (Musshoff and Madea, 2007a)	Hair/ 50 mg	H_2O, Acetone, petroleum ether	MeOH	SPE	MSTFA	GC-EI-MS	morphine: 0.1/0.2
COC and metabolites (Cognard *et al.*, 2005)	Hair/ 50 mg	CH_2Cl_2, H_2O and MeOH	0.01M HCl	HS-SPME	-	GC-CI-MS/MS	COC: 0.005
COC and metabolites (2005) (Musshoff and Madea, 2007a)	Hair/ 50 mg	CH_2Cl_2	MeOH	-	MSTFA:NH_4I:DTE	GC-MS/MS	-
COC and CE (Bermejo *et al.*, 2006)	Hair/ 50 mg	Tween 80	Enzymatic hydrolysis	SPME	-	GC-EI-MS	COC: 0.08/0.4
Opiates, A, COC and metabolites, diazepam (Cordero and Paterson, 2007)	Hair/ 10-50 mg	CH_2Cl_2, Isopropanol and acetone	0.1M HCl	SPE	MBTFA, MSTFA/TMS	GC-MS	COC: 0.2
6-MAM, morphine, A, methamphetamine (MA), (MDA), 3,4-methylenedioxymethampthetamine (MDMA), BE, ephedrine and COC (Gottardo *et al.*, 2007)	Hair/ ~100 mg	Tween 20	0.1 M HCl	LLE	-	CZE-MS	< 0.1
COC, BE (Barroso *et al.*, 2008)	Hair/ 20 mg	CH_2Cl_2, deionized water and MeOH	MeOH : HCl (2:1)	SPE	MSTFA/TMCS (5%)	GC-EI-MS	COC: 0.02 - 0.05

A ,Diazepam and its Metabolites, COC and its Metabolites and Opiates (Miller *et al.*, 2008)	Hair/ 10 mg	0.1% SDS, deionized water and dichloromethane	Phosphate buffer pH 5	-	-	LC-ESI-MS/MS	0.02 - 0.09
morphine, codeine, 6 – MAM, COC and BE (Huang *et al.*, 2009)	Hair/ 20 mg	CH_2Cl_2 H_2O	MeOH	-	-	LC-MS/MS	COC: 0.001 morphine:0.01
Opiates, COC and metabolites (Fernández *et al.*, 2009)	Hair/ -	Tween 80, H_2O	MAE	-	-	HPLC–DAD	-
Opiates and COC (Musshoff *et al.*, 2009)	Hair/ 50 mg	H_2O, petroleum benzene, CH2Cl2	MeOH	SPE	MSTFA/pyridine	GC–EI-MS	COC: 0.01/0.11 morphine: 0.03/0.11
Morphine and GHB (Rossi *et al.*, 2009)	Hair/ 20 mg	GHB: methylene chloride morphine: CH_2Cl_2	GHB : NaOH 0.01M morphine: KOH 0.1M	GHB: - morphine: LLE	GHB: BSTFA/TMCS morphine: PFPA/PFPOH	GC-EI-MS	0.01/0.1
COC and its metabolites, opiates and some stimulants (Bucelli *et al.*, 2009)	Hair/ 20-50 mg	CH_2Cl_2	phosphate buffer (pH 5.0)	SPE	-	LC-MS/MS	0.005 – 0.08/ 0.02 – 0.25

Opiates, A, MDA, MDMA, methadone and benzylpiperazine (BZP) (Guthery *et al.*, 2010)	Hair/ 20 mg	MeOH	0.1M HCl	SPE	MTBSTFA TFAA	GCxGC/TOF-MS	-
A, ketamine, methadone, COC, cocaethylene and THC (Merola *et al.*, 2010)	Hair/ -	Deionized water, acetone	1M HCl	HS-SPME	acetic anhydride MSTFA	GC-MS	0.01-0.12/0.02 - 0.37
Codeine, morphine and 6-MAM (Moller *et al.*, 2010)	Hair/ 10 mg	-	MeOH	HS-SPME	BSTFA + 1%TMCS	GC-MS	morphine: 0.01
A, analgesics, antidepressants, antipsychotics, benzodiazepines, COC and opioids (Nielsen *et al.*, 2010)	Hair/ 20 mg	-	methanol : acetonitrile : ammonium formate	Minivials with PTFE filter	-	UPLC-TOF-MS	COC: 0.01-0.05 morphine: 0.03-0.50
6–MAM, morphine, codeine, 6 – acetylcodeine and tramadol (Barroso *et al.*, 2010)	Hair/ 20 mg	CH_2Cl_2, deionized water and MeOH	MeOH	SPE	MSTFA/TMS	GC-EI-MS	Morphine: 0.05
Opiates and COC (Míguez-Framil *et al.*, 2011)	Hair/ 50 mg	Shampoo, H_2O	Enzymatic hydrolysis	SPE followed by Matrix solid phase dispersion (MSPD)	-	ESI-MS/MS	COC: 0.007 morphine: 0.04

A, Amphetamine; BSTFA/TMCS, Bis (trimethylsilyl) trifluoroacetamide / Trimethylchlorosilane; BSTFA/TMCS, Bis (trimethylsilyl) Trifluoroacetamide/ Trimethylchlorosilane; BSTFA, Bis (trimethylsilyl) trifluoroacetamide; CH_2Cl_2, Dichloromethane; COC, Cocaine; CZE, Capillary Zone Electrophoresis; CZE-MS, Capillary Zone Electrophoresis – Mass Spectrometry; DTT, Dithiothreitol; ESI-MS/MS, Electrospray Ionization – Tandem Mass Spectrometry; GC - ion trap – MS, Gas Chromatography – ion trap – Mass Spectrometry; GC - MS/MS, Gas Chromatography - Tandem Mass Spectrometry; GC-EI-MS, Gas Chromatography - Electron Impact ionization – Mass Spectrometry; GC-NCI-MS/MS, Gas Chromatography - Negative Chemical Ionization- Tandem Mass Spectrometry; GC-PCI-MS, Gas Chromatography - Positive Chemical Ionization - Mass Spectrometry; GCxGC/TOF-MS, Gas Chromatography x Gas Chromatography /Time-of-Flight MassSepctrometry; GHB, gamma-Hydroxybutyric Acid; GHB, gamma-Hydroxybutyric Acid; H_2O, Water; HBFA, Heptafluorobutyric anhydride; HCl, Hydrochloric acid; HFPOH, Heptafluoropropanol; HPLC–DAD, Liquid Chromatography - Diod-Array Detector; HPLC-FL, High Pressure Liquid Chromatography with Fluorescence; HPLC-MS, High Pressure Liquid Chromatography - Mass Spectrometry; HS-SPME, Headspace – Solid Phase Microextraction; LC-APCI-MS/MS, Liquid Chromatography - Atmospheric Pressure Chemical Ionization– Tandem Mass Spectrometry; LC-ESI-MS/MS, Liquid Chromatography - Electrospray Ionization- Tandem Mass Spectrometry; LC-MS, Liquid Chromatography - Mass Spectrometry; LC-MS/MS, Liquid Chromatography – Tandem Mass Spectrometry; LLE, Liquid-Liquid Extraction; MAE, Microwave-Assisted Extraction; MDA, 3,4-methylenedioxyamphetamine; MDE, 3,4-methylenedioxy-*N*-ethylamphetamine; MDMA, 3,4-methylenedioxymethampthetamine; MBTFA, *N*-methyl-bis(trifluoroacetamide); MeOH, Methanol; MeOH/TFA, Methanol/ Trifluoroacetic acid; MSTFA, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide; MSTFA/TMCS, N-methyl-N-(trimethylsilyl) trifluoroacetamide/ Trimethylchlorosilane; MSTFA/TMS, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide Trimethylsilyl; MSTFA:NH_4I:DTE, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide:ammonium iodide: dithioerythritol; MTBSTFA; *N*-methyl-*N*-(t-butyldimethylsilyl)trifluoroacetamide NaOH, Sodium Hidroxyd PAA; Propionic Acid Anhydride PFPA, Pentafluoropropionic anhydride PFPA, Pentafluoropropionic anhydride; PFPA/HFIP, Pentafluoropropionic anhydride/ *Hexafluoroisopropanol;* PFPA/PFPOH, Pentafluoropropionic anhydride / Pentafluoropropanol; PFPOH, Pentafluoropropanol; PTFE, Polytetrafluoroethylene; SDS, Sodium Dodecyl Sulfate; SDS, Sodium Dodecyl Sulfate; SFE, Supercritical Fluid Extraction; SPE, Solid-phase Extraction; TFAA, trifluoroacetic anhydride; TMCS, Trimethylchlorosilane; TMCS; Trimethylchlorosilane; TMS/BSTFA; Trimethylsilyl/ Bis (trimethylsilyl) trifluoroacetamide; TMSIM, Trimethylsilylimidazole; UPLC-TOF-MS, Ultra-Performance Liquid Chromatography - Time-of-Flight – Mass Spectrometry;

