Assessment of prenatal exposure to mercury: standard operating procedures

Abstract

Mercury is toxic for humans, and the toxic effects of different forms of mercury have been extensively studied. Human biomonitoring is recognized as the most effective tool for evaluation of cumulative human exposure to mercury. In-utero development is the most vulnerable stage for the long-term adverse neurodevelopmental effects of mercury. Characterizing prenatal exposure is critical for evaluating public health impacts of mercury and assessing public health benefits of exposure reduction measures. Approaches to estimating exposure to mercury include measuring mercury levels in different biological matrices. The level of mercury in tissues can be an indicator of exposure to various types of mercury. The validity, usefulness and meaning of such measurements depend on the form of mercury exposure, type of tissue measurement and other factors. This document consists of standard operating procedures describing the assessment of mercury in hair, cord blood and urine. Quality control is essential to get reliable results. The document also provides information on alternative methods that can be used for analysis of mercury.

Keywords

Biomarkers – analysis
Mercury – analysis
Prenatal Exposure Delayed Effects – analysis
Maternal Exposure – adverse effects
Environmental Exposure

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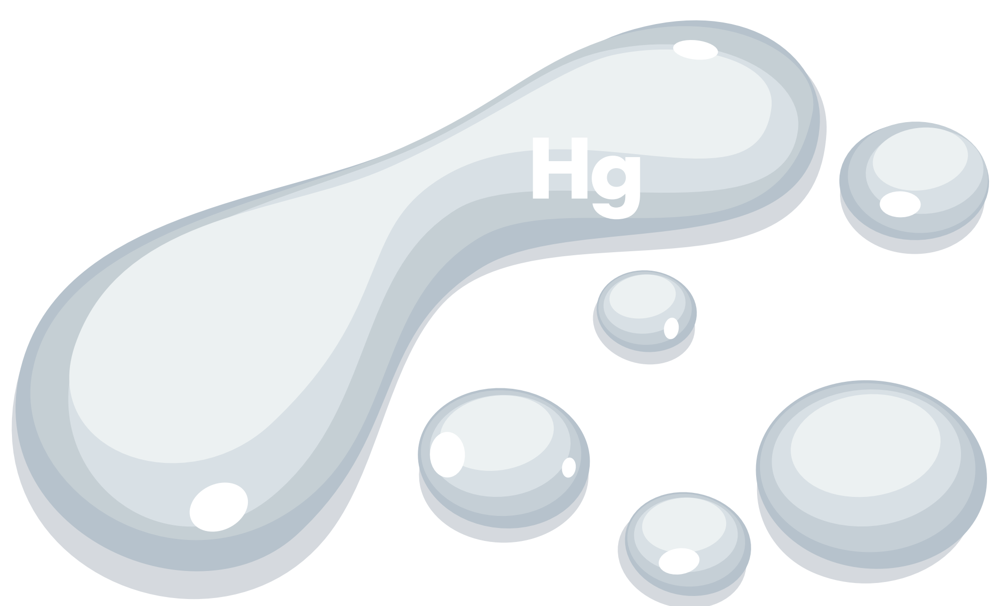
Acknowledgments

The Standard Operating Procedures for assessment of prenatal exposure to mercury were developed in the framework of the project "Development of a Plan for Global Monitoring of Human Exposure and Environmental Concentrations of Mercury" funded by the Global Environment Facility.

The WHO Regional Office for Europe gratefully acknowledges technical support provided by the UN Environment at all stages of the project, from the project planning, through coordination among the project components at the implementation stage, to the organization of the final discussions on the documents developed in the frame of the project.



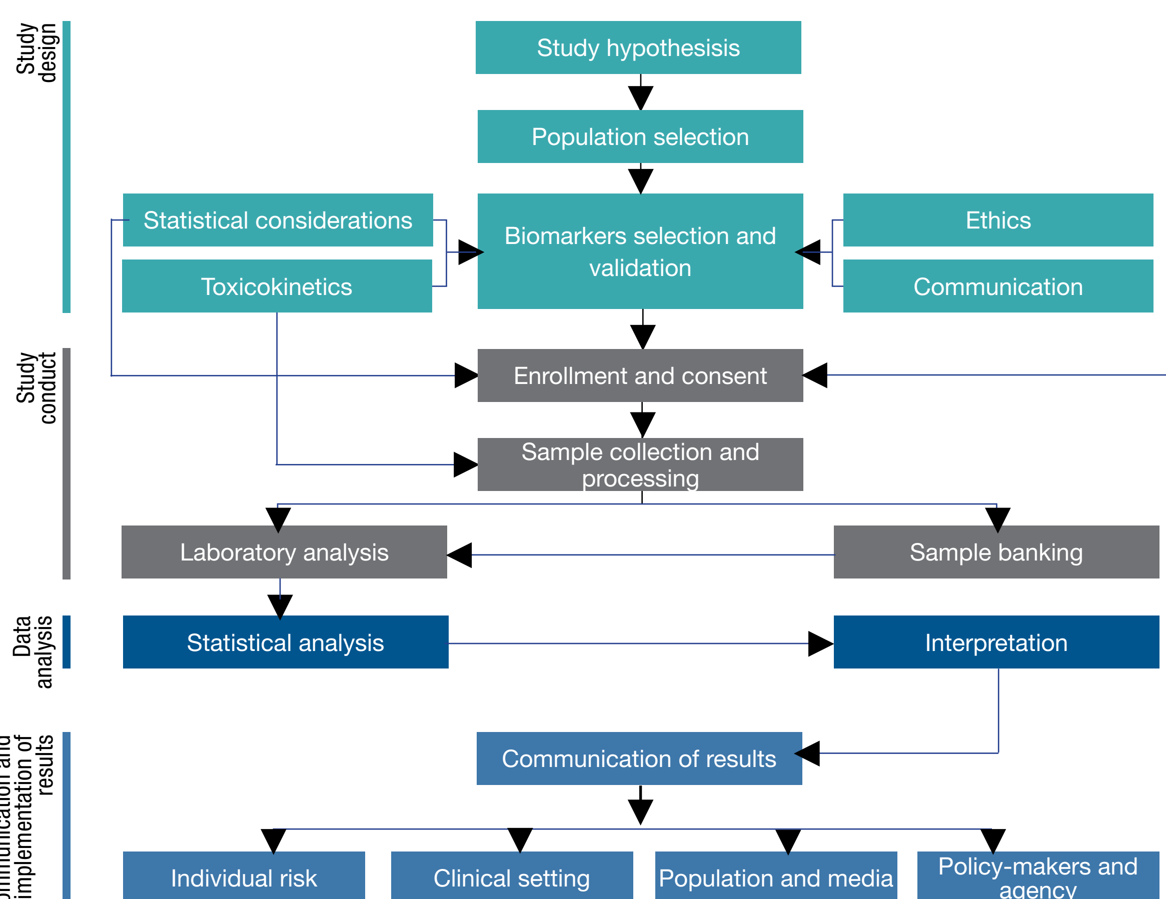
Introduction

Although human biomonitoring (HBM) has been widely employed in the framework of occupational exposure, it has only recently been used to assess the exposure of the general population to environmental pollutants. The extension of HBM to this field of application over the past few years has been boosted by, among others, different initiatives focused on increasing our understanding of the relationship between the environment and health.

The potential of HBM in the field of public health is an accepted fact, although the lack of harmonization between the different HBM studies/programmes can considerably limit the comparison of results, their global interpretation and subsequent translation into policy. It is, therefore, fundamentally important to develop a harmonized framework that allows the most efficient use of data obtained in HBM studies, such as in the European Union-supported projects Development of a coherent approach to human biomonitoring in Europe (ESBIO), Consortium to Perform Human Biomonitoring on a European Scale (COPHES) and its twin feasibility study DEMOCOPHES.

The organization of an HBM survey is a complex process involving professionals with different technical skills (epidemiologists, analytical chemists, toxicologists, statisticians, physicians and communications specialists), all of whom contribute to specific stages of the study. They work together to deal with the interactions between the various disciplines concerned (Fig. 1).

Fig 1. Stages of a biomonitoring study



Source: National Research Council of the National Academies *(1)*.

Quality control programme for mercury human biomonitoring

Abstract

The objective of the document is to define an effective system for performing quality-control activities to ensure the reliability of mercury human biomonitoring (HBM) results. These activities are focused on the pre-analytical and analytical stages of the mercury HBM. The measures described should be seen as a general recommendation for use when planning and implementing HBM surveys at national, regional and international level. The document should be considered for use together with relevant standard operating procedures for sampling and analysis of mercury in human scalp hair, cord blood and urine.

Keywords

Mercury – analysis
Methylmercury compounds – analysis
Biomarkers - analysis
Maternal exposure
Maternal-fetal exchange
Infant, newborn
Environmental exposure
Quality control
Public health

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Abbreviations

HBM	human biomonitoring
ID	identification

Introduction

Commonly, quality control measures tend to focus on the analytical phase. All laboratories employ measures such as blanks, calibration curves and control samples during analysis in order to guarantee the reliability of their results. Control measures are, however, often missing in other stages of an HBM study that may be equally, or even more, important from a quality control standpoint.

It is important to note that all the precautions and control measures taken during chemical analysis are useless if the samples have been contaminated or altered during sampling, transport or processing. In view of this, HBM should not start in the laboratory; quality control measures must cover all steps in the pre-analytical phase, especially sampling (materials, vessels, procedures and documentation), transport (temperature, shipping requirements), sample pretreatment (centrifugation, extraction), aliquoting process and storage. Further down the line, this control should be extended to chemical analyses and subsequently to the data analysis process, by applying quality control to the databases generated.

1. Quality control at the pre-analytical phase

The pre-analytical phase has important implications for sample integrity in all studies involving biological specimens. Generally speaking, two kinds of factor can alter the sample before it is analysed.

- Influencing factors appear before the sample is collected and are specific for each biomarker. Examples of influencing factors that can modify the biomarker concentration include the biological half-life of a chemical, alcohol consumption, medication intake or individual habits such as diet. These issues should, therefore, be taken into account during the design of a study when describing the study population, statistical considerations, sampling strategies, recruitment or biomarker/matrix selection as well as during interpretation of the results.
- In the case of interfering factors, the concentration of the biomarker is modified after sampling due, for example, to external contamination, physical or chemical changes in the biomarker during transport or storage, or changes in the biological matrix such as coagulation or sedimentation. Several precautions can be taken to avoid these alterations and potential contamination during sampling and the transport, processing and storage of samples. Additionally, appropriate training of the fieldworkers is highly beneficial in this respect.

In HBM studies involving the general, presumed non-exposed population, control of the pre-analytical phase is even more important than in other kinds of study that involve biological samples due to the characteristics of such HBM studies, especially the types of substance analysed and the concentration ranges, which are usually measured in HBM. Thus, when measuring an environmental chemical there is a risk of sample contamination due to the presence of this chemical in the environment. This is particularly important in the case of ubiquitous chemicals that may even be present in the sampling material *(2)*. Additionally, as exposure to environmental chemicals occurs

at low concentrations, their levels in biological matrices also tend to be low, so that the influence of potential contamination on the results is high.

