Chromatographic Methodologies for Analysis of Cocaine and Its Metabolites in Biological Matrices

Maria João Valente[1], Félix Carvalho[1], Maria de Lourdes Bastos[1], Márcia Carvalho[1,2] and Paula Guedes de Pinho[1]
[1]REQUIMTE, Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto,
[2]CEBIMED, Faculty of Health Sciences, University Fernando Pessoa, Porto
Portugal

1. Introduction

Cocaine is the main active alkaloid extracted from the leaves of the coca plant, *Erythroxylum coca*. It is a widely abused psychotropic drug, for its immediate neurological effects, including euphoria, reduced fatigue and increased mental acuity and sexual desire (Devlin & Henry, 2008; Goldstein et al., 2009; Small et al., 2009). However, cocaine abuse is usually followed by many pathophysiological consequences, namely central and peripheral neurochemical changes that result in hypertension-related morbidity and mortality, including myocardial infarction and cerebrovascular accidents, as well as liver and kidney toxicity, tissue ischemia and adverse psychotic effects such as paranoia and hallucinations (Devlin & Henry, 2008; Glauser & Queen, 2007; Heard et al., 2008; Karch, 2005; Lombard et al., 1988; Ndikum-Moffor et al., 1998; Tang et al., 2009; White & Lambe, 2003).

According to a recent report on drug abuse, and despite a visible decrease of production and consumption in the last few years, in 2008 cocaine abuse still affected up to 0.5% of the adult population (15-64 years old) worldwide. Cocaine remains the second most problematic drug in the world, after opiates (UNODC, 2011).

In Europe, cocaine ranks second in most abused illicit drugs, after cannabis. It revealed a mean prevalence of 1.3% of the adult population by the same year, with national prevalence reaching over 6% of the young adult population (15-34 years old) (EMCDDA, 2010).

In this chapter we will point out the clinical and forensic relevance of measuring cocaine and its metabolites in different biological matrices, and provide a bibliographic review on techniques for sample preparation and existing chromatographic methodologies for cocaine analysis.

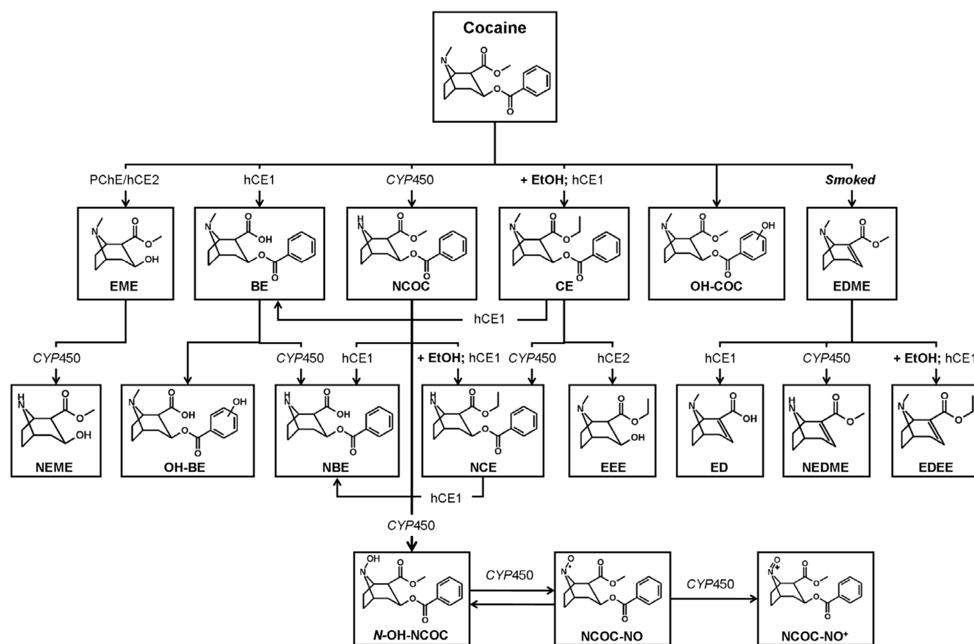
2. Toxicokinetics

Chemically, cocaine may exist in two forms: a hydrochloride salt or a free-base rock (“crack”). “Crack” melts at 98 ºC and volatizes above 90 ºC, but is not very soluble in water,

making it possible to smoke but not to inject. In contrast, the salt is easily soluble in water but has a high melting point (195 ºC) and decomposes when smoked, being suitable for intravenous (i.v.) injection, nasal insufflation (or snorting) or ingestion (Cook, 1991; Favrod-Coune & Broers, 2010).

Subsequently to absorption, cocaine is easily diffused in the blood into most body organs including heart, brain, liver, kidneys and adrenal glands (Favrod-Coune & Broers, 2010; Fowler et al., 1989; Volkow et al., 1992). However, its bioavailability depends on the route of administration, being of about 90% if injected or smoked, 25 to 94% when snorted, depending on the dose, and only up to 30% after ingestion (Cook, 1991; Leikin & Paloucek, 2008).

The onset of action, the intensity and the duration of the effects experienced by consumers are also affected by the type of consumption. For the smoked form, the onset occurs almost immediately, and the intensity of the neurological effects is nearly two-fold higher than for the other means of abuse (Favrod-Coune & Broers, 2010; Freye & Levy, 2009), most likely the reasons why "crack" is the most consumed form of cocaine.



BE - benzoylecgonine; CE - cocaethylene; *CYP*450 - cytocrome P450; ED - ecgonidine; EDEE - ecgonidine ethyl ester; EDME - ecgonidine methyl ester; EEE - ecgonine ethyl ester; EME - ecgonine methyl ester; EtOH - ethanol; hCE1 - human carboxylesterase type 1; hCE2 - human carboxylesterase type 2; NBE - norbenzoylecgonine; NCE - norcocaethylene; NCOC - norcocaine; NCOC-NO - norcocaine nitroxide; NCOC-NO+ - norcocaine nitrosonium; NEDME - norecgonidine methyl ester; NEME - norecgonine methyl ester; *N*-OH-NCOC - *N*-hydroxynorcocaine; OH-BE - hydroxybenzoylecgonine; OH-COC - hydroxycocaine; PChE - pseudocholinesterase.

Fig. 1. Cocaine metabolic pathways.

The i.v. injection takes a few seconds (15 - 30 s) to onset the first effects, following the snorted form with over a minute, and finally the oral form, the most unusual one among addicts, which takes over 20 minutes to produce effects (Freye & Levy, 2009; Heard et al., 2008; Jeffcoat et al., 1989).

The psychotropic effect usually lasts 2 to 3 hours after cocaine ingestion, approximately 1 hour when snorted, and less than 30 minutes for the injected and smoked forms (Favrod-Coune & Broers, 2010; Jeffcoat et al., 1989).

Figure 1 represents cocaine metabolic profile, which strongly depends on both form of consumption and administration route.

Following administration, cocaine is primarily metabolized into two major metabolites, benzoylecgonine (BE) and ecgonine methyl ester (EME), and two minor metabolites, norcocaine (NCOC) and *m*- and *p*-hydroxycocaine (OH-COC) (Goldstein et al., 2009; Maurer et al., 2006; Zhang & Foltz, 1990).