PART II: General and specific objectives of this thesis

The general objectives of this study was to develop and validate an analytical method that allows the simultaneous determination of cocaine and morphine in human hair samples using GC method coupled a mass spectrometry detector with acceptable LOD and LOQ. In this sense, for development and validation of GC-MS method for quantification of cocaine and morphine in hair numerous studies and preliminary experiments were developed that led to the most appropriates and satisfactory results:

I. To study different conditions of GC-MS with methanolic solutions of COC and morphine in order to obtain the best peak resolution and separation of the two compounds;
II. To study derivatization conditions taking into consideration the derivatized agent, incubation times and temperatures;
III. To optimize the extraction procedure;
IV. To study the selectivity, sensitivity, LOD, LOQ, accuracy and precision of the developed method;
V. After the successful development and validation, the application of the GC-EI-MS method to real samples. These real samples were collected in volunteers attending at Support Center for Detoxification (Centro de Atendimento a Toxicodependentes (CAT)), Valongo and Gondomar - Porto.

PART III: Experimental Part

Chapter I

Materials and methods

1. Materials and methods

1.1. Reagents and materials

All chemicals and reagents were of analytical grade. Methanol (MeOH), trifluoroacetic acid (TFA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), Bis (trimethylsilyl) trifluoroacetamide / Trimethylchlorosilane (BSTFA+1%TMCS) and cocaine (Míguez-Framil *et al.*) were obtained from Sigma–Aldrich (St. Louis, MO). Ammonium hydroxide was obtained from Panreac (Barcelona, Spain). morphine and ethylMorphine (EM) (Internal Standard) were obtained from Lipomed (Arlesheim, Switzerland). Isopropanol, hydrochloric acid, potassium dihydrogen phosphate were acquired from Merck (Darmstadt, Germany). OASIS® MCX cartridges, 10 mg, 1 mL capacity were obtained from WATERS (Milford, MA).

1.2. Hair samples

Authentic hair samples were collected from drug abusers admitted to centers for detoxification treatment (CAT, Valongo and Gondomar – Porto). Drug-free control hairs were taken from volunteers. All the volunteers declared they had never used drugs such as opiates or cocaine. Hair was collected from the vertex posterior area as close as possible to the scalp and the proximal and distal ends were carefully identified has recommended (Jurado and Sachs, 2003; Agius and Kintz, 2010; Dinis-Oliveira *et al.*, 2010). The collection method is exemplified in **Fig. 6**.

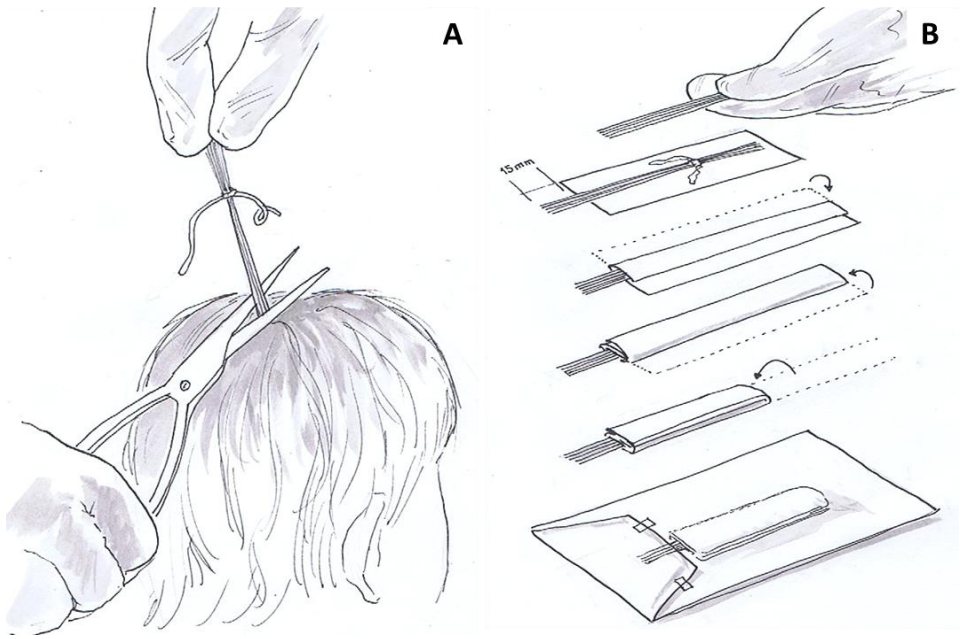

Fig. 6 – A- A lock of hair about the width of a thin pencil, or several locks of hair are cut from the posterior vortex region of the scalp or the back of the skull as close as possible to the scalp. **A –** Holding the sample tightly, the cut root ends of the sample should be aligned and carefully placed flat on a piece of aluminum foil (previously folded once or twice) with the cut root ends projecting ~15 mm beyond the end of the foil; **B –** The root end of the foil should be marked and the foil folded around the hair and pinched tightly to be kept in place (Reproduced from Dinis-Oliveira *et al.*, 2010).

1.3. Preparation of stock solutions

Stock solutions of the analytes and internal standards in MeOH (1 mg/mL) were prepared and stored at - 20ºC until use. A working solution of the internal standard at 15 µg/mL was also prepared in MeOH. Working standard solutions were prepared daily at different concentrations by dilution of stock solutions with MeOH. For this purpose, a concentration of 40 µg/mL and 1 µg/mL were prepared in MeOH in order to prepare by dilution a range of different concentrations between 5 – 200 ng/mL of COC and morphine corresponding to 0.25 – 10 ng/mg. The concentrations of the methanolic solutions in hair (ng/mg) were obtained by spiking 20 mg of hair, with different concentrations of cocaine and morphine.

To prepare 0.1M potassium dihydrogen phosphate (pH 7), 13.61g of potassium dihydrogen phosphate was weight and dissolved in a final volume of 1 L of deionized water. The pH adjustments were made using NaOH 1 M. This solution was stored at 4ºC.

1.4. Sample preparation for GC-MS analysis

1.4.1. Hair decontamination

Before analysis the hair samples were decontaminated by washing successively whole strands in 5 mL CH_2Cl_2, 5 mL of deionized water and 5 mL of MeOH (twice, sequentially). After MeOH removal,hair was incubated at 40ºC for 30 minutes until completely dryness and then cut into small segments. The last two washes with MeOH was analyzed as describe below to verify the absence of external contamination.

1.4.2. Drug extraction from hair

The drugs are fixed inside the hair matrix; therefore an extraction procedure is required. Approximately 20 mg aliquots of hair cut into small pieces were weighed into 10mL glass tubes, and 3 mL of a mixture of methanol/hydrochloric acid (2:1, v/v) solution and 40 µL of IS (15 µg/mL) were added, and incubated ON (~16h) at 65ºC (**Fig. 7**). After centrifugation at 3500 rpm for 5 min, the methanolic solutions were transferred to clean glass tubes, and 5 mL of KH_2PO_4 0.1 M (pH 7) were added. The samples were homogenized by rotation/inversion movements. Optimization procedures and different approaches to the extraction method are discussed in the results.

