UNODC
United Nations Office on Drugs and Crime

Guidelines for Testing Drugs under International Control in Hair, Sweat and Oral Fluid

MANUAL FOR USE BY NATIONAL DRUG ANALYSIS LABORATORIES

Laboratory and Scientific Section
UNITED NATIONS OFFICE ON DRUGS AND CRIME
Vienna

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Note

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Contents

Introduction

Background

The present *Manual* is a revision of the United Nations Office on Drugs and Crime (UNODC) manual *Guidelines for Testing Drugs Under International Control* (ST/NAR/30/Rev.2). This version has been prepared taking into account recent developments in analytical technology to detect conventional and new, unconventional drugs and is based on up-to-date scientific knowledge of the physiology and pharmacology of the so-called "alternate" biological specimens, which today may offer important information, complementary to the analysis of the traditional biological specimens (blood and urine).

In the intervening time period since the publication of the previous revision of this *Manual,* there have been significant advances in the analytical techniques used for the analysis of drugs under international control in hair, sweat and oral fluid. Concurrently, there has also been an increase in the number of substances that are encountered in drug analysis laboratories, which can vary considerably from country to country and also from region to region within the same country [1]. The drugs appearing in markets in recent years include not only traditional drugs already under international control, but also new psychoactive substances (NPS), or combinations of biologically active compounds distributed in the form of clandestine preparations. Concurrently, it has been noted that there has been an expanding abuse of substances and drugs used for medical purposes, such as benzodiazepines, antidepressants and therapeutic substitutes for opioids.

National institutions as well as clinical and forensic toxicology facilities are required not only to analyse seized materials, but also to detect and measure the abused compounds and their metabolites in biological specimens. In the clinical environment, toxicologists are usually required to promptly identify drugs and drug metabolites to support the physician in the diagnosis and treatment of acute intoxications. Also, in non-clinical settings (forensic, epidemiological, etc.) the toxicologist is often the cornerstone of policies and legislative provisions as well as of court decisions.

As a result of the changes described above, laboratories must be able to deal with an ever increasing number of substances and use analytical methods coupling sensitivity and specificity with the widest analytical spectrum, assuring both rapid

response and robust operation at the same time. Taking into account the analysis of biological specimens, additional challenges must be faced, such as the need for high sensitivity and for high selectivity towards numerous potential endogenous interferences. Furthermore, the rapid decrease of drug concentrations in biological fluids due to the metabolic changes of the parent compounds poses additional problems to the toxicologist.

Given the above considerations, it is clear that an efficient exchange of information between laboratories, as well as between laboratories and regulatory agencies at the national and international levels will offer a harmonization of methods, which forms the basis of an effective global control of the phenomenon of drug abuse. In particular, the validation of analytical methods according to international standards (e.g. ISO/IEC 17025 or 15189) and participation in proficiency testing programmes are recommended to define the accuracy and the trueness of the laboratory's tests, allowing an efficient comparison between results obtained by different laboratories.

Alternative specimens for analysing drugs of abuse

It is generally accepted that chemical testing of biological specimens is the only "objective" means of diagnosis of exposure to therapeutic and non-therapeutic drugs (including toxicants, abused drugs, doping compounds and other xenobiotics). For this purpose, urine testing has been by far the most common toxicological approach because relatively high concentrations of drugs and metabolites are generally present in this biological matrix. However, urine analyses are essentially limited to testing for and reporting on the presence (or the absence) of a drug or its metabolites over a short retrospective period [2]. Blood, in which the presence of many compounds is limited to a few hours, is generally considered the biological sample of choice to detect drugs in the actual phase of biological activity, i.e. to test the drug-related impairment of a subject. The relatively low concentrations and short half-life of exogenous compounds in the blood places important demands on the analytical techniques, which should be 10- to 100-fold more sensitive than for urine. Since the beginning of routine drug analysis in biofluids, the standard approach has been based on immunoassay screening in urine, followed by confirmation with gas chromatography-mass spectrometry (GC-MS) [3]. Even if performed with the most rigorous analytical procedure, an intrinsic weak point of the analysis of drugs in biofluids is the limited detection window (from hours up to a few days) and the prevalence of metabolites versus the parent drug.

In the late 1970s [4], hair was first reported to be suitable for retrospective toxicological analyses, and since then, numerous new applications of innovative analytical techniques have been reported for the detection and measurement of many analytes in hair, including, among others, ketamine, Δ^9-tetrahydrocannabinol (THC),

11-nor-Δ^9-THC-9-carboxylic acid (THCCOOH), doping agents, synthetic cannabinoids and cathinones [5]. Therefore, hair analysis is now considered to be the most efficient tool to investigate drug-related histories, particularly when the period of use needs to be tested back to many days or even months before the sampling [6]. On these grounds, following recent suggestions from international associations, such as the Society of Hair Testing, hair analysis can become not only a fundamental tool in forensic toxicology and medicine, but also a way to find traces of illicit drugs in subjects claiming abstinence for months before sampling. Following the success of advances in hair analysis, other "alternative biological specimens", such as sweat and oral fluid, have gained popularity as forensic specimens, being able to provide information in specific circumstances. As depicted in table 1 [7], these alternate matrices offer different detection windows. In most instances, they show significantly different metabolic profiles when compared to traditional blood and urine testing.

Table 1. Detection windows for drugs in various biological matrices [7]

Specimen	*Detection window*
Blood (serum)	Several hours to 1-2 days
Urine	Several hours to 3 days
Oral fluid	Several hours to 1-2 days (or more for basic drugs)
Sweat (patch)	Weeks
Hair	Months/years

In addition to differences in metabolism and pharmacokinetics, the various biological matrices show other peculiarities, particularly relevant in the forensic environment. First of all, there are issues with the possibility of urine substitution, dilution, and adulteration during sample collection. These problems are much less likely for hair and the other alternate specimens compared to urine. This advantage has promoted the popularity and use of these specimens for drug testing [3]. Also, in comparison to blood, the alternate matrices have the undoubted advantage of a minimally invasive collection procedure, which can potentially be performed in a non-medical setting.

The analysis of drugs in hair was first reported outside the field of forensic toxicology in 1954 [8]. However, only in 1979 [4] was a radioimmunoassay for morphine detection reported and used to document chronic opiate-abuse histories. To date, a literature search for the string of terms "hair and analysis and toxicology" results in about 600 publications, 160 of which were published in the last three years (using the PubMed search in MEDLINE for example). The international interest in the subject is also exhibited by the publication of a number of books [3, 6, 9-11] and by the existence of an international society, "The Society of Hair Testing" (SoHT), founded in 1995. As mentioned above, the major practical advantage of hair testing compared to urine and blood testing for drugs is its larger detection window, ranging from weeks to several months (depending on the length of hair shaft analysed). In practice, by combining the detection windows offered by blood, urine and hair, a

toxicologist can gather objective information on drug use/exposure within an extended time frame.

While the great majority of publications concern the "National Institute on Drug Abuse (NIDA) five" (marijuana, opioids, cocaine, amphetamines and phencyclidine) [3, 6, 10, 11] the number of drugs/metabolites whose detection in hair has been published is increasing constantly, including ethyl-glucuronide and fatty acid ethyl esters (alcohol metabolites), lysergic acid diethylamide [12, 13], ketamine [14], antidepressants [12, 15-17], mephedrone [18], gamma-hydroxybutyric acid [19, 20], synthetic cannabinoids [21-25] and other new psychoactive substances [26]. Hair analysis has also been used for the determination of a large number of pharmaceutical drugs [11] and chemical compounds [27].

Purpose and use of the *Manual*

The present *Manual* is focused on the application of up-to-date techniques of analytical toxicology to the biological specimens, hair, sweat and oral fluid. These biological matrices, having a different composition to more traditional biofluids, i.e. urine and blood, require robust analytical methodologies based on unequivocal determination of the analytes of interest obtained by accurate qualitative and quantitative techniques. The major issues that are still open regarding the interpretation of the qualitative and quantitative results will be discussed

The present *Manual* is one in a series of similar publications dealing with the identification and analysis of various classes of drugs under international control. These manuals are the outcome of a programme pursued by UNODC since the early 1980s, aimed at the harmonization and establishment of recommended methods of analysis for national drug analysis laboratories.

In accordance with the overall objective of the series, the present *Manual* suggests approaches that may assist drug analysts in the selection of methods appropriate for the sample under examination and provide data suitable for the purpose at hand. It should be noted that due to the nature of the analytes and requirements for extraction and analysis, the detection of drugs in biofluids generally requires the need for relatively advanced techniques such as gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. The reader should be aware, however, that there are a number of other methods, including those published in the forensic science literature, which may also produce acceptable results. Any new method that is to be used in the reader's laboratory must be validated and/or verified locally prior to routine use.

UNODC's Laboratory and Scientific Section welcomes observations on the contents and usefulness of the present *Manual*. Comments and suggestions may be addressed to:

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All manuals, as well as guidelines and other scientific-technical publications, may be requested by contacting the address above.

1. The analysis of drugs in hair

1.1 Anatomy and physiology of hair

1.1.1 Structure of hair [28]

Hair is a complex tissue, whose biology and physiology are not yet completely understood. Hair is a keratin-containing appendage that grows from a root located in a cavity of the skin called the follicle [28]. The hair follicles extend from the dermis through the epidermis and the stratum corneum at the surface of the skin, and in humans, cover a high percentage of the body surface. A fully formed hair fibre contains a number of different structures. At its surface, hair has a thick covering consisting of one or more layers of flat overlapping scale-like structures, collectively called cuticles, that function to protect and anchor the hair shaft to the follicle [29]. The cuticle layers surround the cortex, which contains nearly all the fibre mass and fibrous proteins, consisting of spindle-shaped cells that are aligned longitudinally. The third structure is the cell membrane complex. The medulla is the fourth structure, which contains the medullar cells and is detectable only in the hairs with a large diameter (in the human beard, the medulla is complex and, in some instances, a double medulla may be observed).

Chemically, hair is a cross-linked, partially crystalline-oriented polymeric network, which contains different chemical functional groups, with the potential for binding small molecules. It is composed of approximately 65-95 % proteins, 15-35 % water, 1-9 % lipids and 0.25-0.95 % minerals. In particular, the lipids are derived mainly from sebum and the secretion of the apocrine glands of the skin and consist of free fatty acids, mono-, di- and triglycerides, wax esters, hydrocarbons and alcohols. The colour of the hair is related to the different amount and distribution of hair pigments, principally melanin[29]. There are basic structural similarities between hair of different colour, ethnic origin and body region. The fundamentals of hair composition, anatomy and physiology have been described in articles by Harkey [29] and Cone and Joseph [6], and are summarized in the following sections.

1.1.2 Mechanism of formation of hair and hair growth [28]

Normal hair grows in a series of three stages: the anagen (growing stage), the catagen (transition stage) and the telogen (resting stage). During the anagen stage, the papilla

promotes the hair growth. Hair is formed in the zone of cell and protein synthesis. It then moves up to the zone of differentiation, where the melanocytes produce the hair pigment, which is incorporated into each fibre inside the cortical and medullary cells by means of a phagocytosis-mediated mechanism. The intermediate filament proteins, consisting of helical di-sulphur bond dimers of acidic and basic-neutral filaments (keratins), are the first proteins synthesized in the differentiating cortical cells. These dimers, at a later stage, pair to form tetramers that combine, becoming protofilaments (important subunits of the cortex). The cuticle is built higher up in the follicle and consists of cysteine rich proteins. The resulting cell complex, temporarily tied by desmosomes, tight and gap junctions, is finally fixed by the cell membrane complex. Cells are in active proliferation in the root, whereas in the hair shaft any residual metabolism is lost. During the catagen stage, the metabolic activity of the root slows down and the base of the bulb migrates upwards in the skin through the epidermal surface. In the telogen phase, growth stops, the follicle atrophies and finally the hair is lost. The length and the rate of growth of hairs depend on the duration of the stages described above. The duration of the stages varies from person to person, between the scalp hair and the hair of other body regions and even between different areas of the scalp of the same person (mosaic pattern growth).

1.1.3 Incorporation of drugs into hair

The exact mechanism by which chemicals are incorporated and bound into hair is not completely known. By the analysis of experimental data, a multi-compartmental model has been proposed which identifies four ways for the incorporation of drugs into hair [30]:

- Passive diffusion from the blood supplying the hair follicle, as the hair follicle is surrounded by a dense capillary network;
- From sweat, sebum and from the secretion of apocrine glands (only for pubic and axillary hair). Sweat is secreted by eccrine glands on the surface of the body, and can be considered a vehicle for the excretion of drugs. Sebaceous and apocrine gland ducts exit directly into the tunnel of the hair follicle just below the surface of the skin, thus the hair shaft (except for beard hair) is in tight contact with these secretions until it emerges from the skin;
- Because of its high surface-to-volume ratio, the hair may be easily contaminated by the external environment, by exposure to chemicals in aerosols and smoke or by direct contact with powders;
- Directly from the skin to the zone of hair synthesis.

The chemical and physical properties of the compounds affect their susceptibility to incorporation into the hair structure. In general, small molecular dimensions, high hydrophobicity and the presence of basic ionizable groups tend to favour binding into the hair structure. In particular, several potential binding structures for xenobiotics

have been identified in the hair matrix [6]. Among these, a prominent role is exerted by keratin with its carboxyl, amine, phenol, hydroxyl and sulphydril groups, as well as multiple hydrogen bonds. In general, basic drugs, such as cocaine, nicotine and amphetamines are incorporated into hair to a much greater extent than acidic/phenolic molecules, such as GHB, aspirin or Δ^9-tetrahydrocannabinol (THC). Melanin is also widely recognized as a binding "site" for drugs. It may be considered to behave like a weak cationic exchanger and consequently tends to bind basic drugs.

Other factors affecting drug incorporation/elimination are:

- Hair colour (type and concentration of melanin and other pigments) [31-33];
- Ethnic type [6, 33, 34];
- Cosmetic treatments (shampooing [6], bleaching, dyeing, waving, relaxing or exposing hair to strong bases that may cause hair damage with drug loss or affect drug stability [11]);
- External conditions (sunshine, rain or wind can damage the hair shaft, affecting drug concentration [35]);
- It is generally observed that drug concentrations decrease along the hair shaft with increasing distance from the root. Pragst and Balikova report that at 12-15 cm from the root, hair can retain only about 4 % of the original drug concentration (23 different drugs or metabolites were tested) in regularly shampooed hair not exposed to cosmetic treatments [35].

1.1.4 Types of hair [29]

On the human body there are three types of hair, which differ in length, texture, colour, diameter and shape:

- Vellus hair: short, non-pigmented with a small cross-sectional area, which be found on the apparently "hairless" skin, in the region of eyelids or forehead
- Terminal hair: coarse, long, pigmented, with a large cross-sectional area which can be found in the hairy skin areas such as the scalp, beard, eye-brow, eyelash, axillary and pubic areas
- Intermediate hair: intermediate in length and shaft size, which can be found on the arms and legs of adults

Scalp, beard, pubic and axillary hair have been proposed for hair analysis. Several publications describe a lower drug concentration for opioids, methadone, cocaine, methamphetamine and cannabinoids in scalp hair compared to other types of hair [35]. Therefore, the biological differences between hair sampled from different locations must be considered for a proper interpretation of the results of hair testing.

Scalp hair is easy to collect, even under supervision by non-medical personnel. There are considerable variations of growth rate in the different regions of the scalp. Thus, for the sake of uniformity, sampling from the vertex posterior area is recommended. Average values for the anagen stage in scalp hairs are 4-6 years, the catagen stage lasts a few weeks and the telogen stage about 4-6 months, with an overall growth rate ranging from 0.7-1.5 cm per month, as generally reported in the literature.

In the interpretation of toxicological results of scalp hair, the following factors should be carefully taken into account:

- As the duct of the sebaceous glands discharge directly into the hair follicle, the hair shaft is "bathed" in sebaceous secretions until its emergence from the skin.
- The scalp hair is exposed to sweat.
- Contamination from the environment by powders, fumes, solutions or dust is highly possible.
- Cosmetic treatments can alter the incorporation of drugs or can facilitate their elimination.