BE is mainly produced in the liver, through human carboxylesterase type 1 (hCE1), whereas EME may be formed in the liver by hCE2, and in the plasma via a pseudocholinesterase (PChE), namely butyrylcholinesterase (Goldstein et al., 2009). Both free metabolites are excreted in urine, together representing up to 95% of the excretion products (Kanel et al., 1990).

NCOC results from hepatic *N*-demethylation of the drug through the cytocrome P450 (*CYP*450) system, in particular *CYP*3A4 in human liver, and represents no more than 5% of the administered dose (Goldstein et al., 2009; Kloss et al., 1983; LeDuc et al., 1993). The same enzyme mediates further oxidations, yielding the secondary metabolites *N*-hydroxynorcocaine (*N*-OH-NCOC), norcocaine nitroxide (NCOC-NO) and norcocaine nitrosonium (NCOC-NO^+), which are described as responsible for cocaine-induced hepatotoxicity (Kovacic, 2005; Ndikum-Moffor et al., 1998; Pellinen et al., 1994; Thompson et al., 1979).

Regarding OH-COC, despite being produced at very low levels (less than 12% that of NCOC in hepatic microssomes), the isomer *p*-OH-COC was proven to be pharmacologically active in mice (Watanabe et al., 1993).

Polydrug abuse is a common pattern among cocaine users. In fact, by 2009, over 40% of them simultaneously consumed ethanol (UNODC, 2011). From this combination results the formation of the biologically active metabolite cocaethylene (CE), transesterification product via hCE1 between cocaine and alcohol (Harris et al., 2003; Hearn et al., 1991; Laizure et al., 2003).

Like cocaine, CE can undergo further *N*-demethylation via *CYP*450 or hCE2-mediated hydrolysis, yielding two unique ethanol-related cocaine metabolites, norcocaethylene (NCE) and ecgonine ethyl ester (EEE), respectively (Boyer & Petersen, 1990; Dean et al., 1992; Wu et al., 1992). NCE may also be a NCOC transesterification product in the concurrent use with alcohol (Maurer et al., 2006).

Besides cocaine, both EME and BE can undergo a *N*-demethylation as well, producing norecgonine methyl ester (NEME) and norbenzoylecgonine (NBE). This last metabolite can also be formed by hydrolysis of NCOC or NCE (Maurer et al., 2006).

During “crack” smoking, ecgonidine methyl ester (EDME) is formed in large quantities as a thermal breakdown product of cocaine (Jacob et al., 1990; Kintz et al., 1997). EDME may be metabolized by identical pathways as for cocaine: it can be oxidized into norecgonidine methyl ester (NEDME) via *CYP*450, or hydrolyzed through hCE1 into ecgonidine (ED) or ecgonidine ethyl ester (EDEE) in the presence of ethanol. This last one may be analyzed as a specific biomarker of the concomitant use of “crack” and ethanol (Fandino et al., 2002).

3. Clinical and forensic relevance of cocaine analysis

Over decades, cocaine abuse has reached epidemic proportions, and health complications related to cocaine use continue to be a major social burden worldwide.

According to the World Drug Report 2011 (UNODC, 2011), drug of abuse-related deaths are estimated between 104,000-263,000 per year, and they include fatal overdoses (over 50% of all deaths), accidents, suicides, deaths from infectious diseases transmitted through the use of contaminated needles, including hepatitis C and HIV, or complications due to chronic use, namely organ failure and myocardial infarction (Kloner et al., 1992; Shanti & Lucas, 2003; UNODC, 2011).

In Europe, cocaine-related deaths represent 21% of all deaths related to illicit drug abuse, with a report of approximately 1,000 deaths per year (EMCDDA, 2010; UNODC, 2011).

Of note, the reported mean purity of traded cocaine rounding 50% by 2009 and a lowering trend along the years, as well as the common mixture with several active adulterants like painkillers, may complicate the scenario of cocaine intoxications (EMCDDA, 2010; UNODC, 2011). In addition, since the polydrug use includes approximately 62% of cocaine users, drug combination often results in complex clinical patterns which are difficult to discriminate and treat (UNODC, 2011).

Thus, a thorough methodology for detection and quantification of cocaine, alongside with other drugs, may be crucial for an accurate evaluation of cocaine intoxication cases and contribute for a positive outcome.

For human performance forensic toxicology purposes, also defined as behavioral toxicology, cocaine is frequently tested in urine samples and swabs of oral fluid from drivers and applicants for driving licenses with a history of drug use (Brookoff et al., 1994; Gjerde et al., 2008; Montagna et al., 2000; Samyn et al., 2002; Tagliaro et al., 2000; Wylie et al., 2005).

Cocaine detection is also a common procedure in the context of workplace drug testing, more often in pre-employment and post-accidental screening, but also in random screenings, usually in urine samples (George, 2005; Verstraete & Pierce, 2001; Zwerling et al., 1990).

Another area of forensic toxicology is *postmortem* forensic toxicology, which involves in suspected drug-related deaths. These may include suspected drug intoxication cases (overdoses or accidental), suicides, homicides, motor vehicle accidents, arson fire fatalities and apparent deaths due to natural causes. In these cases, cocaine and its metabolites may be analyzed in several specimens including blood, vitreous humor, bile, urine, stomach

contents or organ tissues (Bertol et al., 2008; Darke & Duflou, 2008; Dias et al., 2008; Garlow et al., 2007; Graham & Hanzlick, 2008; Simonsen et al., 2011).

4. Determination of cocaine and its metabolites in biological specimens

The development of a procedure for the quantitative analysis of a biological matrix includes several steps, from sampling, to sample preparation, chromatographic analysis and finally analysis of the results (figure 2).

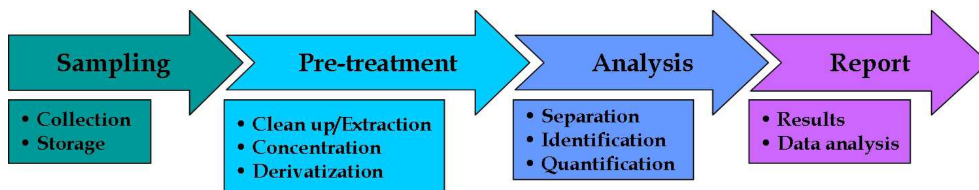


Fig. 2. Schematic representation of the steps included in the overall procedure for analysis of exogenous compounds in a biological specimen.

One of the main concerns regarding biological sampling for cocaine determination involves its instability in many matrices. At room temperature, cocaine can be quickly hydrolyzed into BE, and it is even more susceptible in cholinesterase-containing samples, including plasma and whole blood, in which the parent drug easily degrades into EME (Garrett & Seyda, 1983; Isenschmid et al., 1989).

The stability issue is not as significant in urine specimens as it is for plasma or blood. While in blood stability appears to be dependent on cocaine initial concentration, in urine it depends mainly on pH (Baselt, 1983). It was shown that cocaine concentration in urine may fall down to 37% when stored at -20 ºC, for a 12-month period of time (Dugan et al., 1994), but by acidifying the samples to a pH of 5.0, cocaine and BE levels in the frozen urine samples may be stable for at least 110 days (Hippenstiel & Gerson, 1994). In these samples, the use of preservatives, such as sodium fluoride, appears to have only minor effects on the specimen stability (Baselt, 1983). In blood and plasma without preservation, most cocaine is hydrolyzed into EME. This may be prevented with the addition of a PChE inhibitor (Isenschmid et al., 1989).

