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SIBioC DOCUMENT

Standard operating procedures for the determination of abuse substances in biological matrices

Document of the SIBioC Study Group "Clinical and forensic toxicology, and doping"

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ABSTRACT

This document, endorsed by the Italian Society of Clinical Biochemistry and Clinical Molecular Biology - Laboratory Medicine (SIBioC) presents the Standard Operating Procedures (SOPs) for the determination of drugs and psychoactive substances in biological matrices, developed to ensure consistency and reliability in forensic and clinical toxicology. The SOPs address key aspects such as sample collection, chain of custody, laboratory safety, and analytical methodologies, including screening and confirmatory tests. Given the legal implications of forensic toxicological analyses, the procedures emphasize traceability, accuracy, and adherence to national and international guidelines. Biological matrices covered include urine, saliva, blood, and hair, each selected based on the required detection window and medico-legal context. The document also outlines best practices for laboratory personnel training, data interpretation, and result reporting, with specific threshold values for drug detection. Additionally, it discusses the regulatory framework governing in Italy toxicological assessments in contexts such as workplace drug testing, driving under the influence (DUI) investigations, and fitness evaluations for firearm licenses and child custody cases. These SOPs aim to standardize toxicological procedures across laboratories, enhancing the quality and legal validity of analytical results.

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The Italian version of this document is provided in Supplementary Digital Material 1 (Supplementary Text File 1).

Introduction

The activities of the toxicology laboratory serve a dual role depending on its objectives, whether clinical or forensic. The identity, authenticity, and selection of a specific analytical method are essential prerequisites for the reliability of analytical results and their correct interpretation.[1, 2] Since forensic toxicological analyses, unlike those conducted for clinical purposes, may serve as legal evidence, they must provide clear and unambiguous answers, ensuring traceability of every operational phase to prevent any dispute in judicial proceedings. Generally, the determination of psychoactive substances for medico-legal purposes primarily concerns cases of driving under the influence of drugs and/or alcohol (Article 187 and 186 of the Italian Highway Code, respectively), monitoring of workers in high-risk jobs (Drug testing in the Workplace *Provvedimento18 settembre 2008 "Procedure per gli accertamenti sanitari di assenza di tossicodipendenza o di assunzione di sostanze stupefacenti o psicotrope in lavoratori addetti a mansioni che comportano particolari rischi per la sicurezza, l'incolumità e la salute di terzi".GU Serie Generale n.236 del 08-10-2008)*, and verifying physical and mental fitness for obtaining, renewing, or confirming the validity of a driving license for vehicles or motorcycles by Local Medical Commission for issuing certificates of fitness to drive (CLM).[3] Additionally, analytical assessments are also required for issuing firearm licenses and in child custody and adoption cases to demonstrate the non-use of illicit substances and psychoactive drugs.

These Standard Operating Procedures (SOPs) were developed out of the need for a shared document that outlines the analytical protocols and threshold values applicable to the analysis of drugs and psychoactive substances in various biological matrices, particularly when such investigations serve as legal evidence. The absence of specific regulations has led individual laboratories in Italy, over the years, to adopt their own operational methods tailored to their needs, resulting in methodological inconsistencies among laboratories performing forensic analytical testing. The aim of this Document is to standardize and harmonize, at the national level, the analytical and organizational procedures of laboratories within the National Health Service involved in substance analysis, providing practical support to laboratories that intend to perform (or already perform) drug and psychoactive substance analyses in various biological matrices. This ensures that they meet the necessary requirements for delivering high-quality services to users. These SOP will address general aspects (matrices, chain of custody, screening, and confirmation tests), with reference to the analytical assessments stipulated by legislation (workplace analysis, Articles 187 and 186 Italian Highway Code, license issuance/renewal).

Laboratory organization

Laboratory safety

Laboratories conducting analyses to detect drugs of abuse in biological matrices must have a solid security system in place to ensure that unauthorized personnel are prohibited from accessing the laboratory or areas where samples or related documents are stored. Documents and samples must be stored in locked cabinets, refrigerators, or freezers, with constant temperature monitoring. The laboratory must maintain a log documenting the entry and exit of visitors to the protected areas of the laboratory.

Laboratory personnel

Only properly qualified personnel, whose competence has been formally recognized, can work within the laboratory, although one individual may hold multiple roles. The laboratory must maintain a register documenting the competencies of staff members based on their assigned duties. The documents, whether paper or digital, must include an up-to-date curriculum vitae. Each laboratory staff member assigned to a specific function must have the necessary preparation and experience commensurate with their responsibilities, as documented by specific training related to the technology used in this particular laboratory field. All laboratory personnel must have received adequate training in workplace health and safety.[4]

Chain of custody procedures

The chain of custody is a procedure designed to document the collection, transportation, storage, analysis, reporting, and disposal of a particular biological sample. The goal is to ensure that the authenticity and integrity of the sample are maintained from collection to disposal. A laboratory must be able to answer the following questions:

- Where are the samples currently being analyzed?
- Who currently possesses the samples to be analyzed?
- When did the laboratory receive the samples for analysis?
- Where were the samples stored?

• Who has had possession of the samples to be analyzed?

The chain of custody must specify where and by whom the samples were collected, processed, and stored, and demonstrate that they have not been tampered with or altered.

Laboratories conducting drug analyses, regardless of the biological matrix used, must assume responsibility for the chain of custody of the submitted samples, documenting their traceability from the moment they are accepted by the laboratory until the completion of the analysis, including reporting, storage, and eventual disposal of any remaining material. The records related to the chain of custody should be kept on paper and/or in digital format for a period of no less than five years or as recommended by current regulations.[4]

Forms

Sample collection report form

The sample collection report form, which must be drafted in triplicate, must include the following:

• details of the person responsible for the collection;

• details of the individual undergoing the analytical examination (personal information, residence, ID number, etc.). If the individual does not possess a valid ID, identification can be carried out with the help of an authorized supervisor or a witness with an ID. If the individual's identity cannot be verified, the responsible person cannot proceed with the sample collection;

• information relating to the collection for the unique identification of the sample (identification code, department/area where the sample was collected, etc.);

• a list of any medications that the individual may have taken or been administered in the days preceding the sample collection;

• signatures of both the person who performed the sample collection and the individual undergoing the analytical examination;

• of the three copies of the collection report, one is delivered along with the samples to be analyzed and the chain of custody form (see below) to the analysis laboratory, one is retained by the facility/person who performed the blood collection, and one is provided to the individual undergoing the analytical examination.[4]

Chain of custody form

The chain of custody form tracks every movement of the sample, from its collection to its arrival at the laboratory conducting the analysis.

