Italian Guidelines for Forensic Toxicology

GUIDELINES FOR THE DETERMINATION OF NARCOTIC AND PSYCHOTROPIC SUBSTANCES IN BIOLOGICAL SAMPLES IN FORENSIC TOXICOLOGY AND MEDICO-LEGAL CONTEXTS

SCIENTIFIC SOCIETY ITALIAN FORENSIC TOXICOLOGISTS GROUP (GTFI)

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Introduction

This document sets out the Guidelines and best standard practices drawn up by the Scientific Society Italian Forensic Toxicologists Group (GTFI) in testing biological samples for narcotic and psychotropic drugs, in forensic toxicology and medico-legal contexts.

By narcotic and psychotropic drugs (NPDs) we mean all chemical compounds and their metabolites, of natural, synthetic or semi-synthetic origin, pharmacologically active, capable of altering the psychic and behavioural sphere to varying degrees, with psycholeptic, psycho-analeptic and psycho-dysleptic effects, and capable of generating tolerance, addiction and dependence symptoms. This classification therefore includes traditional narcotic substances, new psychoactive substances (NPSs), active ingredients of drugs having a psychoactive action, alkaloids and other organic or inorganic substances having a psychoactive action or of general toxicological interest.

The Guidelines are intended for forensic toxicology laboratories that conduct qualitative-quantitative analyses of NPDs on biological specimens (taken from living or cadavers), and are revision no. 6 of the Guidelines originally drafted in 2000.

This revision of the Guidelines was drafted by the GTFI Board of Directors, consisting of the following members:

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And was presented to GTFI assembly on June, 8th 2022.

The Guidelines consist of the following sections:

1. Purpose and applications;
2. Terms and definitions;
3. Laboratory requirements;
4. Laboratory procedures;
5. Analysis requirements;
6. Sample acceptance, collection, moving and handling;
7. Analytical methods;
8. Analysis report;
9. Quality assurance;
10. References.

1. Purpose and Applications

Testing NPDs in biological samples for forensic toxicology and legal medicine purposes needs constant analytical improvements, due not only to the consolidation of new methodologies and instrumentation, but above all to scientific progress in the identification of new markers of exposure and the use of new, alternative or complementary biological matrices.

In view of the fact that they can serve as evidence in administrative or criminal law matters, these tests should meet the requirements of certainty and reliability (which can be proven by documenting and tracing each step in the analysis) as well as transparency and, if possible, national consistency.

A high level of quality in the results of the above-mentioned testing is achieved not only by using appropriate analytical techniques and proven analysis procedures and methods, shared by the national and international forensic toxicology community, but also by ensuring that the results originate from facilities that are qualified, efficiently organized, highly reliable over time, and constantly updated.

Purpose

The purpose of these Guidelines can be summarized in the following points:

- To disseminate and promote a knowledge of forensic toxicology analytical procedures and especially in the critical interpretation of the results, according to their biological and statistical significance:
- To help laboratories that conduct forensic NPD testing meet the requirements for an efficient, effective, and reliable organization.
- To provide the aforementioned laboratories with a reference tool for a correct analytical approach that guarantees a quality standard, based on the harmonisation and comparability of results, providing indications and recommendations on the management of analytical processes, and a reference for correct reporting of forensic toxicology and medico-legal tests.

Applications

The Guidelines, designed as an element of self-discipline and a fundamental requirement of a quality management system, which guarantees the evidentiary value of the analytical data provided, are intended as an essential component of a sought-after process of 'excellence accreditation' for laboratories conducting forensic NPD testing.

Therefore, these laboratories must adopt a quality management system that embodies and monitors a quality policy, based on the following principles:

- Organisational effectiveness;
- Excellence in results;
- Constant improvement of the quality standards;
- Empowerment of the staff to ensure the quality of their work and to disseminate quality policy
- Constant review of the quality policy and its objectives.

Scope of application

These Guidelines must be implemented and applied by all laboratories, with documentable characteristics referred to in paragraph 1, that wish to test biological samples for NPDs for the purpose of applying legal provisions set out in the applicable regulations. The facilities that operate in this area must therefore comply with the principles set out in these Guidelines, in terms of organisation and methodology, in order to comply with the requirements of consistency and auditability for quality assurance in terms of:

- Organizational structure, scientific qualification, duties and responsibilities of staff;
- Sample acquisition, management and storage procedures;

- Procedures for developing, validating and applying analytical methods;
- Minimum criteria for identifying and quantifying NPDs in biological samples;
- Internal and external auditing of analysis reliability;
- Drawing up and issuing the analysis report (report), including interpretation of results and indication of the scope of their usability, also in relation to applicable regulations.

2. Terms and Definitions

Accuracy (or Precision): Closeness of the average analyte concentration result obtained by a quantitative method to the true concentration value. It is expressed as a percentage error (E%).

Quality certification: A process aimed at continuous quality improvement, whereby a laboratory undergoes assessment by an independent body to check its performance according to predetermined requirements.

Analysis: In this context, in short, the term refers to forensic laboratory tests to detect NPDs in biological samples collected from living or dead persons.

Screening: Preliminary analysis, generally performed by immunochemical techniques, which provides a presumptive result (probable negativity or presumed positivity / non-negativity -) of a sample against a substance/ class of substances also, but not necessarily, by reference to a cut-off value where established by law, rule or regulation. By definition, a result obtained by screening alone has no legal (forensic) value.

Confirmatory analysis: Analysis that must be performed by a method of higher specificity than the screening, possibly based on different physico-chemical principles, in order to specifically identify a substance and/or its metabolites presumptively identified by the screening.

Revision analysis (or counter-analysis): Analysis performed on a revision sample (counter-sample) with a method having specificity and sensitivity characteristics equal to or greater than those of the analytical method used for the disputed analysis. The subject being tested has the right to be present, through its own lawyer and/or through its own technical advisor, at the identification of the sample, the verification of its integrity and all the procedures of the revision analysis. The revision analysis may be performed by the same Laboratory that performed the first-instance analysis, or by laboratories specifically identified on the basis of an objective external assessment, officially recognised for this purpose, and documenting full compliance with these Guidelines. In the latter case, the transfer of the sample between the two laboratories must be strictly carried out while maintaining the chain of custody.

Qualitative analysis: Analysis that can provide a result expressed in terms of presence/absence of an analyte, also in relation to a cut-off value where provided for by specific regulations, or in any case a predetermined one.

Quantitative analysis: Analysis that can measure the concentration of one or more analytes with a predetermined level of precision and accuracy.

Quality Assurance: Compliance with Documented Procedures (managerial and technical) through strict application of operating instructions and continuous monitoring of the various phases of the analysis process.

Batch: Group of samples examined in sequence or simultaneously, analysed within the same analytical session.

Blank (or blank sample): Biological sample previously submitted for analysis and found negative for one or more substances of interest (analyte content below the identification limit, LOD).

Sample: Determined quantity or volume of biological matrix to be submitted for analysis.

Calibrator: Sample containing a defined amount of analyte, known to the operator, prepared in a biological matrix equal or similar to that of the samples to be analysed, to be used for the preparation of the calibration curve.

Carry-over ('carry-over' or 'memory effect'): Undesirable presence of one or more analytes of interest observed during instrumental analysis of a biological sample, measurable through the analysis of a blank following the analysis of a sample containing a certain amount/concentration of analyte. If the analysis of such a blank produces a result below the identification limit, LOD, the method carry-over is acceptable.

Chain of custody: A documented procedure to guarantee the authenticity, integrity and traceability of a biological sample from the moment it is taken/collected to its disposal; it must allow sample traceability (from collection to acceptance and subsequent management within the laboratory), to document its storage conditions at all stages, to protect it from voluntary or involuntary tampering and adulteration, and to identify all movements and processing by recording the date and the personnel who carried them out.