Urine specimens are the most commonly used for general drug screening (Leyton et al., 2011; Marchei et al., 2008; Zwerling et al., 1990). However, for cocaine detection, there are some limitations, including limited window of detection, occurrence of false-negatives as a consequence of very low cocaine concentrations in samples, specific requirements for storage, possibility of sample dilution *in vivo* by excessive fluid ingestion, requirement of collection under observation to avoid adulteration or sample exchange, or even absence of urine specimens in *postmortem* cases (Cone et al., 1998; Cone et al., 2003; Musshoff et al., 2006; Polla et al., 2009).

The analysis of oral fluid swabs, sweat patches and hair samples has become a viable substitute to urinalysis, specifically in the context of behavioral toxicology and workplace drug testing (Samyn et al., 2002; Toennes et al., 2005; Verstraete, 2005).

The main advantages of oral fluid include not only the non-invasiveness of the collection, but also the higher concentration of the parent drug found in saliva when compared to blood and urine. For the same individual, cocaine concentration in oral fluid is approximately three-fold of that found in plasma and over five-fold in urine (Cone et al., 1994a; Moolchan et al., 2000; Samyn et al., 2002; Schramm et al., 1993). In addition, cocaine elimination half life is lower in saliva, which makes oral fluid analysis suitable for determination of very recent use (Dolan et al., 2004; Jufer et al., 2000). Moreover, saliva can provide an unequivocal screen result within minutes and has demonstrated a good correlation with impairment symptoms of drivers under the influence of drugs, reasons that make saliva the preferred matrix for roadside analysis (Kidwell et al., 1998; Verstraete, 2005).

However, oral fluid use has some limitations, such as the limited volume of specimen when compared to urine sampling, especially considering that recent use often results in the production of little amounts of saliva or even none at all, and the variability of salivary pH (Cognard et al., 2006; Kidwell et al., 1998; Verstraete, 2005).

Similarly to oral fluid, sweat is occasionally chosen for on-site testing (Samyn et al., 2002; Samyn & van Haeren, 2000). The sweat samples may be collected as skin swabs or through patches similar to bandages attached to the skin (Kacinko et al., 2005; Kidwell et al., 2003). These patches can be worn comfortably for several days (usually one week). This allows an accumulation of cocaine in the patch over the days, which is very useful, for example, for monitoring patients on drug-abuse treatment or epidemiologic surveys on cocaine-use in a given population (Burns & Baselt, 1995; Chawarski et al., 2007; Kidwell et al., 1997; Preston et al., 1999).

The main limitations of sweat analysis include the lower amount secreted at a given time in comparison to saliva, the great variability of results between doses and individuals, the variation of drug disposition between sites of collection and collection devices, and the occurrence of false positives from prior skin contamination or external patch contamination. Part of the drug may as well be reabsorbed into the skin or degraded in the patch (Burns & Baselt, 1995; Donovan et al., 2011; Huestis et al., 1999; Kidwell et al., 1998; Kidwell et al., 2003).

An early controlled study demonstrated that cocaine is detected in sweat samples up to 48 hours after administration (Cone et al., 1994b), but subsequent works suggested a window of detection as long as one week (Burns & Baselt, 1995; Kintz, 1996). Nonetheless, cocaine concentration in sweat is an indicator of a relatively recent use (Chawarski et al., 2007; Kidwell et al., 1997).

For past drug abuse, hair samples present the wider window of detection, allowing a higher rate of positive results than urine (Dolan et al., 2004; Kline et al., 1997; Scheidweiler et al., 2005). A study on hair cocaine and BE incorporation showed that a single 25-35 mg intravenous cocaine dose may be detected in hair for up to 6 months (Henderson et al., 1996).

A segmental hair analysis, meaning a determination of cocaine content in the length of the hair shaft, provides useful information about the individual history of drug abuse and may be used to estimate time of exposure back to a few months (Scheidweiler et al., 2005; Strano-Rossi et al., 1995). This characteristic makes hair analysis a suitable alternative matrix for long-term studies such as monitoring relapses during treatment programs or follow-up of treatment outcomes (Moeller et al., 1993; Simpson et al., 2002; Strano-Rossi et al., 1995; Wish et al., 1997).

Hair samples are not easily adultered and collection procedure does not violate the individual privacy. The hair fibers are preferentially obtained from the posterior vertex area of the scalp and as close as possible to the skin. Due to its stability, there are no specific criteria for transportation or storing, although it is recommendable to wrap the samples in aluminum foil to avoid contamination and store at room temperature.

A general critical step of hair analysis is the interpretation of the results. At this point, a few issues must be taken into account. One potential problem inherent to hair cocaine interpretation concerns the racial bias. Some studies have demonstrated that ethnicity must be considered, since the incorporation of cocaine into the human hair seems to be more extent in non-Caucasian than in Caucasian subjects, possibly due to pigmentation differences (Henderson et al., 1998; Joseph et al., 1996; Joseph et al., 1997; Reid et al., 1996). Hair cosmetic treatments, like bleaching or dying, can also interfere with the analytical results as they may affect the drug stability, leading to a partial or total loss of hair cocaine contents (Skender et al., 2002; Wennig, 2000).

Hair cocaine may reflect not only chronic cocaine abuse, but also environmental contamination. This last one includes passive contamination, for example cocaine from dust or sprays deposited on the hair surface, and passive ingestion, which may be related to passive “crack” smoking or unknowingly oral ingestion, by contact with persons who have consumed cocaine or with contaminated objects (Mieczkowski, 1997).

Several studies have demonstrated that the inclusion of an efficient washing step prior to hair analysis, typically with an organic solvent such as dichloromethane, will effectively eliminate the environmental drug contamination component (Kintz, 1998; Koren et al., 1992; Schaffer et al., 2002; Skender et al., 2002). However, Kidwell & Blank (1996) showed that heavy hair cocaine contamination cannot be completely eliminated with any of the washing solutions tested (from water and methanol, ionic or non-ionic solutions, to dimethylformamide). Romano et al. (2001) also demonstrated that even a rather small amount of cocaine (10 mg) applied to the hair persists despite using decontamination procedures.

In order to distinguish systemic exposure from environmental contamination, Koren et al. (1992) suggested the determination of the major metabolite BE in hair samples, which allegedly is detected only as a result of cocaine abuse and not contamination, whereas Cone et al. (1991) identified NCOC and CE more suitable to classify hair cocaine as a reflection of drug abuse.

Postmortem cocaine determination and interpretation can involve additional problems. As defined by Mckinney et al. (1995), “the interpretation of *postmortem* cocaine concentrations is made in an attempt to estimate drug concentrations present at the time of death and thus infer not only drug presence but also drug toxicity”.

For instance, when the *postmortem* interval is excessively prolonged, or when the autopsy or laboratory analysis takes too long to be processed, cocaine can be completely hydrolyzed, chemically or enzymatically. Moreover, *postmortem* cocaine redistribution and release from tissues is a reality and has to be taken into account (Drummer, 2004; Yarema & Becker, 2005).