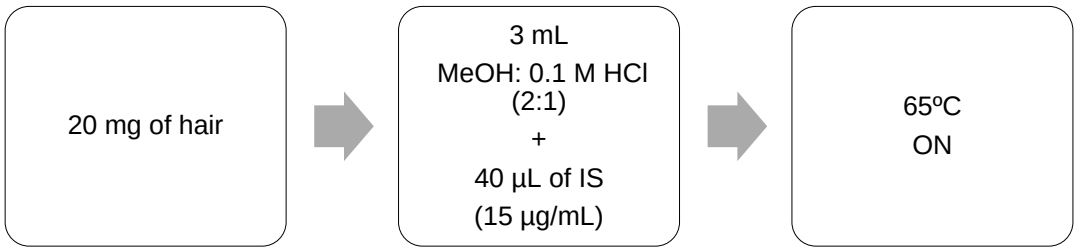

Fig. 7 – Optimized extraction procedure

1.4.3. Solid Phase Extraction (SPE)

The purification of standards, blanks (supernatant without analytes) and positive samples were performed in mixed-mode extraction cartridges (MCX), which had been previously conditioned with 2 mL of MeOH and 2 mL of deionized water. After the sample had passed through, the cartridges were washed sequentially with 2 mL of each of the following: hydrochloric acid 0.1 M and MeOH. After drying under full vacuum for 1 minute, the analytes were eluted with 2 mL of a mixture of dichloromethane: isopropanol (80:20, v/v) with 2% of NH_4OH (**Fig. 8**). The obtained solutions were evaporated to dryness in an acid – resistant Centrivap concentrator (Labconco).

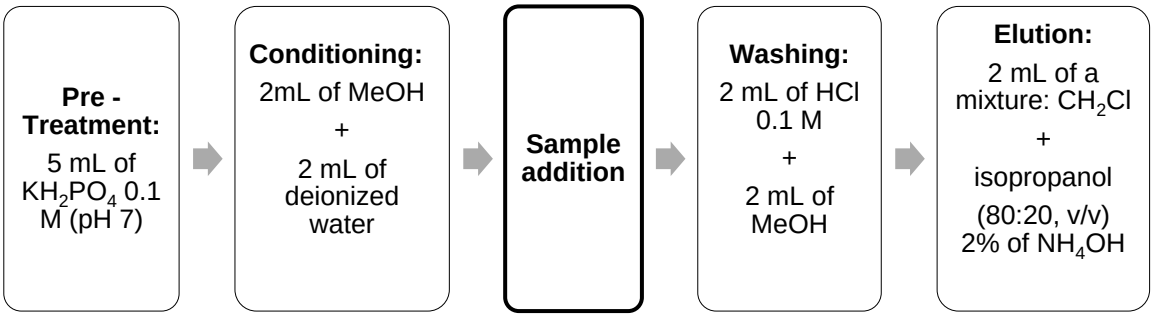

Fig. 8 - SPE conditions used in the present work.

1.4.4. Derivatization procedure

Fifty µL of MSTFA were added to the evaporated solution, mixed and heated for 30 minutes at 80ºC. After cooling to room temperature, the samples (2 µL) were injected into the GC-EI-MS system (optimization of the derivatization conditions is discussed in chapter 2).

1.5. Gas-chromatography mass spectrometry conditions

Quantitative GC-EI-MS analyses were performed on a Varian CP-3800 gas chromatograph equipped with a VARIAN GC/MS/MS Saturn 2200 ion trap mass detector. Chromatographic separation was achieved using a capillary column VF-5ms (30m×0.25mm×0.25m) from VARIAN and a high purity helium C-60 (Gasin, Portugal) as carrier gas. Two µL of each derivatized sample was injected. An initial temperature of 80ºC for 1 min, increased to 300ºC at 10ºC/min, held for 4 minutes giving a total run time of 27 minutes. The flow of the carrier gas (helium) was maintained at 1.0 mL/min in constant flow mode. The injector port was set at 280ºC. The analysis was performed in full scan in splitless injection mode. The obtained full scan chromatogram was reprocessed using the selected qualifier ions and retention times presented in T**able 6**.

Table 6 - Detection parameters of COC, morphine and IS by GC-EI-MS

Analytes	**Retention time (t_r)**	**Fragments** (*m/z*)
COC	18.176	82; 182 and 303
Morphine	20.421	429
IS	20.375	385

1.6. Method validation

The validation of the method was performed accordingly European Medicines Agency, EMA (EMA, 2009). The limit of detection (LOD), limit of quantification (LOQ), accuracy, recovery, linearity and intra-assay and inter-assay precision of the method, were obtained. In order to obtain these validation data, calibration samples were prepared by spiking blank hair with appropriate volumes of cocaine and morphine standard solutions.

1.6.1. Selectivity

Blank samples (no analyte or internal standard added) were extracted as described above and analyzed by GC-IT/MS to detect possible interferences (matrix-related or other resulting from the experimental procedure) with cocaine and morphine. Chromatographic selectivity was evaluated by the presence or absence of co-eluting peaks at the retention times of the analytes. Three independent experiments were performed.

1.6.2. Linearity

The method linearity was determined by evaluation of the regression curve (ratio of analyte peak area and IS peak area versus analyte concentration)w and expressed by the squared correlation coefficient (r^2) using spiked samples. Three independent calibration curves (y = mx + b) were obtained using six different concentrations of cocaine and morphine (0.25, 0.5, 1, 2.5, 5 and 10 ng/mg) and the mean slopes were obtained in order to calculate the concentration of real samples (unknown concentrations). These concentrations were prepared daily as mentioned in section 1.3.

1.6.3. Limit of detection (LOD) and Limit of quantification (LOQ)

Limits of detection and quantification were determined based on the standard deviation of the response and the slope. The limit of detection (LOD) may be expressed as: $\mathrm{LOD} = \frac{3.3\sigma}{S}$ and the limit of quantification (LOQ) as: $\mathrm{LOQ} = \frac{10\sigma}{S}$ where σ is the standard deviation of the response and S the slope of the calibration curve.

1.6.4. Precision and accuracy

The repeatability precision data was quantified by analyzing the areas of 3 replicates of each concentration (0.25, 1 and 10 ng/mg) and calculating the coefficient of variation on the same day (% CV intraday). The areas of the three consecutive injections performed at each concentration on 3 different days were used to calculate the interday repeatability (% CV interday). The accuracy of the method was evaluated by spiking blank matrix with three different COC and morphine concentrations (0.25, 1 and 10 ng/mg, corresponding to 5, 20 and 200 ng/mL) and through the calculation of the percentage deviation between the calculated value and the nominal value [Accuracy () = () × 100].

1.6.5. Recovery

The recovery was evaluated analyzing two sample groups of the same concentrations (0.25, 1 and 10 ng/mg) in triplicate, but differently processed. In the first group, COC, morphine and IS were analyzed following the extraction and SPE procedures mentioned above. In the second group, the samples were spiked with COC and morphine before extraction while the IS was only added to the extract after the SPE procedure, but before drying. The recovery was evaluated by the comparison of the mean response of the two groups. The response of the unextracted group represents 100% recovery.

1.6.6. Proof of applicability

Several samples collected from drug abusers attending the support center for detoxification (CAT, Gondomar - Porto) were analyzed by the validated method. The presence of cocaine and morphine was confirmed by the clinical information of each volunteer. The hair samples were collected, stored and analyzed as described above.