Beard hairs are thicker, have bigger follicles and have the slowest growth rate (0.27 mm/day versus 0.2-1.12 mm/day in scalp hair). Their follicles are characteristic because the sebaceous gland duct exits from the skin in a channel separate from the channel of the hair shaft; thus beard hair may be somewhat less contaminated by sebaceous secretions. However, as the sample is commonly obtained by shaving, inherently, the specimen can be contaminated by pieces of epidermis. Similarly to scalp hair, beard hair may be contaminated by the external environment.

Pubic hair grows slower than scalp hair (0.3 mm/day versus 0.2-1.12 mm/day), has a longer resting phase and may also be contaminated by urine. Pubic and axillary hairs are less suitable as specimens, as they are curled rather than straight (thus segmental analysis is more difficult) and are also exposed to sebum, sweat and apocrine gland secretions (discharged into the hair follicle).

1.2 Specimen collection

A proper specimen collection protocol forms the basis of a correct interpretation of the toxicological results. According to the guidelines of the Society of Hair Testing (SoHT) [9], sample collection should be performed by a responsible authority respecting the legal, ethical and human rights of the person being tested. A competent individual, but not necessarily a medical practitioner, should collect hair samples.

1.2.1 General recommendations for sample collection [9]

Collection location: collection must be performed within a secure contamination-free facility with access restrictions in place.

Collection devices and consumables:

- A chain of custody form;
- A foil and a collection envelope;
- A security seal;
- A transportation envelope.

The subject should be informed of the procedure and identified by an ID.

The collector must wear gloves and use clean tools to avoid any risk of inter-individual contamination.

Collection site: the posterior vertex region of the head is the preferred sampling site as this region is associated with the smallest variation in growth rate. As an alternative, or in addition to scalp hair sampling, pubic, beard or axillary hair can be collected, taking into account the interpretation issues posed by these differing specimens.

Sample amount: to carry out screening and confirmatory routine tests, and, if required, to repeat the analyses (including counter-analysis by a second laboratory), the amount of sample collected is critical. As a rule of thumb, a "lock of hair with the thickness of a pencil" is adequate. The sampling of a suitable amount of hair from a single site can result in a visible "bald patch" on the scalp, especially in children or subjects with baldness or thinning hair. This can be avoided by collecting several smaller samples from multiple sites of the scalp, possibly within the vertex region.

Collection procedure: a lock of hair should be tied and cut as close to the skin as possible.

Description (depending on the purpose of the test): the case history, relevant anamnesis data, the colour, length, site of collection and any cosmetic treatments should be recorded.

Packaging: the root end of scalp hairs must be aligned and clearly identified. The sample must be firmly secured, e.g. with an aluminium foil, to maintain integrity and avoid contamination.

Storage: hair samples must be stored in a dry, dark environment at room temperature. Direct exposure to sunlight must be avoided. Storage at low temperature is not recommended, as it can result in hair swelling, growth of moulds and drug loss. Storage in plastic bags must be avoided because of contamination by plastic softeners. Plastic is also potentially capable of extracting lipophilic substances. A simple procedure for storage is to wrap the hair sample in aluminium foil and seal it in a paper envelope.

A form of chain of custody must be used throughout the procedure.

1.2.2 Additional recommendations for sample collection

Post-mortem collection

For the collection of post-mortem hair, the sampling procedure detailed above is complemented with the following additional recommendations:

- The collection should be performed at the beginning of the autopsy to avoid external contamination with post-mortem body fluids or tissues leaked during the dissection.
- To correctly package the sample, depending on the decomposition stage of the body, a robust vessel may be preferable to paper envelopes and aluminium foil.
- As information about recent drug exposure can be derived from the analysis of the hair root, this part of the hair could also be collected (e.g. by pulling the hair).

Collection in drug-facilitated crimes

Recently, reports on the use of drugs to modify the behaviour of a victim for criminal gain have increased. Pharmaceuticals (e.g. benzodiazepines, hypnotics, sedatives or anaesthetics) and drugs of abuse (e.g. GHB, ecstasy and LSD) have been reported to have been used, often mixed with alcohol. A low dose is generally needed for the impairment of the victim (except for GHB) and thus it can be relatively straightforward to administer these drugs surreptitiously. Typically, victims report forms of amnesia during and after the event, resulting in a delay in the notification of the crime.

In these instances, the toxicologist may be asked to detect, by means of hair analysis, a single drug exposure having occurred weeks or months before sample collection [15]. Although the body of literature supporting this type of application is limited and interpretation controversial, in sample collection it is mandatory to complement the general recommendations with the following additional key points:

- The hair sample must be collected a minimum of 4-6 weeks after the alleged incident. This period is adequate to ensure that the hair shaft incorporating the drug emerges from the bulb area in the follicle to a height above the skin surface sufficient for collection.
- The collection of more than one sample is recommended following a positive result to corroborate the finding.
- Due to possibly differentiating drug concentration along the hair shaft, segmental analysis is mandatory.
- The victim must avoid cosmetic treatments or haircuts until the investigation has been concluded.

1.3 Sample preparation

1.3.1 Choice of analytical method and target drug according to case history

Prior to hair analysis, a proper evaluation of anamnesis data, case history and circumstantial information is mandatory. Also, the characteristics of the hair sample should be considered in order to decide the pre-analytical and the analytical strategy. In order to avoid generic screenings, which inherently not only sacrifice sensitivity and selectivity, but also require higher sample quantities, it is generally preferable to focus on one or more targeted drugs and consequently identify the most appropriate analytical methods. Indeed, depending on the molecular characteristics of the targeted drugs, different procedures in terms of decontamination, solubilization, extraction, derivatization and analysis are needed.

1.3.2 Division into segments

It is generally agreed that analyses of scalp hair cut into measured segments of 10-30 mm may provide a "profile" of the toxicological history of the subject.

However, in particular cases, such as drug facilitated crimes, the analysis of even smaller segments has been reported to give a detailed account of the time of drug intake, even in instances of single intake [9]. Prior to cutting the hair sample, the expected distribution of drugs along the hair shaft, in respect to the distance from the root, must be taken into account for a proper decision on the length of the segments.

Because of exposure to degrading agents for a longer period of time and to repeated washings, the analysis of segments of hair very far from the root may provide a low diagnostic sensitivity. Moreover, considering that uncertainty in correlating hair length with the time of drug incorporation increases along with hair age, it has been recommended to investigate progressively longer segments of hair, starting from the scalp end. For a more detailed discussion of the strategy for segmental hair analysis, the reader can refer to the review by Pragst et al. [36].

1.3.3 Decontamination procedure

Washing the hair sample before the analytical process must be carried out with two main aims [9,35]:

- To reduce possible analytical interferences and improve the extraction recovery by the removal of external residues on the hair surface, such as hair care products, sweat, sebum and dust;

- To remove possible "external contamination" from drugs present in the environment and passively deposited onto the hair shaft surface (including the possibility of sample contamination inside the laboratory).

The hypothesis of differentiation between drug use and environmental contamination by applying suitable decontamination procedures was already proposed in the first years of application of hair testing [4], but the problem has not yet found a widely accepted solution. The most recent methods that aimed at excluding that a "positive" result is affected by external contamination have been proposed by Cairns et al. [37] and Tsanaclis and Wicks [38]. Both methods are based on the analysis of the wash solutions in parallel with the analysis of the hair. Moreover, it should be highlighted that the washing procedure must be validated in each laboratory, taking into account that an unsuitable decontamination process strongly affects the interpretation [3, 11, 39].

The following general concepts are useful for developing a proper decontamination procedure:

- The purpose of the washing step is to remove external impurities as completely as possible while simultaneously avoiding extraction of the drug from the hair matrix. Since the achievement of this goal is practically impossible, the analyst should find the most favourable compromise by tuning the wash/rinse procedures. Non-protic washing solvents (e.g. dichloromethane, acetonitrile) do not cause hair to swell and consequently are believed to remove external contamination without loss of compounds incorporated in the hair matrix. In contrast, protic washing solvents (e.g. water, methanol) cause the hair matrix to swell and therefore may also remove part of the embedded compounds [40].
- The washing procedures can include a combination of cycles with organic solvents and aqueous solutions with or without detergents [40,41].
- In the case of massive external contamination, no washing procedure can completely remove the contaminating compounds from the hair.
- The practical relevance of the discrimination between environmental contamination and drug use depends on the purpose for which hair testing is applied. (This problem will be discussed in section 1.6, on Interpretation).

Among the solvents suggested in decontamination procedures, the following have proved to be effective, particularly when combined: surfactants (e.g. 0.1 M sodium docecylsulfate), phosphate buffers, acetone, diethylether, methanol, ethanol, dichloromethane, hexane and pentane. Many different procedures have been proposed in the literature, none of which has gained general acceptance. However, the following simple and general method is proposed [12, 35, 42, 43].

Analytical Notes: Decontamination

Procedure with organic solvent

1. Take a hair strand (~ 100 mg).
2. Wash with 5 ml dichloromethane for 2 min.
3. Dry with adsorbent paper.
4. Wash again in 5 ml dichloromethane for 2 min.
5. Dry again.

Procedure with aqueous solvent

1. Take a hair strand (~ 100 mg).
2. Wash with 10 ml of 0.1 % SDS in water (w/v) for 3 min.
3. Rinse twice with 10 ml of water for 3 min.
4. Rinse with 10 ml of acetone for 3 min.
5. Dry in oven at 60°C for 30 min.

1.4 Extraction and clean-up [9, 35]

The direct detection of drugs in the integer hair shaft is difficult, although matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has shown this potential [44], and usually drugs must be extracted from the keratin matrix by digestion and/or elution. Laboratories introducing new hair testing methodologies must investigate the efficiency of different extraction conditions to optimize their in-house procedures, while minimizing hydrolysis of labile drugs. Furthermore, as a large panel of substances are usually screened in hair, sample preparation should be fairly unselective.

The first step in sample preparation is the homogenization that is performed by cutting hair into snippets of 1-3 mm or by grinding (e.g. in a ball mill). In order to ensure sample homogeneity, it is recommended that at least 20-30 mg of hair be subjected to the above process. In this step, care should be taken to avoid carry-over (e.g. by contamination of the scissors) and disposable vials should be used. The compounds embedded into the hair matrix can be solubilized by using a variety of extraction methods, whose efficiency and selectivity must fit both the characteristics of the target drugs and the analytical techniques, as discussed in the following paragraphs.

1.4.1 Extraction with methanol

Methanol dissolves neutral, hydrophilic and moderately lipophilic compounds; because of its hydrophilic character it penetrates the hair, producing swelling of the matrix and the liberation of the drugs. The sonication of the sample in an ultrasonic bath enhances the extraction process.

Advantages

- Almost all drugs can be extracted by methanol.
- It is effective towards hydrophilic and lipophilic compounds.
- This procedure is "mild" towards unstable compounds that easily undergo hydrolysis (e.g. heroin and cocaine).
- Direct injection of the extract in GC-MS or LC-MS is possible when the drug concentration is sufficiently high.
- Methanol/aqueous organic acid mixtures have been proven to enlarge the panel of drugs that can be efficiently extracted [45-47].

Disadvantages

- Methanol extracts often incorporate interfering substances, and a clean-up procedure (such as liquid/liquid or solid phase extraction) is recommended in routine use.
- The recovery of ionizable drugs is incomplete and lower than in other extraction procedures.

1.4.2 Extraction by aqueous acids or buffered solutions

Incubation in aqueous 0.01-0.50 M HCl or in 1 M phosphate buffer at pH 6.4-7.6 is usually performed at 56°C or 60°C overnight. When needed (e.g. to exclude external contamination), morphine glucuronides, representing a minor fraction of total morphine, can be determined by comparing morphine concentration before and after treatment with glucuronidase/arylsulfatase [48, 49].

Advantages

- The extract is generally cleaner than methanol extracts.
- Basic drugs are efficiently extracted.

Disadvantages

- Hydrolysis of the following molecules has been reported:
  - Partial conversion of cocaine to benzoylecgonine;

– Conversion of heroin (diacetylmorphine) to 6-monoacetylmorphine (6-MAM);
– Hydrolysis of 6-MAM to morphine.

1.4.3 Digestion in diluted NaOH

1 M NaOH is added to hair and incubation is typically performed for one hour at 80°C, or overnight at 60°C. It is suitable only for drugs that are stable under alkaline conditions.

Advantages

- Particularly advantageous for nicotine, amphetamines and some neuroleptics.
- Very effective for quantitative recovery.
- Can be used in combination with headspace solid phase microextraction (HS-SPME) for the detection of semi-volatile compounds (e.g. amphetamine derivatives, local anaesthetics, barbiturates, diphenhydramine, ketamine, methadone, phencyclidine, phenothiazines, tramadol, and tricyclic antidepressants).
- Can be useful for the detection of drugs which have very low concentrations such as metabolites of cannabinoids.

Disadvantages

- It is not suitable for drugs that are unstable under alkaline conditions (e.g. cocaine, benzodiazepines).
- The partial or complete solubilization of the hair matrix produces a “dirty” solution which needs careful clean-up before instrumental analysis.

1.4.4 Clean-up of hair extracts

Except for the direct application of immunoassays (see 1.5.1), the solutions obtained from the extraction of hair samples are substantially incompatible with analytical procedures such as gas chromatography coupled to mass spectrometry (GC-MS), which has been the standard analytical technique used in this field. Therefore, clean-up procedures by using liquid-liquid phase extraction (LLE) or solid-phase (SPE) extraction have been used since the early times of hair analysis [50].

SPE methods are generally preferred over LLE, because of their higher ease of automation and a generally higher "cleanliness" of the extracts. One drawback of these techniques is the cost of the extraction consumables (disposable cartridges). SPE has recently been miniaturized in the form of solid-phase microextraction (SPME), in which the extraction process occurs on the surface of a polymeric fibre. Fibres with different chemistries are available and the selectivity of the extraction towards the targeted drug can consequently be tuned. The fibre is then desorbed and regenerated in the high temperature GC-MS injection port. SPME has found particular success in hair analysis as headspace SPME (HS-SPME) [15].

In general, it should be stressed that the selectivity of the clean-up strongly affects the results of the subsequent analytical procedures. Highly selective clean-up gives rise to cleaner extracts, enhancing the detectability of the target analyte(s), but may exclude other classes of analytes from being detected because of poor recovery. Thus, a trade-off must be found between selectivity and versatility of the clean-up procedure. A general review of the modern sample pre-treatment methods for many classes of analytes can be found in Wada et al. [51]. One of the most commonly used SPE clean-up procedures for opioids and cocaine is described hereafter.

Analytical notes

- Treat 30-50 mg of washed hair by extraction or digestion/extraction as described above (sections 1.4.1-3).
- Extensively centrifuge the extracts.

SPE clean-up

1. Condition an SPE column (e.g. Bond Elute Certify) with methanol (2 ml) and phosphate buffer 0.1 M, pH 6 (2 ml).
2. Transfer the supernatant hair extract to the column, taking care to avoid transferring the precipitate.
3. Wash with 2 ml of water.
4. Wash with 3 ml of 0.1 M HCl.
5. Dry the column at maximum vacuum for 10 minutes.
6. Wash with 5 ml of methanol.
7. Elute with 2 ml CH_2Cl_2/Isopropanol (80:20) at 2 % of NH_4OH.
8. Dry the organic eluate under nitrogen at 40°C; if gas chromatography-mass spectrometry (GC-MS) is planned (see below), derivatize by adding N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) + 1 % of trimethylchlorosilane (TMCS) at 75°C for 20 minutes).