Coefficient of Variation Percentage (CV%) or Relative Standard Deviation (RSD): Index of dispersion of analytical measurements, used to measure the precision of quantitative testing, determined by the percentage ratio between the standard deviation of a set of measurements, taken on different portions of the same sample, and the value of the arithmetic mean of these measurements.

Counter sample (review sample): Sample taken from the same subject at the same time as the sample being analysed, for any counter-analysis.

Control: Sample containing a defined and known amount of analyte, preferably different from that of the calibrators, prepared in biological matrix equal or similar to that of the actual samples.

Blind control: Undisclosed control to verify compliance of an analysis with the relevant documented procedure. If the concentration is unknown to the Laboratory or to one or more of the laboratory personnel (blind control for internal use), it is used to assess whether, and to what extent, the analysis result yielded meets predetermined quality characteristics (external or internal quality control).

Identification and quantification criteria: Set of predetermined criteria that must be simultaneously and compulsorily met in order to assign the required degree of specificity for the identification of an analyte and/or precision and accuracy for its quantification.

Calibration curve: Graphical and mathematical evaluation of the relationship between the quantity or concentration of an analyte and the signal it produces.

Cut-off or Threshold Value or Decision Threshold: Concentration limit defined, in a conventional manner, to establish the negativity or the positivity (non-negativity in the case of screening analysis) of a sample. The cut-off value, therefore, may vary depending on the scope of the analysis.

Matrix effect: Combined effect of all components of a biological sample, or an extract thereof, other than the analyte, on the measurement of the amount of the analyte itself.

Measurement uncertainty: Parameter associated with the result of a measurement that characterises the dispersion of values reasonably attributable to the measurand and expressed in the same unit of measurement.

Documented Information (DI): Information that the organisation (the Laboratory) believes it must manage to ensure system effectiveness, communicate information, support the operation of its processes, and maintain information so that it can ensure that processes are conducted as planned and evidence of compliance can be provided. They can be in paper or electronic format.

Calibration (or linearity) interval: Range within which a method can yield quantitative results that meet predetermined acceptability criteria.

Lower Limit of Quantification (LLOQ): The smallest concentration or amount of analyte that the method can measure with adequate accuracy and precision.

Limit of Detection (LOD): Amount of analyte present in a sample that can produce a discernible signal from that produced by a negative (blank) control.

Upper Limit of Quantification (ULOQ): Highest concentration or amount of analyte that an analytical method can measure with adequate accuracy and precision.

Quality Manual: Collection of documentation concerning the quality of all laboratory activities; contains documented management and technical procedures. According to 2015 revision of ISO 9001 a quality manual is no longer required, but the management system must be supported by "documented information".

Certified Reference Material (CRM): A biological matrix sample, which is consistent and stable over a specified period of time, containing known and certified quantities or concentrations of one or more analytes.

Precision: A characteristic of an analysis method relating to the dispersion of a series of repeated measurements on different portions of the same sample. It can be estimated from the coefficient of variation obtained from such measurements. It is generally measured within an analytical session (intra-batch precision, repeatability) and between different analysis sessions or even between different laboratories (inter-batch precision, reproducibility).

Documented Procedures (See Documented Information): Written procedures relating to all management and technical activities of the laboratory.

Proficiency Testing (PT): Pathway for improving laboratory quality, generally on a voluntary basis, carried out through the periodic testing of blind controls in order to identify errors of a systematic or random nature and adopt the necessary countermeasures, the effectiveness of which can be assessed in subsequent controls. Participation in external PTs allows verification of the quality of laboratory performance.

Report or Analysis Report: Final summary documentation of the analysis process, containing the results and, where appropriate, the relevant interpretative comments.

Negative Result: Result below a cut-off/Threshold/Decision Value or a reference value chosen by the laboratory (e.g. below the LLOQ or the LOD).

Positive result: Identification of an analyte according to predetermined identification criteria, being present in the sample at a concentration greater than or equal to the cut-off/Threshold/Decision value, once the measurement uncertainty is subtracted, or a reference value chosen by the laboratory (e.g. greater than the LLOQ).

Robustness: Characteristic of an analytical method relating to its ability to yield valid and stable results over time, even after slight, deliberate variations (e.g. different technicians, different laboratories).

Analytical specificity (or selectivity): Ability of an analysis method to identify a given analyte in the presence of other substances (such as other xenobiotics of similar structure or composition, metabolites, degradation products, endogenous components of the biological matrix, impurities, etc.).

Stability: Measurement of the analyte's susceptibility to biotic (in the biological sample, after collection) and/or abiotic (exposure to light, heat, pH, freeze/thaw cycles) degradative or hydrolytic processes.

Calibration: Definition of the measurement characteristics of a measuring instrument by comparison with a reference quantity or instrument.

Validation of an analytical method: A set of tests designed to assess the ability of an analysis method to achieve the objectives it was designed for.

External Quality Assessment (EQA): External monitoring of the analytical reliability of a laboratory carried out by an independent body, assessed by examining the qualitative and quantitative results obtained from the analysis of a set of blind controls. Differently from a Proficiency Testing, participation in an EQA can be made compulsory in relation to certain standards: in this case, it can lead to restrictive and/or sanctioning measures against laboratories that do not comply with the minimum standards required by the EQA.

3. Laboratory Requirements

Laboratory management, Director

Laboratory management involves taking on professional, organizational, teaching, and administrative responsibilities.

This position requires a degree in scientific disciplines, together with specific knowledge of analytical toxicology and forensic toxicology, acquired through suitable and documentable university training, or through proven experience in the field for a continuous period of at least five years, and documented by relevant scientific publications and continuous updating.

Organizational structure, scientific qualification, duties and responsibilities of staff

Laboratory staff must have specific knowledge of toxicology analysis and forensic toxicology, combined with documented professional training appropriate to their specific responsibilities, and must be thoroughly familiar with current regulations concerning NPDs, especially in relation to testing biological samples for NPDs.

Training and refresher courses for laboratory staff must be documented and stored. The number of persons in the staff must be appropriate for the number of tests conducted in the laboratory.

In addition to the Director, the presence of at least one other manager with a degree in suitable scientific disciplines, with adequate experience in analytical and forensic toxicology (documented by training, experience, refresher courses and relevant scientific publications) is recommended to coordinate and supervise the staff's activities, ensuring compliance with procedures and verifying quality requirements.

Minimum safety standards

Procedures must be put in place in the Laboratory to protect the technician safety and, in particular, adequate information and indications must be provided on the risks, the measures necessary for their prevention and, in general, for the technician safety pursuant to the applicable regulations.

The Director, or his/her representative, who is entrusted with the role of "Safety Officer", must ensure that these provisions are strictly complied with. The handling and disposal of risk materials must be regulated by specific procedures, pursuant to the applicable regulations.

4. Laboratory Procedures

Overview

The laboratory must draw up and store in a documented form the procedures relating to all management and technical activities carried out.

Documented Procedures (or Documented Information)

The Documented Procedures provide a detailed description of all the activities necessary for the correct performance of each type of analysis that the laboratory declares to carry out; they also contain the analysis methods and establish orderly sequences of actions and events so that all the processes described therein, carried out in a consistent and reproducible manner, enable each analysis to be performed under standardised conditions.

The ***Management Activities*** require Documented Procedures concerning:

- Characteristics and purpose of the analyses and their results;
- Sample acceptance and chain of custody;
- Use, routine maintenance, and calibration of measuring instruments and analytical instrumentation;
- Drafting, delivering/sending the report (analysis report);
- Protection and confidentiality of sensitive personal and judicial data and results;
- Archiving and storage of analytical documentation and related data;
- Use of internal and external quality controls, monitoring and quality improvement;
- Staff qualification, training and refresher courses.