The information that must be included in the chain of custody form includes:

• adhesive labels with a barcode or alphanumeric code, identical to those affixed to the sample collection report and the containers of the three sample aliquots;

• place, date, and time of collection;

• any relevant sample information;

• name, address, email address, and phone number of the analysis laboratory;

• name and signature of all individuals who have had custody of the sample aliquots during the transport from the collection site to the final destination (analysis laboratory);[4]

• place, date, and time of the sample receipt in the laboratory.

Informed consent form

The informed consent form is an essential document as it formalizes the patient's affirmative expression of willingness to undergo the required examinations with full awareness. In the case of minors, the informed consent must be provided by the parents or legal guardians, or by the appointed guardian, taking into account the minor's will in relation to their age and level of maturity.[4] Since these investigations are always accompanied by a specific request with the corresponding medico-legal question, if consent is not provided for the investigation, the person making the request should be notified to manage the issue. For the same reason, consent for the transmission of the analytical data to the requester should also be obtained in advance.

Types of biological samples

Toxicological analyses for the detection of xenobiotics (drugs, toxic compounds, narcotics, or psychotropic substances) in biological matrices are typically requested for clinical and/or medico-legal purposes. Since such investigations are frequently used in various fields (diagnosis of substance dependence, assessment of fitness to drive, fitness for work, fitness for carrying firearms, eligibility for specific competition and/or contractual requirements, child custody, or national or international adoption), or may even serve as judicial evidence, they must meet specific criteria for certainty and reliability, including the documentation and traceability of each analytical phase, as well as transparency and consistency through the application of clear procedures.

The type of matrix to be used is critical for addressing the medico-legal question, as this choice determines the time window in which abused substances can be detected.

In cases where recent consumption of narcotics or psy-

chotropic substances needs to be evaluated, urine is the matrix of choice. However, it is important to emphasize that the result of an analysis of a drug obtained from a urine sample cannot be correlated with the possible psycho-physical impairment of the individual at the time of sample collection. If an assessment of current impairment is required, blood is the appropriate matrix to use.

The alternative biological matrix to blood is saliva, which is suitable for the determination of xenobiotics and/or abused substances and/or their metabolites in clinical settings, to demonstrate current use in driving, performing specific work tasks (*e.g.*, before starting a work shift), and in medico-legal contexts.

If it is necessary to extend the detection window for a particular substance, hair is the appropriate matrix. In fact, while a substance (*e.g.*, a drug of abuse and/or its metabolite) can be detected for several hours in blood and a few days in urine, the same substance can be detected in the hair matrix for several months, depending on the location and length of the hair.[2]

Urine

Urine is a preferred biological matrix for the analysis of psychoactive or psychotropic substances. Its use offers advantages such as a non-invasive collection method, the possibility to sample large volumes, and the possibility of detecting the parent substance and/or its metabolites several days after ingestion. A positive result indicates that the substance was ingested several hours or days before the sample collection, but it cannot correlate the possible psycho-physical impairment with recent ingestion.

The main limitation of this matrix is the limited clinical relevance of quantitative analysis, as the concentrations of analytes vary depending on the dose, mode of administration, time since ingestion, hydration, and individual metabolism.[2, 4]

Sample collection procedures

Urine collection must be carried out according to a procedure that ensures, while respecting the privacy of the person undergoing the test, the identity, integrity, and authenticity of the sample. When the sample is collected for administrative and/or judicial purposes, the sampling must be performed under direct observation by a qualified and authorized healthcare professional, who must explain the sample collection procedure to the person being tested. The containers must be equipped with an airtight, tamper-evident seal, or sealed with a non-reusable adhesive seal, on which both the individual and the sample collector affix their signatures. The materials used for sample collection must remain intact and sealed until opened for the collection.

The urine sample, which should be between 40 and 60 mL in volume, must be collected in appropriate disposable plastic containers, and the collection should take place in areas free from potential contamination sources, such as water, soaps, or detergents. A notable disadvantage of toxicological analysis using urine samples is that the sample can easily be subject to adulteration and/or dilution attempts. For this reason, it is recommended to collect the sample under supervision, with temperature measurement (32-38 °C) and creatinine level determination (20 mg/dL) to verify the sample's authenticity. If any issues arise during the collection process, a "non-compliance" report must be filled in. A urine sample collected for medico-legal purposes must be divided into three aliquots, labeled A (for screening analyses), B (for confirmatory analyses), and C (for review analyses).

From the moment the sample is accepted by the laboratory, or by the competent physician in the case of high-risk jobs, an internal chain of custody procedure must be implemented.[2, 4]

Saliva

Saliva, used in the preliminary phase of testing (screening tests), can be considered a biological matrix for detecting current use. Saliva samples must be collected in duplicate, using two different collection kits/tubes, in preparation for possible confirmatory testing.

Sample collection procedures

Saliva collection must follow a procedure that ensures, while respecting the privacy of the person undergoing the analytical assessment, the identity, integrity, and authenticity of the sample. Saliva samples can be collected without stimulation in standard tubes with airtight closures, although, in recent years, specific saliva collection kits have been marketed. These kits are equipped with containers that do not contain additives and are capable of collecting a known and constant volume of saliva via an embedded volume indicator in the collection device. The manufacturers of these devices must ensure that they do not interfere with the detection of abused substances carried out by laboratories. Despite these efforts to standardize the collection process, saliva collection may take more time than with other matrices, and some difficulties may arise, often influenced by the subject's reduced saliva production, which can be linked to their emotional state.

A saliva sample collected for medico-legal purposes must be divided into two aliquots, labeled A and B, with at least 1.5 mL collected for each sample. Alternatively, the collected saliva sample should be divided into two aliquots of at least 1.5 mL. Aliquot A is immediately used for screening tests, and if positive, it will be used for confirmatory tests. Aliquot B is stored in a freezer at -20 °C for potential review analyses requested by the individual undergoing the analytical assessment.[5]

Blood

Blood is the preferred biological matrix for demonstrating recent consumption, suitable for the determination of xenobiotics and/or abused substances and/or metabolites in both clinical and medico-legal contexts. The concentration of the substance in the blood and/or plasma allows for the establishment or exclusion of recent ingestion and is directly correlated with the psycho-physical state of the subject at the time of sample collection. Blood sampling, performed by healthcare personnel at the request of the Criminal Police (*e.g*., in the case of a traffic accident), constitutes an act of judicial police, for which the individual must be notified of their right to be assisted by a defense attorney (Art. 114 disp. att. Criminal Procedure Code, Artt. 354, 356 Criminal Procedure Code).[6, 7] If consent for the investigation is not granted, the Metropolitan Police Service must be notified to manage the situation accordingly.