It is, therefore, essential to identify and avoid possible sources of contamination, such as:

- exogenous contamination at the sampling location;
- contamination from the sampling equipment or vessels;
- contamination due to absorption of the components to be analysed into the walls of the vessel employed.

The influencing factors for the target biomarker must be identified and a sampling strategy designed to take them into account. Finally, the information required to ensure correct interpretation of the results must be recorded.

Although various tools can be employed to achieve good quality control, standard operating procedures (SOP) tend to be the most useful. An SOP is a clear, concise, comprehensive and detailed step-by-step written description of a sampling or recruitment procedure or analytical method.

SOPs can be applied at all stages of a study to provide the basic information for quality control. Their use helps different laboratories/research teams to obtain comparable results. In view of the above, SOPs for the selection of participants and the recruitment protocol should be developed together with those for sampling and the transport, processing and storage of samples in order to control, as far as posible, all the factors that can affect the sample during the pre-analytical phase.

Other control measures include the use of field blanks during fieldwork or the collection of replicate samples. Different kinds of blank can be used during fieldwork to assess potential contamination of a sample during sampling or transport until its arrival at the laboratory. An empty vessel or tube (from the same batch as the rest of the material) can be considered a collection blank. These blanks are especially useful when prescreening of the material has not been carried out and for identifying environmental contamination. Blanks can also be prepared during the aliquoting process. Blanks should be treated and manipulated as though they were real samples in order to evaluate potential contamination during the real process.

It is also essential to ensure that the sample collected is representative and reflects the composition of the original. Thus, during the aliquoting process, all samples must be homogenized before being divided. Checklists containing the necessary materials or important points to be checked are also good control tools.

Special attention must be paid to the sampling and storage materials as different kinds of interference have been described between the materials from which the vessels or tubes where samples are collected were made and the target chemical. For example, glass must be avoided when metals are analysed (3). Likewise, some types of plastic can increase biomarker concentration values, for example, in the case of bisphenol A or phthalates *(2)*.

Control of the sampling and storage materials is crucial in HBM because, as noted above, the concentrations measured are usually in the range of parts per million or parts per billion (or even lower), meaning that a minimal background contamination can have important consequences for the final results. In order to control this potential source of errors, the following approaches can be designed to control the sampling material.

- Prescreening of the sampling material, consisting of screening a batch of collection tubes or vessels prior to sampling, should be undertaken to ensure that the background contamination is negligible (< limit of detection) and will not contribute to the final measurement. This precaution should be extended to the storage material.

- In some situations, the sampling and storage materials can be precleaned to eliminate any potential background contamination. For example, vessels employed to collect urine for metals analysis can be washed with a dilute nitric acid solution to eliminate any traces of metal from them. The effect of such a pretreatment should be checked by analysing 5% of the pretreated material.
- Materials certified to contain less than a minimum concentration of the target biomarker can be used. Some commercial materials are provided with certificates indicating the absence or minimum content of a specific chemical. For example, special tubes are available for trace metals analysis in blood samples.

The sampling time is a highly critical point during the pre-analytical phase (4). Correct sample collection requires an SOP containing detailed step-by-step instructions. Likewise, a written record of every event that occurs during sampling and all sample-related parameters (date and time of collection, volume, length and colour) are other useful quality control measures. Such steps can help to identify, for example, cross-contamination of a sample (for example, a urine sample contaminated with blood due to maceration caused by delivery). Additionally, well-documented fieldwork facilitates communication and helps to avoid misunderstandings and errors in the fieldworkers' team and between fieldworkers and laboratory staff.

Finally, sample traceability must be guaranteed, necessitating unambiguous identification of the specimens and related documents (questionnaires, personal data).

The quality of the labels used should, therefore, be checked to ensure that the identification (ID) code remains legible irrespective of temperature and humidity and, of course, that the label remains stuck to the tube, vessel or document.

After sampling, the samples collected should be transported under the conditions required to maintain their integrity. This is another critical control point. Transport to the laboratory must be done in compliance with the shipping regulations for biological materials.

The final step in the pre-analytical phase is sample storage and biobanking (if such is planned), although a previous step, namely reception of the samples and the acceptance/rejection criteria, should not be overlooked. Although these aspects are sometimes forgotten, they are crucial control points.

When samples arrive at the laboratory, the integrity of the packaging and the conditions of the sample tubes and vessels should be checked. Any problem encountered, such as broken or damaged packaging or a spilled sample, must be documented. To ensure that this is carried out correctly, it is advisable to establish a sample reception protocol that specifies the items to be checked and allows problems to be recorded on a sample registration sheet (Annexes 1 and 2).

The requirements for sample transport should be defined beforehand so as to establish the critical points to be checked. Checking should be performed from the outside in, that is, first verifying the state of the packaging and then opening it and continuing the process. If the biological specimens are accompanied by questionnaires or other documents, these should also be checked during the reception of the sample, when the previously defined sample acceptance/rejection criteria should be applied.

Although SOPs are essential support tools, they are not a comprehensive solution and should be complemented by trained laboratory staff and fieldworkers.

2. Quality control at an analytical phase

From an analytical point of view, it is essential to establish a quality assurance/quality control programme to ensure the reliability and comparability of results. Such programmes should cover both the basic quality assurance/quality control measures routinely applied in analytical laboratories as well as external action to ensure the comparability and quality of the results.

Internal quality controls are a basic tool in analytical laboratories as it is essential to harmonize control activities as well as the SOPs used to obtain laboratory results. Quality control activities must, therefore, be one of the fundamental points described in the working procedure, and tolerance criteria must be well-established before any assays are performed.

Refinement of the method must take into account that blank controls, repeatability controls, reproducibility controls or veracity are parameters that must be considered when evaluating the performance of the method. No external results should be reported in the absence of correct results from internal quality controls associated with the analysis and confirmation from the laboratory of compliance with these requirements.

The present procedure is concerned with the performance of quality controls associated with instrumental methods, typically methods based on the preparation of working curves onto which the results of test samples can be interpolated.

Interlaboratory comparison can be seen as a measure of the capacity of a laboratory. In order to obtain enough information about the performance of a laboratory, at least three rounds must be considered: before, during and after analysis of the study samples. In this way, the accuracy of the results of the participants can be evaluated and the validity of the study ensured.

Participation in each of these rounds must be evaluated according to the defined criteria. Unsatisfactory results in some of the rounds must be investigated and the possible causes of malfunction must be eliminated and corrected. In this respect, interlaboratory exercises can be used to demonstrate the adequate performance of laboratories in comparison with others.

2.1. Internal quality controls

2.1.1. Standards

Internal quality controls should be performed using certificated standards, when these are available. Such standards must be certified confirming their traceability to international standards. In addition, they must have an associated uncertainty in order to evaluate the confidence intervals and allow the laboratory to determine the accuracy of its results.

Any manipulation of these standards (such as dilution in order to obtain lower concentrations of the nominal value) means that the laboratory must calculate the new uncertainty based on the initial uncertainty of the standard and all contributions associated with the volumetric equipment used during preparation. If the analytical method has been suitably validated, these uncertainties will have been considered and consequently included in the defined tolerances of the validation.

2.1.2. Equipment

Equipment that may affect the result of the assay must be calibrated. In this regard, laboratories should have previous predefined tolerances that can be used to accept or reject the results of these calibrations.

Volumetric equipment must achieve the tolerance established for its class although, as a general rule, only class A volumetric material should be used.

Precision balances must be used to weigh standards or samples, when required. For example, in the case of hair analysis, no less than 30 mg of the sample or standard should be weighed if the balance has a resolution of 0.1 mg (four decimal point balance). If the laboratory has a five decimal point balance, it should be used to weigh no less than 3 mg. Weight measurements below these values introduce errors that could affect the uncertainty in the final result or increase the error in the analysis.

2.1.3. Sample conservation

Sample conservation is critical in obtaining valid results. The laboratory must have written procedures to prevent sample degradation or contamination. Storage conditions (temperature, luminosity, air- and water-tightness, humidity and storage time) should be defined.

Urine or blood samples must be stored refrigerated (<5 °C) in the dark, in an air- and water-tight container, prior to analysis. Hair samples can be stored at room temperature but must be kept away from moisture.

Laboratories must ensure the impossibility of sample contamination. Sample manipulation must be carried out in clean areas. Blank determinations, which should be treated the same as samples, can give a good indication of the cleanliness of the process.

2.1.4. Preparation of calibration curves

The laboratory must prepare a calibration curve at least every three months and use it until the next one is prepared. Curves with a minimum of five points must be prepared. The range of the curve should cover the expected values for all samples, or at least the vast majority of them.

If the laboratory has a method validation in which parameters associated with the curve have been obtained, with tolerances for these parameters, the working calibration curve must comply with the acceptance criteria obtained.

If no method validation is available, the laboratory must establish beforehand acceptance criteria for at least two of the parameters regression coefficient, linearity coefficient and slope.

Regression coefficient

The regression coefficient *(r)* is a way of determining the proportion of the total variability of a dependent variable (y) in relation to its average that is explained by the regression model. This parameter is a good measure of the goodness-of-fit of the regression curve.

The regression coefficient is considered to be adequate if it is higher than that indicated in Table 1 for the permitted confidence level and with the corresponding degrees of freedom. Usually, a 95% confidence interval is accepted as being appropriate. This interval corresponds to the column with the value 0.05. N is the number of points that have been used to construct the curve.

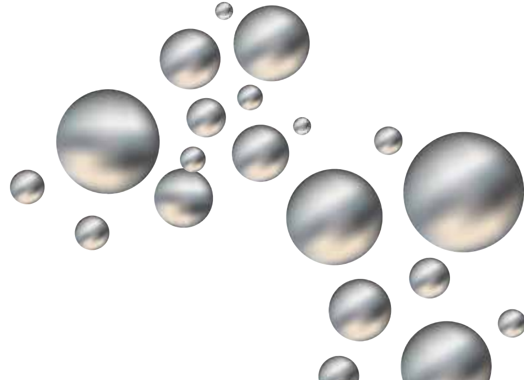


Table 1. Critical values for Pearson's r in a unilateral test according to the degrees of freedom (N-2)

N-2	0.05	0.025	0.01	0.005
1	0.988	0.997	0.9995	0.9999
2	0.900	0.950	0.980	0.990
3	0.805	0.878	0.934	0.959
4	0.729	0.811	0.882	0.917
5	0.669	0.754	0.833	0.874
6	0.622	0.707	0.789	0.834
7	0.582	0.666	0.750	0.798
8	0.549	0.632	0.716	0.765
9	0.521	0.602	0.685	0.735
10	0.497	0.576	0.658	0.708
11	0.476	0.553	0.634	0.684
12	0.458	0.532	0.612	0.661
13	0.441	0.514	0.592	0.641
14	0.426	0.497	0.574	0.623
15	0.412	0.482	0.558	0.606
16	0.400	0.468	0.542	0.590
17	0.389	0.456	0.528	0.575
18	0.378	0.444	0.516	0.561
19	0.369	0.433	0.503	0.549
20	0.360	0.423	0.492	0.537
21	0.352	0.413	0.482	0.526
22	0.344	0.404	0.472	0.515
23	0.337	0.396	0.462	0.505
24	0.330	0.388	0.453	0.496
25	0.323	0.381	0.445	0.487
26	0.317	0.374	0.437	0.479
27	0.311	0.367	0.430	0.471
28	0.306	0.361	0.423	0.463
29	0.301	0.355	0.416	0.456
30	0.296	0.349	0.409	0.449
35	0.275	0.325	0.381	0.418
40	0.257	0.304	0.358	0.393
45	0.243	0.288	0.338	0.372
50	0.231	0.273	0.322	0.354
60	0.211	0.250	0.295	0.325
70	0.195	0.232	0.274	0.302
80	0.183	0.217	0.256	0.283
90	0.173	0.205	0.242	0.267
100	0.164	0.195	0.230	0.254

Source: Valencia University *(5).*

Linearity coefficient

The linearity coefficient *(Cm)* is a measure of the goodness-of-fit compared with a straight line

$$Cm = \left(1 - \frac{Sm}{m}\right)100$$

where

Sm – the deviation of the slope and m is the slope.