Several studies have demonstrated the lack of predictability of *postmortem* redistribution rates of cocaine and its metabolites over time. Also, *postmortem* blood and urine cocaine and its metabolites levels do not reflect the *antemortem* or *perimortem* values, and thus should not be used to establish cause of death (Karch et al., 1998; McKinney et al., 1995; Stephens et al., 2004; Yarema & Becker, 2005).

In alternative, samples from gastric contents and vitreous humor, nails, either fingernails or toenails, bone, and tissues such as brain, lung, liver and muscle may be analyzed to determine *postmortem* drug levels (Garside et al., 1998; McGrath & Jenkins, 2009; Stephens et al., 2004; Yarema & Becker, 2005).

Due to its isolation in the eye cavity, vitreous humor seems to be less susceptible to *postmortem* redistribution and putrefaction than other biological fluids. Despite the small amount of sample that can be collected, this specimen can be useful when the body undergoes massive bleeding or burning, or when it is in a state of prolonged decomposition (De Martinis & Martin, 2002).

4.1 Sample preparation

Due to the short half-life of cocaine in most biological specimens and its extensive metabolism, it is important to include into the analysis cocaine metabolites as well, increasing thus the detection window for drug abuse.

In order to obtain "clean" samples for analysis and increase the chromatographic sensibility towards specific drugs and their metabolites, most biological matrices require pre-treatment and concentration steps prior to chromatographic analysis. This is accomplished by extraction procedures that include mainly liquid-liquid extraction (LLE), solid-phase extraction (SPE) and more recently solid-phase microextraction (SPME).

4.1.1 Extraction procedures

The variation of acid-base properties among cocaine and its metabolites, as displayed in table 1, may challenge the selection of the most efficient extraction procedure.

The LLE consists on the separation of analytes based on their solubilities, with extraction occurring between two liquid immiscible phases (one aqueous and one organic) by adding adequate solvents.

Analyte	**Acid-base properties**
Cocaine	weak base; pKa = 8.6
Benzoylecgonine	amphoteric; pKa = 2.2, 11.2
Ecgonine methyl ester	weak base; pka > 8.0
Norcocaine	weak base; pka = 8.0
Hidroxycocaine	weak base
Cocaethylene	weak base; pka > 8.0
Ecgonidine methyl ester	weak base
Hydroxybenzoylecgonine	amphoteric
Benzoylnorecgonine	amphoteric
Norcocaethylene	weak base
Ecgonine ethyl ester	weak base
Ecgonidine	amphoteric

Table 1. Acid-base properties of cocaine and its metabolites.

Through LLE, the weak base analytes, such as cocaine, NCOC and EME, are the most easily extracted from biological matrices. On the other hand, isolation of amphoteric compounds, including BE, is more complex and requires a careful choice of the appropriate solvent and regulation of the pH.

Wallace et al. (1976) described a method for cocaine and BE determination in urine samples of patients who undergone surgery with cocaine anaesthesia. After extraction into a chloroform-ethanol solution (80/20%), the organic phase was evaporated to dryness at 55 ºC, under a stream of filtered air. Recovered extracts were analyzed by gas chromatography (GC) coupled to a flame ionization detector (FID), and using this LLE method it was attained a recovery of 93 and 65% for cocaine and BE, respectively, and a limit of detection (LOD) of <0.1 and 0.2 μg/mL.

This relatively low recovery of amphoteric species may be a great limitation of LLE. However, it can be useful when the aim is to quantify the parent drug in a matrix where one or more metabolites are known to be present in large amounts. An example of this application is the determination of cocaine levels in urine samples, in which BE is the major analyte present.

With this purpose, Garside et al. (1997) reported a single-step LLE method using petroleum ether as the only solvent for quantification of cocaine in urine through GC coupled to mass spectrometry (MS) detection. The method has a considerably low cost and since only cocaine and other non-polar metabolites were isolated, it was not necessary to use the time-consuming and expensive derivatization step. However, a mean recovery of only 48.8% for cocaine was achieved.

A following study by Farina et al. (2002) using as solvent an ethyl ether-isopropanol mixture led to a 74.4% recovery of cocaine from urine samples, as measured by a GC method with nitrogen-phosphorous detector (NPD).

LLE was also efficiently applied to cocaine and its metabolites determination in other matrices including hair (Kintz & Mangin, 1995), nails (Engelhart & Jenkins, 2002), serum (Williams et al., 1996), plasma (Dawling et al., 1990), whole blood (Gunnar et al., 2004) and organ tissues (Hime et al., 1991).

Nonetheless, there are obvious limitations inherent to the LLE, including the use of large amounts of possibly hazardous solvents and the low recovery as a result of poor separation of the organic and aqueous phases or even formation of emulsions (Ferrera et al., 2004; Franke & de Zeeuw, 1998; Ulrich, 2000).

The SPE technique has been efficiently used to extract cocaine and its metabolites from several biological matrices, including whole blood, plasma, urine, saliva, hair and sweat, with recoveries over 80% for all analytes (Badawi et al., 2009; Bjork et al., 2010; Brunet et al., 2008; Cordero & Paterson, 2007; Lin et al., 2001; Ohshima & Takayasu, 1999). Despite the advantages, SPE still requires organic solvents, though in lower quantities compared to LLE, and the columns' price can increase the costs of the extraction procedure. When comparing extraction efficiencies of LLE and SPE applied to the same samples, for the same purpose, it is generally observed that both recovery and quality of chromatograms are superior for the SPE technique (Clauwaert et al., 1997).

For both liquid chromatography (LC) and GC, SPE appears to be the preferred extraction method through which all cocaine analytes may be isolated from a single sample with very reasonable recovery rates.

SPE allows the extraction of compounds dissolved in a liquid matrix by adsorption of the analytes in a solid porous phase. The compounds are then separated based on their affinity to the stationary phase. Therefore, the selection of the appropriate SPE column type depends on the analytes chemical and physical properties. For cocaine analysis the most usual phases used include strong cation-exchange phases, non polar C8 or C18 and mixed-mode phases that combine the other two, allowing the extraction of both polar and non polar cocaine analytes in the same column.

In a recent study, Jagerdeo and Abdel-Rahim (2009) compared the specificity and extraction efficiency of different SPE columns for cocaine and its metabolites from urine samples. They showed that a non polar C8 sorbent efficiently extracted the parent drug and CE, but no EME and only a trace amount of BE. On the other hand, both divinylbenzene copolymers ENV+ (for aliphatic and aromatic polar analytes) and Oasis MCX (strong cation-exchange phase) enabled the extraction of all analytes, with improved signal to noise ratio but with a lower extraction rate than C8. The mixed-mode phase showed the best results, with better recoveries, cleaner chromatograms and great mass accuracy.

In the last few decades, a solvent-free extraction method, the solid-phase microextraction (SPME), first designed for isolation of volatile chlorinated organic chemicals in water (Arthur & Pawliszyn, 1990), has been applied to the analysis of biological samples.

