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DOCTORAL THESIS

Identification and quantitative determination of xenobiotics in hair by new, highly specific mass spectrometry approaches

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Contents

	page
Summary	9
1. Aim of the study	11
2. Background	13
2.1 Hair anatomy and physiology	13
2.2 Mechanisms of incorporation and elimination of drugs in hair	14
2.3 Hair analysis	18
<i>2.3.1 Decontamination</i>	18
<i>2.3.2 Separation of drugs from hair matrix</i>	19
<i>2.3.3 Clean-up of hair extracts</i>	20
<i>2.3.4 Detection and quantification</i>	20
<i>2.3.5 Interpretation of analytical results</i>	21
<i>2.3.6 Practical applications</i>	21
3. Highly-specific quantification of ergotamine in hair samples by LC-MS/MS	25
3.1 Ergotamine	25
3.2 Materials and methods	28
<i>3.2.1 Chemicals and reagents</i>	28
<i>3.2.2 Preparation of standard solutions</i>	28
<i>3.2.3 Sample collection and extraction</i>	28
<i>3.2.4 Calibration</i>	29
<i>3.2.5 LC-MS/MS procedure</i>	30
<i>3.2.6 Validation protocol</i>	31
<i>3.2.7 A case of iatrogenic ergotism</i>	33
3.3 Results and discussion	34
<i>3.3.1 MS and MS/MS</i>	34
<i>3.3.2 LC-MS/MS</i>	36
<i>3.3.3 Sample preparation</i>	38
<i>3.3.4 Validation results</i>	38
<i>3.3.5 Application to a case of iatrogenic ergotism</i>	39
4. Determination of citalopram, escitalopram and their demethylated metabolites in neonatal hair samples by liquid chromatography – tandem mass spectrometry	43

4.1 Citalopram/Escitalopram	43
4.2 Materials and methods	45
4.2.1 <i>Chemicals and reagents</i>	45
4.2.2 <i>Preparation of standard solutions</i>	45
4.2.3 <i>Sample collection and extraction</i>	45
4.2.4 <i>LC-MS/MS procedure</i>	46
4.2.5 <i>Validation protocol</i>	47
4.3 Results and discussion	48
4.3.1 <i>MS and MS/MS</i>	48
4.3.2 <i>LC-MS/MS</i>	51
4.3.3 <i>Validation results</i>	52
4.3.4 <i>Application to real cases</i>	54
5. A fast screening MALDI method for the detection of cocaine and its metabolites in hair	57
5.1 Cocaine in hair	57
5.2 MALDI-MS	60
5.3 Materials and methods	63
5.3.1 <i>Chemicals and reagents</i>	63
5.3.2 <i>Sample preparation for GC-MS analysis</i>	63
5.3.3 <i>GC-MS analyses</i>	64
5.3.4 <i>Sample preparation for LDI/MALDI experiments</i>	64
5.3.5 <i>LDI/MALDI experiments</i>	65
5.4 Results and discussion	68
5.4.1 <i>Standard solution analyses</i>	68
5.4.2 <i>Hair samples analysis</i>	71
6. Conclusions	77
7. References	79

Summary

The analysis of drugs in hair samples has become very popular in recent years with possible applications in forensic and in clinical toxicology. Hair testing can complement conventional blood and urine analysis as it enlarges the window of detection and, by segmentation, permits differentiation between long-term therapeutic use and single exposure. The use of high specific and sensitive analytical procedures is therefore required to achieve low limits of detection. In this study new analytical procedures were developed for the accurate and specific quantification of xenobiotics whose determination in hair would be of toxicological or forensic interest. The research program was developed in three phases: in the first phase a new selective and sensitive method, based on liquid chromatography-tandem mass spectrometry (LC-MS/MS), was developed and validated for the quantitative determination of ergotamine in hair, a highly toxic drug, used to treat vascular headaches. The validated method was successfully applied to a case of iatrogenic ergotism resulting from the prolonged intake of ergotamine tartrate for recurrent headaches. The second phase consisted of the development and validation of a new LC-MS/MS method for the assay of citalopram and escitalopram, two highly selective serotonin reuptake inhibitors, used for the treatment of depression and anxiety disorders, and their demethylated metabolites, in 10-mg hair samples. This method proved to be suitable for neonatal hair analysis and was applied to two real cases of gestational exposure to the drugs. In the third phase, a fast screening method, based on matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was developed for the detection of cocaine and its metabolites, benzoylecgonine and cocaethylene, in hair samples. Different MALDI sample preparation approaches have been tested and the employment of a multi-layer procedure yielded the best results; the same approach was subsequently applied to hair samples that were known to contain cocaine and metabolites, as determined by a classical gas chromatography-mass spectrometry (GC-MS) method.

1. Aim of the study

Hair testing has become very popular in recent years with possible applications in forensic and in clinical toxicology. The use of highly specific and sensitive analytical procedures is therefore required to achieve low limits of detection.

The aim of this study was the development of new analytical procedures for the accurate and specific determination of xenobiotics in hair, based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) or matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The main goals of the research program were the identification of xenobiotics, such as pharmaceuticals or drugs, whose determination in hair would be of toxicological or forensic interest; the development and validation of new mass spectrometric procedures for the identification and quantitative determination of those xenobiotics and their metabolites; the quantitative determination, by means of the validated procedures, of the analytes of interest in hair, from subjects exposed to the xenobiotics. The analytes selected for this study were: ergotamine, a highly toxic drug, used to treat vascular headaches; citalopram and escitalopram, two highly selective serotonin reuptake inhibitors, used for the treatment of depression and anxiety disorders, and their demethylated metabolites; cocaine and its metabolites, benzoylecgonine and cocaethylene.

2. Background

2.1 Hair anatomy and physiology

Hair is a complex tissue whose structure and biology are only partially understood. It is not a homogeneous fiber, but consists of keratinized cells glued by the cell membrane complex that together form three concentric structures: cuticle, cortex and medulla (Figure 1a).¹ The pigmented cortex is responsible for the stretching stability and color composition, whereas the 5–10 layers of shingle-like cells of the non-pigmented cuticle are responsible for high chemical and physical resistance and shine. Hair originates from the hair follicle (Figure 1b) located 3– 5 mm below the skin surface. The hair follicle is surrounded by a rich capillary system that provides the growing hair with the necessary metabolic material. The germination center around the hair bulb papilla is formed by matrix cells (keratinocytes and melanocytes) present on the basement membrane.

Hair color is produced by melanocytes located in the basal layer in contact with the basal membrane (Figure 1c); melanocytes produce melanin pigments in melanosomes. Melanocytes and pigmentation play an important role in the incorporation of basic drugs into hair. Each hair also belongs to a sebaceous gland with the duct leading to the upper part of the root to ensure that the mature hair is bathed in sebum for two to three days prior to reaching the skin surface. The eccrine sweat glands are nearby but separated from the hair root. The sweat glands wet the hair shaft and can contribute to the incorporation of hydrophilic drugs. The hair growth cycle is composed of the anagen (active growing), catagen (transition) and telogen (resting) stages. The individual length of hair depends on stage duration and growth rate. The average values for these stages are 4–8 years, a few weeks, and 4–6 months, respectively. Scalp hair growth ranges 0.6–1.4 cm per month in general.^{2,3} It should be noted, however, that there are significant differences both in the proportions anagen/telogen hair and in growth rate at various anatomical sites; both parameters are dependent on race, sex, age and state of health. At any given time,

approximately 85% of adult scalp hair is in the growing phase (anagen) with the remaining 15% in the remaining phase (telogen). The main consequence of cyclic growth is hair age heterogeneity with respect to distance from the skin.

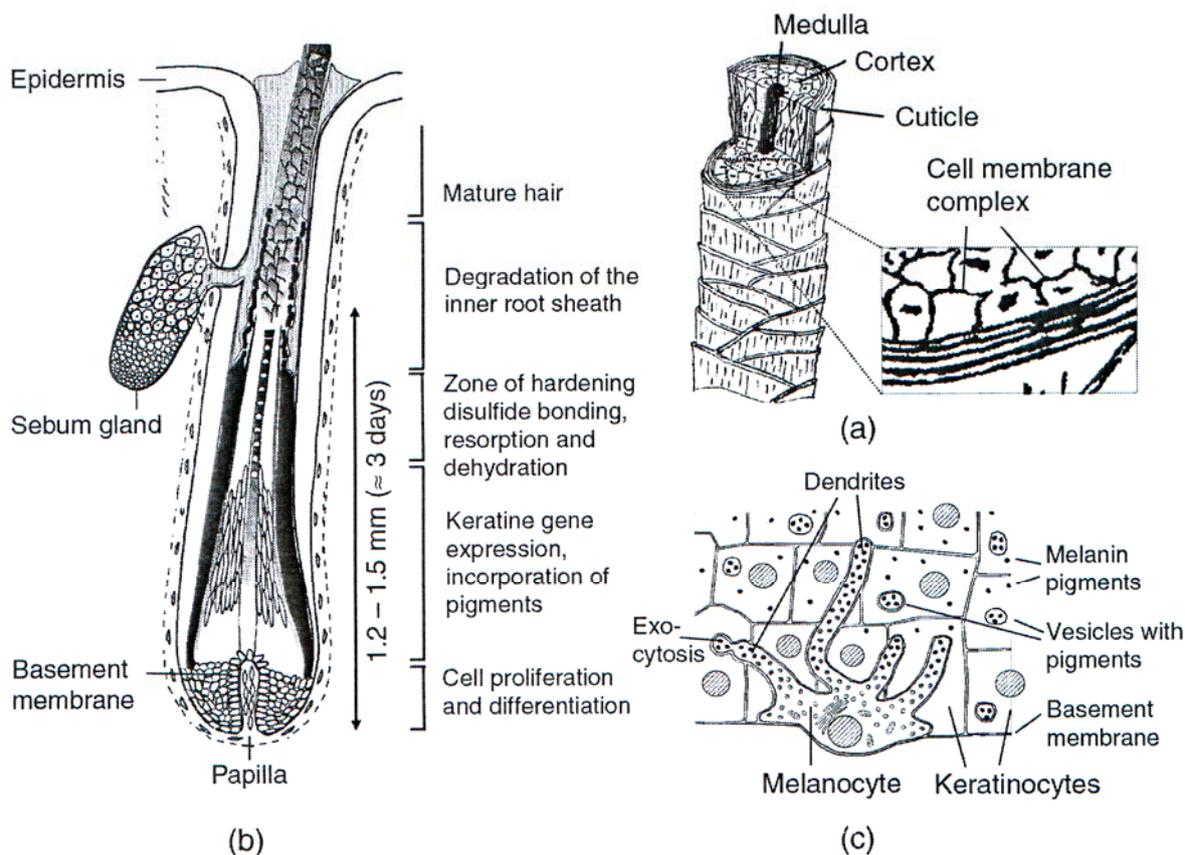


Figure 1: (a) Structure and constituents of the human hair shaft; (b) Formation of hair in a follicle from matrix cells on the basement membrane to the mature hair shaft; (c) Melanocytes on the basement membrane of the cortex synthesize melanine in melanosomes that are discharged in vesicles into keratinocytes by an exocytotic mechanism. There, the membranes of the vesicles and melanosoms are digested and remain the melanin pigments.¹

2.2 Mechanisms of incorporation and elimination of drugs in hair

The precise mechanisms involved in the incorporation of drugs into hair remain unclear requiring further investigation. Incorporation models typically assume that drugs or chemicals enter hair by passive diffusion from blood capillaries into growing cells over a

length of 1.2 to 1.5 mm between the level of matrix cells and end of the keratinization zone of the hair follicle. This period would correspond to a timetable of drug exposure of about three days. Experimental data, however, indicates that drugs enter hair by various mechanisms in a variety of locations, times and sources (Figure 2). Besides incorporation from blood, substances can be incorporated, although with some time delay, from deep skin compartments during hair shaft formation. The most important alternative mechanism is, however, deposition by diffusion from sweat or sebum secretions into the completed hair shaft. In addition, substances can be deposited from the external environment. This multi-compartment model has been specifically demonstrated by Henderson.⁴ It is important to note, however, that the nature of the incorporated substance (structure, chemical properties) as well as the physical/physiological characteristics of the individual strongly influence the likely dominant mechanism. From a structural point of view, incorporation of drugs is influenced by the melanin content of hair and by the lipophilicity and basicity of the substances. Despite root exposure to the same drug concentration in blood, the concentration of basic drugs in pigmented hair was about 10-fold higher than non-pigmented hair.⁵⁻⁷ In controlled studies, similar results were obtained by comparison of black, brown, blond and red colored hair⁸ as well as in Caucasians and non-Caucasians.⁹ Results related to melanin concentration^{10,11} were also confirmed in several studies using animals¹² and *in vitro* experiments.^{13,14} In contrast, no differences were observed for drug incorporation for pigmented and non-pigmented hair for neutral compounds such as carbamazepine.⁷ Generally, the incorporation of drugs into hair from blood is controlled by the pharmacological principles of drug distribution. Lipophilic (uncharged) organic molecules can easily penetrate membranes and diffuse according to the concentration gradient in matrix cells. However, for hydrophilic molecules or organic ions of medium molecular mass, membranes form an impermeable barrier. Basic or acidic drugs ionized to a high degree at physiological pH can reach matrix cells following deprotonation or

protonation, respectively, to a neutral state. As such, the pKa of the compound and pH of the matrix cells are both important. The intracellular pH of keratinocytes has been found to be more acidic than plasma¹⁵ and the pH of melanocytes has been reported to be between 3 and 5.¹² Furthermore, a significant melanin affinity for basic drugs has been demonstrated *in vitro*.^{13,14} Both effects, lower pH and binding to melanin, lead to the accumulation of lipophilic and basic drugs in matrix cells with clear preference for pigmented hair, while acidic drugs or metabolites are found only in very low concentrations in hair.

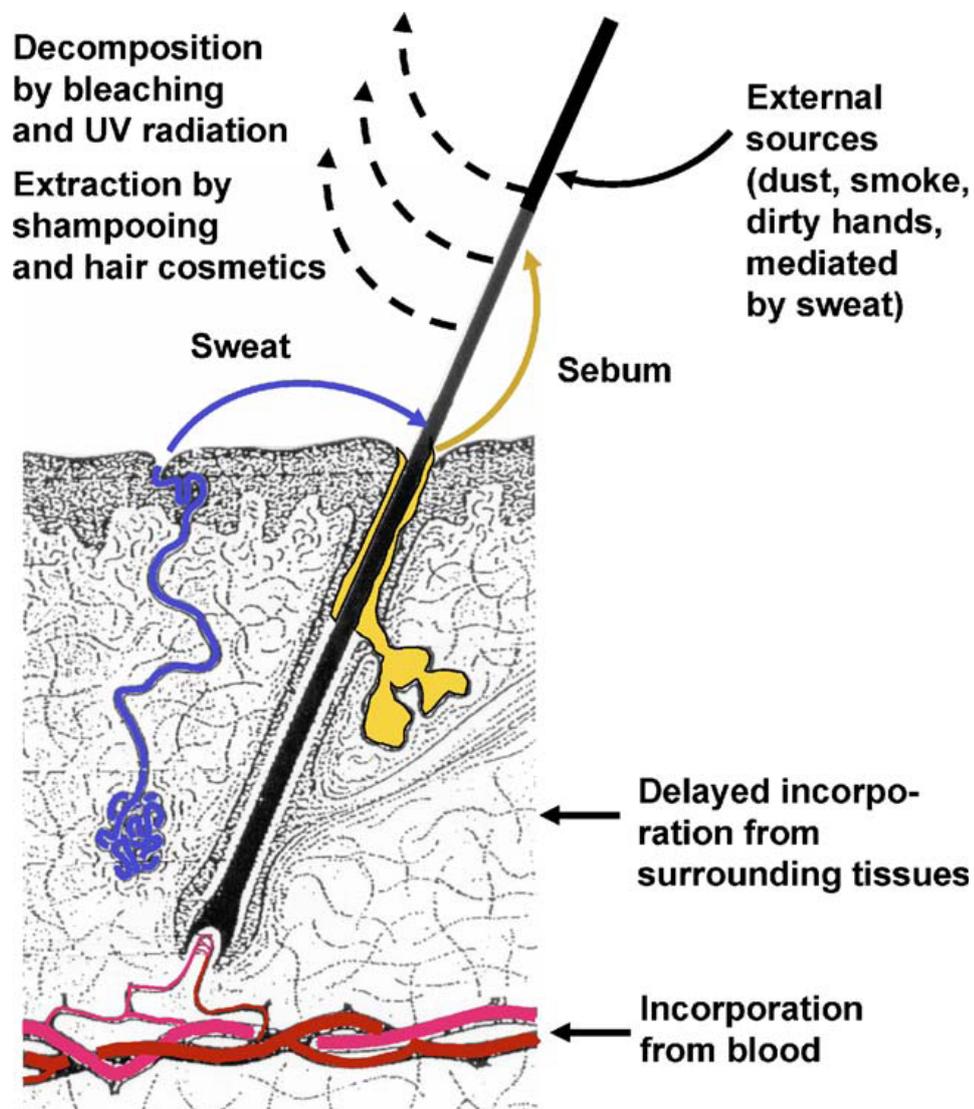


Figure 2. Incorporation and elimination of drugs in hair.¹

In most cases, drug metabolism leads to increased hydrophilicity. Polar metabolites such as benzoylecgonine, morphine and amphetamine enter hair to a lesser extent than their lipophilic precursors (cocaine, 6-monoacetylmorphine and methamphetamine, respectively); similarly, the tricyclic antidepressants amitriptyline, clomipramine, doxepine and imipramine accumulate more in hair than their corresponding non-metabolites.¹⁶

Although the concentration of many illicit and therapeutic drugs in hair has been described, only a small fraction of these investigations were adequately controlled studies, i.e., in which daily dose and duration of drug intake were actually known.

Retention and stability of drugs in hair is considered good. This is clearly demonstrated in hair segments of patients receiving constant dosage. The duration of substances in normally kept hair depends on the chemical structure and increases with increasing polarity. Therefore, as a rule, the concentration ratio metabolite/drug increases from proximal to distal.¹⁷ However, for metabolites formed by ester hydrolysis, this longer detection of the metabolite can also be caused by a slow hydrolytic degradation of the drug within hair. In regularly shampooed hair, i.e., not treated by aggressive cosmetic agents such as oxidative dyeing, bleaching or permanent wave, drugs are usually well detected at least one year after intake. Although the cuticle becomes more susceptible to damage by mechanical stress and increasingly penetrable for drug elimination, it is affected to a much higher degree by the cosmetic treatments described above.¹⁸⁻²⁰ In fact, decreases of 1–90% of the original drug concentration can occur; the extent of drug decline following cosmetic treatment is dependent on its initial concentration and the properties of the hair matrix.

2.3 Hair analysis

The analysis of drugs in hair samples has become very popular in recent years with possible applications in forensic and in clinical toxicology as well as in work-place drug testing.

The major, practical advantage of hair testing compared to urine or blood testing for drugs is that it has a wider detection window (weeks to months, depending on the length of the hair shaft, against 2-4 days, for most drugs). For short-term information on an individual's drug use, blood and urine are preferred specimens, while long-term histories are only accessible through hair analysis. Hence, these tests complement each other. The assessment of chronic exposure to drugs is achieved by segmental hair analysis. In fact, hair grows at approximately 1 cm per month, and is possible to associate the drug distribution pattern in the analyzed segments with a period in the past, taking into account both variable hair growth rates and intra- and inter-individual differences. Furthermore, drugs are very stable within the hair matrix for long periods of time. Another advantage of hair analysis when compared to blood or urine is the non-invasive collection procedure; furthermore the sample can not easily be adulterated.

However, hair analysis suffers from several drawbacks and it is necessary to observe some recommendations for obtaining correct results. The main steps to follow are reported below.