1.5 Methods for the analysis of drugs in hair [9, 11, 35]

1.5.1 Screening techniques

When compared to urine testing, methods for hair analysis have to cope with lower drug concentrations and a limited amount of sample collected. In hair matrices, parent drugs and their intermediate metabolites are generally prevalent, whereas in urine very often only end metabolites are detectable. A screening procedure to distinguish between presumptively positive samples and negative ones can be performed by either immunological or GC-MS methods. However, more advanced techniques such as liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) or high resolution mass spectrometry (LC-HRMS) provide the substantial advantage of simultaneously "screening" and identifying a wider range of drug classes/analytes in a single analysis [52]. The most common screening methods are briefly discussed below.

Immunological methods

These methods are based on the molecular recognition between an antibody and its antigen(s) (drugs or metabolites) and are poorly affected by biological matrix interferences. They are most suitable under controlled conditions, e.g. when the sample solution has a pH and composition compatible with the reactivity of antibodies and the reactions used for the detection. The associated clean-up of the hair extracts can then be simplified or avoided.

Laboratories that use immunological methods should ensure that:

- Screening assays have adequate sensitivity to detect the drug concentrations present in the liquid obtained by extraction/digestion of hair;
- Screening assays have adequate specificity and cross-reactivity to deal with the drug/metabolite ratios present in the hair, which are mostly different from urine;
- The hair matrix does not interfere with the immunoassay;
- All presumptively "positive" samples should be confirmed by a technique based on chromatographic separation and mass spectrometry.

Chromatographic methods

Although GC-MS is traditionally considered the "gold standard" of confirmation procedures in hair testing, it can also be employed for screening, allowing the simultaneous analysis of several classes of drugs (e.g. amphetamines, cocaine,

opioids) whenever the extraction/clean-up and derivatization procedures can be unique [53]. A wider screening has recently been reported using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) methods. When in combination with high resolution mass spectrometry (HRMS), LC-MS in full scan and alternating ionization modes (positive and negative) can be a powerful screening procedure for both targeted and untargeted analytes.

1.5.2 Confirmation techniques

The purpose of "confirmation" is the unequivocal identification of the analytes detected in screening and their accurate quantification. A confirmation technique should be based on chromatography to separate the target analytes from other xenobiotics and the endogenous interferences from the matrix, and should be coupled (hyphenated) to a highly specific detection method. In principle, any analytical technique used for confirmation of the results should have both higher sensitivity and specificity than screening and its qualitative/quantitative accuracy should be widely known in the field of application.

In hair analysis (and generally in pharmaco-toxicological analysis of biosamples), GC-MS and LC-MS/MS are by far the most recognized techniques, due to the high specificity and selectivity of the MS or MS/MS detection. However, laboratories using GC-MS and/or LC-MS for confirmation of results should ensure that their confirmation techniques:

- Have sufficient sensitivity to detect low drug levels, well below the suggested cut-offs;
- Are able to describe the drug/metabolite ratios in hair, where relevant;
- Have an adequate accuracy for quantification in real conditions;
- Can identify drugs not included in the traditional screening panels by a wider detection window.

Gas chromatography–mass spectrometry (GC-MS)

Capillary gas chromatography-mass spectrometry has been by far the method most frequently used in hair analysis. The main advantages of GC-MS are:

- High resolution of the capillary GC;
- High specificity of electron impact ionization (EI) mass spectra, producing diagnostic fragment ions that can be used in library comparisons;
- Little or no ion suppression/ion enhancement effects;

- High selectivity of measurement in the selected ion monitoring (SIM) or, when GC is coupled with tandem mass spectrometry (MS/MS) in multiple reaction monitoring (MRM) mode enhancing signal-to-noise ratios;
- Possibility of using cold isotope labelled (e.g. deuterated) internal standards to control crucial phases of the analytical process, such as extraction recovery, injection, ionization efficiency, etc.

Modern GC-MS and GC-MS/MS instruments allow the measurement of a large number of substances in the same analytical run, by subdividing the acquisition time into time windows in which different SIM or MRM experiments are performed.

To undergo GC-MS, the targeted analytes must be volatile and thermally stable. Derivatization helps to analyse compounds which are not inherently suitable for GC-MS. In GC-MS, the EI mass spectra are highly informative, due to extensive fragmentation of molecules upon ionization. Both positive and/or negative chemical ionization (GC-PCI/MS and GC-NCI/MS) can also be used. The latter approaches, often more selective than EI, could nonetheless produce a loss of information power due to lower fragmentation of ionized species. In this case, the use of MS/MS (either in triple quadrupoles or ion traps) on precursor ions ($M+H^+$ or $M-H^-$) increases specificity and sensitivity by reducing the non-specific noise originating from the matrix and enhancing the signal-to-noise ratios. As in every GC-MS analytical method, analytes must be identified by comparison to the retention times and the relative abundances of the qualifier ions of the same analytes in a positive quality control sample run in the same analytical session. For quantification, the ratio of the abundance of quantifier ion to the quantifier ion of a deuterated internal standard must be used. Standard calibration curves can be obtained by adding pure standards, prepared in methanol, to pulverized control hair (e.g. blank hair obtained from children or laboratory personnel).

Liquid chromatography-mass spectrometry (LC-MS)

LC, operating in liquid phase and at room temperature, has a much wider analytical applicability than GC, being able to separate polar, thermally labile, high molecular weight or ionized molecules. On the other hand, LC exhibits lower chromatographic performance than GC, though recent developments in ultra-high performance liquid chromatography (UHPLC) have greatly enhanced resolution. Following the development of atmospheric pressure ionization (API) and related "soft" ionization techniques (e.g. electrospray-ESI and atmospheric pressure chemical ionization—APCI), LC-MS coupling has been realized and has extensively entered all fields of analytical chemistry, including forensic toxicology. However, because of limited selectivity of the chromatographic separation and the lack of fragmentation produced by ESI and APCI, LC-MS tends to be less selective than GC-MS. For this reason, MS/MS instruments (ion traps or triple quadrupoles or hybrid instruments) or high resolution MS (time of flight, ion cyclotron resonance, Orbitrap) are generally coupled to LC instead of single quadrupole instruments, which lack the ability to carry out MS/MS analyses.

A number of papers dealing with LC-MS/MS in hair analysis have been published [35, 51, 54, 55], which provide the following points of interest:

- A multitude of substances of toxicological interest (drugs, pharmaceutical drugs, endogenous compounds, pollutants, metabolites, etc.), can be determined by LC-MS/MS techniques, including illicit drugs, methadone, buprenorphine, benzodiazepines, neuroleptics, performance enhancing drugs, doping agents ethanol metabolites.
- A wide range of compounds, of largely different chemical characteristics can be detected simultaneously (hundreds of drugs in a single chromatographic run).
- With some limitations, the clean-up steps can be simplified in comparison to GC-MS.
- Despite its advantages, LC-MS/MS is generally more expensive and less standardized than GC-MS and consequently its use has been limited to a relatively small number of laboratories.
- Some methods suitable for the identification of the principal classes of drugs are described in 1.5.6. They are based on the most "classical" LLE for clean-up of hair extracts and GC-MS techniques. For a hair decontamination procedure, see 1.3.3.

1.5.3 Amphetamines by GC-MS (adapted from Frison et al.*) [56]*

Although different methods based on the coupling of a highly efficient separation technique with mass spectrometry can be used for this purpose, a relatively straightforward and cost effective GC-MS method is herein proposed.

Solubilization/extraction

1. To 30-50 mg of decontaminated, powdered or cut hair, accurately weighed, add 1 ml 1 M NaOH and 1 ng/mg of deuterated amphetamine derivatives.
2. Allow to stand for 10 min.
3. Incubate at 45°C overnight (or in an ultrasonic bath for a shorter time).
4. Add 5 ml ethyl acetate and homogenize.
5. Agitate on a horizontal shaker or by inversion for 20 min.
6. Centrifuge for 15 min. at 3,500-4,000 rpm.
7. Collect the organic phase, add 2 drops of methanol-HCl (99:1 v/v) and evaporate to dryness. Do not overdry.

Derivatization

1. Add 15 µl of 2,2,2-trichloroethylchloroformate.
2. Add 35 µl of ethyl acetate.

3. Incubate for 15 min. at 80°C.
4. Re-evaporate to dryness.

Dry solvents and reagents must be used. Solvents can be dried using molecular sieves.

Analysis

- Dissolve the derivatized extract in 50 ml ethyl acetate.

GC-MS operating conditions

GC oven conditions:	Column temperature initially set at 50°C and held isothermal for 0.5 min., the temperature is then ramped to 200°C at 30°C/min., then ramped to 300°C at 10°C/min. and held isothermal for 2 min.
Column:	5 % phenylmethyl silicone column, 30 m length, 0.25 mm ID, 0.25 µm film thickness
Injection parameters:	Splitless, 1-2 µl injected
Carrier gas:	Helium, 0.9 ml/min.
MS Detector:	Single quadrupole, electron ionization (EI) mode
Acquisition:	Selected ion monitoring (SIM), see table 4 for specific ions to monitor target analytes and deuterated internal standards (IS)

Table 2. GC-MS diagnostic ions of studied derivatives [56]

Analyte	*Ionic species*						
	*m/z**	*m/z*	*m/z*	*m/z*	*m/z*	*m/z*	*m/z*
Amphetamine[a]	218	220	274	288	309		
Methamphetamine[a]	232	234	236	238	288	323	
MDA[b]	218	220	353	355	135	206	
MDMA[b]	232	234	367	369	135	206	
MDEA[c]	246	248	250	252	381	383	
MBDB[c]	246	248	250	252	381	383	177
BDB[c]	232	234	367	369	177		

*Quantifier ion.

[a]SIM ions for amphetamine can be acquired in the same time segment as methamphetamine.

[b]SIM ions for MDA can be acquired in the same time segment as MDMA.

[c]SIM ions for MDEA, can be acquired in the same time segment as MBDB and BDB.

Abbreviations: MDA: 3,4-methylendioxyamphetamine; MDMA: 3,4-methylendioxymethamphetamine, MDEA: 3,4-methylendioxyethylamphetamine; BDB: 3,4-methylenedioxyphenyl-2-butanamine; MBDB: 3,4-methylenedioxyphenyl-N-methyl-2-butanamine.

1.5.4 Cannabis by GC-MS

Although different methods based on the coupling of a highly efficient separation technique with mass spectrometry can be used for this purpose, as widely feasible in forensic toxicology laboratories, a relatively straightforward and cost effective GC-MS method is herein proposed.

Determination of THC, CBD, CBN

Extraction

To 30-50 mg decontaminated, powdered or cut hair add:

1. 100 ng of tri-deuterated Δ^9-tetrahydrocannabinol (Δ^9-THC-d3).
2. 2 ml 1M KOH (or NaOH).
3. Incubate for 30 min at 95°C (or overnight at 45°C).
4. Cool the sample.

LLE clean-up

1. Add 5 ml iso-octane.
2. Agitate by inversion for 15 min.
3. Centrifuge for 15 min. at 3,500-4,000 rpm.
4. Collect the organic phase in a glass vial.
5. Evaporate to dryness under nitrogen at 40°C.
6. Dissolve the extract in 40 µl iso-octane.

GC-MS operating conditions

GC oven conditions:	Column temperature initially set at 60°C and held isothermal for 0.5 min. The temperature is then ramped to 300°C at 15°C/min. and held isothermal for 15 min.
Column:	5 % phenylmethyl silicone column, 30 m length, 0.25 mm ID, 0.25 µm film thickness
Injection parameters:	Splitless, 1-2 µL injected
Injector temperature:	250°C
Carrier gas:	Helium, 0.8 ml/min.
Detector:	Ionization mode, positive EI mode
MS parameters:	Selected ion monitoring (SIM), see table 3 for ions to monitor

Table 3. GC-MS: typical retention times (RT) and fragment ions used for analyte identification and quantification [27]

Analyte	*RT (min.)*	*m/z**	*m/z*	*m/z*
Cannabidiol	9.3	231	246	314
Tetrahydrocannabinol (THC)	9.6	299	271	314
THC-d3 (IS)	9.6	302	274	317
Cannabinol	9.9	295	238	310

*Quantifier ion.

Determination of THC-COOH

Extraction

To 30-50 mg decontaminated, powdered or cut hair add:

1. 100 ng of tri-deuterated Δ^9-tetrahydrocannabinol (Δ^9-THC-d3)
2. 2 ml 1 M KOH (or NaOH)
3. Incubate overnight at 45°C
4. Cool the sample

LLE clean-up

1. Add 1 ml HCL 1M pH 4 and 3 ml hexane: ethylacetate 9:1.
2. Agitate by inversion for 15 min.
3. Centrifuge for 15 min. at 3,500-4,000 rpm.
4. Collect the organic phase in a glass vial.
5. Add again 3 ml hexane: ethylacetate 9:1.
6. Agitate by inversion for 15 min.
7. Centrifuge for 15 min. at 3,500-4,000 rpm.
8. Collect the organic phase in a glass vial.
9. Put together both organic phases.
10. Evaporate to dryness under nitrogen at 40°C.
11. Derivatize with 50 µl N,O-Bis(trimethylsilyl)trifluoroacetamide, (BSTFA), 1 % trichloromethylchlorosilane (TCMS) at 70°C for 30 min.

GC-MS operating conditions

GC oven conditions:	Temperature initially set at 140°C and held isothermal for 2 min.; the temperature is then ramped to 250°C at 20°C/min., held isothermal for 10 min., then ramped to 290°C at 10°C/min. and held for 5 min.
Column:	5 % phenylmethyl silicone column, 30 m length, 0.25 mm ID, 0.25 µm film thickness
Injection parameters:	Splitless, 1-2 µl injected
Injector temperature:	250°C
Carrier gas:	Helium, 0.8 ml/min.
Detector:	Single quadrupole, electron ionization (EI) mode
Acquisition:	Selected ion monitoring (SIM); For THC-COOH m/z 473, 488,* 489; For its trideuterated analogue (internal standard) m/z 476, 491,* 492

*Quantifier.

Alternative methods using negative ion chemical ionization (NCI) and GC-MS/MS have also been proposed in the literature [57-59].

1.5.5 Opioids and cocaine by GC-MS

Although different methods based on the coupling of a highly efficient separation technique with mass spectrometry can be used for this purpose, as widely feasible in forensic toxicology laboratories, a relatively straighforward and cost effective GC-MS method is proposed.

Extraction

To 30-50 mg of decontaminated powdered or cut hair, accurately weighed, add:

1. 1 ml 0.1 M HCl.
2. 1 ng/mg of deuterated opoid and cocaine derivatives (e.g. benzoylecgonine-d3, cocaine-d3, morphine-d3).
3. Add a magnetic stir bar and incubate for 16 h at 56°C while stirring.

Clean-up

1. Neutralize the extract, adding 1 ml of 0.1 M NaOH and 2 ml of 1 M phosphate buffer, pH 8.4.
2. Add 10 ml dichloromethane-isopropanol-n-heptane (50:17:33, v/v) and homogenize.
3. Agitate on a horizontal shaker or by inversion for 20 min.
4. Centrifuge for 15 min. at 3,500-4,000 rpm and separate the organic layer.
5. Add 5 ml 0.2 M HCl, extract and collect the organic layer.
6. Add 1 ml 1 M NaOH, 2 ml 1 M phosphate buffer, pH 8.4, 5 ml dichloromethane, extract and collect the organic phase.
7. Evaporate the extract to dryness.

Derivatization

1. To the dried extract add 30 µl bis(trimethylsilyl) trifluoroacetamide (BSTFA) or N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) plus 1 % trimethylchlorosilane (TMCS).
2. Incubate for 30 min. at 70°C.