- If historic values for the linearity coefficient are available, the acceptance criteria should be established as the mean of the Cm values $\overline{Cm}$ for the series of curves available minus $t_{Student}$ times the standard deviation for these Cm values $SD_{\overline{Cm}}$ (lower limit) and 100 (upper limit) ($t_{Student}$ obtained from the number of values used).

$$\overline{Cm} - (t_{Student}\ x\ SD_{\overline{Cm}}) \leq Cm_{current} \leq 100$$

- If no historic values are available, chromatographic methods must exceed a Cm value of 0.97 to be acceptable. Non-chromatographic methods must exceed a Cm value of 0.95.

Slope

The slope is the tangent of the angle of the straight line with the X axis and is a way of evaluating the sensitivity of the response obtained.

- If historic values for the slope are available, the acceptance criteria should be established as the mean of the slopes for the series of curves available minus $t_{Student}$ times the standard deviation for these slopes (lower limit) and the mean of the slopes for the series of curves available plus $t_{Student}$ times the standard deviation for these slopes (upper limit) ($t_{Student}$ obtained from the number of values used).

$$\overline{Slope} - \left(t_{Student}\ x\ SD_{\overline{Slope}}\right) \leq Slope_{current} \leq \overline{Slope} + \left(t_{Student}\ x\ SD_{\overline{Slope}}\right)$$

Quality controls for points on the calibration curve

A calibration curve can be used for a period of three months instead of a daily working curve. For daily measurements during this period, at least two points on the curve must be checked (one in the low range and the other in the high range) prior to starting the working series.

- If a method validation is available, the result obtained for these curve controls must lie between the acceptable values derived upon validation of the point concerned.
- If a slope history is available but there is no method validation, the result obtained must lie in the range

$$X_{point} \pm (t_{Student}\ x\ SD_{point})$$

where

X_{point} – the mean value obtained upon reading the point

SD_{point} – the standard deviation for the values obtained upon reading the point

$t_{Student}$ – obtained from the number of values used to obtain SD_{point}.

- If an estimation of the method reproducibility is available but there is no slope history, the result obtained must lie in the range

$$V_{point} \pm (t_{Student} \times SD_{repro})$$

where

V_{point} – the value obtained for the control point on the curve

S_{Drepro} – the estimated standard deviation in reproducibility

$t_{Student}$ – obtained from the number of values used to obtain SD_{repro}.

2.1.5. Analysis of test blanks

An *initial test* blank must be analysed prior to commencement of the daily series of assays.

If a method validation is available, the result obtained for the blank must be lower than the values obtained for the limit of detection for the method estimated during validation.

If historic blank readings are available but there is no method validation, the acceptance criterion for the blank is that the signal obtained must not exceed the mean for the series of blanks by more than three times the standard deviation for these values.

If a series of measurements for samples with a very low concentration of the analyte of interest is available but there are no historic blank readings, the acceptance criterion for the blank is that the signal obtained must not exceed three times the standard deviation obtained for these samples of very low concentration.

If the first initial blank measurement does not meet the acceptance criteria, the system must be cleaned. A new test blank must be measured after this cleaning. This process must be repeated until an acceptable value is obtained. Once such an acceptable value has been obtained, a second blank reading must be performed to confirm the validity of the result. Consequently, if no acceptable value is obtained after measurement of the initial blank, two successive measurements that comply with the acceptance criteria must be obtained in order to be able to proceed with routine testing.

A *test blank* must be measured at least every five samples, using the same criteria as for the initial blanks.

If programming of the sample series is automatic and the results are collated at the *end of the series*, it may be necessary to increase the number of repeats of the blank (for example, three consecutive repetitions instead of just one) to ensure a correct reading.

A series of blank repetitions (for example, three) must be measured once the sample series has been completed to ensure the cleanliness of the system.

2.1.6. Duplicate samples

One out of every 10 samples must be repeated at different times during the series. If the sample is analysed in duplicate or triplicate, this repetition must consist of re-analysing in duplicate or triplicate. The results must be compared with each other.

- If a method validation is available, the results must comply with the reproducibility criteria obtained during validation.
- If no method validation is available, a compatibility index must be applied:

$$IC = \frac{|x_1 - x_2|}{\sqrt{(2SD_1)^2 + (2SD_2)^2}}$$

Source: ISO/IEC Guide 43-1:2007

where

x_1 and x_2 are the mean values obtained for each repetition of the sample; and

SD_1 and SD_2 are the standard deviations obtained for the duplicates, triplicates (or more) for each of the repetitions.

- If the sample is repeated using a single analysis rather than in duplicate or triplicate, a maximum deviation for each of the samples with respect to the mean must be established (for example, a maximum of 10% of the mean value) to consider the result acceptable.

2.1.7. Quality controls

The laboratory must perform quality controls for at least four points in the test range: high, mid-point, low and limit of quantification. These test control points must differ from the control points for the curve.

One of these quality controls must be inserted, at random, every five samples to ensure that all have been sufficiently analysed as part of the laboratory's operations. In any case, and if the values for all samples to be analysed fall within a very narrow range, the quality control can be repeated at the point closest to this range of samples.

- If a method validation is available, the results must comply with the criteria for the quality control points obtained during validation.
- If a history of results associated with the various control points is available but there is no validation, the acceptance criterion is that the value for the quality control must fall within the range

$$X_{point} \pm (t_{Student} \text{ x } SD_{point})$$

where

X_{point} – the mean value obtained upon reading the control point;

SD_{point} – the standard deviation for the values obtained upon reading the control point; and

$t_{Student}$ – obtained from the number of values used.

- If no results history is available, a maximum deviation for each of the samples with respect to the mean must be established (for example, a maximum of 10% of the mean value) to consider the result acceptable.

Blind samples

The laboratory must organize tests with blind samples at least once a year. To this end, the lead technician must prepare samples of a known concentration (but not known to the laboratory) for analysis. This blind sample must be prepared using certified standards, remnants of intercomparison samples or well-characterized samples.

- If a method validation is available, the real value for the sample must fall within the range

$$V_{SBlind} \pm I_{test}$$

where

V_{SBlind} – the value obtained upon analysing the blind sample; and

I_{test} – the expanded uncertainty obtained during validation of the method.

- If reproducibility values are available for the method but there is no method validation, the real value for the sample must fall within the range

$$V_{SBlind} \pm (t_{Student} \text{ x } SD_{repro})$$

where

V_{SBlind} – the value obtained upon analysing the blind sample;

SD_{Repro} – he value obtained for the standard deviation in reproducibility; and

$t_{Student}$ – obtained from the number of values used to obtain SD_{repro}.

- If no other values are available, a maximum deviation with respect to the real value must be established in order to accept the result. This deviation can be estimated from the literature or the experience of the laboratory with similar methods or analytes.

2.2. External quality controls

Interlaboratory comparisons[1] are widely used for various purposes at national, regional and global scale. Examples of typical purposes for interlaboratory comparisons include:

(i) evaluating the performance of laboratories as regards conducting specific tests or measurements and monitoring the performance of laboratories over time;

(ii) identifying problems in laboratories and initiating improvements which, for example, may be related to inadequate testing or measurement procedures, ineffective staff training and supervision or calibration of equipment;

(iii) establishing the efficacy and comparability of testing or measurement methods;

(iv) providing additional confidence to the laboratories' clients;

(v) identifying differences between laboratories;

(vi) instructing participating laboratories on the basis of the results of such comparisons;

(vii) validating the stated uncertainty estimations;

(viii) evaluating the operational characteristics of a method;

(ix) assigning values to reference materials and evaluating their suitability for use in specific testing or measurement procedures;

(x) supporting equivalency declarations for measurements from national institutes of metrology by way of key comparisons and complementary comparisons performed on behalf of the International Bureau of Weights and Measures and associated metrological associations.

The procedures described below are mainly applicable for laboratories organizing an intercomparison study, such as reference laboratories at national level. They are also fully applicable for participants in interlaboratory comparisons.[2]

Proficiency tests[3] comprise the use of interlaboratory comparisons to determine the performance of laboratories, as indicated in points (i) to (vii). Proficiency tests are not normally concerned with purposes (viii), (ix) and (x) as it is assumed that laboratories are competent in these applications. However, they can be used to provide independent proof of the competence of a laboratory.

The steps prior to performing an intercomparison exercise are related to:

- assigning the value to the sample:
- determining the standard deviation parameter for the proficiency test, which will subsequently be needed for the calculations in the exercise;

[1] Interlaboratory comparison: organization, performance and assessment of measurements with the same or similar items by two or more laboratories according to predetermined conditions.

[2] Participant: laboratory, organization or person who receives the proficiency testing items and provides the results for review by the proficiency test provider.

[3] Proficiency test: assessment of the performance of participants with respect to previously established criteria by way of interlaboratory comparisons.

- determining the number of repetitions to be performed by each participant; and
- confirming the validity of the sample to be analysed by way of homogeneity and stability tests. These parameters must be calculated by the organizer of the exercise.

Before sending the samples to the various participants, the organizer must prepare detailed and documented instructions.

Obviously, the organizer must analyse the quantity of samples required to conduct the exercise, taking into account the number of participants, the homogeneity and stability tests that need to be performed and the possibility of repetition, loss or damage to the sample during the transport phase. A quantity that exceeds the strictly calculated requirements should, therefore, be considered.

2.2.1. Assigning the value to the sample

The criteria used to obtain the value against which the results submitted by the laboratories will be compared must be determined before the exercise is carried out. These criteria are as follows.

- The value for a certified reference material or a spiked sample is obtained when:
  - a sample of certified reference material is used for the proficiency test; the value of the property, and the uncertainty in this value, must be known; or
  - a raw sample spiked with quantities of the test substance is used; spiking may be performed by the organizer or by the participating laboratory using concentrated solutions supplied by the organizer.
- The result is obtained as the mean value obtained by a group of expert laboratories which have tested the sample or samples using previously accepted test methods that can be considered to be "absolute" or "reference" methods. Atypical results must be eliminated prior to calculating the mean.
- The result is obtained as the mean value calculated by the group of participating laboratories after elimination of atypical values or as the mean obtained using robust statistical methods (such as algorithm A; see below). This is a riskier system in free-access intercomparison systems as erroneous data may affect the mean, meaning that the elimination of outliers must be efficient.

