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Curriculum Igiene Industriale e Ambientale

A research route in the actions against the abuse of psychotropic substances

New analytical tools for determination of drugs of abuse by means of Liquid Chromatography – tandem Mass Spectrometry

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ABSTRACT

The research route of this PhD started from workplace drug testing, an emerging need for safety and hygiene at work. The aim of the work consisted of developing and validating analytical methods: innovative tools for drug addiction monitoring and for illicit drugs testing.

Different classes of psychotropic substances are examined in relation to their chemical and pharmacological effects, with a special focus on new psychoactive substances (NPS) and new trends of abuse. The most important biological matrices, suitable for analysis, are studied and the peculiarities of each matrix are highlighted. Analytical methods were developed for drugs determination in biological samples, such as human urine, plasma, oral fluids and hair. All these matrices are equally important for forensic purposes as they give different kind of information about the time of assumption and they show some distinctive features. Sample preparation is a critical step and is a key factor in determining the success of analysis: the task of chemist is to develop the best strategy to ensure that the system, consisting of 3 variables, *matrix - target analytes - instrumental equipment*, will be solved consistently with the ultimate purpose of the analysis. Therefore in this project appropriate sample preparation techniques have been investigated with special regard to each different matrix. The manual operations associated with these processes are often expensive, labor-intensive and time-consuming and affect the uncertainty of the final analytical data, for these reasons in this work new procedures based on innovative sample preparation were investigated and developed. The goal has been to provide short sample preparation times, automation of the clean-up step and low sample volumes, minimizing the consumption of solvents and chemicals.

Development of methods based on liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) was a crucial component of this work. LC–MS is considered to be the benchmark for quantitative/qualitative bioanalysis, providing specificity, sensitivity and speed to the method. Different chromatographic conditions have been evaluated for the optimal separation and detection of the investigated compounds. As a new trend, the application of columns with superficially porous packing materials has been investigated, which allowed fast chromatographic runs and high efficiency.

The versatility of the LC-MS/MS has been proven by providing a single analytical method for the determination of 11 drugs of abuse in biological fluids: conventional matrices, such as plasma and urine, and unconventional ones, such as oral fluids.

The research route was then directed to hair analysis and for the first time pressurised liquid extraction (PLE) was shown to be effective for the extraction of drugs belonging to different classes from hair samples. Experiments were carried out to ensure correct interpretation of the analytical data by studying the phenomenon of external contamination (a critical point in the analysis of hair for forensic purposes).
To improve the methods selectivity, multi reaction monitoring (MRM) acquisition modes were used for quantification of the investigated compounds in both cases.

Finally analytical target was extended to NPS: a method for the screening of methylenedioxyamphetamine and piperazine-derived compounds in urine was developed. These substances, characterized by possessing common moieties, are screened using precursor ion and neutral loss scan mode and then quantified in MRM acquisition mode.

All the methods developed have been validated according to specific international guidelines to ensure reliable results, unambiguously interpreted and useful for forensic purposes.

List of publications

1. **Micro-solid phase extraction coupled with high-performance liquid chromatography–tandem mass spectrometry for the determination of stimulants, hallucinogens, ketamine and phencyclidine in oral fluids**
   Sergi, M.; Compagnone, D.; Curini, R; D'Ascenzo, G.; Del Carlo, M.; Napoletano, S.; Risoluti, R. *Analytica Chimica Acta,* 2010, 675 p. 132-137
   DOI 10.1016/j.aca.2010.07.011

2. **Determination of illicit drugs in urine and plasma by micro-SPE followed by HPLC–MS/MS.**
   Napoletano, S.; Montesano, C.; Compagnone, D.; Curini, R.; D'Ascenzo, G.; Roccia, C.; Sergi, M., *Chromatographia*, 2012, 75 p. 55-63
   DOI 10.1007/s10337-011-2156-6

3. **Analysis of illicit drugs in human biological samples by LC-MS^n**
   Sergi, M.; Napoletano, S. book section in "*Liquid Chromatography – Mass Spectrometry in Drug Bioanalysis*", Q.A. Xu and T.L. Madden, Editors. 2012, Springer US. p. 349-398
   DOI 10.1007/978-1-4614-3828-1_12

4. **Pressurised-liquid extraction for determination of illicit drugs in hair by LC–MS–MS.**
   Sergi, M.; Napoletano, S.; Montesano, C.; Iofrida, R.; Curini, R.; Compagnone, D., *Anal. Bioanal. Chem.* 2013, 405 p. 725-735
   DOI 10.1007/s00216-012-6072-x

5. **Screening of methylenedioxyamphetamine and piperazine-derived designer drugs in urine by LC–MS/MS using neutral loss and precursor ion scan**
   Montesano, C.; Sergi, M.; Moro, M.; Napoletano, S.; Romolo, F.S.; Del Carlo, M.; Compagnone, D.; Curini, R. *Journal of Mass Spectrometry*, 2013, 48 (1) p. 49-59
   DOI 10.1002/jms.3115

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INTRODUCTION

The abuse of alcohol and drugs is a social problem discussed for some time, but the analysis of the consequences in the field of safety and hygiene at work is relatively recent. The implications of the continued abuse of illicit drugs (see also box 1.0) in the workplace represents a high cost to society. Vittadini and Lanfranco analyzed alcoholism and drug addiction in the workplace: it is reported that alcohol and drugs would be responsible for even 47% of accidents occurred [1]. Hence the need of appropriate analytical tools for effective drug testing and monitoring, as also highlighted in the Mandatory Guidelines for Federal Workplace Drug Testing guidelines of SAMSHA [2].

Illicit drugs testing in biological matrices encompasses a variety of procedures, that range from general unknown analysis for a wide variety of drugs and screening procedures for well-defined drug classes to the specific confirmation and quantification of individual compounds.

Screening of drugs is usually achieved by means of immunoassays; enzyme immunoassays (EIA and EMIT), enzyme linked immunoassays (ELISA) and fluorescence-polarization immunoassays (FPIA) are the most diffused. However, in the last years screening tests are often performed with techniques reserved in the past only to confirmation procedure (for quantitative purposes), i.e. liquid chromatography (LC) and gas chromatography (GC) coupled with mass spectrometry (MS) capabilities [3, 4]. In fact, after screening and identification, the relevant analytes must be quantified; according to SOFT-AAFS guidelines [5] all quantification assays must be performed by GC–MS(MS) or LC–MS/MS and they have to be fully validated. Confirmation procedures may involve the targeted analysis of well-defined drug classes or multi-analytes approaches. In this respect LC–MS and especially LC–MS/MS is increasingly used avoiding the expensive and time consuming derivatization step that is required for the analysis of a number of drugs, especially metabolites, in GC–MS.

The core of the PhD project has been the development of novel analytical method with special regards to innovative techniques for sample preparation. Both single-class and multi-analytes quantification methods have been developed by LC–MS/MS in the most important biological matrices. The analytes taken into account belong to different classes, namely opiates, cocainics, amphetamines, cannabinoids and hallucinogens which are discussed in **§1,** a focus box on novel psychotropic substances (NPS) closes the topic. Biological matrices

and their specific features are treated in **§2** and guidelines for the development of an analytical method are briefly mentioned in **§3**. Sample preparation techniques, i.e. solid phase extraction (SPE), liquid liquid extraction (LLE) and protein precipitation (PP) are discussed in **§4**.

The experimental work has been divided in three sections, following the research route; section I is reserved to determination of illicit drugs in biological fluids using μ-SPE for sample pretreatment; in section II extraction of drugs from hair by pressurised liquid extraction (PLE) is investigated; section III is dedicated to screening method for identification of new methylenedioxyamphetamine- and piperazine-derived designer drugs.

1. Target compounds

1.1 Opiates

Opiates are derived from opium, a naturally occurring product obtained from the plant *Papaver somniferum*. The major psychoactive components of opium are morphine, codeine and thebaine. Diacetylmorphine (heroin) has been synthetically derived from morphine and is the most commonly opiate sold in the illicit market. This substance has an extremely short half-life (about 5 minutes) and it is rapidly metabolized to 6-monoacetylmorphine (6-MAM) and further to morphine. Morphine is then largely metabolized by conjugation to morphine-3β- and 6β-glucuronides (**Figure 1**). While the former metabolite is inactive, the latter shows an analgesic activity even greater than the parent compound [6].

Other misused opiates include dihydrocodeine and codeine, which is available in over-the-counter preparations but can also be found in street heroin, together with 6-acetylcodeine. Detection of heroin's metabolite, 6-MAM, is important from a forensic point of view since it allows the differentiation between heroin and morphine or codeine consumption because heroin itself is rarely found in any conventional biological matrix. However since 6-MAM half-life is of about 40 minutes it is detectable in urine at maximum for 8 hours after heroin assumption [7]. For this reason the cut-off value of 6-MAM in urine is very low, i.e. 10 ng mL^{-1}. This metabolite is more likely to be detected in oral fluid (OF), blood and hair. In hair care should be taken to prevent analyte hydrolysis during extraction.

Opiates have a wide range of pharmacological effects: analgesia, sedation, respiratory depression, decreased gut motility, and cough depression. They are clinically used in the

relief of moderate to severe pain and as cough sedatives; they are abused for their complex psychotropic effects producing tolerance and physical dependence.

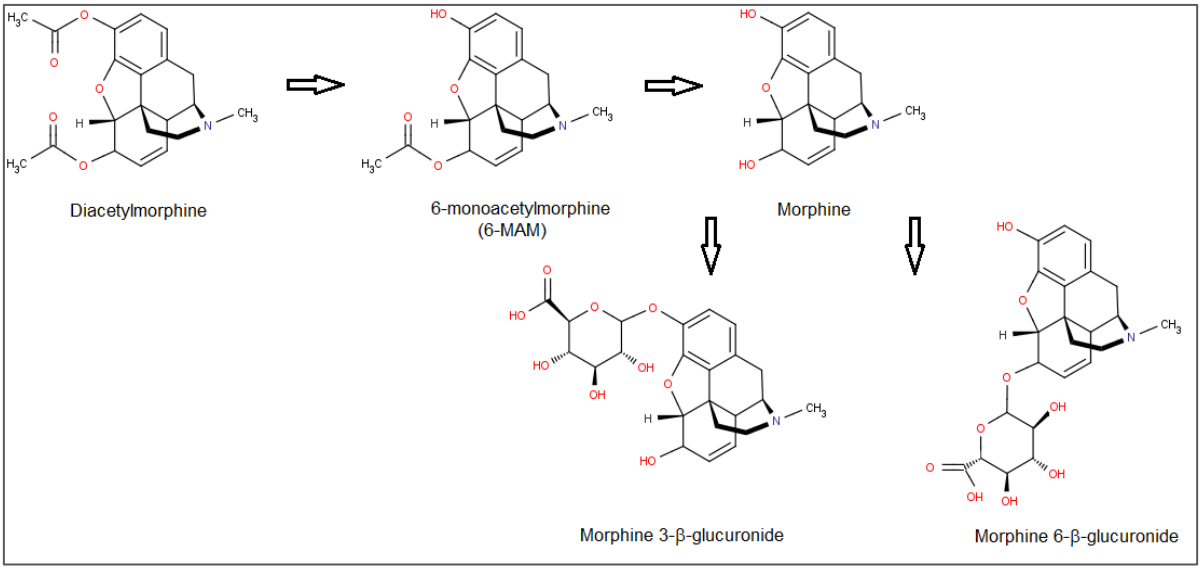


Figure 1. Metabolic pathway of diacetylmorphine (heroin)

Synthetic opiates, such as fentanyl, buprenorphine and methadone are also becoming important; they are not necessarily structurally related to morphine but they show agonistic effect at the same receptors, producing similar psychoactive effects; they are often used in the treatments of heroin dependence [8].

Testing urine for heroin and morphine usually begins with hydrolysis of the conjugates to liberate free morphine prior to sample clean-up and chromatography; hydrolysis can be performed either enzymatically or by addition of strong acids. The performance of different kind of enzymes and of acid hydrolysis on the cleavage of morphine conjugates was tested by *Zezulak et al.* and *Lin et al.*, acid hydrolysis resulted always more effective. Direct analysis of conjugates is also possible by LC often coupled with MS(/MS) [9] and has been stimulated by the recognition of the role of morphine 6β-glucuronide (M-6G) as an active metabolite. In fatal cases the ratio of morphine to its glucuronides has been shown to be a useful tool to determine survival time between ingestion and death [7]. In plasma morphine glucuronides levels exceed those of morphine 0.5 hours after dosing and remain higher at all times thereafter [10].

Very often opiates determination is included in multi-class methods together with all the most common drugs of abuse like cocaine and metabolites, amphetamines, benzodiazepines etc [11].

1.2 Cocaine

The primary source of cocaine is the *Erythroxylum coca* plant that grows abundantly on the eastern slopes of the Andes Mountains in Peru and Bolivia where as early as 3000 years ago coca leaves were chewed primarily to enhance physical performance and to decrease the need for food and rest. Illicit cocaine use grew slowly for many years, and then increased at a rapid pace in the 1970s; today is the second most used illicit drug after cannabis due to its powerful addictive stimulant action [12].

In humans, cocaine (COC) is extensively metabolized by both enzymatic and nonenzymatic pathways. Its primary metabolites include benzoylecgonine (BZE) and ecgonine methyl ester (EME) while other metabolites such as the active norcocaine (NCOC) and benzoylnorecgonine (BNE) are usually detected at considerably lower concentrations. Cocaethylene (CE) is another metabolite that may serve as biomarker for co-ingestion of alcohol because the transesterification of COC to CE is faster than hydrolysis to BZE in the presence of ethanol. The cocaine metabolites are excreted in urine usually for 36–48 hours, so the presence of cocaine metabolites in this matrix indicates recent drug use [13]. The main metabolites are shown in **Figure 2.**

The amount of parent drug and its metabolites varies notably depending on the selected biological matrix. In hair and oral fluids (over the first 4 h) cocaine is more concentrated than its metabolites which are more polar and so less liable to passive diffusion compared to the parent drug [14], in urine cocaine is often not detectable so BZE and EME are markers of its consumption. In plasma BZE is the primary detected metabolite while EME is generally detected only at low concentrations following cocaine administration by the intranasal, intravenous and smoked routes [15] however, following controlled oral administration, EME was present in plasma at concentrations up to four times that of cocaine. Also NCOC was found to be present at substantial concentrations following oral administration [16].

The analysis of cocaine in biological specimens may be complicated by its instability particularly in blood which contains cholinesterase, while BZE and EME are more stable; in hair testing time-consuming “soft digestion” are preferred to prevent any conversion of cocaine to BZE.

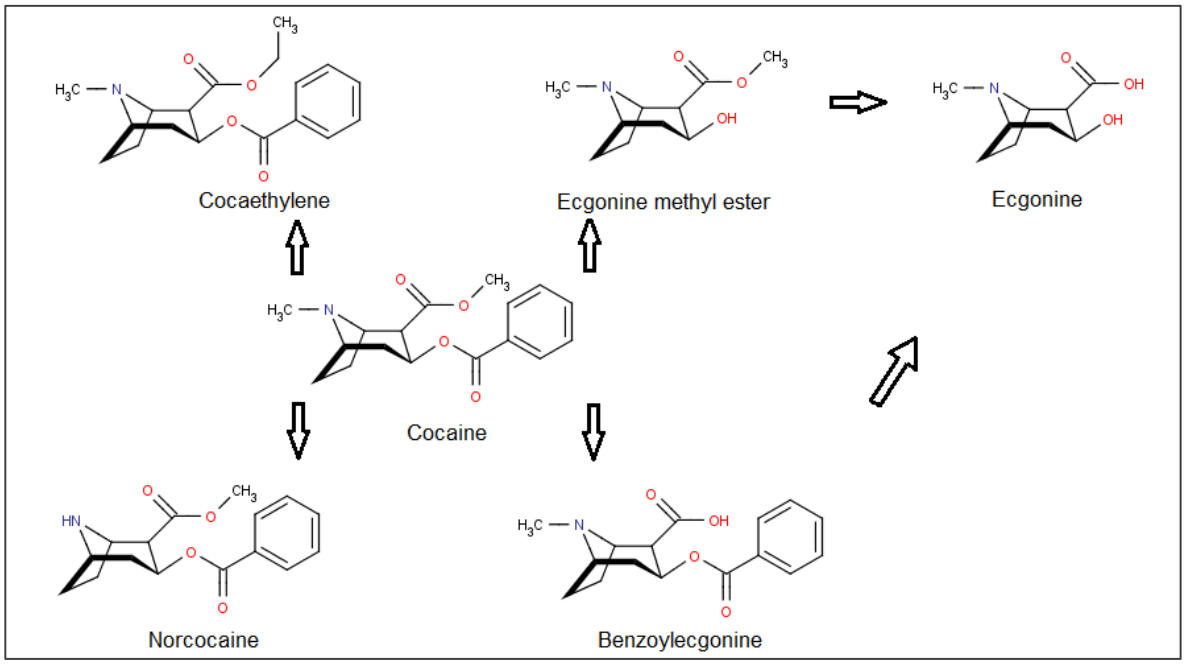


Figure 2. Metabolic pathway of cocaine

1.3 Amphetamines

Amphetamine and methamphetamine are powerful stimulants of the central nervous system by acting as indirect sympathomimetic drugs which have been in use since the early 1900s. These drugs have a single asymmetric centre and therefore they exist as two enantiomers, each of which has different pharmacological activities [17].

Over the years, several substitutions have been made to the parent molecules to modify their activity. A group of related drugs, often referred to as ‘designer amphetamines’, includes several methylenedioxy analogues which are often found on the illicit market as an abused drug. These compounds include 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) or “ecstasy” and 3,4-methylenedioxyethylamphetamine (MDEA). The structures of amphetamine, methamphetamine, MDA, MDMA and MDEA are shown in **Figure 3**.

The analysis of amphetamine, methamphetamine and related parent compounds requires no hydrolysis since they do not undergo to conjugation; the main metabolic pathway involves aromatic and aliphatic hydroxylation, N-demethylation and oxidative deamination [18]. Analysis of the phenolic metabolites, that are partly excreted as conjugates, does require

hydrolysis which is typically accomplished using either acid or enzymatic procedures but in most methods, only the parent compounds are analyzed.

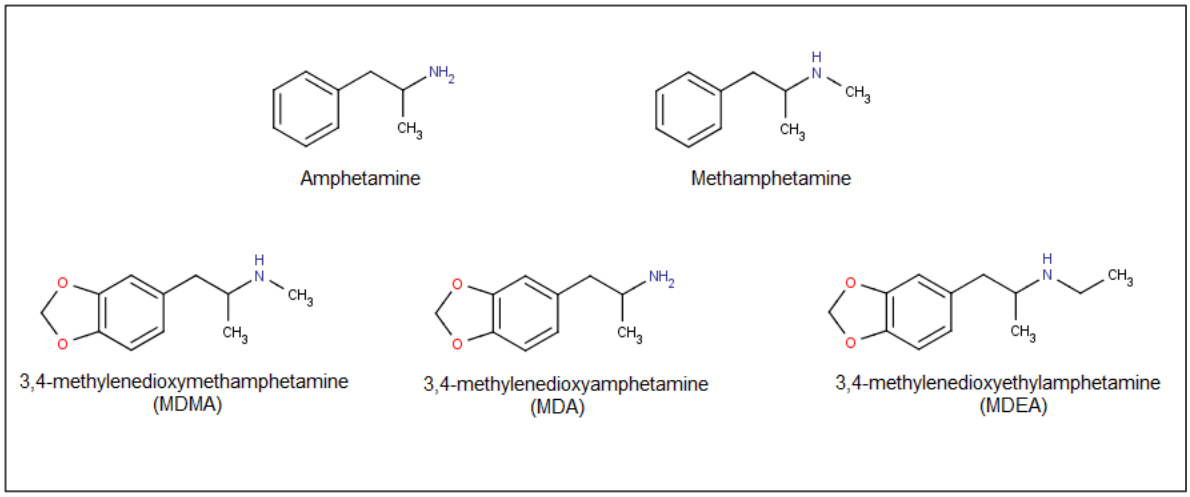


Figure 3. Amphetamines structures

Amphetamine-derived designer drugs undergo predominantly two overlapping metabolic pathways: O-demethylenation to dihydroxy derivatives (catechols) followed by methylation of one of the hydroxy groups, and further degradation of the side chain to N-dealkyl and deaminooxo metabolites [18]. MDA, MDMA, and MDE are additionally metabolized to glycine conjugates of the corresponding 3,4-disubstituted benzoic acids, so-called hippuric acid derivatives. Their half-lives are shorter compared to amphetamine and methamphetamine.

Amphetamines are basic drugs, therefore their excretion is strongly influenced by urine pH and their excretion profile is not easily predictable being amphetamines half-lives very variable; for instance if the pH of the urine is neutral, approximately 30% of the ingested amphetamine is eliminated unchanged, but if the pH is 5, up to 74% is eliminated unchanged [19]. Analysis of enantiomer composition can, in some cases, provide significant additional information for interpretation. The S(+) enantiomer is the most active form of amphetamine and methamphetamine [20]. In fact there is ample evidence, that the two enantiomers are metabolized at substantially different rates being the S(+) enantiomer metabolized more rapidly than the R(-) enantiomer. For this reason, the proportion of the S(+) to the R(-)

enantiomer changes with time and can be helpful in the determination of how long since the drug was taken [17].

Concentrations of amphetamines in OF are usually higher than in blood and so they can easily be detected, also in small volumes of sample; amphetamines can be detected for 20 to 50 hours with detection limits of 10 ng mL^{-1} [19]. Similarly in hair, concentrations are high and a good within-subject correlation between dose and methamphetamine and amphetamine hair concentrations was reported by *Polettini et al.* [21]. Amphetamines are generally stable to strong basic and acid conditions, so alkaline digestion or acid extraction are the most diffused method for their extraction from hair.

BOX 1.0 Spot on Law Enforcement action against illicit drugs diffusion

This box provides an overview of psycotropyc substances intercepted by a territorial branch of the Forensic Police Service of Central Anti-Crime Directorate of National State Police (Department of Public Safety – Ministry of Interior), the Interregional Forensic Police Office (GIPS) of Ancona. The data shown in Tables B1.1 and B1.2 are not involved with an epidemiological study on the consumption of psychotropic substances, but they are a warning on the continued spread and abuse of those illicit drugs, which already represents for years a high social cost.

It should be underlined that the GIPS has exclusive competence for the investigations carried out by the State Police and often gives also scientific support to Financial Police in their investigations, while the scientific support for the investigations carried out by Carabinieri is guaranteed by their own specialized units.

Analysis of illicit drugs is performed by GIPS only in criminal proceedings (and not in administrative ones) on express request of the competent court. This activity of forensic support reflects the priorities of the investigative offices which in turn reflect the territorial peculiarities. So from Fermo and Ascoli P. judicial authorities will require more probably analysis of street drugs, ready for the end users (psychoactive substance diluted with cutting agents); instead from Ancona, requests most likely will concern large quantities of marijuana and/or heroine, due to the presence of the port connected with the area over-Adriatic sea; in these cases, psychoactive substances are more concentrated, these will be stored for later distribution throughout the national territory or only for transit; greater criminal organization

will be probably involved. These concepts are found in the tables below, in particular on the percentage of marijuana (Table B1.1) and the average percentage of diacetylmorphine (Table B1.2) in the data referred to Ancona.

Table B1.2 it also show the low significance of amphetamine-type substances and even less for the so-called NPS (see box 2) than other illicit drugs. The legitimate question is: the spread and abuse of these substances is reflected in this data (albeit geographically restricted and with the limitations described above) or there is the need to do more and better both from the point of investigation both from point of forensic analysis to have full cognizance of the phenomenon?

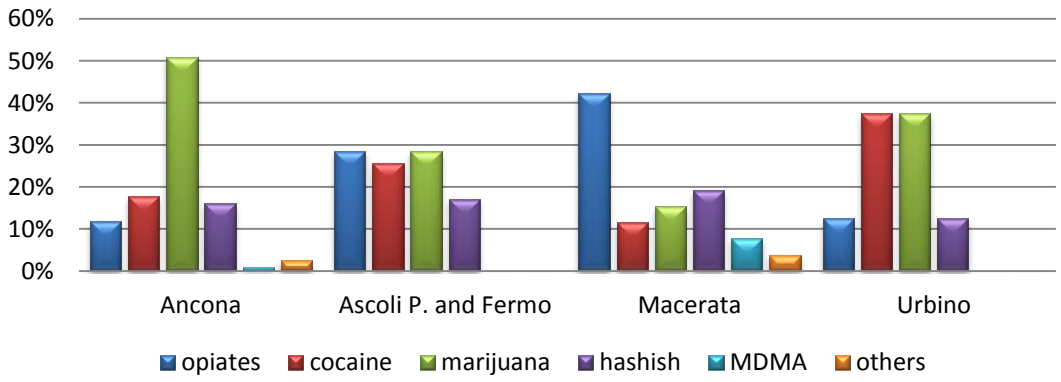
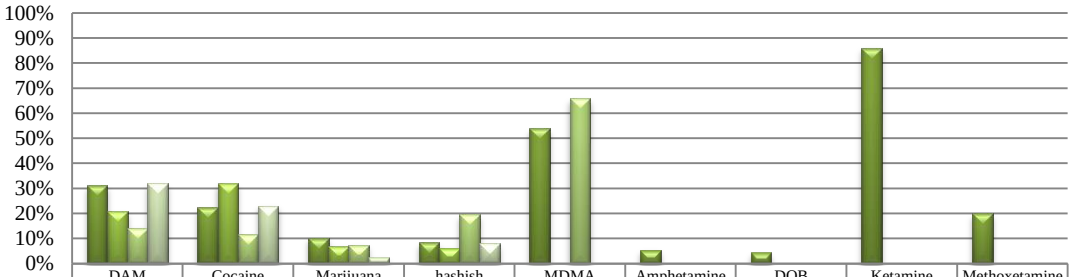


Table B1.1. Overview of drugs of abuse detected by GIPS of Ancona related to the different cities (data expressed as a percentage). Reference periods 2013-2014.



	DAM	Cocaine	Marijuana	hashish	MDMA	Amphetamine	DOB	Ketamine	Methoxetamine
Ancona	31%	22%	10%	8%	54%	5%	4%	86%	20%
Ascoli P. and Fermo	21%	32%	7%	6%					
Macerata	14%	12%	7%	20%	66%				
Urbino	32%	23%	2%	8%					

Table B1.2. Average percentage of psychoactive substance found in the samples analyzed by GIPS of Ancona in the period 2013-2014. In Marijuana and Hashish psychoactive substance quantified was THC.

1.4 Cannabinoids

Cannabinoids are a group of compounds unique to the plant *Cannabis sativa L.*. This plant has been known since centuries not only for medical purposes, but also as a "recreative" drug, widely consumed among young and adults in the last fifty years. Nowadays it is the most used illicit drug in the world [12]. Three cannabis preparations are illicitly trafficked: herbal cannabis (marijuana), cannabis resin with fine plant particles (hashish), and cannabis extract (cannabis oil or hash oil).

The psychotropic effects of this plant, are due to Δ^9-tetrahydrocannabinol (THC) which together with cannabinol (CBN) and cannabidiol (CBD), is the main representative of a group of about 70 terpenophenolic compounds, collectively known as phytocannabinoids [22]. THC is rapidly absorbed, passes into the blood stream and, due to its lipophilicity, can spread throughout the body and penetrate the brain, interacting with the molecular targets, called cannabinoid receptors (CBR) [23, 24].

Cannabinoids are rapidly and extensively metabolized by hepatic enzymes [25, 26]. The major pathway involves hydroxylation to 11-hydroxy-THC (THC-OH), a psychoactive metabolite, followed by further oxidation to inactive 11-nor- Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH). THC and its metabolites subsequently undergo phase II biotransformation to glucuronide conjugates. THC and its hydroxylated metabolites are conjugated through ether bonds; THC-COOH metabolite forms this ether bond as well, but predominately forms an ester bond between the glucuronide and carboxyl moiety [27] (**Figure 4**).