GC-MS operating conditions

GC oven conditions:	Column temperature initially set at 70°C and held isothermal for 1 min., the temperature is then ramped to 220°C at 30°C/min., then further ramped to 300°C at 30°C and held isothermal for 5 min.
Column:	5 % phenylmethyl silicone column, 30 m length, 0.25 mm ID, 0.25 µm film thickness
Injection parameters:	Splitless, 1-2 µl injected
Injector temperature:	250°C
Carrier gas:	Helium, 1.2 ml/min.
Detector:	Single quadrupole, electron ionization (EI) mode
Acquisition:	Selected ion monitoring (SIM), see table 4 for specific ions to monitor target analytes and deuterated internal standards (IS)

Table 4. GC-MS: typical fragment ions used for analyte identification and quantification [48]

Analyte	*Ionic species*				
	*m/z**	*m/z*	*m/z*	*m/z*	*m/z*
Cocaine	303	182	272	82	
Cocaine-d3(IS)	306	185	275	82	
Benzoylecgonine	361	346	240		
Benzoylecgonine d3(IS)	364	349	243		
Codeine	234	313	343	356	371
Morphine	324	414	429		
Morphine-d3 (IS)	327	417	432		
6-MAM	324	340	399		

*Quantifier ion; 6-MAM = 6-monoacetyl morphine.

1.5.6 Opiates and cocaine by LC-MS [54]

Decontamination

1. Wash 20 mg of hair three times with 1 ml dichloromethane.
2. Dry the sample at 40°C.

Homogenization/pulverization

1. Cut the sample into small segments.
2. Put it into a stainless steel smashing vial immersed in liquid nitrogen.
3. Pull a magnetic plunger back and forth in the vial to pulverize the sample
4. Alternatively, pulverize in a ball mill.

Extraction

1. Add 10 µl of a 1000 ng/ml of deuterated internal standard solution (benzoylecgonine-d3, cocaine-d3, codeine-d3, morphine-d3, 6-MAM-d6).
2. Add 990 ml methanol.
3. Incubate for 4 h at 40°C under ultrasonication.

Analysis

1. Transfer approximately 800 ml of supernatant into an auto-sampler vial.
2. Inject 10 µl into the LC/MS-MS.

LC-MS operating conditions

Column:	4.5 mm (ID) x 150 mm, 3.5 µm phenyl column
Mobile phase:	95 % methanol, 5 % 10 mM ammonium acetate adjusted to pH 4 with 99 % formic acid in isocratic mode
Flow rate:	0.5 ml/min.
Injection volume:	10 µl
Acquisition:	Triple quadrupole in positive ion electrospray ionization (ESI) mode; Multiple reaction monitoring (MRM) mode, parameters as described in table 5, monitoring two transitions for the analytes and one for the internal standards

Table 5. Optimized MRM parameters for analytes and internal standards [60]

Analyte	*Precursor ion MH^+ m/z*	*Product ion (transition I) m/z*	*Product ion (transition II) m/z*
Morphine	286	165	128
Codeine	300	165	152
6-MAM	328	165	211
Heroin	370	165	152
Morphine-d3	289	152	—
Codeine-d3	303	165	—
6-MAM-d3	331	165	—
Heroin-d9	379	212	—
Cocaine	304	182	77
Benzoylecgonine (BZE)	290	168	105
Ecgonine methylester (EME)	200	182	82
Norcocaine	290	168	136
Cocaethylene (CE)	318	196	82
Cocaine-d3	307	185	—
Benzoylecgonine-d3	293	171	—
EME-d3	203	185	—
CE-d3	321	199	—

1.6 Interpretation of results

The interpretation of hair analysis generally aims at elucidating the following key points in order to:

- Establish if the individual used or was exposed to drugs;
- Identify the drugs that were used;
- Distinguish between single, occasional, repeated or chronic use;
- Identify the time of drug intake.

A critical examination of the case history considering the hypothesis of external contamination, site of hair collection, variability of hair growth, drug pharmacology and a thorough review of the literature [35] must accompany the interpretation of analytical results. Because of its inherent complexity, interpretation of the results from hair analysis should only be performed by specifically experienced personnel [61].

1.6.1 Analysis of hair in comparison with other conventional biological specimens

Drug testing in hair as compared to blood and urine exhibits the following peculiarities:

1. *The sampling procedure*. Blood collection is a relatively invasive procedure and in most countries can be carried out only by a medical doctor. Conversely, urine collection can be performed by nurses/non-medical personnel, but may interfere with privacy, and therefore strict surveillance of the procedure can be problematic and cause medical/psychological problems (e.g. shy bladder). On the other hand, elusive manoeuvres on the part of the subject, such as adulteration and substitution of urine, are possible. There is a debate on the legitimacy of strict direct surveillance (particularly by non-medical personnel), or video controls. In contrast, sampling of scalp hair is only minimally invasive, and can be performed by an experienced individual, not necessarily a medical practitioner. Whereas pubic hair poses collection problems similar to urine in terms of privacy, the risk of adulteration and substitution of the hair sample is lower.

2. *The time window of detection*. As previously reported in section 1.2, the detection window of drugs in plasma/serum (3 hours to 2 days) and urine (6 hours to 3 days) is considerably lower with respect to hair (> 3 days to months/year). Furthermore, because of the slow kinetics of drug decomposition in hair, the repetition of collection to obtain a fresh, substantially equivalent hair sample is possible when there is a claim of specimen mix-up or breach in the chain of custody. However, factors such as location of the collection, growth rate of the hair and cosmetic treatments may not allow substantially equivalent samples to be collected.

3. *The deterioration of the sample and its storage*. After sampling, blood and urine must be refrigerated or frozen to avoid deterioration, whereas hair can be stored at room temperature for years. Because of its high surface-to-volume ratio, hair is extremely susceptible to contamination from the environment during storage and care should be taken to avoid exposure to humidity and direct sunlight.

1.6.2 Drug consumption and external contamination [37,38]

Since the early years of hair testing, the differentiation between personal use and external contamination by drugs has been an issue. This problem is particularly relevant for those illicit drugs that are prone to contaminate the environment because of their availability in powder form (e.g. cocaine and heroin), or because of their modes of consumption (smoke, inhalation, snorting). The environmental exposure can lead to:

- Direct external contamination of the hair with minimal penetration under the hair surface;
- Inadvertent inhalation/intake of small amounts of drug, resulting in systemic adsorption and leading to trace levels of the drug in question and its metabolites inside the hair.

External contamination is likely to occur in the household of a drug user or a drug trafficker. Otherwise, external contamination is also possible in personnel professionally exposed to drugs or in subjects working in clandestine laboratories [62, 63]. However, occupational exposure can be avoided by the use of proper protective equipment [64, 65].

As previously discussed, when interpreting a positive result from hair testing, the toxicologist should always be aware of alternative explanations, and particularly of external contamination. In the process of interpretation, the toxicologist should preliminarily consider historical, circumstantial and clinical data. In the analytical phase, in order to minimize the possibility of misinterpretation, the following precautions have been suggested to differentiate external contamination (Cairns et al. and Tsanaclis and Wicks [37, 38]):

(a) Perform a preliminary decontamination of the hair by washing. Determine drugs in the washing and calculate the ratio between drug concentration in the last washing residues and in the hair matrix;

(b) Verify the coexistence of drug and its relevant metabolites in adequate proportions;

(c) Use of cut-off levels;

(d) When available, calculate the ratio between drug concentrations in hair collected from different body sites (e.g. scalp hair and pubic hair).

Laboratories should use a wash step of the hair sample for decontamination before analysis; however, there is no consensus or uniformity in the washing procedures, which need to be validated in each laboratory [3, 11, 39]. The analysis of the wash residues and comparison with the drug concentration detected in the hair can be useful to assist differentiation between drug use and environmental contamination. The detection of drug metabolites in due proportions is another important piece of evidence to confirm drug use or contamination, even if still not definitive [66, 67].

Typical examples of the use of criteria for drug metabolites are:

- The detection of 11-Nor-Δ^9-THC-9-carboxylic acid (THC-COOH) and 11-hydroxy-Δ^9-THC (THC-OH) in addition to the parent drug Δ^9-tetrahydrocannabinol (THC) [58, 59];

- The determination of benzoylecgonine (BE), cocaethylene (CE), ecgonine-methylester (EME) and norcocaine (NC) together with cocaine (COC) [66] with suggested ratio metabolite/parent drug: BE/COC > 0.05[7] (varying ratios as a function of drug use were reported by Pepin and Gaillard [68]);

- The determination of 6-monoacetylmorphine (6-MAM) morphine, acetylcodeine and codeine together with the parent drug heroin (rarely detected in hair), with suggested ratios: 6-MAM/morphine > 1.3 [7] (varying ratios as a function of drug use were reported by Pepin and Gaillard [68]);

- The detection of amphetamine (AP) and methylenedioxyamphetamine (MDA) respectively for the parent drug methamphetamine (MA) and methylenedioxymethamphetamine (MDMA) (ratios in function of the concentration of the parent drug were reported by Han et al. [69]).

As to the use of interpretation/decisional levels of drugs in hair, the following cut-offs are recommended by the Society of Hair Testing to identify "chronic drug use" [9].

Table 6: Cut-offs recommended by the Society of Hair Testing to identify "chronic drug use" [9]

Screening		*Confirmation*	
Group	*Cut-off (ng/mg)*	*Target analyte*	*Cut-off (ng/mg)*
Amphetamines	0.2	Amphetamine	0.2
		Methamphetamine	0.2
		MDA	0.2
		MDMA	0.2
Cannabinoids	0.05	THC	0.05
		THC-COOH	0.0002

Screening		Confirmation	
Group	Cut-off (ng/mg)	Target analyte	Cut-off (ng/mg)
Cocaine	0.5	Cocaine	0.5
		BE, EME, CE, NC	BE/COC 0.05
Opioids	0.2	Morphine	0.2
		Codeine	0.2
		6-Acetylmorphine	0.2
Methadone	0.2	Methadone	0.2
		EDDP	0.05
Buprenorphine	0.01	Buprenorphine	0.01
		Norbuprenorphine	0.01

Abbreviations: MDA: methylendioxyamphetamine; MDMA: 3,4-methylenedioxy-N-methylamphetamine; THC: Tetrahydrocannabinol; THC-COOH: 11-Nor-Δ^9-THC-9-carboxylic acid; BE: Benzoylecgonine; EME: ecgonine methyl ester; CE: cocaethylene; NC: Norcocaine; COC: Cocaine.

These values are not limits of quantification or detection, but "decisional" values and must be used with special attention to the specific issue to which the test is applied. For example, in cases of drug facilitated crimes, the expected concentrations can be much lower than the above cut-offs due to single exposure to an impairing drug. THC-COOH, the specific metabolite of THC that would allow to discriminate between passive, external contamination and active intake of *Cannabis I.* preparations, may be present in hair at extremely low concentrations (in the low pg/mg range). Consequently, very sensitive and specific methods must be used to detect THC-COOH in hair, exhibiting a lower limit of quantification < 0.2 pg/mg; a specific extraction such as bead-assisted liquid-liquid extraction on pulverized hair is required, followed by derivatization and GC-MS/MS detection in negative ion chemical ionization (NICI) conditions with methane as a reagent gas. In order to detect THC-COOH at such low concentrations, the performance of the triple quadrupole detectors used must be optimized, and their use cannot be proposed here as a routine methodology available in the laboratories all around the world [58, 59].

1.6.3 Interpretation of the relation between drug dose-drug concentration in hair [11, 35]

The possibility of establishing a relation between the amount of drug intake and drug/drug metabolites concentrations in hair remains highly controversial and is not recommended.

Various factors, out of the control of the toxicologist, are responsible for that controversy, among which:

- The available studies in humans are generally retrospective and based on self-reported data that are inherently untrustworthy;
- Prospective studies with a strict control of drug intake are uncommon;
- The overall drug intake is usually uncertain due to the unknown purity of the clandestine drug preparations available on the market;
- Drug incorporation depends on hair type and structure;
- Drug elimination/degradation depends on the type and frequency of washing and cosmetic treatments as well as on the hair structure.

A limited number of studies concerning the relation between the amount of drug intake and their concentrations in hair have been performed under strict control for heroin, methadone and THC. This opportunity was offered by heroin maintenance programmes with controlled intravenous administration of pharmaceutical grade heroin. In this context, Kintz et al. observed the impossibility of inferring the amount of drug used on the basis of hair concentrations [32]. However, in contrast, Musshoff et al. reported an actual correlation between the dose and the total opiate concentration in the hair. Furthermore, considering individually heroin and its metabolites, 6-acetylmorphine and morphine, it was noted that the coefficient of correlation increased in correspondence to the respective plasma half-life [70].

Himes et al. studied the concentration of methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in hair on a population of opiate dependent pregnant women. They concluded that methadone and EDDP concentration in hair do not agree with the total methadone dose administered [71].

Huestis et al. observed different detection rates of THC and THC-COOH between daily and non-daily cannabis users [72]. Other papers, although based on self-reports, would support the existence of a dose-concentration relationship, albeit weak for heroin, methadone, THC and, additionally, for methamphetamine [73] and cocaine [68, 74].

1.6.4 Time of drug intake [35]

The identification of the time of drug intake requires sectional analysis that should consider the drug history of the subject and the growth rate of sampled hair (1.1 ± 0.2 cm/month for scalp hair, 0.03 cm/day). The rationale of identification of the time of drug intake is that, under ideal conditions, incorporation occurs only in the hair root and the location of the drug molecules in the hair shaft does not change over time. According to Pragst et al. [36], knowing the growth rate of hair, the calculation of the date of drug intake is a function of the distance of the molecule from the root and of the hair growth rate. This can be expressed by the following equation:

$$\mathbf{Ti = Ts - Li/Vh - Lr/Vh - To}$$

Ti = Time of the drug intake

Ts = Time of the hair sampling

To = Time between incorporation of the drug into the hair root and appearance at the skin surface

Li = Distance of the drug position in hair from the proximal end of the hair sample

Lr = Length of the residual hair shaft from the skin surface after sampling

Vh = Hair growth rate

As incorporation occurs between matrix cells and the end of the keratinization zone, covering a distance of 1.2-1.5 mm, the time resolution is about 3-4 days, but can increase for drugs with a longer half-life in blood. Several examples seem to confirm a correlation between the drug history and the drug concentrations in seriate hair segments. However, other reports, which take into consideration the variability of hair growth and of the mechanisms of drug incorporation, emphasize the following limits of the model explained above:

- Incorporation of drugs or metabolites from sweat or sebum leads to an extension of the drug zone in the distal direction.
- In a lock of hair the population of individual hairs is heterogeneous, including growing elements and other elements in resting telogen phase, in uneven proportions. Consequently, the drug distribution along the shaft is non-homogeneous and the measured drug concentration in a section of the lock is the average of that in the single hairs. This greatly reduces the time resolution of the segmental analysis.

For long hair, two additional key points must be considered:

- The mean drug concentration decreases with the increase of the distance from the hair root (i.e. the increase of age of the hair). For example, after more than one year, only about 4 % of the original amount is reported to remain for 23 drugs of abuse [35].
- The time resolution of a hair segment decreases with increasing distance from the root, thus it is generally recommended to investigate progressively longer hair segments.

1.7 Applications of hair analysis in the forensic field

Hair analysis in the forensic field is mainly applied in the following settings, with a special focus on drugs under international control and their metabolites:

(a) Investigating drug abuse histories in criminal and civil contexts;

(b) Workplace drug testing;

(c) Driving licence re-granting;

(d) Divorce, child custody;

(e) Testing for previous intentional/unintentional drug use around a certain date;

(f) Determination of gestational drug exposure;

(g) Investigation of doping practices;

(h) Post-mortem toxicology:

- Drug-related deaths;
- Health impairments caused by chronic drug abuse;
- Tolerance in opioid death cases;
- Chronic drug use and fatal accidents;
- Criminal poisoning;
- Contribution to identification of a corpse;
- Demonstration of external contamination;

(i) Drug-facilitated crimes (robbery, sexual assault, child abuse);

(j) Alcohol abuse.

Investigation of drug-use history [3, 11, 35]

A retrospective timetable of an individual's drug use can sometimes be provided by segmental hair analysis. It is a valuable aid to evaluate the reliability of self-reported history and to provide substantial evidence of the past behaviour of the subject, including compliance to detoxification treatments and switching from one drug to another. The knowledge of the drug abuse history on an objective basis, such as by using hair testing, can be particularly useful especially when a reliable drug history cannot be obtained, such as in the case of psychiatric patients.