SPME can be used both in laboratory context and on-site, and it consists of a syringe-like device with a fused silica fiber coated with a polymeric stationary phase, like polyacrylate or polydimethylsiloxane, which adsorbs the analytes by direct immersion on liquid samples or by head-space (HS) extraction. The fiber is then placed in the injection port of a chromatography equipment and the analytes are recovered through desorption at elevated temperatures (Manini & Andreoli, 2002).

For cocaine analysis, SPME allows the detection of cocaine analytes at parts per billion (ppb) levels (ng/mL) in variable specimens such as urine, plasma, sweat, saliva and hair (Alvarez et al., 2007; Bermejo et al., 2006; Follador et al., 2004; Yonamine & Saviano, 2006; Yonamine et al., 2003).

Besides the low LOD values, SPME is considered easy to automate and involves little equipment. It can be used for the extraction of either liquid or solid matrices and it can be performed on very small samples (Ulrich, 2000). However, there are several disadvantages inherent to SPME technique, namely the possibility of carry-over from one sample to next one, the cost and fragility of the fiber, and the prolonged equilibration time prior to extraction (Ferrera et al., 2004).

Table 2 presents the main advantages and limitations of each extraction method.

The choice of extraction method will depend on the matrix to be analyzed, the analytes to detect, and the budget and material existent in the laboratory.

Method	Advantages	Limitations
LLE	Inexpensive May be good for solid matrices	Large amounts of organic solvents Difficult separation Poor and variable recoveries Emulsion formation Not appropriate for extraction of chemically different analytes
SPE	Fast Easy to automate Good for extraction of chemically different analytes Clean extracts Handles small samples	Use of organic solvents May require derivatization Expensive
SPME	Solvent-free Easy to automate Little equipment required May be used on-site Adequate for solid matrices High sensitive Small volume samples	Expensive Fragile polymer coating Prolonged extraction Requires procedure optimization (extraction time and temperature) Possible carry-over between samples Low recoveries

LLE – liquid-liquid extraction; SPE – solid-phase extraction; SPME – solid-phase microextraction.

Table 2. Advantages and limitations of the extraction procedures.

4.1.2 Derivatization procedures

Derivatization is a reaction by which a compound is chemically modified through reaction with a so called derivatizing agent, with a specific functional group. The reaction product is a compound (or derivative) with new properties that include different volatility, solubility, aggregation state or reactivity. It may be performed for several reasons, such as increasing compatibility with the chromatographic equipment (e.g. by decreasing polarity and increasing volatility), improve separation and resolution efficiency and attain lower detection limits (Wang et al., 2006).

Derivatization can also be useful when isotopically labeled analogs of the analytes are chosen as internal standard (IS). In these cases, it is required that the analytes and the IS generate sufficiently separated peaks, and that derivatization of the analytes allows the elimination of the phenomenon of "cross-contribution", i.e. "contribution of the analyte and the IS to the intensities of ions designated for the IS and the analyte" (Chang et al., 2001).

For GC analysis, the derivatizing agents include silyl, acyl or alkyl groups that will substitute the proton from a terminal -N-H, -S-H and/or a -O-H polar group, producing non-polar and more volatile derivatives (Segura et al., 1998; Wang et al., 2006).

The ability of the analytes to form silyl or acyl derivatives depends on their functional group. While the TMS derivatives have large affinity towards hydroxyl and carboxyl groups and much lower towards amines, the acylating agents promptly targets highly polar groups including amines and both alcohols and phenols (Segura et al., 1998; Soderholm et al., 2010).

The overall derivatization technique is described in figure 3.

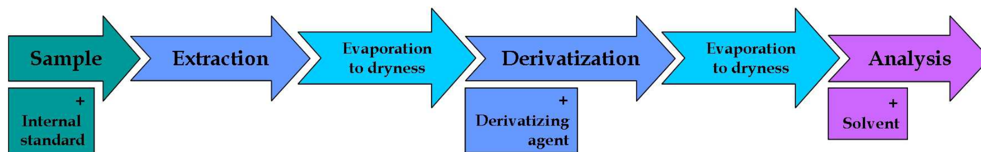


Fig. 3. Schematic representation of the steps included in the overall derivatization procedure of the analytes.

After adding the IS to the sample, extraction procedure is carried out. Subsequently, the solvent is evaporated to dryness with a gentle nitrogen stream, optionally with heating. The derivatizing agent is added and the derivatization of the analytes is performed by heating the sample. For silylating agents, the procedure ends at this time point and the sample is ready to analyze, right after cooling to room temperature. When performing acylation or alkylation, the samples have to be evaporated once more to eliminate the excess of agent, and the residue is further recovered by a solvent for posterior chromatographic analysis.

Cocaine and CE are not prone to derivatization. On the other hand, all *N*-demethylated metabolites, such as NCOC, NBE and NCE, can produce derivatives from the –N-H substitution, while BE, EME and the metabolites OH-BE and OH-COC may undergo a hydroxyl substitution.

Table 3 summarizes some studies on determination of cocaine and its metabolites in biological samples using either acylation, alkylation or silylation as derivatization methods for GC analysis.

The most usual agents for silylation are the trimethylsilyl (TMS) derivates, which confer to the new compounds high volatility and stability. Several TMS derivates with different chemical and physical characteristics have been produced and commercialized so far, but the TMS-amides *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) are still the most commonly used, generally and particularly for cocaine analysis in biological specimen (Brunet et al., 2008; Romolo et al., 2003; Segura et al., 1998).

In addition, many studies employ a catalyst like trimethylchlorosilane (TMCS) with BSTFA (Brunet et al., 2008; Brunetto et al., 2010; Cone et al., 1994a; Kintz & Mangin, 1995; Romolo et al., 2003), or less commonly t-butyldimethylchlorosilane (TBDMCS) with the silylating agent *N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) (Lowe et al., 2006), to improve the silylating potential of the derivatizing agents (Segura et al., 1998).

Acylation is frequently applied to cocaine and its metabolites determination as well, however the acidic by-products generated in this reaction requires the elimination of the excess of derivatizing agent prior to analysis, while silylating agents can be directly injected into the GC equipment for analysis. The shorter time of preparation and the less amount of solvents required for analysis are the main advantages of silylation over acylation (Segura et al., 1998).

Sample	Analytes	Derivatizing agents	LOD	Reference
Blood Urine	Cocaine, BE	PFPA/HFIP	20 ng/mL	(Aderjan et al., 1993)
Plasma Saliva Urine	Cocaine, BE, EME Other metabolites	BSTFA/TMCS	1 ng/mL 3-6 ng/mL	(Cone et al., 1994a)
Hair	Cocaine, BE	HFBA/HFIP	0.03 ng/mg	(Jurado et al., 1995)
Hair	Cocaine, BE, EME, CE	BSTFA/TMCS	0.05 – 0.8 ng/mg	(Kintz & Mangin, 1995)
Hair	Cocaine, BE	BSTFA/TMCS	0.05 – 0.2 ng/mg	(Romolo et al., 2003)
Blood Urine Muscle tissue	Cocaine, BE, EME, CE Other metabolites	PFPA/ PFPOH	2 ng/mL 2-640 ng/mL	(Cardona et al., 2006)
Brain tissue	Cocaine, BE, EME, CE, EDME, EEE	MTBSTFA/TBDMCS	50 ng/g	(Lowe et al., 2006)
Urine	Cocaine, EME	PFPA/PFPOH	12.5 - 50 ng/mL	(Saito et al., 2007)
Sweat	Cocaine, BE, EME, EDME	BSTFA/TMCS	2.5 ng/patch	(Brunet et al., 2008)
Urine	Cocaine, BE	BSTFA/TMCS	3 - 10 ng/mL	(Brunetto et al., 2010)
Adipose tissue	Cocaine, BE, EME, CE	BSTFA	5 - 20 ng/g	(Colucci et al., 2010)
Hair	Cocaine, CE	MSTFA	0.08 – 0.09 ng/mg	(Merola et al., 2010)