2.2.2. Determination of the standard deviation for proficiency testing $\hat{\sigma}$[4]

Various options are available for assigning the value of the standard deviation for proficiency testing.

Prescribed value

The standard deviation for proficiency testing can be assigned on the basis of compliance with standard values. This method has the advantage of best representing the purpose of the method.

Perceived value

The standard deviation for proficiency testing can be established on the basis of the prior experience of the coordinator and his/her collaborators using values obtained in the past.

When the standard deviation for proficiency testing ($\hat{\sigma}$) is obtained by prescription or perception, there is a possibility that the value selected is not realistic as regards the reproducibility of the

[4] Standard deviation for proficiency assessment: measure of the dispersion used to evaluate the results of a proficiency test based on the information available.

measurement method. The following test can, therefore, be applied to ensure that the value of $\hat{\sigma}$ corresponds to the repeatability and reproducibility values obtained using the method

if

$\hat{o}_R$ – the standard deviation of reproducibility, and

$\hat{o}_r$ – the standard deviation of repeatability.

The interlaboratory standard deviation is calculated as:

$$\sigma_L = \sqrt{\sigma_R^2 - \sigma_r^2}$$

The value of the factor ϕ is subsequently calculated by substituting the values of σ_L and σ_r and the value selected for $\hat{o}$ in the following equation:

$$\hat{\sigma} = \sqrt{(\emptyset\ x\ \sigma_L)^2 + \left(\frac{\sigma_r^2}{n}\right)}$$

where

n is the number of replicates that each laboratory will perform.

If the value obtained for ϕ is less than 0.5, the value selected for $\hat{\sigma}$ corresponds to a degree of reproducibility that, in practice, the laboratories will be unable to meet, in which case this value will have to be increased.

Value based on a general model

The value of the standard deviation for proficiency testing can be derived from the reproducibility value obtained for the measurement method.

For example, Horowitz has proposed the following model for evaluating the standard deviation of reproducibility using the concentration:

$\sigma_R = 0{,}02\ c^{0{,}8495}$

where c – the concentration of the measure to be determined as a percentage (mass fraction).

Value based on the repeatability and reproducibility results

When the values for the standard deviations of reproducibility and repeatability are available, the standard deviation for proficiency testing can be obtained as follows:

σ_R – the standard deviation of reproducibility, and

σ_r – the standard deviation of repeatability.

The interlaboratory standard deviation is calculated as:

$$\sigma_L = \sqrt{\sigma_R^2 - \sigma_r^2}$$

The value of $\hat{\sigma}$ is calculated as:

$$\hat{\sigma} = \sqrt{\sigma_L^2 + \left(\frac{\sigma_r^2}{n}\right)}$$

where

n is the number of replicates that each laboratory will perform.

Value based on the data obtained in a proficiency test round

The value of the standard deviation for proficiency testing can be derived from the value derived from the results reported by the participants in this round of tests. The standard deviation must be the robust standard deviation for the results reported by all participants, as calculated using algorithm A.

Order the p data in ascending order:

$x_1, x_2, \dots, x_i, \dots, x_p$

Order the robust means and robust standard deviations (x* and s*) for these data.

The initial values for x* and s* are:

$x^* = \text{median of } x_i \quad (i = 1, 2, \dots, p)$

$s^* = 1.483 \text{ median of } |x_i - x^*| \quad (i = 1, 2, \dots, p)$

Update the values of x* and s* as follows. Calculate:

$\delta = 1.5\, s^*$

For each x_i ($i = 1, 2, \dots, p$) calculate:

$$x_i^* = \begin{cases} x^* - \delta, if\ x_i < x^* - \delta \\ x^* + \delta, if\ x_i > x^* + \delta \\ x_i\,, other\ cases \end{cases}$$

Now recalculate the new values for x* and s* as:

$$x^* = \sum \frac{x_i^*}{p}$$

$$s^* = 1{,}134 \sqrt{\frac{\sum (x_i^* - x^*)^2}{(p-1)}}$$

Summing over i.

The robust estimation of x* and s* is derived from an iterative calculation until the process converges. Convergence is assumed when no changes occur between one iteration and the next in the third significant figure of the robust standard deviation and the equivalent figure of the robust mean. A computer can be programmed to carry out this method.

2.2.3. Criteria for selecting the number of measurements to be performed by each participating laboratory

Variations in method repeatability mean that biases may appear in proficiency tests. When the variation in repeatability is too high compared with the standard deviation for proficiency testing, there is a risk of obtaining unreliable results. In these circumstances, a laboratory may have a very high bias factor in one round but not in another, which would make finding the cause more difficult.

If we wish to limit the influence of variations in repeatability, the number of replicates (n) performed by each laboratory should be chosen such that:

$$\frac{\sigma_r}{\sqrt{n}} \leq 0{,}3\, \hat{\sigma}$$

where

σ_r – the standard deviation of repeatability established prior to the exercise (either by way of an experimental interlaboratory exercise or determined by the organizing laboratory).

If this condition is met, the standard deviation of repetition does not represent more than 10% of the standard deviation for proficiency testing.

In addition, all participating laboratories must perform the same number of replicates when participating in intercomparison tests.

2.2.4. Homogeneity test procedure[5]

When it is acceptable not to perform homogeneity tests for all measurands, a measurement method and characteristic measurand that are sufficiently sensitive to the heterogeneity of the samples will be selected.

Prepare and package the samples for a proficiency testing round, ensuring that there are sufficient samples to perform both the proficiency test and the homogeneity tests.

Select a number (g) of packaged samples at random, where $g \geq 10$. The number of samples included in the homogeneity test can be reduced if historic data for these homogeneity tests performed according to the same procedures are available.

Prepare two test portions for each sample, minimising the intratest differences as far as possible.

Take these 2g test portions at random and perform the test for each one, completing the test series under repeatability conditions.

Calculate the mean (x) intrasample standard deviation (s_w) and intersample standard deviation (s_S) as follows.

The data for a homogeneity test are represented by $x_{t,k}$

where

t represents the sample (t = 1, 2, …, g)

k represents the portion of sample (k = 1, 2).

The mean for each sample is defined as:

$$x_{t,.} = \frac{x_{t,1} + x_{t,2}}{2}$$

and the range of intertest portions as:

$$w_t = \left| x_{t,1} - x_{t,2} \right|$$

The general mean is calculated as:

$$\bar{x}_{.,.} = \sum \bar{x}_{t,.}/g$$

The standard deviation of the general mean is calculated as:

$$s_x = \sqrt{\sum \left(x_{t,.} - \bar{x}_{.,.}\right)^2/(g-1)}$$

The intrasample standard deviation:

$$s_w = \sqrt{\sum w_t^2/(2g)}$$

where the summation covers all samples (t = 1, 2, …, g).

[5] According to International Organization for Standardization 13528:2005.

Finally, to calculate the intersample standard deviation:

$$s_S = \sqrt{s_{\bar{x}}^2 - (s_w^2/2)}$$

Assessment criteria for the homogeneity test

Compare the intersample standard deviation (s_S) with the required standard deviation for proficiency testing ($\hat{\sigma}$). The samples comply with an appropriate homogeneity criterion if:

$$s_S \leq 0{,}3\,\hat{\sigma}.$$

If this criterion is met, the intersample standard deviation does not represent more than 10% of the overall standard deviation for proficiency testing. If this criterion is not met, the coordinator may consider one of the following possibilities.

- The method used to prepare the samples to make any necessary improvements could be examined.
- A number of samples could be distributed to each participant in the intercomparison exercise in order to perform a measurement for each sample. The heterogeneity of these samples will increase the intrasample standard deviation to a value:

$$\sigma_{r1} = \sqrt{\sigma_r^2 + s_S^2}$$

This value σ_{r1} can be used to increase the number of replicates for each participant in the exercise.

The intersample standard deviation could be included in the standard deviation for proficiency testing, calculating $\hat{\sigma}$ as

$$\hat{\sigma} = \sqrt{\hat{\sigma}_1^2 + s_S^2}$$

where

$\hat{\sigma}_1$ – the standard deviation for proficiency testing without including any tolerance for sample heterogeneity.

2.2.5. Stability test procedure

The same laboratory that performs the homogeneity test must perform the stability tests. The same method and same product as in the homogeneity tests must be used.

Perform the stability tests after the homogeneity tests. The time difference between the former and the latter should be similar to the time that is estimated to pass between the preparation of samples for the intercomparison exercise and the maximum period during which the participants must present their results.

Take a number (g) of samples, where $g \geq 3$.

Prepare two test portions of each sample as described for the homogeneity tests.

Take the $2g$ portions at random to obtain a measurement result $y_{t,k}$ for each sample, performing all measurements under repeatability conditions.

Calculate the mean $\bar{y}$ (.,.) of all measurements.

Assessment criteria for the stability test

Compare the mean obtained in the homogeneity test with the mean obtained in the stability test. The samples are considered to be stable if:

$$\left|\bar{x}_{..} - \bar{y}_{..}\right| \leq 0{,}3\ \hat{\sigma}$$

If this criterion is not met, sample preparation and storage must be assessed and improved if possible.

2.2.6. Instructions for participants

Prior to sending the proficiency test items, the proficiency test provider will notify participants of the expected date of arrival of the items and the date by which the results must be returned by the participating laboratory with sufficient notice.

The proficiency test provider must provide detailed and documented instructions to all participants. These instructions will include:

- the need to treat the proficiency test items in the same manner as the majority of samples tested routinely (unless the specific requirements of the programme require some deviation from this principle);
- the storage conditions;
- the test methods to be used, or allowed, when applicable;
- the procedure for preparing and conditioning the proficiency test items;
- handling instructions, including safety requirements;
- the specific environmental conditions under which proficiency testing must be performed and, if necessary, the requirement that participants must notify the pertinent environmental conditions during the measurement;
- specific instructions regarding the way in which results must be reported (such as measurement units, number of significant figures or decimal places) and instructions regarding the uncertainty in the result (if required); in the latter case, the coverage factor and, if possible, the probability of that coverage must be included;
- deadline for reporting the results;
- contact information for the provider in the event of any questions; and
- instructions regarding return of the proficiency test ítems (if applicable).

2.2.7. Calculation of statistical parameters associated with the proficiency test results

Estimation of participant bias

If x is the result (or mean of the results) reported by a participant for the measurement of one of the parameters to be determined in a proficiency testing round, the bias (D) can be calculated as:

$$D = x - X$$

where

X – the assigned value.

If a participant obtains a result that gives a bias higher than 3.0 $\hat{\sigma}$ or lower than -3.0 $\hat{\sigma}$, the result will be considered and marked as an “action signal”. Similarly, if a participant obtains a result that gives a bias higher than 2.0 $\hat{\sigma}$ or lower than -2.0 $\hat{\sigma}$, the result will be considered and marked as a “warning signal”.