Workplace drug testing [35]

Since the late 1980's, some countries have adopted policies to ensure a "drug-free workplace" in order to protect public safety, corporate security, and minimize losses in productivity. In this context, employees may undergo drug testing to exclude drug abuse during work hours. Additionally, workers employed in safety-sensitive jobs often are requested to provide objective evidence excluding any form of drug abuse to obtain a certificate of fitness-to-work. In these contexts, urine analysis is by far the most common tool. However, in special cases, hair analysis is preferred because of its wider surveillance window compared to urine, even if higher costs and

operative complexity may hinder its adoption. In this field, the risk of false positive results caused by environmental contamination may, however, generate cause for litigation. It should also be noted that false negative results may occur, especially for infrequent users of cannabis, because of the poor incorporation of THC-COOH in the hair matrix.

Driving licence re-granting [3, 11, 35]

On the basis of the recognized ability of hair testing to unravel long-term histories of drug use, some countries (e.g. Italy and Germany) use hair analysis in the process of re-granting driving licences to subjects, following its previous confiscation for "driving under the influence" or for having been found in possession of illicit drugs. Applicants for a driving licence with a history of drug abuse must give evidence to show they have stopped drug use and show no risk of relapse [75]. Therefore, hair analysis has been included in the panel of clinical and laboratory tests adopted to verify fitness to obtain a driving licence in these cases.

Divorce, child custody [9]

In addition to an evident usefulness in civil litigations, including divorce and child custody, in which an accusation of drug use/addiction may be relevant, a particular application of hair analysis can be found when an illicit treatment of a child with therapeutic (generally sedative) or clandestine drugs is hypothesized.

In this field, however, the following key points should be considered for a proper interpretation of the results:

- Mothers who continue to misuse drugs as well as alcohol during pregnancy are a source of exposure for the unborn fetus.
- Hair growth starts in the third trimester of pregnancy.
- Drugs are transferred to maternal milk and hence to newborn infants during breastfeeding.
- Infants, during their first movements, (crawling or walking) can gain access to unattended drugs or drug residues in contaminated areas.
- Additional potential sources include people smoking drugs in the house of the child and the sweat of persons or siblings in close contact with infants.
- Average hair growth in infants differs considerably from the hair growth of an adult [28]; many newborns at birth have hair of varying lengths and varying life span.

Testing for previous intentional/unintentional drug use around a certain date [35]

This inquiry frequently occurs in cases of manslaughter, murder or armed robbery. When the suspect is arrested and, to obtain mitigating circumstances, claims to have been under the influence of drugs during the crime. Hair analysis cannot prove

retrospective drug use accurately at a specific time, but can be used for inferring an approximate time frame during which drug intake was more relevant. After the identification of the approximate segment of the hair shaft corresponding to the date of the crime, a segmental analysis should be performed in at least three segments, one corresponding to the approximate time of the crime and both proximal and distal segments. Agreement with the alleged drug use is obtained by the identification of a higher drug concentration in the hair shaft segment corresponding to the approximate crime date. However, to the best of our knowledge, the diagnostic specificity and accuracy of this approach has not been fully established.

Determination of gestational drug exposure [3, 11, 35, 76]

Drug abuse, as well as alcohol and smoking during pregnancy, may lead to miscarriage, premature birth, increased perinatal and neonatal mortality rates, retarded physical and mental development, learning difficulty or hyperactivity. In addition, gestational opiate exposure often results in neonatal withdrawal syndrome.

Drug analysis on meconium is an effective approach to investigate in utero drug exposure only if the specimen is sampled promptly during the perinatal period. If the drug abuse is suspected later, the segmental analysis of hair obtained from the baby and the mother can be a viable strategy. As discussed previously, segmental analysis of the neonatal hair and interpretation should consider the distinctive traits of an infant's hair growth [28].

Verification of doping practices [3, 35]

Hair testing can complement, but not substitute for urine drug testing. For example, a positive result from hair testing can demonstrate exposure during the period prior to sample collection, even in case of one or more negative urine test. On the contrary, a positive urine drug result cannot be overruled by a negative hair test. The rationale of this interpretation is that, as mentioned in section 1 on "Drug-facilitated crime", there is no evidence that most drugs can be detected in hair after a single intake. For example, large bio-molecules, such as many doping agents (e.g. growth hormone, insulin and erythropoietin), cannot be transferred from the blood capillaries to the growing hair, though some methods for detection of anabolic steroids in hair have been published [77,78]. Another potential problem is that the administered parent compound is not necessarily the target compound in hair [79].

Post-mortem toxicology [9, 35, 76]

Numerous applications of hair analysis in post-mortem toxicology have been published in the literature. Hair should be sampled routinely during autopsies, according to the collection recommendations previously reported in the section 1.2.2, "Post-mortem collection".

In general, the detection of drugs in hair is not believed to be sufficient for proving a lethal intoxication, although the collection and analysis of the hair root may

provide important information regarding acute poisoning, particularly in cases of advanced decomposition or delayed death when conventional specimens are scarcely available.

If the drug abuse history of the deceased is unavailable and circumstantial information and/or morphological (macroscopic and microscopic) data are suggestive of chronic drug abuse, hair testing (with its wide detection window) is suitable to confirm the suspicion of long-term drug abuse or chronic poisoning, even in the presence of negative results from the toxicological analysis of blood and urine.

Pharmacological tolerance to opioids can increase by orders of magnitude during chronic use. Thus, a certain drug concentration may be harmless to a long-term addict, while at the same time be lethal to a first-time user (first exposure or use after a long period of abstinence). In this context, suitable hair analysis to investigate semi-quantitatively the abuse history is mandatory to verify the hypothesis of a drug-related death.

In case of fatal traffic or work accidents, it is important to investigate retrospectively by hair analysis if the deceased was a drug user. In addition, if the individual survived for a certain time and conventional specimens were not collected, segmental hair analysis could also be useful to verify chronic or repeated drug intake at the time of the accident. Hair analysis can contribute to the identification of an unknown corpse, as it can assist characterization on the basis of chronic consumption of substances (e.g. psychiatric, smoker, non-smoker, drug abuser).

Drug-facilitated crimes [3, 9, 11, 35, 76]

As mentioned in section 1.2.2, there has been an increase in reports about the use of drugs to modify the behaviour of a person to facilitate a criminal action (for example, robbery, sexual assault or child abuse). For this purpose, a high potency of the drug is needed to allow the use of a low dose for the impairment of the victim, which can be administered surreptitiously. Moreover, victims often report a loss/deficiency of recent memory leading to a delay of notification (particularly in cases of sexual assault). Multi-segmental analysis of hair allows for discrimination between a single drug intake (although with uncertain diagnostic specificity) and long-term use. Confirmation of a single drug exposure, in agreement with the hypothesis of a drug-facilitated crime, can be obtained by the identification of a single positive hair segment corresponding to the alleged time of the crime, with all the adjacent segments being negative. However, this could not be considered absolute proof, as segmental analysis cannot determine the exact day of intake. In other words, if the subject took the drug shortly after the alleged fact, the result would be the same. Moreover, a recent review of the literature [79] suggests that some drugs are not detected in hair after a single exposure, confirming the above-mentioned problems of the lack of sensitivity of this approach. Several reasons can account for a negative result of hair testing after single administration. Factors hampering drug incorporation into the hair can include hydrophilicity, large molecular mass

(> 800 Da), lack of basic moieties, presence of acidic functions, lack of N-alkyl chains and/or N-benzene rings in the molecule.

Alcohol abuse

Although the problem of alcohol abuse is outside the scope of this *Manual*, alcohol use is often combined with drug use. Hair testing has recently been proposed for the diagnosis of chronic excessive alcohol consumption by consensus of the Society of Hair Testing [80], however, the direct determination of ethanol itself in hair is not possible, and as such, its minor metabolites, ethyl glucuronide (EtG) and/or fatty acid ethyl esters (FAEE), should be measured. The use of hair analysis to investigate the pattern of alcohol use is particularly delicate [81] and it is not advisable to use the results of hair testing in isolation as a marker for alcohol use. Also, the definition of "excessive ethanol consumption" is nosologically equivocal and should be adapted to the different contexts in which the analysis is applied. In addition, the different and often severe consequences in the major judicial systems related to a diagnosis of alcohol abuse or alcohol dependence cannot be overlooked. Furthermore, multiple factors may affect its elimination from the body and more information regarding the distribution of EtG and FAEE in the hair of the "normal" population, including different ethnic and geographical origins, is needed. Thus the suggested cut-off concentrations should be considered with caution when they are used for forensic purposes. Finally, false positive results for EtG and FAEE have been reported after use of hair lotions that contain alcohol and for FAAE in women on estroprogestin therapy [82].

2. The analysis of drugs in sweat

Sweat analysis has thus far found limited application in forensic and clinical toxicology, mainly because it is extremely difficult to collect sweat samples without contamination from skin. Currently, the major application of sweat analysis uses a commercially available device, developed in the United States, in some rehabilitation programmes. However, despite severe limitations in the practical feasibility of the test and difficulties in interpretation, sweat analysis is worthy of attention and research in this field is necessary to further establish analytical applications.

2.1 Anatomy and physiology [3, 83]

2.1.1 Secretion of sweat

The main function of sweat in the human body is the control of thermal homeostasis. The skin layers involved in the production of sweat are:

- The epidermis, the external layer, which is composed of stratified epithelium, 75-150 μm thick (except in palms of the hands and soles of the feet, where it is thicker). Its outer surface, the stratum corneum, acts as a barrier preventing the loss of water and solutes across the skin;

- The dermis, the inner layer, is a dense fibroelastic connective tissue framework, supporting a multitude of structures such as vessels, nerves and specialized excretory and secretory glands.

The secretion of sweat occurs by at least two distinct pathways: by passive diffusion, producing the so-called insensible sweat, and by glandular secretion, with the process of sweating. The former is based on the passive diffusion of fluids, including water, through the skin. The rate of fluid loss depends on the extension of the body surface, ambient temperature, body temperature, and the relative humidity of the environment. The latter pathway produces the sensible sweat through secretion by eccrine sweat glands. The secretory elements are located in the innermost part of the gland and, when stimulated, discharge their secrete into either the lumen or the duct, from where the secretion emerges to the skin surface.

A multitude of stimuli, including exercise, stress (both mental and emotional) and thermal stress, trigger the eccrine glands. The maximum rate of sensible sweat production can be as high as 2-4 l/h. The variability of the above mentioned factors as well as the irregular glandular distribution on the body surface hamper the systematic sampling of sweat.

The apocrine and sebaceous glands are irrelevant to thermoregulatory homeostasis, but their secretion on the body surface can mix with sweat and therefore affect its composition. The sebaceous glands, distributed on the whole body surface (except on palms and soles), are associated with hair follicles and produce a viscous, yellow-white oily fluid (sebum), mostly composed of lipids. Apocrine glands are sited in the axillae, pubic and mammary skin, and their function in humans is not clear. Their secretion is a viscous, cloudy, yellow-white liquid which is composed of lipids.

2.1.2 Composition of sweat

As explained above, the specimen sampled on the skin surface is a mixture of the secretion of eccrine or sweat glands, apocrine and sebaceous glands, therefore the term "sweat-testing" is potentially misleading. The real composition of sweat should be considered by the toxicologist for a proper analysis and interpretation of the results.

The average composition of sweat is:

- Water, 99 %;
- Na^+ and Cl^-, the major ions, ranging from 5 to 80 mM;
- Amino acids, biogenic amines and vitamins, in trace amounts;
- Cholesterol, triglycerides and fatty acids secreted from the apocrine glands, in trace amounts;
- Triglycerides and wax esters secreted from the sebaceous glands, in trace amounts;
- Lactic acid, whose concentration strongly affects the pH of sweat, ranging from 4 to 6.

2.1.3 Incorporation of drugs

Researchers have known for more than a century that drugs are excreted in sweat [84, 5]. The mechanism by which drugs are incorporated into this fluid has not been fully clarified, however, the pH of sweat and the pKa of the drugs are of the utmost importance. In short, the following model has been proposed.

Sweat originates from the water fraction of blood (plasma), and its composition is largely determined by reabsorption and exchange mechanisms. Drugs and their metabolites are delivered to the skin glands (sweat, eccrine and apocrine glands), where a diffusion process occurs through membranes. The rate of diffusion is proportional to the oil/water partition coefficient of the non-ionized drug, therefore the passage is facilitated for drugs with a low degree of ionization at physiological pH. In the case of basic drugs, excretion in sweat is increased because of the acidic nature of sweat [85, 86]. In addition to the glandular-mediated excretion of drugs, a transdermal liquid transport has also been reported.

2.2 Specimen collection [3, 83]

2.2.1 General aspects of sweat sampling

Several collection methods have been proposed for sweat sampling. Some examples are: the collection of sweat using a dry gauze covered with waterproof plastic; the use of pads impregnated with salts; cotton swabs, polyvinyl shirts, perspiration stains from clothing, drug wipes, and a transcutaneous chemical collection by band-aid-like devices equipped with a water/gel matrix with the function to absorb compounds. In some experimental studies, the sweat process was boosted by thermal or pharmacological (i.e. pilocarpine) stimuli. By using these sampling techniques, various drugs, including methadone, phenobarbital, morphine, cocaine, THC and methamphetamine have been analysed.

However, the interpretation of results obtained under the above-mentioned conditions is affected by relevant sources of bias:

- Occlusive covering of the skin by waterproof material obstructs the perspiration of the underlying epidermis, and therefore changes significantly the physiologic sweat production.
- Similar considerations apply to the triggered sweat production.
- Transcutaneous chemical collection by means of water/gel matrix devices is not representative of sweat deposition on skin surface in normal conditions.
- Specimens obtained by means of instant sampling (i.e. swabbing sampling) are representative only for a limited and unpredictable window of detection.
- The development of a non-occlusive sweat collection device (sweat patch) that is wearable for a prolonged interval and allows the evaporation of the water content of sweat has allowed the above reported limitations to be overcome.

2.2.2 Collection by sweat patches

Sweat patches have been designed to allow the evaporation of the water contained in sweat, while at the same time retaining its solutes. The functions of the skin barrier are preserved, since irritation of the skin due to the prolonged contact with moisture does not occur. The most common sweat patch is the Pharmchek™ patch [87]. Its use was approved in 1993 by the United States Food and Drug Administration for analysis of cocaine, opoids, phencyclidine, amphetamines and cannabinoids.

According to the producer, the PharmChek™ Sweat Patch is made of three components:

1. A "3M's Tegaderm™ 1625 transparent wound dressing" polyurethane/adhesive layer, that is approximately 6 cm wide, 7 cm long, and 0.025 mm in thickness and is a hypoallergenic, water-resistant adhesive. When it is stuck to the skin, it infiltrates the exfoliated stratum corneum cells. Therefore, when it is removed, the cells adhere to the adhesive and prevent resticking, providing a tamper-evident mechanism. It is marked by a unique identification number, avoiding substitution issues.

2. A collection pad approximately 3 cm wide, 5 cm long and 0.7 mm thick composed of medical grade cellulose. It retains the non-volatile components of sweat.

3. The release liner, a very thin medical-grade tissue paper (approximately 3 cm wide, 5 cm long, and 0.003 mm thick) interposed between the polyurethane film and the absorption pad. It allows the release of the collection pad from the adhesive after patch wear.

Reportedly, the PharmChek™ provides an adequate amount of sample after it has been worn for a minimum period of 24 hours. This interval is effective to collect a sufficient volume of insensible sweat, however the wear time can be shortened if trigger factors, such as exercise, provide an additional quota of sensible sweat.

The application on skin areas subject to movement-related tension should be avoided, because the adhesion of the patch is quite aggressive and elective areas are the upper arm, the lower rib cage area and the upper back. Prior to application, the skin should be cleaned using, e.g. two isopropanol wipes. The alcohol should be allowed to completely evaporate before application to avoid skin irritation due to isopropanol trapped beneath the sweat patch. Generally, the patch is applied on the upper arm, which should be flexed during application to reduce the tension of the skin [88].