Chapter II

Results and Discussion

2. Results and Discussion

2.1. Choice of suitable derivatization temperature

Morphine due to its hydroxyl groups (-OH) needs to be derivatized to originate a more volatile compound and consequently a better peak resolution (Niessen, 2001), cocaine's chemical stability during the derivatization process must be also evaluated (since both drugs are analyzed simultaneous); several variables concerning derivatization were taken into consideration (derivatization temperature, time and agent).

Three different temperatures of silylation, a low (40ºC), medium (55ºC) and a high (80ºC) temperature were analyzed in order to evaluate the effectiveness of the derivatization procedure. For each temperature, triplicates of supernatant spiked with 500 ng/mL of COC and morphine were analyzed also with IS at final concentration of 200 ng/mL. Dried residues were submitted to derivatization with 50 µL of MSTFA and incubated at the different temperature conditions and different time of incubation (20, 30 and 40min).

The obtained results (**Fig. 9**), showed that at different temperatures and time points COC chemical stability is slightly different, at 80ºC after 30 min the abundance of cocaine increases corresponding to a more adequate stabilization. The same temperature and time presented better results for morphine which proved to be the appropriate conditions for derivatization.

Another derivatizing agent, BSTFA which incubation temperature was 70ºC for 20 minutes, was also studied aiming the comparison of the obtained results from MSTFA derivatization; based on the results of ratios between analyte peak area / IS peak area presented in **Fig. 9**, the possibility of BSTFA as derivatizing agent was excluded despite the fact that BSTFA is one the most cited in literature. In the present work, MSTFA, temperature of 80ºC and 30 minutes of derivatization were chosen regarding the observed results.

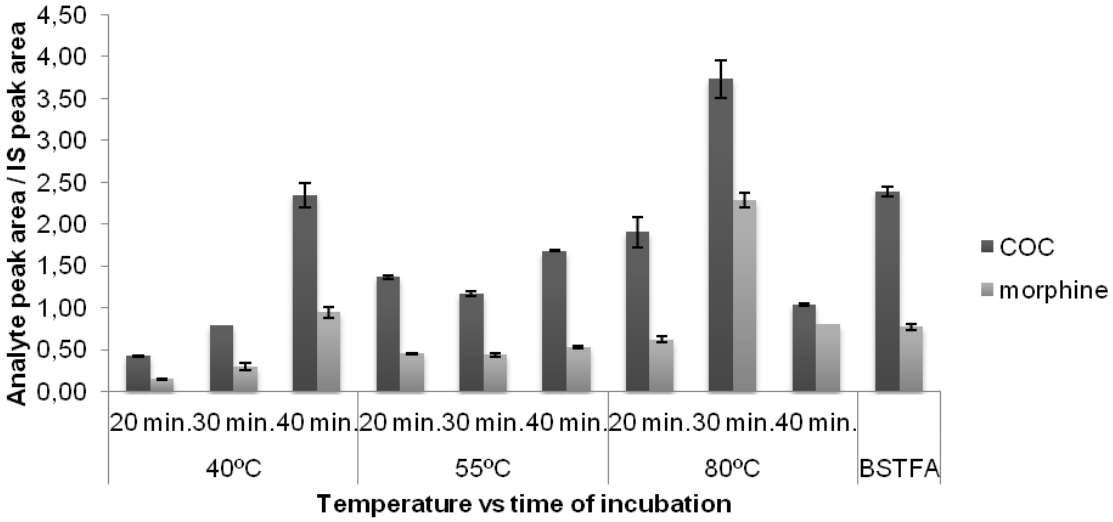

Fig. 9 - Suitable derivatization temperature time of incubation and derivatizing agent. Different temperatures and times corresponds to MSTFA derivatizing agent

2.2. Optimization of drug extraction

As already mentioned in Part I, the drug extraction from the matrix is the most consuming and rigorous step because the drugs are firmly enclosed in the hair structure (Tagliaro *et al.*, 1997; Romolo *et al.*, 2003; Kronstrand *et al.*, 2004; Barroso *et al.*, 2008; Pragst and Balikova, 2008; Barroso *et al.*, 2010; Nielsen *et al.*, 2010).

There were found several approaches in literature concerning drug extraction, methanolic extraction with or without ultrasonic bath and HCl 0.1M (Kauert and Röhrich, 1996; Gentili *et al.*, 2004; Kronstrand *et al.*, 2004; Scheidweiler and Huestis, 2004; Cordero and Paterson, 2007; Gottardo *et al.*, 2007; Musshoff and Madea, 2007b; Huang *et al.*, 2009; Musshoff *et al.*, 2009; Guthery *et al.*, 2010; Moller *et al.*, 2010).

A mixture between MeOH and acid was used to accomplish the extraction of COC and morphine from hair. The first mixture consisted in using 3 mL of MeOH:0.1M HCl (2:1) as suggested by (Barroso *et al.*, 2008; Barroso *et al.*, 2010). Extraction time effect was studied (3h, 5h and ON at 65ºC) due to its great relevance and thoroughness of the method.

The other mixture used was 3 mL of MeOH:TFA (9:1) (Nakahara, 1999; Musshoff and Madea, 2007b; Favretto *et al.*, 2011). The main differences among the two methods are the acid used and the incubation temperature, 65ºC for MeOH: 0.1 M HCl and 45ºC for MeOH:TFA with a previous ultrasonic bath (room temperature, 1h).

Besides all the parameters taken under consideration during an extraction procedure, pH is a crucial factor since COC is easily hydrolyzed to BE under alkaline

conditions (Kintz and Mangin, 1995; Montagna *et al.*, 2000; Cordero and Paterson, 2007; Gottardo *et al.*, 2007; Kintz, 2007; Musshoff and Madea, 2007b; Guthery *et al.*, 2010), which support the applied acidic mixtures chosen. The extraction procedures are summarized in **Fig. 10**.

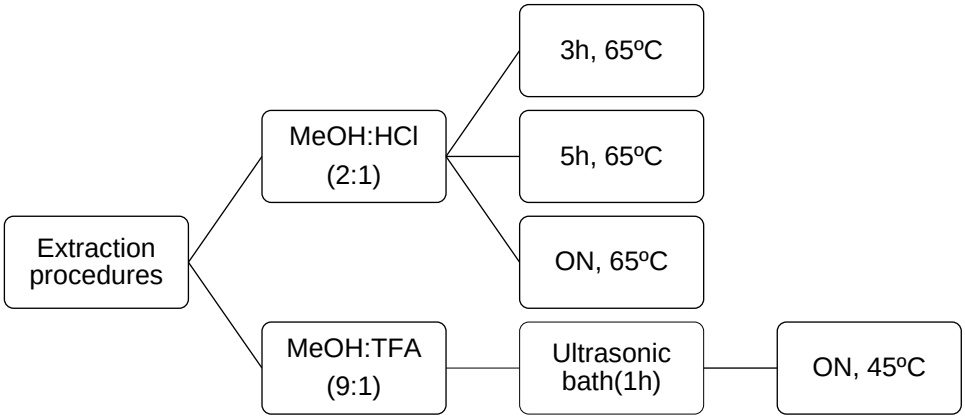

Fig. 10 - Representative scheme of the studied extraction conditions.

Spiked hair cannot simulate adequately an authentic hair sample, therefore in order to optimize the extraction method, soaked (fortified) hair was used exposing 1 g pool of a blank sample to a COC and morphine aqueous solution at 40 µg/mL (35ºC, 72h) (Barroso *et al.*, 2008; Lee *et al.*, 2008). This soaked sample was analyzed at different times of incubation (0h, 24h, 48h and 72h) to verify if there were incorporation of the drugs into hair at an early incubation time. The incorporation of the analytes within the matrix was only verified after 72h of incubation. The possibility of adsorption was excluded by the analysis of the several washes made with MeOH. The steps of the referred procedure are described below:

→ 1g blank hair + 4 mL of an aqueous solution of COC and morphine at 40 µg/mL
→ Incubation at 35ºC during 72 hours.
→ Decontamination: CH_2Cl_2 + deionized water + MeOH (the hair was rinsed with MeOH until no analyte could be detected in the GC-EI-MS chromatogram. To achieve this condition the hair was washed with MeOH seven times.