BE - benzoylecgonine; BSTFA - *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; CE - cocaethylene; EDME - ecgonidine methyl ester; EEE - ecgonine ethyl ester; EME - ecgonine methyl ester; HFBA - heptafluorobutyric anhydride; HFIP - 1,1,1,3,3,3-hexafluoroisopropanol; MSTFA - *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; MTBSTFA - *N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide; PFPA - pentafluoropropionic anhydride; PFPOH - 2,2,3,3,3-pentafluoro-1-propanol; TBDMCS - *t*-butyldimethylchlorosilane; TMCS - trimethylchlorosilane.

Table 3. Studies on determination of cocaine and its metabolites in biological samples by gas chromatography using derivatization procedures.

For acylation of cocaine analytes, the haloalkylacyl derivates, particularly the fluorinated ones like pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA), are widely applied to several biological matrices, like blood and urine (Aderjan et al., 1993; Cardona et al., 2006; Saito et al., 2007), and hair and tissues (Cardona et al., 2006; Jurado et al., 1995).

Due to the weak reaction of acyl derivatizing agents with carboxyl groups, most of the studies on cocaine determination using a GC method combines to the acylating agent an alkylating one, such as 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and 2,2,3,3,3-pentafluoro-1-propanol (PFPOH) (see table 3), which easily displace the reactive proton of carboxyl groups, increasing thus the efficiency of the derivatization of all hydroxyl, carboxyl and amine functional groups (Cardona et al., 2006).

4.2 Chromatographic analysis

Over the years, several chromatographic methods have been developed to determine cocaine analytes in biological samples. For screening, one of the easiest and less expensive methods is the thin-layer chromatography (TLC), presenting a good alternative to immunoassays. Gas chromatography (GC) and liquid chromatography (LC) are more appropriate for confirmation and quantification.

4.2.1 Thin-layer chromatography

Since early before the 1980's, TLC has been systematically used for urine drug screening. Through this method, cocaine, the major urinary metabolite BE, and the transesterification product CE, can be detected. However, the method presents low sensitivity, with LOD values over 1 μg/mL, even when methylating BE back into the parent drug is performed (Bailey, 1994; Budd et al., 1980; Wolff et al., 1990).

The simplicity of the method, the rapid analysis time, and the ability to detect not only the parent drug but also metabolites and other interfering drugs made TLC very useful for forensic purposes. However, due to its proven low sensitivity and lack of specificity, as the conventional methodology may not distinguish cocaine from other compounds usually present in biological samples (for example, nicotine and caffeine), TLC is not as much applied to drug screening as immunoassays are (Janicka et al., 2010; Yonamine & Sampaio, 2006).

More recently, an improved and computerized TLC technique denominated high-performance thin-layer chromatography (HPTLC) was developed. HPTLC presents better resolution, allowing the separation of cocaine and its metabolites from interferences, and is more sensitive, reaching LOD values down to 50-550 ng/mL. In addition, the association to an advanced densitometer and a detector, such as the ultraviolet (UV) detector, makes HPTLC suitable for quantitative analysis in cases of high cocaine doses, as for example in cocaine overdoses (Antonilli et al., 2001; Yonamine & Sampaio, 2006).

4.2.2 High-perfomance liquid chromatography

For many decades, LC has been widely applied to the separation of organic compounds. The separation through LC is based on the analytes distribution between a liquid mobile phase and a stationary phase. Nowadays, the LC is usually equipped with pumps that apply relatively elevated pressures to force the mobile phase through the very small packing particles that forms the stationary phase, being referred as high-performance liquid chromatography (HPLC). Table 4 summarizes some studies on cocaine and its metabolites determination in biological material through LC or HPLC, with variable detection equipment.

Sample	Analytes	Column	Mobile phase	Detector	LOD	Reference
Urine	Cocaine, BE	C18	(A) NH_4HCO_3 (B) CH_3OH 5-90-5% B in A	MS/MS	1 – 1.4 ng/mL	(Berg et al., 2009)
Serum	Cocaine, BE	PFPP	(A) 0.1% HCOOH + $HCOONH_4$ 1mM (B) CH_3CN + 0.1% HCOOH + $HCOONH_4$ 1mM	MS/MS	0.1 -0.4 ng/mL	(Bouzas et al., 2009)
Urine	Cocaine, BE, CE	C18	(A) CH_3COONH_4 in H_2O:CH_3OH:CH_3CN (8:1:1) (B) CH_3COONH_4 in H2O:CH_3OH:CH_3CN (1:2:2) 100-47.2% A in B	DAD	20 ng/mL	(Clauwaert et al., 1996)
Plasma	Cocaine, BE, CE	C8	(A) CH_3CN (B) PO_4^{3-} buffer 10-50-10% A in B	DAD	10 ng/mL	(Fernandez et al., 2006)
Blood Urine	Cocaine, BE, EME, CE, NCOC	C18	(A) 5% CH_3CN + 0.05% HCOOH (B) 100% CH_3CN + 0.05% HCOOH 95-60% B in A	MS/MS	0.001 – 0.003 mg/kg	(Johansen & Bhatia, 2007)
Hair	Cocaine, BE	C18	(A) 0.01% HCOOH (B) CH_3OH (C) CH_3CN 10% B + 70-30-70% A + 20-60-20% C	MS/MS	1 – 10 pg/mg	(Lopez et al., 2010)
Hair	Cocaine, BE	C18	CH_3OH:CH_3CN:KH_2PO_4 buffer (10:15:75) + 0.25% $N(CH_2CH_3)_3$	FD	1 ng/mL	(Mercolini et al., 2008)
Saliva	Cocaine, BE	Phenyl	(A) CH_3OH + $HCOONH_4$ 10 mM (B) H_2O + $HCOONH_4$ 10 mM 6-41.2% A in B	MS/MS	0.22 - 0.29 ng/mL	(Mortier et al., 2002)
Urine	Cocaine, EME	PFPP	CH_3CN + $HCOONH_4$ 5mM + HCOOH	MS/MS	1.6 – 2.8 pg on column	(Needham et al., 2000)
Plasma	Cocaine, BE, EME, NCOC	C18	$HOC(COOH)(CH_2COOH)_2$ 0.05 M:Na_2HPO_3 (4:1) + 18% CH_3CN + 0.3% $N(CH_2CH_3)_3$	UV	35 – 90 ng/mL	(Virag et al., 1996)
Serum	Cocaine, CE	Cyanopropyl	CH_3CN: PO_4^{3-} buffer (38:62)	UV	25 ng/mL	(Williams et al., 1996)

BE - benzoylecgonine; CE - cocaethylene; DAD - diode array detector; EME - ecgonine methyl ester; FD - fluorescence detector; MS - mass spectrometry; NCOC - norcocaine; PFPP - pentafluorophenylpropyl; UV - ultraviolet.