2.3 Drug extraction

Many studies have been devoted to detecting the presence of drugs in sweat including amphetamines, methadone, phencyclidine, barbiturates, cocaine, heroin and codeine. Target compounds can be eluted from the pad placed in a vial with 2.5, 4 or 6 ml of methanol: 0.25 M (pH 5.0) sodium acetate buffer (25:75, v/v) [89, 90] or with 5 ml methanol [91] or acetonitrile [92]. The vial is shaken for 30 min. and the extraction fluid is collected after forcing the collection pad to the bottom of the vial. Prior to analysis, the elution fluid generally undergoes further clean-up and concentration steps. These extraction procedures, in principle, should not differ from those applied to other, more complex biological fluids, such as urine or plasma/serum, and will not be detailed further.

Table 7. Extraction methods [3, 93]

Extraction method	*Analyte/class of analytes*
Liquid-liquid extraction	Amphetamines
	Methamphetamines
	Phencyclidine
Solid-phase extraction	Cannabinoids
	Cocaine
	Opioids
Solid-phase micro extraction	Cocaine
	Cocaethylene

2.4 Methods for the analysis of drugs in sweat

2.4.1 Screening [3]

In order to increase analytical productivity, the fluids obtained by patch devices (also defined as patch eluate) are usually screened by immunochemical techniques to preliminarily identify the potential presence of drugs.

The ELISA technique (OraSure Drugs-Of-Abuse Assays, OraSure Technologies, Inc.) [3] has been found suitable for the screening of the eluates from the PharmChek™ sweat patch. This procedure consists of the following steps:

Analytical notes

1. Load the sample and the enzyme-labelled hapten conjugate in the antibody-coated microtitre wells.
2. After a predetermined time, when the competitive binding between the drug and the antibody coated on the surface of the microplate is complete, wash the wells six times with de-ionized water to remove the enzyme conjugate and the excess of the drug.
3. Add 3,3′,5,5′-tetramethylbenzidine (substrate).
4. Incubate for a further 30 min.
5. Stop the reaction by adding 100 µl of 4 M sulfuric acid.
6. Read the microplates by means of a microplate reader at 450 and 630 nm wavelength (calculate differential radiation absorption).

As in all competitive immunoassays, the signal intensity is inversely proportional to the amount of drug or metabolite(s) in the solution. ELISA, for example is based on the drug-antibody interaction and therefore is affected by cross-reactivity, and should be employed only as a qualitative screening technique. The cut-off value should be established by a preliminary evaluation of a suitable number of "negative" samples. Positive samples, and in many instances also "critical" negative samples, should always be submitted to more accurate, confirmation analysis, generally by GC-MS or LC-MS/MS.

2.4.2 Confirmation

GC-MS or LC-MS/MS analyses are performed, analogously to urine, blood or hair analysis, with the following purposes (see section 1.5.2 for experimental procedures):

- Since immunoassays allow the identification of mere classes of compounds (drugs and/or metabolites), confirmation techniques should identify unambiguously the specific analyte(s).
- As immunoassays are limited by the availability of antisera, GC-MS or LC-MS/MS can be used to screen and identify compounds for which no antisera are available.
- Because immunoassays, due to different cross-reactivity towards structurally related compounds, are unsuitable for quantitative determinations, GC-MS or LC-MS/MS are instead used to accurately quantify the analytes.

Gallardo published a comprehensive review providing details on methods for simultaneous quantification of drugs and metabolites in sweat by GC-MS [91]. The readers

may refer also to the works of Brunet [92] and DeMartinis [94, 95] for details on the analysis of cocaine and metabolites, opioids, methadone, amphetamine, methamphetamine and ring-substituted congeners. Although GC-MS is more widely used, LC-MS/MS is proposed by PharmChem as a confirmatory technique [88]. To the best of our knowledge, however, LC-MS/MS methods allowing the simultaneous determination of multiple compounds in sweat extracts have only recently been reported [96, 97].

LC-MS operating conditions

Sample preparation:	Vigorous shaking of patches in 20 mM ammonium formate buffer, pH 7.0: methanol (50:50 vol/vol). Direct injection of 15 µL of the filtered solution
Column:	2.1 mm (I.D) x 50 mm, 5 µm. HyPURITY Aquastar column (ThermoFisher)
Mobile phase:	Gradient from 95 % A (20 mM ammonium formate, pH 7.0) 5 % B (methanol) to 100 % B
Flow rate:	0.5 ml/min.
Injection volume:	10 µl
Acquisition:	Triple quadrupole in positive ion electrospray ionization (ESI) mode
	Multiple reaction monitoring (MRM) mode; for detailed parameters, see reference [97]

2.5 Interpretation of results

As in the analysis of hair, the parent drug is often the major component present in sweat [94, 98-101]. Following administration, drugs appear in sweat after approximately 2 h and remain detectable for 24-48 h [3, 88]. As previously discussed, a sufficient amount of sample is provided by a patch during a minimum wear interval of 24 h, although in some cases, such as in cocaine use, a shorter wear interval was reported as adequate [102]. While data on dose-concentration studies using sweat patch analysis are still scarce, Cone et al. reported a positive result from sweat analysis following the administration of as low as 1 mg of cocaine [103]. However, the few available studies reveal a large variability of concentrations in sweat following the administration of the same dose of drug. Therefore, sweat analysis should not be employed as a means to infer the dose of the ingested drug [103-106]. In another study, a relation between the application site of the patch and the concentration of drug determined was also reported [100]. In addition, as the patch collects and holds drugs over a time interval, ranging from 1 to 10-14 days, its analysis cannot distinguish between a single or a repeated drug intake. External contamination at the

time of patch application on skin or during the wear interval can reasonably occur. The former contamination can be avoided by cleaning the skin twice with isopropanol wipes or with isopropanol followed by water and soap. The latter contamination was reported to occur only in some experimental studies, but under "normal life" conditions is unlikely to occur, because it requires prolonged contact with important amounts of drugs deposited on the skin [3].

Sweat patch drug results can be expressed as ng/ml of reconstituted acetate buffer/methanol or, as proposed by the Substance Abuse and Mental Health Services Administration (SAMHSA) guidelines, as ng/patch. The following cut-off levels have been proposed by the manufacturer, PharmChem, Inc. [88], and by SAMSHA limited to workplace drug testing [107].

Table 8. Cut-off levels proposed by PharmChem, Inc. [88] and by SAMSHA [108]

Screening levels Enzyme immunoassay			*Confirmation levels GC-MS or LC-MS/MS*		
Drug class	*PharmChem ng/ml*	*SAMSHA ng/patch*	*Drug/metabolite*	*PharmChem ng/ml*	*SAMSHA ng/patch*
Marijuana	1.5	4	THC parent drug	0.5	1
Cocaine	10	25	Cocaine	10	25
			Benzoylecgonine	10	25
Opioids	10	25	Heroin	10	—
			6-Acetyl Morphine	10	25
			Morphine	10	25
			Codeine	10	25
Phencyclidine	7.5	20	Phencyclidine	7.5	20
Amphetamines	10	25	Amphetamine	10	25
			Methamphetamine	10	25
			MDA	—	25
			MDEA	—	25
MDMA	—	25	MDMA	—	25

Abbreviations: MDA: 3,4-methylendioxyamphetamine; MDMA: 3,4-methylendioxymethamphetamine; MDEA: 3,4-methylendioxyethylamphetamine;

2.6 Applications of sweat analysis

The practical use of sweat patch testing is strictly related to its inherent features:

- The sampling is less invasive than in the collection of conventional specimens (blood, urine) and even less than alternate specimens considered in this *Manual* (hair and oral fluid).

- As urine and oral fluid collection require the active participation of the subject for sample production, sweat sampling is more independent of the subject behaviour.
- It is less invasive compared to blood sampling, which requires venipuncture and also less invasive than urine testing.
- As previously discussed, the PharmChek™ sweat patch is designed to avoid evasive manoeuvres, such as substitution or removal.
- The wear interval of the patch can last up to 10-14 days, providing constant monitoring for any drug use over this time period and thus avoiding a less comfortable monitoring by multiple urine sampling in the same time period.
- Both parent drug and metabolites are generally detected by patch sweat analysis.

Despite the above-discussed advantages of sweat sampling with respect to other conventional and unconventional specimens, an effective correlation between the amount of the ingested drug and its concentration in sweat has not been well established. Moreover, in some instances, the need to wear the patch for a prolonged period could be problematic (e.g. skin irritation, allergy).

In regard to the above mentioned features, sweat patch testing is generally employed for monitoring drug abstinence and, in special circumstances, in workplace drug testing. Its application is also a deterrent to drug use and for this reason it is particularly advantageous in the former field. However, some issues were reported in workplace drug testing because the patch also monitors the off-duty behaviour of the employee, and this could be considered an invasion of privacy.

2.6.1 Rapid on-site immunoassay testing [85]

At the time of publication of this *Manual*, a kit for rapid on-site "sweat testing", in the form of skin surface analysis, is available from the Drugwipe® device. It consists of an immunoassay-based device that allows the detection of trace amounts of controlled drugs on a surface. The "Drugwipe® K" is expressly developed for drug testing on skin, supposedly deposited through perspiration or sweat. The analysis lasts about 5 minutes and consists of the following steps:

- Collect the sample by multiple swiping of the adsorbent pad on the targeted area of skin surface, a sample volume of less than 10 µl is adequate for analysis.
- Fill the water container with tap water.
- Immerse the adsorbant pad in the filled water container for 15 seconds.
- After 2-5 minutes, depending on drug concentration, read the results on the readout window of the device.

A positive result is indicated if a red line, together with the control line, appears in the readout window.

The cut-off values of the Drugwipe® device, according to the manufacturer, are reported in the following table. To the best of our knowledge, these values have not received any external validation so far and therefore, their practical meaning is uncertain.

Table 9. Cut-off values of the Drugwipe® K device [85, 109]

Drugwipe® cut-off values (ng/ml)		
Cannabis	Δ^9-Tetrahydrocannabinol	30
Cocaine	Cocaine	50
Opioids	Heroin	20
	Morphine	20
Amphetamines	d-Amphetamine	200
	Methylenedioxymethamphetamine	100
	d-Methamphetamine	100
	Methylenedioxyampthetamine	100
	Methylenedioxyethamphetamine	500
Benzodiazepines	Aminoflunitazepam	5
	Flunitrazepam	10
	Nitrazepam	10
	Temazepam	10
	Diazepam	10

A computer-controlled Drugwipe® reader can be employed to record the results digitally, with the following advantages:

- To allow the reading under suboptimal light conditions
- To avoid a visual and inherently subjective reading
- To store the results on a digital support and subsequently to analyse them, obtaining a quantitative result, although not valid in the forensic field.

Good accuracy, sensitivity, specificity and positive predictive value were reported for Drugwipe® sweat testing [110].

Regarding the interpretation of the results, the toxicologist must bear in mind that:

- An interval of about 30-90 minutes between drug ingestion and Drugwipe® testing is necessary to obtain a drug concentration on skin above the cut-off values;
- The device does not prove drug use, but only gives evidence of a subject's exposure to drugs.

3. The analysis of drugs in oral fluid

Saliva, or more appropriately, oral fluid, is an alternate specimen that already has applications in clinical and forensic toxicology. It is currently receiving interest for monitoring drugs of abuse, due to a better understanding of the oral fluid/blood relationship and as a result of technical improvements in methods for sampling and analysis [111-117].

3.1 Production and composition

Although saliva is most commonly used to describe fluids from the oral cavity, the broader term oral fluid is preferred. Oral fluid represents a mixture of different salivas, produced by the serous and mucous cells of salivary glands (parotid (25 %), submandibular (71 %), sublingual (4 %), and 800-1000 minor glands located throughout the oral cavity), and other oral fluids (e.g. gingival crevicular fluid, lymph). In addition, blood and cells from the oral mucosa may be present. Serous cells secrete a colourless aqueous fluid with a high electrolyte content and mucous cells secrete a more viscous fluid containing proteins, glycoproteins and polysaccharides.

Oral fluid is composed of 98 % water, 0.7 % proteins (mainly amylases), 0.26 % glycoproteins (mucins) and electrolytes. Mechanical stimuli produce serous saliva, whereas food stimuli produce mucous-containing saliva. Typical daily oral fluid secretion is 500-1500 ml, at an average flow of 0.6 ml/min. (range 0.1–1.8 ml/min.; which during sleep is 0.05 ml/min.). Production rates for stimulated saliva have been reported to increase up to 2 ml/min. on average, but have reached as high as 7 ml/min. Oral fluid pH is typically slightly acidic, but increases with flow rate from a low of 5.5 to a high of 7.9. Consequently, when oral fluid production is stimulated, pH changes can affect the oral fluid plasma partition rate of drugs.

3.2 Oral fluid as a biofluid for drug analysis

Since oral fluid is effectively an ultra-filtrate of blood, all of the organic compounds present in plasma may be detected in oral fluid, although in trace amounts for some analytes. The first experiments to measure biological analytes in oral fluid date back

to the mid-19th century, with ethanol apparently first reported in oral fluid in 1875. Further experiments in the 1930s demonstrated the role of lipophilicity and ionizability of solutes in their partitioning from blood to oral fluid. Since then, a wide variety of analytes, including steroids, hormones, enzymes, antibodies, DNA therapeutic drugs, has been determined in oral fluid, mainly in clinical settings.

Since the 1970s, the possibility of using oral fluid as a biological matrix for detecting drugs in forensic cases has been studied. In recent years, in the interest in using oral fluid has increased especially for detecting drugs in driving under the influence (DUI) cases [115, 118, 119]. At the moment, oral fluid is used for on-site initial screening of drugs, which is followed by blood sampling for quantification of drug concentrations. In addition to on-site initial screening [120], oral fluid is used as a confirmation matrix in DUI cases for some psychoactive substances, when per se laws for drugs and driving are in place [121-123]. Oral fluid can also be used to evaluate drug use in selected cohorts or populations in epidemiological studies [124].

3.3 Advantages and disadvantages

Oral fluid is regarded as a non-invasive specimen that is easily accessible, does not raise privacy or gender concerns and exhibits a minimal possibility of specimen adulteration. The presence of higher concentrations of parent drugs than metabolites and the correlation of oral fluid drug concentrations to free drug concentrations in blood provide the pharmacologic significance of measurement of active drug and assessment of probable impairment. When on-site immunoassays are used, with the potential of immediate test results, oral fluid for drug testing seems appropriate for roadside DUI scenarios [119]. However, for the majority of drugs, the correlation between oral fluid and blood concentrations is not close, and one cannot estimate the blood concentration from an oral fluid concentration [117]. Due to large inter-individual variation seen in the oral fluid/blood (OF/B) drug concentration ratios, the drug findings in an oral fluid sample should not be directly used to estimate the concentration of drugs in whole blood, even when using a sampling device that does not chemically stimulate salivation. Nevertheless, detection of drugs in oral fluid is a sign of recent drug use, and the qualitative examination of results indicates that for several substances the findings in oral fluid are in accordance with the findings in whole blood, especially when the metabolites are also taken into account. By optimizing the cut-off concentrations in both matrices, the number of false negative findings in oral fluid as compared with blood can be reduced [117].

There are also disadvantages to the use of oral fluid for measuring drugs: the small specimen volume and the low analyte levels; variations of drug concentration due to pH changes; and contamination of oral fluid from drugs taken by the oral and/or intranasal routes, resulting in distorted oral fluid plasma ratios (OF/P). Another possible disadvantage is the short time course for detectability of drugs in oral fluid, as most drugs disappear from oral fluid within 12 to 48 hours of administration, or even

longer for basic compounds, thereby preventing the detection of "historical" drug use. However, this is an advantage for detection of recent drug intake. Consequently, oral fluid is useful in the detection of recent drug use in drivers or accident victims and for testing employees prior to beginning safety-sensitive activities in the workplace.