Sample collection procedures

The collection of the sample must be conducted according to a procedure that ensures, while respecting the privacy of the individual undergoing the analytical assessment, the identity, integrity, and authenticity of the sample. Blood sampling must be performed in dedicated rooms exclusively used for collection, in compliance with health and safety regulations. The skin should be cleaned with non-alcoholic products. The collection device must contain an anticoagulant (*e.g*., sodium fluoride/potassium oxalate) and must be capable of collecting a known and constant volume of blood (at least 5 mL). As in the case of other samples, for medico-legal purposes, the collected blood sample must be divided into three aliquots, labelled A, B, and C. Aliquot A will be used for possible screening analysis, aliquot B for confirmatory analysis, and aliquot C will be stored in a freezer at -20 °C for potential review analyses requested by the individual undergoing the analytical assessment. All three vials must be sealed with a tamper-evident seal, duly signed, to ensure the integrity of the sample.[6, 7]

Hair matrix

Toxicological analyses for the detection of xenobiotics (drugs, toxic compounds, narcotic or psychotropic substances) and biomarkers of alcohol use and abuse in the hair matrix are typically requested for clinical and/or medico-legal purposes. The request for toxicological analysis on the hair matrix is primarily linked to the possibility of extending the detection window for a given substance. While the proximal part of the hair, close to the scalp, can reveal recent exposure, moving toward the distal part (toward the tip) indicates exposure further in the past. Moreover, given that hair grows at a rate of approximately 1 cm per month during the anagen phase, segmental analysis of hair, measured by centimeter, can provide information about the history and type of substance use over the months corresponding to each analyzed segment. Hair sample collection is non-invasive, and transportation and storage do not require special precautions.[2, 8]

Sample collection procedures

It is preferable to collect the sample from the posterior area of the head (*vertex*), as close to the scalp as possible, since this region is associated with minimal inter-individual variability in hair growth rate. When the sample is collected from a child or an individual with noticeable hair thinning, smaller locks can be taken from different areas of the head, although focus should remain on the posterior vertex region. Hair is the preferred matrix for drug analysis; however, when hair collection is not possible (*e.g*., baldness, shaved head), alternative collection sites such as the chest, pubic area, armpits, or face (beard hair) can be used. Collecting samples from these intimate body areas requires careful consideration of the subject's privacy, while ensuring that the correctness of the collection process is not compromised. Additionally, it is important to note that the growth rate and dormancy (telogen phase) of hair from these areas differ from that of scalp hair. Therefore, it is not possible to determine a precise window for substance use, but it can confirm or exclude previous use. The amount of hair needed for analysis is a lock whose thickness should be approximately the size of a pencil and can be divided into two parts (aliquot A and B). It is important to collect enough hair to perform both screening and confirmatory analyses (aliquot A), as well as to store part of the sample for potential review (counter-analysis, aliquot B). The lock of hair should be secured with a thread as close to the scalp as possible before cutting. Once cut, the hair lock should be placed in a designated aluminum foil sheet, preferably with millimeter markings, with the root portion securely

tied with string and extending out of the sheet. If the length of the hair is insufficient for the procedure described above, the sample can still be collected. In this case, the cut lock should be fixed to a sheet with a clip, indicating with an arrow the portion closest to the scalp.[8]

Preparation of hair matrix sample

A hair matrix sample is significantly different from urine, blood, or saliva samples because it is a solid matrix; therefore, the preparation process involves several initial phases, including washing, drying, and possible segmenting of the lock. The washing procedure is essential to remove surface contamination and is usually performed with organic solvents such as methylene chloride, methanol, or acetone, which have minimal interference with the extraction of analytes from the matrix. The washing process should be quick (1-2 minutes maximum) and use a small volume of washing solution. The laboratory must define its own standardized procedure for washing hair samples before conducting qualitative and quantitative analysis of the active ingredients potentially contained within. The samples are then left to dry at room temperature, finely chopped using scissors, and finally weighed (30-50 mg, depending on the required analysis). The detection of xenobiotics in a hair sample requires an initial "digestion" phase to release the analytes from the keratin matrix. It is important to emphasize that the use of acidic or basic aqueous buffers during digestion may hydrolyze (either partially or completely) chemically labile compounds such as cocaine and heroin. On the other hand, incubation of the hair matrix with methanol or aqueous solutions buffered to neutral pH, although it does not cause hydrolysis of chemically labile compounds, may affect the yield of the analytes, which may generally be lower compared to results obtained with digestion in acidic or basic buffers. However, while digestion in acidic or basic environments always requires subsequent liquid-liquid or solid-phase extraction, methanolic or aqueous extracts at neutral pH can be analyzed directly. The extraction following digestion ensures better cleanliness of the extract. The efficiency of a digestion and extraction method should therefore be verified during the general validation phase of the analytical method.[8]

Total testing process

Pre-analytical phase

Acceptance of biological samples

The aliquots of biological samples, marked A, B, and C (in cases where a third aliquot is required), must arrive at the laboratory inside a refrigerated thermal container. For hair samples, they must be placed in a sealed envelope immediately after collection. Along with the thermal container and/or the envelope containing the samples, the corresponding chain of custody forms and sampling records must be sent to the laboratory. The following checks are essential at the time of sample acceptance:

- integrity of the packaging to exclude any tampering with the sample during transport;
- verification that the information on the containers and/or the envelope of the aliquots matches the information on the chain of custody form;
- acknowledgment of the chain of custody document, including the date, time of acceptance; and the signature of the laboratory personnel responsible for accepting the sample.

In case of discrepancies or missing information, the laboratory is required to prepare a non-conformity report and notify the requesting institution of the non-compliance.

Sample storage procedures

Once collected, biological samples should be stored at +4 °C if the time between collection and the initiation of analytical procedures spans several days. For long-term storage (months/years), samples must be kept at a temperature of -18/-22 °C.[3, 9] Keratinous samples should be stored in a dry place at room temperature.[8] Stored samples must be locked and inaccessible except to authorized personnel handling the samples.

Non-conformities making samples unacceptable for analysis[4, 5, 7-9]

Urine

- Absence of barcodes or mismatched barcodes;
- sample without attached documentation;
- absence of informed consent from the person undergoing the analytical assessment;
- broken or tampered security seals on sample containers or transport containers;
- absence of security seals;
- receipt of only one or two aliquots;
- insufficient sample volume for analysis;
- non-intact containers with evident sample loss;
- inconsistency between the urine sample and the type of request received;
- creatinine <20 mg/dL; 1.7 μmol/L;
- specific gravity <1003 mg/L or >1035 mg/L;
- pH <3 or >10.

A urine sample may be considered adulterated if nitrites (≥500 μg/mL), chromium (VI) (≥50 μg/mL), a halogen

(bleach, iodine, fluoride), glutaraldehyde, pyridine (pyridinium chlorochromate), or a surfactant are present.

Blood and saliva

- Absence of barcodes or mismatched barcodes;
- sample without attached documentation;
- absence of informed consent from the person undergoing the analytical assessment;
- broken or tampered security seals on sample containers or transport containers;
- absence of security seals;
- receipt of only one or two aliquots;
- insufficient sample volume for analysis;
- non-intact containers with evident sample loss.