2.3.1 Decontamination

Cleaning the hair sample of external contamination is necessary for two reasons. Firstly, residues of hair care products (wax, shampoo, hair sprays) as well as sweat, sebum and dust, typically present on hair lead to increased analytical noise/ background. Secondly, drugs from the environment of the individual could adhere thus contributing to false positive test results. To exclude that a positive analytical result originates from this type of environmental contamination, the first wash solution should be tested or stored for

subsequent analysis. In addition, the detection of metabolites in a typical ratio to the parent drug can be considered as a definite proof of consumption.

Solvents used for hair decontamination should remove external impurities as completely as possible, but not extract drugs from the hair matrix. Different hair washing sequence can be followed: 0.1% sodium dodecylsulfate, distilled water and acetone; one or two washes with dichloromethane;²¹ sequences of different organic solvents²² or a brief wash with methanol.²³

2.3.2 Separation of drugs from hair matrix

There are currently no direct methods for the detection of organic drugs in hair matrix. Therefore, drugs must be extracted by solubilisation or digestion of the hair matrix itself. In order to make the appropriate choice, the chemical structure of the drug and its sensitivity to agents used for sample preparation must be considered. Prior to extraction, the hair is typically cut to 1–3 mm lengths or alternatively, hair may be processed by grinding.

A procedure compatible with almost all drugs is methanol extraction (5–18 h) in an ultrasonic bath.²⁴ Hydrophilic methanol penetrates the hair matrix leading to swelling and drug liberation via diffusion and dissolves neutral and lipophilic compounds. Ultrasonication causes a strong degradation of the hair structure and contribute to the process of extraction. Drugs sensitive to hydrolysis such as heroin²⁵ and lipophilic drugs such as THC²⁴ can be determined from the same extract. A disadvantage of this approach, however, is a relatively high impurity level in the extract. Therefore, a second clean-up procedure involving liquid/liquid extraction or solid-phase extraction is generally recommended.

Basic drugs (opiates, cocaine and its metabolites, amphetamines, methadone) are well extracted by aqueous 0.01 to 0.5 M HCl²⁶ or phosphate buffer (pH 6.4 or 7.6)²⁷ due to protonation.

An enzyme mixture of glucuronidase/arylsulfatase is occasionally used in order to extract any deposited metabolites. These aqueous extracts are much cleaner than methanol extracts but, despite this improvement, partial hydrolysis of cocaine to benzoylecgonine and 6-monoacetylmorphine to morphine may occur.

Hair extraction of drugs in aqueous medium is facilitated in the presence of denaturants such as urea (8 mol/L) and thioglycolate (0.2 mol/L) under acidic conditions (pH 3.2). This approach has proven particularly successful for the extraction of benzodiazepines.²⁸

The enzymes pronase and proteinase K can be used to hydrolyze hair protein. For drugs that are stable under alkaline conditions, a convenient method of hair extraction is digestion with aqueous NaOH (1 mol/L) for one hour at 80 °C. Basic extraction is particularly advantageous for nicotine,²⁹ amphetamines,³⁰⁻³³ THC,³⁴⁻³⁹ antidepressants and neuroleptics.⁴⁰⁻⁴²

Generally, quantitative recovery of drugs from the hair matrix can be assumed.

2.3.3 Clean-up of hair extracts

Following the extraction step, which is normally the most time-consuming step in hair analysis, a sample cleanup step is often required, to minimize any interference caused by endogenous compounds, which is particularly important in the case of liquid chromatography-based methods because of ion suppression/enhancement effects. This sample cleanup procedure is usually performed using liquid-liquid extraction (LLE)⁴³⁻⁴⁶ or solid-phase extraction (SPE).⁴⁷⁻⁵³ However, solid-phase microextraction (SPME),^{37,54-56} solid-phase dynamic extraction (SPDE)⁵⁷ and supercritical fluid extraction⁵⁸⁻⁶⁰ have also been reported.

2.3.4 Detection and quantification

Instrumental methods used in hair analysis must be suitable for unambiguous drug identification and quantitation. Hair analysis can begin with a general screening by

immunoassays, followed by a confirmation using chromatographic techniques. Gas chromatography coupled to mass spectrometry (GC-MS) is by far the most widely used analytical tool for drug determination in hair specimens. Nevertheless, liquid chromatography-mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) are becoming more important in this field, owing to their better sensitivity for thermo labile compounds, yielding lower limits of detection and quantitation, and the fact that time-consuming derivatization steps are not necessary prior to analysis.

2.3.5 Interpretation of analytical results

Hair analysis for drugs is preferentially performed in forensic cases. Because of its serious consequences, the analyst assumes a high responsibility for obtaining a correct result. Therefore, the whole process from sampling to result interpretation must be well organized and precisely performed to avoid any potential error.

The most serious pitfalls of hair analysis are not in the practical performance but in the interpretation of the results, regarding both drug concentration and time of drug use. A main problem is that there is no inter-individual correlation between frequency of drug use and concentration in hair: incorporation efficiency differs strongly between individuals and it is influenced by hair pigmentation, particularly for basic drugs.

2.3.6 Practical applications

Drugs seriously interfere with human physical and/or psychological performance. Such interference has serious negative consequences for many common activities ranging from industrial safety to driving ability. Recently, drugs use has become highlighted as a mean to enhance sports performance. Because of its potential as a long-term index of drug use history, hair analysis provides a mechanism to monitor and control abuse in workplace drug testing, drug test in context of driving ability examination, doping control.

Hair analysis is also frequently used in criminal cases in order to establish whether the accused regularly used drugs during the period in which the crime was committed.

These cases typically involve drug trafficking, crimes committed to support personal drug consumption and crimes committed by addicts under the influence of drugs. The diagnosis of drug addiction is an essential part in the examination of the accused by a forensic psychiatrist and may be supported or dismissed by the hair drug testing results.

Hair samples are particularly useful to prove chronic exposure to drugs or other poisons. Therefore, it can be used as a diagnostic tool for clinical detection of drugs abuse, for gestational drug exposure in neonates and for elucidation of other chronic poisonings from many sources including long-term environmental pollution, food adulteration or unknown criminal activity.

Hair samples of patients in withdrawal treatment were analyzed in several studies in order to determine the identity of abused drugs before treatment and to control change in consumption behaviour after treatment.^{22,27,61-64} Segmental hair analysis can provide a retrospective calendar of an individual's drug use or period of abstinence. Furthermore, the knowledge of previous drug abuse is important to obtain an accurate and correct diagnosis of psychotic patients. Alcohol, smoking and illegal drug use during pregnancy are serious hazards to the fetus and may lead to miscarriage, premature birth, increased peri- and neonatal mortality rate, retarded physical and mental development, learning difficulty or hyperactivity. A neonatal withdrawal syndrome may manifest in cases of maternal opiate or methadone abuse. Meconium analysis is well-established approach to investigate *in utero* drug exposure.⁶⁵ However, if drug abuse is suspected only at some later time analysis of hair obtained from the baby and mother can be performed. When performing hair analysis on the neonate fetal hair growth and hair cycle³ and the steady substance exchange with amniotic fluid before birth must be taken into account.

Another important application of hair analysis is in *post-mortem* toxicology. Over the last years hair samples are regularly collected during autopsy. Although hair is not generally useful for proving a lethal intoxication, it has been demonstrated that acute

poisoning can be established with hair root examination.⁶⁶ However, the predominant value of hair analysis in *post-mortem* cases is to support death diagnosis by proving or excluding chronic substance abuse. Other applications include information regarding pathological alterations of internal organs, to provide evidence for drug tolerance in cases of opiate or methadone overdose, to explain withdrawal symptoms or as an indication that, in case of a longer survival following an accident, the deceased was probably under the influence of drugs. Finally, hair analysis can contribute to the identification of an unknown corpse.

Hair testing is useful also in criminal assault cases. It is not a new phenomenon to use drugs to govern other person's perception and behaviour. In this context, drugs are often used to achieve a number of goals including personal criminal profit, (i.e., robbery, sexual assault and child abuse). Drugs involved in such crimes can be pharmaceutical (sedatives, hypnotics and anesthetics), ethanol or drugs-of abuse (cannabis, LSD, GHB, ecstasy, etc).

The potential use of hair analysis in therapeutic drug control has also been explored in several publications.⁶⁷⁻⁶⁹ The opinion of a number of authors range from “a worthless tool”⁶⁸ to a proposal to establish standardized methods of hair analysis for all newly introduced pharmaceuticals.⁶⁹ Hair testing is unsuitable for individual adjustment of drug dosing (therapeutic drug monitoring, TDM). Detailed monitoring to ascertain therapeutic compliance is difficult due to enormous intra- and inter-individual variation and relatively poor relationship between dose, plasma concentration and hair concentration. Several investigations have, in fact, been performed with antiepileptics and different groups of psychopharmaceuticals.^{5,16,70-84} From a practical point of view, several experimental approaches may warrant continued pursuit.

Although alcohol is the most frequently abused substance, it has played only a minor role in hair analysis because it is a volatile substance and not durably incorporated.

Therefore, a hair alcohol test must be able to differentiate social drinking from abuse. In addition, other routine and relatively low cost laboratory biomarkers for chronic alcohol abuse have been well established including gamma-glutamyltransferase (GGT), mean corpuscular volume of erythrocytes (MCV), carbohydrate deficient transferrin (CDT). Because of these limitations, alcohol abuse via hair analysis is limited and generally associated with determination of its minor metabolites including fatty acid ethyl esters and ethyl glucuronide.⁸⁵

3. Highly-specific quantification of ergotamine in hair samples by LC-MS/MS.

3.1 Ergotamine

Ergot alkaloids form a sizable group of indolic alkaloids isolated from the ergot fungus *Claviceps Purpurea*, a plant parasite that mainly infests rye grains. In the Middle Ages, consumption of grain contaminated by the fungus caused a painful and debilitating syndrome, called Saint Anthony's fire, or ergotism,⁸⁶ and characterized by painful convulsions and gangrene of the limbs. Ergotamine was the first alkaloid to be isolated from ergot in 1920^{87,88} and used for therapeutic purposes, first in obstetrics and gynecology to control postpartum hemorrhage due to its vasoconstrictor effects, and later for the treatment of vascular headaches. Ergotamine has a pronounced action on the monoaminergic receptors due to its structural similarity to the adrenergic, dopaminergic and serotonergic neurotransmitters.⁸⁹ The vasoconstrictor action of ergotamine and of its semi-synthetic derivative dihydroergotamine was long believed to be the basis for their clinical effects, but recent advances in the understanding of the pathophysiology and pharmacology of migraine revealed that they both have inhibitory effects on the trigeminal nerves.⁹⁰ Figure 3 shows the chemical structure of ergotamine.

Ergotamine tartrate is currently available in injectable, oral and rectal preparations. The drug is poorly absorbed after oral administration; the extent and rate of absorption is increased when it is administered together with caffeine, and combination of the two drugs is available as oral preparations. Ergotamine is rapidly metabolized in the liver and excreted mainly in the bile as metabolites; only traces of unchanged drug are excreted in the urine or feces. After a single oral dose of 2 mg of ergotamine tartrate administered to 11 subjects, peak plasma concentrations of 100 to 900 pg/mL (mean 600) were observed after 0.5 to 3 h; following an i.m. dose of 0.5 mg given to 10 subjects, peak plasma concentrations of 900 to 4000 pg/mL (mean 2000) were reached in about 0.5 h and the

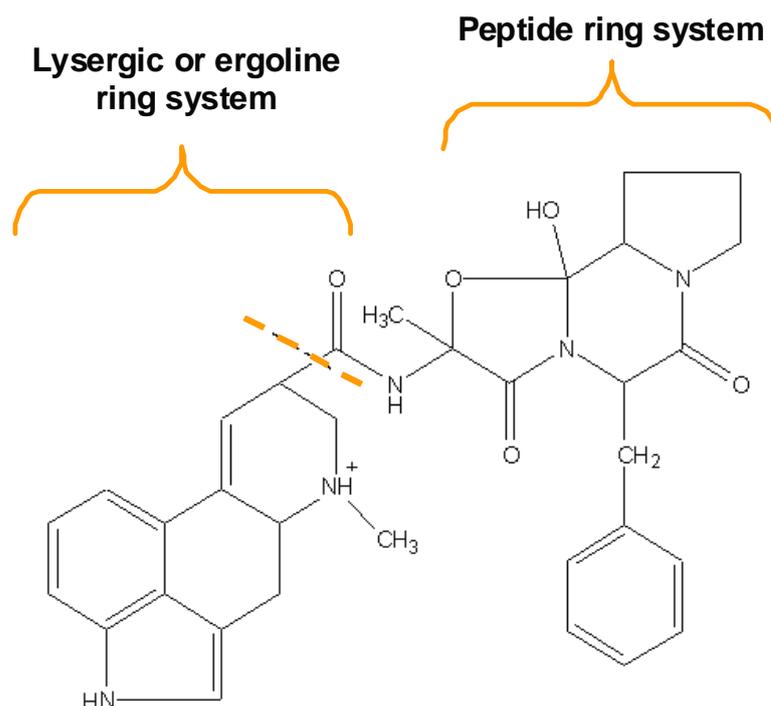


Figure 3. Chemical structure of ergotamine

steady-state plasma concentration resulting in a 50% of maximal effect was calculated to be 240 pg/ml.^{91,92}

Ergotamine is highly toxic; in large, repeated doses it can produce all the symptoms of ergot poisoning. Fatal poisoning has occurred after the oral administration of 26 mg of ergotamine over a period of several days, and also following single injections of only 0.5 to 1.5 mg. In 12 cases of non-fatal ergotamine overdose, mean plasma concentrations of 8000 pg/mL were reported 1.7 to 5 h after ingestion.⁹³

The pharmacological specificity of the ergot alkaloids can be significantly increased if their dosages and plasma concentrations are carefully controlled, and methods for accurately determining ergotamine in biological fluids have been reported in the past based on the use of tritium-labeled alkaloids⁹⁴⁻⁹⁶ or radioimmunoassays (RIA).^{97,98} As an alternative to RIA, Edlund⁹⁹ was the first to suggest measuring ergot alkaloids in plasma using high-performance liquid chromatography (HPLC) and fluorescence detection with a

method capable of detecting concentrations down to 100 pg/mL in a 3 mL sample after an extensive purification of the sample. Further improvements were made to the HPLC-fluorescence method^{100,101} and it has been routinely used for pharmacokinetic studies.

However, the quantitative analysis of drugs exhibiting a therapeutic activity at pg/mL concentrations in biological fluids benefits from the use of mass spectrometry (MS) which has unique specificity and selectivity features and is the ideal confirmation technique in forensic toxicology analyses. The first MS method developed for quantifying ergotamine in human plasma was obtained by direct inlet injection in negative ion chemical ionization (NCI) mode after a time-consuming pre-treatment of the sample.^{102,103} A simpler method for identifying and quantifying ergotamine in plasma or serum, based on gas chromatography-mass spectrometry (GC-MS), was described by Feng et al. in 1992.¹⁰⁴ However, the GC-MS method developed could only measure the product of decomposition of the target analyte since ergotamine is a non-volatile, thermally labile compound which decomposes during gas chromatography. Casy¹⁰⁵ made the point that ionization conditions *softer* than EI are needed for a definite identification of ergolines carrying peptide-based C8 substituents (as ergotamine), an ideal mass spectrum for identification purposes being the one which provides evidence of both molecular weight and characteristic fragment ions. A method for the determination of ergotamine in whole blood and hair samples has not been yet described in the literature.

The most advanced liquid chromatography-mass spectrometry (LC-MS) techniques have recently been applied to the quantification of different ergot derivatives in human fluids or food,¹⁰⁶⁻¹¹⁰ and we recently applied liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the identification of the ergot semi-synthetic derivative lysergic acid diethylamide (LSD).¹¹¹ The application of LC-MS/MS in the quantification of ergotamine should in principle yield the following advantages with respect to methods based either on HPLC-fluorescence, direct inlet NCI or GC-MS: higher specificity; lower

limits of detection with reduced amount of sample and without extensive, time-consuming sample pre-treatment; detection of the target analyte instead of its decomposition product.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Ergotamine tartrate was purchased from Sigma (St. Louis, MO, USA); trideuterated lysergic acid diethylamide (d3-LSD, internal standard, IS) was obtained from Radian International (Austin, TX, USA). All chemicals were analytical reagent grade and all solvents were HPLC grade. HPLC water was prepared using a Milli-Q Plus (Millipore, Molsheim, France) system.

3.2.2 Preparation of standard solutions

A primary standard solution of ergotamine tartrate (44 µg/mL, calculated as free base) was prepared in HPLC water and divided immediately into 400 µl aliquots, which were then stored in the dark at -20°C. Working standard solutions were prepared daily at concentrations of 100 and 10 ng/mL in acetonitrile. The IS (d3-LSD) working solution was prepared in acetonitrile at a concentration of 100 ng/mL.

3.2.3 Sample collection and extraction

Drug-free hair samples were collected from volunteers.

Head hair segments were collected from the back of the head, washed with CH₂Cl₂ and pulverized in a MM2000 ball mill (Retsch, Haan, Germany); 50 mg aliquots were put in PET tubes and, after adding 4 mL of methanol and 20 µL of IS working solution, they were ultrasonicated for three hours and stirred for another three hours at 45°C. After centrifugation at 1814 x g, supernatants were transferred to clean tubes and dried under a nitrogen stream at room temperature in the dark. Dried residues were dissolved in 2 mL water and, after adding 200 mg of ammonium carbonate and 8 mL of chloroform, liquid/liquid extraction was performed for 15 min in the dark. After centrifugation, the

organic layer was removed, transferred to clean tubes and dried under a vacuum at room temperature in the dark. The dried residues were dissolved in 50 μL of solvent A. The sample preparation procedure is schematized in Figure 4.

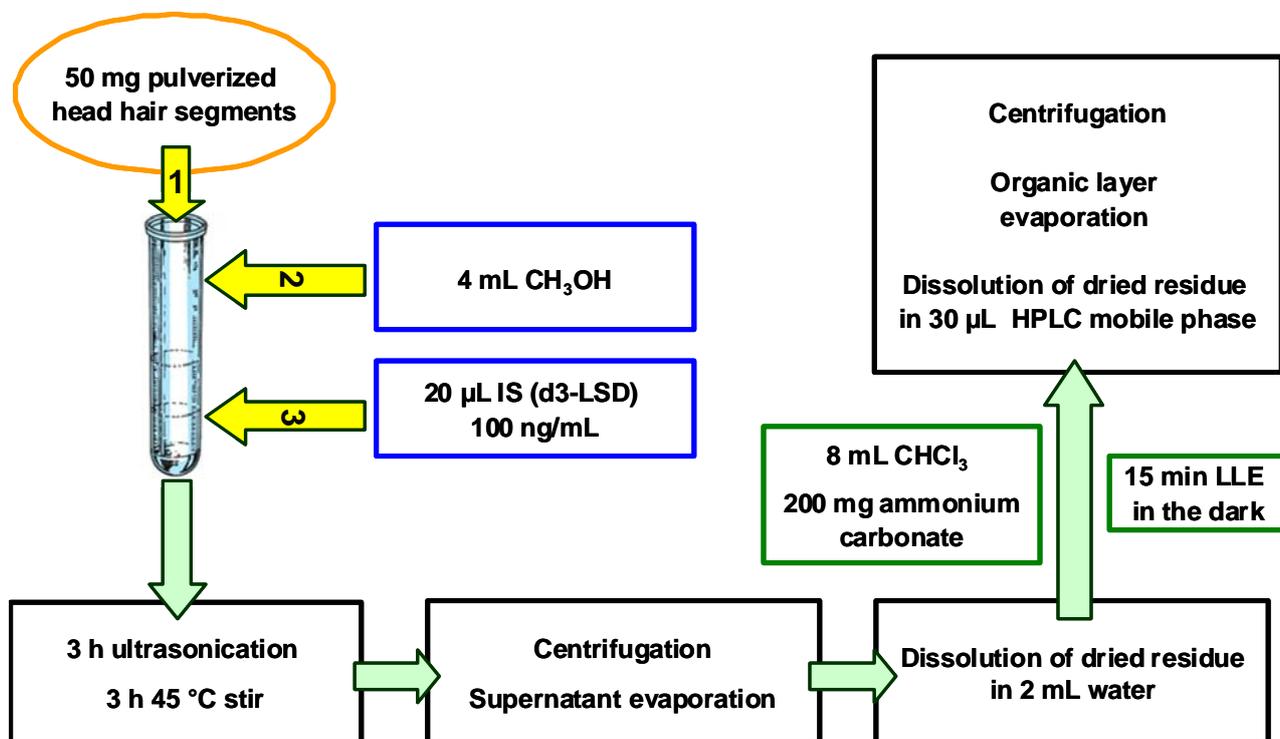


Figure 4. Hair sample preparation for LC-MS analysis: extraction and clean-up procedures.