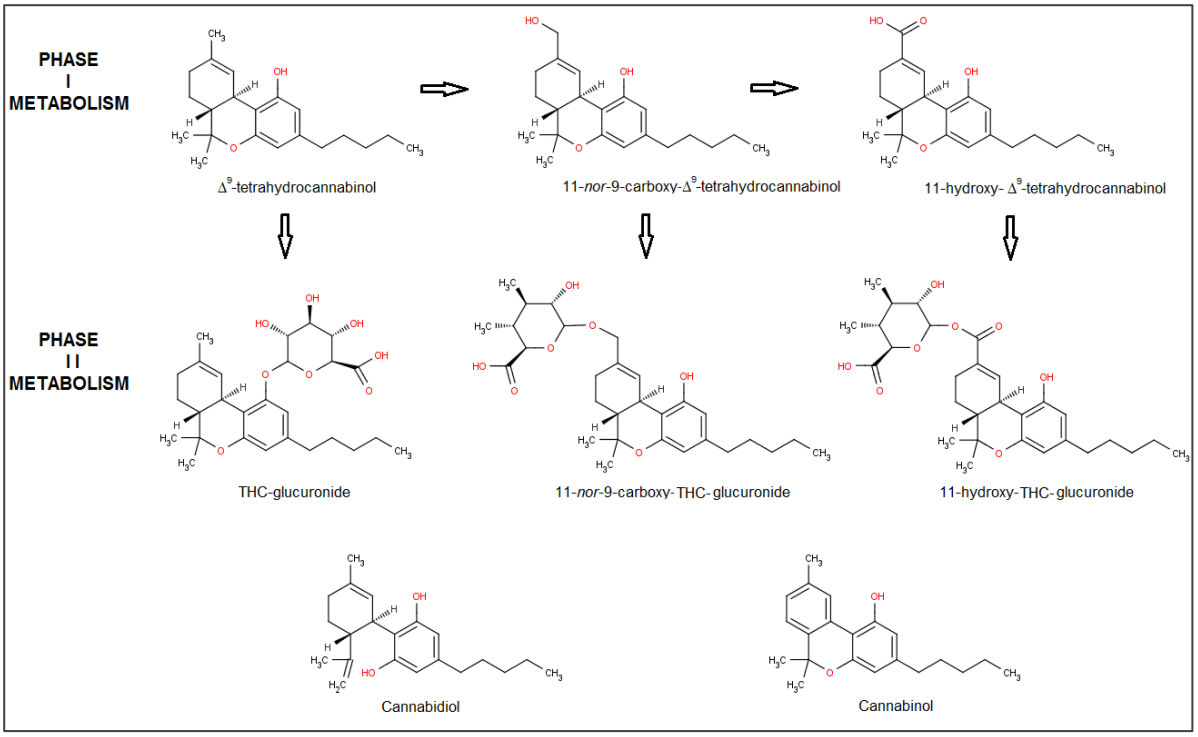


Figure 4. Metabolic pathway of THC and structure of main cannabinoids.

The usual target compounds in blood samples are THC and its major metabolites, THC-COOH and THC-OH). Some methods also detect THC-COOH-glucuronide [28], since this metabolite has been suggested as a marker of a recent cannabis use [29].

Cannabinoids analysis in hair is not straightforward because of the low concentrations of THC and of its major metabolite THC-COOH, its acidic properties not favouring incorporation into the cortex of the hair strand (fg mg^{-1}) [30-32]. Despite the low concentrations in hair samples, the determination of THC-COOH has been shown to be crucial to distinguish between passive drug exposure and active consumption since this metabolite is formed exclusively within the body.

In urine the target analyte for cannabinoids analysis is THC-COOH which is primarily excreted as its glucuronic conjugate. The detection windows can be very large: about four days in occasional smokers [33] and up to months for chronic users [13]. THC and THC-OH, excreted as well in the form of glucuronides, may be present in lower concentrations. A procedure of hydrolysis is generally performed before analysis to convert the conjugated drugs in their free form. Cleavage of the glucuronic bond can be accomplished by basic or

enzymatic hydrolysis. However the basic hydrolysis is not sufficient to deconjugate the glucuronide of THC and THC-OH [34]. The direct analysis of THC conjugates can also be performed by LC–MS/MS [35-39].

In OF, THC and THC-COOH have been shown to remain longer that other drugs of abuse, up to 22 h; CBD and CBN can instead be used as marker of recent cannabis use [40]. High concentrations in OF primarily arise from oral mucosal contamination during smoking and minimal partitioning into blood is observed; OF/P ratio is variable over time, generally high ratios (>10) are found within a few hours and afterwards a decrease is observed [41].

Very often cannabinoids are not included in multi-class methods, since their lipophilic nature involve specific sample preparation. Their analysis is complicated by the possible absorption on laboratory vessels and by their low concentrations in biological samples.

1.5 Hallucinogens

Typically, a hallucinogen causes the user to have a heightened state of awareness of sensory input (audio, visual, etc.) and diminished control over the experience.

Hallucinogens are usually plant alkaloids or derivatives and can be divided into several categories. In this work we just focused on ketamine, mescaline, phencyclidine (PCP), psilocybin and psilocin (**Figure 5)**.

All these drugs have been included in several multi-analyte detection methods [42-45].

Mescaline is a naturally occurring alkaloid found in the peyote cactus *Lophophora williamsii* but it is also synthesized for illicit use.

Psilocybin is a naturally occurring compound found in mushrooms of the genus *Psilocybe* commonly referred to as “magic mushrooms”. The drug is typically ingested by eating the mushrooms. In addition to psilocybin, the mushroom also contains small amounts of psilocin which is nearly twice as potent as psilocybin. It has been shown that in human psilocybin showed rapid dephosphorylation to psilocin which is primarily excreted as the glucuronide conjugate rather than the free compound [46, 47]. Enzymatic hydrolysis with β-glucuronidase from *E. coli* has been shown to be optimal for conjugates hydrolysis [48]; in acid conditions the analytes are not stable while with basic hydrolysis no cleavage is observed.

PCP and ketamine are classified as anesthetics and were initially developed for that purpose; they have the same arylcyclo-alkylamine skeleton and are capable of producing some hallucination effects. Plasma, serum and urine are the most widely used biological fluids for PCP. Typically, only the parent drug is analyzed, however the parent drug is rapidly and extensively metabolized within the body and only small concentrations are detectable in urine and plasma; hair is for this reason a valuable specimen [49].

Ketamine is often used in drug facilitated crimes [50], it has relatively short distribution and elimination half-life; its major metabolite is nor-ketamine, which, together with the parent compound can be detected in the different biological matrices [51].

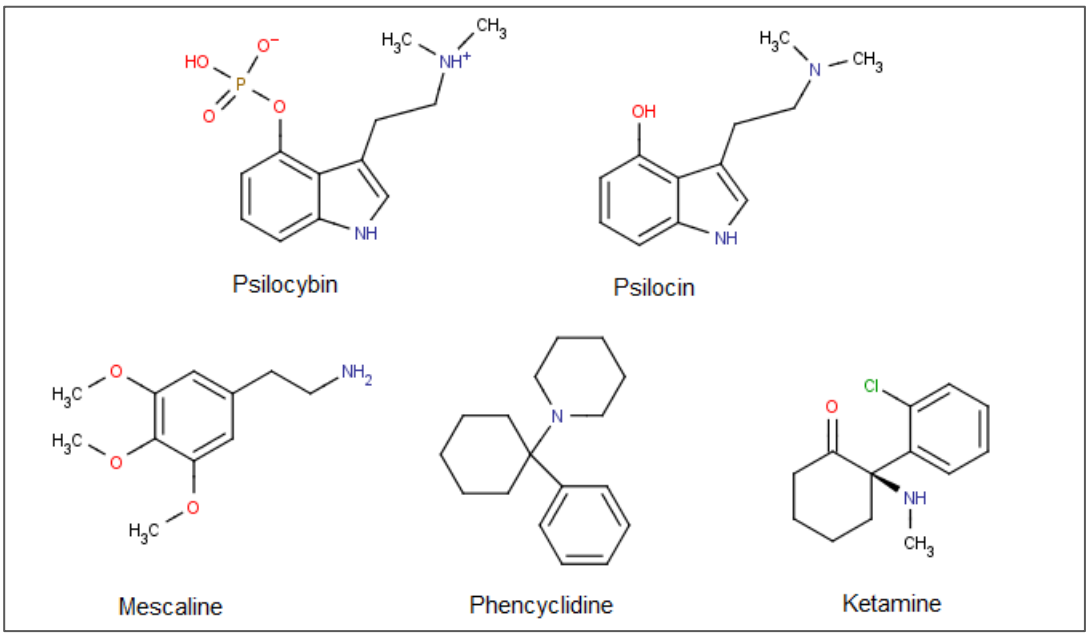


Figure 5. Chemical structures of some hallucinogens

BOX 2.0
Focus on Novel Psychotropic Substances

This box provides an overview of the so-called Novel Psycotropic Substances (NPS). The first part discusses the phenomenon of global dissemination, while the second part shows the chemical classification of substances with some examples represented in **Figure B2.1**

NPS: worldwide diffusion and Italian "silent tsunami"

Since the late 1990s and early 2000s there has been an explosion in clandestinely produced designer drugs of abuse that have considerably changed the illicit drug market. Whereas in earlier decades drug abuse was restricted to a clear number of substances, in the last years more than 100 new designer drugs of abuse have been introduced. The continuous search for and synthesis of designer drugs and their widespread consumption result in a growing number of reports about seizures, abuse and intoxication. The term "new (or novel) psychotropic substances" (NPS) relates to psychotropic substances that are either synthetically changed natural compounds, modified molecular structures of existing drugs or completely different, designed, chemicals; the term 'new(or novel)' means that these drugs have recently appeared on the illicit drug market and that they are newly abused, it does not necessarily mean that they have been synthesized or discovered recently.

Over the last century an extensive survey of structural variations has been conducted by pharmaceutical companies, universities and other researchers from which some new designer drugs have been derived. Sometimes these drugs can be regarded as 'failed pharmaceutical agents' that already have been described in scientific literature but did not find their way to legal medical use (e.g., benzylpiperazine [BZP] or synthetic cannabinoids)[52].

Due to the legal situation in many countries, which try to ban drugs or to regulate their use by scheduling them, producers and users are looking for ways to bypass illegality. As substances usually have to be structurally defined to be scheduled, minor structural changes of common drugs are sufficient to outsmart the official law, at least for some time. Novel substances can appear directly in response to legislative action to replace a similar recently banned drug.

The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) coordinates information exchange and creates risk assessments for single substances that are basis for national legal acts. EMCDDA was created in 1997 and reinforced in 2005, after the underestimation of the phenomenon of MDMA diffusion. An early warning system (EWS)

was created and it reports on newly encountered psychoactive substances facilitating the sharing and disseminating of information among EU member states. Griffith has recently identified in synthetic opioid the class of substances that currently represents the latest warning in Europe (in Romania they replaced heroin)[53]. EMCDDA studies underlined that NPS, because of the greater pharmacological effectiveness (i.e. 5 grams of 25I-NBOMe, a derivative of phenethylamine, are sufficient for 10000 doses), are imported / distributed at lower concentrations then classical drug of abuse, so it is required a greater sensitivity in detection systems. In England and Ireland NPS abuse has actually already exceeded that of traditional drugs of abuse, furthermore the abuse of NPS in Northern Europe is clearly established [53, 54].

A system similar to European EWS exists in the USA to monitor nationwide diffusion of NPS. O'Brien, recently showed that two SCN were among the 10 most detected drugs of abuse in USA already in 2012 (namely two synthetic cannabinoids: XLR-11 and AM-2201)[55]. United Nations Office on Drugs and Crime (UNODC) publish annually the World Drug Report, a document reporting a detailed overview of recent world drug trends, trafficking routes and regional consumption patterns and gives statistics about production, seizures, prices, consumption and treatment. Gerra, member of UNODC, about NPS phenomenon recently underlined these points [56]:

- in some countries, mephedrone is replacing heroin (without overcoming the intake by injection and the possible hazards derived);
- in Australia there is a growing abuse of ketamine;
- an increasingly important share of consumers consists of teenagers;
- in USA there is a worrying abuse of NPS among military forces;
- the NPS were increasingly widely used as adulterants of traditional drugs of abuse.

Global widespread access to the internet is an important supporting factor. The internet is a big marketplace for designer drugs because it facilitates distribution of information, networking, ordering chemicals and selling drugs worldwide. Often, new designer drugs are marketed as 'legal highs' or 'research chemicals.'

This new illicit market, revealed by Central Directorate of Anti-drug Services of the Department of Public Safety-Ministry of Interior, can be outlined as follows.

1. Order online from websites.
2. Payment by credit card / bit-coin / paypal (but also payment on delivery)

3. Shipping is from packaging and storage sites distributed across Europe. Substances stored there were purchased from chemical industries (actually mostly detected in China).
4. Delivery within 48 hours.

In this context data on the diffusion in Italy of NPS abuse are collected by the Department for Antidrug Policies-Presidency of the Council of Ministers (DPA), which instituted the National Early Warning System (NEWS) similar to European EWS.

87 synthetic cannabinoids, 42 synthetic cathinones, 67 phenethylamines, 5 piperazine, 8 tryptamines, 4 ketamine-related compounds are a part of the NPS intercepted in Italy from 2009 to 2013 by NEWS. In contrast to new therapeutic drugs whose metabolism and toxicity are usually thoroughly investigated before application in patients, no systematic risk assessment is performed for new designer drugs. Consequently, it is impossible to predict possible drug–drug interactions, interindividual variations in metabolic profiles, side effects and serious poisonings. At best, limited experiences from self experiments can be found in books such as PIHKAL [57], TIHKAL [58] and internet forums. Lacking dosage finding studies, quality controls and exactly weighed dosages, it is not surprising that the abuse of new designer drugs has led to several intoxications with both nonfatal and fatal outcomes in the last years [52].

NEWS registered in Italy about 600 cases of acute intoxication (35% caused by ketamine and related compounds, and 23% from SCN): 40% of people aged up to 25 years and a significant 60% of people aged over 25 [56]. The pattern of nationwide distribution of these cases is strongly inhomogeneous with some accentuated diffusion gradients, such as the area of the Triveneto. Serpelloni called the phenomenon in Italy a “silent tsunami” because of the lack of attention by media. The National Action Plan of the DPA for NPS provides a network of collaborating centers tightly interlaced with each other through the management structure of the NEWS. There are two specialist areas in NEWS, one for analytical and bio-toxicological aspects (National Institute of Health) and one for clinical-toxicological aspects (Poison Control Center of Pavia) [54].

The role of the chemical laboratory and the analytical identification of NPS is empathized in this National Action Plan. Laboratory must have suitable analytical tools dedicated to determination of NPS, which is essential for the sequels of competence (i.e. diagnosis, medical treatment, monitoring of the abuse or for eventual criminal or administrative

proceedings). In fact, action of the DPA provided for the upgrading of law enforcement laboratories throughout the country[54].

NPS: chemical classification [52]

Phenylalkylamines. The basic structures of amphetamine and 3,4-methylenedioxy-N-methylamphetamine (MDMA) have been the starting point for a lot of modifications that were made mainly at the aromatic system, less frequently at the amine function and the alkyl chain. The generated derivatives on the basis of their substituents can be divided into smaller subgroups: dimethoxyphenylpropanamines (i.e DOB), dimethoxyphenylethanamines (so-colled 2C family), Trimethoxy derivates, β-keto-compounds (i.e. mephedrone), methylenedioxyamphetamine-derived (i.e. MDPA), ecc.

Tryptamines. Tryptamine is the basic structure of hallucinogenic psilocybin, the psychoactive ingredient in 'magic mushrooms' and bufotenin, another naturally occurring psychoactive alkaloid. Designer drugs with tryptamine structure have an effect because of their similarity to serotonin.

Piperazines. Next to the phenylalkylamines, the representatives of the piperazine group have gained most significance, as can be seen in the scientific literature, which is full of analytical methods, case reports and papers that deal with analytical profiling of confiscated or purchased substances. Structurally the piperazines can again be divided in two subgroups: BZPs and phenylpiperazines. Since the beginning of the 21st century BZP and 3-trifluoromethylphenylpiperazine (TFMPP) have been circulating among drug consumers, sometimes in a combination that is said to have ecstasy-like effects but rather resembles to an amphetamine–mescaline mixture. Later, mCPP, MDBP and MeOPP appeared and other derivatives followed. The piperazine derivatives act as stimulants (e.g., amphetamine) and can also produce euphoria. Additionally, TFMPP and mCPP have small hallucinogenic potential that may be led back to an interaction of 1-aryl-piperazines with serotonin receptors.

Phenylcyclohexyl derivates. These compounds are structurally derived from phencyclidine (PCP) and probably act as NMDA channel blockers, as does PCP.

Synthetic cannabinoids. A large variety of compounds divided in 4 subgrpoups: 'classical cannabinoids', which are Δ^9-THC analogues like HU-210; cyclohexylphenols , a result of research of Pfizer, such as CP 497,47-C8, which was discovered in the most famous herbal blend 'Spice'; so-called JWH compounds, a large series of naphthoylindoles and naphthoylpyrroles and other naphthalin-containing structures like JWH-018 and JWH-073; Eicosanoids that resemble endogenous cannabinoids, as well as other species that do not share any structural characteristic with the three other groups.

Pyrrolidinophenones are structurally related to cathinone and the β-keto compounds, but differ from those in the pyrrolidine heterocyclic ring system that embeds the nitrogen atom of the amine function.

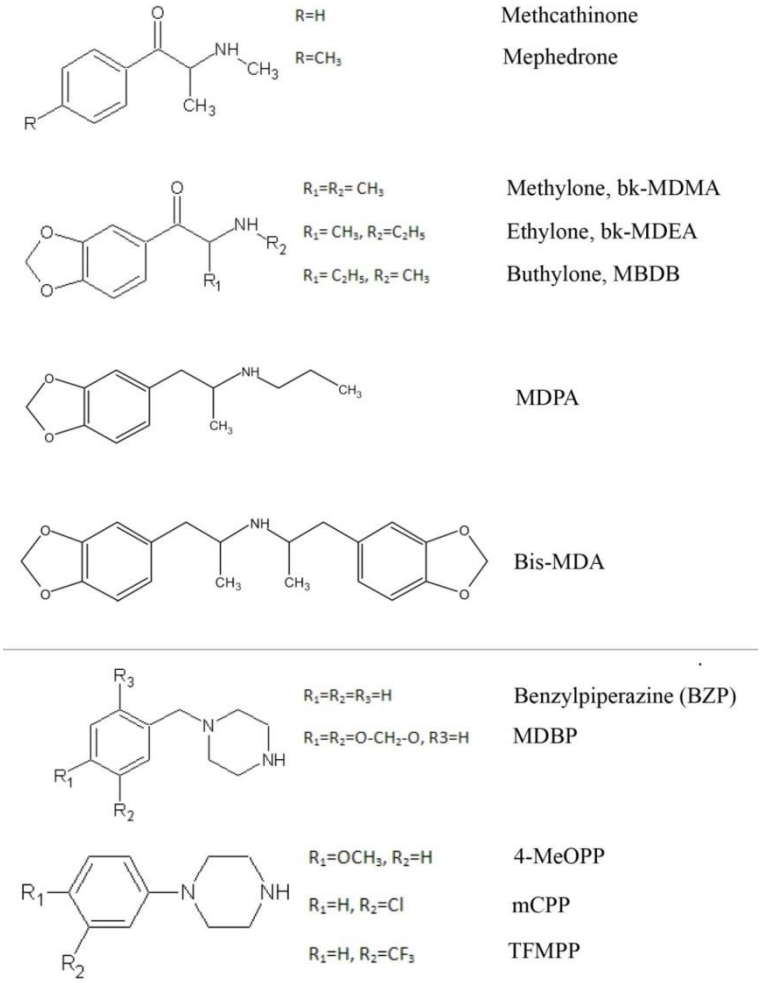


Figure B2.1 Chemical structures of some NPS. Structures of some phenylalkylamines: β-keto compounds and methylenedioxyamphetamine - derived (*top*). Structures of some piperazines: benzylpiperazines and phenylpiperazines (*bottom*)

2. Biological matrices

Biological samples are extremely complex matrices, containing many components that can interfere with chromatographic separations and/or mass spectrometer response; sample preparation is thus an important aspect of bioanalytical estimation.

Biological matrices can be divided into two groups, i.e. conventional and alternative matrices. In forensic analytical chemistry, analysis is typically conducted on fluids such as urine, serum and plasma which thus are conventional matrices. Besides these, alternative matrices such as oral fluid, hair, sweat or meconium have become important.

All these matrices are equally important for forensic purposes as they give different kind of information about the time of assumption and they show some distinctive features. In general, the detection time is longest in hair, followed by urine, oral fluid, and blood (**Figure 6.**).

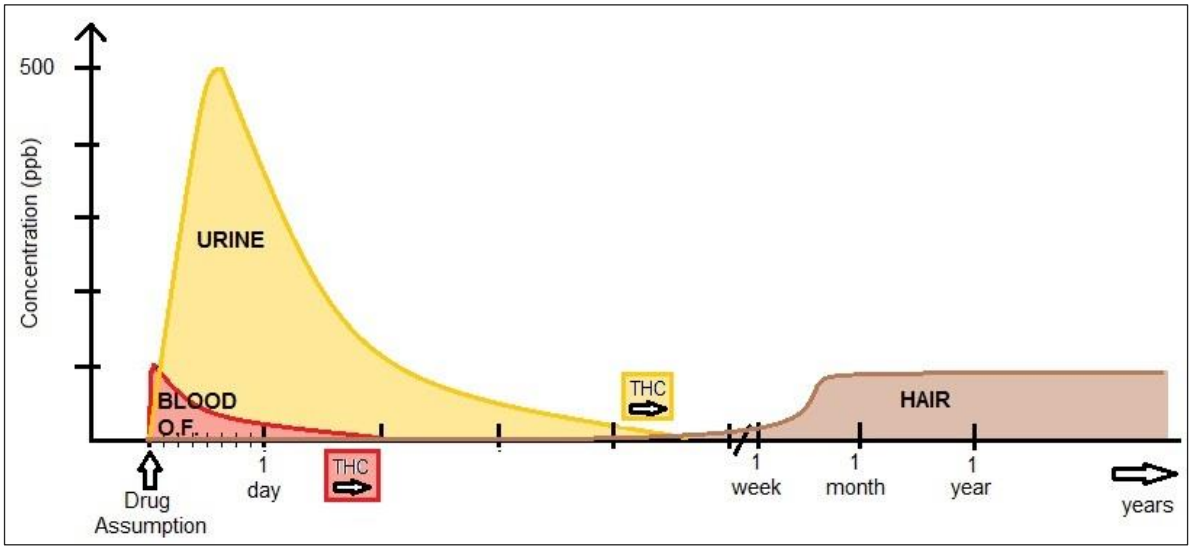


Figure 6. Median time windows of drugs in the most important biological matrices.

2.1 Blood, plasma and serum

Blood is the transporter of many vital substances and nutrients for the entire body (an adult has a blood volume of about 5 L), thus it contains many endogenous and exogenous compounds in a wide range of concentrations.. Whole blood (WB) consists mostly of water but also contains dissolved proteins, mineral ions, glucose, hormones, platelets and cells. The physiological pH is maintained at 7.4 by a complex buffer system and a minimal change

produces serious health consequences. The variation of physiological parameters within narrow limits makes blood an advantageous testing-matrix.

Plasma is the liquid part of blood which is prepared by spinning fresh blood containing an anticoagulant. Like WB it contains dissolved proteins which can lead to protein binding of the analytes and may be detrimental for the separation and detection.

Serum is the liquid fraction of WB and it is collected after the blood is allowed to clot; it has the same composition of plasma but it is deprived of fibrinogen and other clotting factors.

Most drugs are not equally distributed between the subcompartments of blood, it depends on the affinity of drugs for erythrocytes; consequently, the concentration in serum or plasma may differ from that in whole blood. Whole blood/plasma ratio (WB/P) typically vary between 0.5 to 1.2 for most drugs of abuse [59].

Determination of illicit drugs in WB, plasma and serum is of great importance in forensic toxicology because the substances can be detected just after the exposure, prior to metabolism. For this reason detection of drugs in blood can be very useful to evaluate the degree of impairment. Among the tested matrices blood presents the shorter time windows; drugs of abuse can be detected at the low nanogram per milliliter level for 1 or 2 days. However the detection time is influenced by many factors: the dose, the preparation and route of administration, acute versus chronic use, the nature of the molecule [19]. For example, the detection window for heroin is very short, amphetamines are detectable for about 48 hours in plasma while THC-COOH, the main metabolite of THC has an half-life of 2-5 days and can be detected during a period of 12 days in chronic users [19].

Drug determination in human plasma is not straightforward because of the high protein content which can lead to protein binding of the analyte, and is complicated by the low concentrations that are often found (0.1 – 10 ng mL^{-1} level). An extra problem posed by blood testing is the low sample volume that is normally available.

2.2 Urine

Compared with blood, urine is relatively free of protein, thus making it possible for direct extraction; simple dilution and even direct injection after centrifugation [60] are possible avoiding long sample preparation procedures.

Urine contains high concentrations of metabolites and provides for most drugs a longer detection time compared to blood, from several days for opiates and cocaine, up to months for chronic cannabinoid use. Urine is the matrix of choice for monitoring drug exposure in workplace testing and for screening and identification of unknown drugs also because their concentrations are relatively high. In addition its collection is physically non-invasive and it is often available in large volumes.

Limitations of urine drug testing are well known adulteration tactics, the pH variability that can affect the excretion pattern of certain drugs (amphetamines for example) and the low concentration or even lack of the parent drug [61]. In this case metabolites, which often consist of conjugates with glucuronic acid or sulphate must be detected. Another limitation of urine is that the amount of drugs are not well correlated with drug concentrations at the effector sites and with impairment. The presence of drug in urine is only a marker of previous use, and this does not necessarily mean that the person is under the influence; even though roadside drug screening is still performed using urine sample, the results are often of no legal usefulness.

Most drug conjugates are highly hydrophilic and not amenable to normally performed extraction methods. Thus, pretreatment procedures generally include gentle enzymatic hydrolysis or fast acid hydrolysis of such metabolites; alkaline hydrolysis is only suitable for cleavage of conjugates with an ester bond like THC-COOH glucuronide. Compared to enzymatic cleavage, acid and basic hydrolysis are less time consuming and cheaper, however not all the analytes are stable under the strong conditions required.

2.3 Oral fluid

Oral fluid is a composite of a number of fluids (including saliva) secreted into the oral cavity primarily by three glands: the parotid, submaxillary, and sublingual glands, as well as by other smaller glands. Limitations in oral fluid testing are its variable viscosity, pH and flow depending on a range of physiological variables. In addition external contamination (i.e. positivity does not arise from active drug consumption but rather from passive exposure) can be an issue; in these cases detection of metabolites can be crucial.

The transport mechanism of drug from blood to oral fluid is largely by passive diffusion particularly of unionized drug in blood plasma that is not bound to proteins [62]. Oral fluid

predominantly contains the parent drug because of its higher liposolubility that favours passive diffusion. For the same reason, drugs with high liposolubility tend to have higher concentrations. The concentration of drug in oral fluid is also dependent on the respective pH of the two fluids and the pKa of the drug in question. Basic drugs which are usually unionized at salivary pH (6.2-7.4) have a relatively high concentration in oral fluid compared to drugs with a low pKa.

Detection windows of drugs in OF (5-48 h) is similar to blood. OF testing has gained increased attention in the last years, especially in driving under the influence of drugs (DUID) investigations, first of all because it can be obtained easily by non-medical personnel in a non-invasive and observable way and also because for most drugs there is a good correlation with plasma concentration and with degree of impairment. In practice the OF/blood concentration ratio is not constant and for some drugs, especially THC, absorption in the mucosa of oral cavity results in poor correlation; THC has been shown to be detected in OF for up to 22 hours [40], long after a person might be adversely affected by the drug.

A common way to collect OF is the insertion in the mouth of a sorbent material on and around the tongue similar to a cotton bud. The device is placed in the mouth for a few minutes until sufficient fluid has been absorbed. This is then added to a diluent (aqueous buffered solution) that dilutes the OF and makes it more amenable to analysis but may be inconvenient if analytes are in very low concentrations. Furthermore most devices have been reported to have a significant effect on drug concentration found in OF [63, 64]: one of the potential pitfalls related to the use of absorbent swabs is that there may be some loss of drug on the swab itself. Expectoration or passive drool may be preferred in this respect, because, in addition to the lower costs, they better reflect real drug concentrations in excreted saliva [65]. However, some drugs have been reported to have lower stability in expectorated OF compared to OF collected with devices (Quantisal) [66]; in addition samples are often viscous and can be contaminated with food and other debris.