A single action signal or two consecutive warning signals signify that the laboratory must start an investigation into the bias found in its results.

Percentage differences

If x is the result (or mean of the results) reported by a participant for the measurement of one of the parameters to be determined in a proficiency testing round, the percentage difference (D%) can be calculated as:

$$D\% = 100\ (x - X)/X$$

where

X is the assigned value.

If a participant obtains a result that gives a percentage difference higher than 300 $\hat{\sigma}$/X% or lower than -300 $\hat{\sigma}$/X%, the result will be considered and marked as an action signal. Similarly, if a participant obtains a result that gives a percentage difference higher than 200 $\hat{\sigma}$/X% or lower than -200 $\hat{\sigma}$/X%, the result will be considered and marked as a warning signal.

A single action signal or two consecutive warning signals signify that the laboratory must start an investigation into the bias found in its results.

2.2.8. z-score

The z-score value is calculated as:

$$z = \frac{(x - X)}{\hat{\sigma}}$$

where

x – the value reported by the participant

X – the assigned value, and

$\hat{\sigma}$ – the standard deviation for proficiency testing.

If a participant obtains a result that gives a z-score higher than 3.0 $\hat{\sigma}$ or lower than -3.0 $\hat{\sigma}$, the result will be considered and marked as an action signal. Similarly, if a participant obtains a result that gives a z-score higher than 2.0 $\hat{\sigma}$ or lower than -2.0 $\hat{\sigma}$, the result will be considered and marked as a warning signal.

If the proficiency test involves a small number of participants (for example, fewer than 10 laboratories), the significance of the z-score for the individual rounds must be considered with great care. In such cases, it is preferable to evaluate the combination of results from different rounds when assessing the performance of each laboratory.

2.2.9. E_n number

This parameter is calculated as:

$$E_n = \frac{x - X}{\sqrt{U_{lab}^2 + U_{ref}^2}}$$

where

X – the assigned value

U_{ref} – the expanded uncertainty in X

U_{lab} – the expanded uncertainty in x, the result obtained by the participant.

A value higher than 1.0 or lower than -1.0 is equivalent to a z-score value above or below 2.0, respectively, therefore a result of this type must be treated as defined in the z-score assessment.

2.2.10. z'-score

The z'-score value is calculated as:

$$z' = \frac{(x - X)}{\sqrt{\hat{\sigma}^2 + u_X^2}}$$

where

u_X – the (non-expanded) uncertainty of the assigned value X.

The z'-score results are interpreted in the same manner as the z-score values.

A comparison of the z-score with the z'-score shows that the z'-score values in one round may be lower than the corresponding z-score values, in accordance with a constant factor of

$$\frac{\hat{\sigma}}{\sqrt{\hat{\sigma}^2 + u_X^2}}$$

If

$$0{,}96 \leq \frac{\hat{\sigma}}{\sqrt{\hat{\sigma}^2 + u_X^2}} \leq 1{,}00$$

then the z'-score will be very close to the z-score, in which case it can be concluded that the uncertainty in the assigned value is negligible.

2.2.11. Zeta-score *(ζ)*

This parameter can only be used if the value assigned to the proficiency test has not been calculated using the results from the participating laboratories.

$$zeta\ score = \frac{(x - X)}{\sqrt{u_x^2 + u_X^2}}$$

where

u_x – the value of the standard uncertainty (not expanded) estimated by the participating laboratory, and

u_X – the standard uncertainty (not expanded) of the assigned value X.

The ζ-score is interpreted in a similar manner as the z-score.

If successive ζ-scores higher than 3.0 are obtained, this may mean that the participant is underestimating the sources of uncertainty.

If a laboratory presents a very large bias and the uncertainty interval X ± U_X does not include the assigned value, very high values will also be obtained for the ζ-score.

2.2.12. E_z score

The E_z score is defined as

$$E_{Z-} = \frac{x - (X - U_X)}{U_x}$$

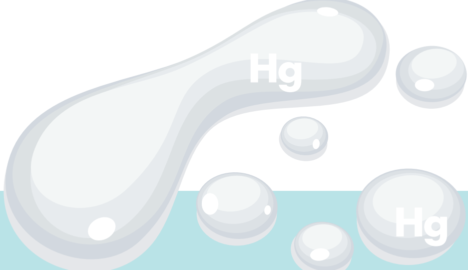
$$E_{Z+} = \frac{x - (X + U_X)}{U_x}$$

In these cases the expanded uncertainty is used.

- If both values (E_{z-} and E_{z+}) fall within the range -1.0 to 1.0, the result is considered to be satisfactory.
- If one of the two E_z values falls within the range -1.0 to 1.0, the result is doubtful.
- If both values are less than -1.0 or greater than 1.0, the result is unsatisfactory.

3. Evaluation of laboratory proficiency

Each laboratory can evaluate its performance using the check-list in Annex 3 which includes a series of questions organized into sections for collecting data from laboratories and the criteria for applying them for evaluation. Information should be collected for this purpose about equipment, level of expertise, experience of participating in intercalibration studies and accreditation.



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Annex 1. Registration of reception of a sample

1. ORIGIN OF THE SAMPLE:

Centre:
City/Country:
Date of sampling:

2. SAMPLE RECEIVED:

Signature of reviewer:

☐ Urine
☐ Hair ☐ Cord blood

3. SAMPLE RECEPTION:

Date (dd/mm/yy) Time (hh:mm)

A) PACKAGING

☐ NO PROBLEMS DETECTED
☐ PROBLEMS DETECTED:

☐ Packaging damaged
☐ Cooling agents defrosted
☐ Others:______________________

B) SAMPLES

☐ NO PROBLEMS DETECTED
☐ PROBLEMS DETECTED:

☐ Spilled sample/broken vessel
☐ Insufficient amount/volume (*specify the matrix*)___
☐ Inconsistency in the ID codes
☐ Others:______________________

C) DOCUMENTS

☐ NO PROBLEMS DETECTED
☐ PROBLEMS DETECTED:

☐ Absence of the registry of collected samples
☐ Absence of the hair sampling questionnaire
☐ Absence of the urine sampling questionnaire
☐ Absence of the cord blood sampling questionnaire
☐ Absence of the study questionnaire
☐ Inconsistency in the ID codes
☐ Others:______________________

4. DATE OF STORAGE/BIOBANKING:

5. COMMENTS:

ID CODES FOR RELATED SAMPLES

Urine Hair Cord blood
☐ ☐ ☐

Annex 2. Registration for collected samples

ORIGIN:	CENTRE: ADDRESS: CITY/COUNTRY: CONTACT (NAME AND PHONE):		
ID code	Sampling date (dd/mm/yy)	Sampling questionnaire attached(Y/N)	Comments

Shipment date:

Name and signature of fieldworker:

Annex 3. Self-evaluation of laboratory competence

Laboratory evaluation questionnaire

GENERAL INFORMATION

1. Data for the person completing the questionnaire.

Name: ....

Position: ....

Company: ....

Address: ....

City: ....

Post code: ....

Country: ....

email: ....

Telephone: ....

2. Which analyses do you perform in your laboratory?

☐ Mercury in hair ☐ Mercury in urine ☐ Mercury in cord blood

3. Which analytical technique(s) do you use?

....

4. Please specify the type, manufacturer and model of your analytical apparatus

....

5. What is the minimum amount of scalp hair/urine/blood required for measurements? mg or mL

....

METHOD INFORMATION

6. Is the analytical procedure for mercury in scalp hair/urine/cord blood accredited?

☐ NO ☐ Yes

If yes, please include your Technical Annex number:

....

7. Do you have a general standard operating procedure for the validation of analytical methods?

☐ NO ☐ Yes

8. Is there a standard operating procedure for the analysis of mercury in .... in your laboratory?

☐ No ☐ Yes

9. Do you have a validated method for the analysis of mercury in ....?

☐ No ☐ Yes

10. Please complete the following information about your analytical method.

Interseries repeatability	..................%
Limit of quantification	....
Limit of detection	....
Accuracy	....
Uncertainty	....

11. How do you calculate your interseries repeatability?

....

12. How do you calculate your limit of quantification?

....

13. How do you calculate your limit of detection?

....

14. How do you calculate your accuracy?

....

15. What components do you use to calculate your uncertainty?

....

QUALITY CONTROL

16. Do you have an internal quality control system?

☐ No ☐ Yes

17. Do you apply the following quality controls?

....

Straight line controls[6]	☐ No	☐ Yes *Please specify the frequency.*.........................
Regression coefficient	☐ No	☐ Yes
Linearity coefficient	☐ No	☐ Yes
Slope	☐ No	☐ Yes
Blanks	☐ No	☐ Yes *Please specify the frequency.*.........................
Quality controls	☐ No	☐ Yes *Please specify the frequency.*.........................
Blind samples	☐ No	☐ Yes *Please specify the frequency.*.........................
Duplicate samples	☐ No	☐ Yes *Please specify the frequency.*.........................

18. Do you use certified reference materials?

☐ No ☐ Yes *Please specify the manufacturer and concentration:*

....

19. Do you use reference materials?

☐ No ☐ Yes *Please specify the manufacturer and concentration:*

....

20. Do you use calibrated/verified equipment?

☐ No ☐ Yes

21. Do you have an annual equipment calibration plan and programme?

☐ No ☐ Yes

22. Do you have records of sample storage conditions, when necessary?

☐ No ☐ Yes *Please specify the manufacturer and concentration:*

23. Do you have an annual intercomparison programme?

☐ No ☐ Yes

[6] This is related to the frequency of calibration to confirm that the curve parameters fulfil the validation criteria defined.

contd. QUALITY CONTROL

24. How often do you participate? (Please indicate organizer and number of times per year)

....

25. The evaluation of your interlaboratory results is based on:

z-score	☐ No	☐ Yes
En number	☐ No	☐ Yes
z'-score	☐ No	☐ Yes
Zeta-score	☐ No	☐ Yes
Ez-score	☐ No	☐ Yes
Other	☐ No	☐ Yes *Please specify*....................................

Evaluation criteria

Question Number	Explanation
6	YES: Accredited laboratories must be considered expert laboratories; however, in this case, most of the other questions must be answered in the affirmative for this to be considered. NO: The proficiency of the laboratory can be estimated on the basis of the remaining questions.
7	YES: The first step when developing a particular validation should be the drafting of the general procedure for that validation. NO: If the method for the determination of mercury (question 9) has been validated, an appropriate proficiency could be acceptable.
8	YES: The second step when developing a particular validation should be the drafting of the particular procedure for mercury validation. This should be the initial step in the validation. NO: If the method for the determination of mercury (question 9) has been validated, an appropriate proficiency could be acceptable.
9	YES: A validated method is a necessary step when evaluating the proficiency of the laboratory. In addition, if quality control questions are appropriately answered, and question 10 offers suitable statistical parameters, the laboratory performance could be considered sufficient. NO: The laboratory should be able to validate the method. As a minimum, it is desirable to obtain statistical parameters for accuracy and the limit of quantification.