Hair

- Absence of barcodes or mismatched barcodes;
- sample without attached documentation;
- absence of informed consent from the person undergoing the assessment;
- receipt of a single sample;
- insufficient sample quantity for analysis;
- sample loss.

Key messages

Minimum requirements for the pre-analytical phase	
Dedicated areas	In compliance with privacy regulations.
Trained personnel	Documentation of training pathway.
Completion of chain of custody form and sampling record	Traceability of the sample to ensure its authenticity.
Informed consent	Request for consent for data processing. Request for consent for sampling. Request for consent to send data to the requesting party.
Supervised collection	In the case of urine collection.
Sample storage	Varies depending on the sample matrix. Stored samples must be securely locked and accessible only to authorized personnel responsible for handling them.

Analytical phase

Screening analyses

An initial screening for the detection of classes of abused substances can be performed using both immunochemical techniques that have been validated for the matrices in question and marketed with such indications, as well as chromatographic techniques coupled with mass spectrometry.[2]

Immunochemical tests commonly used for drug screening employ different detection systems but rely on the same antigen-antibody reaction principle, where the analytes (drugs and/or metabolites) compete with an analyte or enzyme present in the screening test. Immunochemical methods are generally characterized by rapid execution times, high or total automation, but, conversely, they exhibit reduced specificity and high inaccuracy in quantitative results, particularly when the sample contains multiple chemical species that can be detected but not discriminated by the method (*e.g.*, unchanged compound and its metabolites). These methods, therefore, produce qualitative results, indicating the probable positivity, or more accurately the "non-absence," in the sample of a particular analyte or more often of a class of substances in relation to a threshold or cut-off value defined by the manufacturer. If the result of a screening test is negative, it is essential to verify that the method minimizes the occurrence of false negatives.

Chromatographic techniques coupled with tandem mass spectrometry or time-of-flight (TOF) analyzers, although slower for screening large numbers of samples, have the advantage of simultaneously identifying a wide range of different analytes in a single analysis. However, these analytical methods are not frequently available in laboratories and require a high level of expertise from the personnel operating them.

All positive screening test results must be confirmed using analytical methodologies specific to the analytes being tested, such as chromatographic separation techniques coupled with detection methods like mass spectrometry. No semi-quantitative values should be reported, and the report should only indicate "non-absence" (*e.g.*, "not absent").[2]

Screening threshold values for biological matrices

Table I reports the threshold values for screening tests as recommended in the *Provvedimento* "*Procedure per gli accertamenti sanitari di assenza di tossicodipendenza o di assunzione di sostanze stupefacenti o psicotrope in lavoratori addetti a mansioni che comportano particolari rischi per la sicurezza, l'incolumità e la salute di terzi*" (GU Serie Generale n.236 del 08-10-2008),[10] by the Society of Hair Testing (SoHT)[11, 12] and by the Italian group of Forensic Toxicologists (GTFI)[13] for different biological matrices (Table II, III).

Table I.—*Screening test threshold values for urine matrix as indicated in the* Provvedimento *"*Procedure per gli accertamenti sanitari di assenza di tossicodipendenza o di assunzione di sostanze stupefacenti o psicotrope in lavoratori addetti a mansioni a rischio*" (G.U Serie Generale n.236 del 08-10-2008) and by the Italian Group of Forensic Toxicologists (GTFI).*

Substance class	Cut-off workplace analysis (ng/mL)	Cut-off GTFI (ng/mL)
Amphetamines	500	500
Cannabinoids	50	50
Cocaine and metabolites	300	300
Opiates and metabolites	300	300
Methadone (EDDP)	300	300
Buprenorphine	—	5

EDDP: ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidin.

Table II.—*Screening test threshold values for the saliva matrix recommended by the Italian Group of Forensic Toxicologists (GTFI).*

Substance class	GTFI (ng/mL)
Amphetamines	40
Cannabinoids	10
Cocaine and metabolites	30
Opiates 6-MAM	4
Opiates Morphine	40
Methadone	50
Buprenorphine	5

6-MAM: 6-monoacetylmorphine.

Table III.—*Screening test threshold values for the hair matrix recommended by the Society of Hair Testing (SoHT).*

Substance class	Cut-off SOHT (ng/mg)
Amphetamines	0.2
Cannabinoids	0.05
Cocaine and metabolites	0.5
Opiates and metabolites	0.2
Methadone	0.2
Buprenorphine	0.01

Confirmation analysis

Confirmation methods must ensure the accurate identification and quantification of the substances of interest (parent substances and/or their metabolites) with appropriate sensitivity and specificity. The identification and quantification of the analytes of interest are achieved using chromatographic separation methods coupled with a specific and sensitive identification technique, such as mass spectrometry (MS), which identifies compounds based on their molecular weight and the typical fragments obtained through collision with a known energy electron beam or a high-pressure gas inside a vacuum collision cell (gas chromatography-mass spectrometry [GC-MS] or liquid chromatography coupled with mass spectrometry [LC-MS] and/or tandem mass spectrometry [LC-MS-MS]). The use of an internal standard (preferably deuterated) is essential, as when added prior to the preparatory phase, it ensures the reliability of the analytical data both in relation to the extraction process and the instrumental separation.

It is important to note that the threshold value of a confirmation test must necessarily be lower than that of a screening test. Samples containing the substances of abuse and/or their metabolites at concentrations equal to or higher than the pre-established cut-off values are considered positive. The aliquots B and C of a sample that tests positive must be stored in a dedicated locked freezer for a period agreed upon with the requesting party (clinical, administrative, or judicial authority), or according to legal regulations. This storage period must be documented in the SOP.[8]

Threshold values for confirmation tests for biological matrices

In the following tables are reported the threshold values for confirmation tests recommended in the *Provvedimento* "*Procedure per gli accertamenti sanitari di assenza di tossicodipendenza o di assunzione di sostanze stupefacenti o psicotrope in lavoratori addetti a mansioni che comportano particolari rischi per la sicurezza, l'incolumità e la salute di terzi*" (GU Serie Generale n.236 del 08-10-2008)[10] (Table IV), and the minimum requirements* outlined in the GIFT guidelines for the different biological matrices[13]

Table IV.—*Threshold values of confirmation tests for the Urine matrix as indicated in the* Provvedimento *"*Procedure per gli accertamenti sanitari di assenza di tossicodipendenza o di assunzione di sostanze stupefacenti o psicotrope in lavoratori addetti a mansioni a rischio*" (Rep. Atti n. 178/CSR) (GU Serie Generale n.236 del 08-10-2008).*