2.4 Hair

Hair is mainly composed of protein (65–95%, essentially keratin), water, lipids, and minerals. The hair shaft consists of an outer cuticle that surrounds a cortex; it originates from a follicle which is surrounded by a rich capillary system that provides the growing hair with necessary metabolic material. The growth of hair occurs in cycles, alternating between

periods of growth (anagen phase) and periods of quiescence (catagen and telogen phases). Head hair is produced at a rate of approximately 0.6–1.42 cm.month^{-1} [67]. Pubic hair, arm hair, and axillary hair are possible alternative sources for drug detection when scalp hair is not available; however, the data interpretation in these cases is more complex.

The precise mechanism by which chemicals are incorporated in hair is still unclear, but it is generally considered that xenobiotics can enter hair by at least three mechanisms: passive diffusion from blood capillaries into growing hair cells, deposition by diffusion from sweat or sebum secretions into the completed hair shaft, and from the external environment [68]. According to the passive diffusion model, excretion of drugs in hair should be delayed a few days because new hair takes some time to emerge from the skin surface and be available for shaving [21]. The chemical properties of the incorporated substance as well as the physical/physiological characteristics of the individual strongly influence which mechanism will dominate. Three key factors influence the drug incorporation. These include the melanin content of hair and the lipophilicity and basicity of the substance itself: pigmented hair will contain higher drug amount and similarly to OF, lipophilic and basic drugs will be more concentrated.

In abusers most drugs are expected to be found in the nanogram per milligram range; cannabinoids are usually found at lower concentrations (pg mg^{-1}) and THC-COOH is even less concentrated. In the last years it has been shown that single drug exposure is detectable in hair for most drugs, concentrations in the low picogram per milligram are expected [69, 70].

Hair differs from other materials used for toxicological analysis because of its unique ability to serve as a long-term storage of foreign substances, due to its large detection window which is weeks to months depending on the length of the analysed hair shaft. Other advantages are that it is easy to collect, store and transport. Moreover, segmental analysis of hair strands allows the determination of the historic pattern of drug use. Consequently, hair analysis of illicit drugs and medicines is currently employed to address a wide range of challenges, such as drug abuse history, workplace testing, post-mortem toxicology, therapeutic drug monitoring or drug facilitated assault (DFA) investigations [71]. In DFA cases, single exposure must be detected and hair is of particular value, because the crimes are often reported long time after any drugs have been eliminated from blood or urine.

The most crucial issue facing hair analysis is the avoidance of false-positive results caused by passive exposure to the drug. For this reason SoHT recommends that hair analysis starts with a wash step to remove external contamination [71]. Following this step hair is generally incubated in solvent medium where drugs are transferred and then extracted. Only the detection of drug metabolite(s) in hair establishes that internal drug exposure has occurred, verifying the positivity of the sample.

Extraction of drugs from hair is usually performed by enzymatic or alkaline digestion with concentrated NaOH (only possible when the analytes are stable under the required strong conditions), by methanol extraction which is compatible with almost all drug classes but is often incomplete, longer and gives dirt extracts or by aqueous acids or buffer extraction [68].

3. LC-MS Analysis

The toxicological analysis is essential in the clinical and medico-legal process, being aimed at seeking evidence of the presence of psychoactive substances in biological fluids and tissues. The fields of intervention of a clinical toxicology laboratory are mainly two:

- *clinical toxicology*, where the analysis are made with a therapeutic purpose*;*
- *forensic toxicology*, which performs analysis for administrative and forensic purposes.

Since errors in analytical forensic toxicology may lead to legal consequences for both the offender and the injured, obtain a meaningful and reliable analytical data is mandatory.

LC–MS or LC-MS/MS has helped this field closing the gap with respect to hydrophilic, thermolabile and non-volatile analytes that were not sufficiently covered by the established gold standard technique (GC–MS) [72]. Jenkins et al. compared with each other LC-MS, LC-UV and GC-MS determination of the specific case of MDMA showing how the coupling LC-MS technique is very versatile, sensitive and selective analysis of drugs of abuse and pretreatment is relatively simple [73]. LC-MS and LC-MS/MS are thus largely replacing GC-MS and GC-MS/MS in the analysis of drugs of abuse, because it does not require derivatization, is capable of simultaneous determination of both conjugated drugs of abuse that free forms in a single analysis without the intermediate step of hydrolysis pretreatment of the sample: these are important advantages in terms of analysis time and performance [74].

3.1 Development of an analytical method: guidelines

Specific guidelines or procedures have been adopted in order to assist the chemist and directing him towards the practical applications, for which a new analytical method is being created, including measures of verification and external assessment. In 1996 the American Board of Forensic Toxicology launched the Forensic Toxicology Accreditation Program based on the guidelines of the Society of Forensic Toxicologists in American Academy of Forensic Sciences (SOFT/AAFS): the final document was adopted and the latest version was published in 2006 [75]. In Europe there is a document relating to the performance of analytical methods and interpretation of results aimed to identification of drug residues in foodstuffs, which is sometimes used: the regulation 2002/657/EC [76]. The German guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh) are, instead, of a more exquisitely forensic. In 2006 a document entitled "Drugs of Abuse Testing Guidelines" was published by AGSA (Swiss Working Group for Drugs of Abuse Testing Guidelines) and supported by the major public institutions in Switzerland. However, there are also those issued by the Food and Drugs Administration (FDA) relating bio-analytical method [77], sometimes supplemented with EMEA's homologue document [78] and SOFT-AAFS or 2002/657/EC [76]. SOFT-AAFS guidelines also assist and guide the laboratory on the procedure to be followed before chemical analysis: from sampling to acceptance of sample, rules to be followed to ensure traceability: documentation that proves adequate chain of custody, without which the following analytical results would be unusable in a court.

All guidelines specify that in the case of analysis aimed at medico-legal purposes, the analytical steps envisaged are basically two: the *screening tests* and the *confirmatory tests*.

Screening tests allow to quickly analyze several samples, even by untrained personnel, in an economic, efficient and standardized to prevent a negative samples: these ones identify samples in which the concentration is below a threshold value defined cut-off (a reference value, expressed in concentration, above which the result of an analytical test is considered positive and below which the test is considered negative). These methods, therefore, should aim to limit the error of false negative below a certain threshold.

3.2 Validation

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data is related to the criteria used for the validation. Reliable analytical data are a prerequisite for correct interpretation of

toxicological findings in the evaluation of scientific studies, as well as in daily routine work. Unreliable analytical data might not only be contested in court, but could also lead to unjustified legal consequences for the defendant or to wrong treatment of the patient. Therefore, new analytical methods to be used in forensic and/or clinical toxicology require careful method development and thorough validation of the final method.

The fundamental parameters for this validation include accuracy, precision, selectivity, sensitivity, reproducibility, and stability [77]. Other parameters are: limits of detection (LODs) and quantification (LOQs), linear dynamic range (LDR) [75]. In the case of LC-MS/MS based procedures, appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method, as outlined by several authors [79-84]

3.3 Quality management and accreditation

Quality management and accreditation have become matters of increasing relevance in analytical toxicology in recent years. Forensic laboratory accreditation is based upon international standards (ISO/IEC 17025:2005 [85]) which include requirements for method validation and allows to control and monitor overtime the laboratory performance and its analysts by proficiency tests [86].

4. Sample preparation and matrix effect

To produce meaningful information, the analysis has to be performed on a representative sample of the material from which it was taken. Biological samples cannot normally be injected directly into the analysing system without sample preparation.

Sample pretreatment is thus of utmost importance for the adequate analysis of drugs and it is performed to dissolve or dilute the analyte in a suitable solvent, removing the interfering compounds and pre-concentrating the analyte. However, this is a difficult step which takes 50-75% of the total time of the analysis; conventional sample preparation involves PP and/or SPE and LLE.

When LC–MS(/MS) is the selected technique for detection the main function of sample preparation is the reduction of matrix effect (ME) by removing co-eluting interfering compounds; in fact one limitation associated with LC–MS, especially using electrospray (ESI) ion source, is its susceptibility to ME.

In this case ME is defined as the effect of co-eluting residual matrix components on the ionization of the target analyte. Typically, suppression or enhancement of analyte response is

accompanied by diminished precision and accuracy of subsequent measurements. ME thus limits the utility of LC–MS for quantitative analysis and is a major issue in method development.

MEs usually interfere with the analyte response proportionally to analyte concentration, they occur through reactions or interactions between the analyte(s) and some matrix constituents resulting in species that can either suppress or rarely enhance signal response in the MS. Ion suppression in ESI has been explained by different mechanisms [87], these include:

- competition between matrix components and analyte ions that are co-eluting in the sprayed solution for access to the droplet surface for gas-phase emission;
- matrix interferences that compete for available charge;
- co-precipitation of the analytes with non-volatile materials;
- neutralization of analyte ions through gas phase acid/base reactions.

Current validation guidelines for bioanalytical methods require the evaluation of ME as a part of method development; there are two common methods to assess MEs, i.e. the post-column infusion and the post-extraction addition methods. The former, initially proposed by *Bonfiglio et al.* [88], method provides a qualitative assessment of MEs by the identification of regions in the chromatogram that are most subjected to suppression or enhancement. This is carried out by monitoring the signal of a constant infused solution of the analyte(s) into the eluent from the analytical column when a blank sample is injected, by a post-column tee connection. In the absence of matrix interferences the continuous post column infusion led to a constant signal in the detector, while elution of compounds that can enhance or suppress the analyte signal led to increased or decreased signal. In contrast, the post-extraction addition approach allows a quantitative assessment of the MEs by comparing the response of the analyte in a standard solution with the response of the analyte spiked into a blank matrix sample after all the extraction procedure. Generally MEs are assessed at different concentrations over the linear range (at least 2); an alternative approach to evaluate MEs over the entire range is to compare the slope of two calibration curves, one prepared in solvent and one in matrix (post-extraction). *Peters et al*. have highlighted in a recent review [89] the importance of assess the variability of ME (RSD%) using at least 6 different matrix sources and they suggest that the variability of MEs between samples is at least as important as the average extent of these effects.

Residual matrix components in biological materials which cause ion suppression are mainly endogenous phospholipids, sugars or salts, creatinine in urine, amino acids and proteins. Because phospholipids have been identified as a major source of MEs when dealing with biological materials, multiple researchers have shown the convenience of monitoring the levels of various phospholipids in the samples to assess the effectiveness of the clean-up. In fact it has been shown that even if phospholipids do not co-elute with drugs, their presence can result in retention time shifts, elevated baselines, and divergent curves, thus influencing assay performance [90]. Single phospholipids can be monitored [91] or alternatively in-source collision induced dissociation (CID) with a high cone voltage and a low collision energy can be used to monitor the 184 and 104 ions only, which are characteristic of glycerophosphocholines (GPChos) and 2-lyso glycerophosphocholines (2-lyso GPChos) respectively, resulting in total ion chromatograms (TIC) representing all compounds containing the phosphocholine head group [92, 93].

Clearly large differences in the extent of matrix effects can be observed between different matrix types and between different sample preparation techniques, including direct injection, dilution, protein precipitation (PP), solid-phase extraction (SPE) and liquid‑liquid extraction (LLE).

The reduction of MEs can be obtained by changing chromatographic parameters so that the analyte does not fall into suppression zones, by the use of atmospheric pressure chemical ionization (APCI) instead of ESI which is less susceptible to matrix effect [94] or better by optimizing the sample preparation step.

4.1 Protein precipitation

PP is commonly used for fast sample clean-up in illicit drugs testing. This technique is based on the removal of proteins from the matrix by denaturing them directly in the initial sample, commonly by the addition of a water miscible organic solvent (e.g. methanol, ethanol, acetonitrile or acetone), a strong acid, such as trichloroacetic acid (TCA) and trifluoroacetic acid (TFA) or less frequently by means of salts and metal ions. The denatured proteins are then removed from the sample by centrifugation. The precipitant agent is often able to disrupt protein-drug binding, allowing drug direct determination.

Precipitants exert specific effects on proteins to facilitate their precipitation from solution. Protein solubility in biological fluids results from polar interactions with the aqueous

solvent, ionic interactions with salts and repulsive electrostatic forces between equally charged molecules.

Polson et al. [95], comparing different protein precipitation approaches, found that the most efficient protein precipitants for protein removal are zinc sulphate, acetonitrile and TCA when the ratio to plasma volume is 2:1 or greater; lower ion suppression is obtained with acidic precipitants since they extract less interfering compounds, however when dealing with lipophilic drugs, acidic precipitants result in lower recoveries. Often different protein precipitants such as zinc sulphate/methanol [96] can be combined to obtain optimal performances; in the specific case it has been shown that the use of the two combined precipitants provided samples with a content of phospholipids 2.5 times lower, compared to other precipitants.

PP is commonly used for analysis of drugs of abuse in plasma/serum and OF [97], it is the fastest and simplest clean-up approach and is applicable for both hydrophilic and hydrophobic compounds, however it is not a very efficient technique due to its non-selective nature, in addition the analyte(s) may be adsorbed on the proteins, and a significant amount of un-precipitated plasma components may still remain. A number of authors, compared different sample preparation techniques and found that PPT is the least effective and causes significant ion suppression for many compounds [83, 91]. Very often PP is used prior to SPE to have cleaner extract.

a. Ultrafiltration

Ultrafiltration uses a membrane filter to separate the proteins from the sample. A centrifugal force is applied to accelerate the diffusion and, thus, the entire process. Low molecular weight compounds are able to pass through the filter, which has pores of a certain size range, while the proteins are retained on the filter.

Ultrafiltration has been shown to be a suitable technique for clean-up applications since it allows the reduction of matrix effect with a minimal handling. Compared with classical PP, the clean-up appears more efficient in terms of matrix effect reduction and automation of the procedure [98].

b. Protein precipitation plates

PP plates are membrane-based filter firstly developed by *Biddlecombe et al*. [99]. Structurally, these plates contain tubes with a membrane filter and are attachable to vacuum

filtration; after precipitation of protein, filtration can be carried out in the same well without centrifugation and supernatant transfer steps. Generally they are available in 96-well format, which enables both manual and robotic automation for the entire process.

It has been shown, than compared to PP, use of PP plates leads to more reproducible results [100]. Moreover, significant reduction in the sample preparation time is obtained.

This approach has recently been used for the rapid analysis of drugs belonging to different classes in OF [101]; high recoveries were obtained for all analytes however significant matrix effects were found for a number of drugs.

4.2 Liquid liquid extraction

LLE is a classical technique involving the partitioning of analytes between two immiscible liquids, based on the octanol-water partition coefficient. The choice of the extraction solvent is crucial, it should match the analytes polarity while still being immiscible with water and it should preferably be compatible with the detection method. Other factors, such as pH and ionic additives may greatly affect the extraction efficiency; extraction is performed at a pH value at which the analyte is not ionized.

This technique is quite easy however some shortcomings: emulsion formation, use of large sample volumes and toxic organic solvents make LLE expensive, time-consuming and environmentally harmful. Another drawback of LLE is its unsuitability for hydrophilic compounds such as many drugs metabolites. In addition organic solvent do not provide a selective extraction and when dealing with complex matrices such as blood, LLE has the potential for increased matrix effects compared to SPE [91].

Despite its disadvantages, LLE is still widely used in the sample preparation of biological fluids for the analysis of illicit drugs and can be convenient when a wide range of different analytes must be extracted [102].

a. Miniaturized techniques

The miniaturization of LLE has led to the development of a number of new methodologies. Miniaturized LLE techniques use negligible volumes of extracting solvent and a minimum number of steps.

Single-drop micro-extraction (SDME) is based on the use of a micro-drop (1-10 μL) of extraction solvent immiscible with the sample. In direct injection (DI)-SDME the micro-drop is suspended in a stirred aqueous solution from the tip of a micro-syringe needle. After

extracting for a given period of time, the drop is retracted back into the micro-syringe needle and finally injected into the chromatographic system. Head space (HS)-SDME allows to work with more volatile analytes, the extraction is performed by exposing the solvent drop to the head-space above the sample. Another sub-category of SDME is constant-flow micro-extraction (CFME) [103], in this case the extraction is carried out by a micro-syringe which is introduced in an extraction chamber where the sample is continuously pumped at a constant flow-rate. Due to the continuous contact with the flowing fresh sample solution, the extraction efficiency and concentration factor are higher than in static extraction [104]. Liquid-liquid-liquid micro-extraction (LLLME) is also based on a single drop extraction and is suitable for ionisable analytes. The analyte is firstly extracted into an organic layer which possesses a lower density than water and then back-extracted into an aqueous micro-drop. The pH of the aqueous solution and the aqueous micro-drop can be adjusted to obtain firstly the neutral form of the analyte, extractable by organic solvent and then ionize it to be extracted into aqueous drop [105].

In hollow fibre liquid phase micro-extraction (HF-LPME), analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent immobilized within the pores of a porous hollow fibre, and into an acceptor solution inside the lumen of the hollow fibre [106]. The whole assembly is placed in a sample solution and the target analytes are extracted from the sample through a supported liquid membrane; the micro-extract is not in direct contact with the sample solution. As a result, samples may be stirred or vibrated vigorously without any loss of the micro-extract. Analyte enrichment factors are very high without the need for evaporation and reconstitution.

Dispersive liquid-liquid micro-extraction (DLLME) is based on a ternary component solvent system in which an appropriate mixture of extraction solvent and disperser solvent is quickly injected into the aqueous sample with a syringe. The mixture is then gently shaken and a cloudy solution is formed; after centrifugation, the fine particles of extraction solvent are precipitated in the bottom of a conical test tube and collected with a syringe. The water-immiscible extracting solvent should have a higher density than water, while the disperser solvent should be miscible in the extracting solvent and the aqueous sample, usually acetone, acetonitrile and methanol are used for this purpose [107]. The fine droplets of extraction solvent are dispersed throughout the aqueous sample, allowing interaction with the analyte.

All the cited LLME techniques are quite diffused in forensic toxicology for the extraction of multiple analytes from urine [108-113] and hair [114-117].

b. Pressurized liquid extraction

Pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE®) involves extraction with solvents at high pressure and temperature without reaching their critical point, so that they are kept in the liquid state. The use of high pressures allows employing solvents above their atmospheric boiling points to increase solvation power and extraction kinetics. The utilization of organic solvent can thus be reduced or even eliminated as the dramatic changes in the physical-chemical properties of water enhances its usefulness as an extraction solvent.

PLE can be performed both in the static and dynamic modes; in the first mode sample and solvent are maintained for a defined time at constant temperature and pressure while in dynamic mode fresh solvent is continuously introduced to the sample improving the extraction but diluting the sample. Optimization of the extraction process generally begins with an appropriate choice of the extraction solvent. A single solvent, a mixture of solvents of different nature or solvents modified with additives such as surfactants can be used. PLE extraction is generally not selective thus resulting in the co-extraction of different matrix components, thus PLE eluent is generally submitted to a clean-up procedure.

PLE has advantages over other extraction methods, including better reproducibility, the reduced use of extraction solvent, reduced time and automation of sample preparation [118]. PLE can be used exclusively with solid samples thus limiting its application in forensic toxicology where analysis are usually conducted on liquid matrices; applications in drug testing are limited to airborne particles [119] and wastewater particulate [120, 121]. In the present study PLE has been tested for the extraction of drugs from hair with excellent results [122].

c. Salting-out LLE (SALLE)

The concept of salting-out is well-known, when an inorganic salt is added to a mixture of water and water-miscible solvent it leads to the separation of the solvent from the mixture, forming a biphasic system. Salting-out in bioanalysis using acetonitrile as organic solvent was firstly introduced by *Rustum et al.* [123]. The salt-induced phase separation between acetonitrile and aqueous solution may be induced by the addition of a variety of inorganic

and organic electrolytes, mainly magnesium, ammonium or calcium sulphate, sodium or calcium chloride but also ammonium acetate which is more "LC–MS friendly".

Overall, SALLE offers several advantages over conventional LLE such as applicability to a broad range of drugs from low to high lipophilicity, improved recoveries and analyte enrichment, availability of a range of salts, making it possible to comply efficiently with LC–MS/MS methods. A recent application of SALLE in forensic toxicology has regarded the extraction of synthetic cannabinoids metabolites from urine [124]

4.3 Solid phase extraction

SPE is the most popular sample pre-treatment approach in drug testing, its main advantages are: high recoveries, need for less organic solvent (compared to LLE), possibility of pre-concentration, ease of operation and greater possibility of automation [118].

The technique involves passing a liquid sample through a solid sorbent bed, usually consisting of modified silica particles. The aim is to retain the analytes in the sorbent bed, wash away interferences and finally elute the analytes as a clean extract in a small volume. One advantage of SPE over LLE is that it requires lower amounts of organic solvents for the extraction, which is important for environmental and health reasons. SPE also often offers higher selectivity for the target compound(s) due to the wide range of sorbent types available. Other advantages of SPE are its higher enrichment factors and the possibility of automation.

Different formats and SPE sorbents which provide various selectivity is available. The choice of sorbent phase is the key factor in SPE, by far the most widely used phase is chemically bonded silica C_{18} and less C_8; in the last years polymer-based sorbents (polystyrene, divinylbenzene and many others) are more and more diffused, their advantages being stability over the entire pH range and higher surface areas. Ion exchange materials, grafitized carbon and mixed mode sorbent (containing both nonpolar and strong cation/anion exchange) are also quite diffused. Recently new sorbents based on increased selectivity, such as molecular imprinted polymers (MIPs) and aptamers (reviewed in the next paragraphs) have been introduced.

The analyte can be retained on the sorbent surface by a range of different types of interaction, such as hydrogen bonds, dipole-dipole or dipole-induced dipole mechanisms,

dispersive, charge transfer or ionic interactions. The three main modes of SPE for organic compounds utilizing one or several of these interactions are: reversed-phase, normal-phase, and ion-exchange systems.

In spite of all advantages stated above, SPE in some cases is time-consuming and relatively expensive, as SPE cartridges are manufactured for single use only. They sometimes have poor batch-to-batch reproducibility and still require a relatively large amount of organic solvent.

a. Miniaturized techniques

SPE devices are available in a variety of formats, and in the last years there has been increasing attention towards miniaturized devices. Miniaturized SPE offers the big advantage of reducing the volumes of sample and solvents used in the process, in addition new formats allow to minimize the manual operations associated with SPE reducing time required for extraction.

µ-SPE or disposable pipette extraction (DPX) uses a standard pipette tip which contains a sorbent loosely packed between two frits. Being a pipette tip, is it easy to take the solvent in or out through this dispersive sorbent, without requiring vacuum neither for loading or elution, as it occurs in classic SPE. In addition using automatic pipettes several specimens can be processed simultaneously, improving laboratory throughput.

The steps involved with µ-SPE are similar to those of SPE, but normally the sample is withdrawn/aspirated through the tip several times to obtain an effective extraction both in the loading and elution phase. Commercially tips are available with a variety of sorbents ($C_{18,}$ C_8, anion and cation exchange) and capacity (200 µL to 5 ml). µ-SPE has been used in several fields, recent applications with drugs of abuse are found in the literature. A method for the analysis of several drugs of abuse in urine has been reported [125], while in other studies DPX was used for the extraction of opiates from vitreous humour [126] or THC and THC-COOH from blood and urine using 200 µL of sample [127]. µ-SPE has been also used by our research group for the extraction of a wide variety of drugs from OF [42] , plasma and urine [128] whose optimization has been part of the PhD project.

Another new technique which is based on the miniaturization of SPE is micro-extraction on packed sorbent (MEPS) which has been firstly introduced by *Abdel-Rehim et al.* [129]. In

MEPS the sorbent bed is reduced to a few mg and inserted in the barrel of a syringe (100-250 µL). The technique is designed for sample volumes ranging from 10 µL to 1000 µL and provide easy automation by directly insert the syringe in an autosampler. Similarly to µ-SPE, samples can be drawn and ejected several times to increase extraction efficiency and is also possible to work with a large volume, by aspiring multiple times from the same vial if pre-concentration of the sample is required.

MEPS protocol involves pre-treatment of complex samples such as dilution and centrifugation. Dilution with water followed by centrifugation is recommended for plasma/serum and whole blood samples. One of the most significant advantages of MEPS is that the same sorbent bed can be reused many times just by washing with water and an appropriate solvent; it has been reported that the same needle can be reused up to 120 times without any loss of performance [130]. All the sorbent normally found in SPE can be used with MEPS, included MIPs to obtain an increased selectivity [131, 132] . Recent applications of MEPS in forensic toxicology include the rapid screening of opiates [133] and cocaine [134] in urine. In the experimental part (section I) the optimisation of a method for the extraction of cannabinoids from OF using MEPS will be described [135].

Solid phase micro extraction (SPME) is the first miniaturized SPE technique, which has been introduced in the 1990 by Arthur and Pawliszyn [136]. Today is very diffused and used as frequently as the traditional preparation techniques. SPME devices consist of a modified syringe that contains a fused-silica fibre tip which is coated with an organic polymer within its syringe needle. In contrast to conventional SPE, SPME syringe assembly allows combination of all the steps of sample preparation into one step, thus reducing sample preparation time, use of organic solvents and disposal cost. There are two types of extraction modes for SPME: direct immersion of fibre into liquid sample matrix, (DI-SPME) and head-space (HS-SPME) extraction in which the liquid sample matrix is heated in a vial to volatilize the analytes and the fibre is placed just above the sample matrix. In a typical protocol, the two essential steps are extraction and desorption. Conditions such as pH, salt concentration, sample volume, sample agitation/stirring speed, extraction temperature and extraction time strongly affect the extraction efficiency and must be optimized. For desorption SPME offers various options, when coupled to GC thermal desorption within the GC injector is possible while in LC desorption is carried out by a suitable solvent. A great

advantage of SPME is that derivatization and extraction can be performed simultaneously on the fibre.

Various commercially available fibre coatings are polydimethylsiloxane, polyacrylate, polydimethylsiloxane-divinylbenzene etc. which allow the extraction of analytes of different polarities. In addition selective microextraction coatings have been developed based on antibodies, aptamers [137], and molecularly-imprinted polymers (MIPs) [138]. The fibres are easy to reuse. SPME is widely used in forensic toxicology and most applications have been reviewed by *Pragst* [139].

Stir bar sorptive extraction (SBSE) is a development of SPME and instead of fibre, the extraction phase is coated onto magnetic stir bars. The stir bar is added to the sample and the solution is stirred, analytes will partition between the sample matrix and the extraction phase; the extraction of an analyte from the aqueous phase into the extraction medium is controlled by its partitioning coefficient between the sorbent phase and the aqueous phase. In SBSE the volume of polymer coated onto the bar is 50-250 times larger than in SPME [140] and so SBSE afford greater recoveries. Similar to SPME, SBSE can be used by direct immersion (DI) and head-space (HS). The main drawback of this technique is the duration of extraction, typically 20-150 min which limits its application in routine analysis. In illicit drug testing SBSE has been applied only in a few studies, for example for the extraction of amphetamines and ketamine from urine [141].

Part I

Versatility

Analysis in biological fluids

µ-SPE for determination of illicit drugs in plasma, urine and oral fluids -

EXPERIMENTAL

1. Chemicals and Reagents

Amphetamine (AMP), methamphetamine (MAMP), MDA, MDEA, MDMA, cocaine (COC), benzoylecgonine (BZE), mescaline (MES), ketamine (K), phencyclidine (PCP), psilocybin (PSY) and internal standards (ISs) methamphetamine- d5, MDMA-d5, benzoylecgonine-d3 and phencyclidine-d5 were purchased from S.A.L.A.R.S. S.p.A. (Como, Italy). The purity of the reference compounds was ≥99%. All standards were provided at a concentration of 1 mg mL^{-1}. Acetonitrile and methanol were of RS-Plus grade. Ultrapure water was produced by a Milli-Q Plus apparatus from Millipore (Bedford, MA, USA). All solvents were obtained from Sigma-Aldrich (Milwaukee, WI, USA).

Individual stock solutions were prepared in methanol at 0.1 mg mL^{-1} (1 μg mL^{-1} for the ISs); working standard mixtures of the analytes were prepared by appropriate dilution of the standards in methanol at concentration of 10 μg mL^{-1} in methanol and stored at -20 °C.