3.2.4 Calibration

Calibration samples were prepared by spiking drug-free hair, with appropriate amounts of ergotamine working solutions at concentration ranges of 10 - 200 $\mu\text{g}/\text{mg}$. These samples were processed as described above. Calibration curves were constructed by plotting the peak area ratios of the selected ion species (for the analyte and IS) versus analyte concentrations, using at least six calibration points per curve (15, 30, 50, 100, 150, 200 $\mu\text{g}/\text{mg}$ for hair). Three sets of spiked samples were prepared, each on a different day. Peak area ratios of target analytes and their respective internal standards were calculated

for each concentration by Xcalibur's LCQuan software (version 1.2). The data were fit to a linear least-squares regression curve with a weighing factor of $1/x$ to reduce heteroscedasticity.

3.2.5 LC-MS/MS procedure

Chromatographic separation was performed on a Luna CN (150 x 2.1 mm, 5 μ m) analytical column (Phenomenex, Torrance, CA). A Spectra System P4000 pump (ThermoFinnigan, San Jose, CA) was used for gradient elution at a constant flow of 0.2 mL/min.

The HPLC solvents were: A (water, 0.1% formic acid, ammonium formate 2 mmol/L, pH 3) and B (acetonitrile, 0.1 % formic acid, ammonium formate 2 mmol/L). The mobile phase was programmed as follows: original conditions 20% B, linear gradient to 80% B in 8 min, 80% B hold from 8 to 12 min. Re-equilibration time was 4 min.

All mass spectrometric measurements were performed on an LCQ Duo (Finnigan, San Jose, CA) ion trap mass spectrometer equipped with an electrospray ionization source (ESI) working in positive ion mode. The Thermo-Finnigan Excalibur software (version 1.2) was used for system control, data acquisition and quantification. The ESI parameters were: spray voltage 3.30 kV, capillary temperature 220°C, capillary voltage 8 V, sheath gas (nitrogen) 40 arbitrary units (a.u.), auxiliary gas (nitrogen) 20 a.u. Collision-induced dissociation (CID) experiments used a supplementary radio frequency voltage ("tickle voltage") in the range of 0-5 V applied to the end caps of the ion trap. Helium was used as the target gas, at a pressure of 1.46×10^{-3} Pa. Acquisition was done in product ion scan mode, splitting the acquisition time into two stages, scanning product ions of m/z 327 (MH^+ of LSD-d3) in the range m/z 125-327 for the first 7.5 min, and then product ions of m/z 582 (MH^+ of ergotamine) in the range m/z 160-582 for the next 4.5 min.

The percentage deviation of the relative intensities of product ions was determined by duplicate analysis of six hair samples spiked with concentrations of 25 and 50 pg/mg of

ergotamine, analyzed on two separate days (n = 12 for each concentration). A percentage deviation $\leq 15\%$ for ions whose relative intensity is $> 10\%$ in the product ion spectrum was set as an acceptance criteria.

The product ions at m/z 268 for ergotamine and at m/z 226 for IS were used for quantification.

3.2.6 Validation protocol

The following criteria were used to evaluate the method: specificity, recovery, matrix effect, sensitivity, linearity, intra- and inter-assay precision, accuracy.

Six different blank hair samples of different origin were extracted without supplementation of the IS and analyzed to determine the extent to which endogenous components may contribute to the interference at the retention time of analyte and the internal standard. The chromatograms were evaluated by a unique combination of retention time, precursor and fragment ions for both the analyte and the internal standard.

"Spike-after-extraction" samples were prepared by extracting ergotamine-free hair, and afterwards adding working solutions of the analyte and the IS to the sample extracts.

Recoveries (extraction efficiency, %) were determined at three concentrations by comparing the chromatographic peak areas obtained for extracted spiked samples (15, 50 and 200 pg/mg) with those obtained for the spike-after-extraction samples at the same concentration. The experiment was performed in multiple replicates (n = 3) at each concentration. The acceptance range for recovery was established to be 70 - 120 % for both the IS and the analyte.

In order to evaluate ion suppression, the peak areas of spike-after-extraction samples (15 and 50 pg/mg) were compared with those obtained by injection of the same amount of ergotamine and IS in solvent A/solvent B (50/50, vol/vol). The experiment was performed in multiple replicates (n = 3) at each concentration. The loss of signal was then calculated as follows:

$$\% \text{ Loss of signal} = (A_n - A_m)/A_n \times 100$$

where A_n is the peak area for the analyte in neat solution and A_m is the peak area for the analyte in matrix. The acceptable limit was established to be 15% for both the analyte and the IS.

The linearity of the method was investigated by calculation of the regression line by the method of least squares and expressed by the correlation coefficient (R^2). The acceptance criteria were: 20 % deviation of the lowest limit of quantitation (LLOQ) from nominal concentration; 15 % deviation of standards other than LLOQ from nominal concentration; at least four out of six calibration standards should meet the above criteria including the LLOQ and the standard at the highest concentration.

Intra- and inter-assay precision (as % relative standard deviation, RSD) was assessed by extracting and analyzing 5 replicates of spiked samples (at 15 pg/mg, 50 pg/mg and 200 pg/mg), on three consecutive days ($n = 15$). An estimate of the intra-assay precision was obtained using one-way analysis of variance (ANOVA) with day as grouping variable.

Accuracy (as % relative error, RE, i.e. the percentage deviation of the mean result for all analyses from the nominal value of a fortified sample) was determined on the basis of the total data set ($n = 15$) at three concentrations: 15, 50 and 200 pg/mg.

The following acceptance criteria for quality controls above the lowest limit of quantitation were used: accuracies must average within $\pm 15\%$ of the target value with a RSD of $\leq 15\%$.

The LLOQ was the lowest concentration at which the RE and the RSD were $\leq 20\%$. The limit of detection (LOD) was the concentration with signal-to-noise (S/N) ratio of 3. Both parameters were determined empirically by analysis of a series of decreasing concentrations of the drug-fortified samples, in multiple replicates ($n = 5$).

3.2.7 A case of iatrogenic ergotism

A 40-year-old woman came to the Casualties Department complaining of a 7-day history of a general feeling of weakness, bilateral, symmetric anesthesia of hand fingers, intense pallor and feeling cold, especially after exertion. Clinical evaluation revealed intense pain and cyanosis of the right thenar eminence, upper extremity edema and vasospasm. It was not possible to measure her arterial tension in the upper limbs and her peripheral pulses dropped in a generalized manner. There was no evidence of chronic peripheral vascular disease or neurological pathologies. Her past medical history was significant for recurrent headaches, for which she took an oral caffeine-ergotamine preparation for prophylaxis and as a rescue treatment. This preparation (Cafergot, Novartis Farma, Italy) contains 1 mg ergotamine tartrate and 100 mg caffeine per tablet. The patient was admitted to the Vascular Surgery Department for further evaluation. An angiography study showed segmented stenosis of arteries in the upper and lower limbs; ergotamine use was discontinued and she was treated with oral nifedipine and i.v. hydration. The patient's condition improved during her 72 h stay: the symptoms disappeared and the physical examination was normal; because the patient was improving and a diagnosis of iatrogenic ergotism was reached, she was discharged home without a control angiography study.

Several cases of limb ischemia caused by ergotamine use, either acutely or chronically, have been reported in the literature;¹¹²⁻¹¹⁵ the diagnosis of iatrogenic ergotism was generally reached after obtaining a detailed medication history. In the presented case, in order to support the clinical diagnosis, urine, blood and hair samples were collected from the patient at the Vascular Surgery Department and analyzed at the Forensic Toxicology and Antidoping Unit. Hair 30 cm long was collected from the back of the head (vertex posterior); roots (proximal end) and tips (distal end) of the head hair strands were

marked, and segments 4 cm long were collected from both the proximal and the distal ends.

3.3 Results and discussion

3.3.1 MS and MS/MS

Ergotamine is a high-molecular-weight (MW = 581), polar molecule with a peptide substituent at C8 (see Figure 3). An aqueous solution of ergotamine at 100 ng/mL, injected into ESI ion source, produces abundant MH^+ ions at m/z 582 providing evidence of the analyte's molecular weight. No fragment ions from *in source* thermal- or electro-induced dissociation¹¹⁶ could be seen, confirming Casy's hypothesis¹⁰⁵ that soft ionization techniques are suitable for peptide-containing ergot alkaloids. To achieve a higher degree of specificity, however, information on the molecular weight of the target analyte should be accompanied by characteristic fragment ions, as obtainable by collision-induced dissociation (CID) on pre-selected ions. To optimize the experimental conditions for CID on MH^+ of ergotamine, the aqueous solution of ergotamine at a concentration of 100 ng/mL was infused directly into the ESI ion source of the LCQ, while multiple mass spectrometry experiments were conducted by varying the collision voltage amplitude and time. Figure 5 shows the CID spectrum of MH^+ of ergotamine obtained at 0.5 V and a collision time of 30 ms, its chemical structure and cleavages giving rise to the observed fragments. These results are similar to those obtained by Lehner et al.¹¹⁷⁻¹¹⁹ in triple quadrupole experiments determining ergotamine in food; as a matter of fact, by CID on the ion trap the same product ions are obtained, but their relative abundance differs from those obtained by the triple quadrupole. This is not surprising considering that the collisional parameters (voltage, time, target gas, mechanism of energy deposition) on the two instruments are quite different. The abundant ion at m/z 564 is attributed to the loss of a water molecule from the peptide ring system presumably after protonation of the tertiary

alcohol oxygen atom. The ion species at m/z 320 can derive from the cleavage of the peptide ring system at both the amide and ether bonds followed by rearrangement and cyclization,¹¹⁹ while the ion at m/z 268 (chosen for quantification) corresponds to loss of the whole lysergic amide. The neutral loss of the whole peptide ring system from MH^+ yields the ergoline ring derivative at m/z 223.

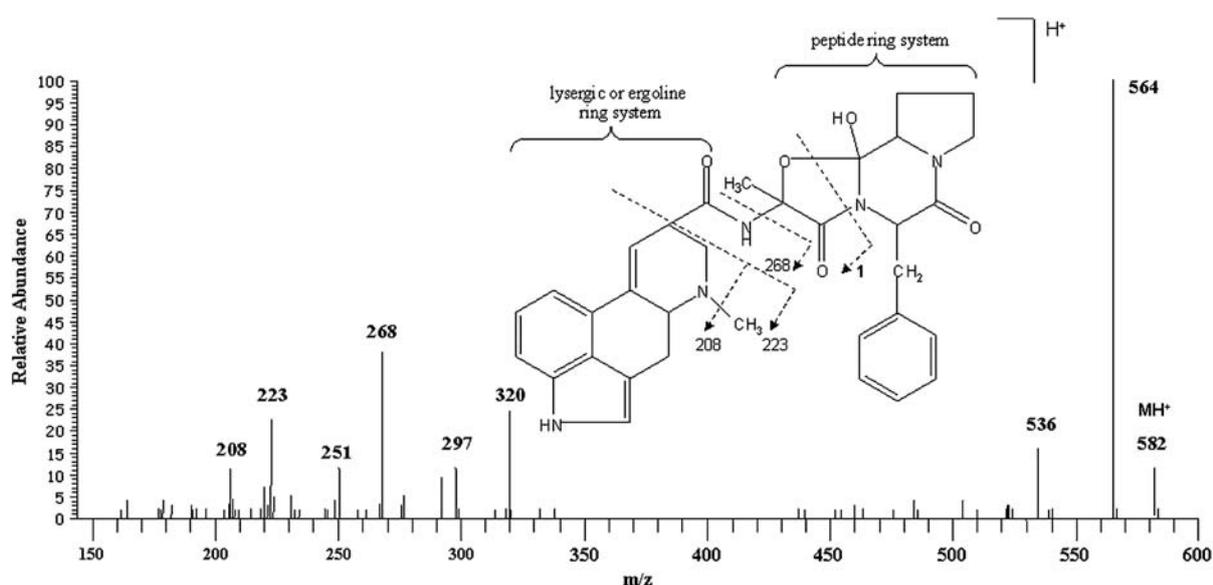


Figure 5. Product ion spectrum of MH^+ ion of ergotamine (m/z 582) obtained at 0.5 V. m/z 582 \rightarrow m/z 564, loss of water from peptide ring system; m/z 582 \rightarrow m/z 536, loss of water and CO; m/z 582 \rightarrow m/z 320, cleavage of peptide ring system in amide and at ether (cleavage 1) followed by H rearrangement and cyclization¹¹⁹; m/z 582 \rightarrow m/z 297, loss of water and lysergic amide; m/z 582 \rightarrow m/z 251, loss of peptide ring system and ammonia. The position of H^+ in the protonated molecule has not been set in the figure, since the observed fragmentations gave evidence that ESI-induced protonation¹²⁰ occur either on the alcoholic oxygen or nitrogen atoms.

This last fragmentation pathway is similar to the one observed in the spectrum of LSD and similar compounds under ESI and CID of the resulting MH^+ ions through the loss of the diethylamide moiety.¹¹¹ In the absence of a deuterated ergotamine analogue, we hence decided to use d3-LSD (available at the lab) as the IS. Its MS/MS spectrum is shown in Figure 6 together with its chemical structure and the cleavages giving rise to the observed fragment ions.

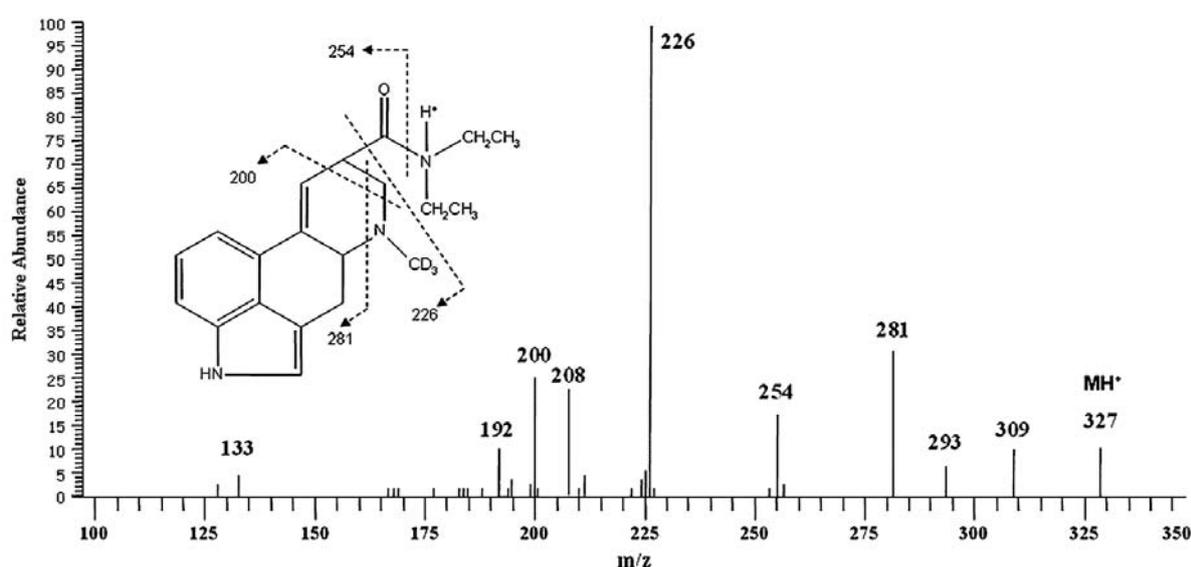


Figure 6. Product ion spectrum of MH^+ ion of d3-LSD (m/z 327) obtained at 0.5 V; m/z 327 \rightarrow m/z 309, loss of water; m/z 327 \rightarrow m/z 208, loss of CD_3NCH_2 and $NH(C_2H_5)_2$.

3.3.2 LC-MS/MS

Having established the best MS/MS conditions, chromatographic separation was optimized by injecting pure standard solutions. Retention times were 7.0 and 8.5 min (\pm 2%, RSD, estimated from 10 replicates on the same day and on three consecutive days) for the IS and ergotamine, respectively. The choice of a slightly polar phase (cyanopropyl) allowed the use of a mixed reversed and normal-phase separation mechanism so that ergotamine and d3-LSD could be separated efficiently by gradient elution relatively quickly (in less than 16 min, including the re-equilibration time).

Spiked hair samples were then run in LC-MS/MS conditions as detailed above. Figure 7 shows the chromatograms obtained for: a) a blank hair sample; b) a spiked hair sample at 15 pg/mg; c) a spiked hair sample at 100 pg/mg. The drug is identified in MS/MS mode by nine ions with a relative abundance > 10% at m/z 582, 564, 536, 320, 297, 268, 251, 223, and 208 (see Figure 5). The percentage deviation of the relative intensities of all product ions, monitored as described in the 3.2.5 paragraph, was always < 15%.

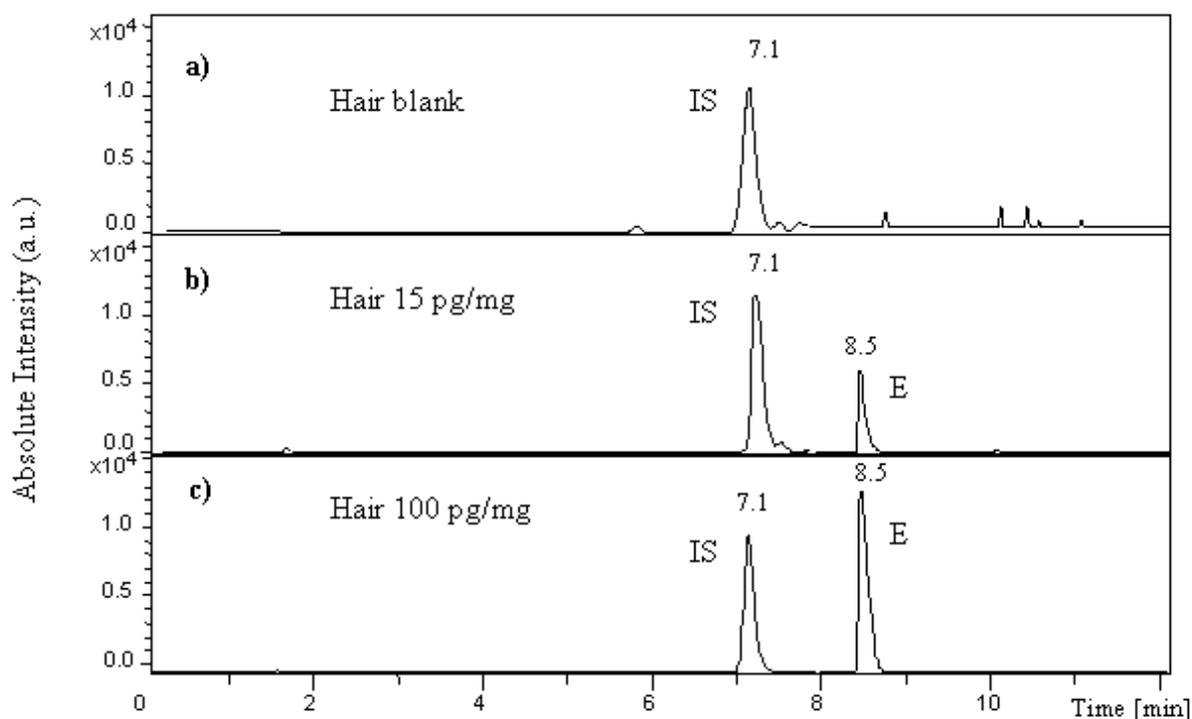


Figure 7. Reconstructed ion chromatograms of the two transitions m/z 327 \rightarrow m/z 226 (IS) and m/z 582 \rightarrow m/z 268 (ergotamine, E) obtained for: a) blank hair sample; b) spiked hair sample at 15 pg/mg; c) spiked hair sample at 100 pg/mg (a.u. = arbitrary units).