→ 20mg (triplicate) of the hair pool were used to proceed with the optimization procedure.

The effectiveness of the extraction procedure and time of extraction was established by confronting the area counts of COC and morphine obtained of each extraction method, whereas the MeOH: 0.1M HCl presented the highest areas without significant changes for COC but with a more pronounced increase in morphine. Taking into account the possible conversion of COC into BE, the chosen conditions were, 65ºC ON which goes in agreement with the majority of the consulted literature as already mentioned.

After each tested time, the extracts were analyzed following the same procedures mentioned in methods. The results of the optimization extraction procedure are presented in **Fig. 11**.

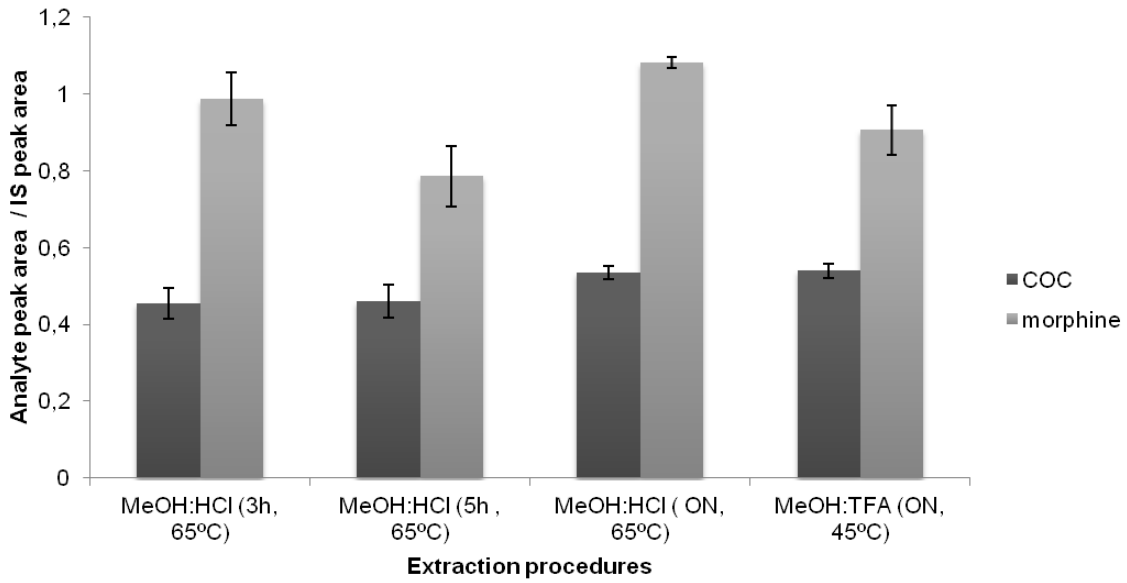

Fig. 11 - Effects of the different extraction procedures and times of extraction on the extracted analytes.

2.3. Method Validation

2.3.1. Chromatographic separation, selectivity and specificity

The purification of the spiked sample by SPE was performed essentially to eliminate all the possible interferences in the chromatogram as a result of the presence of endobiotics in hair samples. Nevertheless, even after the clean-up of samples the chromatograms revealed some interference derived from hair constituents or other substances without any relevance for this study.

Preliminary tests were performed to obtain the best peak resolution and separation of the two compounds, for which several parameters related to the detection were tested such as, flow rate of the carrier gas and column oven temperatures were tested. In order to achieve best sensitivity, the possibility to inject a commonly used sample volume (1 µL) was also analyzed, but a 2 µL volume sample showed enhance of the sensibility without adding significant interferences. It was possible to detect the analytes of interest and the IS at the expected retention times (COC – 18.176 min., morphine – 20.421 and IS – 20.375 min.). Even though a great peak resolution between the IS and morphine was not achieved (**Fig. 12**), although this compound can still be used as an IS, since it is cheaper than the deuterated drugs and more environmental friendly. The integration of the chromatographic peaks for quantitative analysis was performed by monitoring the full-scan chromatogram with the specific ions (SIM mode) allowing more precise peak integration (relevant in small chromatographic areas) (Dinis-Oliveira *et al.*, 2010).

Fig. 12 represents a full-scan chromatogram of a spiked 10 ng/mg methanolic solution of COC and morphine with all the analytes successfully identified as well as the IS.

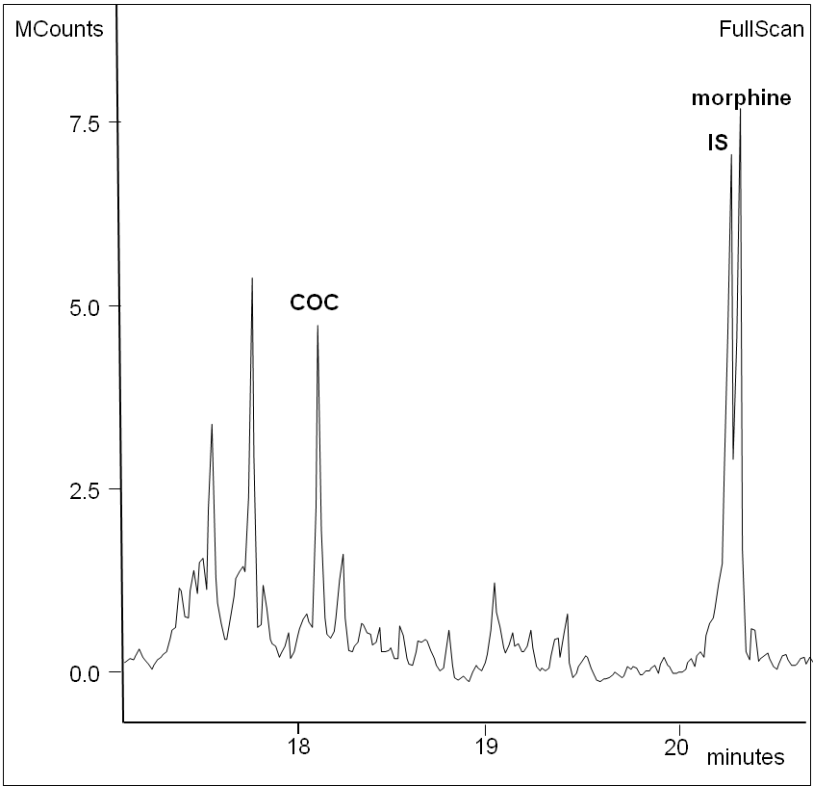

Fig. 12 – Reconstructed GC-EI-MS full-scan chromatogram of a spiked methanolic sample (10 ng/mg)

The GC-EI-MS chromatograms of spiked COC and morphine samples were compared with the chromatograms obtained in a blank sample. Through the analysis of a blank sample, without analytes and IS, it is possible to affirm that no interfering peaks were revealed in the chromatogram at the retention times and selected ions (**Fig. 13**). Consequently, the selectivity and specificity was guaranteed in this method. This demonstrates that co-eluting compounds from the matrix of hair can result in false-positive results (Nielsen *et al.*, 2010).