For the extraction procedure the following devices were used: ultrasonic bath Starsonic 18 from Liarre (Bologna, Italy) and micro-centrifuge D3024 from Scilogex (Berlin, CT, USA).

2. Sample Preparation

μ-SPE extraction was performed using OMIX C_{18} tips from Varian (Palo Alto, CA, USA). The functionalized fibre was initially conditioned by flushing twice an ultrapure water/acetonitrile solution (1:1, v:v) and three times an ultrapure water/methanol solution (9:1, v:v) using an automatic propipette from Phenomenex (Bologna, Italy).

Urine samples were diluted with ultrapure water (1:1, v:v), while plasma and OF were processed without prior manipulation. 180 μL of sample (plasma, OF or diluted urine) were then mixed with 20 μL of methanol containing ISs at a concentration of 1 μg mL^{-1}.

The mixed solutions were sonicated for 6 min at room temperature and centrifuged at 10,000 x g for 5 min to remove the precipitated proteins. 100 μL of supernatant were then collected and passed through the tip by five load/release cycles of the pipette. After a washing step with 100 μL of ultrapure water, analytes elution was achieved using 100 μL methanol containing 10 mM formic acid. Four μL of the eluate was directly injected in the LC–MS/MS system.

3. LC–MS/MS analysis

The HPLC equipment consisted of a Series 200 Micro-LC Pump and a Series 200 autosampler from Perkin Elmer (Norwalk, CT, USA). An API 2000 from PE-Sciex (Toronto, ON, Canada) equipped with a TurboIon-Spray source was used.

The 11 analytes selected for the multi-class method were separated using a reverse phase C_{18} Kinetex column from Phenomenex packed with core-shell particles (2.6 μm, 2.1 x 150 mm). A Phenomenex security GuardUltra Cartridge (packed with C_{18} particles) was also used to protect the column from damaging contaminants and microparticulate. The mobile phases were methanol:acetonitrile (1:1, v:v) (phase A) and 5 mM formic acid in water (phase B); the flow rate was 0.2 mL min^{-1} entirely transferred into the mass spectrometer source. The analyte separation was performed using a gradient elution according to the following steps: increase of the phase A from 0 to 25% in 0.5 min then to 45%, in 7 min and to 100% in another 3 min; afterwards the system is switched back to the initial conditions in 5 min. The complete separation of all substances occurred in 12 min. A timed switched valve (a 10 routes Valco valve connected to the instrument) drived the effluent to the source only from minute 5.0 to 12.5 and then from 16.0 to 18.5. All the analytes were detected in positive ionization (PI) with a capillary voltage of 5,000 V, nebulizer gas (air) at 65 psi, turbo gas (nitrogen) at 35 psi and 400 °C; focusing potentials and entrance potential were set at 400 V and 10 V, respectively.

Two multi-reaction monitoring (MRM) transitions were selected for each analyte. All source and instrument parameters for the monitored analytes were tuned by injecting each single standard solution at a concentration of 100 ng μL^{-1} at 10 μL min^{-1} by a syringe pump; all the source parameters have been checked (and revised as necessary) in flow injection analysis with the optimized chromatographic conditions.

Peak areas for selected ions were determined using PE-Sciex package Multiview 1.5 and quantitation was performed by the IS method. The selected transitions, together with the main HPLC–MS/MS parameters, are reported in Table I.1.

Analyte	R_t (min)	Q1 (amu)	DP (V)	Q3 (amu)	CE (V)	CXP (V)
Amphetamine	5.5	136.1	15	91.2	28	4
				119.1	33	3
Benzoylecgonine	8.2	290.1	50	168.1	30	5
				105.0	44	6
Cocaine	9.6	304.1	23	182.3	29	3
				82.0	49	2
Ketamine	8.0	238.1	21	125.3	42	8
				179.3	26	7
MDA	6.5	180.2	13	163.3	16	5
				105.1	33	5
MDEA	7.6	208.1	21	163.2	20	5
				105.1	37	7
MDMA	6.9	194.2	20	163.2	20	5
				105.1	37	5
Mescaline	6.6	212.0	19	195.2	29	5
				180.2	10	4
Methamphetamine	6.1	150.0	22	91.1	31	3
				119.1	16	2
Phencyclidine	10.7	244.2	10	159.3	21	8
				91.2	58	10
Psilocybin	5.4	285.0	138	58.2	45	6
				225.2	20	5
BZE-d3	8.2	293.1	50	171.1	30	5
MDMA-d5	6.9	199.2	20	165.2	20	5
MAMP-d5	6.1	155.2	22	121.3	16	2
PCP-d5	10.7	249.2	10	164.2	21	8

Table I.1. HPLC–MS/MS conditions for selected analytes and ISs

4. Validation

Methods were fully validated according to SOFT-AAFS guidelines [3] and *Peters et al.* [142]. For this purpose the following parameters were evaluated: limits of detection (LODs) and limits of quantitation (LOQs), linearity, precision, accuracy, recovery, stability, matrix effect, specificity and carryover. Calibration standards were prepared in 10 mM formic acid in methanol, while quality control (QC) samples, used for validation experiments were prepared in authentic matrix.

4.1 Linearity, selectivity and carryover

Calibration standards were prepared at six concentration levels in three replicates each one. Linearity was checked from LOQ to 100 ng mL^{-1} in plasma, LOQ to 200 ng mL^{-1} in OF and LOQ to 1000 ng mL^{-1} in urine.

Analyte responses were normalized to internal standards and quantified from calibration standards by linear least-squares regression.

Two product ions were selected for each compound, one for quantitation and one for qualitative analyte confirmation. For identification quantitative to qualitative ion ratios were required to be within 20% of those in QC samples. The presence of any matrix interferences at the retention time of the selected analytes was observed by analysing ten blank samples of urine, plasma and OF.

Carryover phenomena has been verified by injecting the calibrators, spaced with injections of pure methanol, at increasing and decreasing concentration in triplicate.

4.2 LODs and LOQs

LODs represent the smallest concentration of a drug needed to give a peak height three times the noise level of the background signal from a blank sample. According to SOFT/AAFS guidelines [5], LODs were derived experimentally on the analyte MRM transition with lowest S/N analyzing blank OF samples and blank samples spiked with decreasing amount of standard solution at the appropriate concentration; LOQs were determined similarly as the smallest concentration of a drug needed to give a peak height ten times the noise level of the background signal from a blank sample. In addition quantifier to qualifier ratios within ±20% of mean QC and both accuracy and precision within ±20% were required at LOQ level.

4.3 Accuracy and Precision

Accuracy and precision were determined at three concentration levels:

- 20, 50 and100 ng mL^{-1} in plasma and OF.
- ½ cut-off, cut off, and 1.5 cut off values in urine

Precision was expressed as the relative standard deviation RSD (%); intra-day precision (repeatability) was calculated for each analyte, from the areas of six independent samples spiked before extraction, for each concentration level; inter-day precision (reproducibility) was obtained from the areas of 18 spiked samples analyzed over six days (six independent samples per day). Accuracy was estimated from six samples spiked before extraction step; the concentration relative to the mean peak area (Cc), was calculated by the calibration curve equation and was compared with the theoretical concentration (Ct). Accordingly A% = Cc/Ct × 100.

4.4 Recovery and matrix effect

Recovery (R%) was calculated at three concentration levels (see above § 4.3.).

Six QC samples for each concentration were spiked with the appropriate amount of standard stock solution and processed, while an equal number of samples were processed as blanks and spiked with the same amount of standard stock solutions after the clean-up step. Recovery values were obtained by comparing, for each analyte, the mean peak area of samples spiked before the extraction (A) with the mean peak area obtained when the analytes were spiked after the extraction procedure (B). Accordingly, $R(\%) = A/B \times 100$.

The interference of the matrix on the S/N ratio of each analyte was calculated comparing the slopes of two calibration curves, one obtained in solvent and the other prepared in a pool constituted by 20 independent matrices. Furthermore 6 independent blank samples were spiked at a concentration corresponding to three times the LOQ for each analyte, to assess the variability of the matrix effect.

4.5 Stability

Stability tests were run on processed QC samples fortified with the analytes and standard solutions in methanol both for short term and long term stability (two months). In addition real samples spiked with the analytes and kept at -20°C were analyzed over several days for the full term of 2 months.

To assess short term stability processed samples and standard solutions were kept at -20°C and three different concentration levels were analyzed at t = 0, 1,7, 14 days. For long-term stability, processed samples were analyzed over several days (t = 0, 30, 60) for the full term of 2 months. The influence of three freezing/thawing cycles was also evaluated. Responses were compared to the t = 0 samples amount.

RESULTS AND DISCUSSION

In this part, a miniaturized SPE techniques, namely µ-SPE, has been tested for the extraction of drugs belonging to different classes from urine, plasma and OF. The method, initially developed for OF drugs determination, was later extended and harmonized by including the two conventional matrices.

Different procedures based on liquid chromatography–mass spectrometry (LC–MS) and LC–MS/MS for determination of drugs in OF: an exhaustive review of whole analytical procedures necessary for the determination of drugs of abuse (sampling, pre-treatment, analysis confirmation) has been reported by Samyn [143]. Recent studies on OF have demonstrated the usefulness of LC–MS for the determination of illicit drugs[144] and for the identification and quantitation of amphetamines, cocaine, opiates, and phencyclidine in oral fluid by LC–MS/MS [145]. Ultraperformance LC–MS/MS assay for 29 illicit drugs and medications and simultaneous screening and quantification in OF has been also reported [146, 147]. LC–MS methods, however, present problems related to matrix effects in terms of endogenous compounds such as salts, proteins or metabolites [83, 148, 149]. Thus, because of the high viscosity of OF due to the presence of mucine, a clean-up step is necessary prior to analysis [150] Some authors have also described a direct injection in OF and blood [151]. Many LC–MS methods for the determination of drugs of abuse in plasma have been published, for example, for the determination of cocaine and its metabolites [152, 153], amphetamines and designer drugs [154]; only few methods for the simultaneous determination of amphetamine, BZE and cocaine in plasma have been reported [96, 151, 155]. Sample pre-treatment consisted rarely in a simple protein precipitation (PPT); usually a SPE step was used in addition. Several methods for the simultaneous determination of amphetamine, designer drugs, cocaine and metabolites based on LC–MS/MS in urine have been published [60, 156], but not including hallucinogens; a direct injection (after ten-fold dilution of matrix) LC–MS/MS method for the identification and quantification of amphetamine, methamphetamine, MDA and MDMA in urine drug testing has been developed by Andersonn et al. [157] and a work describing a procedure by SPE followed by UPLC–MS/MS has been also reported [158].

1. LC–MS/MS conditions

To improve selectivity and sensitivity of both methods, Kinetex chromatographic columns, with Core–Shell^{TM} technology with an average diameter of particles 2.6 microns, were used.

The use of columns with superficially porous packing materials is a relatively recent trend in chromatographic separation. These columns allow greater separation efficiency and speed of analysis by reducing the diffusion of the analytes. With reference to the Van-Deemter equation, their use dramatically improves chromatographic efficiency by the combination of a lower axial diffusion (due to the solid core of the particle) and a lower eddy dispersion term (due to narrow particle size distribution). The advantage of using columns with superficially porous packing materials is their greater efficiency in high speed analysis without the generation of high back-pressures, which is typical in UPLC and requires for special LC equipment.

In the multi analyte method here developed, the main problem in the chromatographic set-up was the separation of the amphetamine type molecules that exhibited the same ion fragments. We selected a column of 15 cm which gave a better resolution, compared to an identical column of 10 cm. In addition to obtain an increased resolution the influence of a certain amount of acetonitrile in the organic phase, consisting solely of methanol, has been tested. These tests, carried out at different percentage, resulted in an increase in selectivity using 10% acetonitrile, no further improvement for higher amounts was achieved. The stability of the LC method was evaluated by calculating the retention time variations. The RSD, calculated from retention times obtained over 30 injections, proved to be less than 2.0% for all compounds. The performances of core shell columns were evaluated comparing a Gemini C_{18} column, previously used in our laboratory, with a Kinetex. It was verified that the latter column allowed to reach detection limits substantially lower (about three fold) for the considered analytes in biological samples. In Fig. I.1. the extracted ion currents for the selected analytes, obtained from the analysis of a fortified blank sample are reported.

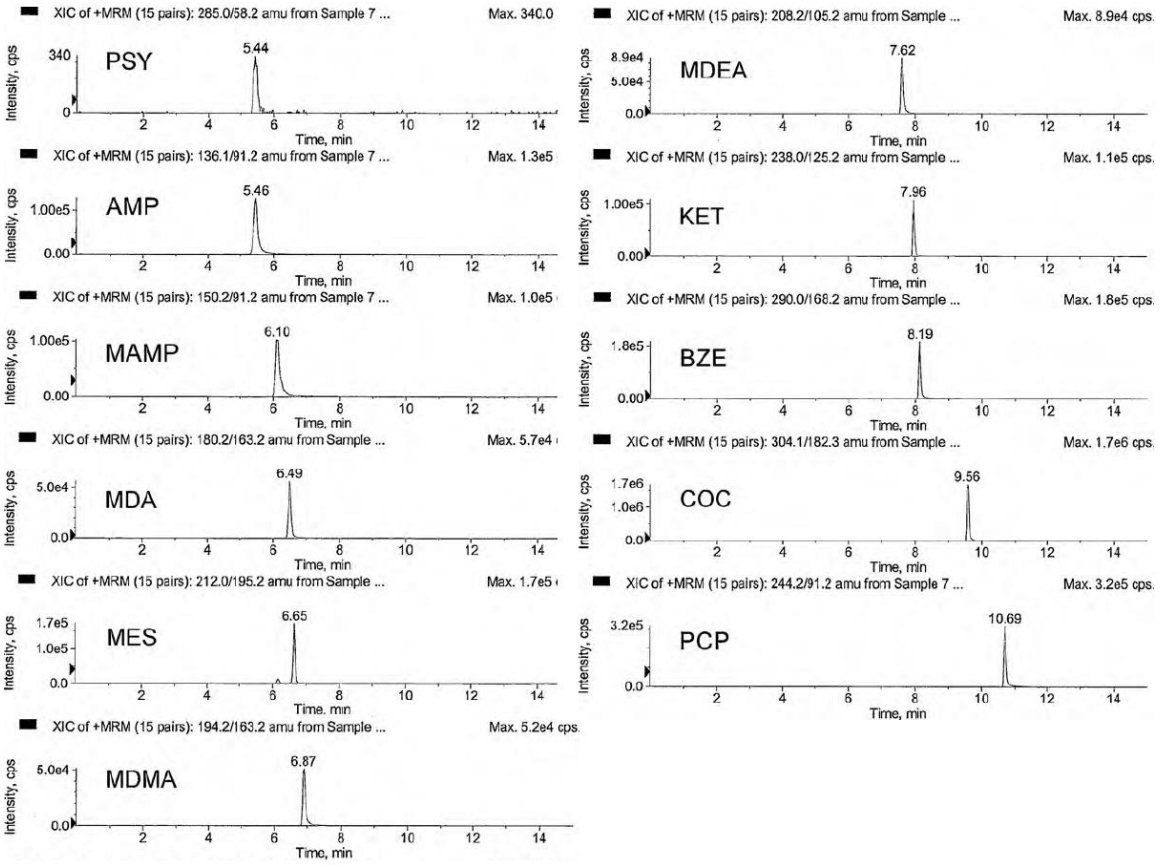


Figure I.1. Extracted ion currents of the selected MRM transitions (only quantifier) obtained from the analysis of a plasma sample spiked with the analytes.

2. Clean-up optimization

This procedure has been applied on plasma, OF and urine samples. Compared to classic SPE this technique allows the sample pretreatment in a shorter time and it allows to use only 100 µL of sample; this is particularly useful when dealing with plasma samples.

The procedure was optimized similarly to a traditional SPE. The maximum allowed concentration of organic solvent for sample pre-treatment (partial protein precipitation) was initially studied: a sensitive decrease in the analyte retention was observed using methanol concentrations higher than 10%, particularly for polar molecules such as amphetamine or mescaline and for this reason 10% was finally selected. The optimal number of aspirate/dispense cycles was also evaluated, testing 1 to 10 cycles and it was found that no further increases were obtained with more than 5 cycles.

The extraction performances were evaluated by measuring the analyte content in five fractions: the first was the analyte loading solution, ultrapure water:methanol solution (9:1,

v:v), and represented the percentage of the analyte unretained by the tip; the second was the analytes lost during the washing step with water; while the third, fourth and fifth fractions were the amount of analyte recovered after three subsequent acidified methanolic fraction elutions (100 µL containing 10 mM formic acid). The absolute recoveries are lower for more polar substances such as amphetamine and psilocybin (55–60%), while they can be considered almost quantitative for cocaine and phencyclidine. Capacity of the tips for the specific analyte was checked along the concentration range of analysis, especially for urine in which the range of concentrations investigated is wider. The analytical results have shown that there are no saturation phenomena in the tested range of concentration .

The present procedure has been attempted also for other classical drugs of abuse. However, retention of opiates was not satisfactory in the optimized conditions (lower than 50%), while detection of cannabinoids was achieved below the suggested cut-off levels. For these reasons both types of drugs were not included in the validation study.

3. Validation

3.1 Linearity, LODs and LOQs

Methamphetamine-d_5, MDMA-d_5, BZE-d_3 and phencyclidine-d_5 were used as ISs for their analogues and also for the other compounds with a similar chromatographic behavior or structure affinity. The linearity was satisfactorily described by un-weighted least-squares linear regression; as reported in Table I.2, the average determination coefficients (r^2) are above 0.99 as required by the SOFT/AAFS guidelines [5].

Linear range has been selected in order to include the concentrations normally found in real samples; concentrations up to 1000 ng mL^{-1} are tested only for urine, where high concentrations of drugs are normally found.

The blank samples injected between calibrators always exhibited no carryover phenomena; QCs were always within the limits of precision of the method. LODs were derived experimentally as suggested by the SOFT/AAFS guidelines to better determine the true LOD in chromatographic assays [5] and are reported in Table I.2. To ensure identification of the analytes even at the level of LODs, the latter were calculated for each analyte on the MRM transition having the lowest S/N. LOQs were calculated in a similar way: an acceptable relative standard deviation (±20%) can be routinely achieved at the lowest concentration of calibrator.

Analyte	Internal std	Equation	r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Plasma (range= LOQ-100 ng mL^{-1})					
Amphetamine	Methamphetamine-d5	y=0.0319x-0.0054	0.9990	0.6	1.9
Benzoylecgonine	Benzoylecgonine-d3	y=0.0476x-0.0309	0.9988	0.5	1.6
Cocaine	Benzoylecgonine-d3	y=0.0306x-0.0309	0.9995	0.05	0.2
Ketamine	Methamphetamine-d5	y=0.0353x+0.0157	0.9994	0.2	0.7
MDA	MDMA-d5	y=0.0148x+0.0074	0.9996	1.8	5.9
MDEA	MDMA-d5	y=0.0219x+0.0205	0.9995	1.0	3.3
MDMA	MDMA-d5	y=0.0198x-0.0053	0.9992	0.9	2.7
Mescaline	Methamphetamine-d5	y=0.0357x+0.0372	0.9993	0.5	1.7
Methamphetamine	Methamphetamine-d5	y=0.0897x-0.0712	0.9994	0.8	2.4
Phencyclidine	Phencyclidine-d5	y=0.0309x-0.0368	0.9990	0.3	1.0
Psilocybin	Methamphetamine-d5	y=0.0210x-0.0046	0.9996	1.2	3.9
OF (range=LOQ-200 ng mL)$^{-}$					
Amphetamine	Methamphetamine-d5	y=0.0289x+0.0140	0.9980	0.3	1.5
Benzoylecgonine	Benzoylecgonine-d3	y=0.0420x+0.0052	0.9999	0.2	0.8
Cocaine	Benzoylecgonine-d3	y=0.0320x+0.0039	0.9956	0.05	0.2
Ketamine	Methamphetamine-d5	y=0.0321x+0.1101	0.9980	0.3	1.0
MDA	MDMA-d5	y=0.0261x+0.0038	0.9985	1.2	2.9
MDEA	MDMA-d5	y=0.0471x+0.0210	0.9982	0.4	2.3
MDMA	MDMA-d5	y=0.0170x+0.0011	0.9970	0.3	1.9
Mescaline	Methamphetamine-d5	y=0.0182+0.0157	0.9943	0.1	1.0
Methamphetamine	Methamphetamine-d5	y=0.0101+0.0098	0.9978	0.2	0.6
Phencyclidine	Phencyclidine-d5	y=0.0268+0.01253	0.9986	0.1	0.4
Psilocybin	Methamphetamine-d5	y=0.0184+0.00521	0.9954	1.0	3.0
Urine (range=LOQ-1000 ng mL^{-1})					
Amphetamine	Methamphetamine-d5	y=0.0248x+0.0340	0.9986	0.7	2.3
Benzoylecgonine	Benzoylecgonine-d3	y=0.0863x+0.0359	0.9979	0.4	1.3
Cocaine	Benzoylecgonine-d3	y=0.0392x+0.0359	0.9994	0.02	0.06
Ketamine	Methamphetamine-d5	y=0.0209x+0.0271	0.9997	0.3	1.0
MDA	MDMA-d5	y=0.0992x+0.0541	0.9992	2.0	6.6
MDEA	MDMA-d5	y=0.0161x+0.0028	0.9980	0.5	1.7
MDMA	MDMA-d5	y=0.0132x-0.0675	0.9993	1.0	3.3
Mescaline	Methamphetamine-d5	y=0.0220x+0.0015	0.9995	0.3	1.0
Methamphetamine	Methamphetamine-d5	y=0.0371x-0.0376	0.9993	1.0	3.3
Phencyclidine	Phencyclidine-d5	y=0.0585x-0.0012	0.9995	0.1	0.3
Psilocybin	Methamphetamine-d5	y=0.2212x-0.0015	0.9992	142	5.6

Table I.2. Regression data, LODs and LLOQs for selected analytes in plasma, OF and urine

These low detection limits have been achieved using the best conditions for the detection together with the clean-up strategy that allows the enrichment of the analytes maintaining a low matrix effect. The LOQs obtained for all the analytes in urine are lower than the cut-off values reported by SAMSHA [2].

3.2 Accuracy and precision

Accuracy and precision were assessed using fortified drug-free matrix. Accuracy is very close to 100% for most analytes and always within ± 15% . Intraday and intermediate reproducibility, in term of RSD, were constantly < 15%.

In urine precision, accuracy and recovery have been calculated at three concentration values, corresponding to ½ cut off, cut off and 1,5 cut off for each analyte. For some analytes cut-off values were not available and the three concentrations were chosen over the entire range. The experiments showed that the analytical performances of the method were independent of concentration. All precision and accuracy data are reported in Table I.3

Analyte	Plasma						
	Nominal Conc (ng mL^{-1})	Intra-day (n=6)		Inter-day (n=18)	Recovery (%)	ME	
		RSD%	A%	RSD%			RSD%
Amphetamine	20	4	98	7	55	1.09	6
	50	6	98	6	56		
	100	9	100	5	56		
Benzoylecgonine	20	5	100	7	73	1.08	6
	50	7	101	7	79		
	100	3	101	4	75		
Cocaine	20	2	101	3	82	1.06	5
	50	3	102	3	85		
	100	1	101	2	79		
Ketamine	20	6	102	6	80	1.06	5
	50	5	103	6	84		
	100	4	102	4	82		
MDA	20	7	98	12	63	1.09	4
	50	7	97	7	65		
	100	7	99	9	66		
MDEA	20	2	105	6	78	1.08	7
	50	2	102	3	79		
	100	2	103	4	80		
MDMA	20	7	100	8	70	1.06	7
	50	5	101	7	68		
	100	5	100	6	72		
Mescaline	20	4	103	5	62	1.07	6
	50	7	100	5	63		
	100	5	99	4	63		
Methamphetamine	20	3	99	4	67	1.06	9
	50	3	101	4	69		
	100	5	101	6	71		
Phencyclidine	20	2	99	3	85	1.07	8
	50	2	100	4	87		
	100	2	100	3	86		
Psilocybin	20	7	97	7	61	1.07	9
	50	5	98	5	60		
	100	3	97	4	63		

Table I.3. Intra and inter day precision (expressed as RSD%), accuracy (A%), recovery and matrix effect (ME) data for selected analytes in plasma, OF and urine *(see next pages)*

Analyte	OF						
	Nominal Conc (ng mL^{-1})	Intra-day (n=6)		Inter-day (n=18)	Recovery (%)	ME	
Amphetamine	20	6	95	7	64	1.06	6
	50	6	97	5	65		
	100	4	97	6	64		
Benzoylecgonine	20	7	101	6	82	1.05	6
	50	6	99	7	79		
	100	5	100	7	80		
Cocaine	20	6	103	8	84	1.06	5
	50	4	101	5	87		
	100	5	98	5	81		
Ketamine	20	6	102	8	85	1.03	9
	50	4	100	5	80		
	100	4	105	7	83		
MDA	20	3	97	5	70	1.05	6
	50	5	97	4	68		
	100	4	99	4	70		
MDEA	20	6	96	8	79	1.08	5
	50	5	100	7	80		
	100	6	97	5	76		
MDMA	20	4	99	7	74	1.03	9
	50	5	98	6	72		
	100	3	100	6	75		
Mescaline	20	7	100	8	64	1.06	7
	50	7	98	7	63		
	100	6	102	8	63		
Methamphetamine	20	5	100	6	75	1.04	7
	50	3	99	4	73		
	100	2	102	4	70		
Phencyclidine	20	7	103	8	87	1.03	4
	50	5	101	6	81		
	100	4	98	5	83		
Psilocybin	20	6	95	6	63	1.07	6
	50	4	97	4	60		
	100	3	95	5	61		

Table I.3. Intra and inter day precision (expressed as RSD%), accuracy (A%), recovery and matrix effect (ME) data for selected analytes in plasma, OF and urine *(see previous and next page)*

Analyte	Urine						
	Nominal Conc (ng mL^{-1})	Intra-day (n=6)		Inter-day (n=18)	Recovery (%)	ME	
Amphetamine	250	7	98	8	58	1.08	5
	500	8	97	9	61		
	750	6	96	7	59		
Benzoylecgonine	75	8	103	7	83	1.06	4
	150	3	101	4	88		
	225	7	99	7	80		
Cocaine	0.1	3	100	4	85	1.08	7
	5	1	101	2	85		
	100	2	102	3	86		
Ketamine	250	5	102	5	84	1.07	5
	500	10	104	3	86		
	750	3	103	3	88		
MDA	250	7	97	10	70	1.09	5
	500	3	98	8	72		
	750	7	96	7	76		
MDEA	250	5	101	6	78	1.08	9
	500	6	100	7	81		
	750	5	102	5	83		
MDMA	250	7	100	7	76	1.01	2
	500	3	99	3	79		
	750	3	100	4	81		
Mescaline	250	4	97	4	66	1.08	5
	500	5	96	7	65		
	750	2	96	2	66		
Methamphetamine	250	6	100	7	74	1.07	6
	500	4	99	4	73		
	750	3	101	4	79		
Phencyclidine	12.5	3	99	4	89	1.02	6
	25	1	100	2	88		
	37.5	1	102	2	90		
Psilocybin	12.5	7	96	7	57	1.06	8
	25	5	97	5	63		
	37.5	5	95	6	65		

Table I.3. Intra and inter day precision (expressed as RSD%), accuracy (A%), recovery and matrix effect (ME) data for selected analytes in plasma, OF and urine *(see prevous pages)*

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3.3 Matrix effect and selectivity

During the optimization of the multi-class method, dilution of matrix with ultrapure water was tested to reduce ion suppression (1:1, 1:2, 1:5 and 1:10 dilution). Dilution 1:1 v:v, for urine was a good compromise to reduce matrix effects with acceptable loss of sensitivity, taking into account that the SAMHSA cut-off values are relatively high. Plasma dilutions caused considerable decrease of sensitivity without gain in terms of matrix effect. The variability of the matrix composition has proved to be a critical point in OF: this phenomenon was not evident analyzing pools of saliva, but it was observed on samples from individuals, among which there were also possibly positive cases. This was one of the major issues to address in the development of the μSPE procedure. To assess the variability of the matrix effect between samples, which can be considered as important as ME value [89], six different blank matrix sources were spiked with the analytes after the extraction step and were analyzed. The RSD% is shown in table I.3 together with the ME obtained by the ratio between the slopes of the calibration curves in solvent and in matrix. The proposed analytical procedures have demonstrated to strongly reduce matrix effect: signal ion suppression affected the sensitivity always less than 15%. These analytical results indicated that it is possible to use a calibration curve in solvent, as previously described, for the quantitation of the selected analytes in the three matices.