Question Number	Explanation
10	These values allow the performance of a laboratory to be evaluated. A comparison between different laboratories allows the reliability of each one to be determined.
11	This question allows the statistical proficiency of the laboratory to be determined.
12	This question allows the statistical proficiency of the laboratory to be determined.
13	This question allows the statistical proficiency of the laboratory to be determined.
14	This question allows the statistical proficiency of the laboratory to be determined.
15	This question allows the statistical proficiency of the laboratory to be determined. In this particular case, the possibility of underestimating the uncertainty in the measurement must be evaluated as this could affect the ability to obtain comparable results.
16	YES: It is necessary to evaluate the scope of internal quality controls in order to ensure that any deviation will be detected. NO: The first step to be able to trust in the reliability of the results must be to have an internal quality-control system.
17	YES: It is not necessary to implement all controls, but a higher number of controls ensures better results. NO: The laboratory should try to implement at least some of the controls, for example a calibration curve control and some type of sample control.
18	YES: The use of certified reference material ensures an assigned value. Possible manipulations (dilutions…) must be considered in order to obtain the real final value in every case. NO: As a minimum, reference materials (question 19) must be used.
19	YES: The laboratory can use suitable materials provided that these materials have been appropriately characterized. NO: This question can be ignored if question 18 is affirmative.
20	YES: Equipment calibration ensures instrumental repeatability and avoids equipment-related errors. NO: Calibration is the first step in any equipment control. No measurements should be performed prior to the calibration of critical equipment.
21	YES: An annual calibration plan and programme ensures that all equipment is operating correctly. Intermediate verifications should be carried out when necessary. NO: All equipment must be calibrated before the analysis is conducted.
22	YES: Measurement traceability is essential for suitable control of the environmental conditions. NO: Temperature, humidity and other aspects must be monitored when necessary. If not, the final results should be considered to be unreliable.
23	YES: Annual intercomparison programmes show the willingness of the laboratory and must be considered to be a favourable answer. NO: Only long-term participation provides the laboratory with an effective tool for evaluating its results.

Question Number	Explanation
24	A long participation time must be evaluated positively, irrespective of the results of this participation.
25	The z-score could be an insufficient means of determining the proficiency of the laboratory. Additional methods show a better capacity of the laboratory.

The criterion for evaluating laboratories should be based on the information collected from questions 6–25 of this questionnaire. However, this criterion can vary and can be applied more or less strictly depending on specific requirements and situations. In view of this, the following criteria can be applied.

- Laboratories reporting negative answers to questions 16 or 20 should be automatically excluded.
- Laboratories reporting fewer than nine positive answers must improve their quality system, for example by adopting some of the activities referred to in the evaluation criteria table. In particular, method validation should be the final goal for all participants, and it is strongly recommended to obtain quality control criteria from this validation.
- For laboratories reporting fewer than 18 positive answers, special attention should be paid to the answers to questions 10–15 and 24 as these allow proficiency to be evaluated and therefore the laboratory to be considered as a candidate.
- A positive response to more than 18 questions indicates a good analysis proficiency profile. The laboratory can, therefore, be evaluated as a candidate for performing the analysis. However, it should participate in the specific interlaboratory comparison exercises for the WHO study.

Standard operating procedure for assessment of mercury in human scalp hair

(sampling, analysis of total mercury, interpretation of results)

Abstract

This standard operating procedure (SOP) describes the process of assessing prenatal exposure to mercury through human biomonitoring using scalp hair as a biological matrix. Sampling of scalp hair, analysis of total mercury and interpretation of results are detailed in this document.

Keywords

Mercury – analysis
Methylmercury Compounds – analysis
Biomarkers – analysis
Hair – chemistry
Maternal Exposure
Maternal-Fetal Exchange
Infant, Newborn
Environmental Exposure

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Abbreviations

CRM	certified reference materials
HBM	human biomonitoring
Hg	mercury
IAEA	International Atomic Energy Agency
ID	identity
LOD	limit of detection
LOQ	limit of quantification
SOP	standard operating procedure

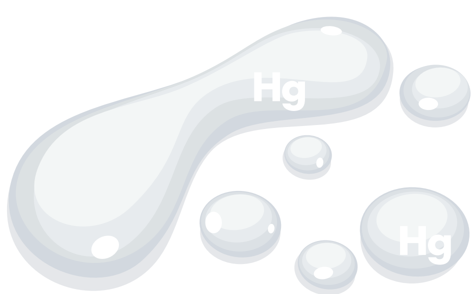
Introduction: human hair as a matrix for mercury human biomonitoring

Environmental chemicals absorbed by the body can be incorporated into hair. Human hair has been widely employed in different scientific areas, such as forensic and clinical toxicology, occupational medicine and doping control. In the last few years, it has also been used in the human biomonitoring (HBM) of environmental chemicals. The use of this matrix in HBM presents some advantages such as non-invasiveness; ease of sampling, transport and conservation; and no requirement for special materials or specific health-care personnel to take the sample. Although is not a suitable matrix for many chemicals, it is particularly useful for the study of mercury (Hg) exposure due to fish consumption *(1)*, and a number of studies in different populations have employed hair samples for this purpose *(2).*

Hair is generally the preferred choice to document methylmercury exposure as it provides a simple, integrative and non-invasive sample. Indeed, once incorporated into the hair, mercury cannot return to the blood, thus providing a good long-term marker of exposure to methylmercury. Most mercury in hair is in the form of methylmercury, especially among populations that consume large amounts of fish. Hair incorporates methylmercury during its formation and the levels contained show a relatively direct relationship with blood mercury levels, thus providing an accurate and reliable method for measuring methylmercury intake levels *(3)*.

Hair is a biological material that grows in cycles, alternating between periods of growth and quiescence. It is widely accepted that hair grows at a rate of 1 cm a month, although this rate can change depending on the hair type and body location. Structurally speaking, hair is a cross-linked, partially crystalline, oriented polymeric network containing different functional chemical groups that can bind small molecules. It is composed of approximately 65–95% proteins, a high proportion of which are sulphur-rich. Water accounts for approximately 15–35% and lipids 1–9%. The mineral content of the hair is less than 1% *(4,5).*

This standard operating procedure (SOP) provides detailed instructions for collection and analysis of human scalp hair samples and interpretation of results. Quality control throughout mercury HBM is described in a separate SOP and should be considered at each stage.



1. Human scalp hair sampling

The hair sampling procedure does not require sophisticated technical material and fieldworkers will be able to collect the samples properly after a simple training. The procedure described avoids aesthetic problems even in the case of short hair and therefore minimizes possible rejections by volunteers for this reason.

The hair sampling procedure varies slightly depending on the length of the hair and the mobility of the volunteer. The method described covers the different possibilities.

Special attention should be paid to the amount of hair collected (too small an amount of hair may compromise the analysis) and lock immobilization.

The quantity of the sample collected depends on the amount required for subsequent chemical analysis. This will vary depending on the analytical method and the limit of quantification. These issues must be discussed in advance and defined with the laboratory responsible for the analysis.

Immobilization of the lock is a critical step in hair sampling as the end closest to the scalp must be unequivocally identified. This SOP describes different possibilities for performing this immobilization. In the event of using adhesive tape for the immobilization, special attention should be paid to the segment of the sample to be analysed, which must be free from adhesive tape.

This SOP proposes control points during sample reception in order to allow routine control for acceptance or rejection of the samples.

Detailed instructions are given for preparing the human scalp hair sample for mercury analysis.

1.1. Scope of the method

This method is used to collect samples of human scalp hair of different lengths:

- shorter than 3.5 cm (1.4 in)
- 3.5–5 cm (1.4–1.97 in)
- longer than 5 cm (1.91 in).

The sample preparation and aliquoting method also takes into account the length of the collected samples, considering two situations: immobilized samples and non-immobilized samples.

1.2. Safety precautions

The following safety precautions should be taken for hair sampling.

- No special safety precautions for biological hazards need be taken when working with hair.
- Gloves and suitable scissors should be used when taking the samples.

[1] The length cut-off values can be modified depending on the segment of the sample to be analysed.

1.3. Materials required

Table 1 shows the materials required for hair sampling, the rationale for using them and any possible alternatives.

Table 1. Material for hair sampling for mercury analysis

Material	Rationale	Alternative
Alcohol and cotton	Used as a hygienic precaution.	
Latex gloves (powder free)	Used as a hygienic precaution.	Similar single-use powder-free disposable gloves made of other materials
Scissors	Although different methods can be used to cut the sample, it is advisable to employ scissors specially designed for hair cutting. As the lock should be cut very close to the scalp, scissors with blunt ends are useful to avoid damage.	Any clean and sharp scissors of an appropriate size
ID labels	Samples must be unequivocally identified.	Writing the ID code directly on the paper envelope with a permanent marker pen
Permanent marker pen	Needed to indicate the extreme closest to the scalp. Common pens do not write well on the adhesive tape.	Any other writing material which ensures that the mark will remain clearly legible
Adhesive tape	Used to immobilize the lock.	Any other material which ensures that the lock remains immobilized
Paper bags	These are the primary sample container. Paper materials avoid problems resulting from static electricity. The size should be in accordance with the sample (e.g. 8x14 cm; 12x20 cm).	Paper envelopes
Zip-lock plastic bags	This second container protects the sample from liquids. The size should be in accordance with the sample (e.g. 8x14 cm; 12x20 cm).	Any other type of plastic bag that ensures the sample remains isolated

ID = identity. Note: a pre-sampling checklist is available in Annex 4.

1.4. Preparation/pre-treatment of the sampling material

The sampling material required for hair collection does not need any special preparation or pre-treatment. However, for hygiene purposes, the scissors should be cleaned prior to each sample collection. All material for collecting hair samples should be ready and easily available for the fieldworker in charge of hair sampling.

The procedure for scissors cleaning is as follows.

1. Put on a pair of single-use disposable gloves.
2. Moisten a piece of cotton with alcohol.
3. Wipe the scissors with the moistened cotton (Photo 1).

Photo 1. Cleaning the scissors. © Instituto de Salud Carlos III

1.5. Sampling procedure

The procedure for hair sampling varies somewhat depending on the length of the hair. This will determine how the lock should be immobilized. Note that this document has been developed assuming analysis of the 3 cm closest to the scalp. If sample analysis is performed using a piece of different length, it must be ensured that this piece is free from adhesive tape.

The materials required for hair sampling should be ready and easily available for the person or team in charge of hair sample collection.