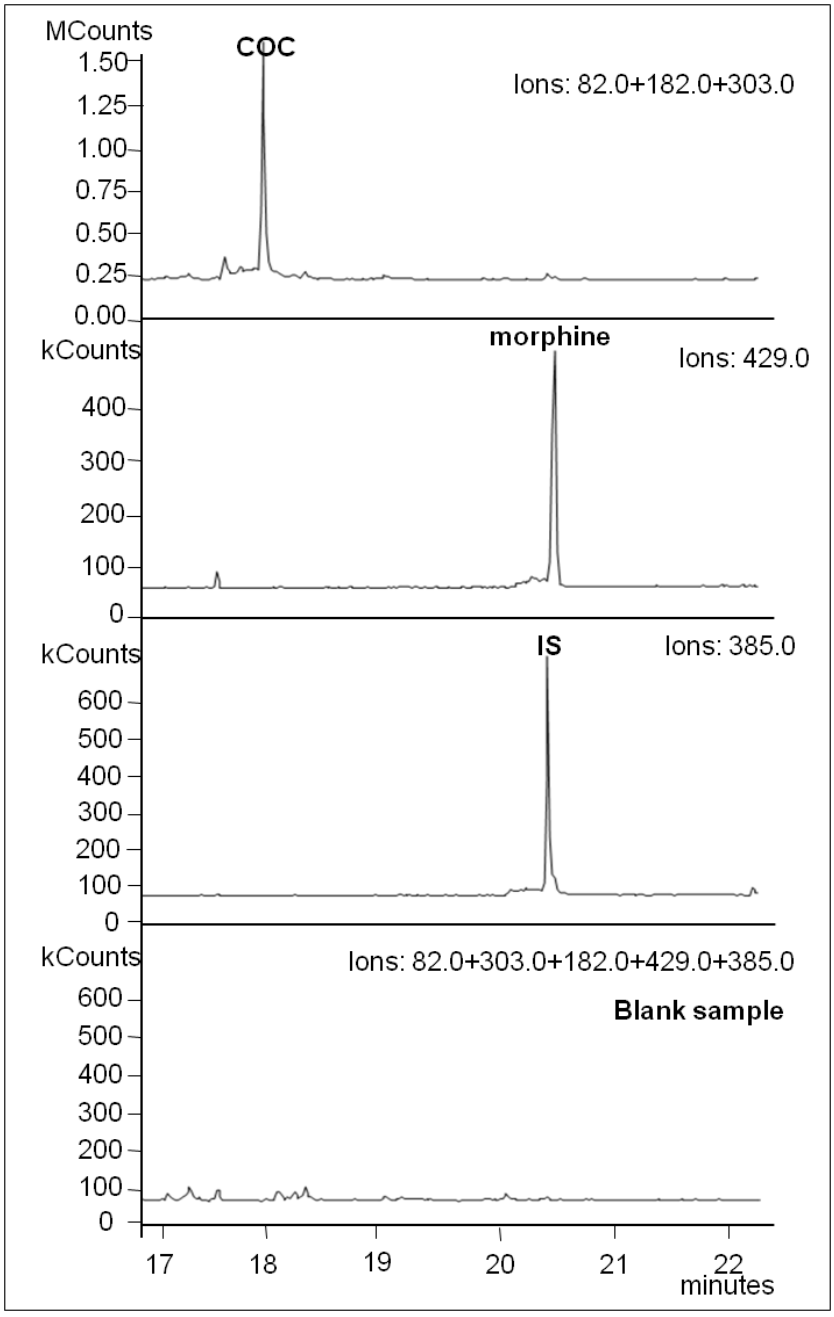

Fig. 13 – Reconstructed GC-EI-MS SIM Mode chromatogram of a blank and spiked methanolic sample (10 ng/mg); It is represented the ion chromatogram of COC (*m/z* 82,182 and 303); ion

chromatogram for morphine (*m/z* 429); Ion chromatogram for the IS (*m/z* 385) and finally the monitoring chromatogram of all the ions of interest in blank sample.

There was no relevant difference between the fragmentations of the standard analytes (without hair) and the spiked solutions. The fragmentation pattern of COC and morphine produced by GC-EI-MS are shown in **Fig. 14**.

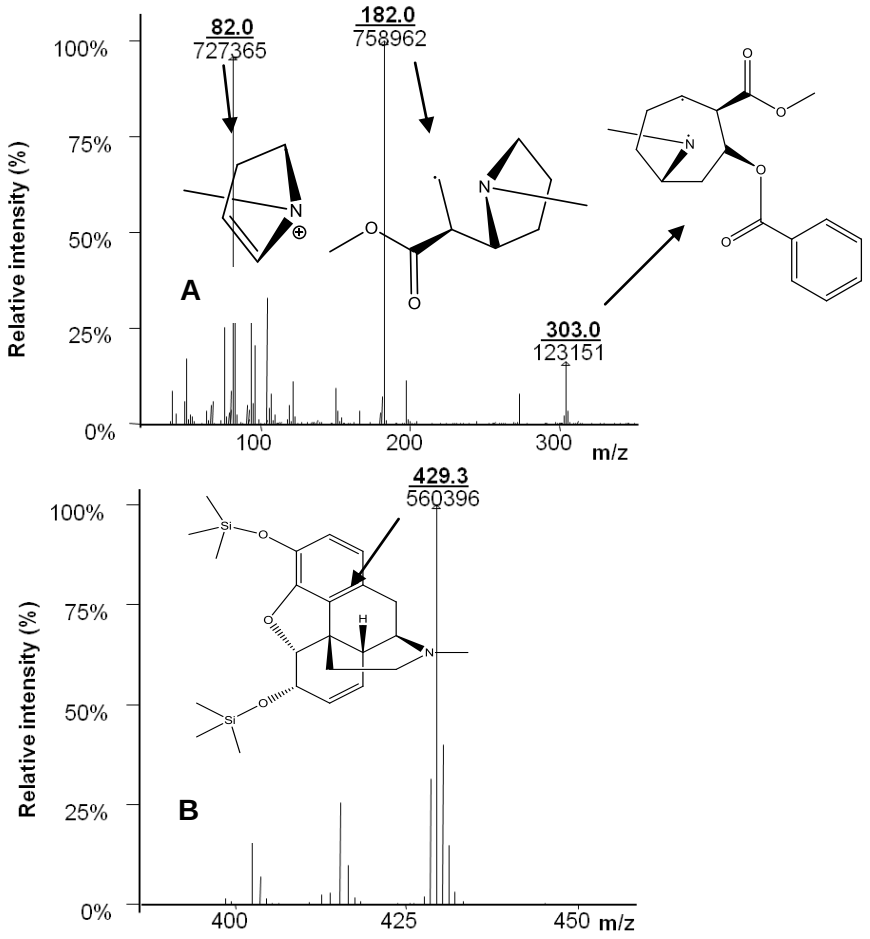

Fig. 14 – Reconstructed mass spectra of COC and morphine with the respective fragments, **A -** the *m/z* 82 ion [base peak, *m/z* 182 $[M-121]^+$ and 303 $[M]^+$ of reference COC; **B -** the *m/z* 429 ion $[M]^+$ of reference TMS-morphine; The *m/z* of ions selected for quantitative analysis are underlined.

2.3.2. Linearity, LOD, LOQ, Precision, Accuracy and Recovery

The linearity of each compound was investigated in triplicate in the range of 0.25 – 10 ng/mg. The calibration curves were obtained with six calibration concentrations all prepared in MeOH to a 20 mg of drug-free hair, further the IS was added and samples

were analyzed following the described procedure (Chapter 1). The GC-EI-MS chromatogram peak areas/IS peak area were plotted against the known concentrations of the standard solutions to establish calibration equations (**Fig. 15)**. A linear regression equation was calculated by the least squares method (**Table 7 and 8**).