Table 4. Studies on cocaine and its metabolites determination in biological samples by liquid chromatography.

The separation of cocaine analytes is usually performed in reversed-phase columns, such as C8 and C18. However, other stationary phases may be used, depending on the properties of the analytes in study. For example, Needham et al. (2000), after observing the unsuccessful retention in a C18 column of EME, a very polar cocaine metabolite, demonstrated that a pentafluorophenylpropyl (PFPP) bonded silica column increased the retention and improved the peak shape of both metabolite and parent drug.

Among the detection equipment used with chromatographic methods, two of the most popular for LC cocaine analysis include the UV detectors and the fluorescence detector (FD), due to their low cost and easy automation (Janicka et al., 2010).

The weak UV absorption of polar cocaine metabolites diminishes the usefulness of an UV detector. Nonetheless, some studies have shown acceptable results using an UV or a diode array detector (DAD), but with visible lack of sensitivity when compared to other detectors (see table 4).

Mass spectrometry (MS) greatly improved the detection and identification of analytes after chromatographic elution, providing identification based on mass-spectral data. More common than the simple MS detection, many LC methods use tandem MS (or MS/MS, or MS^2) in which multiple steps of MS selection enable a more accurate identification.

The elevated sensitivity of MS allows detection of compounds at concentrations below ppb levels, as found for several biological specimens (see table 4).

4.2.3 Gas chromatography

GC is a widely used methodology for drug abuse analysis. In this chromatographic technique the mobile phase is a carrier gas, typically an unreactive gas like nitrogen, hydrogen or helium. The sample is carried through a liquid or a polymeric stationary phase bounded to a solid support inside a column. This column is located inside an oven that controls the temperature of the mobile phase, and the analytes in the sample are separated based on polarity and vapor pressure differences.

Either liquid or gaseous (extracted through HS-SPME) samples may be analyzed by GC, however, only volatile compounds can be detected. Thus, while cocaine and its non polar metabolite CE are easily determined in biological samples extracts without prior preparation techniques (Cognard et al., 2005; Hime et al., 1991), most of the other cocaine analytes requires previous derivatization.

Like LC techniques, GC presents high selectivity and low detection levels. Table 5 presents some analytical studies on cocaine and its metabolites by several GC techniques in different biological matrices.

Before the development of the MS detector, cocaine analysis in biological samples by GC methods used essentially a nitrogen-phosphorus detector (NPD). This detector is moderately priced and provides a quite sensitive analysis, with cocaine LOD values below 100 ng/mL.

Among the studies using GC-NPD, the use of extraction methods slightly improves the sensitivity of the chromatographic method towards cocaine analytes. Urine samples extracted by SPME shows a somewhat lower cocaine LOD than urine samples treated by LLE (12 vs. 15 ng/mL), while the analysis of non extracted blood samples presents lower

sensitivity than the other two (LOD = 20 ng/mL) (Farina et al., 2002; Hime et al., 1991; Kumazawa et al., 1995).

Sample	Analytes	Extraction/ Derivatization	Detector	LOD	Reference
Plasma	Cocaine, CE	SPME/-	MS	11 – 19 ng/mL	(Alvarez et al., 2007)
Hair	Cocaine, BE	SPE/MSTFA+TMCS	MS	15 – 20 pg/mg	(Barroso et al., 2008)
Blood Urine Muscle tissue	Cocaine, BE, EME, NCOC, CE, EDME, NBE, NCE, OH-BE, EEE	SPE/PFPA+ PFPOH	MS	2-640 ng/mL	(Cardona et al., 2006)
Hair	Cocaine, EME, CE, EDME	SPE/-	MS/MS	5 – 50 pg/mg	(Cognard et al., 2005)
Saliva	Cocaine, EME, CE, EDME	SPE/-	MS/MS	0.1 – 0.5 ng/mL	(Cognard et al., 2006)
Urine	Cocaine	LLE/-	NPD	15 ng/mL	(Farina et al., 2002)
Blood	Cocaine, CE	-/-	NPD	20 ng/mL	(Hime et al., 1991)
Placenta	Cocaine, BE, CE	SPE/MSTFA	MS	0.2 – 0.7 ng/mL	(Joya et al., 2010)
Urine	Cocaine	SPME/-	NPD	12 ng/mL	(Kumazawa et al., 1995)
Saliva Urine	Cocaine, BE, EME	LLE/PFPA + PFPOH	MS	2 ng/mL	(Strano-Rossi et al., 2008)
HPTECs	Cocaine, BE, EME, NCOC	SPE/MSTFA	MS	0.4 – 20.9 ng/mL	(Valente et al., 2010)
Nails	Cocaine, BE, NCOC	SPE/PFPA + PFPOH	MS	3 – 3.5 ng/mg	(Valente-Campos et al., 2006)
Skin biopsy	Cocaine, BE, EME, NCOC, CE, EDME, NCE, EEE	SPE/MTBSTFA+TBD MCS	MS	1.25 – 5 ng/biopsy	(Yang et al., 2006)

BE - benzoylecgonine; CE - cocaethylene; EDME - ecgonidine methyl ester; EEE - ecgonine ethyl ester; EME - ecgonine methyl ester; HPTECs - human proximal tubular epithelial cells; LLE - liquid-liquid extraction; MS - mass spectrometry; MSTFA - *N*-methyl-*N*-trimethylsilyltrifluoracetamide; MTBSTFA - *N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide; NBE - norbenzoylecgonine; NCE - norcocaethylene; NCOC - norcocaine; NPD - nitrogen-phosphorus detector; OH-BE - hydroxybenzoylecgonine; PFPA - pentafluoropropionic anhydride; PFPOH - 2,2,3,3,3-pentafluoro-1-propanol; SPE - solid-phase extraction; SPME - solid-phase microextraction; TBDMCS - *t*-butyldimethylchlorosilane; TMCS - trimethylchlorosilane.

Table 5. Gas chromatography studies measuring cocaine and its metabolites in biological samples.

MS development for GC analysis greatly improved the detection of cocaine analytes. In fact, when comparing equal specimens analyzed by GC-NPD and GC-MS, LOD values for the parent compound using the second method may be ten-fold lower than those seen with the conventional NPD. Taking the example of blood samples once more, while Hime et al. (1991) described a cocaine LOD of 20 ng/mL by GC-NPD analysis, Cardona et al. (2006) obtained a cocaine LOD of 2 ng/mL using a GC-MS equipment with prior SPE extraction and combined acylation/methylation of the analytes.

GC-MS is more often applied to the analysis of less conventional biological matrices than LC. These include nails and even biopsy material (Joya et al., 2010; Valente-Campos et al., 2006; Yang et al., 2006). Moreover, GC-MS allows the determination of metabolites of specific consumption patterns, like EDME and EEE for "crack" abuse, and CE and NCE for concomitant use with alcohol, and other secondary minor metabolites, such as NBE and OH-BE (Cardona et al., 2006; Yang et al., 2006).