Analytes	Cut-off workplace analysis(ng/mL)
Amphetamine	250
Methamphetamine	250
MDA	250
MDMA	250
MDEA	250
THC	15
THCCOOH	15
Cocaine	100
BEG	100
Morphine	100
Codeine	100
6-MAM	100
Methadone	100
EDDP	—
Buprenorphine	5
Norbuprenorphine	—

MDA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; MDEA: methyldiethanolamine; THC: delta-9-tetrahydrocannabinol; THC-COOH 11-nor-9-carboxy-delta-9-tetrahydrocannabinol; BEG: benzoylecgonine; 6-MAM: 6-monoacetylmorphine; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

TABLE V.—*Threshold values of confirmation tests recommended by SiBioC for the saliva matrix (included in the new Italian Highway Code) and those recommended by Italian Group of Forensic Toxicologists (GTFI) according to the EWTDS guidelines (European guidelines for workplace in oral fluid, 2015-11-01, version 2.0) to be used for high-risk tasks.*

Analytes	Minimum performance requirements* SIBioC (CdS)(ng/mL)	GTFI workplace analysis (ng/mL)
Amphetamine	2	15
Methamphetamine	2	15
MDA	2	15
MDMA	2	15
MDEA	/	/
THC	1	2
THCCOOH	/	/
Cocaine	2	8
BEG	2	8
Morphine	2	15
Codeine	2	15
6-MAM	2	2
Methadone	2	20
EDDP	2	20
Buprenorphine	2	1
Norbuprenorphine	2	1

*"Minimum performance requirements," referring to the concentrations of analytes in the biological fluid under investigation that the laboratory must be capable of quantifying with accuracy, and which are suitable for assessing the applicability of a method for a specific toxicological-forensic analytical purpose, even in the absence of specific regulatory requirements.
MDA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; MDEA: methyldiethanolamine; THC: delta-9-tetrahydrocannabinol; THC-COOH: 11-nor-9-carboxy-delta-9-tetrahydrocannnabinol; BEG: benzoylecgonine; 6-MAM: 6-monoacetylmorphine; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

(Table IV, V, VI) and the threshold values used to detect chronic and excessive alcohol consumption or verify abstinence from narcotics, as established in a consensus document by the SoHT[11, 12] (Table VI, VII). Regarding the analyses of hair matrices, if the assessment concerns an offense facilitated by the unconscious consumption of pharmacologically active substances by the victim, the threshold value considered is the limit of quantification (LOQ) of the analytical method used, which should be in the picogram range per milligram of hair (pg/mg). Additionally, for certain substances present in the body (gamma-hydroxybutyric acid [GHB], testosterone, cortisol...), it is essential to conduct segmental hair analysis to demonstrate any difference in the substance's concentration across various examined segments, in order to possibly differentiate the peak related to intake or administration from endogenous production. Regarding blood matrices, the recent revision of the Italian Highway Code (*Legge 25 novembre 2024, n. 177 G.U. Serie Generale - n. 280 del 29-11-2024)* substantially modifies the approach. Until now, investigations had to determine or exclude recent consumption linked to the subject's psychophysical state at the time of sampling. The new Code shifts from "driving under the influence" to "driving with substance use," thus the threshold value should be the one listed in Table VIII.[13]

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TABLE VI.—*Threshold values for confirmatory tests for the keratin matrix recommended by the Society of Hair Testing (SoHT) and those indicated for high-risk duties (provision 19/09/2008).*

Analytes	SOHT (ng/mg)	Cut-off workplace analysis (ng/mg)
Amphetamine	0.2	0.2
Methamphetamine	0.2	0.2
MDA	0.2	0.2
MDMA	0.2	0.2
MDEA	0.2	0.2
THC	0.05	0.1
THCCOOH	0.0002	0.1
Cocaine	0.5	0.5
BEG	*	0.05
Morphine	0.2	0.2
Codeine	0.2	0.2
6-MAM	0.2	0.2
Methadone	0.2	0.2
EDDP	**	–
Buprenorphine	0.1	0.05
Norbuprenorphine	***	–

*The presence of benzoylecgonine, norcocaine, cocaethylene, hydroxylcocaine, or hydroxybenzoylecgonine should be considered to confirm the use; **the confirmation of EDDP demonstrates the use of methadone; ***the confirmation of norbuprenorphine demonstrates the use of buprenorphine.
MDA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; MDEA: methyldiethanolamine; THC: delta-9-Tetrahydrocannabinol; THC-COOH: 11-nor-9-carboxy-delta-9-tetrahydrocannnabinol; BEG: benzoylecgonine; 6-MAM: 6-monoacetylmorphine; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

TABLE VII.—*Threshold values for alcohol abuse markers in hair matrix recommended by the Society of Hair Testing (SoHT).*

Substance class	Analytes	Cut-off for chronic excessive consumption (ng/mg)	Cut-off for verification of abstinence (ng/mg)
Fatty acid ethyl esters (FAEEs)	Ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate	≥0.5 (as the sum of the concentrations of the four ethyl esters) in the proximal 0-3 cm segment ≥0.1 in the proximal 0- 6 cm segment	≤0.2 (as the sum of the concentrations of the four ethyl esters) in the proximal 0-3 cm segment ≤0.4 in the proximal 0-6 cm segment
Ethyl glucuronide	Ethyl glucuronide	≥0.03 both in the 0-3 cm and 0-6 cm proximal segments	≤0.007 both in the 0-3 cm and 0-6 cm proximal segments

TABLE VIII.—*Threshold values for confirmation tests related to substances of abuse in the Blood matrix recommended by the Italian Group of Forensic Toxicologists (GFTI) (New Italian Highway Code).*

Analytes	Minimum performance requirements GTFI (Italian Highway Code) ng/mL
Amphetamine	2
Methamphetamine	2
MDA	2
MDMA	2
MDEA	2
THC	1
THCCOOH	2
Cocaine	2
BEG	2
Morphine	2
Codeine	2
6-MAM	2
Methadone	2
EDDP	2
Buprenorphine	2
Norbuprenorphine	2

*"Minimum performance requirements," referring to the concentrations of analytes in the biological fluid under investigation that the laboratory must be able to quantify accurately, and that are suitable for evaluating the applicability of a method for a specific toxicological-forensic analytical purpose, even in the absence of specific regulatory requirements.
MDA: 3,4-methylenedioxyamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; MDEA: methyldiethanolamine; THC: delta-9-tetrahydrocannabinol; THC-COOH: 11-nor-9-carboxy-delta-9-tetrahydrocannnabinol; BEG: benzoylecgonine; 6-MAM: 6-monoacetylmorphine; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

has deemed it appropriate to standardize the threshold values for saliva and blood matrices to make the analytical data consistent for a proper medico-legal evaluation.