Only a small number of interfering signals, at different retention times, were recorded from the analyses of drug-free samples, demonstrating that the developed methods provided good selectivity for all the considered analytes.

3.4 Stability

Stability tests were run for working standard solutions and processed samples. Keeping solutions at −20◦C during the inter-day assays, both for long term and short term stability experiments, the intensity of signal of the MRM transitions were no significantly different for all the analytes. The analytes were not affected even by repeated freezing-thawing cycles: the calculated concentration of storage and freshly prepared samples were within intermediate reproducibility values of the methods.

Part II

Hair

Analysis in unconventional biological sample

Pressurized liquid extraction for the extraction of illicit drugs from hair

EXPERIMENTAL

1. Chemicals

Standards of AM, MA, MDA, MDEA, MDMA, COC, BZE, MES, codeine (COD), morphine (MOR), 6-acetylmorphine (6-MAM), K, PCP MOR-d3, MA-d9, MDMA-d5, CO-d3, BE-d3, and PCP-d5were purchased from S.A.L.A.R.S. S.p.A. Norcocaine (NCOC) was obtained from Cerilliant Corporation (Round Rock, TX, USA). THC, THC-COOH, CBD, CBN, OH-THC, THC-d3, THC-COOH-d3 and cocaine-d3 (COC-d3) were purchased from LGC Standards (Wesel, Germany). The purity of the reference compounds was ≥99 %. All standards were provided at a concentration of 1 mg mL^{-1} with the exception of ISs that were at a concentration of 0.1 mg mL^{-1}.

Individual stock solutions were prepared in methanol at 0.1 mg mL^{-1}, except for the ISs which were prepared at 1 μg mL^{-1}. Working standard mixtures were prepared by appropriate dilution of the standards in methanol. All solutions were stored at -20°C in the dark.

Acetonitrile and methanol were of RS-Plus grade. Ultrapure water for LC was produced by a Milli-Q Plus apparatus from Millipore. All solvents, hydrochloric acid, and potassium dihydrogen phosphate, used for preparation of aqueous buffer, were purchased from Carlo Erba. Formic acid, sodium hydroxide and sodium dodecylsulphate (SDS) were from Sigma–Aldrich

2. External decontamination

Crudely cut hair (~1 cm) was placed in a 50 mL Falcon cone tube and 5 mL phosphate buffer (0.1 mol L^{-1}, pH 6) was added. The mixture was vortex mixed for 1 min and, after removal of the aqueous buffer, the hair was washed sequentially with 5 mL of isopropanol and 5 mL of dichloromethane.

The last wash was collected in a vial and evaporated under a gentle stream of N_2; the residue was reconstituted with 1 mL of 10 mmol L^{-1} formic acid in methanol and was checked for external contamination.

3. Pressurised liquid extraction

Extraction of target analytes from hair was performed by pressurised liquid extraction (PLE) using a Dionex ASE 200 (Sunnyvale, CA, USA) accelerated-solvent-extraction system. Two different extraction methods were developed: a **multiclass** method including 14 analytes and a specific one for **cannabinoids**. A sample of hair (50 mg) cut into 1–2 mm segments was homogenized with Varian Hydromatrix bulk sorbent (Palo Alto, CA, USA) by means of a mortar. The mixture was then placed in a 1 mL pressure resistant stainless steel cell, which was sealed at both ends with glass fibre filters. Void volumes in the cell were filled with Hydromatrix bulk sorbent, and 25 μL of methanol containing the ISs were added. PLE was performed with a single extraction cycle, using as extraction solvent SDS 25 mM in water:methanol (90:10, v/v) for cannabinoids extraction and water:methanol (80:20, v/v) for the other analytes. The extraction conditions were: pressure, 100 bar; temperature, 150 °C; preheat time, 1 min; heat time, 7 min; static time, 5 min; flush volume, 0 %; purge time, 60 s. The PLE extract (6–8 mL) was automatically collected in glass vial with cap and solvent-resistant PTFE septa.

4. Clean-up of extracts

1.1 Multi-class method

The PLE extract was collected in a graduated tube and 4 mL were cleaned by SPE on a polymeric reversed-phase cartridge (Strata X 30 mg/1 mL from Phenomenex, Torrance, CA, USA). The cartridge, installed on the Visiprep vacuum system, had previously been conditioned with 1 mL methanol and 1 mL of water:methanol (80:20, v/v) After the loading phase, the cartridge was washed with 1 mL of ultrapure water and the analytes were eluted with 0.5 mL of 10 mmol L^{-1} formic acid in methanol, collected in a vial for analysis; two μL were injected,

1.2 Cannabinoids

PLE extract was collected into a graduated tube and centrifuged at 6000 g for 5 min at 25°C (ALC PK 121R, Thermo Scientific Inc., Bremen, Germany). Five mL were then cleaned up by SPE using polymeric reversed phase cartridges Strata X 30 mg/1 mL from Phenomenex (Torrance, CA, U.S.A.). The cartridge, installed on vacuum system Visiprep, was previously conditioned with 1 mL of methanol and 1 mL of water-methanol (90:10 v/v). Following the loading phase, cartridge was washed with 3 mL of water-methanol (50:50 v/v) and after drying under vacuum the analytes were eluted with 1 mL of ammonium hydroxide 50 mM

in methanol. The eluate was collected into a glass tube, evaporated to dryness under gentle nitrogen flow and the residues were finally reconstituted in 200 µL of methanol for injection in the LC-MS/MS system (10 µL).

5. Preparation of a fortified matrix by soaking

Positive hair was prepared in our laboratory following an existing procedure with slight modifications. Briefly, different types of hair (smooth, curly, coloured, treated, grey, etc.) were roughly cut and mixed. This mixture (8 g) was immersed in a mixture of 60 mL distilled water containing the substances of interest, and 60 mL 0.02 mol L^{-1} HCl in dimethyl sulfoxide. The hair, totally immersed and periodically mixed, was soaked in this solution for three weeks.

It was then vacuum filtered on a Buckner funnel and let to dry. Dried hair was washed to remove traces of the analytes on its surface and then underwent the external decontamination procedure.

6. LC–MS/MS analysis

A PerkinElmer (Norwalk, CT, USA) series 200 micro-LC pump with autosampler (equipped with a 5 µL loop) and vacuum degasser system was used for chromatography. The LC system was coupled to a PerkinElmer Sciex API 2000 triple quadrupole mass spectrometer equipped with a TurboIonSpray source for the multi-class drugs analysis while an API 4000 Qtrap® was used for the detection of cannabinoids.

For both methods the analytes were separated on a reversed phase C_{18} Kinetex XB column (10 cm x 2.1 mm ID) from Phenomenex, packed with 2.6 µm average diameter particles; a KrudKatcher ultra HPLC in-Line filter 0.5 µm, also from Phenomenex, was used to protect the column.

For the muli class-method the mobile phases were methanol:acetonitrile (90:10, v/v) (phase A) and 7 mmol L^{-1} formic acid in water (phase B); the flow rate was 0.4 mL min^{-1}, entirely transferred into the mass spectrometer source. The gradient was: 0 % to 15 % A in 0.2 min, increased to 35 % A in 3.2 min, and then increased to 100% in 2.6 min. This was maintained for 2 min and then switched back to the initial 0 % in 2 min. Complete separation of all the substances was achieved in 6 min. A timed switch valve (a 10-port Valco valve connected to the instrument) was used to drive the effluent to the source from 1.5 to 6 min and then from 8 to 10 min only. All the analytes were detected in positive-ionization mode with a capillary

potential of 5500 V, focusing potential of 400°C, nebulizer gas at 40 psi, turbo gas at 90 psi, and curtain gas at 30 psi; the source temperature was 400 °C. The MRM ion currents were acquired in two acquisition periods, to ensure maximum sensitivity of MS analysis. In the first period (from 0 to 3.65 min) the analytes detected were MOR, COD, 6-MAM, MES, AM, MA, MDA, MDMA, and MDEA; in the second period (from 3.65 to 6min) KE, CO, NCO, BE, and PCP were detected.

For cannabinoids analysis the mobile phases were: (A) methanol and (B) water both containing 1.25 mM ammonium acetate. The flow rate was 0.35 mL min^{-1} entirely driven into the ion source. The following gradient elution scheme was used: phase A was increased from the initial 60% to 75% in 3.4 min, then up to 90% in 1.7 min and in the following 0.6 min brought to 100%. The latter was maintained for 2.7 min and then switched back to the initial 65% in 2.5 min. The complete separation of all substances occurred in 8 min. A timed switch valve (a 10-PORT Valco valve connected to the instrument) drives the effluent to the source only from minute 4.5 to 8 and then from 10 to 12. All the analytes were detected in positive ionization, except THC-COOH that was analyzed in negative ionization, with a capillary voltage of 5500 V (-4500 V for THC-COOH), nebulizer gas at 60 psi, turbo gas (nitrogen) at 40 psi and curtain gas at 30 psi; the source temperature was 475°C.

In both methods for each analyte two multiple reaction monitoring (MRM) transitions were selected. All source and instrument conditions for the monitored analytes were tuned by injecting each single standard solution at a concentration of 0.1 ng μL^{-1} at 10 μL min^{-1} by use of a syringe pump. All the source conditions were checked (and revised as necessary) by flow-injection analysis with the same chromatographic conditions (flow and solvent composition).

Peak areas for the selected ions were determined by use of the PE-Sciex package Multiview 1.5 and quantification was performed by the internal standard method. The selected transitions, with the main LC–MS/MS conditions, are reported in Table II.1. and II.2.

Period	Analyte	t_R (min)	Q1 (amu)	DP (V)	EP (V)	Q3 (amu)	CE (V)	CXP (V)
1	Morphine	1.72	286.0	42	11	201.2	35	8
						153.2	53	10
	Codeine	2.29	300.0	90	12	165.1	55	5
						215.1	33	4
	6-MAM	2.79	328.0	40	11	165.2	55	7
						211.1	37	5
	Mescaline	2.78	212.1	17	4	195.2	15	8
						165.2	32	6
	Amphetamine	2.44	136.1	14	8	91.1	25	11
						119.2	14	5
	Methamphetamine	2.61	150.0	18	9.2	91.1	26	10
						119.2	16	5
	MDA	2.74	180.1	18	8	163.1	15	7
						105.1	30	8
	MDMA	2.87	194.1	18	6	163.2	17	5
						105.2	35	7
	MDEA	3.36	208.0	18	6	163.2	17	6
						105.1	35	10
	Morphine-d3	1.72	289.0	42	11	165.0	54	10
	MDMA-d5	2.87	199.1	18	6	165.1	17	2
2	Ketamine	3.95	238.0	22	6	125.0	37	6
						207.1	17	6
	Benzoylecgonine	5.18	290.0	25	10	168.2	25	6
						105.2	40	12
	Cocaine	4.50	304.0	26	8	182.2	26	5
						82.2	45	9
	Norcocaine	4.90	290.0	20	9.2	168.2	21	5
						136.2	31	4
	Phencyclidine	5.37	244.1	11	8	86.1	18	11
						91.1	42	11
	BZE-d3	5.18	293.1	25	10	171.1	25	6
	COC-d3	4.50	307.0	26	8	184.9	26	5
	PCP-d3	5.37	249.0	11	8	91.0	18	11

Table II.1.HPLC–MS/MS parameters for the selected analytes (multi-class method)

Period	Analyte	t_R (min)	Q1 (amu)	DP (V)	EP (V)	Q3 (amu)	CE (eV)	CXP (V)
1	THC-COOH	2.84	343.1	-95.1	-8	299.5	-29.7	-12.3
						345.2	-38.2	-10
	THC-COOH-d_3	2.84	346.1	-95.1	-8	302.5	-29.7	-12.3
2	THC	6.00	315.1	64.9	10.8	193.1	30.1	11.5
						123.2	45.1	6.1
	CBN	5.59	311.1	86.7	11	223.1	30.7	14.1
						293.3	24.1	13.3
	CBD	3.95	315.1	64.9	10.8	193.1	30.1	11.5
						123.2	45.1	6.1
	OH-THC	3.71	331.1	69.9	10.9	193.0	33	11.6
						201.2	33	12.6
	THC-d_3	6.00	318.1	64.9	10.8	196.1	30.1	11.5

Table II.2. HPLC–MS/MS parameters for the selected analytes (cannabinoids)

7. Validation

The method was validated according to SoHT guidelines [71] and Peters et al. [142] with a few modifications. Linearity, recovery, matrix effect, precision, accuracy, limits of detection (LODs) and limits of quantification (LOQs) were evaluated.

Calibration standards were prepared in methanol. Quality control samples (QC) were prepared by spiking the standard solution on the hair in the ASE cell; in addition certified reference material (CRM), real positive hair samples and positive hair prepared in our laboratory by soaking, were used for accuracy, precision and recovery studies.

The CRM was Medidrug DHF 1/08-A H-Plus, distributed by LGC Standards (Wesel, Germany). It is a powdered hair control, prepared from spiked hair samples of human origin. This material only contained some of the analytes of interest (MOR, 6-MAM, AMP, MAMP, MDA, MDMA, MDEA, CO, BZE, THC, CBN, CBD).

7.1 Quantification and identification

Linearity was investigated using six calibration standards for each group in the following concentration ranges:

- from LOQ to 10 pg mg^{-1} for THC-COOH and to 200 pg mg^{-1} for CBD, CBN, THC and OH-THC:
- from LOQ to 12.5 ng mg^{-1} for COC, to 1.25 ng mg^{-1} for BZE and NCOC and to 5 ng mg^{-1}.for the remaining analytes.

Replicates (n=3) at each concentration level were analyzed during two days. The calibration curves were derived by plotting the peak area of analytes to ISs versus the hair concentration using a least squares regression model.

Two product ions were selected for each analyte, one for quantitation and one for qualitative analyte confirmation. Quantitative to qualitative ion ratios were required to be within 20% of those in QC samples.

7.2 Recovery

The recoveries (RE) and extraction efficiencies were determined at two concentration levels:

- 0.2 and 10 pg mg^{-1} for THC-COOH, 2 and 200 pg mg^{-1} for THC, CBN, CBD and OH-THC
- 4 pg mg^{-1} and 12.5 ng mg^{-1} for COC; 7 pg mg^{-1} and 1.25 ng mg^{-1} for BZE; 30 pg mg^{-1} and 5 ng mg^{-1} for the other analytes.

Five hair samples of different types (blond, black, straight and curled) were used. For each concentration and hair type, RE were obtained by comparing the absolute peak area for each analyte when spiked into hair before extraction (A) with the absolute peak area for each analyte of samples processed as hair blanks and spiked after the extraction (B). Accordingly, RE(%) = A/B*100.

7.3 Matrix effect, selectivity and carry-over

Interferences from endogenous hair components were evaluated by the analysis of 10 different drug-free hair samples collected among workers in the laboratory. If analytes were not detected (<LOD), the method was considered selective. In addition two samples, just spiked with the internal standards, were analyzed to check for the absence of the respective analyte peaks.

Carry-over effects were assessed by analyzing a blank hair sample after injection of the highest calibrator.

The interference of the matrix on the S/N ratio of each analyte was calculated comparing the slopes of two calibration curves, one obtained in solvent and the other prepared in a pool constituted by 20 independent matrices and processed by PLE. Furthermore 6 independent blank hair samples were spiked at a concentration corresponding to three times the LOQ for each analyte, to assess the variability of the matrix effect.

7.4 LODs and LOQs

The LOD was estimated on the basis of four repeated analyses of fortified hair samples at decreasing concentrations. The LOD was calculated as the concentration where the signal to noise ratio (S/N) of 3:1 for the lower MRM transition was given.

The LOQ was defined as the lowest concentration of fortified hair samples with both precision and accuracy within 20% with fulfillment of the retention time and ion ratio tolerances. Furthermore, a S/N above 10 was required.

7.5 Accuracy and precision

The method precision and accuracy were determined at two concentration levels on fortified hair samples (see above § 7.1.2).

Each sample was analyzed four times on three days. The concentrations of the analytes were calculated from the daily calibration curves. Accuracy was calculated for each analyte in terms of bias, as the percent deviation of the mean calculated concentration at each concentration level from the corresponding spiked concentration. Within-day precision (repeatability) and inter-day precision (intermediate precision) were calculated as relative standard deviation (RSD%).

The RSD% and bias were generally accepted at maximum values of 15% or 20% near LLOQ.

Precision was further evaluated by analyzing positive real hair samples and positive hair prepared by soaking with varying content of the analytes. The determination was based on two replicates analyzed over four days.

Accuracy was further evaluated with CRM for the analytes that were included in the sample.

7.6 Stability

Stability tests were performed on processed samples and standard solutions in methanol. Tests were performed on short term storage solutions and for processed samples stored at -20°C, by analysis of the solutions on different days; long term stability was checked for a total time of two months.

The effect of freeze–thawing cycles was also evaluated, as suggested by Peters et al. [46].

RESULTS AND DISCUSSION

1. LC–MS/MS optimization

GC–MS has been the method of choice for confirming the detection of illicit drugs in hair; however, GC–MS requires analyte derivatization and, in fact, only a few multiclass methods have been reported. LC–MS does not need a derivatization step and, thus, facilitates multiresidue and multiclass analysis. Recently, with the introduction of ultra-high performance liquid chromatography (UPLC), the simultaneous screening and quantification analysis of 96 common drugs of abuse has been reported [159].

As regards cannabinoids analysis, to date the only techniques able to reach the cut-off of 0.2 pg mg^{-1} recommended by SoHT [71] for THC-COOH determination in hair are gas chromatography coupled with tandem mass spectrometry (GC–MS/MS) using electron impact (EI) ionization mode [160, 161] or negative ion chemical ionization (GC-NCI–MS/MS) that allows a further increase of the sensitivity [30-32] and GC/GC–MS [162]. LC–MS/MS has been used in cannabinoids hair analysis just in the last few years [163-165], THC-COOH was included only in one study [166] but the LOQ obtained is significantly higher than the cut-off values and the method can be applied only for chronic use studies.

In this project LC–MS/MS was selected as detection technique for both multi-class method and cannabinoids; the same column was used for the two methods.

In multi-class method chromatographic optimization the chromatographic separation was a critical aspect of method development, considering the number of analytes in this study and the similar structures of some compounds. Different types of HPLC column were tested: Gemini C_{18} (3 μm, 100 mm×2.0 mm), Kinetex C_{18} (2.6 μm, 150 mm×2.1 mm); Kinetex C_{18} XB (2.6 μm, 100 mm×2.1 mm); and Ascentis (2.7 μm, 150 mm×2.1 mm), all packed with C_{18}. Peak separation and symmetry obtained with the Kinetex columns were higher and the XB was selected because of the opportunity to work at higher flow rates, thus reducing run time.

Methanol as mobile phase was found to be better than acetonitrile in terms of sharpness of peaks, although addition of 10 % acetonitrile to methanol resulted in better selectivity. Addition of formic acid, at a final concentration of 7 mmol L^{-1}, was necessary to obtain symmetrical peaks, especially for amphetamine-like compounds.

Optimization of the gradient revealed it was essential to start the run with 100 % aqueous phase to avoid widening of morphine peak. As suggested in previous studies [167], to obtain

a better selectivity for amphetamine-like compounds a pH gradient was used by addition of formic acid to the aqueous phase only. Under these optimized conditions the total runtime (including the re-equilibration step) was 10 min. Extracted ion currents for all analytes are reported in Fig. II.1.

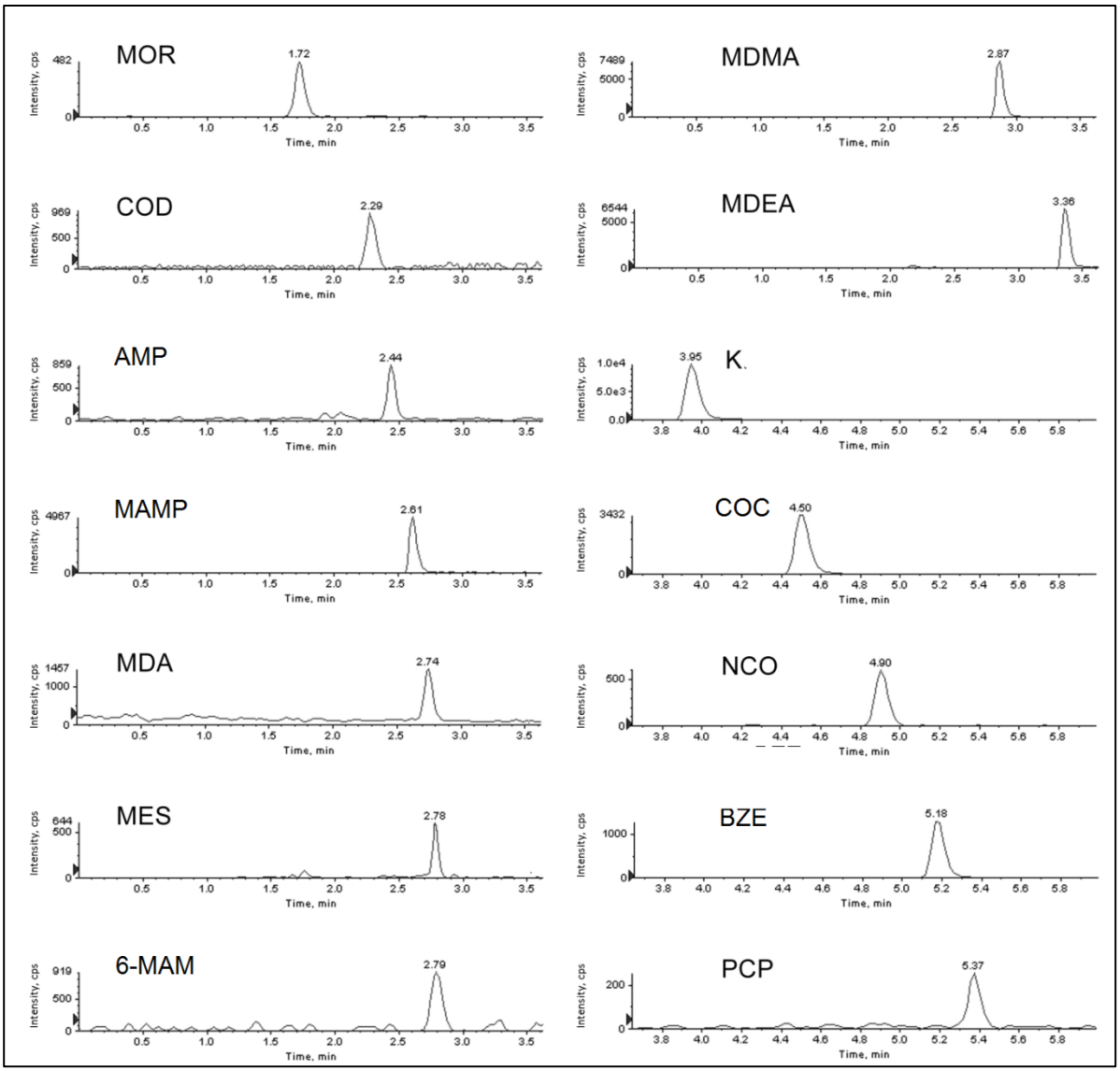


Figure II.1. Extracted ion currents of the selected MRM transitions obtained from analysis of the fortified drug-free matrix created ad hoc in our laboratory

For cannabinoids separation particular attention has been paid to the separation of analytes with common fragments and to the separation of THC-COOH from the other analytes, which was crucial to introduce an acquisition period for this analyte, since it is better ionized in negative acquisition mode contrarily to the others that are detected in positive mode.

Sensitivity for the analytes, particularly for THC-COOH, was properly tuned to the conditions given in the experimental section by direct flow injection analysis (FIA). A fixed amount of standards was injected using methanol as mobile phase at different flow rates, and the areas of the different analytes were monitored by MRM.

Different columns, all packed with C_{18} particles were tested. Firstly the experiments were conducted on a Phenomenex Kinetex XB C_{18} (50 x 2.10 mm) packed with 5 µm particles. The mobile phase used was methanol since the signal was significantly suppressed using acetonitrile.

Chromatography optimization for cannabinoids was found to be crucial also for matrix effect optimization. The XB C_{18} of 5 cm packed with 5 µm particles was optimal for analysis in model solution, but significant matrix effect was obtained with real samples, since the separation was very fast. For this reason we decided to select a longer column XB C_{18} (100 x 2.10 mm) packed with smaller particles (2.6 µm) which offered increased resolution and efficiency.

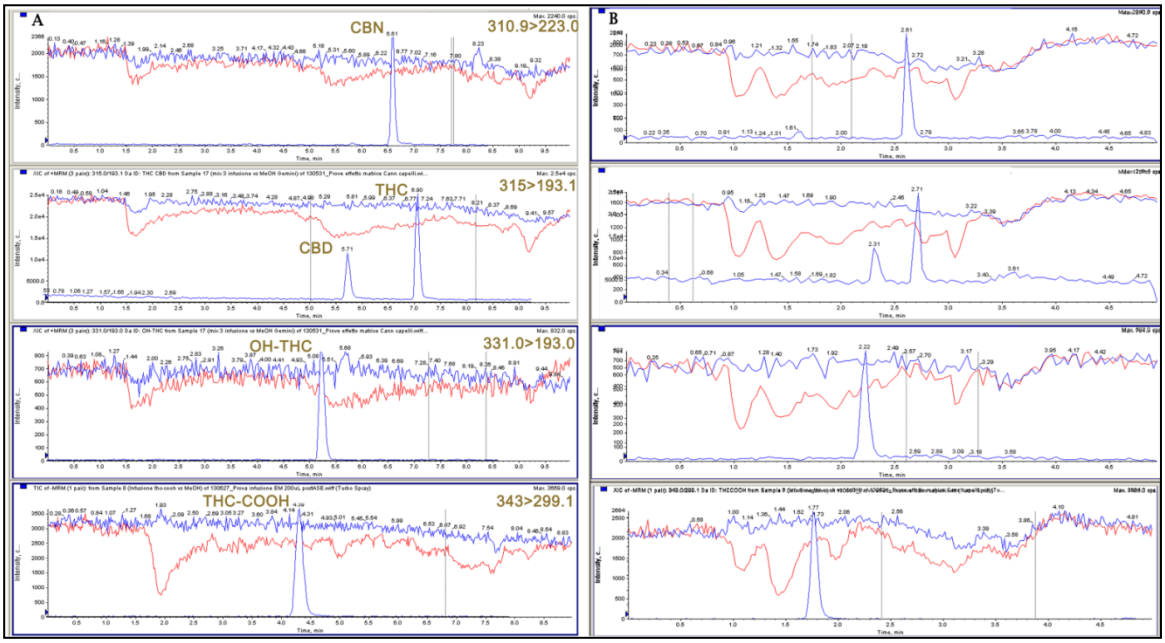


Figure II.2. Analyte separation by means of a XB-C_{18} column of 100x2.10 mm (A, on the left) and 50x2.10 mm (B, on the right)

With this column a better separation of analytes and interfering compounds, still present in the sample despite the strong clean-up, was achieved, significantly reducing the matrix effect (fig II.2).

In the optimized conditions all the analytes were separated in 8 min. Extracted ion currents for all analytes are reported in Fig. II.3.