Furthermore, and as described for LC techniques, the detection of analytes eluted through GC can be performed by tandem MS as well. MS/MS improvement over MS is visible when comparing equal samples analyzed by both methods. For instance, saliva samples analyzed by GC-MS showed a cocaine LOD of 2 ng/mL, whereas GC-MS/MS was sensible for cocaine concentrations below ppb levels (0.1 ng/mL) (Cognard et al., 2006; Strano-Rossi et al., 2008).

In our laboratory, we have recently developed and validated a GC method for detection and quantification of cocaine and its metabolites in primary cultured human proximal tubular epithelial cells (HPTECs) (Valente et al., 2010). As far as we know, this was the first chromatographic technique described for the analysis of cocaine analytes in a cellular matrix.

This *in vitro* cellular model, which was previously optimized and characterized by our group (Valente et al., 2011a) as well, was used to evaluate the specificity and sensitivity of a GC-MS method for the quantification of cocaine, its major metabolites BE and EME, and the minor metabolite NCOC, particularly known for its cytotoxic effects on the liver.

Samples of confluent cells cultured at physiological conditions (supplemented medium, at 37 ºC and a humidified environment with 95% O_2 and 5% CO_2) were used as matrix for analysis in which standard solutions of cocaine and its metabolites were prepared. Extraction was then performed through strong cation-exchange phase SPE columns (OASIS MCX), allowing the pre-concentration of the cocaine analytes in the samples. The compounds were then submitted to derivatization with MSTFA, which generated well resolved chromatographic peaks for all the analytes in study.

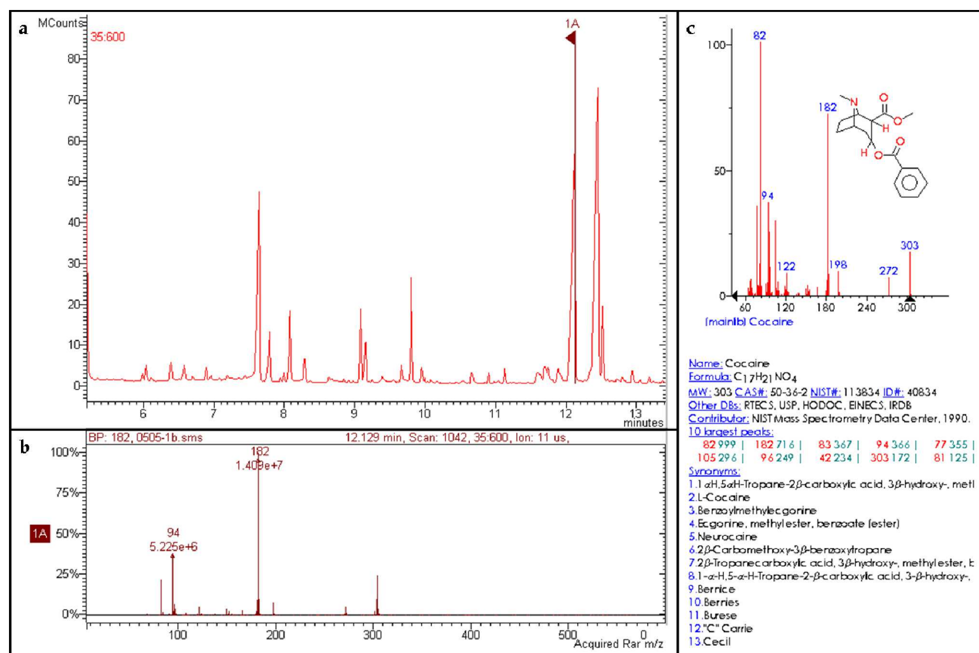
The method was proven to be accurate, linear for a wide range of concentrations (0 - 100 µg/mL) and specific for cocaine analytes. It provided very low LOD values for all cocaine analytes (0.4 – 20.9 ng/mL).

This validated GC-MS technique was further successfully applied to a toxicokinetics study on renal cocaine metabolism, in which we were able to demonstrate that, unlike what happens in the liver, cocaine is metabolized in the kidney into EME and NCOC in lesser extent, but not into BE (Valente et al., 2011b).

This study demonstrated the usefulness of GC, and particularly GC-MS, not only for the determination of drugs of abuse in biological samples, for either clinical or forensic purposes, but also for physiological evaluations and development of toxicological models.

4.3 Data analysis

After a complete chromatographic separation, a chromatogram is obtained as the example shown in figure 4a. The identification of each peak in the chromatogram can be attained through comparison of the retention times of the compounds in the sample and standard compounds analyzed at the same chromatographic conditions. Another way is the comparison of the mass spectrum of the analyte, provided by a MS detector, with the existing mass spectra in a database.



(a) full scan chromatogram, (b) mass spectrum of indicated peak and (c) cocaine identification through a mass spectrum database (National Institute of Standards and Technology, NIST 05 database).

Fig. 4. Analysis of a biological matrix containing cocaine and its metabolites, through gas chromatography with detection by mass spectrometry.

Figure 4 represents the identification of the cocaine peak in a biological sample eluted in a GC-MS equipment. In figure 4a is pointed out a peak (1A) and the respective mass spectrum in figure 4b, indicating the relative abundance of each mass-to-charge ratio (m/z) in that peak. The m/z profile of the selected peak is then compared to those existing in the database, and the compounds with approximated spectrum are presented in a decreasing order of similarity. In this case, cocaine m/z profile is given as the most resembling to the 1A peak (figure 4c).

Independently of the detector used, the quantification of an analyte requires the use of calibration curves obtained from standard solutions of the compounds in study analyzed at the same chromatographic conditions of the samples, and preferably prepared in an equal matrix to eliminate matrix effects.

To avoid miscalculations resulting from errors inherent to steps prior to analysis, for example injection of variable sample volumes in the chromatographic equipment, it is recommendable to use an IS. The IS is added to each sample at the same time point, its concentration should not alter with further preparation procedures, and the IS chromatographic peak cannot interfere or elute at the same time of any analyte of the sample.

Using an appropriate IS, for both samples and SS, the determination of the compounds takes into account the area of the IS chromatographic peak, and the calibration curves are presented as [standard solution area/IS area = f(concentration of the standard solution)]. Finally, the concentration of each analyte will be extrapolated using the ratio [analyte area/IS area].

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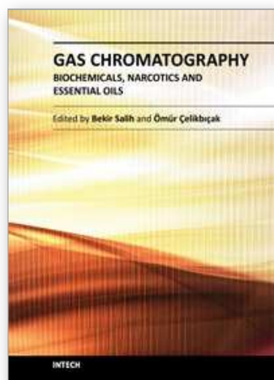

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Gas Chromatography involves the study of various vaporizable molecules in chemistry and the other related research fields. This analytical method has a number of features and advantages that make it an extremely valuable tool for the identification, quantification and structural elucidation of organic molecules. This book provides detailed gas chromatography information to applications of biochemicals, narcotics and essential oils. The details of the applications were briefly handled by the authors to increase their comprehensibility and feasibility. This guide should be certainly valuable to the novice, as well as to the experienced gas chromatography user who may not have the enough experience about the specific applications covered in this book. We believe this book will prove useful in most laboratories where modern gas chromatography is practiced.

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University Campus STeP Ri
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51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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