For ***Technical Activities,*** the Documented Procedures must detail:

- Purpose of the analysis (diagnostic objectives and scope of the analysis; list of individual analytes or classes of substances that the analysis can supposedly detect or identify specifically and/or quantify; biological matrix to which the analysis is applied; any cut-off value);
- Principles of analysis methods with any bibliographical references;
- List of validation parameters of the methods used and respective values obtained;
- Operational details with reference standards, reagents (composition, preparation, precautions for use, storage conditions, instability or deterioration, shelf life), solvents and other consumables;
- Qualitative and quantitative characteristics of the biological matrix required to perform the analysis and any repetitions;
- Procedure for setting up the sample and controls, for their identification and positioning in the analytical batch;
- Instrumentation used with reference to the relevant procedures for routine maintenance, verification of functionality and calibration, as well as their periodicity;
- Predetermined criteria for acceptability of the results of an analysis session;
- Minimum criteria for the identification and/or quantification of each analyte or class of substances.

Each Technical Documented Procedure for a screening or confirmation method must include, for each analysis session, a number of positive and negative controls appropriate for the number of samples to be tested (at least one positive control and one negative control for every ten samples) in order to ensure the quality of the results yielded and to take corrective action if the requirement of acceptability of results is not met.

5. Analysis Requirements

Analysis quality management system

The laboratory must provide its services and develop its processes under controlled conditions and must adopt a quality management system

for all activities relating to forensic toxicology and medico-legal analysis processes.

Diagnostic purposes and biological matrices

Forensic toxicology analyses for diagnostic purposes involve the examination of multiple biological matrices taken from living or dead persons, the respective results of which, either individually or in combination, provide useful elements for a correct diagnosis having forensic toxicology or medico-legal value in various fields, such as, for example, the drug driving test, the fitness to drive test, the fitness for workplace test, the fitness to keep and bear arms test, testing of fitness for specific competition and/or contractual regulations, the diagnosis of use/abuse (also in connection with child custody or international adoptions), the diagnosis of intoxication (currently experiencing the biological effect or *"under the influence of"*), the diagnosis of drug addiction, the diagnosis of acute fatal intoxication, the diagnosis of drug facilitated crimes etc.

Therefore, the laboratory that declares its expertise in conducting testing for forensic toxicology and legal medicine purposes must demonstrate that it is able to perform the analyses at least on the following biological samples: whole blood or plasma and serum, saliva, urine, hair; the Director must also be able (due to the characteristics described above) to assess, depending on the various needs and requests, the type of biological matrix required and the methodology to be adopted.

The following are examples of frequently observed situations:

- In cases where the topicality of the use of NPDs, i.e. the subsistence of their biological effects, must be assessed, testing must be strictly conducted on blood samples. Saliva (more appropriately the fluid of the oral cavity) can also be used for this purpose, albeit taking into account the different time window of detectability compared to blood; it is unacceptable to use only the urinary matrix to diagnose the biological effect produced by NPDs for forensic toxicology and medico-legal purposes (e.g. a state of psycho-physical alteration due to drug use). This is because the detectability of a substance and/or its metabolites in the urine may extend well beyond its complete elimination from the blood (and thus the cessation of its biological effect).

- For testing 'recent' use of NPDs (with a time window of detection of hours-days depending on the pharmacokinetic characteristics of the substance in question) the sample of choice is urine. This sample may also be used for testing chronic intake status if the analysis is extended to several samples collected on different days and 'unannounced' (i.e. with as short a notice to the person concerned as possible, in any case not exceeding 24 hours);

- Chronic intake, as well as past use/abuse behaviour, can be checked by testing hair (head hair and/or body hair samples). Segmental head hair analysis allows reconstruction of the chronology of intake, albeit with a margin of uncertainty. The analysis of hair from other body regions (e.g. armpits, chest, pubic hair) does not allow serial chronological assessments, although it does provide evidence of previous use or exposure. Furthermore, considering the overall time of hair turnover in a sufficiently large body area, a 'time window' of several months can be assessed in relation to the type of hair and its natural growth (chest, pubic, underarm, etc.). Naturally, the more or less recent shaving of body hair influences this time window. For pubic hair, the assessment of the quantitative levels of substances and metabolites present becomes more complex, given the possibility of contamination through the subject's own urine.

Biological samples

The minimum amount of biological sample and counter sample considered sufficient for conducting each analysis must be indicated by the laboratory in the corresponding Documented Procedure. It must take into account the possible need to examine the sample more than once, also in relation to the number of analytes being tested, the qualitative and/or quantitative purpose of the test, or the need for any reason to repeat the analysis.

The following table shows recommended volumes and quantities of biological matrices obtained from living samples for multiple screening and confirmatory analyses. Even smaller volumes/quantities than those indicated in the table may allow screening and confirmation analyses to be conducted, but the repeatability of the analysis must always be guaranteed by the laboratory by storing sufficient portions of the biological matrix being sampled.

For each biological sample, the methods of collection, transport and storage before analysis, as well as the conditions and storage time after analysis, must be clearly indicated in a specific Documented Procedure.

Table 1. Minimal volumes or quantities of the different biological matrices recommended for testing and confirmation.

Biological Matrix	Sample	Counter Sample	Volume
Urine	10 mL	10 mL	20 mL
Blood for alcohol test	3 mL	3 mL	6 mL
Blood for other NPDs	5 mL	5 mL	10 mL
Hair[a]	100[b] mg	100[b] mg	200 mg
Saliva (oral fluid)	1 mL	1 mL	2 mL

[a] In the case of segmental analysis, the quantity refers to each segment; in the case of analysis of alcohol markers, ad hoc portions of suitable origin and length must be provided.

[b] Quantity allowing the use of several analysis methods for different classes of compounds.

Maintenance, monitoring and calibration of measuring instruments and analytical instrumentation

For each weight, volume, temperature and pH measuring instrument, as well as for NPDs identification instrumentation, the laboratory must establish and indicate in a special Documented Procedure the methods and frequency of calibration, routine maintenance and performance monitoring.

For example, refrigerators and freezers must be equipped with a manual (at least daily) or automatic temperature monitoring system. To this end, the Laboratory must have, directly or indirectly, at least one thermometer certified by Calibration Laboratories (CL) to be used for calibrating the other thermometers used, one or more certified reference weights for calibrating analytical balances, and reference buffers for calibrating pH-meters.

Calibrations may be entrusted to certified bodies. Calibration certificates must be recorded in paper and electronic format, where available, and stored for at least three years.

Traceability of analytical and other sample documentation

The laboratory must implement a system for recording and archiving, in paper and electronic format, where available, all information relating to instrumental testing conducted (e.g. chromatograms and mass spectra of the sample and the positive and negative controls used), so that each is fully traceable and documentable.

How and how often back-up copies of the analysis documentation are to be created must be set out in the Documented Procedure.

In addition to the analysis documentation, the laboratory is required to store:

- The paper documentation relating to the samples (e.g. request/identification, acceptance, collection, transport forms or reports);

- Paper and electronic documentation, where available, relating to the sample chain of custody;

- A copy of the analysis report;

- The documentation relating to the certification (or verification) of the degree of purity and validity period of the reference standards used;

- Data on the analysis, maintenance, monitoring and calibration of measuring instruments and analytical instrumentation. It must be stored for at least three years from the date of issue of the report, unless otherwise indicated by specific regulations;

- Copies of the documentation concerning the entry/exit/use of the NPDs used in the form of reference materials.

This documentation must be kept in paper or electronic format for at least three years, unless otherwise provided for by specific regulations.

6. Sample acceptance, collection, moving and handling

Access to the Laboratory

Access to the laboratory must only be granted to authorized persons; the laboratory must take measures to ensure that outsiders cannot access the laboratory either during or outside working hours.