3.4 Oral fluid-blood partition [125]

The mechanisms by which drugs are incorporated into oral fluid are passive diffusion, ultrafiltration and/or active secretion from the blood. Passive diffusion represents the most important route of entry for most psychoactive substances, with the possible exception of ethanol, which, due to its low molecular weight, ultrafiltrates. The major factors affecting drug entry into oral fluid are molecular weight, lipid solubility and degree of ionization at oral fluid pH, and consequently that passage is hindered for molecules with a molecular weight greater than 500 Da, ionized or protein-bound. Plasma and oral fluid pH, drug pKa and degree of protein binding will control the oral fluid to plasma (OF/P) partitioning of ionizable drugs. Saliva flow rate greatly influences salivary pH, so that stimulating saliva flow for specimen collection can alter the partitioning of drugs between blood and oral fluid. Acidic drugs with a pKa < 5.5 generally have a OF/P ratio lower than 1.0 (e.g. THC-COOH and benzodiazepines [126]). Neutral drugs with pKa > 5.5 and < 8.5 have an OF/P ratio of about 1.0. Basic drugs, drugs that are actively transported from tissues to oral fluid (digoxin, penicillin) and ionized drugs are found at higher concentrations in oral fluid than in blood (OF/P > 1). The mean oral fluid-blood partition concentration ratios for specific drugs and medications were reported by Langel et al. and are summarized in table 10 [117].

Table 10. Mean oral fluid-blood partition concentration ratios for specific drugs [117]

Substance	*Mean oral fluid-blood partition concentration ratios*
Amphetamines	
Amphetamine	23
Metamphetamine	29
Benzodiazepines	
7-Aminoclonazepam	0.43
Alprazolam	0.41
Bromazepam	0.31
Clonazepam	0.19
Diazepam	0.056
Lorazepam	0.10
Nordiazepam	0.053
Oxazepam	0.15

Table 10. *(continued)*

Substance	*Mean oral fluid-blood partition concentration ratios*
Z-drugs	
Zolpidem	0.43
Zopiclone	2.5
Cocaine	
Benzoylecgonine	3.3
Cocaine	20
Opioids	
Codeine	8.8
Methadone	2.9
Morphine	9.8
Tramadol	13
Cannabis	
THC	31
Others	
Amitriptyline	1.5
Mirtazapine	3.4
Trazodone	0.31

It should be noted that OF/P ratios increase temporarily more than 100-fold with respect to the theoretical in the early phase, when the drug is smoked or snorted (cocaine, heroin, cannabis) or given as a liquid preparation (methadone) or sublingual tablet (buprenorphine). The levels of ethanol in oral fluid have been shown to demonstrate excellent correlation with blood alcohol levels, with a ratio close to 1, and for this reason, the use of oral fluid as a specimen for initial alcohol testing is authorized under the United States Department of Transportation (DOT) programme as well as under several driving statutes [127-129].

3.5 Methods for the analysis of drugs in oral fluid

3.5.1 Specimen collection and sampling

The analysis of drugs in oral fluid is challenging. Due to the limited volume of specimens, there are limitations to repeat and/or multiple tests. Both on-site and laboratory-based methods have been developed [130, 131]. The most common collection techniques are spitting into vials or polypropylene tubes or absorption of oral fluid with an absorbent material (cotton roll, plastic pad). To collect a sufficient

volume, the flow of saliva can be stimulated through a variety of techniques, such as chewing paraffin, or by use of chemical stimulants, such as citric acid. However, paraffin may absorb highly lipophilic compounds, causing a reduction in measured oral fluid levels, and stimulated saliva appears to have an increased, narrow pH range (approximately 7.4). Variation in oral fluid pH can significantly impact the oral fluid plasma ratio for certain drugs, depending on their pKa.

When collection is made by spitting, a dilution/preservative buffer and an indicator of volume may be added to the plastic tube. When collection is made by an absorbent pad, it is placed in the mouth for few minutes and then is placed in a tube of buffer [132]. On-site methods may similarly collect the specimen with an absorbent pad, from which the specimen is applied to an immunoassay device. Specimen handling is relatively critical. Oral fluid has been shown to be a source of infectious microorganisms, and appropriate precautions should be taken in the handling of oral fluid [133] considering that a significant length of time may pass between sample collection and the confirmation test. Preservatives to stabilize the drugs, and buffers to release the sample and drugs from the absorbing pad, are added to the oral fluid collection devices.

In the framework of the European programme called "Driving Under the Influence of Drugs" (DRUID), devoted to acquisition of data on the prevalence of psychoactive substances in the general driving population in thirteen European countries, according to a uniform study design, particular attention was paid to oral fluid drug testing and correlated technologies for their suitability in roadside testing [134]. Evaluation of oral fluid screening devices was accomplished in one phase, and a study of "equivalent cut-offs" [135] between blood and oral fluid was conducted in a second phase. Equivalent concentrations for blood and oral fluid were developed within the DRUID project, to be used for the decision on whether a sample was positive for a substance or considered negative, solving the problem of two different specimens being collected in the roadside surveys, ensuring that the drug prevalence in samples of blood and oral fluid taken simultaneously is equal.

All participating countries used a StatSure Saliva Sampler device for oral fluid collection, except for one country, where oral fluid was collected by means of ordinary spit cups. Blood samples were collected in four countries using glass tubes containing sodium fluoride and potassium oxalate. Extraction of the substances was based on liquid-liquid (LLE) or solid phase (SPE). Chromatographic separation was performed by gas chromatography (GC) or liquid chromatography (LC), and detection was done by mass spectrometry. In total, 23 substances were included in the "core substance list" and in total more than 50,000 drivers of passenger cars and vans from the driving population in the participating countries gave an oral fluid sample, a blood sample or both. Cut-off values from the DRUID programme are reported in table 11. However, a significant variation in these values occurs in different jurisdictions and between different applications using oral fluid testing.

Table 11. Equivalent cut-offs for the DRUID project core substances [136]

Substance	*Equivalent cut-offs*	
	Whole blood (ng/ml)	*Oral fluid/saliva (ng/ml)*
Alprazolam	10	3.5
Amphetamine	20	360
Benzoylecgonine	50	95
Clonazepam	10	1.7
Cocaine	10	170
Codeine	10	94
Diazepam	140	5.0
Ethanol	0.1 (g/l)	0.082 (g/l)
Flunitrazepam	5.3	1.0
Lorazepam	10	1.1
MDA	20	220
MDEA	20	270
MDMA	20	270
Methadone	10	22
Methamphetamine	20	410
Morphine	10	95
Nordiazepam	20	1.1
Oxazepam	50	13
THC	1.0	27
Zolpidem	37	10
Zopiclone	10	25
Tramadol	50	480
6-Monoacetylmorphine (6-MAM)	10	16
7-Amino-clonazepam	10	3.1
7-Amino-flunitrazepam	8.5	1.0

Abbreviations: MDA: 3,4-methylendioxyamphetamine; MDMA: 3,4-methylendioxymethamphetamine; MDEA: 3,4-methylendioxyethylamphetamine.

3.5.2 Analytical methods for detection of drugs in oral fluid

Oral fluid samples diluted with buffer do not require special pre-treatment and can be analysed directly by immunoassays or liquid chromatography-mass spectrometry (LC-MS). To reduce matrix interferences or concentrate analytes, solid-phase extraction (SPE) or liquid-liquid extraction (LLE) can be used. Although oral fluid has a reduced protein content compared to blood, one possible source of error may be the binding of drugs to mucoproteins and co-precipitation by centrifugation during

specimen processing [137]. Mucin interferences can be removed by several freeze-thaw cycles. A short review of the screening and confirmation methods for oral fluid analysis can be found in the review by Gallardo and Queiroz [91].

Screening

For the determination of drugs in oral fluid, antibodies must react with the parent drugs and lipophilic metabolites (e.g. heroin and 6-MAM or cocaine and ecgonine methyl ester or THC) that predominate in oral fluid. When the drug is smoked, the pyrolytic products may pass from oral tissues to oral fluid. LC-MS has been proposed as a valuable technique for the direct screening/confirmation of drugs in oral fluid, though at a higher complexity than immunoassays. A variety of on-site point-of-care devices have been introduced, based on lateral flow of the oral fluid through a membrane impregnated with lines of labelled immobilized antibodies.

Confirmation

Different methods based on the coupling of a highly efficient separation technique with mass spectrometry can be used for the analysis of oral fluid. GC-MS and LC-MS methods can be used that do not differ substantially from those employable for hair or sweat (see section 1.5). However, considering the limited volume of oral fluid that is often collected, only GC-MS/MS and LC-MS/MS instruments, exhibiting greater sensitivities and lower limits of quantification than single quadrupole GC-MS instruments, guarantee the possibility of repeated analysis. Currently, multi-analyte methods are suggested to overcome the frequent problem of low sample amounts.

GC-MS and a LC-MS/MS methods for the determination of multiple drugs are proposed hereafter.

GC-MS determination of 30 drugs of abuse [138]

The GC-MS method employs mixed-mode solid-phase extraction (SPE), optimized derivative formation and fast gas chromatography/electron impact mass spectrometry.

SPE extraction

1. To 250 µl of oral fluid add 2 ml of phosphate buffer solution (pH 4.1) containing internal standards (IS) at appropriate concentrations, centrifuge at 2,000 g for 5 min., condition an SPE isolute® HCX with methanol (2 ml) and purified water (2 ml).
2. Introduce the sample at constant flow-rate (1-1.5 ml/min.) under slight vacuum.
3. Wash with purified water (2 ml).
4. Adjust pH with phosphate buffer solution (pH 4.1).

First elution phase

1. Elute the compounds with mainly hydrophobic interactions (Δ^9-THC and most of the benzodiazepines) with 3 ml of toluene–ethyl acetate (80 : 20, v/v) into test-tubes.
2. Centrifuge (2,000 g, 5 min.).
3. Transfer the organic phase to clean test-tubes (fraction 1).

Second elution phase

1. Aspirate the cartridges to dryness with a relatively strong nitrogen flow (10 s).
2. Elute the rest of the analytes with both hydrophobic and ionic properties with 3 ml of freshly prepared acetonitrile-aqueous ammonia (100 : 4, v/v).

Divide the eluent into two parts, transferring 500 µl (1/6 of total volume) for amphetamine-type stimulant analyses into a test-tube (fraction 2). The rest of the eluate (2,500 µl) is left in the collection tube (fraction 3).

Derivatization

Fraction 1 and 3

1. Derivatize fraction 1 (Δ^9-THC and benzodiazepines) by adding 60 µl of freshly prepared acetonitrile (ACN), N-methyl-N-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) (40 : 20, v/v).
2. Derivatize fraction 3 (ionic compounds, e.g. opiates, benzoylecgonine) by adding 80 µl of freshly prepared ACN, N-methyl-N(trimethylsilyl)trifluoro-acetamide (MSTFA) (60 : 20, v/v).
3. Vortex mix each test-tube for 1 second and heat for 30 min. at 85°C in capped vials.
4. Transfer to 2.0 ml autosampler vials containing 200 µl inserts.
5. Introduce a nitrogen atmosphere to remove air humidity and prevent the hydrolysis of the derivatized analytes in the vials containing ACN-MSTFA.
6. Inject 2.0 µl from both fractions 1 and 3 into the GC-MS system.

Fraction 2

1. Evaporate the ionic eluent.
2. Add 50 µl of alkaline buffer prepared by mixing 0.45 ml of 10 M KOH and 2.55 ml of saturated $NaHCO_3$.
3. Add 70 µl of toluene-heptafluorobutyric anhydride (HFBA) (100 : 4, v/v) while strongly vortex mixing (7-8 s).

4. Centrifuge (2,000 g, 5 min.)
5. Transfer the toluene layer to an autosampler vial.
6. Inject an aliquot of 4.0 µl into the chromatographic system.

GC-MS operating conditions: fractions 1 and 3

Instrumentation:	Agilent Technologies (Palo Alto, CA, United States) Model 6890N GC system combined with an inert Model 5973 mass selective detector, fast GC oven and a Model 7673 injector/autosampler
GC oven conditions:	*Fraction 1* Initial temperature set at 90°C and held isothermal for 1 min. The temperature is then ramped to 330°C at 60°C/min. and held isothermal for 4.5 min. *Fraction 3* Initial temperature set at 90°C and held isothermal for 1 min. The temperature is then ramped to 330°C at 45°C/min. and held isothermal for 4.3 min.
Column:	Cross-linked 30 m DB-35 ms (0.32 mm i.d., 0.25 µm film thickness) from J&W Scientific (Folsom, CA, United States)
Injection parameters:	2 µl splitless injecton in pulsed flow mode with a deactivated double-taper liner without glass-wool with an injection pressure of 90.5 kPa for 1.0 min.
Carrier gas:	Helium, 2.0 ml/min.
Detector:	Single quadrupole; electron ionization (EI) mode
Acquisition:	Selected ion monitoring (SIM); see table 12 for specific ions to monitor for target analytes and deuterated internal standards (IS)

GC-MS operating conditions: fraction 2

Instrumentation:	HP 5890 Series II gas chromatograph, Model 5971A mass spectrometric detector and a Model 7673 injector/autosampler
GC oven conditions:	Initial temperature set at 130°C and held isothermal for 3 min. The temperature is then ramped to 300°C at 30°C/min.

Column:	Cross-linked 30 m DB-5ms (0.32 mm i.d., 1.0 µm film thickness) from J&W Scientific (Folsom, CA, United States).
Injection parameters:	4 µl splitless injecton with a deactivated single-taper liner with glass wool at a constant pressure of 78 kPa.
Detector:	Single quadrupole; electron ionization (EI) mode.
Acquisition:	Selected ion monitoring (SIM); see table 12 for specific ions to monitor for target analytes and deuterated internal standards (IS).

Table 12. GC-MS: typical fragment ions used for analyte identification and quantification

Analyte	*Ionic species*		
	(m/z)*	*(m/z**)*	*(m/z)*
	Fraction 1		
Δ^9-THC-d3-TBDMS	374	431 (34.5)	
Δ^9-THC-TBDMS	371	428 (33.7)	431 (34.5)
Nordazepam-d5-TBDMS	332	334 (26.8)	
Nordazepam-TBDMS	327	329 (37.4)	328 (25.6)
Oxazepam-(TBDMS)2	457	459 (47.0)	513 (28.2)
Diazepam-d5	289	261 (127.8)	
Diazepam	284	256 (150.0)	283 (130.9)
Lorazepam-2-TBDMS	491	493 (76.2)	515 (34.8)
Phenazepam-TBDMS	407	408 (25.0)	405 (76.3)
Nitrazepam-TBDMS	338	339 (25.6)	292 (18.3)
Temazepam-TBDMS	357	359 (38.4)	358 (24.8)
Clonazepam-TBDMS	372	374 (38.9)	326 (13.4)
	Fraction 2		
Amphetamine-HFB	240	169 (22.9)	118 (39.9)
Methamphetamine-HFB	254	210 (37.3)	169 (12.4)
MDA-HFB	135	162 (68.2)	240 (12.9)
Methylmexiletine	254	136 (253.7)	
BDB-HFB	135	176 (60.4)	254 (11.9)
MDMA-HFB	254	162 (70.9)	210 (49.3)
MDEA-HFB	268	240 (50.3)	162 (60.0)
MBDB-HFB	268	210 (41.3)	176 (67.2)

Analyte	*Ionic species*		
	(m/z)*	*(m/z**)*	*(m/z)*
	Fraction 3		
Methadone-d9-TMS	78		
Methadone-TMS	72	296 (64.0)	297 (16.3)
Benzoylecgonine-d3-TMS	243	364 (25.5)	
Benzoylecgonine-TMS	240	361 (25.6)	346 (10.0)
Cocaine-d3	185	306 (25.6)	
Cocaine	182	303 (26.8)	272 (12.5)
Morphine-d6-2TMS	435	420 (46.2)	
Morphine-2-TMS	429	287 (49.5)	414 (53.0)
Codeine-d6-TMS	377	349 (18.5)	
Codeine-TMS	371	343 (21.5)	313 (20.5)
Ethylmorphine-TMS	385	357 (36.3)	384 (17.3)
6-MAM-d6-TMS	405	343 (100.8)	
6-MAM-TMS	399	340 (90.5)	287 (62.8)
Midazolam	310	325 (22.5)	312 (33.6)
Fentanyl	245	146 (30.2)	189 (30.6)
Zolpidem	235	236 (18.0)	307 (9.8)
Norbuprenorphine-(TMS)3	468	500 (32.3)	524 (19.4)
Alprazolam-d5	284	209 (156.8)	
Alprazolam	279	204 (79.4)	308 (61.8)
Pholcodine-TMS	114	100 (46.3)	
Buprenorphine-d4-(TMS)2	454	486 (32.0)	
Buprenorphine-(TMS)2	450	482 (32.6)	506 (22.0)

*Quantifier ion.