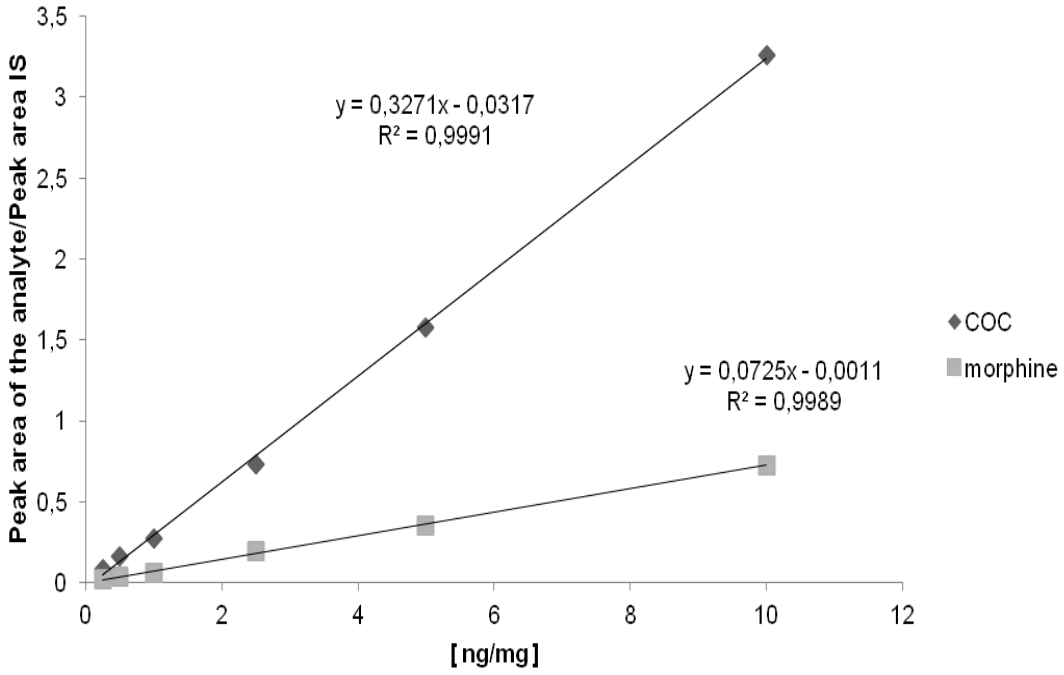

Fig. 15 - Calibration curves for COC and morphine. Plotted peak areas of the analytes/IS peak areas *versus* concentrations (0.25, 0.5, 1, 2.5, 5 and 10 ng/mg).

LOD and LOQ values ranged from 0.01 - 0.03 ng/mg for COC and 0.02 - 0.06 ng/mg for morphine, respectively suggesting good capacity of this method for the quantification of each of the compounds under study. Correlation coefficients (r^2) were higher than 0.9989 over the concentration range confirming the linearity of the method for each compound. The coefficient of variation (%CV) showed good repeatability, with values between 0.83% and 14.6%. The precision of the method for each standard was determined for three concentrations (0.25, 5 and 10 ng/mg) covering the quantification range values. Three non-consecutive injections for each concentration were performed on the same day. The %CV for the intraday repeatability (n=9) are given in **Table 5**. The %CV of interday precision was evaluated, at the same concentrations used for intraday precision, based on three consecutive injections on three different days (n=27) (**Table 7**).

Accuracy measures the effectiveness of all the analytical steps performed, since there is no reference material, the determination of accuracy becomes quite difficult.

Accuracies in the range of 82.3 - 119% for COC and 82.6 - 102% for morphine, which are within the proposed acceptance limits for this parameter (100 ± 20%) (EMA, 2009). The recovery values were determined by triplicate analysis of samples at three concentrations (0.25, 5 and 10 ng/mg) in which the IS were only added after the SPE procedure. The results obtained by comparison with those obtained with the spiked samples (calibration curve) were as follow; 71.7% and 87.3% for morphine and COC, respectively and, associated with lower %CV (7 - 10%), indicated an efficient clean-up procedure (**Table 7**).

Table 7 - Regression curves, linearity, limit of detection (LOD), limit of quantification (LOQ), recovery and precision by GC-EI-MS analysis

Compounds	t_R (minutes)	Concentration range (ng/mg)	Regression Equation Y=mx+b	r^2± SD[a]	LOD (ng/mg)	LOQ (ng/mg)	Recovery (%)[b] (n=9)	Intraday precision (%CV) (n=9)			Interday precision (%CV) (n=27)		
								A	B	C	A	B	C
COC	18.176	0.25 - 10	y = 0.3271x – 0.0317	0.9991 ± 9.07^{-04}	0.01	0.03	87.3	6.98	10.5	3.35	7.44	7.00	3.87
morphine	20.421	0.25 - 10	y = 0.0725x – 0.0011	0.9989 ± 9.45^{-04}	0.02	0.06	71.7	9.47	4.43	4.93	11.3	6.35	6.36

[a] The values of correlation coefficients of the calibration curve of each standard ±Standard Deviation (SD)

[b]Concentrations : **A-** 0.25 ng/mg; **B –** 5 ng/mg; **C –** 10 ng/mg.

Table 8 - Linearity of the slope and intercept of each regression equation associated with the mean relative error

	Linearity[c]	
	Slope(m)±SD	**Intercept(b)±SD**
COC	0.3271 ± 2.51^{-04}	0.0317 ± 2.42^{-02}
Morphine	0.0725 ± 3.72^{-03}	0.0011 ± 2.36^{-03}

[c]Values are the mean of three replicates.

Chapter III

Application of the developed GC-EI-MS method to real hair samples

3. Application of the developed GC-EI-MS method to real hair samples

Real hair samples obtained in July 2011 from 4 volunteers (males) submitted to a methadone program at a center of detoxification treatment (Gondomar and Valongo – Porto), were analyzed by the developed method.

Fig. 16 represents a reconstructed full-scan chromatogram of a real sample (volunteer #2), since that in this sample there were found small amounts of morphine, the corresponding peak could not be identified properly in a full-scan chromatogram. COC was easily identified. Comparing the chromatograms of spiked and real sample, there were no relevant interferences to report.

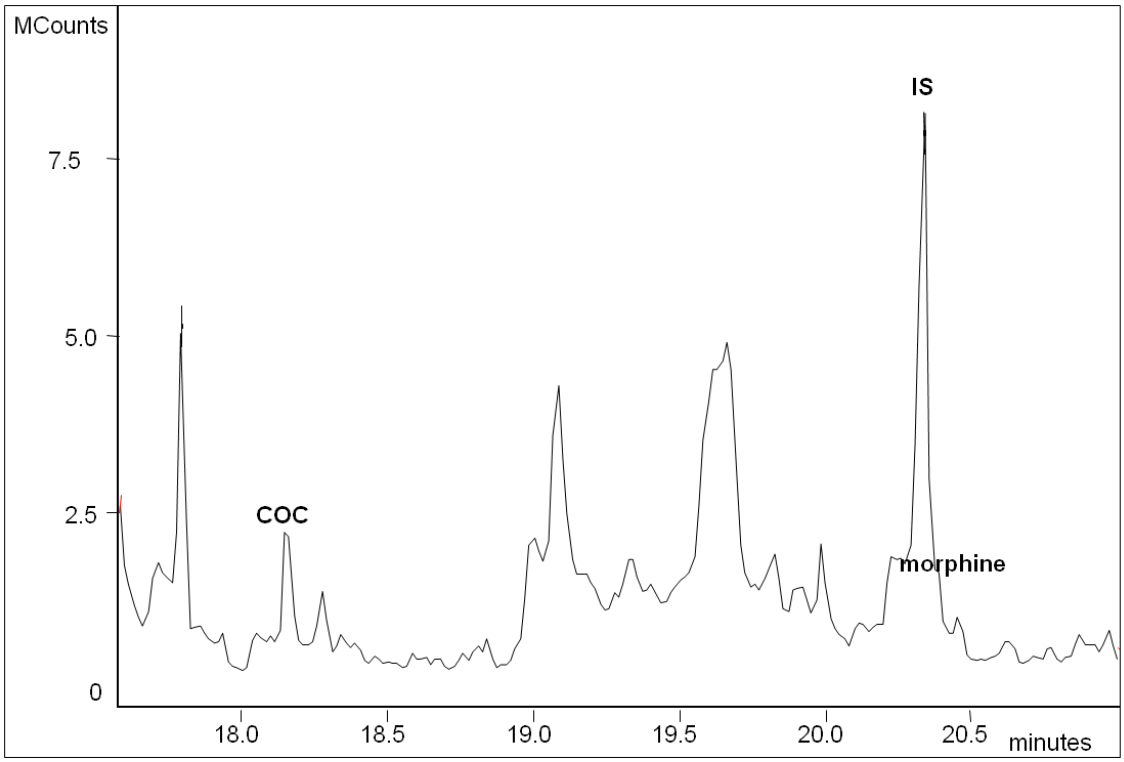

Fig. 16 – Reconstructed GC-EI-MS full-scan chromatogram of a real hair sample positive for COC and morphine (volunteer #2)

In order to quantify COC and morphine in these samples a SIM mode chromatogram analysis were done as shown in **Fig. 17**.