Samples should be collected from the same head area of all volunteers. Two strands of hair should be collected in the case of long hair, one from each side of the head. In order to avoid aesthetic problems, sampling in the case of short hair should be performed by cutting small strands from different places but within the same area of the head.[2]

1.5.1. Hair longer than 5 cm (1.97 in)

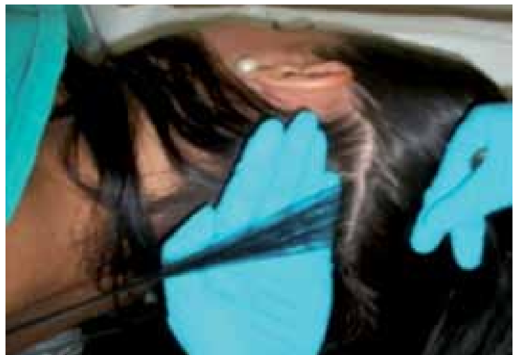
The procedure for sampling hair longer than 5 cm (1.97 in) is described below

1. Grasp the hair from the middle of the back of the head and hold it towards the top of the head (photos 2a and b).

Photos 2a and b. Grasping the hair; (a) sitting, (b) lying. © Instituto de Salud Carlos III

[2] A video of the hair sampling procedure is available on the web page of the Centro Nacional de Sanidad Ambiental, Instituto de Salud Carlos III *(6).*

2. Take several strands of hair horizontally and roll them up to form a lock (photos 3a and b).



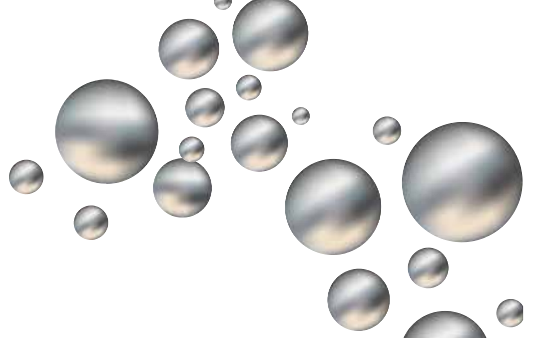
Photos 3a and b. Forming a lock; (a) sitting, (b) lying. © Instituto de Salud Carlos III

3. Fasten the lock with adhesive tape at 5–6 cm (1.97–2.36 in) from the root of the hair (photos 4a and b). Analysis is performed on the 3 cm closest to the scalp; therefore ensure that this fragment is free from adhesive tape.

Photos 4a and b. Fastening the lock with tape; (a) sitting, (b) lying. © Instituto de Salud Carlos III

4. Using the scissors, cut the sample as close to the scalp as possible (photos 5a and b).

Photos 5a and b. Cutting the sample close to the scalp; (a) sitting, (b) lying. © Instituto de Salud Carlos III



5. Seal the end of the adhesive tape and label it with an arrow pointing to the end closest to the root (photos 6a and b).

Photos 6a and b. Sealing the tape (a) and labelling with an arrow pointing to the root (b). © Instituto de Salud Carlos III

Note. The minimum distance of the adhesive tape from the end closest to the scalp depends on the sample to be analysed (in this case the first 3 cm). That piece must be free from adhesive tape.

6. Place the hair sample in a paper envelope and label it with the sample identity (ID) code (Photo 7).

Photo 7. Placing the hair in a paper envelope.
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7. Repeat this process with a second lock from the other side of the back of the head.
8. Place the paper envelope in the zip-lock plastic bag (Photo 8).

Photo 8. Placing the envelope in a zip-lock bag.
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Note. To ensure that the required amount is collected, the locks should have approximately 250 strands. However the weight of the sample can change depending on the sort and length of hair. The minimum amount required for the analysis must be checked with the laboratory that will analyse the sample.

1.5.2. Hair shorter than 3.5 cm (1.4 in)

Hair samples shorter than 3.5 cm should not be immobilized with adhesive tape, to ensure that the sample to be analysed is free from adhesive tape.

The procedure for sampling hair of this length is as follows.

1. Cut 5–10 strands of hair from different places on the back of the head (photos 9a and b).

Photos 9a and b. Cutting strands of hair; (a) sitting, (b) lying. © Instituto de Salud Carlos III

2. Place the hair sample directly in a paper envelope.
3. Repeat until the desired amount of sample has been obtained and label the paper envelope with the sample ID code (photos 10a and b).

Photo 10a and b. Repeating the cutting of strands of hair; (a) sitting, (b) lying. © Instituto de Salud Carlos III

4. Place the paper envelope in the zip-lock plastic bag (Photo 8).

Note. To ensure that the required amount is collected, an example of a scalp hair sample or a picture should be provided by the national survey coordinator or responsible laboratory assistance to field workers taking samples; see example below.

This amount is sufficient for direct analysis of mercury by thermal decomposition amalgamation atomic absorption spectrometry (Photo 11). Note that, depending on the analytical technique, the minimum amount may vary and therefore this must be checked with the laboratory that analyses the sample.

Photo 11. A sufficient amount of hair sample.
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1.5.3. Hair 3.5–5 cm (1.4–1.97 in)

With hair of this length, the manner in which the lock is immobilized is determined by the need to prevent the adhesive tape from touching the 3 cm of hair closest to the scalp. This requirement will change depending on the piece of hair to be analysed.

The procedure for sampling hair of this length is as follows.

1. Cut a lock of hair as close to the scalp as possible, following the instructions shown for hair longer than 5 cm.
2. When fixing the lock, be sure that the 3 cm closest to the scalp are available for analysis. Several means of doing this are possible, three of which are described below.

First option

a. Cut a piece of adhesive tape.

b. Place the end of the lock in the adhesive tape (be careful to ensure that the 3 cm closest to the scalp are free from adhesive tape) (Photo 12).

Photo 12. Placing the lock in the tape.
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c. Place another piece of adhesive tape over the first piece.

Second option

a. Hold the end of the lock closest to the root with a binder (bulldog) clip and a piece of paper (Photo 13).

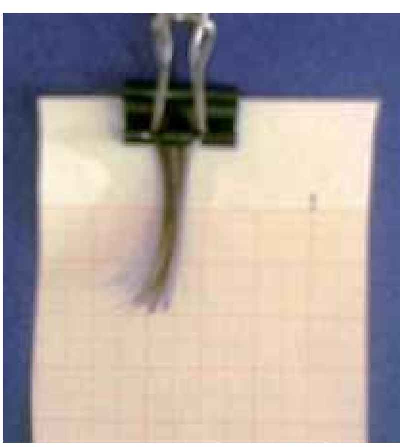

Photo 13. Holding the lock with a binder clip.
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b. Place the hair sample in a paper envelope and label it with the sample ID code.

c. Repeat the process with a second lock from the other side of the back of the head.

d. Place the paper envelope in the zip-lock plastic bag.

Third option

a. Staple the hair sample as tightly as possible (Photo 14).

b. Check that the lock is completely immobilized.

Photo 14. Stapling the hair sample.
© Instituto de Salud Carlos III

1.6. Labelling

The hair sample must be labelled with the ID code and the sampling date immediately after collection. These two entries are useful in the event that one of them is wrongly recorded. The label should be stuck on the first container (paper envelop), and if no label is available the code can be written on it directly.

1.7. Transportation and conservation of the sample

Hair samples do not require any special transportation conditions; they can be transported at room temperature. However, it should be checked that the corresponding documents, including a sheet listing all samples and information concerning any event that occurred during sampling which could affect the sample, have also been included with the samples (Annex 1).

1.8. Sample reception

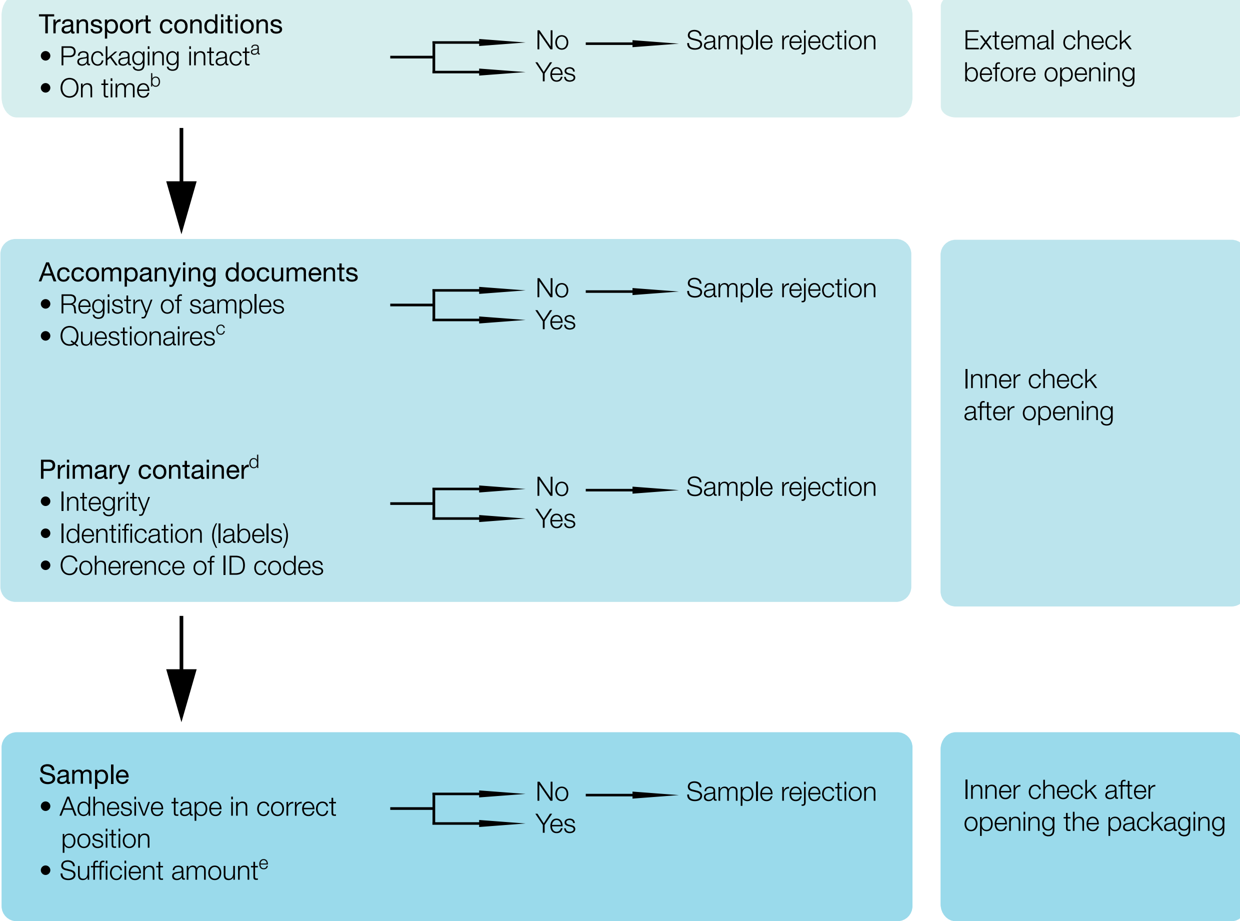
The criteria for accepting or rejecting a sample should be defined in advance and applied during sample reception. These criteria should focus on transportation conditions, accompanying documentation, integrity of the packaging, correct identification and amount of sample (sufficient for analysis and biobanking if samples will be stored and used for other research purposes).

The following points should be checked upon receipt of hair samples.

- Integrity of the packaging: packaging must be correctly sealed and must not have been manipulated; a security seal can be placed on the package at the sampling site.
- Accompanying documents: all samples listed in the registry of collected samples (Annex 1) should be contained in the package; they must be accompanied by the corresponding documents (questionnaires, etc.).
- Correct identification: samples and documents received must be properly identified with the corresponding ID code (Annex 2).
- Amount and quality of the samples: samples must have been properly collected (check position of adhesive tape and amount of hair sampled).