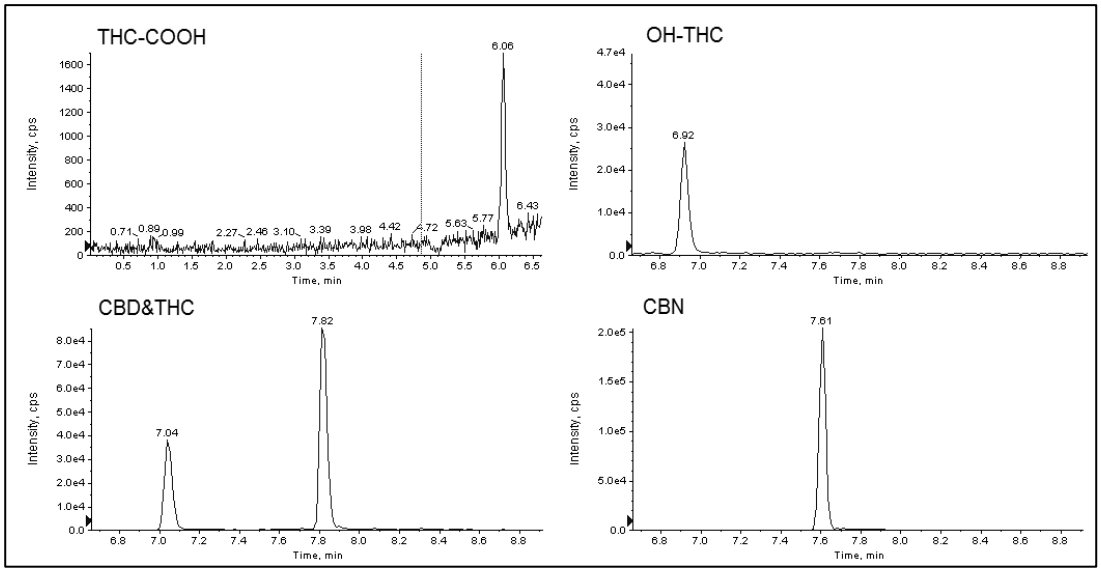


Figure III.3. Extracted ion currents of the selected MRM transitions obtained from analysis of a fortified hair sample

2. External decontamination

Before the extraction, sample washing is crucial to eliminate endogenous (fats residues, sweat, sebum…) or exogenous (shampoo, cosmetics…) interfering compounds. In addition washing step can be useful to exclude external contamination, which can lead to false positives. Removal of traces of analytes that may have been deposited on the hair because of environmental exposure and not abuse (particularly for substances that can be assumed by smoke) is absolutely mandatory. Cannabinoid testing may be difficult for this reason; they are normally assumed by smoke and can condense on hair surface; in addition they are extremely lipophilic compounds and incorporation into the hair matrix via environmental contamination is likely to occur [9]. Consequently external decontamination is important but only the detection of metabolites can definitively exclude passive exposure.

On the other hand, decontamination is also required to wash only the environmental drugs potentially present in the sample and not the one incorporated in order to avoid false negatives.

SoHT guidelines recommend that for washing step, the procedure should include several steps with both organic solvent and aqueous solutions but standardized procedures does not exist [71].

According to their ability to penetrate the keratin structure of hair [168] solvents can be classified as swelling and not swelling; aprotic solvents as dichloromethane and acetone have no extracting capacity, compared to water or methanol that are instead swelling solvents.

Water (phosphate buffer) and dichloromethane were chosen for decontamination with the purpose of minimize all the possible polar and non-polar interfering compounds; an intermediate washing step was carried using isopropanol to avoid immiscible solvent mixing.

For optimization of this step methanol solutions of analytes at concentration of 1 μg mL^{-1} were sprayed on 500 mg of drug-free hair; this sample was submitted to the decontamination procedure to assess its efficiency. The three washing fractions were analyzed and it was founded that the majority of the analytes were removed by treatment with aqueous buffer while cannabinoids were removed by isopropanol; only traces were found in dichloromethane. After the decontamination step, the hair, processed and analyzed as described above, resulted negative for all the analytes tested.

It has also been checked that the decontamination procedure did not actively remove the drugs incorporated into hair; no signal decrease was observed comparing the data obtained from decontaminated and un-decontaminated samples. Despite the swelling ability of water, the short time of contact with the keratin structure hinder the penetration of the "core" of hair, that, thus, remains inaccessible [169].

3. Pressurised Liquid Extraction

The critical step in the analysis of hair is certainly extraction of the drugs from the matrix. Extraction is conventionally performed in two ways [168] solid liquid extraction or alkaline and acid digestion. The extract is then cleaned-up by SPE, SPME or LLE.

Methanol [170-176] or methanol–acid mixtures, to improve the efficiency for basic drugs [177, 178], were initially proposed as extraction solvent. Acidic aqueous solutions, for example dilute HCl [179-182] or phosphate buffer [183, 184] have been also used, as also has acetonitrile, both in combination with methanol [167, 185, 186] and with acidic solutions [187]. The methanolic extracts were found to be rich in interfering compounds whereas the

aqueous extracts are cleaner; in aqueous media, however, drugs of abuse for example heroin, its derivative 6-MAM, and cocaine can easily undergo hydrolysis. The main disadvantage of solid–liquid extraction is the long contact time (16–20 h) between hair and solvent that is needed for good recoveries. Time can be greatly reduced, from 2 to 6 h, when the extraction is coupled with ultrasonic treatment [172, 174] and high temperature [170, 172-175, 177, 184].

Digestion by concentrated NaOH [188, 189] or enzymatic digestion [168], as well as extraction using HCl [190] are also very diffused. Chemical digestion requires shorter times (approx.30 min-1 h) but causes complete hydrolysis of many drugs; enzymatic digestion is prone to low recovery because of instability of the analytes at the pH and temperature required for optimum enzyme activity.

Alkaline digestion is the method normally used for cannabinoids extraction. A drawback is that the stability of the analytes might be affected during the digestion procedure, for example it has been shown that CBD is not stable under the severe conditions of alkaline digestion [191]; other issues include large volume of organic solvent used in traditional LLE procedures tedious sample preparation and poor reproducibility. Also for cannabinoids analysis enzymatic digestion [192] and direct methanol extraction [193-195] have been reported but require more time, up to 5 hours.

Novel approaches such as matrix solid phase dispersion [196] or micropulverized extraction [19] have recently been proposed to reduce analysis time for a number of illicit drugs. However, in the recent years, attention has been focused to assisted extraction techniques, to accelerate and improve the efficiency compared to conventional methods. Supercritical fluid extraction which exploits CO_2 in its supercritical state [197, 198], is extremely useful but limited by its cost [168]. Microwave-assisted extraction has also been developed for opiates, cocaine and their metabolites in hair samples using dichloromethane as organic solvent [199]. This method revealed the potential of assisted extraction methods to reduce analysis time and to automate the whole process maintaining quantitatively acceptable analytical recoveries.

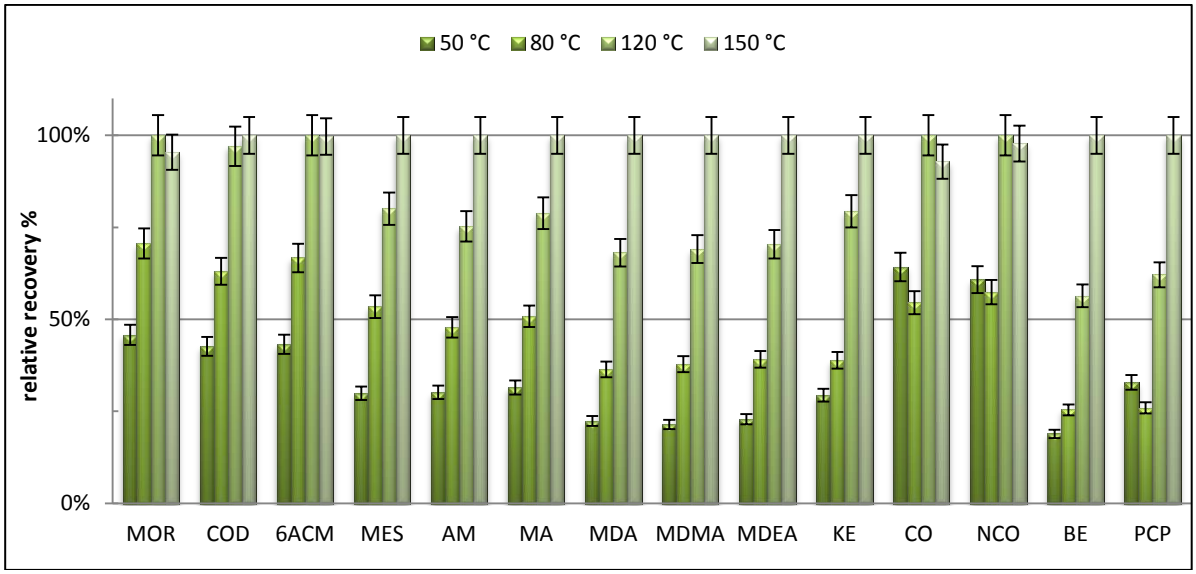
In this work pressurised liquid extraction (PLE) was tested for the extraction of analytes belonging to different drug classes from hair. PLE has previously been used for extraction of metals from hair [41, 42] but no methods for extraction of drugs from this matrix have yet been reported.

Two PLE procedures have been developed: the first allows the extraction of 14 drugs belonging to the class of cocaine, amphetamines, opiates and hallucinogens, the second procedure is able to extract cannabinoids. These analytes have seldom been included in multi analytes methods [163, 165] and their analysis is generally performed separately. The challenges in cannabinoids hair testing arise from the low concentrations of Δ^9-tetrahydrocannabinol (THC) and of its major metabolite 11-nor-9-carboxy-THC (THC-COOH) that is even less concentrated (fg mg^{-1}) [30-32] and from their lipophilicity which requires special attention.

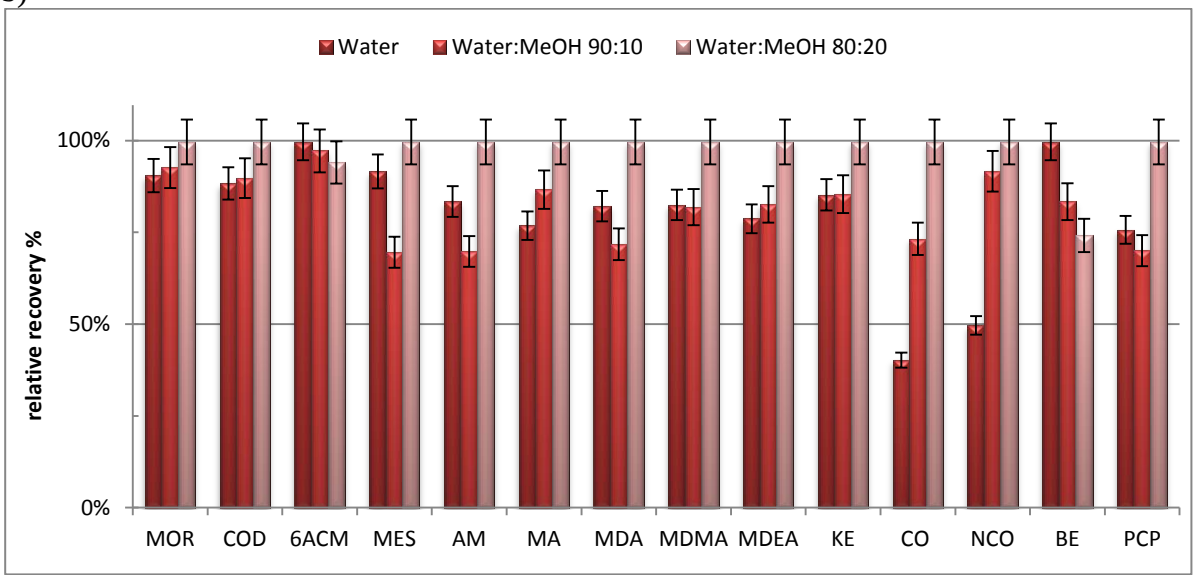
PLE was selected in this study as extraction technique, since it allows to reduce solvent consumption and permit the automation of the extraction step. The extraction procedure was optimized testing temperature, pressure, extraction solvent and time.

Relative recoveries, normalized to the maximum value, were evaluated on fortified hair. The effect of temperature on the recoveries, using water as solvent at pressure of 100 bar, is reported in Figure II.4.a. The highest recoveries were obtained at 150° C, which has been selected as extraction temperature. Considering pressure, its main role is the maintenance of the solvent in liquid phase and its contribution to extraction efficiency is minimal for most of the analytes, this was confirmed carrying out recoveries at 150° C at 70 and 100 bar (data not shown), so the latter was selected. The extraction solvent was later considered: the fortified hair was sequentially extracted with water containing increasing amounts of methanol; the data, normalized to the maximum amount obtained for each analyte, are reported in Figure II.4.b. Cannabinoids were not extracted in all the tested conditions and are therefore not reported in the histograms.

a)


50 °C
80 °C
120 °C
150 °C
100%
50%
0%
relative recovery %
MOR
COD
6ACM
MES
AM
MA
MDA
MDMA
MDEA
KE
CO
NCO
BE
PCP

b)


Water
Water:MeOH 90:10
Water:MeOH 80:20
100%
50%
0%
relative recovery %
MOR
COD
6ACM
MES
AM
MA
MDA
MDMA
MDEA
KE
CO
NCO
BE
PCP

c)

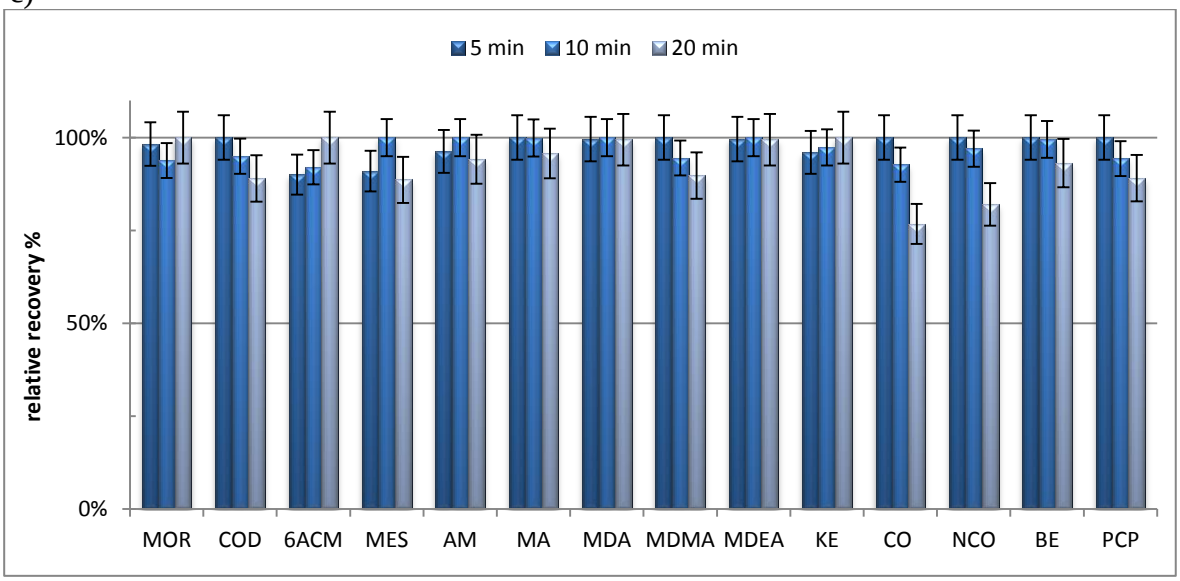

5 min
10 min
20 min
100%
50%
0%
relative recovery %
MOR
COD
6ACM
MES
AM
MA
MDA
MDMA
MDEA
KE
CO
NCO
BE
PCP

Figure II.4. (*Previous page) Effect of (a)temperature, (b) extraction solvent composition, and (c) extraction time on the PLE relative recovery of the analytes included in the multi-class method (data are normalized to the maximum for each analyte). The results were obtained from six replicates*

Larger amounts of analytes were extracted when the proportion of methanol was increased to 20%, indicating that pure water was not able to extract quantitatively all the compounds from the hair matrix. The only analyte with decreased relative recovery in the presence of methanol was BZE. A reasonable explanation is that, during the procedure, COC is partially hydrolyzed to BZE and that a certain percentage of methanol even if small is able to decrease the hydrolysis reaction rate, as shown in fig 2b. No significant improvement in recovery was observed for amounts of methanol > 20%. The extraction time selected was 5 min; in fact, longer times resulted in increased degradation of some analytes (Figure II.4.c).

To allow the extraction of cannabinoids different eluents were tested since no satisfying results were obtained with water:methanol. Increasing the percentage of methanol up to 40% the co-eluting substances made the extract unsuitable for the analysis. Eluents of different nature, such as ethanol, hexane/ethyl acetate were also tested without good results. Different temperatures or extraction times did not produce better recoveries.

Considering that it was not possible to obtain reliable results with physical means, it has been necessary to improve the extraction efficiency towards cannabinoids by adding a surfactant to the extracting solution as reported for environmental samples and plant: surfactants have the ability to form micelles that can solubilise different compounds, including very hydrophobic analytes, without the need for any strong increase in temperature.

Sodium dodecyl sulfate (SDS), anionic, and Triton X-100, non-ionic, have been tested for this purpose (Fig II.5).

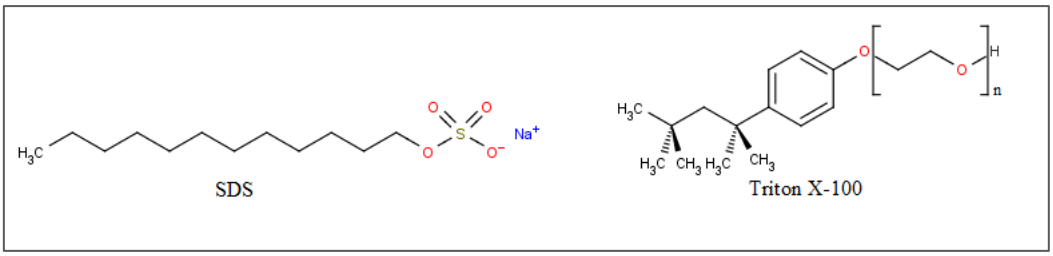


Figure III.5. Surfactants (SDS and triton X-100) chemical strcture

Different concentrations of each surfactant have been added to water to find the optimal conditions; T and P were set at 150°C and 100 bar respectively. At the concentrations tested (0.25-1%), triton showed only a slight improvement in the extraction efficiency and produced a significant ion suppression in the following LC–MS/MS analysis despite the SPE clean-up. On the other hand SDS allowed to reach good results in terms of recovery percentage as reported in Figure II.6. These experiments have been carried out by fortifying blank hair samples

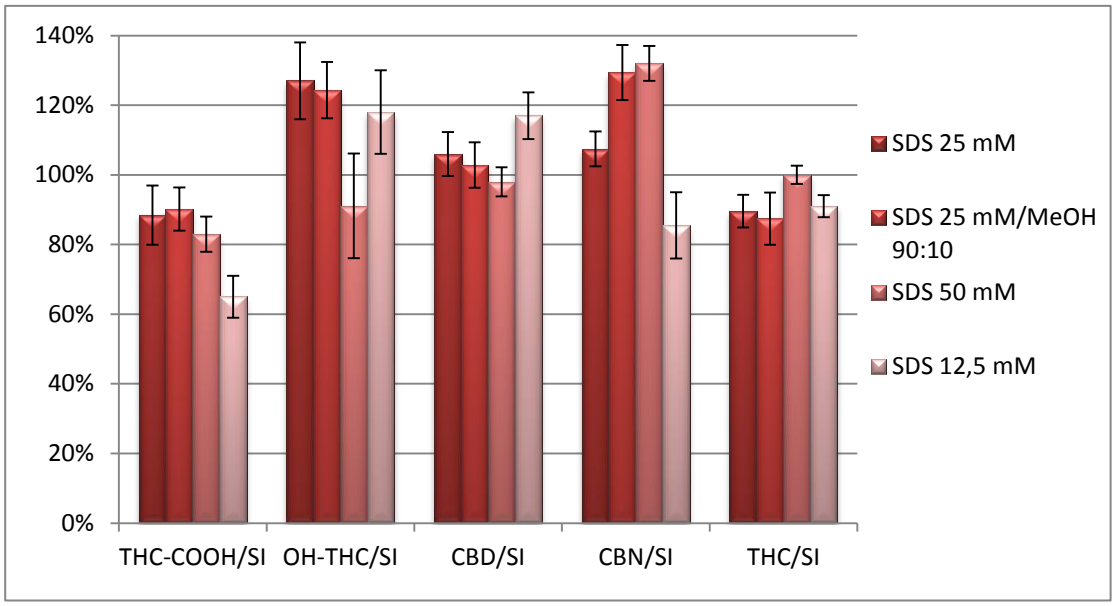


Figure III.6. ASE recoveries obtained with various SDS concentrations in the eluent phase.

In these experiments internal standard were added after the PLE extraction and before the SPE step in order to normalize the losses arising from the clean-up; only the PLE efficiency and not the whole procedure efficiency was evaluated at this stage. In fig. II.6 it can be noticed that the analytes are stable under the P and T conditions tested; SDS at 25 mM provides the best results for THC-COOH, while THC and CBN were better extracted with SDS at 50 mM. Because of the importance of THC-COOH as biomarker of cannabinoid use and its lower cut-off, the concentration of SDS has been set at 25 mM. We also tested the influence of adding methanol in the aqueous SDS solution and it was found that it had little influence; taking into account that a small percentage of methanol was useful in the following SPE step, SDS 25 mM:MeOH (90:10, v/v) was selected for the extraction.

4. SPE clean-up optimization

Because of the presence of potentially interfering compounds in the extracts and the relatively large volume (5–8 mL) obtained in the PLE, a clean-up/preconcentration step is necessary. SPE with reversed phase Strata X cartridges, which contain a modified styrene–divinylbenzene sorbent, has been used for this purpose. In the case of cannabinoids extracts a stronger clean-up was needed to eliminate SDS which is well-known to cause problems in the chromatographic separation as well in the ESI ionization [200]. For this reason different SPE procedures were used for cannabinoids and the other analytes respectively.

For the multi-analyte procedure, the optimization experiments were performed on fortified PLE drug-free hair extracts at a concentration of 100 ng mL^{-1} and recovery values, which include also the matrix effect (discussed later), were calculated relative to methanol standard solution at the same concentration. Maximum extract loading was found to be 4 mL, a further increase produced lower signal due to matrix effect increase. Elution was performed with methanol, because use of acetonitrile resulted in wider chromatographic peaks. Addition of formic acid to methanol improved recovery of the analytes, which possess basic properties. Recoveries, reported in Fig. II.7, were maximized with 10 mmol L^{-1} formic acid in methanol. The minimum volume of eluent, determined by analysis of subsequent fractions of eluate, was 500 μL. In conclusion the SPE procedure resulted in a good clean-up, high recovery with low standard deviations, and enrichment of approximately one order of magnitude (4 mL to 500 μL).

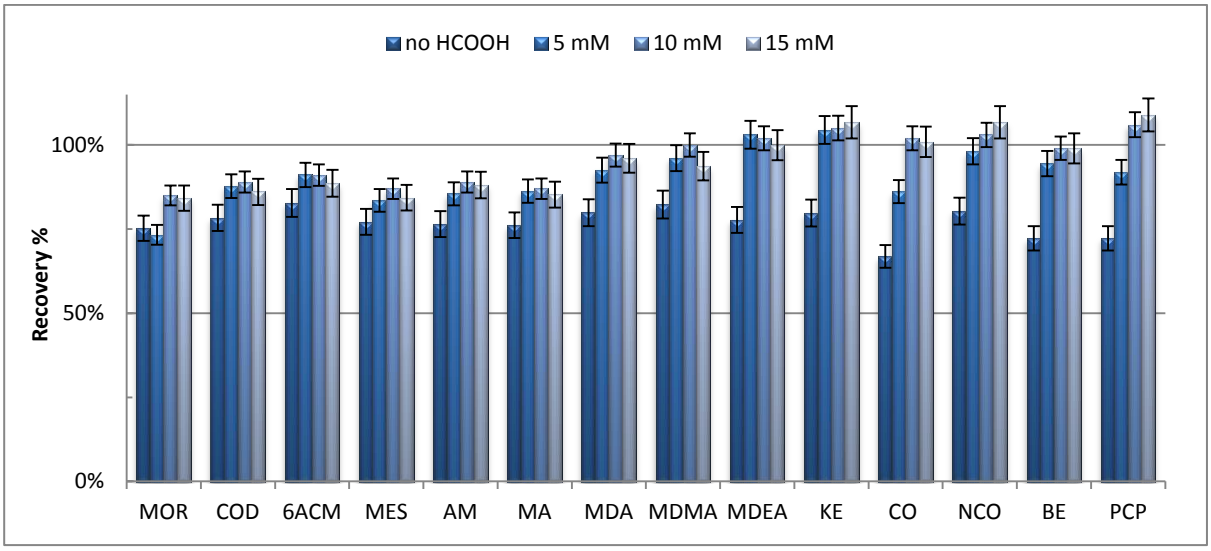


Figure II.7. Effect of formic acid concentration in methanol eluent on SPE recovery. The results were obtained from six replicates

As mentioned above a stronger clean-up was needed for cannabinoids extract to remove the surfactant. The same SPE cartridges were used and particular attention was reserved to the washing step. The first experiments were carried out on model solution containing the selected analytes and SDS at 25 mM in water. The maximum volume of eluate (5-8 mL) which could be loaded into the Strata-X cartridge was initially evaluated: for the preliminary experiments elution was performed with 1 mL of methanol while 1 mL of water was used for washing. As shown in fig II.8 increasing the volume of loading solution, from 1 to 5 mL, the

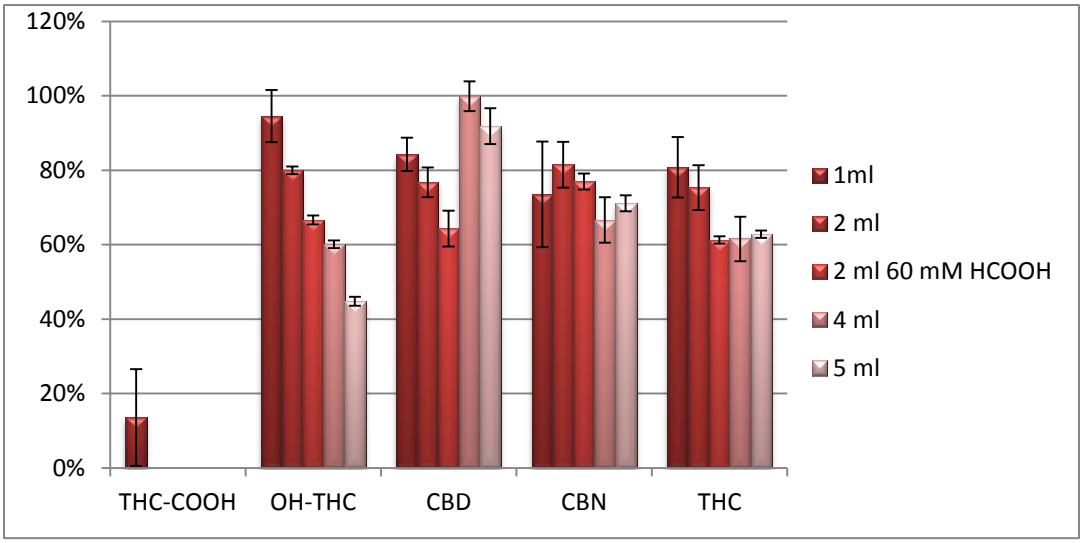


Figure III.8. SPE recoveries obtained with different volumes of loaded SDS solution.

signal of the analytes decreased and for volume higher of 1 mL THC-COOH completely disappeared.

To investigate the cause of these observations a blank sample containing SDS and submitted to the SPE process was analysed by the post-column infusion method (see Introduction §3) to evaluate if matrix effect was responsible for signal decrease.

This experiment showed a severe suppression in correspondence to THC-COOH retention time; further post-column infusion tests using SDS revealed that the surfactant was responsible of the signal drop. Washing step was therefore optimized. In these experiments 6 mL of SDS 25 mM in water were loaded on the cartridge and elution was performed with methanol; increasing percentage of methanol, up to 60% were tested in the washing solution (3 mL). It was found that the sorbent bed of STRATA-X cartridges allowed very strong washing steps, without loose of analytes as shown in fig II.9.