3.3.3 Sample preparation

The extraction procedures proposed to determine ergotamine in body fluids mainly consists of liquid/liquid extraction with varying solvents, back-extraction into sulphuric acid and re-extraction at basic pH.^{89,102} These procedures are time-consuming due to the multiple steps and the low volatility of the organic solvents chosen, and require 3 ml of sample (plasma, serum). In our experiments, liquid/liquid extraction seemed to be the simplest and least expensive procedure, considering that a single extraction step is sufficient for a LC-MS/MS procedure in which the high specificity of the detection balances possible partial purification of the analyte from the matrix. Various solvents were tested to extract spiked hair samples, and chloroform turned out to give the best recoveries and the shortest evaporation time, in accordance with previously published results.¹⁰⁴ Chloroform was chosen in spite of its toxicity since the volume required for extraction is small and the frequency of suspected ergotamine intoxication cases, requiring confirmation, is low. Since ergotamine, as well as other lysergic acid derivatives, is reported to decompose on exposure to air, heat and light, extraction was performed in the dark.¹²¹

3.3.4 Validation results

No interferences were detected during analysis of hair from six different sources, processed as described in the experimental section without supplementation of the analyte or the IS. Peak detection performed by the Excalibur software, based on a unique combination of retention time, precursor ion and product ion spectrum, failed to detect any endogenous interferences at the retention times of the analyte and the IS. Figure 7 demonstrates the selectivity results with the chromatograms of blank hair (sample supplemented with only internal standard) and the peak response of ergotamine at the LLOQ in hair (15 pg/mg).

Results of the recoveries determined for ergotamine ranged from 78 % at 15 pg/mg to 90 % at 200 pg/mg and fulfilled the acceptance criteria.

For ion suppression evaluation, the peak areas measured from the spike-after-extraction samples were found to be in the range 95 - 102% of those obtained from the neat determinations of standard solutions; as a consequence, ion suppression was judged as negligible.

The percentage deviation of the relative intensities of product ions, monitored at two concentrations, was always < 15% thus fulfilling the acceptance criteria.

The response for ergotamine was linear in the range 15 - 200 pg/mg ($r^2 = 0.991$) for hair, using a weighting factor of $1/x$.

An intra-assay precision that was always less than 13,8 % and an inter-assay precision less than 14 % were calculated by ANOVA. The accuracy (% RE) for three fortified levels ranged from 13.5 to 3.2%. Accuracy and precision of all quality control samples met criteria of $\pm 15\%$ for average accuracy with coefficients of variation less than 15%.

The LOD and LLOQ, determined empirically by analysis of a series of decreasing concentrations of the drug-fortified hair samples, were respectively 10 and 15 pg/mg. The validation results are summarized in Table 1.

3.3.5 Application to a case of iatrogenic ergotism

The validated analytical procedure was successfully applied to hair samples obtained in the above-mentioned case of ergotism following the use of ergotamine tartrate for migraine. Hair samples were extracted and analyzed as described above. Ergotamine was detected in both proximal and distal segments at the concentrations of 24 pg/mg and 15 pg/mg, respectively. Figure 8 shows the chromatograms obtained for the proximal and distal hair samples.

When investigating the association between the consumption of a given drug and its long-term effects, anamnestic evidence can suffer from problems of recall bias and under- or over-estimation errors in reported drug use. In the present case, finding ergotamine in

both proximal and distal hair samples bore witness to a prolonged use of the drug (roughly two years, considering an average hair growth of 0,6-1,4 cm/month,¹ which was consistent with the patient's reported clinical history).

A specific and sensitive LC-MS/MS method for analyzing ergotamine in human hair was developed and validated. Positive ion ESI of ergotamine produces abundant MH⁺ ions, which are dissociated into more specific, informative fragment ions by ion trap collisional experiments. The method was validated in hair matrix, exhibiting a linear range of 15 - 200 pg/mg and a LLOQ of 15 pg/mg; it allowed to identify and quantify, for the first time, ergotamine in hair in a case of iatrogenic ergotism.

Sample	Accuracy (% RE)			Precision (% RSD)								
	QC1	QC2	QC3	Intra-assay			Interassay					
				QC1	QC2	QC3	QC1	QC2	QC3			
Hair	13.5	6.2	3.2	13.7	11.2	11.5	13.9	11.5	11.5	9.5	10 pg/mg	15 pg/mg

Quality control (QC) concentrations: QC1 = 15 pg/mg, QC2 = 50 pg/mg, and QC3 = 200 pg/mg.

Table 1. Validation results

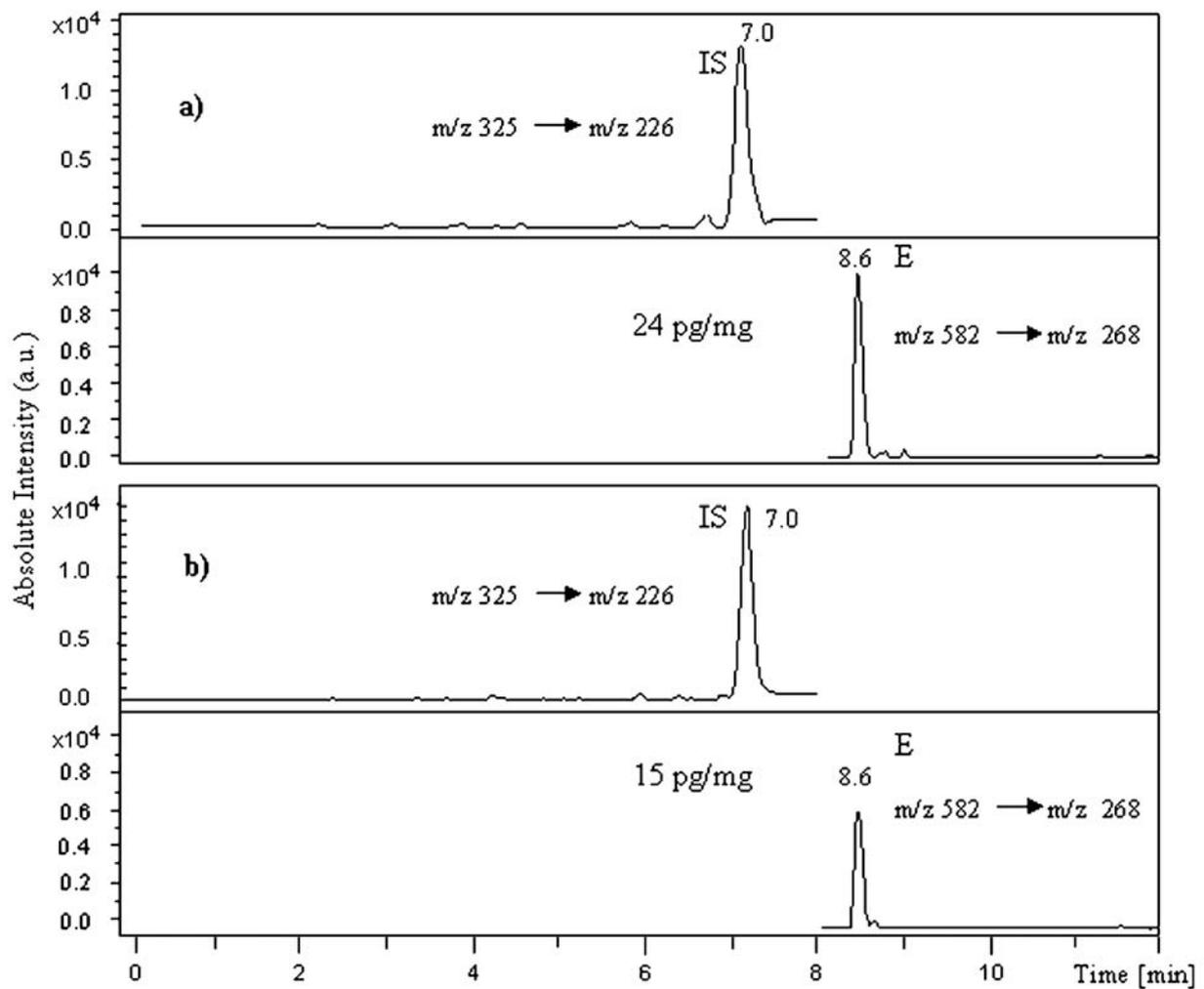


Figure 8. Reconstructed ion chromatograms of the two transitions $m/z\ 327 \rightarrow m/z\ 226$ (IS) and $m/z\ 582 \rightarrow m/z\ 268$ (ergotamine, E) obtained for: a) proximal hair sample and b) distal hair sample of a patient suffering from iatrogenic ergotism (a.u. = arbitrary units).

The method can be used for clinical and toxicological purposes; results from hair testing can be used to assess repeated exposure to ergotamine and possible associations with long-term, drug-induced effects, as in our specific case. Preliminary experiments suggest that it should be possible to extend the method to other body fluids or tissues of interest in forensic toxicological applications.

4. Determination of citalopram, escitalopram and their demethylated metabolites in neonatal hair samples by liquid chromatography - tandem mass spectrometry.

4.1 Citalopram/Escitalopram

Citalopram [(±)-1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile] and escitalopram [S-(+)-1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile] are highly selective serotonin reuptake inhibitors (SSRI) developed for the treatment of depression and anxiety disorders. The S-(+)-enantiomer have a therapeutic activity approximately 30 times more potent than the R-(-) enantiomer¹²². Both citalopram and escitalopram are lipid-soluble and are metabolized in the liver, mainly into demethylated metabolites,^{123,124} which are less powerful *in vitro* than SSRI and less able to penetrate the brain¹²⁵. Both citalopram and escitalopram induce adverse drug reactions and side effects, including tiredness, confusion, dizziness, stomach pain, sweating, nausea, sinus tachycardia and tremor¹²⁶. Exposure to SSRI during pregnancy has been associated with neonatal respiratory distress, cyanotic events, feeding difficulties, hypoglycemia, and a wide spectrum of neurological symptoms¹²⁷. Figure 9 gives the chemical structure of citalopram.

Given its clinical interest, citalopram has been quantified in plasma or serum by HPLC coupled with UV,¹²⁸⁻¹³⁰ DAD¹³¹ or fluorometric detection,¹³²⁻¹³⁵ or gas chromatography – mass spectrometry (GC/MS)¹³⁶. More recently, liquid chromatography with electrospray ionization mass spectrometry or turbulent flow chromatography coupled with tandem mass spectrometry have been used to quantify citalopram¹³⁷⁻¹⁴⁰ and escitalopram¹⁴¹ in human plasma, while GC/MS has been used to assay citalopram in human urine.^{142,143} In the only reported case of citalopram being determined in hair,¹⁴⁴ citalopram and its metabolite desmethylcitalopram, extracted from 50 mg hair by ultrasonication with methanol and solid phase extraction (SPE), were assayed by LC/MS

with in-source collision induced dissociation and, due to the extremely high concentrations (557-1107 ng/mg), were further determined by GC-MS.

Considering the value of documenting gestational exposure to SSRI and the usefulness of hair matrix in revealing exposure to drugs,^{1,145-148} we describe a new sensitive, specific and rapid method validated for the quantification of citalopram, escitalopram and their demethylated metabolites in small amounts (10 mg) of hair. The method entails a rapid extraction from hair after incubation in methanol, a short chromatographic run on a narrow bore achiral column, and tandem MS to enhance specificity.

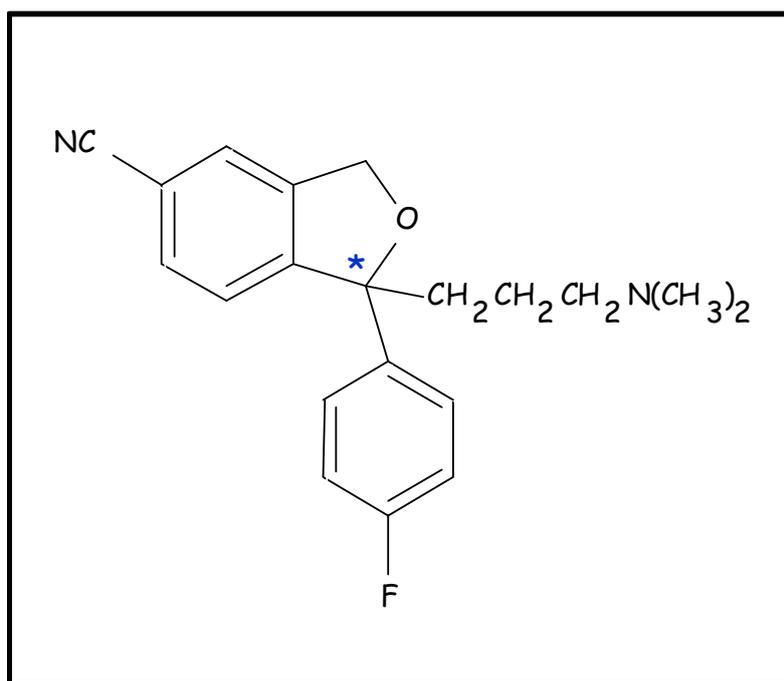


Figure 9. Chemical structure of citalopram.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Citalopram and clomipramine (internal standard, IS) were purchased from Sigma-Aldrich. Escitalopram, desmethylcitalopram and desmethylescitalopram were kind gifts from Lundbeck (Padova, Italy). All chemicals were analytical reagent grade and all solvents were HPLC grade. HPLC water was prepared using a Milli-Q Plus (Millipore, Molsheim, France) system.

4.2.2 Preparation of standard solutions

Primary standard solutions of citalopram, escitalopram, desmethylcitalopram and desmethylescitalopram (200 µg/mL) were prepared in methanol; working standard solutions were prepared daily at concentrations of 1 µg/mL, 100 ng/mL and 10 ng/mL. The IS working solution was prepared in methanol at a concentration of 100 ng/mL from a stock solution of 100 µg/mL.

4.2.3 Sample collection and extraction

Drug-free hair samples were collected from volunteers; specimens from newborns were collected at a Neonatology Critical Care Unit (*Padova University Hospital*).

About 10 mg of hair collected from volunteers or newborns and accurately weighed were cut into small segments and, after adding 10 µL of IS working solution and 2 mL of CH₃OH, they were ultrasonicated for 3 hours. After centrifugation, the supernatant was transferred and dried under a nitrogen stream. The dried residue was dissolved in 1 mL of water and, after adding 200 mg of ammonium carbonate and 5 mL of the diethyl ether/dichloromethane mixture (70:30), liquid/liquid extraction was performed for 15 min, as described by Singh et al.¹⁴³ to determine plasma escitalopram. After centrifugation, the organic layer was removed and dried under a nitrogen stream. The dried residue was then dissolved in 30 µL of solvent A.

In Figure 10 is schematized the sample preparation procedure.

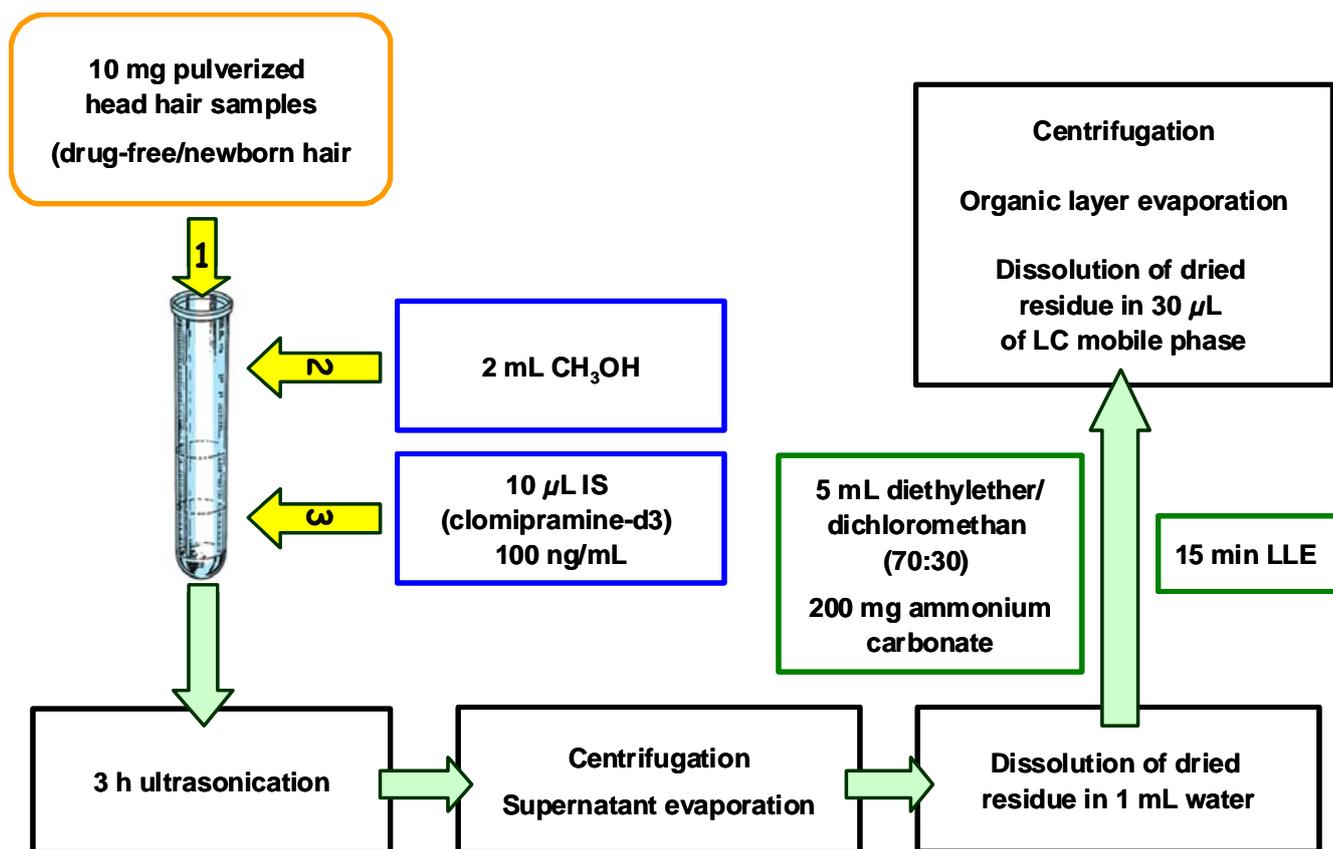


Figure 10. Hair sample preparation for LC-MS analysis: extraction and clean-up procedures.

4.2.4 LC-MS/MS procedure

All measurements were performed on an Agilent 1100 series MSD Trap HPLC-MS system (Agilent Technologies, Palo Alto, CA, USA).

Liquid chromatographic separation was achieved with a Luna C18 (150 x 1 mm, 5 µm) analytical column (Phenomenex, Torrance, CA, USA) with a constant flow rate of 50 µL/min.

The HPLC solvents were solvent A (water, 0.1% formic acid, ammonium formate 2 mM, pH3) and solvent B (acetonitrile, 0.1% formic acid, ammonium formate 2 mM). The mobile phase was programmed as follows: original conditions 20% B, linear gradient to 90% B in 6 min (linear gradient), 90% B hold from 6 to 10 min. Re-equilibration time was 2 min and the injection volume was 8 µL.

The ESI parameters were: capillary voltage -3800 V, nebulizer 25 psi, drying gas 8 L/min and drying temperature 350 °C. For collision-induced dissociation (CID) experiments we used a supplementary rf voltage in the range of 0-1.5 V applied to the end caps of the ion trap.