Key messages

Minimum requirements for the analytical phase	
Dedicated facilities	For screening and confirmation testing
Instrumentation for second-level investigations	Gas chromatography or liquid chromatography coupled with mass spectrometry
Trained personnel	Documentation of the training path
Compliance with cutoff values	Based on the medico-legal inquiry

Post-analytical phase

Communication of analytical results

The analytical result obtained from either a single screening test (negative result) or from a screening test followed by a confirmation analysis (non-negative screening result but negative confirmation, or non-negative screening result and positive confirmation) must be reported by the laboratory director or an authorized collaborator, who must be an expert in the analysis of substances of abuse in biological matrices. They must take into account all the observations and information provided in the collection report regarding any pharmacological treatments the subject may have undergone.[2, 4-6, 8]

The analytical report must include the following identifying information:

- the sample identification number and the personal details of the individual undergoing the test;
- the date of sample collection;
- the date and time the sample was received by the laboratory (if different from the collection date);
- the date of the report;
- the name of the authority or individual who requested the analysis.

The report must also include:

- the biological matrix analyzed;
- the type of analysis performed;
- the analytical method used;
- the results of the analyses performed; if the screening and confirmation analyses detect the presence of drugs and/or metabolites above the predefined threshold value, the report must include the name(s) of the detected substance(s) along with their respective concentrations, and the term "present" must be associated with the quantitative data.
- the cut-off values used;
- the calculation method used to determine the applied analytical uncertainty.

Regarding hair matrices, in addition to the type of matrix analyzed, the report must also include:

- the original length of the sample;
- the length of the analyzed sample segment (or the length of the various analyzed segments);
- any information regarding the color of the hair, as well as any cosmetic treatments applied (*e.g.*, the keratin matrix under examination has undergone a cosmetic coloring treatment).

The analytical data must contain the essential information for its correct interpretation. No additional comments or information should be included unless the authority (or individual) requesting the examination requires an opinion or interpretation of the obtained analytical data. If explicitly requested, the laboratory director will provide a further written opinion on additional relevant information for interpreting the results obtained from the laboratory analysis.

Sample storage

Laboratories conducting analyses for the detection of substances of abuse in biological matrices must store aliquots B and C of positive samples at -20 °C, in accordance with the requirements set by law (or regional circulars governing the analysis of substances of abuse in biological matrices for medico-legal purposes) (for a minimum of 12 months).

Regarding the storage of hair matrix samples, laboratories conducting analyses of substances of abuse and alcohol abuse biomarkers must store aliquot B of the hair sample in a dry, room-temperature, and dark place, in accordance with the relevant laws (*e.g.*, Decree of September 18th, 2008) or regional circulars governing the analysis of substances of abuse in hair matrices for medico-legal purposes. The laboratory must also store and make available all documentation related to the analytical procedures used (for a minimum of 3 years).[2, 4-6, 8]

The documentation must include:

- chain of custody forms;
- records of internal quality control checks performed by the laboratory and external quality assessments in which the laboratory has participated;
- the SOP used by the laboratory for the analysis of primary substances of abuse in biological matrices;
- all analytical results (including those related to method validation with calibration curves and the calculations used to derive the result);
- a copy of the final report.

Samples, as well as documentation related to the chain of custody, must be stored in locked, restricted-access locations, accessible only to personnel authorized to handle them.

Contestation of results

In the case of confirmed detection of one or more illicit substances in the examined biological matrices, the individual undergoing the test may request a counter-analysis on aliquot B of the biological sample, or on aliquot C, if the analytical procedures involve the collection of a third aliquot.

The review test, at the expense of the requester, may be performed at the same laboratory that analyzed aliquot A or B of the biological sample, or at another laboratory chosen by the individual undergoing the test. This aliquot must be accompanied by documentation confirming the chain of custody procedures and must include information regarding the results of the original analysis as well as the cut-off values used in that analysis. All laboratories performing analyses on aliquots B or C of the sample must have documentation demonstrating the use of validated analytical methodologies with precision and accuracy requirements appropriate for the analyses requested. In counter-analyses (or review analyses), only the substances of abuse and/or metabolites found in aliquot A or B of the biological sample should be tested, and the report must be made available in accordance with the applicable laws and regional circulars.[2, 4-6, 8]

Key messages

Minimum requirements for the post-analytical phase	
Trained supervisory staff	Documentation of the training process
Inclusion of identifying information and analytical data in the report	Based on the medico-legal question
Storage of positive samples	Minimum of 12 months
Storage of "chain of custody" documentation	Minimum of 3 years

Quality assurance of analytical testing

Laboratories performing analyses for drugs or psychoactive substances must implement a quality management system that covers all aspects of the analytical process, including:

- sample receipt;
- chain of custody;
- security and communication of results;
- screening and confirmation testing;
- calibration and control certification;
- analytical procedure validation.

Quality assurance procedures must be designed, implemented, and periodically reviewed to monitor the progress of each phase within the analytical process.[2, 4-6, 8]

Analytical method validation

The validation of analytical methods includes all procedures aimed at demonstrating that a particular method, used for the identification and/or quantification of an analyte in a given biological matrix, is reliable and reproducible for the intended use.

Each analytical method routinely used by the laboratory must be pre-validated according to nationally and internationally accepted procedures. For the most commonly used screening methods, validation procedures are typically not required, as the method is validated by the manufacturer. However, each analysis kit is supplied with control calibrators, which, when included in each batch of

samples being analysed, verify the accuracy and precision of the analysis within a preset value. If modifications are made to the manufacturer's guidelines (*e.g*., using the kit for a biological matrix other than the one specified by the manufacturer, altering the LoQ and so on), the laboratory must perform full validation of the modified method/kit.

It is advisable to avoid any modifications to the manufacturer's instructions for kit usage; any modifications should only be made when no other methodologies are available.[2, 4-6, 8]

The validation procedure adopted by laboratories must take into account the following parameters:

• accuracy, defined as the degree of agreement between the mean value obtained from a large number of determinations of a sample and the true or accepted value. Accuracy is expressed as bias or systematic error and is calculated as the difference between the mean value and the true value or as the ratio of this difference to the true value, multiplied by 100 (% bias);

• precision, defined as the degree of agreement between different independent measurements under defined experimental conditions. it can be expressed in terms of imprecision as standard deviation, variance, relative standard deviation (RSD), or coefficient of variation (CV);

• limit of detection (LOD) represents the smallest quantity of analyte in a sample that can be detected but not quantified, with a given probability;

• limit of quantification (LOQ) represents the smallest quantity of analyte that can be quantified with a given imprecision;

• method linearity, referring to the ability of the method to produce analytical results that are directly proportional to the concentration of the analyte being studied;

• matrix effect, a parameter that defines a change in the analyte response due to interference from unidentified components within a biological matrix. For each matrix, accuracy must be within ±15% of the nominal concentration, and precision (%cv) must not exceed 15%;[1, 2, 13, 14]