**Values in parentheses are the relative abundances (%) of qualifier ions with respect of the quantitation ion.

Abbreviations: TMS: trimethylsilyl derivative; HFB: heptafluorobutyrryl derivative; TBDMS: tert-butyldimethylsilyl derivative; MDA: 3,4-methylendioxyamphetamine; MDMA, 3,4-methylendioxymethamphetamine; MDEA: 3,4-methylendioxyethylamphetamine; BDB: 3,4-methylenedioxyphenyl-2-butanamine; MBDB: 3,4-methylenedioxyphenyl-N-methyl-2-butanamine.

Analytes must be identified using a comparison with the retention times and the relative abundances of the qualifier ions of the same analyte in a positive quality control sample run in the same analytical session. For quantification, the ratio of the abundance of quantifier ion to the quantifier ion of a deuterated internal standard must be used. Standard calibration curves can be obtained by adding pure standards, prepared in methanol, to blank control oral fluid.

LC-MS/MS determination of multiple illicit drugs [139]

The LC retention times and the relative abundances of the qualifier ions of the same analyte in a positive quality control sample run in the same analytic fluid was collected using the Intercept® device.

SPE extraction

1. To 250 µl of oral fluid add 25 µl of concentrated HCl, 50 µl of IS working solution at 0.2 mg/l and 750 µl of water.
2. Condition an OASIS MCX SPE cartrdige with 1 ml of methanol and 1 ml of 0.1N HCl.
3. Introduce the sample at constant flow-rate (1-1.5 ml/min.$^{-1}$) under a slight vacuum.
4. Wash successively with 1 ml 0.1M HCl, tetrahydrofuran and a mixture of methanol and water (50:50, v/v).
5. Elute with 0.5 ml of 5 % ammonia in methanol.
6. Dry the cartridges by applying full vacuum for 5 min. Evaporate the eluate to approximately 50-100 µl and reconstitute with 0.95 ml of an ammonium formate buffer (10 mM, 0.01 % formic acid).

LC-MS/MS procedure

Instrumentation:	Alliance 2695 HPLC
Column:	XTerra MS C18 column (2.1 mm × 150 mm, 3.5 µm) (Waters)
Eluition:	10 mM ammonium bicarbonate (pH 10) (A) and methanol (B) starting from 30 % B at 3 min., increase to 50 % B over the next 1 min. From 4 min. to 12 min., linearly increase to 75 % B. At 12 min., increase to 90 % B in 1 min. before returning to initial conditions within 0.1 min. and equilibrating for 6.9 min.
Flow:	0.25 ml/min.
Injection volume:	20 µl

MS parameters

Detector:	Quattro Ultima triple quadrupole mass spectrometer (Micromass UK Limited, United Kingdom) working in electrospray conditions, positive mode (ES+)
Acqusition:	Multiple reaction monitoring (MRM) mode with two transitions; detailed parameters as described in table 13

Table 13. Optimized MRM parameters for analytes and internal standards

Analyte	Precursor ion (m/z*)	Product ions (m/z*)		Cone voltage (V)	Collision energies (eV**)
		Transition I	Transition II		
Amphetamine	136.10	119.10	91.00	20	9, 17
Amphetamine-d11	147.10	98.00		35	18
Methamphetamine	150.10	119.00	91.00	20	9, 20
Methamphetamine-d5	155.10	92.00		20	20
MDA	180.05	105.00	77.00	20	22, 30
MDA-d5	185.00	168.10		20	10
MDMA	194.05	163.05	105.10	40	12, 25
MDMA-d5	199.10	165.10		40	13
Cocaine	304.15	182.10	82.10	20	18, 28
Cocaine-d3	307.15	185.10		22	20
Benzoylecgonine	290.15	168.10	105.00	45	20, 30
Benzoylecgonine-d8	298.10	171.00		45	20
Morphine	286.10	165.10	152.00	75	40, 57
Morphine-d3	289.00	165.10		75	47
6-MAM	328.10	165.00	152.00	80	40, 70
6-MAM-d6	334.20	165.10		75	40
Codeine	300.10	165.10	128.10	70	43, 58
Codeine-d6	306.20	165.10		70	45

*Decimal figures were indicated as per the original reference, but a unit mass resolution MS/MS instrument was used.

**(transition I, transition II)

Abbreviations: 6-MAM: 6-monoacetylmorphine; MDA: 3,4-methylendioxyamphetamine; MDMA: 3,4-methylendioxymethamphetamine.

3.5.3 Data on selected analytes

Cocaine

Cone and colleagues noted that cocaine was the major analyte in oral fluid following all routes of administration (intravenous [140], smoking [141], and intranasal [142]). Benzoylecgonine and ecgonine methyl ester (EME) generally were present in minor amounts and their concentrations usually peaked later than the cocaine concentration. The oral fluid to plasma drug concentration (OF/P) ratio is in the range 0.5-2.5; however, contamination of the oral cavity following the smoking and intranasal routes produced more elevated OF/P ratios in the early 2-3 h period after drug administration. Observation of a significant correlation of oral fluid cocaine

concentrations with plasma concentrations and also with behavioural effects provided the opportunity for development of a new, non-invasive test for cocaine abuse. However, since cocaine is a weak base with a pKa of 8.6, its concentration in oral fluid is highly dependent upon salivary pH and consequently upon salivary flow. Nonetheless, this does not preclude use of oral fluid in forensic testing for evidence of recent use. Moreover, similar concentrations of cocaine BE or EME in oral fluid were reported after collection through different sampling techniques [143]. However, a prolonged occurrence of cocaine in human oral fluid after multiple dosing and high drug exposure was reported by Cone and Weddington [144], and the authors concluded that drug accumulated in the deep body compartments and was slowly released back into the circulation [143].

Amphetamines

The OF/P ratios of amphetamine, methamphetamine and 3,4-methylendioxyamphetamines range from 2 to 20, with a concentration peaking 2-12 h after a 10 mg oral dose, typically in the range 10-60 ng/ml [145-147]. When racemic amphetamine is administered, both D and L isomers are found in oral fluid. The window of detection for methamphetamine has been reported to be from 24-50 h after use [145, 148]. However, although there are perceived advantages of oral fluid for verifying an exposure compared with urine (simple specimen collection and reduced potential for adulteration), oral fluid offers a lower methamphetamine and amphetamine concentration and a shorter window of detection. Hence, when contemplating selection of oral fluid as a test matrix, the advantages related to collection should be weighed against its shorter time of detection and its lower concentration of analytes compared to that of urine [145, 149, 150].

Opioids

The OF/P ratios of opioids after intravenous (i.v.). administration range from 0.1 to 1.9 (heroin), from 0.7 to 7.2 for 6-MAM and from 0.5 to 9.8 for morphine [117].

Heroin is found in the first hour and then 6-MAM and morphine are found for 4-8 h after i.v. injection, but a larger detection window can be envisaged for tolerant individuals. When heroin is smoked, the parent drug can be found up to 24 h and the metabolites, 6-MAM and morphine up to 8 h later [114].

Cannabis [151]

Tetrahydrocannabinol (THC), has been detected in oral fluid following smoking of marijuana cigarettes [131, 152-155], and hashish [156, 157]. The presence of THC in oral fluid appears to be due primarily to contamination of the oral cavity during the smoking process, since THC is highly protein-bound and does not readily pass from blood to oral fluid. In addition, it inhibits salivary excretion. THC detection times in oral fluid are variable and range from 2 to 10 h. Huestis et al. [151] found that THC detection times, averaged 6 and 9 hours after smoking a single 1.75 %

or 3.55 % THC marijuana cigarette, respectively, in comparison with an average detection time in plasma of approximately 5 h [158]. The salivary interval of detection is much shorter than the several days for detection of cannabinoids in urine, but may prove valuable, if an appropriate cut-off is applied, in testing programmes where the goal is to demonstrate a likelihood of impairment. In chronic, daily cannabis smokers a prolonged detection time of THC (up to 48 h) and particularly of THC-COOH (up to 29 days) was reported [159].

3.5.4 Summary

Oral fluid levels of drugs generally correlate better with blood levels than, for example, urine. However, based on a literature review and the recent data acquired in the frame of the European DRUID project, this correlation does not allow a strong prediction of blood levels. This is specifically the case after drug use by oral ingestion, smoking, or nasal insufflation, when contamination of the oral cavity by the drug can lead to elevated drug levels, much greater than corresponding blood levels, at least for several hours. Also, many drugs are weak bases and their oral fluid concentrations may be dependent upon pH. However, there are many potential applications for oral fluid testing for drugs in the general areas of drug detection, treatment and forensic investigations [117, 126].

4. General considerations in the analysis of drugs in hair, sweat and oral fluid

This *Manual* is focused on the application of up-to-date techniques of analytical toxicology to "alternative" biological specimens such as hair, sweat and oral fluid. These biological matrices, evidently different in composition from the more traditional biofluids, i.e. urine and blood, require a robust analytical methodology based on unequivocal determination of the analytes of interest obtained by accurate qualitative and quantitative techniques, including mass spectrometric identification (e.g. GC-MS or LC-MS).

- Only by adopting the accepted strategy in forensic toxicology, based on screening and confirmation on two different aliquots of the same sample, can analytical results obtained in alternative matrices achieve a high degree of reliability which is required in a forensic context.
- In order to manage high routine workloads, a rapid, high throughput screening would be ideal, whereas a confirmation of the results by chromatography coupled to mass spectrometry is always mandatory.
- In addition, it is important to stress that sound analytical data do not always provide for a sound interpretation. In fact, because of current limited knowledge of the modalities/kinetics of incorporation and elimination of xenobiotics, the interpretation of analytical results may still be difficult.
- A few considerations may be helpful for the correct use/interpretation of analytical data.

The sequence of the presence of xenobiotics and their metabolites in both alternate and conventional biological specimens tends to follow this time scheme (increasing time window of detectability): blood → oral fluid → urine → sweat (with patch sampling) → hair. This phenomenon is mostly related to pharmacokinetic properties of active principles, i.e. the mechanisms of adsorption, distribution/incorporation, metabolism and disposal of drugs and metabolites, in which the chemical-physical properties of drugs and their metabolites are crucial. A direct consequence of the diverse time course of xenobiotics in the different biological samples is that the opportunity to detect a drug consumed by a subject is related to the choice of the

most suitable specimen. If analyses are performed in different specimens from the same subject, providing apparently contrasting results (e.g. "positive" urine result vs. "negative" blood result). This can be useful for a more detailed hypothesis on the time and manner of a drug intake. A standard interpretation is not possible and may be misleading. However, some elementary examples of interpretation of results obtained from multiple specimens are presented in table 14 [83].

In summary:

- Each specimen is suitable for particular applications in relation to its specific characteristics.
- Hair can be employed in workplace drug testing, driving licence re-granting, investigation of drug-use history, divorce litigations, child custody hearings, testing for previous intentional/unintentional drug use around a certain date, determination of gestational drug exposure, investigation of doping practices, drug-facilitated crimes and post-mortem toxicology (drug-related deaths, health impairments caused by chronic drug abuse, tolerance in opioid death cases, chronic drug use and fatal accidents, repeated criminal poisoning, contribution to identification of a corpse, demonstration/exclusion of external contamination).
- Oral fluid testing is mostly employed for roadside drug testing and, potentially, can be used to gather information on acute intoxication if blood cannot be collected (such as in workplace drug testing).
- Sweat is still rarely used, mostly for monitoring drug abstinence in "on parole" control programmes and, in limited cases, in workplace drug testing.
- The analysis of multiple specimens can be useful:
  - To better define the time and manner of exposure to one or more xenobiotics;
  - To confirm laboratory results in cases of doubtful clinical and anamnestic histories.
- Qualitative results (i.e. presence or absence of drugs or metabolites) in alternate matrices such as hair, sweat or oral fluid are generally well understood.
- The interpretation of quantitative data, i.e. concentrations of drugs in alternate specimens, is still under debate, especially with regard to the dose-concentration relationship.

Note

Hair, because of the substantial lack of metabolism following incorporation of a drug and the extended "time window" of detectability of drugs, has the potential to

be a useful tool to study the epidemiology of the prevalence of abuse of new psychoactive substances (NPS) (e.g. synthetic cannabinoids, cathinones) in populations. In fact, because of their extensive metabolism, rapid kinetics, excretion and irregular mode of use, their detection in conventional biofluids is extremely inefficient. In this context, the stability of the analytes when incorporated in hair and the negligible biological hazard of hair samples make them suitable for long-term storage and dispatch to reference laboratories hosting the required, highly sophisticated instrumentation. As already mentioned, however, contamination can rarely be ruled out completely, neither can "on-purpose" hair "cleaning" from drug residues. A recent review of the forensic toxicological strategies adopted to face the problems of detection and measurement of new psychoactive substances in biological samples has been published by Favretto et al. [160].

Table 14. Multiple specimen testing: interpretation of apparently discordant results [83]

Matrix					*Possible explanations for disparate results*
Blood	*Urine*	*Oral fluid*	*Sweat*	*Hair*	
+	–				Time of urine collection too close to time of drug use
+		–			Highly protein-bound drugs may be poorly distributed to oral fluid, e.g. benzodiazepines
+			–		Low drug dose; Sampling time outside detection "window"
+				–	Low drug dose; Low binding affinity to hair matrix (e.g. cannabinoids); Hair treatments (e.g. bleaching, straighteners); Sampling time outside detection "window"
–	+				Long interval after dosing; Concentration affects of kidney function
	+	–			Long interval after dosing; Concentration affects of kidney function; Highly protein-bound drug; Sampling time outside detection "window"

Table 14. *(continued)*

Matrix					*Possible explanations for disparate results*
Blood	*Urine*	*Oral fluid*	*Sweat*	*Hair*	
	+		–		Concentration affects of kidney function; Sampling time outside detection "window"
	+			–	Concentration affects of kidney function; Low doses or single dose; Low binding affinity to hair matrix; Sampling time outside detection "window"
–		+			Insufficient time for drug absorption; Residues in oral cavity from the latest drug intake; Sampling time outside detection "window" due to longer detection time in oral fluid
	–	+			Insufficient time for drug absorption, metabolism and excretion; Residues in oral cavity from the latest drug intake
		+	–		Insufficient time for drug absorption, metabolism and excretion; Residues in oral cavity from the latest drug intake
		+		–	Low drug dose; Low binding affinity to hair matrix; Insufficient time for drug absorption, metabolism and excretion; Sampling time outside detection "window"
–			+		Sampling time outside detection "window"
	–		+		Sampling time outside detection "window"
		–	+		Sampling time outside detection "window"

Table 14. *(continued)*

Matrix					*Possible explanations for disparate results*
Blood	*Urine*	*Oral fluid*	*Sweat*	*Hair*	
			+	–	Low drug dose; Low binding affinity to hair matrix; Insufficient time for drug absorption, metabolism and excretion; Sampling time outside detection "window"
–				+	Sampling time outside detection "window"
	–			+	Sampling time outside detection "window"
		–		+	Sampling time outside detection "window"
			–	+	Sampling time outside detection "window"

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