Restrictions and precautions

Laboratories that, in addition to conducting analyses on biological specimens, carry out forensic toxicology analyses on non-biological samples must acquire, handle and store such samples in different environments to those where the biological specimens are accepted and

processed, in order to avoid the risk of environmental contamination by active ingredients, adulterants, diluents, etc.

Acceptance of an analysis request

If biological samples are taken/collected outside the laboratory, the collection and transport methods must first be agreed upon with the relevant facilities, in order to guarantee the chain of custody. In any case, the laboratory's responsibility for compliance with the chain of custody applies to the moment the samples are accepted and to the activities carried out thereafter.

At the time of acceptance, the laboratory must verify:

- The correct way to package and store the sample during transport, especially with regard to the cold chain, when required;
- The suitability of the analysis request and its executability by the laboratory;
- The qualitative-quantitative suitability of the sample in relation to the analysis request;
- The consistency between the sample identification data and the accompanying documentation;
- The collection of informed consent to the testing and information of the subject concerned of the relative defense rights, where such procedures are provided for by the applicable regulations (e.g. in health checks pursuant to Articles 186/186a and 187 of the Highway Code related to DUI).

In addition, the laboratory must record:

- Date and time of sample collection, when known;
- Date and time of sample acceptance;
- Applicant's personal details, address and legible signature;
- Purpose of the analysis;
- Type of sample, its storage protocol and location pending analysis;
- Any clinical, anamnestic and circumstantial data useful for performing the analysis and/or interpreting the result;
- Name and signature of the carrier;
- Name and signature of the laboratory personnel accepting it.

Where biological samples are taken at outpatient clinics on the same premises as the laboratory, the acceptance phase involves:

- Identifying the subject by a valid identity document;
- Informing the subject of the purpose of the analysis, the sampling procedure and the subsequent sample collection, packaging and labelling phases;
- Collecting the subject's written informed consent to sampling and analysis;
- The subject's confirmation, by signature on the sampling form, that he/she has attended all stages of splitting, packaging and labelling of the samples taken with the relevant records mentioned.

Where samples are taken from a cadaver, the laboratory must verify:

- The correct manner in which the autopsy samples are taken, packaged, labelled and stored. If the autopsy is not performed in the same facility as the laboratory, the laboratory staff must be certified as to the maintenance of the cold chain during transport of the samples;
- The qualitative-quantitative suitability of the samples in relation to the analysis request;
- The consistency between the sample identification data and the accompanying documentation

Living person sample collection

Only duly authorized staff and the subject to be sampled may access the sampling location.

The sample collection must always include the collection of a counter-sample for any revision analysis. Specific regulations may require the collection of three equal sample portions. In this case, the three portions must be used for screening (sample A), confirmation (sample B) and revision (sample C) analysis, respectively. This sampling procedure must be used if the screening and confirmatory analysis are performed by two different laboratories.

Urine collection

Urine sample collection must comply with the following procedures:

- Prior to entering the sampling location, the subject is required to leave behind any object, bag, garment likely to conceal material likely to adulterate or tamper the urine sample;
- The subject is required to wash his/her hands thoroughly and dry them;
- Staff must give the subject the urine collection material, inform him/her of the approximate amount of urine to be collected and invite him/her to enter the sampling room;
- The sampling room must allow for direct or indirect observation (closed-circuit television camera, whose presence the subject must be informed of in advance) and it must not (except in the case of observation) contain sources or materials that could be used for diluting or adulterating the sample.

The adoption of these sample collection methods is believed to offer sufficient guarantees against attempts to adulterate or tamper the urine sample. However, additional checks can be made on the sample after collection (e.g. temperature, specific gravity, creatininuria, pH). Checking the specific gravity or creatininuria allows the sample to be checked for dilution. The assessment of these parameters as to the suitability of the sample for analysis is the responsibility of the laboratory director or manager, and is not necessarily linked to standardized chemical-clinical values.

Sampling of head hair or other hair

The laboratory staff collecting the sample must check whether the head hair length is consistent with the analysis request, whether the head hair has visible cosmetic treatments that may interfere with it, and must ask the subject for useful information for performing the analysis and interpreting the analysis result (hygienic treatments, cosmetics, use of lotions, hairsprays, gels or other substances potentially interfering with the analysis) and record all the information collected.

The hair collector, wearing disposable gloves, must isolate a strand of head hair with a diameter of approximately 0.5 - 0.7 cm, preferably in the area of the back vertex of the head, and remove it by cutting it with scissors as close to the skin as possible. Maintaining the alignment of the head hair taken, the operator must divide the lock longitudinally into two approximately equal parts to be used, respectively, for the preparation of the sample and the counter sample. The preparation of the sample, unless the analysis of the whole strand of head hair is requested, must allow unambiguous identification of the proximal end of the strand and prevent misalignment of the head hair. To this end, it is easier if the lock is tied with tape or thread before cutting it, in which case the counter sample will be a second lock taken from the same area. Aesthetic reasons to avoid head hair removal should not be accepted. Furthermore, care must be taken to ensure that the head hair sample is perfectly dry before packaging. If this is not the case, it is necessary to wait until the sample is completely dry by leaving it to air dry after placing it on a clean surface.

If no head hair is available, hair may be taken from other areas of the body, complying for assessment purposes with the specific section in Chapter 5. All details concerning the sampling area must be recorded on the sampling form. The packaging of the hair sample must ensure protection from light and moisture (e.g. hair wrapped in a paper or aluminum foil inside a plastic bag or container, storage at room temperature, in the dark).

Blood sampling

Blood samples must be taken from a vein of an upper limb after disinfection of the skin surface with a non-alcoholic disinfectant. As this is an invasive type of blood sampling, it must be carried out according to the relevant regulations and must be designed to minimize any health risk to the subject undergoing the procedure.

Blood samples must be stored long-term (months/years) at a temperature of -18/-22 °C. In the case of limited time periods between collection and analysis (e.g. a few days) storage at +2/+8 °C is sufficient.

Blood sampling for forensic blood alcohol testing

Blood alcohol testing for forensic toxicology purposes must strictly take the following critical points into account:

- Potential contamination of the sample due to the improper use of skin disinfectants containing ethyl alcohol;
- Possible chemical or biochemical processes favoring the neoformation of ethyl alcohol;
- Handling of the sample (e.g. sieration, centrifugation) such that its original characteristics are altered;
- Possible evaporation of ethyl alcohol from the sample after collection.

For these reasons, blood sampling for forensic blood alcohol testing (a classic example being blood alcohol testing for the assessment of driving under the influence, DUI) strictly requires the following:

- Disinfect the sampling area with a non-alcoholic disinfectant;
- Carry out the sampling by simultaneously collecting, by means of a single venipuncture, at least two portions of blood in separate vacuum tubes;
- Use vacuum tubes containing sodium fluoride as a preservative and an anticoagulant, such as potassium oxalate, equipped with anti-bleeding systems and safety labels;
- Proceed, after sample collection, to repeated inversion of the tubes to avoid separation of blood red cells or clotting.
- Proceed with the storage of the sample and counter sample according to the methods (times and temperatures) described above.

It should be noted that testing blood derivatives (plasma, serum) for ethyl alcohol produces an overestimation (12 - 18 % on average) compared to testing whole blood and is therefore not suitable - for example - with regard to the limits laid down in the Highway Code for alcoholaemia ; therefore, an alcohol test on plasma or serum can only have a clinical, diagnostic value.

Oral fluid collection

Sampling of the oral cavity fluid (saliva) can be performed with a commercial device authorized for sale nationwide, i.e. by collection of the fluid, without stimulation of salivation, in a special container. The division of the sample collection into sample and counter-sample can only be omitted if a blood sample is also taken at the same time as the saliva.

For the activities and measures that must be carried out after the sample has been taken (storage), please refer to the section on blood.