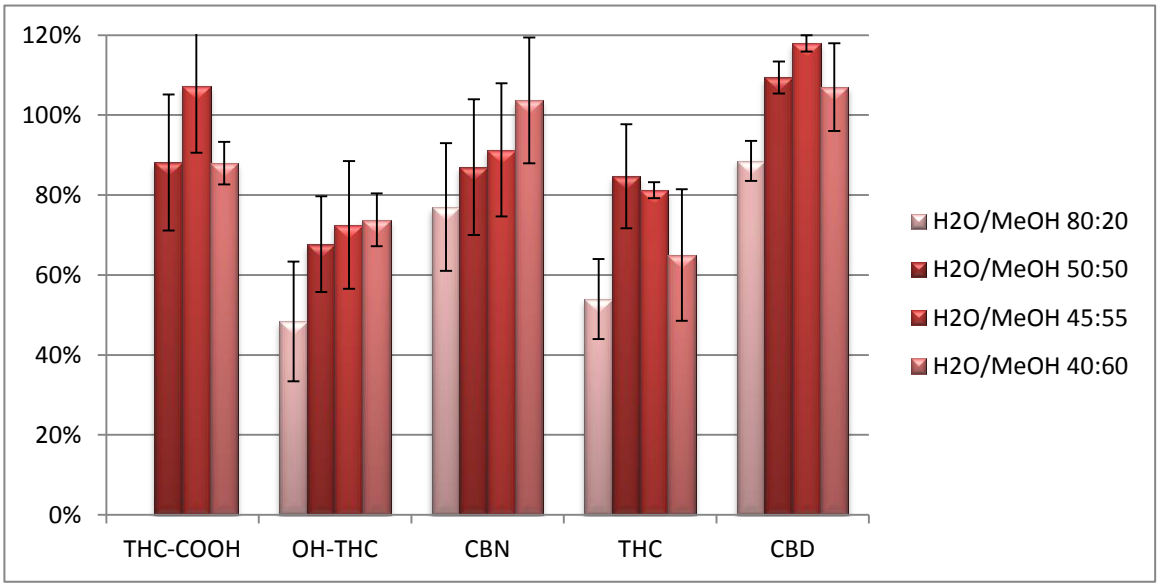


Figure III.9. SPE recoveries obtained with different concentrations of methanol in the washing phase.

The increase of methanol content in the washing solution has been exploited for SDS removal in the eluent; it can be noticed that with 3 mL of water:methanol (45:55, v/v) it was possible to obtain good recoveries avoiding SDS ion suppression. However in the following tests, especially when the eluent volume was reduced by evaporation to dryness, we have

verified that SDS was still present; for this reason it was find optimal to add a further washing step with a low percentage of methanol (80:20, v/v) but a higher volume (6 mL) in order to avoid the analytes loss and increase SDS removal. In addition we found that the addition of a small quantity of formic acid was useful to reduce the losses of THC-COOH in the washing solution since the analyte was completely protonated in these conditions. In summary the washing of the cartridge is performed in two steps: 6 mL of water:methanol (80:20, v/v) and subsequently 3 mL of water:methanol (45:55, v/v) both containing formic acid 5 mM.

Elution step was also optimized and it was found that the addition of NH_3 in methanol allowed a better recovery of the analytes, especially THC-COOH, recoveries were not satisfying using less than 1 mL of eluent (fig II.10).

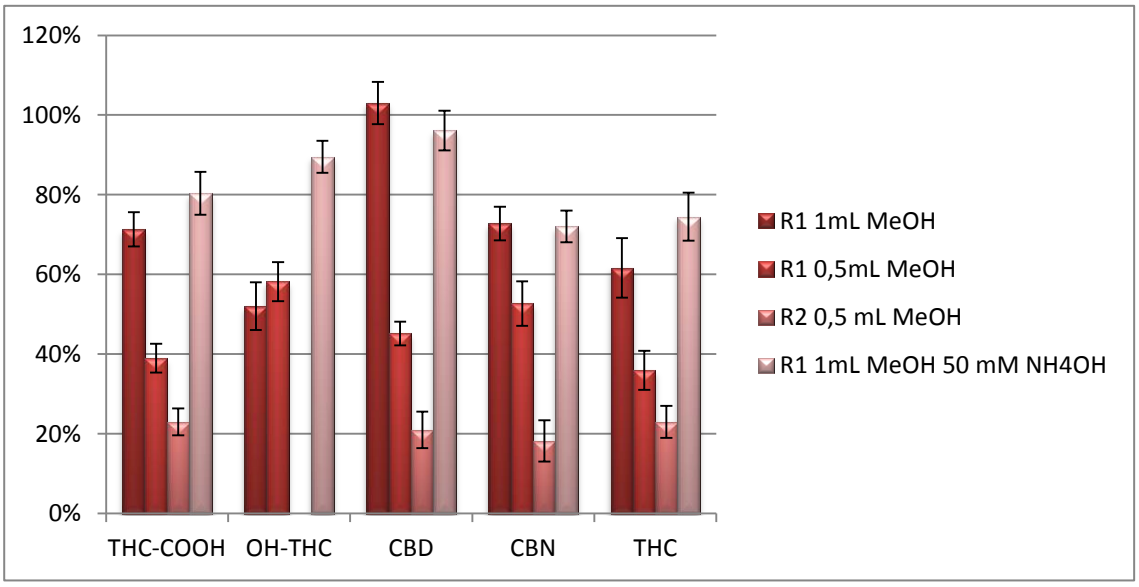


Figure III.10. SPE recoveries obtained with different eluent phases.

Finally to reach the cut-off values of THC-COOH, i.e. 0.2 pg mg^{-1}, drying and reconstitution of the eluent in a smaller volume was crucial, in order to obtain an enrichment of the analytes. After drying the sample was reconstituted with 50, 100, 200 and 250; too small volumes gave bad results because of low recoveries and high matrix effect, 200 µL was a good compromise and allowed to achieve an enrichment factor of 5, suitable to reach the requested cut-off.

5. Comparison of PLE extraction with traditional methods

The evaluation of the effective ability of PLE to extract the analytes from the hair matrix is not easy since it is a solid matrix and traditional spiking does not allow to introduce the analytes inside the keratin matrix but just on its surface. On the other hand certified reference materials (CRM) commercially available do not contain all the drugs and metabolites considered in this study. For this reason an in-house fortified matrix was prepared according to a published procedure by NIST researchers [181], containing all the drugs of the multi-analyte method. In the soaking procedure the ability of dimethylsulfoxide to penetrate the hair matrix was exploited to incorporate the drugs in the hair; a solution containing all the drugs was maintained in contact with the matrix for 3 weeks, assuring the incorporation of the analytes into the hair shaft, however the quantity of drugs effectively incorporated is not known. Matrices obtained with this procedure are normally used as reference materials since they simulate real hair samples; nevertheless there is evidence that the drug does not penetrate as far into the hair from soaking as it would from drug use [201]. Therefore to assess the performance of PLE for the extraction of cannabinoids we used positive real samples which can be easily obtained.

For comparative purposes, different aliquots of real hair samples fortified by soaking and real positive samples (for cannabinoids) were submitted to PLE extraction and to the traditional extraction methods, namely NaOH digestion and HCl extraction. For the latter extraction approximately 50 mg of decontaminated hair fortified by soaking were heated with 2 mL 0.1 mol L^{-1} HCl for 24 h at 45 °C [181]. The extract was then cleaned by SPE (after neutralization with KOH and addition of 2 mL phosphate buffer at pH 6.0). NaOH digestion was performed on 50 mg of decontaminated hair, subjected to basic digestion with 1 mL of 2.5 M NaOH at 60 °C for 25 min [166]; after cooling to room temperature, the solution was neutralised with formic acid. The mixture thus obtained was extracted by vortex mixing with 3 mL of ethyl acetate. After centrifugation the organic supernatant was separated, evaporated to dryness under a gentle flow of nitrogen and the residue was finally reconstituted in 200 μL of methanol.

The choice of two methods was necessary because some analytes (cannabinoids…) are better extracted by NaOH digestion while other analytes (6-MAM, BZE…) are not stable under the strong alkaline conditions; contrarily all the analytes are stable under the PLE conditions. The results obtained demonstrate that PLE is able to extract most analytes from the hair

matrix similarly to traditional techniques as it is shown in table II.3 and II.4 where relative recoveries, normalized to maximum recovery, are shown.

As expected, with NaOH extraction not all the tested analytes are stable. The dilute HCl method resulted in lower recovery compared to PLE for K and 6-MAM. The developed PLE method results in recovery close to the maximum for all the analytes except MOR. For the amphetamine-like compounds MES, K, and PCP, recovery was compared with NaOH digestion, whereas for opiates, cocaine and its metabolites recovery was compared with the dilute HCl extraction method.

Analyte	Extraction recoveries (%)		
	NaOH	HCl	PLE
MOR	-	100	50
COD	-	100	93
6-MAM	-	37	100
MES	100	95	85
AM	79	100	96
MA	91	94	100
MDA	88	95	100
MDMA	90	92	100
MDEA	88	95	100
KE	100	75	89
CO	-	100	92
NCO	-	100	90
BE	-	99	100
PCP	93	85	100

Table II.3. Comparison of relative recovery obtained with NaOH digestion, HCl extraction and PLE. (results are normalized to the maximum recovery for each analyte)

Analyte	Extraction recoveries (%)	
	NaOH	PLE
THC	88	100
THC-COOH	68	100
CBN	100	86
CBD	36	100
OH-THC	93	100

Table II.4 Comparison of relative recovery obtained with NaOH digestion, and PLE. (results are normalized to the maximum recovery for each analyte)

Real comparison of results obtained for MOR and 6-MAM is not easy because of the possible hydrolysis of a significant fraction of 6-MAM to MOR at low pH, leading, then, to underestimation of the PLE extraction efficiency for MOR. This was confirmed by analysis of an sample of certified reference hair by HCl extraction: accuracy of 120 % was achieved for MOR and 32 % for 6-MAM. Accuracy near to 100% was achieved for 6-MAM and MOR with the PLE method.

For the extraction of cannabinoids higher recoveries were obtained for all the analytes except CBN. The differences between PLE and NaOH are particularly significant for CBD which is

known to be unstable under alkaline conditions and high temperatures [191]. In addition with PLE, recovery of THC-COOH is significantly higher and considering the extremely low concentrations found in real sample PLE is particularly advantageous for this analyte.

In summary the performance of PLE for the extraction of most analytes is similar to the traditional techniques; for CBD and 6-MAM, PLE is convenient since these analytes are not stable under acid and basic conditions typical of the traditional techniques tested. In addition with PLE the extraction procedure is automated and the extraction time is very short 5 min vs 30 min and 24 h for NaOH digestion and HCl extraction respectively.

Other procedures based on ultrasonication or incubation with phosphate buffer have recently been proposed [183, 202]. Under our experimental conditions, the performance of these methods is significantly lower than for the PLE method described and with "classical" NaOH digestion and HCl extraction.(from 20 to 50 % for all the analytes tested). In our opinion spiking of the analytes over the hair before extraction is likely to be the main reason for the disagreement found, confirming that the use of CRM or hair fortified by soaking is mandatory to evaluate extraction ability from hair.

6. Validation

6.1 Quantitation and identification

MA-d9, MDMA-d5, BZE-d3, COC-d3, PCP-d5, THC-d3 and THC-COOH-d3 were used as ISs for their analogues and also for other compounds with similar chromatographic behavior or structure. The use, as ISs, of deuterated standards representing a class of drugs rather than single analytes has proved to be optimum, as reported previously [151]. Linearity was satisfactorily described by unweighted least-squares linear regression; reported in Table II.5 and II.6, average determination coefficients (r^2) were >0.99 as required by the SOFT/AAFS guidelines [3].

Different linear dynamic ranges (LDR) were investigated for the analytes because of the different cut-off values reported in the SoHT guidelines. For substances with no reported cutoff, the value given for an analogous compound was used. ULOQ for BZE and NCOC were set a factor of 10 lower than for cocaine because the cut off values are a factor of ten lower than for cocaine; this is because the values expected in real samples for cocaine

metabolites are lower than for the parent drug. A different LDR was investigated also for THC-COOH, since this analyte is found in real samples at a significantly lower concentration compared to the other analytes and for this reason its cut-off value is very low (0.2 pg mg^{-1}).

Blank samples injected between calibrators never revealed carryover phenomena; QCs were always within the limits of precision of the method. LODs were derived experimentally, as suggested by the SOFT/AAFS guidelines, for better determination of the true LOD in chromatographic assays [45], and are reported in Table II.5. To ensure identification of the analytes even at LOD levels, the latter were calculated for the MRM transition having the lowest S/N. LOQs were calculated similarly; for the lowest concentration of calibrator it was verified that an acceptable relative standard deviation (±15%) could be routinely achieved. The LODs and LOQs calculated enable identification and quantification of analytes to the cut-off values reported by SoHT, even for THC-COOH [71].

6.2 Matrix effect and selectivity

Accurate assessment of matrix effects is necessary for bioanalytical methods based on LC–MS, especially in confirmatory analysis. The proposed analytical procedure was shown to strongly reduce matrix effects; ion suppression always affected sensitivity by less than 14% except for PCP, for which sensitivity was reduced by 29 %, indicating the need to use the isotopically labelled PCP-d5 as internal standard. The ratio of the slopes of the calibration curves in solvent and hair are reported in Table II.5 and II.6. The variability of matrix effect, evaluated with 10 different hair samples, was <15% for all the analytes

No interfering signals at the retention time of the analytes were recorded in analysis of drug free samples, demonstrating that the developed method provided good selectivity for all the considered analytes. The effect of melanin (as anionic polymer) on ion suppression was also examined. Samples of white drug-free hair, in which melanin was not present, and pigmented hair were processed separately; two calibration curves from the different matrices were created and the resulting slopes were compared with those from calibration with standard solutions. No statistically significant difference was observed between the calibration curves for white hair and pigmented hair, indicating that the amount of melanin did not affect the analytical response

Analyte	IS	Equation	R^2	LOD (pg/mg)	LOQ (pg/mg)	ME
THC-COOH	THC-COOH-d3	y= 1.42 x + 0.723	0.9921	0.08	0.20	1.11
THC	THC-d3	y= 0.0267 x + 0.017	0.9992	0.8	2.0	0.98
CBD	THC-d3	y= 0.0321 x + 0.002	0.9999	1.5	4.0	0.88
CBN	THC-d3	y=0.040 x + 0.023	0.9971	1.0	3.0	0.91
OH-THC	THC-d3	y= 0.013 x + 0.0132	0.9923	2.0	5.0	0.96

Table II.5. Regression data, LODs, LOQs and ME (cannabinoids).

ANALYTE	IS	Equation	r^2	LOD (pg/mg)	LOQ (pg/mg)	ME[a]
MOR	MOR-d3	y = 0.0247 x + 0.0130	0.9997	7.6	24	0.90
COD	MOR-d3	y = 0.184 x + 0.0417	0.9995	9.4	32	0.93
6-MAM	MOR-d3	y = 0.0031 x - 0.0005	0.9960	8.4	28	0.99
MES	MA-d9	y = 0.0307 x - 0.0456	0.9996	7.4	26	0.95
AMP	MA-d9	y = 0.0297 x + 0.0632	0.9982	9.0	30	0.92
MAMP	MA-d9	y = 0.0429 x - 0.0018	0.9996	2.2	8.6	0.98
MDA	MDMA-d5	y = 0.0191 x - 0.0338	0.9979	7.0	22	0.92
MDMA	MDMA-d5	y = 0.0253 x -0.0132	0.9963	3.0	10.6	0.97
MDEA	MDMA-d5	y = 0.0375 x - 0.0411	0.9984	2.0	9.0	0.86
K	PCP-d5	y = 0.1249 x - 0.1425	0.9968	5.0	16.0	0.93
COC	CO-d3	y = 0.2524 x + 2.1814	0.9994	1.0	3.6	0.87
NCOC	CO-d3	y = 0.1610 x - 0.0299	0.9994	1.8	6.0	0.87
BZE	BE-d3	y = 0.0994 x + 0.0061	0.9992	2.0	7.0	0.87
PCP	PCP-d5	y = 0.1557 x + 0.3351	0.9996	3.0	4.8	0.71

Table II.6. Regression data, LODs, LOQs and ME (multi-class method).

6.3 Accuracy and precision

Accuracy and precision were measured at two concentration values using fortified hair samples and resulted always within the limits of 15%. In addition, to have a better assessment of accuracy and precision CRM, samples prepared by soaking and real samples were analyzed.

Accuracy, measured using the CRM, ranged from 90 to 111 % except for MDEA, which was slightly lower (88 %). For substances not present in the CRM, accuracy reported in table II.7 is the one obtained by spiking over the hair and is close to 100 %, the lowest value being 85 % for MES. Intraday and intermediate reproducibility, expressed as RSD, were constantly ≤13 %; the only values exceeding 10 % were recorded for PCP and MES. The values are reported in table II.8 and II.9.

Analyte	Accuracy (%)
MOR	105
COD†	95
6-MAM	96
MES†	85
AMP	103
MAMP	111
MDA	101
MDMA	103
MDEA	88
K†	91
COC	100
NCOC†	90
BZE	92
PCP†	103
THC	90
CBN	101
CBD	88

Table II.7. Accuracy, calculated for analysis of a CRM. For substances not present in the CRM (†), A% was calculated on a sample obtained by spiking

Analyte	Calculated concentration (ng/mg)	Intra RSD (%)	Inter RSD (%)
MOR	0.11	6	8
	0.19	4	6
COD	0.042	7	6
	0.097	5	7
6ACM	0.070	7	8
	0.037	6	7
MES	0.055	9	8
	0.14	7	11
AM	0.15	7	6
	0.31	4	4
MA	0.40	5	7
	0.83	5	4
MDA	0.20	8	7
	0.43	5	7
MDMA	0.38	5	4
	0.78	3	5
MDEA	0.11	5	8
	0.23	6	9
KE	1.73	4	3
	3.54	3	5
CO	0.18	5	6
	0.34	5	8
NCO	0.097	5	7
	0.24	5	6
BE	0.062	6	5
	0.15	5	7
PCP	0.019	12	13
	0.037	10	12

Table II.8. Intraday and interday precision at two concentrations

Analyte	Conc (pg/mg)	Intra RSD (%)	Inter RSD (%)	Accuracy (%)
THC-COOH	0.2	11	12	89
	10	8	7	91
THC	5	7	8	91
	200	5	9	95
CBD	5	9	11	104
	200	3	5	109
CBN	5	8	7	101
	200	9	10	99
OH-THC	5	12	11	110
	200	9	9	112

Table II.9. Accuracy and precision data for cannabinoids at two concentration levels

6.4 Stability

Good stability of the substances in hair is already well known and is an asset of hair testing for drugs [5]; for this reason stability tests were conducted on working standard solutions and processed samples. In the long term stability experiments the intensity of the signal from the MRM transitions was not significantly different for all the analytes, and no significant alteration was recorded in short term stability, when the solution was kept at −20 °C, during inter-day assays. The analytes are not affected by repeated freeze–thaw cycles: calculated concentrations of stored and freshly prepared samples were within the intermediate reproducibility values of the method. Storage of samples at −20 °C was therefore justified.

Part III

NPS

Extension of the analytical targets

Screening of methylenedioxyamphetamine and piperazine-derived designer drugs in urine using neutral loss and precursor ion scan

EXPERIMENTAL

1. Chemical and reagents

1-phenyl-piperazine (1-PP), 1-(3-methoxyphenyl)-piperazine (3-MeOPP), 1-(4-trifluoromethylphenyl)-piperazine (4-TFMPP), 1-(2-methoxyphenyl)-piperazine (2-MeOPP), 1-(4-fluorophenyl)-piperazine (4-FPP), 4-MeOPP were purchased from Sigma Aldrich (St. Louise, MO,USA); 1-(4-chlorophenyl)-piperazine (pCPP), MDBP, 2-(3,4methylenedioxyphenyl)-piperazine (2-MDPP), 1-(2-fluorophenyl)piperazine (2-FPP), 1-(2-phenylethyl)-piperazine (1-PEP) were obtained from Alfa Aesar (Karlsruhe, Germany); (+)-N-propyl-3,4methylendioxyamphetamine (MDPA), N,N-Di[1-(3,4-methylendioxyphenyl)-2-propyl]amine (Bis-MDA) were purchased from LGC standards (Teddington, UK) while MDA, MDMA and 3,4-methylenedioxy-N-ethylamphetamine (MDEA) have been obtained from SALARS srl (Como, Italy). Acetonitrile and other organic solvents were of chromatography grade from Carlo Erba (Milano, Italy); water was purified using a milliQ system from Millipore (Bedford, MA USA); formic acid and ammonium acetate 98% were obtained from Sigma Aldrich (Milano, Italy).

Stock solutions were prepared by dissolving or diluting each standard in the appropriate amount of methanol in order to obtain individual stock solutions at 1000 mg ml^{-1}. The individual stock solutions of piperazines and methylendioxy-derived compounds were then, respectively, pooled together to obtain 10 mg ml^{-1} solutions in methanol, which was further diluted to obtain standard stock solutions containing 1 and 0.1 mg ml^{-1} of each compound.

All the stock solutions were stored at -20 ° C.

2. LC–MS/MS analysis

Methylenedioxyamphetamine-and piperazine-derived designer drugs analysis was performed by LC–MS/MS. The HPLC equipment consists of a Perkin Elmer (Norwalk, CT, USA) Series 200 Micro-LC Pump with autosampler. A PE-Sciex API 2000 (Toronto, ON, Canada) triple quadrupole mass spectrometer was used for detection.

The analytes were separated using a Gemini C18 column (10 cm x 2.0 mm ID) from Phenomenex (Torrance, CA, USA) packed with particles of 3.0 mm, equipped with a security Guard Ultra Cartridge. The mobile phases were: (A) acetonitrile and (B) 30 mM acetate buffer pH 4.5. The 0.4 ml min-1 flow rate was split 1:4, so only 0.1 ml min-1 were

driven into the ion source. The same mobile phases with different chromatographic runs were carried out for the two classes of compounds. For piperazine-derived compounds, the gradient elution was as follows: increase of the organic phase from 0 to 15% in 13 min and linearly to 65% in the following 2.5 min. Then A was rapidly brought to 100% in 0.1 min, and after 2 min, the column was led to the original ratio within 3 min to enable its equilibration. For methylendioxyderived substances, the organic phase was initially increased from 10 to 30% in 5 min and linearly to 100% in the following 2.5 min. This ratio was maintained for 4 min, and the column was led to the initial conditions within 3 min.

All the analytes were detected in positive ionization with a capillary voltage of 5500 V for methylenedioxyamphetamines and 2500 V for piperazines, nebulizer gas (air) at 20 psi for piperazine and 35 psi for methylendioxy-derived compounds, the turbo gas (nitrogen) at 70 psi and 200°C. All other voltages, including declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) were optimized to provide maximum signal intensity.

Data collection and elaboration were performed by means of Analyst 1.4 software (PE Sciex). Precursor ion scan and neutral loss scan modes were used for the screening of the drugs in human urine. For piperazines, three different experiments were selected: precursor ion scan of m/z 44 (CE 45 V; CXP 6 V) and neutral loss scan of m/z 43 (CE29V; CXP10V)and m/z 86 (CE 30 V; CXP 8 V). For this class of compounds, DP, FP and EP were set, respectively, at 20, 400 and 9 V. For methylendioxyderived compounds m/z 163 (CE 15 V; CXP 8 V), 135 (CE 25 V; CXP 8 V) and 133 (CE 25 V;CXP 8 V) were selected in precursor ion scan mode; DP, FP and EP were set, respectively, at 10, 400 and 9 V.

For both classes, MRM acquisition mode has been used for quantification purposes. The selected masses, together with the main HPLC/MS/MS parameters, are reported in Table III.1.

3. Sample preparation

Urine samples were collected into a polyethylene tube and 3 ml were cleaned up by solid-phase extraction (SPE) using polymeric reversed phase cartridges Strata X 30 mg/1 ml (Phenomenex, Torrance, CA, U.S.A.). Cartridges, installed on Visiprep vacuum system, were previously conditioned with 1 ml of methanol and 1 ml of water. Following the loading phase, cartridges were washed with 1 ml of water–methanol (50:50 v/v) containing 2% of

NH4OH and after drying under full vacuum, the analytes were eluted with 0.5 ml of 10 mM formic acid in methanol. The elute was collected into a vial for the following analysis.

4. Validation

Linearity, matrix effect, precision, accuracy, limits of detection (LODs) and limits of quantification (LOQs) were evaluated, for both methods, according to SOFT/AAFS guidelines [5] and Peters et al. [142] with a few modifications.

4.1 Linearity, LODs and LOQs

Calibration standards were prepared in 10 mM formic acid in methanol. The calibrators were prepared at six levels of concentration (each point has been injected three times), from LOQ to 1000 ng ml-1 for each analyte, and were acquired both in MRM and neutral loss/precursor ion scan modes to assess linearity using both approaches. LODs represent the smallest urine concentration of a compound needed to give a peak height three times the noise level of the background signal from a blank urine sample. According to SOFT/AAFS guidelines, LODs were derived experimentally (not lower than the blank plus three standard deviations) on the analyte MRM transition with lowest S/N analyzing blank urine samples and blank urine samples spiked with decreasing amount of standard solution at appropriate concentration. LOQs were determined similarly as the smallest concentration of a substance needed to give a peak height ten times the noise level of the background signal from a blank urine sample. LODs and LOQs for the screening method were determined similarly on the extracted ion current chromatogram of the best experiment (in terms of signal to noise) for each analyte

4.2 Recovery and matrix effect

Six urine samples were spiked with the appropriate amount of standard stock solution before and after the SPE to obtain a concentration of 200 ng ml^{-1}. Recovery was obtained by comparison of the average peak area of samples spiked before (A) and after SPE (B). Accordingly, R (%) = A/B* 100.

The interference of the matrix on the S/N ratio was evaluated by comparing the calibration curves obtained in blank urine and in 10 mM formic acid in methanol for each analyte. Ion suppression was given by *bs/bm*, where *bs* is the slope of the calibration curve in solvent and *bm* is the slope of the calibration curve in matrix.

Analyte	R_t (min)	Q1 (amu)	EP (V)	DP (V)	Q3 (amu)	CE (V)	CXP (V)
piperazine-derived							
1-PP	9.3	163.0	8	60	120.0	30	8
					77.2	55	10
2-FPP	10.5	181.1	70	7	138.0	28	9
					91.0	45	10
4-FPP	10.9	181.1	57	8	138.0	29	8
					91.0	45	14
1-PEP	10.9	191.3	80	8	105.0	33	10
					76.9	65	12
2-MeOPP	11.9	193.0	60	10	120.0	43	8
					150.0	28	9
3-MeOPP	10.2	193.0	110	8	150.1	29	12
					135.2	38	7
4-MeOPP	11.2	193.0	25	9	150.1	28	7
					122.0	35	6
pCPP	14.9	197.3	100	11	154.1	29	7
					117.9	49	8
2-MDPP	4.3	207.1	75	8	164.1	23	8
					189.9	20	12
MDBP	11.4	220.9	135	7	135.0	30	7
					76.9	58	9
4-TFMPP	15.3	231.1	90	10	188.0	33	12
					119.1	40	10
methylenedioxyamphetamine-derived							
MDA	3.4	180.1	18	8	163.2	15	7
					133.0	25	9
MDMA	3.7	194.1	18	6	163.2	17	6
					133.0	28	7
MDEA	4.1	208.0	18	6	163.2	17	6
					133.0	28	7
MDPA	4.9	222.0	140	8	163.2	21.5	8
					133.0	32	7
Bis-MDA	6.6	342.0	80	9	163.2	27	8
					133.0	40	10

Table III.1. HPLC-MS-MS parameters for selected analytes (MRM)

4.3 Precision, accuracy and selectivity

Precision and accuracy were calculated at three concentration levels (20, 200 and 500 ng ml-1) using freshly prepared calibration curves both for the screening and acquisition modes.

The experimental precision was expressed as the relative standard deviation. Precision, intended as within-laboratory reproducibility, was calculated for each analyte, for three different days from the areas of six urine samples per day spiked before SPE.

Accuracy was estimated from six urine samples spiked before SPE step; the concentration relative to the mean peak area (Cc) was calculated by the calibration curve equation and was compared with the theoretical concentration (Ct). Accordingly A% = Cc/Ct * 100.

The presence of any matrix interferences at the retention time of the selected analytes was observed by processing and analyzing 10 blank samples both in MRM and in neutral loss/precursor ion scan.