Data acquisition was performed in product ion scan mode, splitting the acquisition into two stages and scanning product ions of m/z 325 (MH^+ of (es)citalopram) and m/z 311 (MH^+ of the demethylated metabolites) in the range of 250-330 for the first 7 min, and m/z 315-317 (isotopic cluster of MH^+ of IS, clomipramine) in the range of 300-330 for the next 5 min.

The percentage deviation of the relative intensities of product ions was determined by duplicate analysis of six different hair samples spiked with 25 and 50 pg/mg of (es)citalopram, analyzed on two different days ($n = 12$ for each concentration). A percentage deviation $\leq 15\%$ for ions whose relative intensity was $> 10\%$ in the product ion spectrum was set as an acceptance criterion. The product ion at m/z 262 for (es)citalopram and the desmethyl(es)citalopram, and the precursor ion at m/z 315 for clomipramine were used as quantification ions.

4.2.5 Validation protocol

To validate the method, calibration samples were prepared by spiking drug-free hair with appropriate amounts of working standard solutions in concentrations ranging from 25 to 2000 pg/mg. These samples were processed as explained above. Calibration curves were constructed by plotting the peak area ratios of the selected ion species (for the analytes and IS) versus analyte concentration, using six calibration points per curve (25, 50, 100, 200, 500, 2000 pg/mg). Three sets of spiked samples were prepared, each on a different day.

“Spike-after-extraction” samples were prepared by extracting drug-free hair and then adding working solutions of the analyte and IS to the sample extracts. Recovery

(extraction efficiency, %) was determined at two concentrations by comparing the chromatographic peak areas obtained for extracted spiked samples (50 pg/mg, 500 pg/mg) with those obtained for the spike-after-extraction samples at the same concentration. The experiment was repeated 3 times at each concentration. The acceptance range for recovery was set at 70% for both the IS and the analyte.

Intra- and inter-assay precision (as % relative standard deviation, RSD) was assessed by extracting and analyzing 3 replicates of spiked samples (at 50, 200 and 1000 pg/mg) on 4 consecutive days (n=12). Accuracy (as % relative error, RE, i.e. the percentage deviation of the mean result for all analyses from the nominal value of a fortified sample) was determined on the basis of the total data set (n = 12) at the above 3 concentrations.

The limit of detection (LOD) and the lowest limit of quantification (LLOQ) were determined. The LOD was the concentration with a signal-to-noise (S/N) ratio of 3 and the LLOQ was the lowest concentration at which the RE and RSD were $\leq 20\%$. Both parameters were determined empirically by analyzing a series of decreasing concentrations of the drug-fortified hair in multiple replicates (n = 5).

4.3 Results and discussion

4.3.1 MS and MS/MS

To optimize the experimental conditions for collisionally induced dissociation (CID) on MH^+ of citalopram, a methanol solution of citalopram at a concentration of 1 $\mu\text{g/mL}$ was infused directly into the ESI source, while CID experiments were conducted by varying the supplementary rf voltage in the range 0-1.5 V. Figure 11 shows the MS/MS spectrum obtained for citalopram with a supplementary rf voltage of 0.8 V and the proposed fragmentation pathways. The most abundant fragment ion is produced at m/z 262, while less abundant ions are produced at m/z 307 by the loss of water from the protonated

molecule, and at m/z 280 by loss of the end-of-chain amine. The ion at m/z 262 originates by $\text{NH}(\text{CH}_3)_2$ loss from the ion at m/z 307. These results are consistent with the fragmentation pattern proposed by Smyth et al.¹⁴⁹ on the basis of IT and Q-TOF mass spectrometric experiments. The MS/MS spectrum of MH^+ of escitalopram obtained under the same conditions completely overlaps the one for citalopram: the two isomers have the same retention time in achiral chromatography (see below) and are indistinguishable from their MS/MS spectrum. However, because identifying drugs in neonatal hair is used to corroborate maternal self-reports or clinical history and to document the prenatal exposure to SSRI, no qualitative differentiation between the racemate (citalopram) and the S (+)-enantiomer (escitalopram) was envisaged. Vice versa, their analytical discrimination would unquestionably be necessary when blood concentrations of those two differently potent drugs are to be discussed.¹⁵⁰

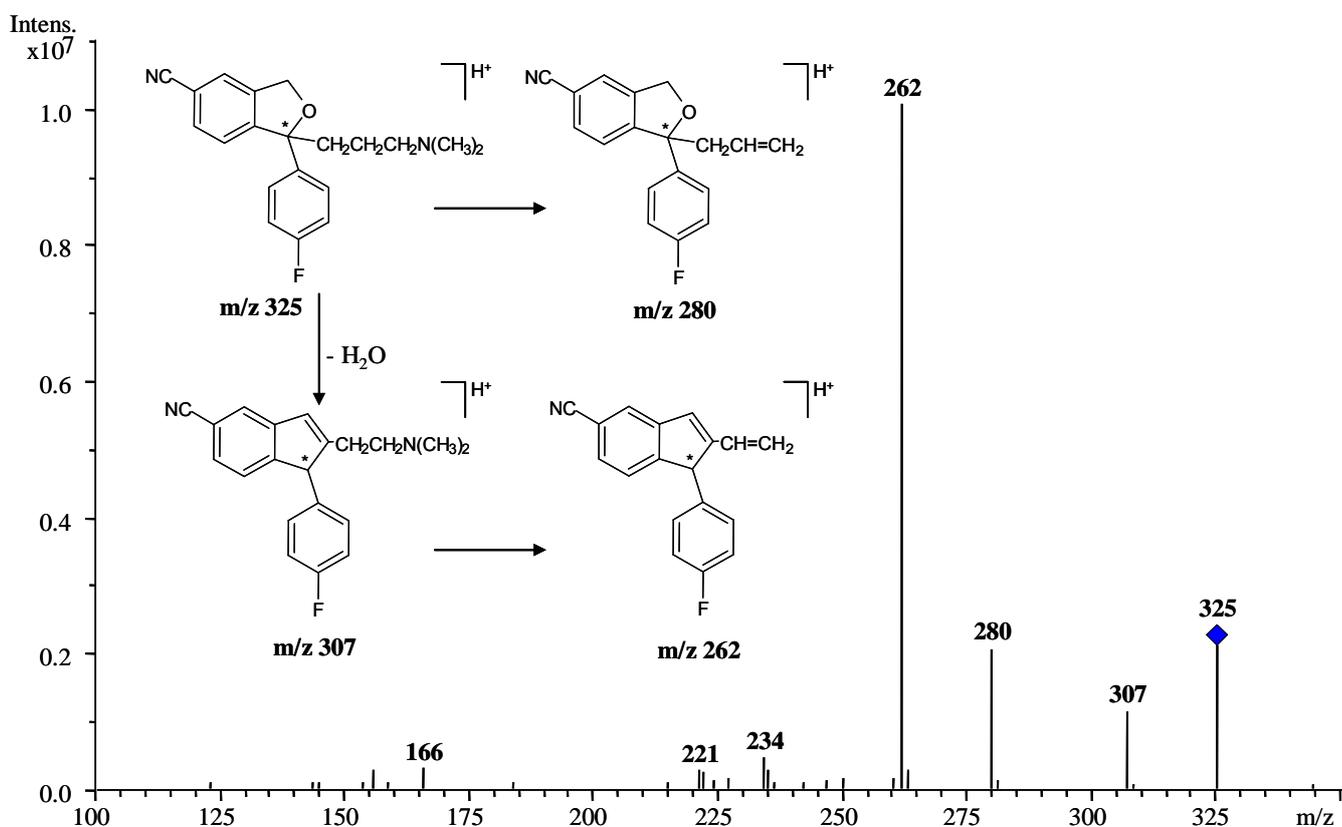


Figure 11. MS/MS spectrum obtained for citalopram with a supplementary rf voltage of 0.8 V and the proposed fragmentation pathway.

The MS/MS spectrum of MH^+ of desmethylcitalopram (m/z 311), reported in Figure 12, shows its most abundant product ion at m/z 262, while less abundant species are observed at m/z 293 and m/z 280 (as seen for citalopram) by the loss of water and of the end-of-chain amine from the protonated molecule. As respectively observed for the two parent drugs, the MS/MS spectra of desmethylcitalopram and desmethylescitalopram are identical, thus hindering their differentiation.

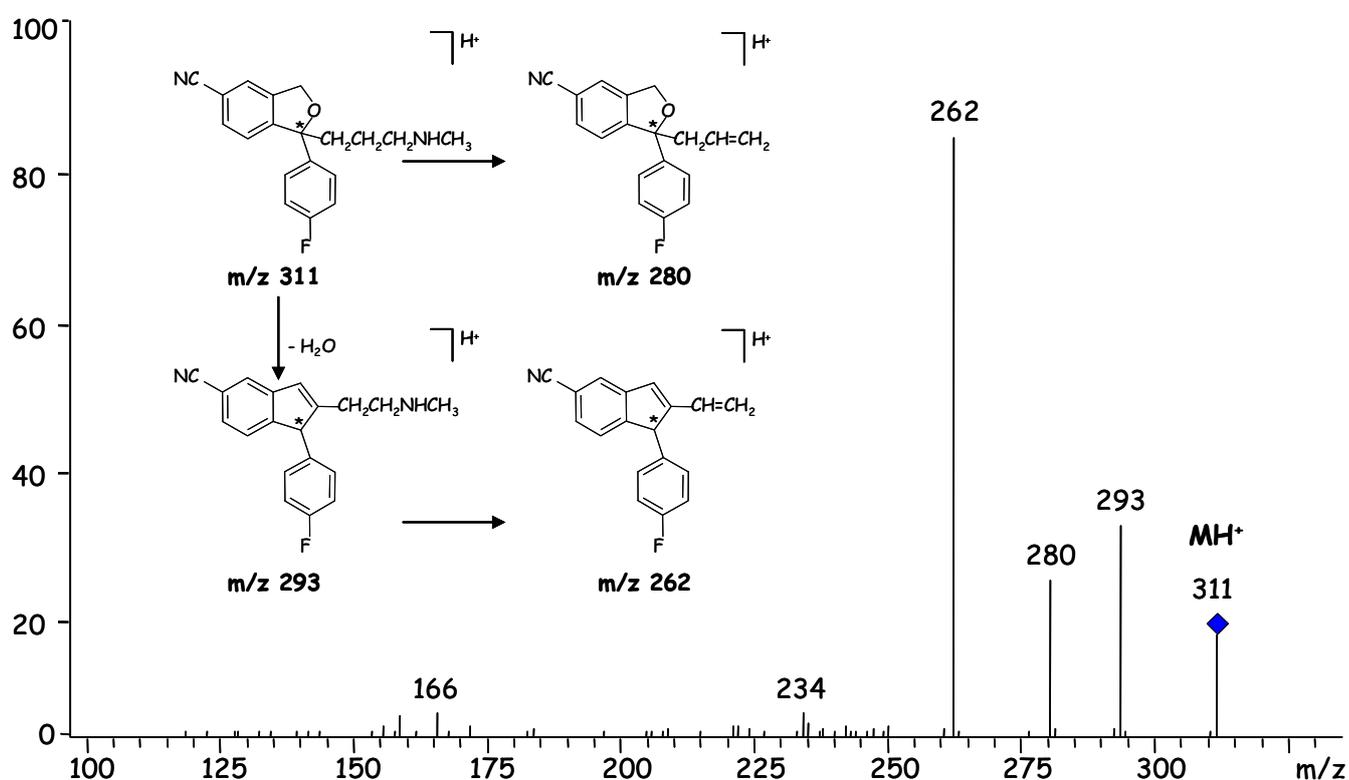


Figure 12. MS/MS spectrum obtained for desmethylcitalopram with a supplementary rf voltage of 0.8 V and the proposed fragmentation pathway.

As for the IS, clomipramine, Figure 13 shows the MS/MS spectrum of its MH^+ ions (m/z 315) obtained at the fragmentation amplitude of 1.00 V. A scarce fragmentation yield is obtained; the two fragment ions at m/z 270 and 242 can be attributed to the cleavage of

the aminoalkyl chain and served as key ions for data interpretation, while the surviving precursor ion at m/z 315 was chosen for quantification.

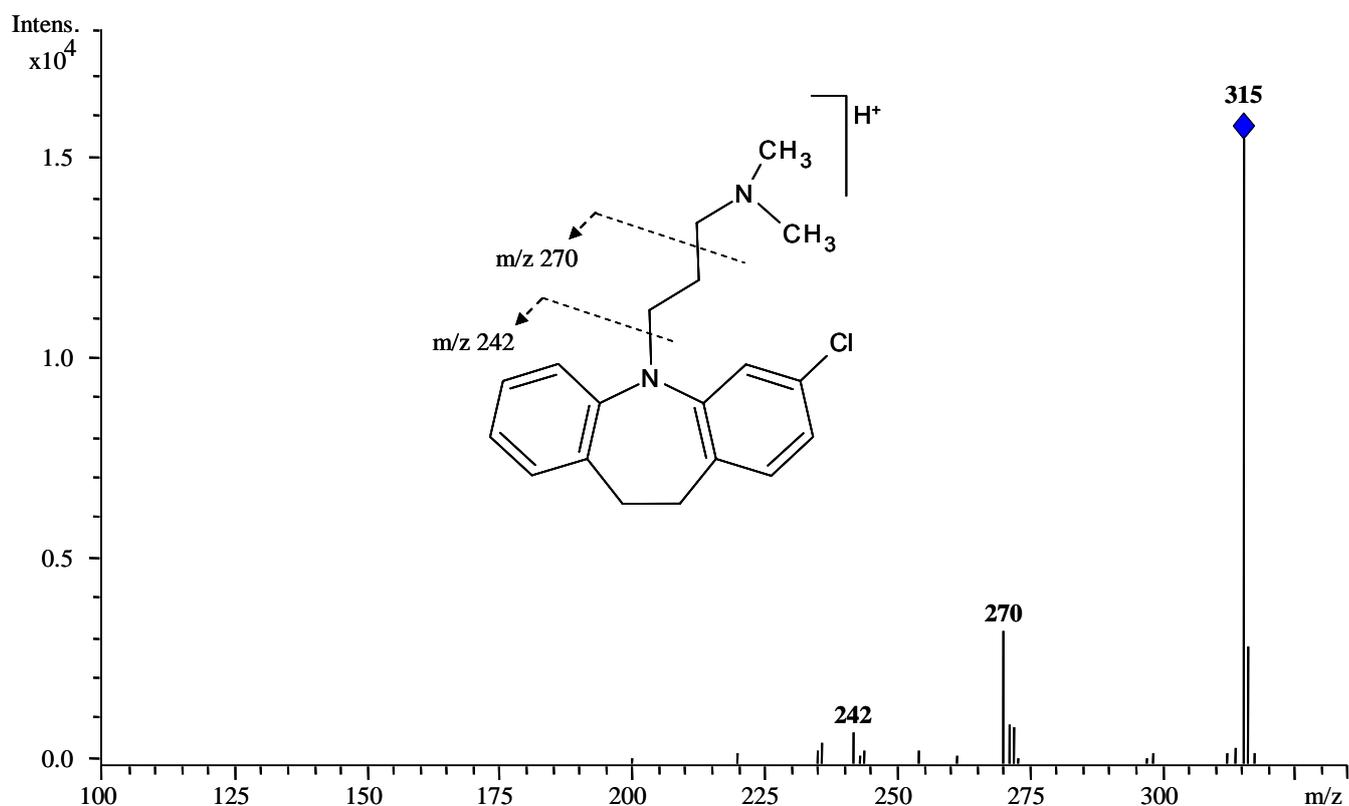


Figure 13. MS/MS spectrum of MH^+ ions (m/z 315) of clomipramine (IS) obtained at the fragmentation amplitude of 1.00 V.

4.3.2 LC- MS/MS

Having established the best MS/MS conditions, chromatographic separation was optimized by injecting pure standard solutions. The column used was a Luna C18 (150 x 1 mm, 5 μ m). Retention times were 6.7 min for citalopram and escitalopram, 6.6 for desmethylcitalopram and desmethylescitalopram, and 7.4 min for IS. As mentioned earlier, escitalopram is the S-(+)-enantiomer of citalopram, and the chromatographic characteristics of the two compounds in an achiral chromatography are identical, so the same method can be used for their quantitative determination as long as no qualitative differentiation between the racemate and the enantiomer is needed. The parent drug is identified in product ion mode by 4 ions at m/z 325, 307, 280, 262 with a relative

abundance > 10%. Similarly, the MS/MS spectrum of the metabolite presents 4 ions at m/z 311, 293, 280, 262 with a relative abundance > 10%. Spiked hair samples were then run in LC-MS/MS conditions, as described above.

4.3.3 Validation results

No interferences were detected during the analysis of hair from 6 different sources, processed as described in the experimental section without supplementing the analytes or the IS. Peak detection based on a single combination of retention time, precursor ion and product ion spectrum failed to detect any endogenous interferences at the analyte and IS retention times. Figure 13 shows the selectivity with the chromatograms of: a) a blank hair sample; b) a spiked sample at 25 pg/mg (LLOQ); c) a spiked hair sample at 500 pg/mg.

The responses for (es)citalopram and desmethyl(es)citalopram in hair were linear in the 25 - 2000 pg/mg range ($r^2 = 0.997$ and 0.998 , respectively) using a weighting factor of $1/x$.

The recoveries determined for (es)citalopram and desmethyl(es)citalopram in hair ranged from 75% at 50 pg/mg to 82% at 500 pg/mg and met the acceptance criteria. The proposed extraction procedures for determining citalopram in body fluids consist mainly of solid phase extraction (SPE) on different sorbents, or liquid/liquid extraction with various solvents. A mixed-mode SPE cartridge was used in the only report on citalopram extraction from hair¹⁴⁴. In our experiments, liquid/liquid extraction seemed to be the simplest and least expensive procedure, considering the high specificity of the LC-MS/MS procedure, which balances any partial purification of the analyte from the matrix. The solvents were chosen on the strength of previously-published results¹⁴⁴ to give the best recoveries and the shortest evaporation times.