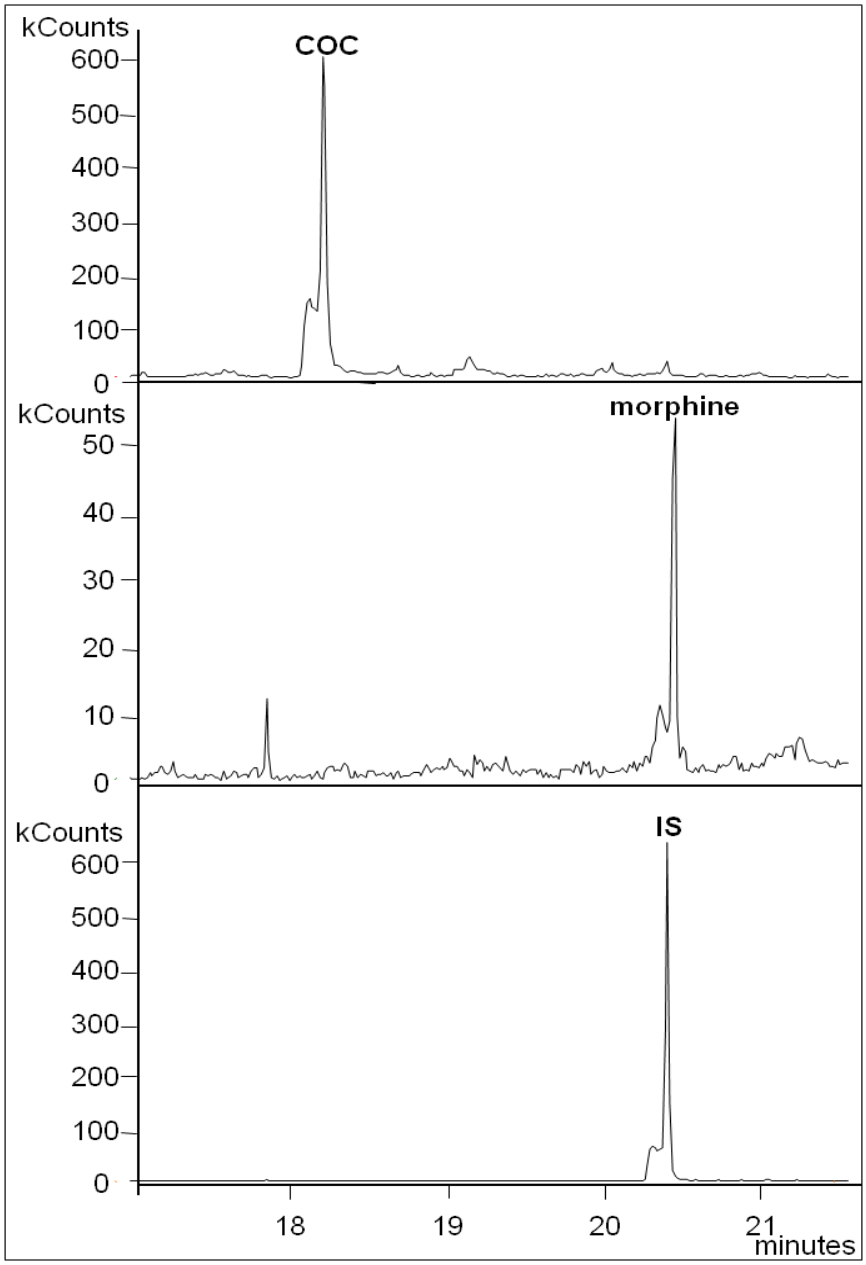

Fig. 17 - Reconstructed GC-EI-MS SIM Mode chromatogram of real hair sample positive for COC and morphine (volunteer #2)

These volunteers are submitted monthly to screening of drugs as a condition for their participation in the referred program. All the information concerning the clinical path taken by these volunteers were collected. The screening of the drugs was performed using an EMIT (enzyme multiplied immunoassay technique). EMIT testing is typically used by employers only as a preliminary drug screening due the possibility of

adulteration of the samples (urine) and cross reactivity with other xenobiotics (Smith, 2005).

All samples analyzed were positive for COC, the concentration range from 2.87 – 19.7 ng/mg. The Society of hair testing recommends the analysis of COC and one metabolite (mainly BE) (Society of Hair Testing, 2004) to confirm the exposure to COC and exclude the possibility of external contamination. However, BE and other COC metabolites may also be formed outside of the body (Musshoff and Madea, 2007b). Since any metabolite was analyzed in this study to exclude the hypothesis of the external contamination, sequential analysis of the washing solvent were performed and neither COC nor morphine peaks were identified in the chromatogram.

The urine from three volunteers was analyzed by EMIT, which revealed positive results for COC. Only volunteer #4 admitted a recent exposure (days before the collection) to COC, which is in accordance to the concentration of COC found in the hair sample (**Table 9**).

Regarding morphine, Society of Hair Testing recommends that when testing for heroin abuse, morphine and 6 – AM should be tested, since morphine can be originated from codeine or heroin consumption (Society of Hair Testing, 2004; Lötsch, 2005) or it may result from therapy with morphine itself.

Therefore, the present study can only confirm an exposure to opiates. The urine from 3 volunteers were EMIT positive for opiates in May, and the volunteer #4 denied the abuse of heroin or treatment with codeine or morphine, which goes in agreement with negative morphine presence.

The results were obtained by using the linear regression obtained from the validated method. **Table 9** presents obtained concentrations of COC and morphine in ng/mg.

Table 9 - Concentration (ng/mg) of COC and morphine in the hair of drug abusers admitted to centers for detoxification treatment

Compound ng/mg	**Hair samples #**			
	1	**2**	**3**	**4**
COC	15.4	5.33	2.87	19.7
morphine	-	1.24	0.28	-

Chapter IV

Conclusions and future perspectives

4. Conclusion and future perspectives

A sensitive, reproducible and relatively simple GC-EI-MS method was developed and fully validated according with EMA to screen and quantify COC and morphine in hair, thus this method meets the sensitivity and selectivity requirements for both clinical and forensic toxicological routine analysis. The applicability of the method was demonstrated by analysis of several real cases.

- The conditions of GC-EI-MS chosen guaranteed a good resolution and separation of both the compounds. Even though the IS and morphine didn't presented a great resolution, this compound, ethylMorphine can be used as alternative and cheapest IS in the quantification of COC and morphine in hair. A suitable derivatization procedure was successfully optimized allowing a sensitive GC-EI-MS analysis;
- Even though the extraction time (ON) still presents a great handicap in hair analysis due to its delay in all the analytical procedure, it guarantees the effective and stable extraction of several drugs simultaneously. The research of shorter periods of incubation as well of different means of extraction prospects new approaches to this study;
- This analytical method was developed and validated by several parameters, linearity, precision, accuracy, LOD and LOQ, which corresponding results are more than satisfactory considering the standard values established;
- This method proved to be applicable to the real samples collected.

Finally, all the general and specific objectives were successfully accomplished.

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