• carry-over effect, which refers to a change in analyte concentration in a sample due to the residue of a previously analyzed analyte remaining in the analytical instrument. Carry-over should be assessed by analyzing blank samples after the calibration standard; the carry-over value in the blank following a high-concentration sample should not exceed 20% of the LOQ and 5% for the Internal Standard (IS). If the analysis of such a blank produces a result below the lod, the method's carry-over is considered acceptable;[1, 2, 15]

• recovery, is a key value for assessing the efficiency of a sample extraction procedure. The analyte recovery does not necessarily need to be 100%, with acceptance criteria being met when results fall between 90-110%;[1, 2, 15]

• stability, measures the susceptibility of the analyte to degradative or hydrolytic processes of a biotic nature (in the biological sample, after collection) and/or abiotic nature (exposure to light, heat, pH, freeze-thaw cycles). Stability evaluations should be conducted to ensure that each step in sample preparation, handling, and analysis, as well as storage conditions, does not affect the analyte concentration;

• dilution integrity test, is essential since sample dilution may be required, and dilution must be part of a validated method. in this case, the resulting inaccuracy and bias must be below 15%. Dilution integrity evaluation should therefore be part of partial method validation.[15]

Internal quality control

Utilizing an internal quality program (IQP) is fundamental to ensure the reliability of analytical results and to avoid random errors that may occur during the analytical and/or pre- or post-analytical phases, rendering the result inaccurate. The frequency of quality checks is related to the definition of an "analytical run (series)" and, as a minimum, is daily or follows the frequency of the analytical series (biweekly, weekly, etc.), but it should be adjusted based on workload, instrument performance, and other considerations. For continuous processes running 24 hours a day, at least two sets of quality controls should be conducted during the 24-hour period. Within the analytical series, the control sample should be inserted randomly among the unknown samples.[2, 4-6, 8, 15]

External quality assessment

A laboratory must participate in appropriate external quality assessment (EQA) programs. Analytical performance that falls outside the criteria established by the EQA program must be promptly addressed. The selection of the program to participate in, should be based on the best possible scientific outcome. Participation may involve the identification of substance classes or individual substances, and their quantification in the case of confirmation analyses, according to the legal cut- off levels or those established by the program administrator. In screening tests, results are typically expressed as "positive" or "negative." In confirmation analyses, it is necessary to provide not only qualitative data but also quantitative data, *i.e*., the detected concentration according to a given calibration curve for the analyte identified in the examined biological matrix.

The results from EQA participation should be reviewed by the laboratory staff and its director to assess the laboratory's performance. If errors are identified, it is essential to determine their causes and implement corrective actions to prevent recurrence. It is recommended to retain the results in both paper and electronic formats for at least three years.[2, 4-6, 8]

Key messages

Minimum requirements for analytical toxicology laboratories	
Analytical quality assurance	Documentation of IQC and EQA for all analytical lines, with retention for at least three years

Toxicological assessments established by legal regulations

As mentioned earlier, toxicological analyses with forensic relevance can often serve as judicial evidence. Therefore, they must provide clear and unequivocal results, ensuring traceability of every operational phase so that they are uncontestable in a court of law. The determination of psychotropic substances for medico-legal purposes mainly concerns cases of driving under the influence of drugs (Article 187 Italian Highway Code) and/or alcohol (Article 186 Italian Highway Code), monitoring workers engaged in risky tasks, and assessing the physical and psychological requirements for obtaining, renewing, or confirming the validity of a driver's license for motor vehicles or motorcycles by the *ad-hoc* Commissions.[2] This also extends to the issuance of firearms licenses and matters related to child custody or adoption. Therefore, in the following paragraphs, we will delve further into some of these issues.

Assessment procedures for workers in risky occupations

Italy is one of the few European countries that with a law concerning toxicological testing for workers. The Legislative Measure of September 18th, 2008, published in the G.U. 236 of October 8th, 2008, titled "*Procedure per gli accertamenti sanitari di assenza di tossicodipendenza o di assunzione di sostanze stupefacenti o psicotrope in lavoratori addetti a mansioni che comportano particolari rischi per la sicurezza, l'incolumità e la salute di terzi*"[10], implemented and made applicable the guidelines provided by the *Italian Unified state-regions and state-city conference and local autonomies* (n. 99/cu 30 October 2007 - G.U. n. 266 November 15 2008). The law emphasizes the employer's obligation to subject specific categories of workers, listed in the Annex I of the Measure, to particular health surveillance. Procedures for assessing the absence of alcohol dependency, excessive alcohol consumption, drug dependency, and/or the use of narcotic substances aim to define and activate safety measures to protect the health and safety of both the worker and third parties. These procedures are primarily designed to prevent accidents related to performing risky tasks.

It is important to note that the procedural process consists of two distinct steps. The first aims to prevent starting a risky activity under the influence of alcohol and/or psychotropic substances, while the second step is critical for excluding excessive alcohol consumption or dependency, even in cases of occasional substance use. The competent physician, once receiving the list of workers to be monitored, has 30 days to determine the date and time for the test. The worker must be notified at least 24 hours in advance (the law does not specify a minimum advance notice).[10]

Assessments for alcohol and/or drug influence in workers at the start of the work shift

For analytical assessments aimed at excluding the influence of alcohol and/or narcotics in workers at the start of their work shift, it is essential to choose a biological matrix that can be easily collected by the competent physician and that can be correlatable to the matrix of choice used to determine current use, *i.e.*, blood. In this regard, it is important to refer to the guidelines for the "*Prevenzione degli infortuni gravi e mortali correlati all'assunzione di alcolici e sostanze stupefacenti, l'accertamento di condizioni di alcol dipendenza e tossicodipendenza e il coordinamento delle azioni di vigilanza*", sent in 2018 by the Ministry of Health to the Permanent State-Regions Conference between the State, Regions, and Autonomous Provinces. These guidelines prescribe the following matrices:

- alcohol: exhaled air using portable devices such as breathalyzers;
- psychotropic substances: saliva, to be used as initial screening tests, followed by confirmation tests if the results are positive.

However, the Italian current legislation provides for the use of exhaled air (breathalyzer) for alcohol, and for substances an examination on urinary matrix (including the possibility by the competent physician of performing analytical immunochemical tests on site, using authorized laboratories).

In the case of urine, three aliquots (labeled A, B, and C) will be collected for screening, confirmation, and possible revision analyses, as described in previous chapters.

The worker who tests positive is temporarily considered

unfit and referred by the competent physician to a healthcare facility, typically the Service Drug Addiction which, in addition to conducting medical assessments, will also perform toxicological testing on new samples.