In addition, the following requirements apply to all sampling situations described in the previous paragraphs:

- The subject must be able to verify that the material needed for the collection is intact, new and sealed;
- All splitting, packaging and labelling of the sample and the counter sample must be carried out in the presence of the person concerned who countersigns the sampling form as well as the sample and counter-sample label;
- The correct protection of the sample from any adulteration, contamination or dispersion must be guaranteed by using suitable and perfectly sealed, tamper-proof or in any case sealable material, which is not likely to break in case of impact during transport, or due to thermal shock during freezing where this is necessary;
- Each analysis step relating to the sample must be reported on the appropriate forms.

Cadaveric sample collection

If samples are not taken at the laboratory's premises, the laboratory must require compliance with the procedures mentioned in Chapter 6.

In terms of the type of post-mortem samples to be taken, it is recommended that samples of peripheral blood, central blood, urine, vitreous humor, bile, muscle, keratin matrices be taken.

Reasons for exclusion and rejection of biological samples

In cases where the biological sample is taken outside the laboratory, it is possible to reject the submitted material if any of the following can be documented:

- Inconsistency, in qualitative or quantitative terms, of the biological sample with the specific analysis request;
- Incorrect storage of the sample during transport;
- Lack of or unverifiable (e.g. illegible) consistency between the sample identification data and the accompanying documentation;
- Evidence of tampering with the sample (e.g. removal or breakage of anti-tampering systems).
- In all cases of rejection, the laboratory director is required to complete a non-compliance report specifically mentioning the reasons for rejecting the sample.

Sample storage, handling and moving

The sample must be properly stored using all precautions and methods to preserve the sample from degradation and ensure the stability of the analytes.

These arrangements must ensure:

- The identification and suitability of the storage locations;
- Storage at the appropriate temperature depending on the type of sample, the storage period before analysis and the purpose of testing. As a general rule, if testing is conducted over limited periods of time (e.g. a few days) between sampling and analysis, liquid samples can be stored at +2/+8 °C, while they must be stored at -18/-22°C, if testing is conducted over longer periods of time (months/years);
- For samples to be stored at -18/-22 °C (blood, saliva and urine), different freezers must be provided for pre-analytical storage and subsequent storage; storage conditions for head hair or other hair must be such that the samples are protected from moisture and light;
- Compliance with the chain of custody;
- Storage of both positive and negative samples until the production of the analytical/reference report, unless otherwise indicated by specific regulations;
- Storage of counter-samples (e.g. aliquots of positive samples after a confirmatory analysis) for at least one year from the date of the analytical report/referral, unless otherwise provided for by specific regulations;
- For samples relating to specific legal proceedings, storage is extended until specific authorization for destruction or disposal by the Judicial Authority. In the event of a request for analysis after long storage times, appropriate cautionary warnings must be given regarding the significance of the results obtained, in relation to the possible instability of the analytes even if the sample is kept under the recommended conditions.

7. Analytical Methods

Overview

A Documented Procedure (Technical Activities) must be defined for all analysis methods used in the laboratory, detailing the information set out in Chapter 4.

Validation test results of the original analysis methods and subsequent revisions must be documented, archived and stored by the laboratory.

The laboratory may only issue reports having toxicological/forensic/ medico-legal value when: 1. analysis results obtained by the application of enzymatic or immunochemical screening are confirmed by a more specific method based on chemical/physical principles other than the above; 2) analysis results are obtained directly by specific methods other than enzymatic or immunochemical ones. Accordingly, the use of mass spectrometry (MS) in its many different methods, preferably in combination with a chromatographic (e.g. gas chromatography, GC; high-pressure liquid chromatography, LC) or electrophoretic (capillary electrophoresis, CE) separation technique, finds general acceptance in the national and international scientific community for the qualitative-quantitative analysis of NDPs.

In general, for each analytical batch, a negative control sample ('blank') and at least one positive reference sample in biological matrix must be processed in order to assess the absence of any interference and instrumental performance.

The analytical injection sequence must always include the following, in this order:

- Analysis of the "blank" sample
- Analysis of unknown samples (or samples to be confirmed)
- Analysis of the positive reference sample(s) and/or the calibration curve.

Screening methods

The use of a screening method is justified in a forensic toxicology laboratory when there is a need to analyze a large number of samples in a short time and at limited cost, with the advantages of high or total automation. Screening methods used for the analysis of biological samples usually employ enzymatic or immunochemical techniques, but chromatographic/mass spectrometric techniques can also be used.

However, immunochemical screening methods are characterized by low specificity (qualitative results) and high inaccuracy (quantitative results), particularly when the sample contains several chemical species that can be detected but not distinguished by the method (e.g. unmodified compound and its metabolites, various types of chemically similar compounds).

Due to their intrinsic characteristics, these methods only produce a presumptive result, i.e. the probable negativity (absence) or positivity (presence, better defined as 'non-negativity') of the sample in relation to an analyte, or more often to a class of substances, relative to a cut-off value predetermined by the method. In any case, whatever the analytical specificity of the screening method, the following statement applies:

A POSITIVE RESULT OBTAINED THROUGH THE EXCLUSIVE USE OF ENZYMATIC OR IMMUNOCHEMICAL SCREENING TECHNIQUES CANNOT HAVE FORENSIC VALUE. IT IS THEREFORE ESSENTIAL THAT SUCH A RESULT BE VERIFIED BY AN ANALYSIS USING A MORE SPECIFIC TECHNIQUE, PREFERABLY A MASS SPECTROMETRIC TECHNIQUE, IN COMBINATION WITH A CHROMATOGRAPHIC TECHNIQUE ON A NEW SAMPLE PORTION

Since the negative result of a screening is generally accepted as such, it is important to verify whether the screening method can minimize the number of false negative results. It must be ascertained (or documented by the manufacturer in the case of immunochemical reagents) that the method does not yield false negatives. In this respect, it is advisable to perform a confirmatory analysis even on a certain randomized percentage of samples found to be negative on screening.

The application of enzymatic or immunochemical screening using kits and calibrators directly supplied by the manufacturers is permitted. It should also be noted that the cut-off value is defined by the manufacturer of an analytical screening kit for a given matrix and may differ from the cut-off values established by specific agreements or regulations. Moreover, even in the field of forensic toxicology there are cases that may require different cut-off values in the use of immunochemical screening, sometimes lower than those suggested by the manufacturers.

It is therefore not correct to unconditionally adopt the manufacturer's suggested cut-off. If a different cut-off value than that suggested by the manufacturer must be identified, the screening method must in any case be revalidated in the laboratory using it, using properly prepared calibrators.

The result of a screening can only be expressed in the form of presumed positivity (presence) or negativity (absence) of an analyte or class of substances in the sample; it cannot be expressed in quantitative terms, being semi-quantitative.

Qualitative and quantitative confirmation methods

The confirmation step, intended as the unambiguous identification of specific analytes, must be able to produce an analytical result as independent as possible from that obtained in the screening phase, when performed by enzymatic or immunochemical techniques. This requires the use of confirmation techniques based on different chemical and/or physical principles from those of screening. Furthermore, the confirmation method must be characterized by higher analytical selectivity and sensitivity than the screening method. In this respect, a quantitative confirmation method capable of achieving a lower limit of quantification, LLOQ, of at least half the cut-off of the screening method is considered acceptable.

The use of a confirmation method that is based on an analytical principle similar or highly related to the screening method, when obtained by enzymatic or immunochemical techniques (e.g. confirmation of immunochemical data by another immunochemical method), is not acceptable. The use of an identical chromatographic technique to confirm a chromatographically obtained datum is acceptable if the detection technique combined with the chromatography is different.

The use of a chromatographic technique to confirm a screening data obtained chromatographically with the same detection system is permitted when the two separation techniques produce poorly correlated results (e.g. two sets of significantly different retention times, use of columns with different selectivity, etc.).