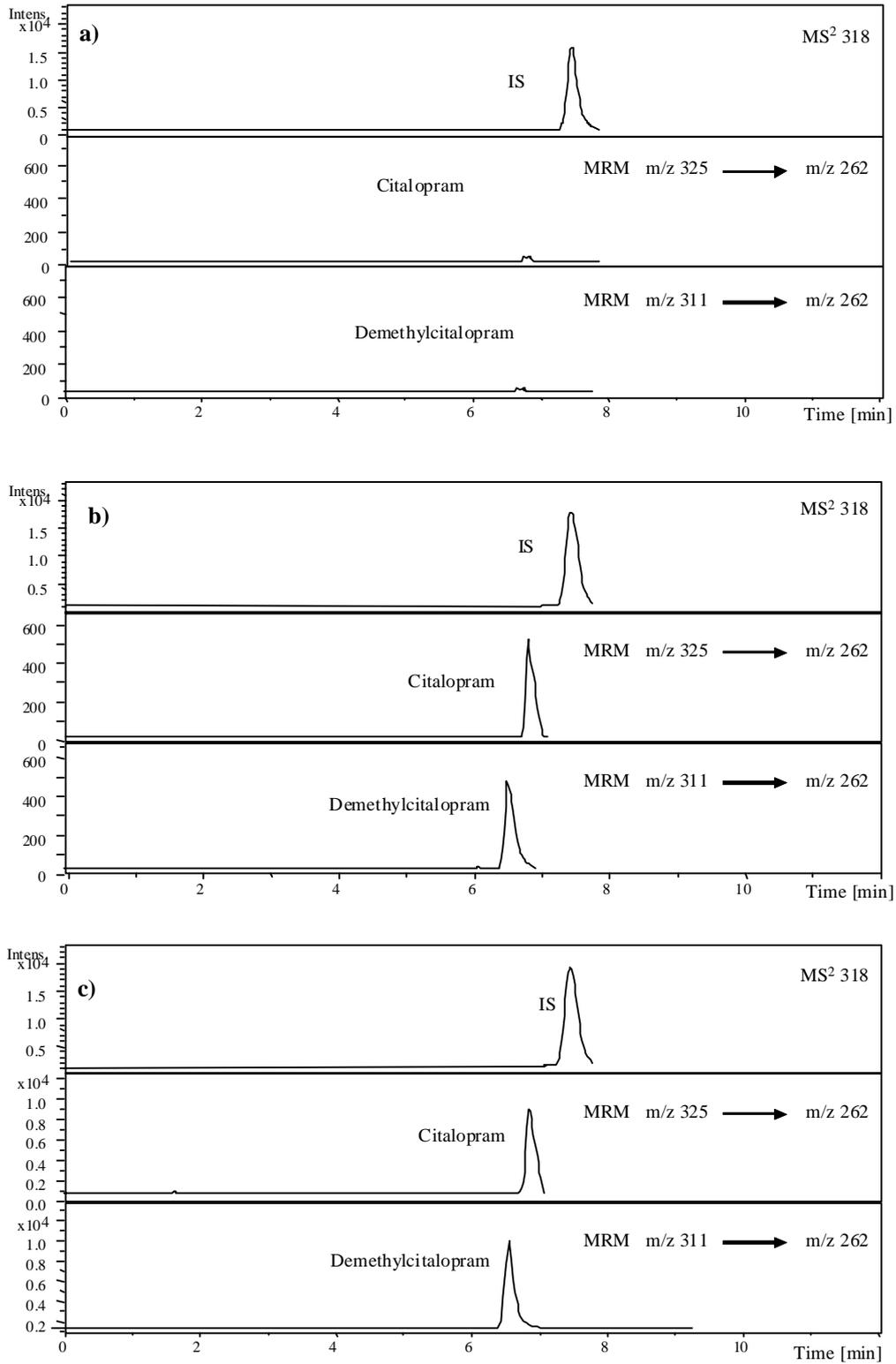


Figure 14. Reconstructed ion chromatograms obtained for a) a blank hair sample; b) a spiked sample at 25 pg/mg (LLOQ); c) a spiked hair sample at 500 pg/mg.

As far as ion suppression or enhancement, the peak areas measured from the spike-after-extraction samples were between 92 and 105% of those obtained from the neat assays of standard solutions, so the matrix effect was judged to be negligible.

Intra-assay precision (% RSD) for citalopram and its metabolite ranged from 12.10 at 50 pg/mg to 9.80 at 1000 pg/mg; inter-assay precision ranged from 13.80 at 50 pg/mg to 11.78 at 1000 pg/mg. The accuracy (% RE) for 3 fortified levels was always $\leq 10\%$. The validation results are summarized in Table 2.

The LOD and LLOQ, determined empirically by analyzing a series of decreasing concentrations of the drug-fortified samples, were respectively 10 pg/mg and 25 pg/mg for both (es)citalopram and its metabolite.

4.3.4 Application to real cases

The validated analytical method was used to analyze hair samples obtained from two cases of prenatal exposure to citalopram (case 1) and escitalopram (case 2).

Hair samples, collected from two newborns exhibiting SSRI exposure symptoms, were extracted and analyzed as described in the Materials and Methods. The analytes were detected in both cases and the concentrations obtained were: 250 pg/mg of citalopram in case 1 and 254 pg/mg of escitalopram in case 2. The desmethylated metabolite was also detected in both specimens, in lower concentrations than the parent drugs. The concentrations of the metabolite were: 24 pg/mg in case 1 and 121 pg/mg in case 2.

Figure 15 shows the reconstructed ion chromatograms obtained: (a) for citalopram and desmethylcitalopram in hair samples from case 1; and (b) for escitalopram and desmethylescitalopram in hair samples from case 2.

	Accuracy* Mean Value (range)			Percent Coefficient of Variation					
				Intraassay			Interassay		
	50 pg/mg	200 pg/mg	1000 pg/mg	50 pg/mg	200 pg/mg	1000 pg/mg	50 pg/mg	200 pg/mg	1000 pg/mg
Citalopram	90 (86–92; n = 12)	95 (90–102; n = 12)	104.1 (103–105; n = 12)	12.10	10.45	10.06	13.80	12.20	11.98
Escitalopram	90 (85–93; n = 12)	104.9 (98–107; n = 12)	104.1 (102–104; n = 12)	12.06	10.40	10.11	13.77	12.23	12.01
Demethylcitalopram	90.2 (86–98; n = 12)	95.5 (94–103; n = 12)	95.9 (92.8–102; n = 12)	11.50	10.86	9.80	12.50	11.44	11.78
Demethylescitalopram	109.8 (100–112; n = 12)	95.5 (96–105; n = 12)	95.9 (93–106; n = 12)	11.53	10.89	9.85	12.46	11.46	11.81

*Percent recovery of the spiked standard; n, number of replicates.

Table 2. Validation results.

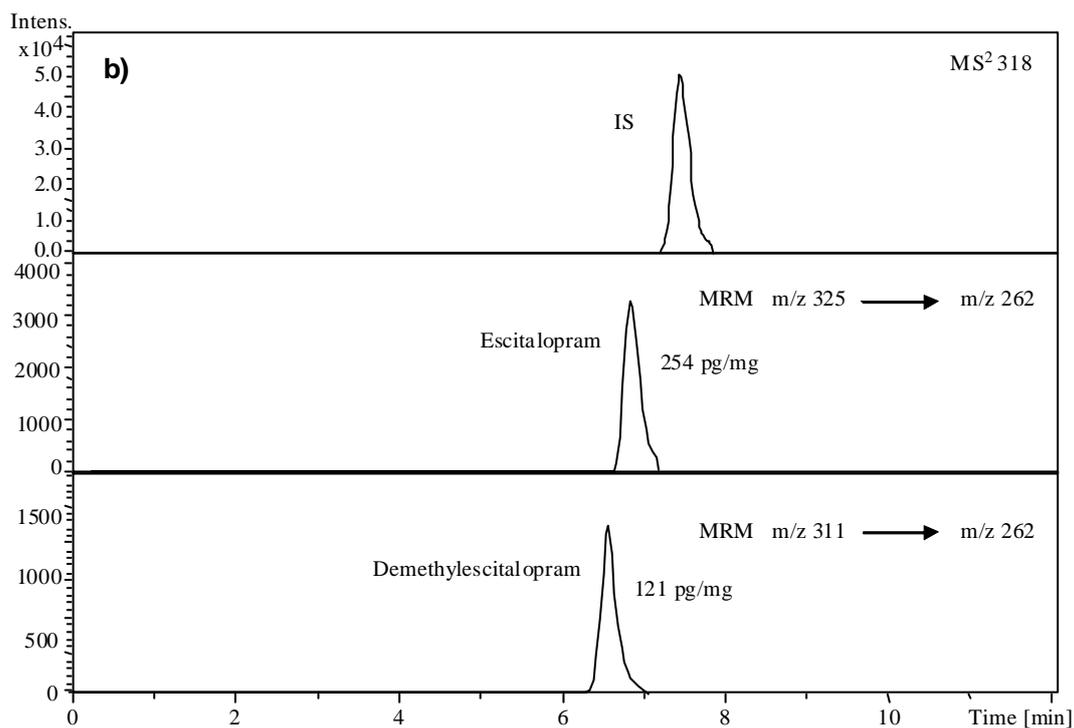
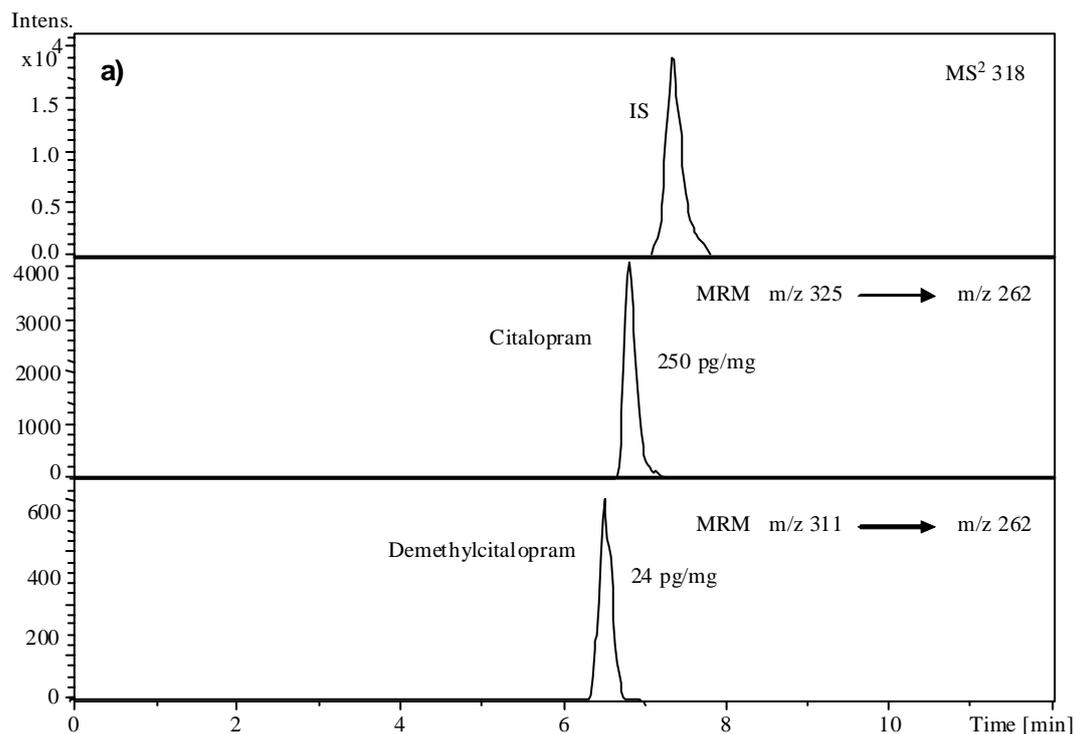


Figure 15. Reconstructed ion chromatograms obtained: (a) for citalopram and desmethylcitalopram in hair samples from case 1; and (b) for escitalopram and desmethylescitalopram in hair samples from case 2.

5. A fast screening MALDI method for the detection of cocaine and its metabolites in hair.

5.1 Cocaine in hair

Cocaine is a powerfully addictive stimulant that directly affects the brain and it is one of the oldest known and more widely used drugs of abuse. It is mainly available in two chemical forms: the hydrochloride salt, a white crystalline powder, that dissolves in water and can be taken intravenously or intranasally, and the freebase ("crack"), a compound that has not been neutralized by acid to make the hydrochloride salt, that is smokable.

It is rapidly inactivated in blood by hydrolysis of the ester linkages to benzoylecgonine and ecgonine methyl ester, the two major metabolites, which are inactive but very useful in detection of cocaine abuse due to their longer half-lives in biological matrices (about five times longer than cocaine). To a lesser extent cocaine is hydrolyzed to norcocaine, an active metabolite, or, by presence of ethanol in the blood, to the toxic cocaethylene.^{151,152} However, it must be taken into account that the hydrolytic metabolites, benzoylecgonine and ecgonine methylester, could also be formed from cocaine outside the body. Only norcocaine and cocaethylene are unambiguously endogenous metabolites. It is therefore recommended for assessment of the severity of cocaine consumption to measure cocaine and both its inactive and active metabolites.

Nowadays, hair analysis is routinely used as a powerful tool for the detection of drug use, not only in forensic science but also in clinical toxicology or in traffic medicine. The widespread use of this kind of analysis is surely related to the progress in separation techniques and the increased sensitivity and selectivity of analytical instrumentation, which allows the detection of low drug concentrations in hair. Nakahara and Kikura¹⁵³ examined in 1994 the incorporation of cocaine, benzoylecgonine, and ecgonine methyl ester into the hair of treated Dark Agouti rats. They determined that cocaine was the major analyte

detected in hair despite higher plasma concentrations of benzoylecgonine; the analyte ratio of cocaine:benzoylecgonine:ecgonine methyl ester was found to be 20:2:1. After administering deuterated metabolites to rats, they also demonstrated that benzoylecgonine present in hair came primarily from the hydrolysis of incorporated cocaine. Very little incorporated cocaine was converted to ecgonine methyl ester in the hair, and the hair ecgonine methyl ester concentrations were very low. They concluded that the incorporation of cocaine from plasma into hair was much greater than that of either benzoylecgonine or ecgonine methylester.

A large number of hair analysis studies have dealt with cocaine (COC) and its metabolites. The molecular structures of these compounds are reported in Figure 16. Different procedures concerning the pre-treatment of hair samples, extraction of analytes from the matrix, isolation from the incubation media, and the analytical techniques have been proposed^{1,154}. Gas chromatography - mass spectrometry (GC-MS) in electron ionization (EI) mode is the most commonly employed analytical technique, however in recent years, chemical ionization (CI) GC-MS and GC- multiple mass spectrometry (GC-MSⁿ) have also been proposed.¹⁵⁵ Moreover, several liquid chromatography - multiple mass spectrometry (LC-MSⁿ) applications for cocaine detection in hair have also been published.¹⁵⁶⁻¹⁵⁹

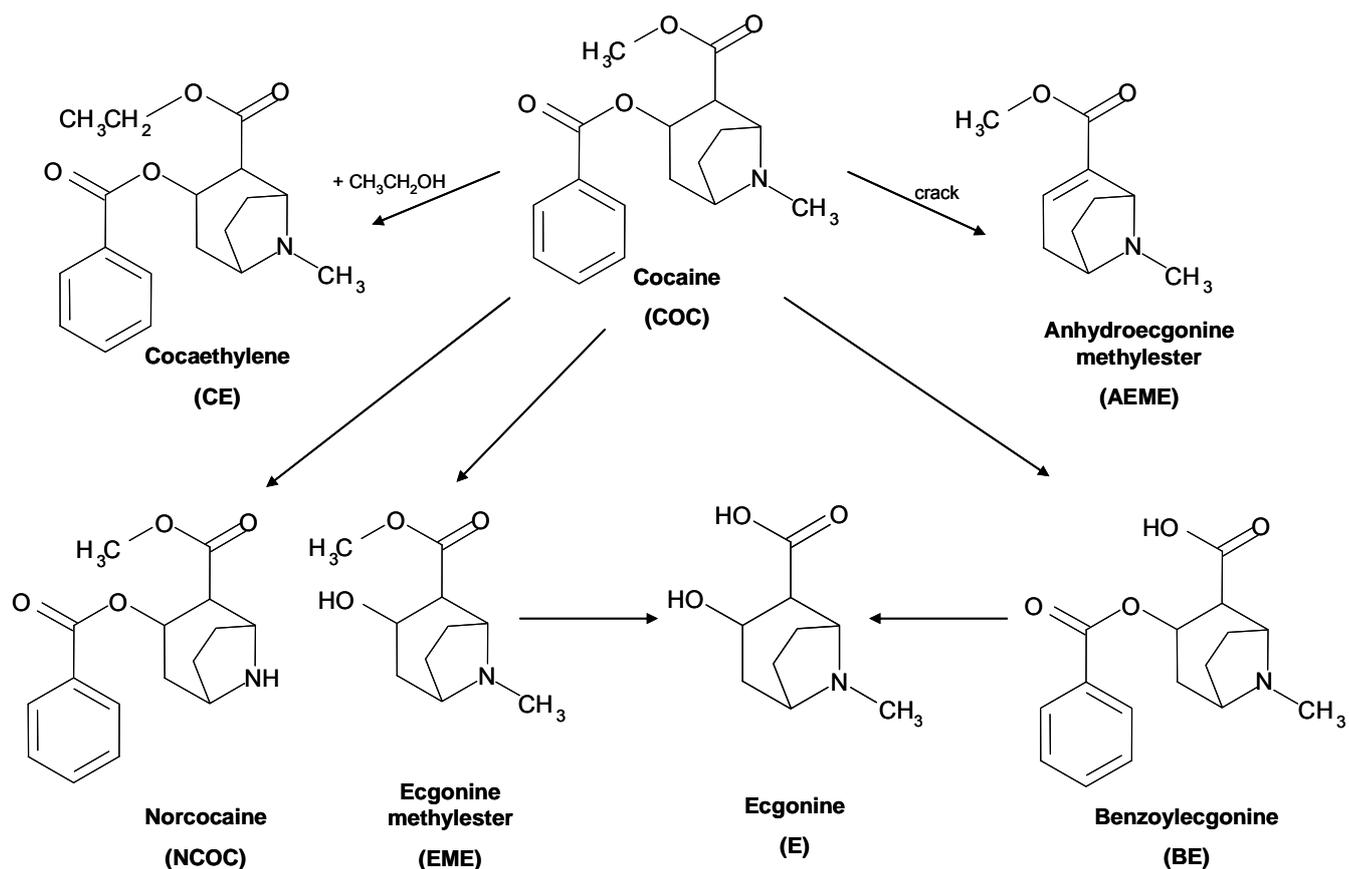


Figure 16. Molecular structures of cocaine and its metabolites.

All of the reported analytical approaches for hair analysis require lengthy extraction steps and chromatographic separations. Thus, considering the increasing abuse of COC, it has become important nowadays to adopt simple and fast screening methods to detect COC and its metabolites in a high number of hair samples. These methods are not necessarily expected to provide quantitative data, however, they should have detection limits sufficient to allow the initial discrimination between negative and positive samples, which should then be confirmed and quantified by classical chromatographic–mass spectrometric methods. Searching the literature, matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) seems to be a very attractive technique to obtain a fast screening method for COC. In fact, it has been successfully applied for analysis of COC in urine, and the results obtained by secondary ion mass spectrometry (SIMS) and MALDI-MS have been compared¹⁶⁰; Nicola et al. have shown the

analytical power of this approach by developing a direct quantitative analysis from thin-layer chromatography obtaining a precision better than 12% and detection limits in the order of 60 pg.¹⁶¹ More recently, MALDI-MS and MALDI-MS/MS were employed for the analysis of COC, directly in rat brain tissue sections.¹⁶² The use of MALDI for analysis of small molecules^{163,164} has even resulted in a dedicated commercial product (FlashQuant Workstation, Applied Biosystems, Forster City, CA, USA). Given several characteristics such as the soft ionisation, the privileged production of molecular species, better tolerance with respect to electrospray ionisation (ESI)-MS to interference from salts and buffers and the simplicity of sample preparation, MALDI-MS is ideally suited for simultaneous, rapid and high throughput analyses of complex mixtures. Therefore, we believed this technique could be applied effectively for the analysis of hair samples for clinical and forensic toxicology purposes. We report here the results obtained in the development of a fast screening method to detect COC and its metabolites benzoylecgonine (BE) and cocaethylene (CE) in hair samples, based on MALDI-MS analysis of powdered hair sample extracts. We performed several experiments, initially by working on standard solutions of the analytes under investigation and subsequently on hair samples that had been found negative or positive by a GC-MS reference method. The initial experiments were conducted to evaluate the outcome of various types of MALDI (and laser desorption/ionisation, LDI) sample preparations and to set the working parameters of the instrument, in order to produce the most reliable results in terms of reproducibility and detection limits.