RESULTS AND DISCUSSION

The consumption of psychoactive is changing in the last years because of the introduction of several new substances on the market, which are gaining popularity [52, 203]; A well-known structural modification, is represented, for instance by the introduction of a methylenedioxy moiety into amphetamine or methamphetamine leading to the illicit drugs 3,4-methylenedioxyamphetamine (MDA) or 3,4-methylenedioxymethamphetamine (MDMA). These drugs themselves have constituted the starting point for a variety of modifications that were directed mainly to the aromatic rings, or, with minor extent, to the amine function and the alkyl chain [52], leading to 'legal highs' (i.e. unregulated psychoactive compounds). In addition to these amphetamine derivatives, piperazine derivatives represent a further class of new drugs of abuse that are unscheduled in many countries [204-206]. Similar to amphetamines, specific structural modifications have been found also for this class of substances [207]: 1-benzyl-piperazine (BZP), 1-(3,4-methylenedioxybenzyl)-piperazine (MDBP), 1-(3-trifluoromethylphenyl)piperazine (3-TFMPP), 1-(3chlorophenyl)piperazine (mCPP) or 1-(4-methoxyphenyl)piperazine (4-MeOPP) are only few examples.

In general, minor structural changes of common drugs are sufficient to bypass legal controls since substances have to be structurally defined to be scheduled. The synthesis of new derivatives represents an ongoing difficulty for analytical toxicologists since most of these new drugs are not detected by established analytical methods.[208]. In fact, the lack of reference mass spectra in the libraries and/or the use of selected ion monitoring (SIM) or selected reaction monitoring targeted at classic drugs of abuse can result in a failure in the detection of these newer designer drugs. In addition, classic screening methods as immunoassays are not often suitable for detection of the amphetamine-derived and new piperazine-derived designer drugs [204, 209, 210]

In this part a method for the screening of methylenedioxyamphetamine-and piperazine-derived compounds in urine by LC–MS/MS has been presented. The substances, characterized by possessing common moieties, are screened using precursor ion and neutral loss scan mode, providing also semi-quantitative data. This kind of approach was previously successful in the determination of different groups of compounds [211-214] and their potentiality for the detection of homologous unknown compounds was also demonstrated [215]. The application of neutral loss and precursor ion scan acquisition modes allows the identification of molecules belonging to the chemical classes of methylenedioxyamphetamine-and piperazinederived compounds. It is not a general unknown

screening, such as IDA or DDA methods, but a screening directed versus aspecific class of substances with defined MS characteristics (i.e. a specific fragmentation pattern).

In this respect, different methods have been proposed: both methylenedioxyamphetamine- and piperazine-derived designer drugs can be generally detected by liquid chromatography (LC) or gas chromatography (GC) coupled with mass spectrometry (MS) [18, 210, 216-220]. These techniques are typically used as confirmation methods, but the scope of applications has expanded to comprehensive target screenings of several compounds or groups of compounds simultaneously. GC–MS, particularly in the full-scan electron ionization mode, is the reference method for comprehensive screening and reliable library assisted identification [221]. Maurer et al. developed a systematic toxicological analysis (STA) for the screening of piperazine and amphetamine derived compounds by GC–MS in full scan [209, 222-225] or SIM mode [226], which were successful for the screening of a number of emerging designer drugs and their metabolites.

Several approaches have been reported on the use of LC in combination with mass spectrometry (LC–MS or LC–MS/MS) for STA. The application of single-stage MS for this purpose is rather limited; instead, LC screenings with MS–MS identification has been reported more frequently[227]. Usually, MS/MS screenings consist of a survey scan to detect the analytes and a dependent scan for measurement of the corresponding MS–MS spectra; the latter are then sent to library search for identification [228, 229]. Other approaches are also possible [3]; recently, some authors employed multiple reaction monitoring (MRM) or SIM to screen a wide range of new designer drugs[218, 230]. The weak point is that no matter how many compounds the procedure covers, it never constitutes ‘general untargeted’ screening because of the selection of the precursor and fragment ions. For this reason, other acquisition modes in MS^2 as precursor ion or neutral loss are particularly interesting. These acquisition modes are very useful for the analysis of molecules that possess similar fragmentation pattern, with at least an identical moiety, and provide useful structural information allowing anyhow lower sensitivity [231].

1. Liquid chromatography

Two different chromatographic runs were optimized for each class of compounds; however, the same mobile phases were selected in order to avoid dead times between the experiments. A reverse phase column was preferred since the method is aimed to known and unknown molecules, and C18 columns are suitable for a wide range of analytes. The optimal conditions were selected using MRM detection mode taking into consideration the separation, the quality and the intensity of the peaks. Various mobile phases and different modifiers were evaluated to optimize the chromatographic separation; the better results were obtained using acetonitrile as organic phase and 30 mM acetate buffer as aqueous phase. Nearly all the piperazines, including some isomers, and methylendioxyamphetamines were well resolved. The optimized gradients present a slow increase of the organic phase in order to separate the maximum number of analytes.

2. SPE optimization

The optimization of the clean-up step allowed the maximization of the extraction recoveries and the minimization of the ion suppression from urine endogenous compounds. Capacity, loading and elution volume experiments were also carried out. A Strata X polymeric stationary phase was selected, since it allowed the pretreatment of a wide range of compounds. The best conditions were initially studied loading water samples, containing both piperazines and methylendioxyamphetamines at concentration of 200 ng ml^{-1}. The addition of formic acid to methanol resulted in an increase of the recoveries, as the analytes possess basic properties; the highest recovery was obtained using a concentration of 10 mM in methanol. The best elution volume was found to be 500 ml.

Matrix effect was assessed comparing the signals of a post-extraction spiked sample and a sample fortified in methanol at the same level. In the above conditions, ion suppression resulted significant, highlighting the need for a careful optimization of the washing step. Increasing the methanol amount up to 50% in water, this matrix effect was considerably reduced without loss of analytes; the addition of 2% ammonium hydroxide was also very useful to minimize the ion suppression.

Loading volume was finally considered: it was possible to load up to 3 ml without matrix effect increase, having, thus, an enrichment factor of 6.

3. Screening approach

Different piperazine and methylendioxyamphetamine-derived compounds having lateral groups of different nature and located in various positions were selected to search for common fragments or neutral losses. Only a few of the molecules included in the study are known to be psychoactive substances (i.e. 4-MeOPP, MDBP, pFPP, MDMA, MDA, MDEA); however, the inclusion of a wide range of molecules belonging to the same class was thought to be useful to improve the ability to detect the common fragments of the two classes since themethod is aimed also to the screening of unknown substances. Product-ion spectra (PIS) of the molecules were collected at different CEs and, then, characteristic neutral losses and product ions were selected to set up the screening methods. The PIS obtained for the piperazine and methylendioxy-amphetamines derived are reported in Fig. III.1 (common fragments and neutral losses are highlighted on the spectra).

It can be noticed that the neutral loss of m/z 43 is characteristic of phenyl-piperazines independent of the side groups, whereas the neutral loss of m/z 86 is obtained for molecules where the piperazine ring is not directly bound to the aromatic ring (i.e. benzyl-piperazines). These characteristic losses have been already reported in previous LC–MS/MS studies for benzylpiperazine (177.0→91.1) and mCPP (197.0→154.1) [230]. The MS/MS fragmentation patterns for the five selected methylendioxy-amphetamines also gave similar spectra with four major fragments (m/z 163, 135, 133 and 105). The considered substances only differ for substitution at the amine function; CID fragmentations for this kind of compounds have been extensively studied [232, 233] These common fragments are likely to be found for all structurally related compounds. For example, the same fragmentation patterns were found for OH-derivatives of MDMA and MDA that have also been sold as new designer drugs in some drug markets [234]; the fragment ion at m/z 135 was found in the fragmentation pattern of N-methyl-1-(3,4methylenedioxyphenyl)-2-butanamine [235], a relatively novel amphetamine-type substance.

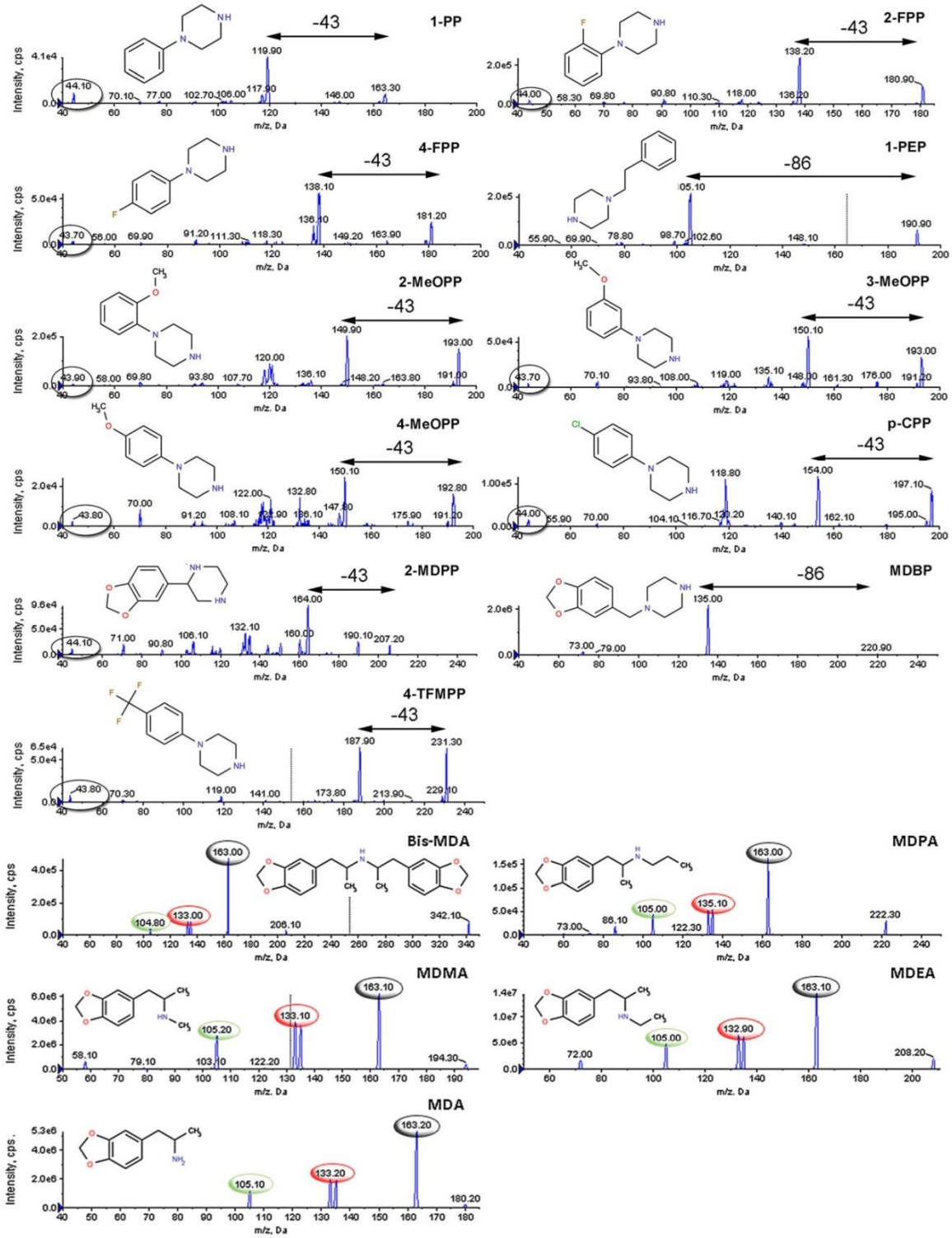


Fig. III.1 Product Ion Spectra for selected analyte (CE= 30 V)s

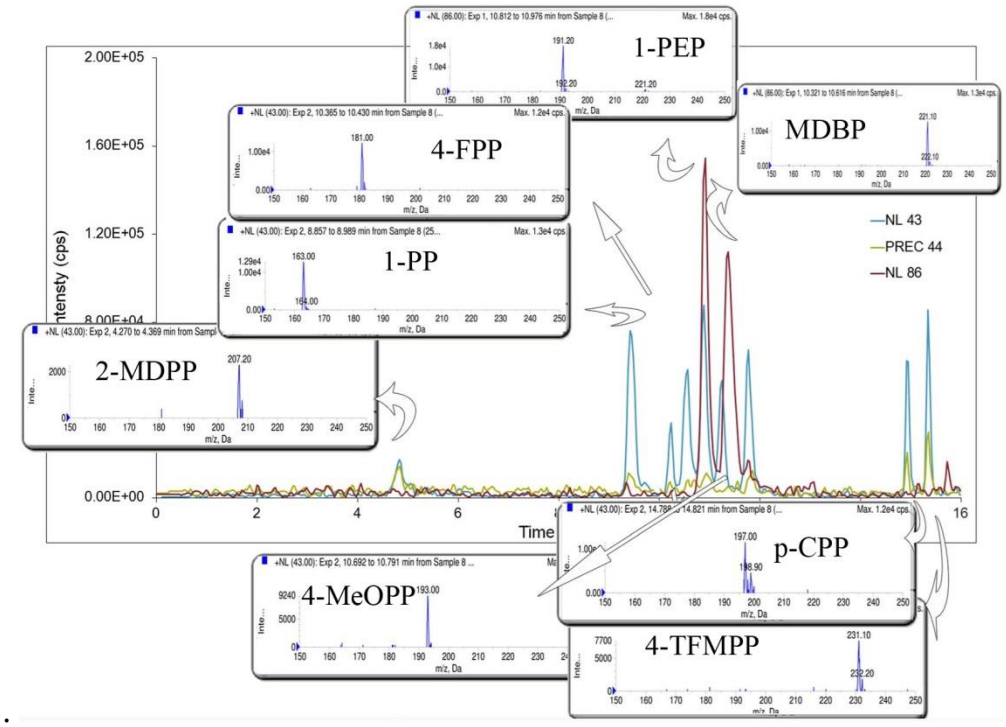
On the basis of the product ions discussed above, neutral-losses and precursor-ion scans were selected to test the method on spiked urine. Three experiments were used for each class of analytes to improve the selectivity for the molecules belonging to the class; detection of piperazines was achieved using precursor ion at m/z 44 and neutral loss of 43 and 86, while detection of amphetamines was obtained scanning the precursor ions at m/z 133, 135 and 163.

The instrumental parameters for precursor and neutral loss have been selected in order to obtain the best performance in terms of sensitivity for each experiment. The critical parameter was the CE, which has been optimized for maximizing the signal of the selected fragment ion for the precursor ion scan experiments, while for maximizing the intensity of the complementary fragment ion for neutral loss scan. As an example for precursor ion at 44 m/z and neutral loss at 43 m/z the collision energies, although they represent the same cleavage, have been set at different values.

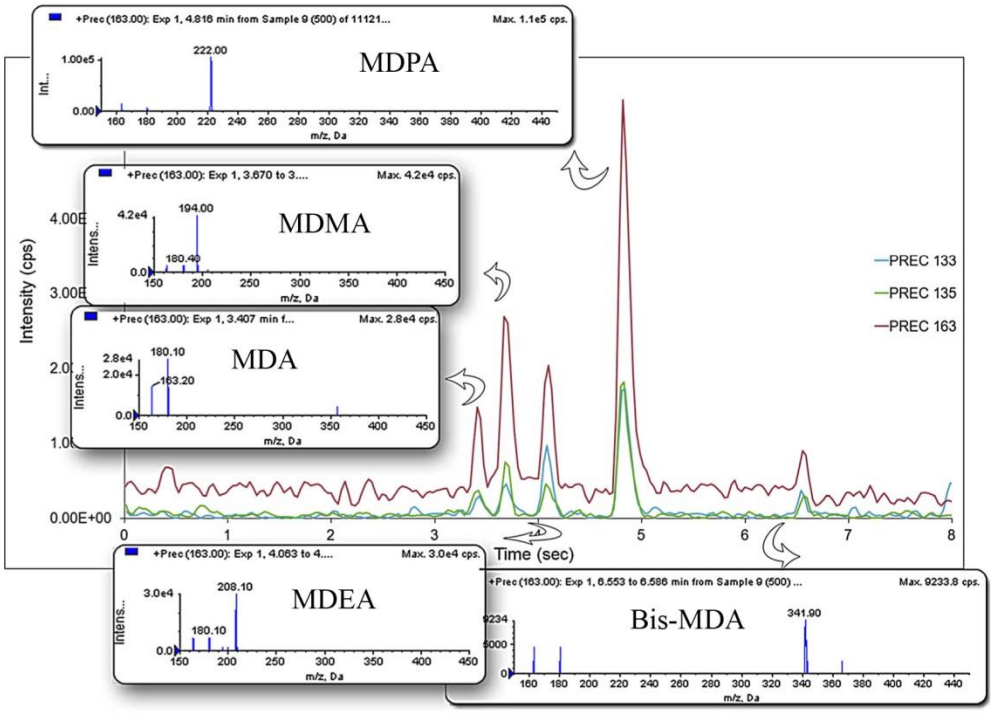
The detection of 'unknown compounds' consists of a set of precursor-neutral loss scans, obtained with the presented screening method. Initially, precursor ion or neutral loss series of experiments are carried out to detect the masses of the potential 'unknown compounds'. In a subsequent phase, product-ion MS/MS spectra of their molecular ions can be acquired to elucidate their structures and an MRM acquisition method for semi-quantitative information can be built. However, we have demonstrated that is possible to obtain reliable semi-quantitative data also in precursor ion or neutral loss scan. The TIC chromatograms reported in Fig. III.2 clearly show the peaks for all the considered analytes. The spectra extracted from each peak reveal the quasi-molecular ions which can be further used in product-ion scan mode to elucidate the structures of the potential unknown compounds. In addition, the mass of the molecular ion can be extracted from the TIC chromatogram, obtaining the XIC profile that can be useful for semi-quantitative purposes. If needed, a confirmation method can be developed selecting suitable MRM transitions.

Since the screening method has been designed for urinary matrix, metabolites have also to be considered. Piperazine designer drugs are mainly metabolized in liver, being the phenyl-piperazines more extensively metabolized than the benzyl-piperazines. Metabolism has been studied only for few piperazines: while BZP and MDBP are mainly excreted as unchanged parent compound, extensive metabolism has been reported for mCPP, TFMPP and MeOPP

[236]. For these last compounds the major metabolic reactions occur at the aromatic moiety (mainly hydroxylation) leaving the piperazine ring unaltered, even after glucuronidation reaction (phase II metabolism) [223]; for this reason, metabolites are likely to be detected by the proposed screening method. As regards amphetamine and related compounds, substantial amounts of unchanged drug are present in urine, especially in acidic urine; MDMA and MDEA are partially metabolized to MDA [237]



a.



b

Fig. III.2 Chromatograms obtained from urine sample spiked with the selected analytes submitted to the SPE step. XIC profiles of neutral loss and/or precursor ion scan are reported for piperazines (a) and methylenedioxyamphetamines (b).

4. Validation

The proposed procedure uses different mass spectrometric acquisition modes to screen compounds belonging to two different chemical classes, leading to a qualitative and a semi-quantitative method. As reported also by Peters et al.[42], 'for qualitative procedures, a general validation guideline is currently not available'. However, they suggest that for qualitative methods, at least selectivity and LODs should be evaluated and that other parameters such as precision, recovery and robustness might also be important. In our validation procedure, we have verified, on the reported known compounds, the method performance for the following parameters: linearity, LODs and LOQs; recovery and matrix effect; precision, accuracy and selectivity.

4.1 Linearity, LODs and LOQs

Calibration standard solutions were prepared over a concentration ranging from LOQ to 1000 ng ml-1 in 10 mM formic acid in MeOH since we have no significant difference in signals between urine and solvent. Linearity data, LODs and LOQs are summarized in Table III.2 and III.3; data obtained by MRM acquisition and screening approach are compared in the same table. The r^2 values are very similar with both approaches and always > 0.99. The values achieved by neutral loss scan and precursor ion scan for LODs and LOQs are up to one order of magnitude higher; this was expected because of the higher noise obtained by these two acquisition methods. In any case, the values are largely below SAMHSA cut off in urine for amphetamines and derivatives that range from 300 to 1000 ng ml^{-1}, allowing the present method suitable for the designed purpose. The data summarized in Table III.2 for the screening approach show that the method can be also employed for semi-quantitative procedures.

Analyte	Equation		r^2	
	MRM	NL/PREC	MRM	NL/PREC
piperazine-derived				
1-PP	$y=177x+1.06\cdot10^3$	$y=2,62\cdot10^3x-3,26\cdot10^3$	0.9967	0.9952
2-FPP	$y=146x+3.73\cdot10^3$	$y=2,29\cdot10^3x+7,01\cdot10^4$	0.9946	0.9929
4-FPP	$y=214x+379$	$y=1,49\cdot10^3x+1,52\cdot10^4$	0.9985	0.9966
1-PEP	$y=359x+1.63\cdot10^3$	$y=4,29\cdot10^3x+4,85\cdot10^4$	0.9946	0.9933
2-MeOPP	$y=86.2x+761$	$y=1,53\cdot10^3x+2,98\cdot10^4$	0.9953	0.9919
3-MeOPP	$y=90.3x+961$	$y=1,72\cdot10^3x+3,93\cdot10^4$	0.9937	0.9929
4-MeOPP	$y=147x+1.33\cdot10^3$	$y=945x+596$	0.9965	0.9985
pCPP	$y=19.3+47.2$	$y=794x+1,26\cdot10^4$	0.9943	0.9933
2-MDPP	$Y=66.7+83.4$	$y=746x-6,52\cdot10^3$	0.9963	0.9929
MDBP	55.7+342	$y=4,25\cdot10^3x+6,06\cdot10^4$	0.9958	0.9935
4-TFMPP	106x+762	$y=1,69\cdot10^3x-6,87\cdot10^3$	0.9976	0.9992
methylenedioxyamphetamine-derived				
MDA	$y=18,8x+175$	$y=1,74\cdot10^3x-5,96\cdot10^5$	0.9929	0.9924
MDMA	$y=148x+2,13\cdot10^3$	$y=2,5\cdot10^3x+3,78\cdot10^3$	0.9961	0.9934
MDEA	$y=170x+3,54\cdot10^3$	$y=2,18\cdot10^3x+7,37\cdot10^4$	0.9931	0.9945
MDPA	$y=103x+886$	$y=6,45\cdot10^3x+2,12\cdot10^5$	0.9972	0.9946
Bis-MDA	$y=135x+2,05\cdot10^3$	$y=135x+2,05\cdot10^3$	0.9979	0.9919

Table III.2 Regression data, for selected analytes in MRM and NL/PREC

4.2 Recovery and matrix effect

Recoveries, reported in Table III.3 resulted always >74% for each analyte. The same SPE procedure has been applied for the two classes of compounds allowing the pretreatment of both in one step. Matrix effect, evaluated on ten different blank urine samples, was greatly reduced as demonstrated by the nearly negligible ion suppression (see Table III.3). This reduction, with no diluting of the urine sample, has been achieved since the selected SPE cartridges allowed the inclusion of up to 50% of methanol in the washing step. Sample

acquired in neutral loss scan and precursor ion scan modes showed ion suppression very similar compared to MRM acquisition mode. This was expected as matrix effect result from the ionization stage that is not influenced by the following acquisition mode.

Analyte	LOD (ng ml^{-1})		LOQ (ng ml^{-1})		Recoveries (%)	ME
	MRM	NL/PREC	MRM	NL/PREC		
piperazine-derived						
1-PP	1	1,5	3	5	81	0.93
2-FPP	1	6	3	20	88	0.86
4-FPP	2	10	6	30	81	0.96
1-PEP	0.2	1,5	0.6	5	86	0.81
2-MeOPP	0.3	6	1	20	85	0.95
3-MeOPP	0.3	3	1	10	85	0.90
4-MeOPP	1	10	3	30	90	0.99
pCPP	2	6	6	20	92	0.86
2-MDPP	2	20	6	60	78	0.75
MDBP	2	2	6	6	94	0.91
4-TFMPP	0.2	3	0.6	10	75	0.75
methylenedioxyamphetamine-derived						
MDA	3	30	9	100	84	0.96
MDMA	0.3	5	1	15	87	0.98
MDEA	0.5	10	1.5	30	82	0.92
MDPA	0.15	1.5	0.5	5	74	0.96
Bis-MDA	0.15	5	0.5	15	80	0.84

Table III.3 LODs, LOQs, recoveries and matrix effect for selected analytes

4.3 Precision, accuracy and selectivity

Precision and accuracy for the quantification and the screening method were very different. The MRM method exhibits a RSD < 15% and accuracy ranging from 90 to 110% for all the analytes, whereas the values reported for neutral and precursor acquisition mode do not respect, for these parameters, the acceptance criteria for a confirmation method (data not shown). Therefore, only semi-quantitative data can be obtained from the screening approach.

The method was found to be selective for all tested analytes. Selectivity experiments with ten different urine samples revealed no interferences of matrix compounds or impurities.

The analytes were injected both as a mixture and singularly in order to evaluate the influence of co-eluting compounds; no significant effect has been detected. However, it must be considered that among piperazine class, some drugs or their positional isomers are also metabolites of therapeutic drugs and might thus result from ingestion of the latter [203]. In cases of doubt, the presence of the respective precursor compounds must be checked to avoid false response (or false positive).

CONCLUSIONS

In the course of the PhD research different methods based on innovative sample preparation techniques or novel analytical approaches were developed for the determination of psychotropic substances in biological matrices by means of Liquid Chromatography – tandem Mass Spectrometry (LC-MS/MS).

Multi-class drugs methods were developed for confirmatory analysis of illicit drugs in urine, plasma, oral fluids (OF) and hair. All these methods are simple, fast and reliable and the sample preparation procedures present significant advantages over the methods reported in the literature; methods have been validated and tested on a number of real samples. Usefulness and applicability of the method is guaranteed by the fact that LODs and, mostly, LOQs for all the analytes were largely below the cut-off values suggested as the lowest necessary by the specific guidelines

A single method was developed for the analysis of 11 illicit drugs from different chemical classes in the biological fluids, despite the greatly different features of the three matrices considered. Miniaturization of Solid Phase Extraction (SPE) led to the reduction of sample volume needed (especially useful for plasma samples). Only 100 µL can be loaded on µ-SPE tips reaching anyhow sufficiently low detection limits. Versatility of the developed method, coupled with its performance as well as the reduced cost, makes it a valuable tool for laboratories involved in routine forensic analysis.

For the first time pressurized liquid extraction (PLE) was shown to be effective for the extraction of drugs belonging to different classes from hair samples. Quantitative extraction of analyte was achieved in a short time, leading to a substantial reduction of analysis time compared with existing extraction techniques. Decontamination step takes into account the complexity of the processes of external contamination and has been proved to prevent positive and negative false results. *Ad hoc*-fortified matrix allowed to take into account the false conclusions that can be obtained by evaluating hair extraction performance when the surface of the hair is spiked with the analytes before the analytical procedure.

Cannabinoids were analysed separately, due to their distinctive chemical characteristics. Their determination was focused on hair, where their analysis is particularly difficult, a surfactant was exploited for cannabinoids extraction leading to high recoveries. The determination of THC-COOH has been shown to be crucial to distinguish between passive

drug exposure and active consumption because this metabolite is formed exclusively within the body; however due to its acid nature its concentration is very low. Nevertheless developed procedure allowed the detection of THC-COOH at its cut-off values in hair.

Analytical target was extended to novel psychotropic substances: an original experimental approach, never previously reported in literature, has been proposed for the determination of new designer drugs, methylenedioxyamphetamine and piperazine-derived. The qualitative and quantitative determination of new derivatives represent an ongoing difficulty for analytical toxicologists since most of these new drugs are not detected by established analytical methods.

The presented method is aimed to the screening of known and unknown substances through the detection of the common fragments of the two classes: characteristic neutral losses and product ions were selected to set up the constant neutral-loss and precursor-ion scan of the two chemical classes considered. These acquisition modes utilized in MS^2 have shown great potentiality for the analysis of molecules that possess similar fragmentation pattern, with at least an identical moiety, and provide useful structural information. The applicability of the screening approach was studied in blank urine spiked with selected analytes and processed by SPE. The ability of the screening method to provide semi-quantitative data was also verified. This method has demonstrated to be a potentially useful tool for the identification of designer drugs derived from piperazines or methylenedioxyamphetamines and can be applied to other drugs classes.

This PhD research route showed that analytical methods can be developed and fully validated obtaining satisfactory low detection limits and forensic identification of psychotropic substances in biological samples without the need of special instrumentations. All the available tools have been exploited to find each time best strategy to solve the system consisting of *matrix - target analytes - instrumental equipment* consistently with forensic purposes (for example a clean-up technique appropriately optimized, the use of columns with superficially porous packing materials to obtain greater efficiency in LC, the use of various and less common acquisition modes in MS/MS).

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