In forensic toxicology, chromatographic separation is however always necessary in a confirmation method.

As already mentioned, the use of MS in combination with a chromatographic (e.g. GC; LC) or electrophoretic (EC) separation technique for the qualitative-quantitative analysis of NPDs is generally accepted as the golden standard by the international and national scientific community.

Therefore:

THE GTFI RECOMMENDS THE USE OF MASS SPECTROMETRY, IN COMBINATION WITH A CHROMATOGRAPHIC TECHNIQUE, AS THE IDENTIFICATION TECHNIQUE OF CHOICE FOR CONFIRMATORY ANALYSIS OF BIOLOGICAL SAMPLES

If the screening method uses chromatographic techniques combined with mass spectrometry, and therefore has a high specificity, confirmation may be performed with a similar chromatographic technique combined with mass spectrometry, while making sure to maximize the information power of the acquisition method (see sections on minimum identification criteria) and the use of quality controls in the analytical sequence.

Use of internal standards

The use of one or more internal standards is strongly recommended for purely qualitative analytical applications, and is mandatory for quantitative analysis methods. Indeed, the internal standard ensures a high degree of control, both in relation to the extraction of the analytes of interest from the original matrix, and in relation to the chromatographic separation and detection system, such as mass spectrometry. Internal standards must be added to the sample and controls before any preparation process. The only exception to this rule is the analysis of hair for which the addition of internal standards must be carried out after washing, comminution (where appropriate) and weighing. When using mass spectrometric detection techniques, the GTFI encourages the use of deuterated internal standards (if possible with a deuterate number ≥3) upon verification that the amount/concentration of the deuterated standard is not such as to significantly interfere with the ionization efficiency (e.g. due to competition phenomena) or quantification (e.g. isotopic contributions) of the analyte. The stability of internal standards throughout treatment and sample analysis must be ascertained or verified in advance.

Minimum identification criteria

The choice of minimum identification criteria, and the respective tolerance ranges, may vary in relation to the instrumental analysis techniques employed by the laboratory, but must in any case comply with the indications of any reference standards, and in any case with what is generally accepted by the scientific community.

The minimum identification criteria for the most common instrumental analysis techniques are defined below:

Chromatographic analysis

- The analyte's relative retention time, as compared to the corresponding internal standard, must be within ± 1% (GC) or ± 2% (LC) of that produced by the corresponding analyte in the positive control.

MS scanning analysis (Full Scan, with electron impact or chemical ionization)

- Presence in the unknown spectrum of all ions of the spectrum of the reference compound (positive control or library spectrum) with intensity, relative to the base peak, ≥10%, including the molecular ion and ions of its isotopic cluster if ≥10%;

- The relative abundances of these ions in the unknown spectrum must be within a tolerance range (±20%) of the corresponding value obtained for the reference compound;

- The mass spectrum of the analyte of interest must contain at least 3 ions with an abundance ≥10%; otherwise, analysis by a second ionization method or derivatization procedure is required;

- The presence in the unknown spectrum of ionic fragments that are absent in the reference spectrum must be explainable by the partial co-elution of matrix components.

MS analysis using Selected Ion Monitoring (SIM)

- At least 3 ion fragments (where possible including the molecular ion or an adduct thereof, depending on the ionization technique used) must be monitored excluding isotopic and non-specific leakage ions. The ionic fragments must be representative of the entire molecular structure and, if possible, correspond to different portions of the molecule;

- If the mass spectrum of the analyte of interest does not contain ionic species with the described characteristics, the analysis must be performed using a second ionization method or a derivatization procedure.

Analysis by multiple mass spectrometry (tandem MS, MS-MS) in Product Ion Scan mode

- The precursor ion must be isolated with the smallest possible amplitude, compatible with the signal strength, in order to exclude interference;

- The same principles of MS analysis in Full Scan also apply.

MS-MS analysis in Selected Reaction Monitoring (SRM) mode

- At least 2 transitions from the precursor ion to the product ion must be acquired;

- The precursor ion of the two transitions can be common, as long as the fragments of the transitions are relatable to different portions of the molecule and, for at least one transition, it must be the molecular ion or an adduct thereof (depending on the ionization technique); product ions must not result from non-specific losses (e.g. loss of H_2O).

MS analysis by scanning, or by monitoring specific ions, in High-Resolution MS (HR-MS) mode

-Accurate mass measurements of protonated molecular ion species (MH+) must be performed under the best allowed conditions of instrumental resolution and accuracy when working in LC-HRMS mode and in full-scan mode; b) the comparison of experimental and calculated isotopic patterns of MH+ ionic species; c) possibly the examination of the fine structure of isotopic patterns of MH+ ionic species (discrimination of isotopic multiplets for M+1, M+2, M+3, and in some cases M+4 isotopic peaks).

- In the coupled-with-LC chromatography mode, and according to the different instrumental modes of monitoring specific ions, the monitoring of ion species chosen with the same criteria as described for low-resolution MS analysis must be carried out under the best allowed conditions of instrumental resolution and accuracy.

Exceptions to these minimum identification criteria must be justified by the physical, chemical and/or structural characteristics of the analytes of interest, or by the limitations of the instrumental analysis technique used. The reasons for deviations from the criteria listed above must be outlined in the Documented Procedure. In such cases, and in general, the identification of specific metabolites of an analyte in the sample and/or the results of other types of analysis can be used to support the identification of the analyte. It should also be noted that it is sometimes possible, for identification purposes, to modify the chromatographic and mass spectrometric behavior of NPDs through numerous derivatization reactions.

When using confirmation methods in mass spectrometry (the elective confirmation technique for GTFI), it is recommended to use the Identification Points (IP) system adopted by European Commission Decision 2002/657/EC in implementation of the Council of the European Union Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (European Union Official Journal L 221, 17.8.2002). If the confirmation method also has quantification objectives, it must meet the minimum quantification criteria outlined above in addition to the minimum identification criteria.

Evaluation of results against the cut-off

If the analysis method has the exclusive value of qualitative confirmation in relation to a predetermined cut-off value, the measurement uncertainty for this value must be known, highlighted and subtracted from the value actually measured, referring the positivity exclusively to those cases in which the measured value, minus the measurement error, is still above the cut-off.

The analysis of positive controls for concentrations close to the cut-off (e.g. cut-off ± 25%) allows the verification of the performance of the qualitative method in this critical area.

The result of a qualitative confirmatory analysis must only be expressed in the form of positivity (presence) or negativity (absence).

Quantitative analysis and minimum quantification criteria

Quantitative analysis involves the establishment of an appropriate calibration curve for each analyte to be quantified, in order to compare the signals obtained from the analysis of an unknown sample (e.g. areas of the chromatographic peaks of the analyte of interest and the corresponding internal standard) with the signals obtained from the analysis of a series of samples of known concentration.

The calibration curve should be such that it covers a variation of at least one order of magnitude of instrumental signal. As for the choice of internal standard and how to use it, the recommendations given in the section on qualitative analysis apply; please refer to it.

For a correct quantification it is necessary that the instrumental signal obtained from the analysis of the unknown sample is equal to or higher than the signal corresponding to the analytical Lower Limit of Quantification (LLOQ) determined during the method validation phase, and in any case within the analytical range of the calibration curve in use.

The recommendations made for qualitative analysis regarding prior verification of the absence of interferents apply. It is essential to have estimates of average recovery of active ingredient obtained on matrices similar or analogous to those for which the absolute amount of active ingredient must be estimated.

The quantitative evaluation of an adequate number of negative controls and positive controls is also required. These controls must have a concentration corresponding to that expected within a predetermined tolerance range declared in the Documented Procedure. If this criterion is not met, the complete calibration curve must be re-examined at the same time as the samples. Positive controls must be evenly distributed within the calibration range, including controls below the cut-off (where applicable) (e.g. -50% of cut-off) and controls at high concentrations (e.g. +200% of cut-off), or in any case compared to the midpoint of the calibration line.