Assessments for the absence of alcohol dependency, excessive alcohol consumption, drug dependency, or substance use

For the analytical assessments aimed at confirming the absence of drug dependency or substance use, the following matrices are used:

• urine: three aliquots (labeled A, B, and C) are collected for screening, confirmation, and possible revision as described earlier;

• keratinous matrices: the sample should be collected by a qualified healthcare professional as outlined before and sent to authorized forensic toxicology laboratories or the appropriate healthcare laboratories.

For assessments to verify the absence of alcohol dependency and excessive alcohol consumption, the following biological matrices are used:

• blood: for detecting the biomarker desialylated transferrin (CDT);

• hair: for detecting the biomarker Ethylglucoronide (EtG).

The threshold values to be applied for risky occupations for the different matrices are indicated in the tables provided earlier.

Assessment procedures for articles 186 and 187 of the Italian Highway Code

Health assessments to evaluate driving under the influence of alcohol and/or narcotic substances aim to protect public health and prevent traffic accidents caused by the use of these substances, both for the driver's safety and for the safety of others (other drivers, passengers, pedestrians). These assessments are carried out in accordance with the Drug-Alcohol Operational Protocol "version 2.2 from February 2005, presented by the Ministry of the Interior, Ministry of Health, and Ministry of infrastructures and transport is in 2005,[16] and in compliance with the new provisions of the Italian Highway Code 2024 (Legge 25 novembre 2024, n. 177 G.U. Serie generale - n. 280 del 29-11-2024).

Matrices used in case of detention

• Exhaled air through instrumental devices provided to law enforcement. for assessing alcohol consumption at the time of detention;

• saliva collected using commercially available instrumental devices provided to law enforcement. for assessing the presence of narcotic substances at the time of detention.

Matrices used in case of traffic accidents with hospital transport

• Whole blood collected by healthcare personnel in hospital settings to determine the presence of unmetabolized substances and/or their metabolites in order to evaluate the presence of the substance at the time of sampling. Blood sampling conducted by healthcare personnel upon request of the Judicial Police (*e.g.*, in the case of a traffic accident) constitutes a judicial police act, for which there is an obligation to inform the individual of their right to be assisted by a defense attorney (Art. 114 disp. att. CPP, Artt. 354, 356 CPP);

• urine collected simultaneously with the blood sample to indicate prior exposure of the subject to xenobiotic substances. Although it may not establish the exact timing, it can be useful for interpreting the results and sufficient to impose an administrative sanction.

It is important to remember that blood and urine samples taken by healthcare personnel should be collected as soon as possible after the request, with the sampling times for blood and urine being as close as possible.

Additionally, the samples collected for toxicological testing must always be accompanied by the correct documentation (analysis request form, chain of custody, informed consent) and stored in thermal containers with refrigerants until they arrive at and are received by the laboratory.

It is essential that biological samples collected by healthcare personnel be sent to the laboratory as soon as possible.

Results

The presence of ethanol and/or psychoactive substances (understood as "parent substance") in blood or saliva highlights what is referred to as "current use." The presence of inactive metabolites of psychoactive substances in blood, or of active ingredients and/or metabolites solely in urine, does not allow the assertion that the individual undergoing testing is intoxicated. In this case, an administrative sanction (Articles 128 and 119 Italian Highway Code) will be applied, rather than a criminal sanction (Article 187 Italian Highway Code Italian Highway Code). It is also important to check that the subject has not undergone medical treatments involving xenobiotics during roadside assistance, prior to the sampling, with such treatments being documented in the chain of custody.

What happens if a person is positive for alcohol and/or narcotics during detention

When an individual is stopped for driving under the influence of alcohol and/or narcotics, in addition to the administrative and/or criminal sanctions provided, the Prefector

orders a review of the psychophysical requirements for driving. This review is a precautionary measure mandated by law and must be conducted by the Local Medical Commission (CML) competent for the individual's place of residence. To evaluate whether the person detained meets the psychophysical requirements for driving and is capable of dissociating driving from the consumption of alcohol and/or abuse substances, the CML arranges for follow-up checks over time, to which the individual is obligated to submit.

Analysis performed for alcohol consumption assessment

If blood is used, the following non-specific markers can be measured: transaminases (AST, ALT), gamma-glutamyl transferase (γ-GT), proteins, complete blood count (CBC).

These assessments are conducted to provide a general overview of liver status and any potential liver damage or a significantly increased red blood cell volume that can be induced by alcohol. However, since these markers can be altered by various other pathologies and are influenced by many biological (age, sex, genetics) and iatrogenic factors, they are not essential for evaluating the required fitness and, therefore, cannot be used alone without direct markers. Due to their nonspecific nature, their use is not recommended for the purpose.

Carbohydrate-deficient transferrin (CDT) is indicative of recent alcohol consumption, with a normal range, a doubtful range, and a range indicating alcohol abuse.

For CDT, each laboratory is advised to define its own decision level or threshold value based on the method used, imprecision, the reference population, and the purpose for which CDT determination is performed. Following IFCC guidelines, the recommended expression is in percentage, with a cut-off value of <2.

If hair is used, ethyl glucoronide (EtG) is a specific and direct marker, being a hepatic metabolite of alcohol. It deposits in the hair and is used to provide a historical perspective on alcohol consumption. In this context, it is considered the marker of choice.

For threshold values of EtG in hair, reference can be made to the tables provided in previous paragraphs.

Analysis performed for psychoactive substance consumption assessment

The biological matrices on which analytical tests are performed are as follows.

Urine

Three aliquots, labeled A, B, and C, will be collected for screening, confirmation, and possible review, as described in previous paragraphs.

Hair

The minimum length to be examined is 3 cm. The presence of the most common and widely consumed narcotics is assessed, including:

- opiates (heroin, morphine, etc.);
- cocaine;
- THC (tetrahydrocannabinols, *i.e.*, all cannabis derivatives);
- methamphetamine;
- ecstasy;
- ketamine;
- methadone;
- buprenorphine.

The procedure includes more stringent checks over time if a second offense (recidivism) occurs. Nevertheless, the medical request issued by the CML on the appropriate form will include the required tests, as well as the matrix site and hair length.

The laboratory must strictly adhere to this request, reporting any inability to comply.

It should be noted that these tests are not covered by the National Health Service (SSN) and are therefore the responsibility of the individual.

Conclusions

The analytical determination of narcotic and psychoactive substances in biological matrices requires particular attention, especially when such determinations have medico-legal implications and are considered as evidence in administrative or criminal contexts. These determinations must meet the requirements of reliability and accuracy, with the analytical data being correctly interpreted. The aim of these Standard Operating Procedures is to provide general indications that can be shared between laboratories conducting forensic investigations, aiming to standardize analytical and organizational procedures in these laboratories. This also serves as a "consultation tool" for laboratories planning to perform analyses of narcotic and psychoactive substances in biological matrices, ensuring a high-quality service for medico-legal data reliability.

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Conflicts of interest
The authors certify that there is no conflict of interest.

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