5.2 MALDI-MS

Matrix-assisted laser desorption/ionization (MALDI)¹⁶⁵ is a “soft” ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as proteins, peptides and sugars) and large organic molecules (such as polymers,

dendrimers and other macromolecules), which tend to be fragile and fragment when ionized by more conventional ionization methods. This technique consists of the interaction of a laser beam with a solid sample constituted by a suitable matrix in which the analyte is present at very low molar ratio (1:10000). This interaction leads to the vaporization of a small volume of the solid sample: in the plume of the high density vapour so generated, reactive species originating from the matrix irradiation react with the neutral molecules of analyte, mainly through protonation/deprotonation mechanisms (Figure 17). A detailed description of the MALDI mechanism is highly complex, due to the presence of many different phenomena:

(i) The matrix is relevant to obtain effective and well reproducible data;

(ii) the solid sample preparation is usually achieved by the deposition on a metallic surface of the solution of matrix and analyte with concentration suitable to obtain the desired analyte/matrix ratio. The solution is left to dry under different conditions (simply at atmospheric pressure, reduced pressure or under nitrogen stream); in all cases what is observed is the formation of an inhomogeneous solid sample, due to the different crystallization rate of the matrix and analyte. Consequently, the 1:10000 ratio is only a theoretical datum: in the solid sample different ratios will be found in different positions and the only way to overcome this is to average a high number of spectra corresponding to laser irradiation of different points;

(iii) the photon–phonon transformation, obtained when a photon interacts with a crystal and giving information on the vibrational levels of the crystal lattice, cannot be applied in the laser induced vaporization observed in MALDI experiments, due to the inhomogeneity of the solid sample;

(iv) the laser irradiance is an important parameter: different irradiance values lead to vapour cloud of different density and consequently different ion–molecule reactions can take place. In other words the MALDI data originate from a series of physical phenomena

and chemical interactions originating by the parameterization (matrix nature, analyte nature, matrix/analyte molar ratio, laser irradiation value, averaging of different single spectra), which must be kept under control as much as possible. However, the results obtained by MALDI are of high interest, due to its applicability in fields not covered by other ionization methods. Due to the pulsed nature of ionization phenomena (an N₂ laser operating with pulses of 102 ns and with a repetition rate of 5 MHz) the analyser usually employed to obtain the MALDI spectrum is the time-of-flight (TOF).

Typical MALDI matrices are carboxylic acids variously substituted (nicotinic, sinapinic, picolinic acid).

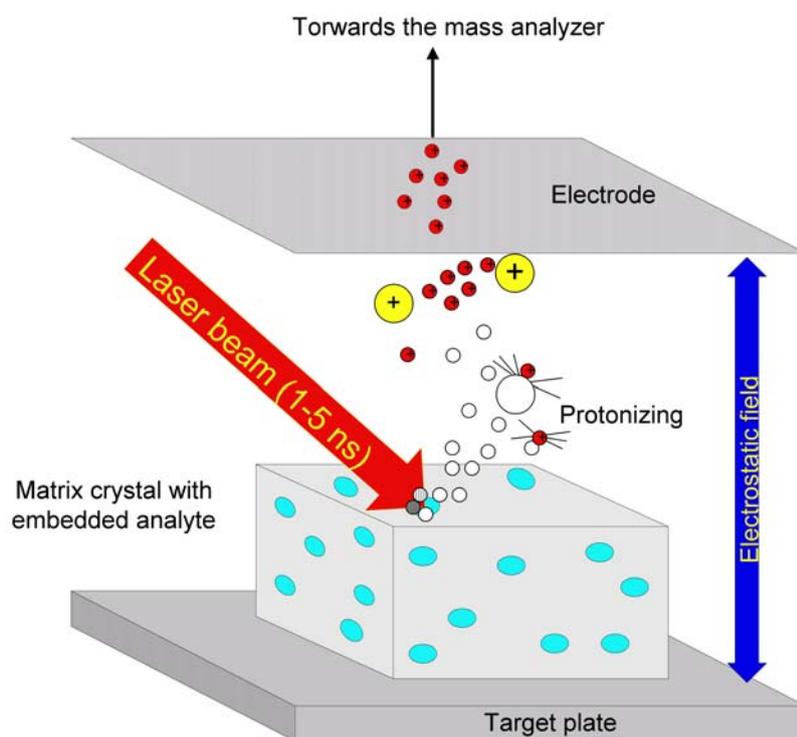


Figure 17. Interaction of a laser beam with a solid sample constituted by a matrix crystal in which the analyte is embedded; the reactive species originating for the matrix irradiation react with the neutral molecules of analyte, mainly through protonation/deprotonation mechanisms.

5.3 Materials and methods

5.3.1 Chemicals and reagents

Standard methanolic solutions of COC, BE and CE at a concentration of 1 mg/mL were purchased from LCG Promochem (Milan, Italy). Working solutions of the three analytes were prepared in methanol at a concentration of 3.3×10^{-6} M (working solution 1) and 3.3×10^{-7} M (working solution 2) for each analyte, corresponding approximately to 1 µg/mL and 0,1 µg/mL respectively.

Analytical-grade acetone, methanol, *n*-hexane, sodium dodecyl sulphate (SDS) and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany). BondElut Certify columns (130 mg/10 mL) for solid phase extraction (SPE) were purchased from Varian (Palo Alto, CA). LC water was provided by a Milli-Q Plus system (Millipore, Molsheim, France).

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) and scopolamine were obtained from Sigma-Aldrich (Milan, Italy).

MALDI matrices 2,5-dihydroxybenzoic acid (DHB), sinapinic acid (SA) and α -cyano-4-hydroxycinnamic acid (HCCA) were obtained from LBL (LaserBioLabs, France).

The hair samples used in this study were originally collected and analyzed for clinical and forensic toxicology purposes at the Forensic Toxicology and Antidoping Unit of the University Hospital of Padova. Control drug-free hair samples were collected from the laboratory personnel.

5.3.2 Sample preparation for GC-MS analysis

Hair samples were decontaminated by washing whole strands in 10 mL of a 10% (w/v) aqueous solution of SDS, followed by washes in 10 mL of water (twice) and 10 mL of acetone. After washing, hair samples were pulverized in a ball mill. 50 mg aliquots were added to 30 ng of scopolamine as an internal standard and 3 mL of a methanol/TFA (9:1) (v/v) solution, ultrasonicated for 60 min, and incubated overnight at 45° C. After

centrifugation, the obtained methanolic solutions were dried under a gentle stream of nitrogen at 35° C, and the residues were reconstituted in 3 mL of a phosphate buffer solution 0.1 M pH 7. SPE was performed with BondElut Certify columns. Eluates were evaporated to dryness and derivatized was accomplished by adding to the residues 50 µL of MSTFA with 1% TMCS, at 75° C for 20 min. Calibration curves for COC, BE, and CE were obtained by using drug-free hair samples spiked with the appropriate amounts of standard analyte solutions.

5.3.3 GC-MS analyses

GC/MS analyses were performed using an Agilent 6890N series II gas chromatograph interfaced with an Agilent 5973 series quadrupole mass spectrometer (Palo Alto, CA, USA) and equipped with an Agilent 7673A autosampler. Analyses were performed under EI conditions with selected ion monitoring (SIM) acquisition modes. The injection (2 µL) was performed in splitless mode at 275° C. We used a Hewlett-Packard HP-Ultra 1 100% methylsilicone capillary column (12 m, 0.2 mm ID, 0.33 µm film thickness) with an oven temperature program set to increase from 50° C (0.5 min) to 300° C at a rate of 20° C/min (20 min) and a carrier gas (He) at a constant flow of 1 mL/min. The interface temperature was 280° C and the electron multiplier was set at 3000 V.

5.3.4 Sample preparation for LDI/MALDI experiments

Hair samples were decontaminated by washing whole strands in 2 mL of water, followed by washes in 2 mL of acetone and 2 mL of *n*-hexane. After drying they were pulverized by means of a ball mill. 1-mg aliquots were suspended in 40 µL of either different CH₃OH/TFA mixtures (70:30, 50:50, 30:70, v/v) or 100% TFA. Following centrifugation, 1-µL aliquots of the obtained solutions were deposited on the MALDI stainless steel sample holder (Figure 18) that was treated as described in the following section.

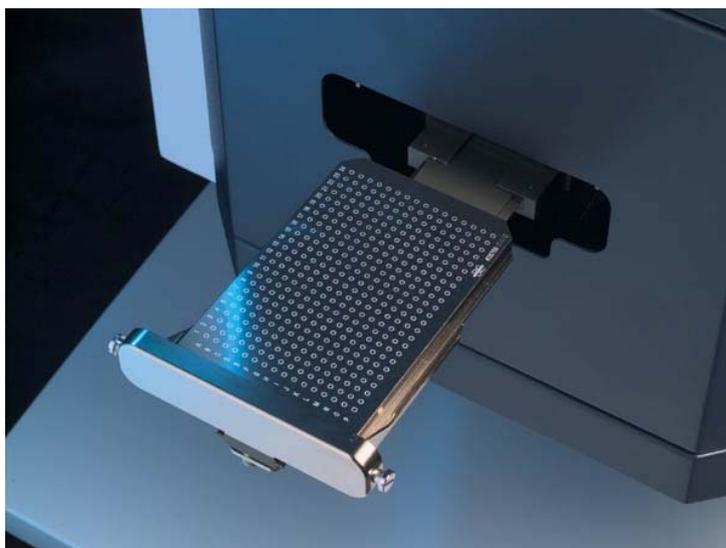


Figure 18. MALDI stainless steel sample holder.

5.3.5 LDI/MALDI experiments

LDI/MALDI analyses were performed using a Bruker Ultraflex II instrument (Bruker Daltonics, Bremen, Germany), operating in a reflectron positive ion mode (Figure 19). The pulsed ion extraction (PIE) conditions were as follows: IS1: 25 kV; IS2: 21.70 kV; reflectron: 26.30 kV; delay time: 150 ns. External mass calibration was performed on the basis of $[M+H]^+$ and $[2M+H]^+$ ions of HCCA at m/z 190.0498 and 379.0924, respectively. The ion selection and mass calibration for the post-source decay (PSD) experiments were performed using the FAST[®] method.

1 μ L of an acetonitrile saturated solution of HCCA or SA or DHB was deposited on the metal sample holder of the instrument and subjected to laser irradiation in order to identify the best matrix composition to be used in the subsequent experiments. Subsequently, various experiments were initially performed on working standard solutions and then on hair samples under different instrumental parameters and types of MALDI sample preparation.

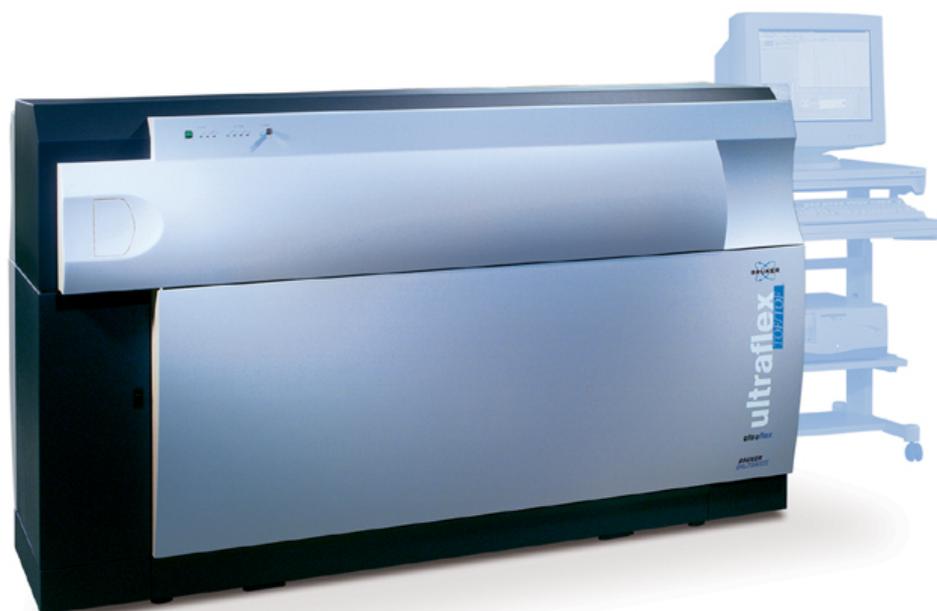


Figure 19. MALDI-TOF Ultraflex II instrument (Bruker Daltonics, Bremen, Germany).

LDI experiments were performed on 1 μL aliquots of the working standard solution 1; classical MALDI experiments were obtained by depositing 1 μL of solution 1 mixed with 1 μL of saturated acetonitrile solution of SA, DHB or HCCA; 'multi-layer'¹⁶⁶ deposition was obtained by depositing the HCCA solution on the sample holder plate, allowing it to dry; then spotting solution 1 as an overlayer, allowing it to dry and depositing another layer of the matrix solution. For experiments with graphite as a matrix/co-matrix, the sample holder surface was treated with graphite derived from pencils of different hardness and solution 1 was deposited on it as an overlayer and allowed to dry; on the sample holder treated with graphite from pencil B, a multi-layer deposition was realised by spotting solution 1 as an overlayer, allowing it to dry, and dropwise depositing or electrospraying the HCCA matrix solution. When HCCA matrix solution was electrosprayed, different spray flow rates (30, 20, 10 and 5 $\mu\text{L}/\text{min}$) and spray times (8, 6, 4, 2 and 1 min) were tested.

The procedures employed for hair samples are herein detailed and summarised in Figure 20.

1. LDI experiments were performed on a 4-cm segment of a single hair from a positive hair sample (sample B, see below), which was fixed on a conductive glass sample holder, usually employed for MALDI-MS ion imaging experiments (step a of Figure 20).
2. MALDI experiments were performed on another 4 cm segment of a single hair from the same positive hair sample, which was fixed on the conductive glass sample holder and spotted with water, 0.1% TFA and HCCA matrix (step b of Figure 20).
3. The sample holder surface was treated with graphite from pencil B, followed by deposition of the powdered hair samples (about 0.2 mg of sample A and B) and spotting with HCCA matrix drops (step c of Figure 20).
4. The sample holder surface was treated with graphite from pencil B. Then powdered hair samples (about 0.2 mg) were deposited on it and HCCA matrix solution was electrosprayed (step d of Figure 20).
5. Finally, 1 mg aliquots of powdered hair samples were suspended in 40 μL of different $\text{CH}_3\text{OH}/\text{TFA}$ mixtures (70 : 30, 50 : 50, 30 : 70, v/v) or 100% TFA. Extraction was carried out at 45 °C over different time intervals (15, 30, 120 and 300 min). Measures of 1 μL aliquots of the obtained solutions were deposited on the sample holder surface treated with graphite from pencil B and let to dry on air; then the HCCA matrix solution was sprayed over (step e of Figure 20).

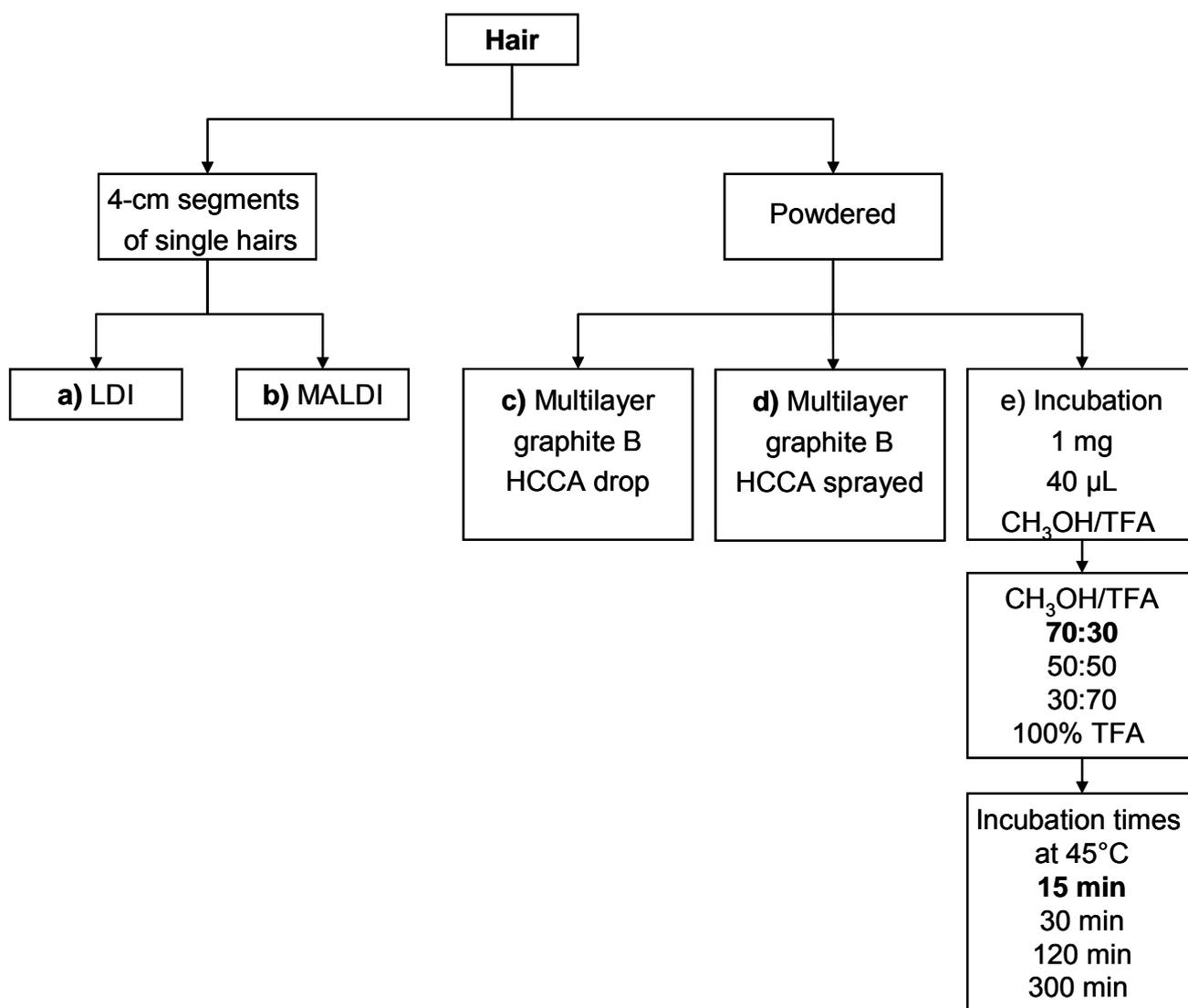


Figure 20. Flow chart of the experimental approaches employed for the analyses of hair samples.

5.4 Results and discussion

5.4.1 *Standard solution analyses*

The first step in the development of a screening method for the detection of COC and its metabolites BE and CE in hair was the evaluation of different LDI and MALDI sample preparation methods that produced the most reliable results in terms of either reproducibility or detection limits. For this purpose, we initially used a methanolic mixture of COC, BE and CE (working standard solution 1) in a molar ratio 1 : 1 : 1. This solution

was first analysed under LDI conditions (1 ng of each analyte were deposited), but the very low intensity of ions corresponding to protonated COC, BE and CE suggested the usefulness of a matrix. To this aim, three different matrices were tested: SA, DHB, and HCCA; the last was chosen due to its lower production of interfering ions in the m/z region of interest (m/z 280–340). The MALDI spectrum obtained by co-crystallisation of analytes and HCCA (Ma) shows the most abundant ions at m/z 379, due to $[2Ma + H]^+$ species; however, ions corresponding to protonated molecules of COC, BE and CE are detectable at m/z 304, 290 and 318 respectively, with a net increase of the signal-to-noise (S/N) ratio with respect to the LDI measurements. Interestingly, although solution 1 was constituted by a molar ratio of 1 : 1 : 1 for the three analytes, the abundance of the three related peaks does not correspond to this ratio and a particularly lower signal is obtained for BE. These results can be explained by a higher proton affinity (PA) of COC and CE, which favours the production of the related MH^+ species under MALDI conditions. In order to improve both sensitivity and S/N ratio, we tested different sample preparation procedures. With a ‘matrix–sample–matrix multi-layer’¹⁶⁶ we obtained better results in terms of intensity (4000–8000 a.u.) and S/N (50 : 1) than those achieved by ‘classical’ sample preparation methods when depositing 1 ng of each analyte. However, when we deposited a lower quantity of the sample (0.1 or 0.01 ng of each analyte), the analyte peaks were confused with the background signal; therefore, the multi-layer procedure was not judged to be particularly effective in achieving a better detection limit.