The calibration curve should be set up with at least five non-zero calibrators.

The standards used for the preparation of positive controls and the quantification of specific analytes (and their internal standards) must be of certified composition and purity and valid. If not commercially available, the use of non-certified standards, e.g. of pharmaceutical grade, is allowed, provided the laboratory has verified and declared their validity in the Documented Procedure.

The results of quantitative analyses must be expressed in such a way as to exclude doubts of interpretation, with units of measurement directly comparable with any reference values and accepted by the International System of Units (SI). The results themselves must preferably be expressed indicating the uncertainty associated with the measurement performed and the comparison with threshold or reference values must take this uncertainty into account. The number of digits with which the result should be expressed is dictated by the magnitude of the uncertainty.

Forensic blood alcohol testing

In view of the information provided in Chapter 6 on blood sampling for blood alcohol testing for forensic toxicology purposes, the GTFI states

THE TECHNIQUE OF CHOICE FOR FORENSIC BLOOD ALCOHOL TESTING IS HEADSPACE GAS CHROMATOGRAPHY (HS-GC), COUPLED WITH FID (FLAME IONISATION DETECTOR) OR MS DETECTION

In this case, the respective method must be able to quantify in the calibration range of at least 0.05 to 3.0 grams/litre (g/L), with imprecision (CV%) and inaccuracy (E%) at the values of 0.05 - 0.1 - 0.5 - 0.8 - 1.5 g/L not exceeding 10%.

The LOD of the method must be less than 0.05 g/L. However, in order to establish the positivity for toxicological-forensic purposes of a blood sample (e.g. according to Art. 186a of the Italian Highway Code) it is suggested to set the decision threshold at 0.1 g/L, in relation to the existence of minimal physiological values of ethanol in the blood, the possible intake of ethanol through food and/or pharmaceutical products, and especially considering that impairment caused by alcohol start to be produced from alcohol levels of 0.1 - 0.2 g/L.

In addition, it is recommended that each laboratory should, similarly to the decision-making threshold of 0.1 g/L, define decision-making criteria to establish whether other specific legal limits are also exceeded (e.g. 0.5 - 0.8 - 1.5 g/L, with reference to Art. 186 of the Highway Code).

In conclusion, it is recommended that each laboratory should estimate the measurement uncertainties associated with the different blood alcohol levels determined, taking into account all the contributions of the different sources of variability affecting alcohol measurements. The estimation of the uncertainties is indeed decisive for the definition of the decision thresholds at each legal limit.

Finally, it should be noted that as far as blood alcohol testing is concerned, any tests performed on blood derivatives are not valid for forensic purposes.

Analytical method validation

Analytical method validation by the laboratory consists of obtaining confirmation, supported by objective evidence, that the requirements for a specific intended use or application have been met.

Analytical methods must be validated prior to their routine application, both for methods developed by the laboratory and for those taken, and applied in whole or in part, from the scientific literature. Validation is a continuous process: each modification of the analytical system requires a new validation phase.

The **validation of a qualitative method** must include at least the following parameters:

- Analytical selectivity/specificity;
- Limit of detectability (LOD);
- Analyte stability;
- Matrix effect evaluation;
- Absence of carry-over.

The **validation of a quantitative method** must include at least the following parameters:

- Analytical selectivity/specificity;
- Analyte stability;
- Linearity in the calibration range;
- Matrix effect evaluation;
- Absence of carry-over;
- Limit of detectability (LOD);
- Lowest and upper limits of quantification (LLOQ and ULOQ);
- Applicability of sample dilution (dilution integrity);
- Precision (at least intra-laboratory repeatability);
- Accuracy;
- Recovery;
- Robustness;
- Uncertainty of measurement;

The choice of parameters that must be included in validation tests must also take into account the frequency of use of an analysis method.

Cut-off and minimum performance requirements

Reiterating the entirely conventional nature of the cut-off value (or Threshold Value or Decision Threshold) for establishing the negativity or positivity of a sample, and also reiterating that it does not necessarily coincide with the values of LLOQ or LOD, the GTFI believes it is necessary to introduce the concept of "Minimum Performance Requirements". Namely, the concentrations of the analytes in the biological fluid under investigation that the laboratory must be able to quantify, with accuracy, and which are suitable for assessing the applicability of a method in relation to a specific forensic-toxicological analytical purpose, where there are no specific regulatory requirements.

TABLE A1 shows the minimum requirements adopted by the GTFI, for the quantitative testing of blood and urine samples for the most frequent classes of NPDs.

The laboratory that intends to carry out quali-quantitative analytical testing for forensic toxicology and medico-legal purposes must be able to ensure the correct quantification of at least the indicated concentrations, commonly obtainable using chromatographic techniques combined with mass spectrometry.

It should be noted that these values are not interpretive cut-offs.

Given the wide variety of screening techniques and methods that can be adopted for different matrices and characterized by often very different performances, it is not appropriate to indicate screening cut-offs, whose values the laboratory will choose according to the performance of the techniques or confirmation methods adopted.

When performing analyses according to specific legal standards or regulations, the decision cut-offs indicated in the standard/regulation must be applied for the evaluation of the quantitative result as positive or negative.

For controls on workers assigned to tasks that expose third parties to health and safety risks, pursuant to art. 41, paragraph 4 of Italian Legislative Decree no. 81/2008, the screening and confirmation cut-offs on urine and hair of the protocol relating to the Provision of the State-Regions Conference of 18 September 2008 (published in the Italian Official Gazette no. 236 of 8 October 2008), which establishes the methods and procedures for controls, shall be used.

For head hair, the concentration values (interpretative cut-offs) adopted in the consensus document, of the **Society of Hair Testing** (SoHT, 2021 revision,) for the identification of the use of the most common classes of NPDs are reported in **TABLE A2** for reference.

In this context, GTFI considers the use of immunochemical screening techniques for the detection of NPDs in hair acceptable but the relevant methods adopted must be validated in the laboratory with special focus on sensitivity (evaluation of true negatives and false negatives).

For oral fluid (saliva), the screening and confirmation cut-offs used by the **European Workplace Drug Testing Society** (EWDTS) are listed in TABLE A3 for reference purposes.

The values listed in Tables A2 and A3 do not, as they stand, have any legal value and allow discrimination between negativity or positivity of a sample with respect to certain substances (and/or their metabolites) according to the interpretation provided by the relevant scientific society. The use of different decision values, after rigorous forensic toxicological evaluation, is possible for specific contexts and analytical purposes (e.g. in cases of investigation on drug facilitated crimes or tests for child abuse/neglect).

8. Analysis Report

The analysis report must be produced in paper format and delivered - unless otherwise provided for by law - to the person requesting the test or to a person holding a written power of attorney issued by the applicant. Additional electronic delivery of the analysis report is allowed (subject to the recipient's written consent, indicated in the analysis request form) if the Laboratory implements a Documented Procedure sufficient to guarantee that the information contained therein cannot be accessed by persons other than the recipient and, in any case, in compliance with the applicable regulations on confidentiality of personal and sensitive data. This procedure must be detailed in the Documented Procedures.

The analysis report must contain at least the following elements:

- Title;
- Identification data of the laboratory;
- Identification number of the analysis report (e.g. sequential number). If the report consists of several pages, they must be numbered consecutively, indicating the total number of pages;
- Identification data of the applicant;
- Identification data of the subject (i.e. anonymous alphanumeric code, if required) from which the samples were taken for analysis;
- Type and purpose of the requested analysis;
- Date and time of sample collection (if known to the laboratory);
- Date of sample acceptance;
- Report date;
- Description of the type of samples (with details of any abnormalities);
- Indication of the types of analyses performed;
- Analytical techniques used;
- Qualitative-quantitative analytical results with relative units and limit of quantification, cut-off, measurement uncertainty, when applicable;
- Legend indicating the meaning of unusual abbreviations or terminologies;
- Interpretation of analytical results, including assessment of the limits of usability of the result, when necessary, within the limits of the information available to the referring Director;
- Name and signature of the Laboratory Director (and optionally the analyst).

9. Quality Assurance

To ensure the accuracy, validity, and usability in forensic toxicology and medico-legal matters of the analyses and their results, the laboratory must adopt an organized system of internal control of facilities, qualification and training of personnel, equipment, methods and analysis procedures. It is advisable to appoint a so-called Quality Manager, who should be entrusted with the organizational tasks of monitoring and improving the laboratory's quality requirements.

Management of the laboratory must be aimed at ensuring that quality requirements are met, i.e. it must provide objective evidence of quality assurance.

Quality assurance takes on a particular role in the analytical activities referred to in these Guidelines, since they are tests for forensic toxicology and medico-legal purposes that comply with regulatory requirements; moreover, the results of such tests may often also serve as legal evidence. Mechanisms must therefore be put in place to identify possible errors and apply consequent remedies.

Quality assurance involves all the processes that take place within the laboratory, from the collection and acceptance of biological samples, to the performance of analyses, the validation of results and their reporting.

Internal and External Quality Control

Internal Quality Control involves a critical and continuous evaluation of all laboratory processes. Indeed, the control must include all stages of the analytical protocol, from sample acceptance to the issue of the analytical report.

The laboratory must have control procedures to monitor the validity of the processes performed. The resulting data must be recorded in such a way as to detect trends and allow statistical analysis for review.

Internal Quality Control must cover at least the following phases:

- Sample acceptance (e.g. type and quantity of samples);
- Storage and security of samples and counter samples (access to storage devices, sample identification systems, temperature monitoring, etc.);
- Analytical instrumentation (functional verification, calibration, routine and extraordinary maintenance);
- Analysis (use of positive and negative control samples to be analyzed together with real samples and control charts);
- Reporting (completeness of compilation).

The use of charts and checklists are important tools of internal quality control for monitoring analytical performance. The laboratory may use them to estimate the measurement uncertainty associated with the results of the analyses performed.

The laboratory carrying out qualitative-quantitative NPD analytical testing should also participate in one or more inter-laboratory External Quality Control schemes (when available, and at least for routine analytical procedures), whereby the results produced by the laboratory are compared with those of other laboratories participating in the same scheme. Usually this is achieved through the supply of homogeneous and stable samples by a third party that collects and statistically analyses the results yielded by the participating laboratories. The main objective of an inter-laboratory scheme is the self-assessment of the quality of the analytical measurements performed and the opportunity to discover variability contributions that would not otherwise be considered.

10. References

[1] UNI EN ISO 9000:2015 standard - Quality management systems - Fundamentals and vocabulary; Requirements.

[2] UNI EN ISO 15189:2013 standard - Medical laboratories - Quality and competence requirements.

[3] UNI CEI EN ISO/IEC 17025:2018 standard - General requirements for the competence of testing and calibration laboratories.

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[5] Procedures for testing employees working in jobs entailing serious risks to the safety, security and health of third parties for drug addiction or use of narcotic or psychotropic drugs pursuant to Order no. 99/cu 30 10 2007. G.U. no. 236 October, 8th 2008.

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APPENDIX

Table A1. Concentrations defining Minimum Performance Requirements for the quantitative analysis of NPDs in blood and urine for forensic toxicology purposes, using chromatographic techniques combined with mass spectrometry

Class of substances or substances	Minimum required performance concentrations (ng/mL)
Opioids	
morphine	*2*
codeine	*2*
6-acetylmorphine	*2*
Cocaine	
cocaine	*2*
benzoylecgonine	*2*
cocaethylene	*2*
norcocaine	*2*
Amphetamine and congeners	
amphetamine	*2*
methamphetamine	*2*
3,4-Methylenedioxymethamphetamine and congeners	
MDMA	*2*
MDA	*2*
MDEA	*2*
MBDB	*2*
Methadone	
methadone	*2*
EDDP	*2*
Cannabinoids	
THC	*1*
11-OH-THC	*0.1*
THC-COOH	*2*
Buprenorphine	
buprenorphine	*2*
norbuprenorphine	*2*

Table A2. Concentration values (interpretive cut-offs) adopted by the Society of Hair Testing (SoHT) to identify the use of different classes of NPDs in head hair samples (3 cm proximal) (from 2021 SoHT Consensus on Drugs of Abuse – DOA - Testing in Hair)

Class of substances or substances	Concentrations (pg/mg) related to interpretive cut-offs
Opioids[a]	
morphine	*200*
codeine	*200*
dihydrocodeine	*200*
6-acetylmorphine	*200*
heroin	*200*
Cocaine[b, c]	
cocaine	*500**
benzoylecgonine	
ecgonine methyl ester	
cocaethylene	
norcocaine	
OH-cocaine	
OH-benzoylecgonine	
Amphetamines and congeners	
amphetamine	*200*
methamphetamine	*200*
MDMA	*200*
MDA	*200*
MDEA	*200*

Table A2 *continued*. Concentration values (interpretive cut-offs) adopted by the Society of Hair Testing (SoHT) to identify the use of different classes of NPDs in head hair samples (3 cm proximal) (from 2021 SoHT Consensus on Drugs of Abuse – DOA - Testing in Hair)

Class of substances or substances	Concentrations (pg/mg) related to interpretive cut-offs
Cannabinoids[d]	
THC	*50***
CBD	*50*
Opioids	
Tramadol[e]	*200*
oxycodone	*100*
Methadone[f]	
methadone	*200*
Buprenorphine[g]	
buprenorphine	*10*
Ketamine[h]	
ketamine	*200*

* Values used pursuant to Law 81/2008, 200 pg/mg of Cocaine and 5 pg/mg of Benzoylecgonine

** Value pursuant to Law 81/2008, 100 pg/mg of cannabinoid metabolites

[a] The use of heroin must be differentiated from that of codeine or morphine by identification of heroin or 6-MAM

[b] The presence of one or more metabolites may be ascertained to confirm use of the substance

[c] For the use of base (crack) cocaine, the presence of anhydroecgonine methyl ester must be considered

[d] Detection of THC-COOH (with LOQ 0.2 pg/mg) strongly supports the use of THC

[e] Confirmation of desmethyltramadol proves the use of tramadol

[f] Confirmation of EDDP proves the use of methadone

[g] Confirmation of norbuprenorphine proves the use of buprenorphine

[h] Confirmation of norketamine proves the use of ketamine

TABLE A3. Recommended concentration values (maximum screening and confirmation cut-offs) for oral fluid analysis in employee testing according to the EWTDS guidelines (from European Guidelines for Workplace in Oral Fluid 2015-11-01 Version 2.0).

Class of substances or substances	Screening cut-off (ng/mL)	Confirmation cut-off (ng/mL)
Opioids		
morphine	*Opiates (morphine) 40* *Opiates (6-MAM) 4*	*15*
codeine		*15*
norcodeine		*2*
6-acetylcodeine		*2*
dihydrocodeine		*15*
6- monoacetylmorphine		*2*
Cocaine and metabolites		
cocaine	*Cocaine + metabolites 30*	*8*
benzoylecgonine		*8*
Amphetamine and congeners		
amphetamine	*Amphetamine class 40*	*15*
methamphetamine		*15*
MDMA		*15*
MDA		*15*
Cannabinoids		
THC	*THC 10*	*2*
Methadone and metabolites	*L-Methadone 50*	*20*
Buprenorphine and metabolites	*5*	*1*

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