Recently, Langley *et al.*¹⁶⁷ have shown that the use of pencil graphite as a co-matrix for MALDI measurements is principally effective, particularly for the analysis of small molecules. For this reason, we also tested the effectiveness of this approach in our studies. In the present case the best results, in terms of ionic yield, were obtained by using a B pencil. The pencil was simply scribbled onto the metal target spot, producing a hydrophobic surface that allows the sample droplets to concentrate in a small spot.^{167,168}

Under these conditions the signal related to BE is strongly depressed, while MH^+ of COC and CE are the most abundant peaks of the spectrum. However, when a dropwise deposition of HCCA on the sample deposited on the graphite surface was realised, enhanced S/N ratios were obtained. The results were even better when HCCA was electro sprayed on the graphite–sample layer, and the best conditions were found by spraying at a flow rate of 5 $\mu\text{L}/\text{min}$ for 1 min.

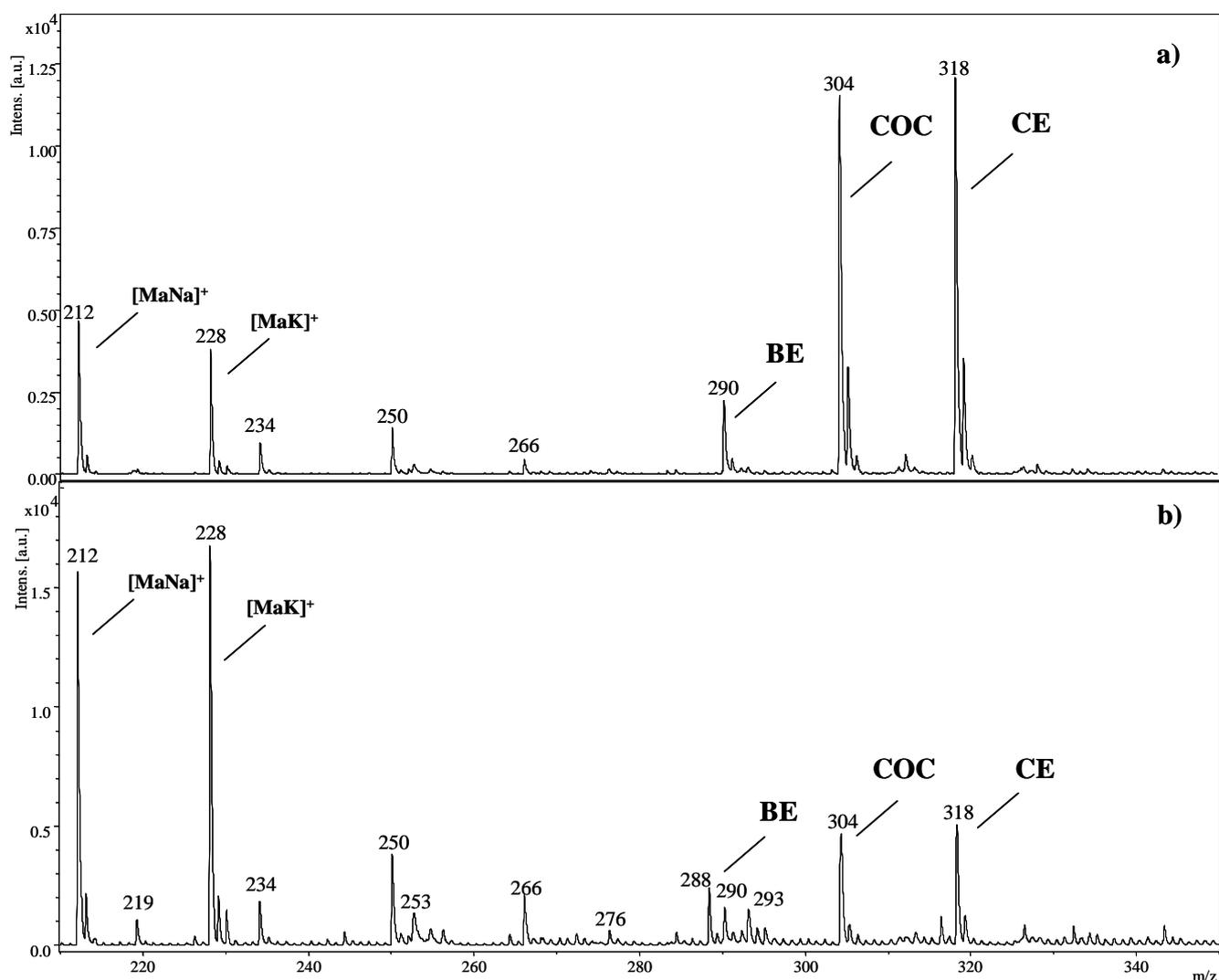


Figure 21. MALDI spectrum obtained after spraying the HCCA solution over the standard samples deposited on graphite when (a) the quantity of each analyte was 1 ng and (b) the quantity of each analyte was 100 μg ; Ma = matrix.

A uniform but discrete matrix spot distribution was observed on the sample and, after laser irradiation upon the surface, we obtained a spectrum which is reported in Figure 21(a). The protonated molecules of the three analytes are detected in high abundance; also in these conditions BE exhibits the lowest ionisation yield. Since the last procedure proved the most efficient, further measurements were performed by depositing decreasing amounts of the analyte mixture. The analysis made with 1 μ L of working standard solution 2 (corresponding to the deposition of about 0.1 ng of each analyte) resulted in the spectrum shown in Figure 21(b). Also in this case, COC and CE produce quite abundant ions, while BE is still detectable, though in a region crowded from interfering ions.

5.4.2 Hair samples analyses

After preliminary investigations at the standard solution of COC, BE and CE, the approaches yielding the best results were employed for the determination of the three analytes in two hair samples (samples A and B). These samples were found to be positive through the GC-MS method, which is described in the Experimental Section and is commonly employed in the authors' laboratory for routine qualitative and quantitative determination of COC and its metabolites. The measured concentrations were 9.5 ng/mg of COC, 2.0 ng/mg of BE and 5.0 ng/mg of CE in sample A; and 100 ng/mg of COC and 30 ng/mg of BE in sample B. The first study was performed on a single hair from sample B. A segment of about 4 cm was fastened with a tape at the extremities on a conducting glass sample holder, normally employed for ion imaging experiments. The hair sample was analysed either without any treatment (*i.e.* in LDI conditions, step a of Figure 20) or after spotting it with water, 0.1% TFA and HCCA matrix (step b of Figure 20). No useful results were obtained in either case. The MALDI spectra obtained are quite complex, with the production of many ionic species in the m/z range relevant for COC, BE and CE detection. An ion at m/z 304 (corresponding to the MH^+ of COC) was randomly detected,

with a little reproducibility. At this point, powdering of the hair sample was considered essential to continue the analysis. The powdering procedure would, in principle, make available a larger sample surface and, consequently, a greater amount of COC, BE and CE to be desorbed. For this reason, hair samples were externally washed avoiding the use of SDS, a surfactant that is often employed for hair decontamination, however, it is deleterious for MALDI processes.

The direct deposition of powdered hair samples (approximately 0.2 mg) onto the stainless steel MALDI plate treated with graphite B, followed by HCCA deposition either as a drop (step c of Figure 20) or as a spray (step d of Figure 20), did not lead to better results either.

In particular, we observed a high variability in the MALDI spectra obtained, possibly due to the non-homogeneity of the sample layer and the consequent irradiation of different portions of the sample surface. Also in this case, the MALDI spectra were quite complex and signals related to COC, BE and CE were found with low signal-to-noise ratio ($S/N \leq 3$). At this stage, we performed the pre-treatment of the powdered hair with different methanol/TFA mixtures or 100% TFA (step e of Figure 20) for 15 min. It must be emphasised that this treatment is very different from that usually employed for hair analysis by GC-MS.

In this treatment, 50 mg aliquots of pulverised hair samples are treated with 3 mL of a $\text{CH}_3\text{OH}/\text{TFA}$ (9 : 1) solution, sonicated for 1 h and left overnight at 45 °C; the extracts obtained are then submitted to SPE, followed by derivatisation of the dried eluates.

In the present study, the 15 min incubation with $\text{CH}_3\text{OH}/\text{TFA}$ 70 : 30 proved to be the most effective for the rapid extraction of COC, BE and CE. The spectra of the positive hair samples, which were obtained by applying the best procedure identified through the standard solution analysis (*i.e.* the overlayer graphite B–sample–sprayed HCCA) are shown in Figure 22(a) and (b).

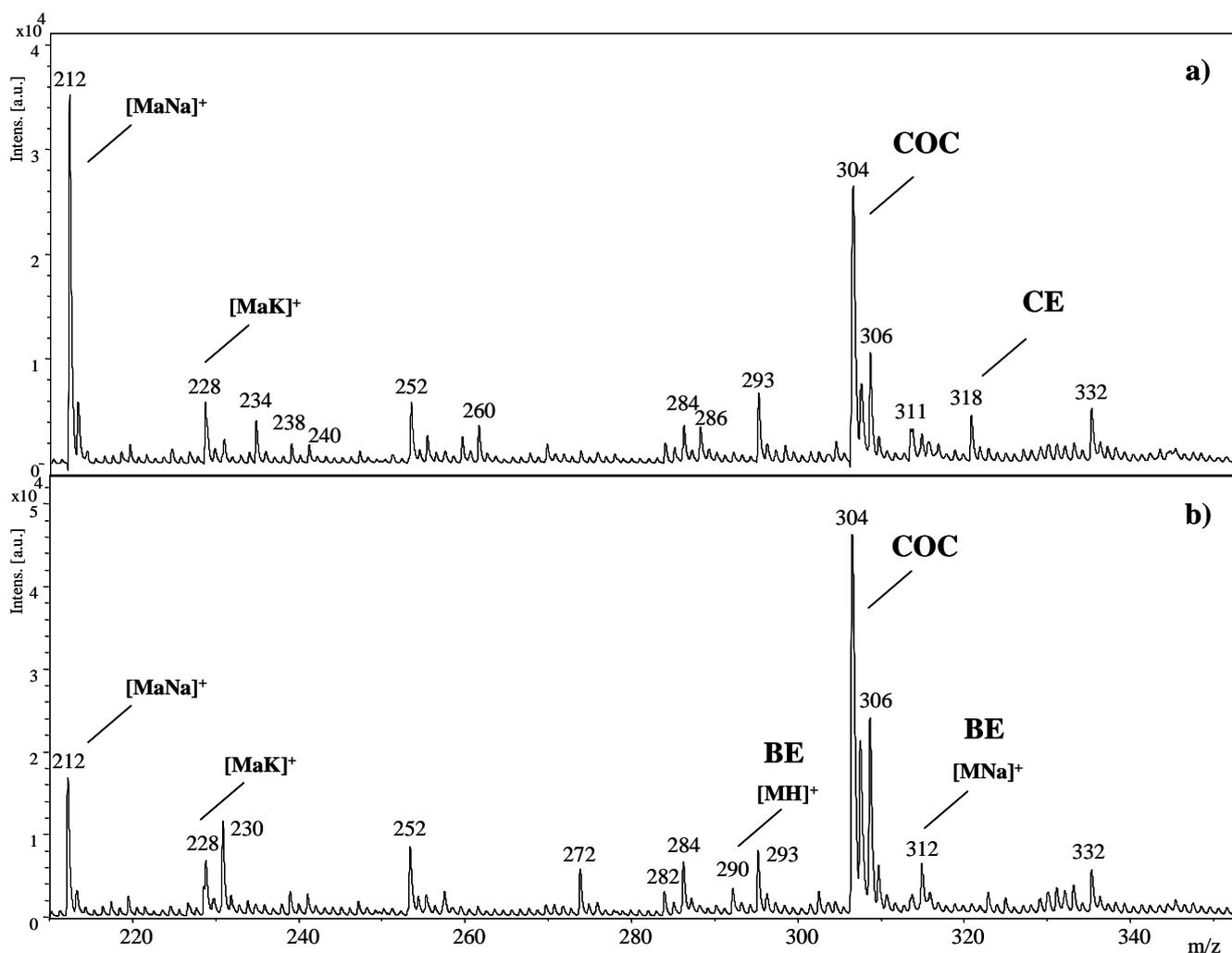


Figure 22. (a) MALDI spectrum obtained for positive hair sample A, obtained by treatment of powdered hair with 70:30 CH₃OH/TFA followed by multi-layer MALDI sample preparation (graphite-sample-spayed matrix); (b) MALDI spectrum obtained for positive hair sample B, obtained in the same conditions.

In both the A and B samples an abundant ion at m/z 304, corresponding to MH^+ of COC, was detected. The MH^+ ion of CE is present at m/z 318 in the MALDI spectrum of sample A, while it is absent for sample B; vice versa, MH^+ and $[M + Na]^+$ species of BE are detectable at m/z 290 and 312 in sample B only, although with a low signal-to-noise ratio.

These data are not in full agreement with those previously obtained by the GC-MS procedure, since they confirm the exclusive presence of COC and BE in B, but they do not reveal the presence of BE in sample A. One possible explanation can be found in the lower concentration of BE (2.0 ng/mg) in sample A with respect to sample B (30 ng/mg),

hampering the detection of BE in the little hair amount employed for MALDI analysis (approximately 0.2 mg).

Moreover, the different sample treatment used in our study for MALDI analysis might have altered the concentration of BE in sample B. The matter can be resolved in future studies, by using a much larger number of hair samples to be analysed by MALDI.

To corroborate the structural assignments of COC, BE and CE, PSD experiments were performed on ions at m/z 290, 304 and 318.

The resulting tandem (MS/MS) mass spectra are superimposable on those obtained from the standard samples, thus proving the validity of the proposed approach for qualitative identification purposes.

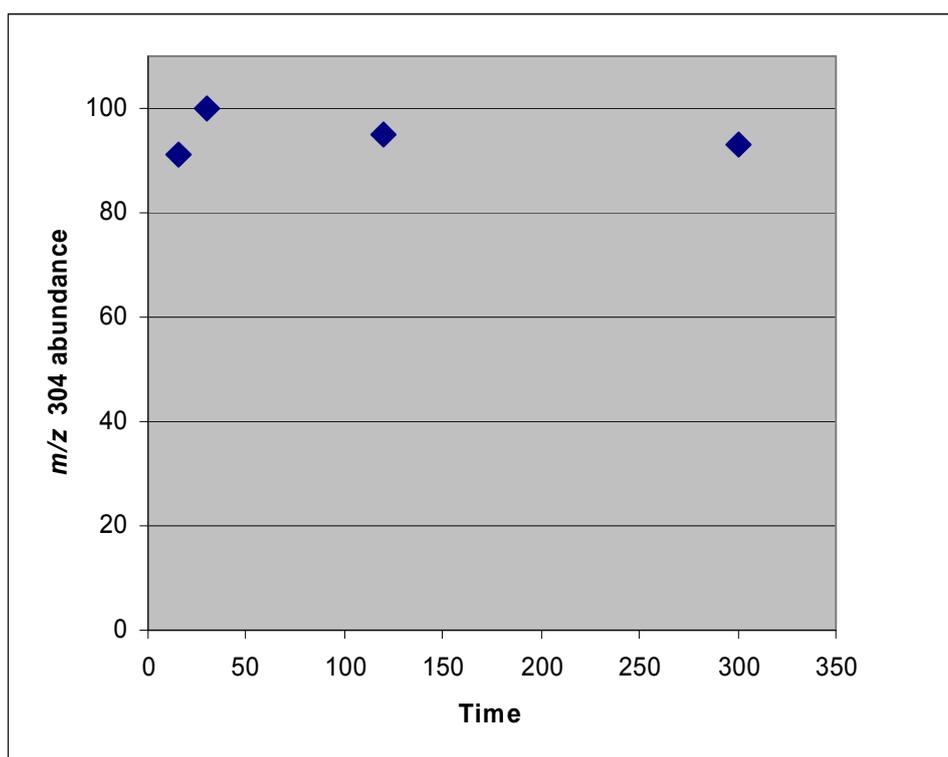


Figure 23. Plot of the abundance of the ion at m/z 304 detected in the MALDI spectrum of hair sample A *versus* extraction time.

From a semi-quantitative point of view, it must be considered that the spectra shown in Figure 22 were obtained by treating 1 mg of pulverised hair sample with 40 μL of $\text{CH}_3\text{OH}/\text{TFA}$ solution. On the MALDI sample holder, 1 μL of supernatant was deposited.

Considering that GC-MS analysis for these samples reveals COC concentration of 100 and 9.5 ng/mg respectively, it follows that the analyte quantities actually deposited on the MALDI plate were 2.5 and 0.25 ng, respectively. It is reasonable to assume that a higher sensitivity can be achieved by reducing the supernatant volume.

We further investigated the yield of the extraction procedure with respect to time. To this aim, the incubation solutions of the powdered hair samples were stored at 45 °C and 1 μL portions of supernatants were drawn at different times (15, 30, 120, 300 min). As shown by the data in Figure 23, obtained for sample A, the abundance of the ion at m/z 304 (MH^+ of COC) remains practically constant (from $4 \pm 0.4 \times 10^4$ a.u. to $5.1 \pm 0.6 \times 10^4$ a.u.), indicating that 15 min are enough to achieve a valid extraction yield.

The whole MALDI hair sample preparation (washing, pulverisation, 15 min incubation) turned out to be significantly more rapid and simpler than that used for GC-MS analysis (washing, pulverisation, overnight incubation, extraction, derivatisation, chromatographic run); furthermore, MALDI analysis is per se much quicker than GC-MS. These are considerable advantages when adopting a high-throughput screening analysis.

6. Conclusions

New analytical procedures were developed for the accurate and specific determination of xenobiotics of toxicological and forensic interest in hair. In this context, LC-multiple mass spectrometry techniques and MALDI mass spectrometry demonstrated to be particularly effective in providing high sensitivity and selectivity.

LC-multiple mass spectrometry was employed in two methods dedicated, respectively, to the quantitative determination of ergotamine and citalopram, escitalopram and their desmethylated metabolites.

The former method allowed, for the first time, the identification and quantification of ergotamine in hair in a case of iatrogenic ergotism. It was validated in hair matrix, exhibiting a linear range of 15 - 200 pg/mg and a lower limit of quantification of 15 pg/mg. The method can be used for clinical and toxicological purposes; results from hair testing can be used to assess repeated exposure to ergotamine and possible associations with long-term, drug-induced effects, as in our specific case. Preliminary experiments suggest that it should be possible to extend the method to other body fluids or tissues of interest in forensic toxicological applications.¹⁶⁹

The latter method, developed for determining citalopram, escitalopram and their desmethylated metabolites in hair, exhibits linearity in the range 25-2000 pg/mg, a lower limit of quantification of 25 pg/mg and a limit of detection of 10 pg/mg, using only 10 mg of specimen. As a matter of fact, the sensitivity of the analytical procedure enables small amounts of hair sample to be analyzed and makes the method suitable for determining the analyte and its metabolite in infant hair. Given the high specificity of the multiple mass spectrometry approach, a rapid liquid/liquid extraction avoids the need for more extensive and time-consuming purification steps. The validated analytical procedure was successfully applied to the quantification of citalopram and escitalopram in two newborn hair samples.¹⁷⁰

Regarding MALDI mass spectrometry, it has to be emphasized that this technique was for the first time applied to the determination of substances of forensic interest in human hair. A screening method for the determination of cocaine, benzoylecgonine and cocaethylene in hair was developed, that gave to promising results. A particularly simple and rapid sample preparation procedure, avoiding overnight hair hydrolysis, is sufficient prior to MALDI analysis. Cocaine and two of its metabolites can rapidly be detected and identified by post source decay experiments. Although it needs to be tested on a much higher samples number and can not be employed for quantitative analysis, the MALDI method is a valuable and feasible technique for detecting drugs and their metabolites in complex biological matrices. Once optimised and validated, it can be proposed as a fast screening procedure to detect the presence of cocaine and its metabolites in hair samples.¹⁷¹

The new analytical procedures above described, objects of the experimental thesis conducted during the graduate school, in the period from 1 January 2006 – 31 December 2008, produced three original articles published in scientific journals of international interest, i.e. *Therapeutic Drug Monitoring* and *Journal of Mass Spectrometry*.¹⁶⁹⁻¹⁷¹

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