In order to follow a unique procedure and apply the same criteria to all samples received, the plan illustrated in Fig.1 can be followed.

Fig. 1. Plan for receipt of samples



[a] The package must be correctly sealed and must not have been manipulated

[b] The maximum time between sample collection and its arrival at the laboratory should be defined beforehand.

[c] If one or more of the questions in the questionnaires are crucial for results interpretation or are an inclusion/exclusion criterion, this should be verified.

[d] The conditions of the zip-lock plastic bag should be checked. All samples must be properly identified and the consistency between sample ID codes and questionnaires should be checked.

[e] The amount of sample is a critical point. If the amount of sample is insufficient to perform the chemical analysis, the sample should be rejected.

An example of a registry of samples reception is in Annex 3 and pre- and post-sampling checklists are in annexes 4 and 5.

1.9. Sample aliquoting/preparation

All accepted samples should be prepared for analysis and stored in tightly closed polypropylene containers in order to avoid deterioration of the target analyte and matrix. The materials to be used in this phase are listed in Table 2.

Only numerical sample identifiers should be used within the laboratory in order to safeguard confidentiality. The unambiguous identification of specimens is necessary to allow the laboratory results to be linked to demographic, dietary and/or lifestyle information also collected for the purpose of the study.

Table 2. Material for hair sample aliquoting/preparation

Material	Rationale	Alternative
Ethanol 70%	For cleaning the tweezers and scissors between sample processing.	
Latex gloves (powder free)	Used as a hygienic precaution.	Similar single-use powder-free disposable gloves made of other materials
Graph paper	The piece of sample for analysis has to be cut from the rest of the strand.	Ruler
Laboratory tweezers	For sample manipulation.	Any other item that allows correct sample manipulation
Scissors	The hair sample to be analysed has to be cut into small pieces.	Any clean and sharp scissors of an appropriate size
Paper pin	Used to immobilize the strand.	Any other object that ensures correct immobilization of the strand
Polypropylene vessel	For storing the hair samples.	Any other container that can preserve the sample from moisture
Labels	Samples must be unequivocally identified.	Write the ID code with a permanent marker pen

ID = identity.

1.9.1. Long hair samples immobilized

Immobilized hair samples (i.e. those longer than 5 cm (1.97 in) and those measuring 3.5–5 cm (1.4–1.97 in)), should be prepared as follows.

1. Remove the lock of hair from the bag in which the sample is provided using tweezers.
2. Place the strand on a sheet of graph paper covering the work surface and immobilize it with the pin clip at the opposite end from that closest to the scalp (Photo 15). The graph paper should be changed between samples.

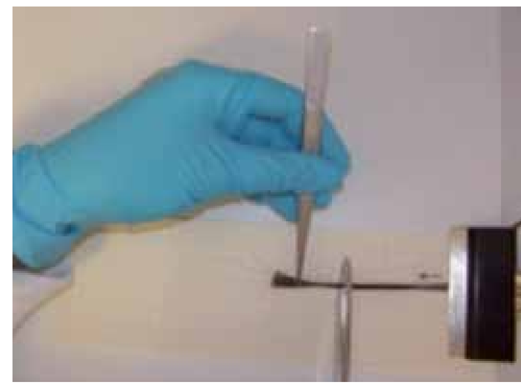


Photo 15. Immobilizing the strand with a pin clip.
© Instituto de Salud Carlos III

3. Cut the first 3 cm (or the defined length for the analysis) closest to the scalp with the help of the laboratory tweezers.
4. Place the segment into the vessel labelled with the sample code. The stopper should be labelled with the same code. The remaining hair should be disposed of as conventional waste.
5. Chop the sample into the smallest possible pieces with the scissors (Photo 16).

Photo 16. Chopping the sample into small pieces. © Instituto de Salud Carlos III

6. Ensure that the final sample is homogeneous (photos 17a and b).

Photos 17a and b. Ensuring a homogenous sample. © Instituto de Salud Carlos III

7. Follow the same procedure for the other samples.
8. Clean the tweezers and scissors with 70% ethanol between samples.
9. To prepare the hair aliquots, weigh the amount required for the laboratory in a polypropylene vessel and label it with the ID code of the sample.

1.9.2. Short hair samples non-immobilized

The preparation procedure for samples of hair which have not been immobilized (i.e. hair shorter than 3.5 cm (1.4 in)), is as follows.

1. Place the hair sample directly in the vessel using tweezers. The vessel and the stopper should be labelled with the same code.
2. Chop the sample into the smallest possible pieces with the scissors.
3. Ensure that the final sample is homogeneous.
4. Clean the tweezers and scissors with 70% ethanol between samples.
5. To prepare the hair aliquots, weigh the amount required for the laboratory in a polypropylene vessel and label it with the ID code of the sample.

1.10. Storage and conservation

Hair samples do not need special storage conditions. As such, they can be stored at room temperature but must be kept away from moisture, for example in a drawer or box.

A database including the sample ID code, aliquot ID code if necessary (e.g. internal code according to an internal quality control system), sampling date, aliquoting date and the amount remaining (approximately) after analysis, should be developed in order to ensure the traceability of samples and aliquots.

1.11. Quality control

1.11.1. Related documents

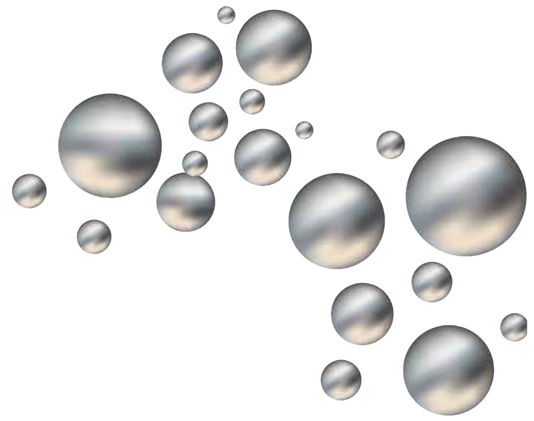
Traceability of the sample throughout the study is crucial, therefore this aspect should be guaranteed. As noted above, correct labelling of the samples and related documents is essential, but it is also necessary to be able to link the sample with the information provided by the volunteer. To this end, all documents related to the samples (questionnaires, registries, etc.) must be labelled with the same sample ID code immediately.

1.11.2. Checklists

Fieldworkers must control each step of the sampling procedure in order to ensure the quality of the samples. Checklists are a useful tool for this purpose and should be developed by the fieldwork team according to each situation.

The following control points should be considered.

- Pre-sampling: check that all material necessary for the sampling and all related documents are ready to be used (see example of a pre-sampling checklist in Annex 4).
- Post-sampling: check that all samples collected are accompanied by the corresponding documents in the shipment packaging. This control should include verification of the correspondence between identification codes and documents and samples. Fieldworkers should check that questionnaires and registries are properly filled out (see example of a post-sampling checklist in Annex 5).



2. Analysis of total mercury in human scalp hair

Numerous analytical methods are available for analysing total mercury in human hair, with cold vapour atomic absorption spectrometry (CVAAS) and cold vapour atomic fluorescence spectrometry (CVAFS) being the most widely used. Some methods, such as neutron activation analysis or X-ray fluorescence, allow segmental analysis along the hair. Also employed for mercury analysis in hair are inductively coupled plasma optical emission spectrometry (ICPOES), inductively coupled plasma atomic emission spectrometry (ICPAES), inductively coupled plasma mass spectrometry (ICPMS), graphite furnace atomic absorption spectrometry (GFAAS) and particle-induced X-ray emission (PIXE). Most of these methods require sample digestion prior to analysis, thereby increasing the possibility of contamination or losses. In contrast, direct solid introduction techniques, where no sample pre-treatment is required, result in very little chemical waste and have a much lower potential for contamination. In addition, the amount of hair required for analysis can be reduced, thus increasing sample throughput. These advantages make the direct analysis of mercury by atomic absorption spectrometry a very useful method for hair analysis in HBM studies *(7)*. This principle combines combustion, gold amalgamation of mercury and detection by atomic absorption spectrometry, and requires minimal sample preparation *(8).*

The washing of hair samples is a controversial issue that has been justified on the grounds of the possibility of the deposition of mercury present in the atmosphere. The ideal washing procedure must only remove external mercury, leaving the endogenous contamination intact. The inclusion of a washing step in hair analysis implies additional manipulation of the sample, and therefore the possibility of a loss of mercury or contamination.

Different washing methods employing a variety of solvents have been tested and some of these have been shown to be capable of removing endogenous mercury *(9–11)*. Consideration should be given to the convenience of washing samples in certain hot-spots where the main source of exposure to mercury is not fish consumption, such as the populations exposed to artisanal gold mining, living near industrial sites (e.g. coal-fired power and heat production, chloralkali plants, etc.) or mercury waste sites *(12)*. Additionally, specific questions to assess this potential exposure should be included in the study questionnaire.

The method described in this SOP permits the reliable and accurate determination of total mercury in hair samples at the typical concentration ranges for environmental and occupational exposure.

As this method does not require any sample pre-treatment or extraction, very little chemical waste is expected and the likelihood of contamination is minimal. The small amount of hair sample used and the short analysis times allow a high sample throughput.

Although a standard sample amount of 3.0–6.0 mg is recommended for this procedure, the laboratory may establish its own value taking into account the equipment used, the development and validation of the method and the expected values for its samples.

Special attention should be paid to the amount of hair received at the laboratory for analysis, as too low an amount of hair may compromise the test. As such, it is highly recommended that a minimum amount of 300 mg of hair be requested.

The limit of quantification (LOQ) for the methods described should be at least 0.01 nanograms of mercury per milligram of hair, in order to avoid mercury quantification problems in populations with low exposure to this contaminant.

An LOQ of 1 ng mercury has been established with the configuration of the two measurement cells described in this document. As the maximum sample weight with the equipment configuration described in this SOP is 100 mg, an LOQ of 0.01ng/mg could be achieved. Lower limits of detection (LODs) can be achieved, if necessary, by using instruments with a third measurement cell.

Special attention must be paid to the recovery rates for the lowest levels, as acceptable recovery rates are always above 80%.

The highest level for the calibration curve included in this method is 25 ng mercury, although calibration levels can be changed by the laboratory during the validation procedure.

Although the mercury analyser can reach levels of up to 1000 ng mercury, such levels are not necessary to determine mercury in hair samples, therefore they have not been considered here.

The linearity, precision, accuracy and uncertainty have been determined for each level of the calibration curve. Each laboratory should establish its own levels for method validation, although at least one concentration close to the LOQ should be included.

Where laboratories have other equipment for the detection of mercury in acid digested samples, it is advisable to follow the instructions provided by the instrument producers. The instructions for sampling and sample handling provided in this SOP are fit for purpose regardless of the instrumentation used for mercury detection. The LOD and LOQ should be checked to be suitable for hair samples.

2.1. Scope of the method

The method described in this SOP allows rapid and accurate quantification of mercury in human scalp hair. The assay range is 1–25 ng total mercury.

2.2. Technical principle

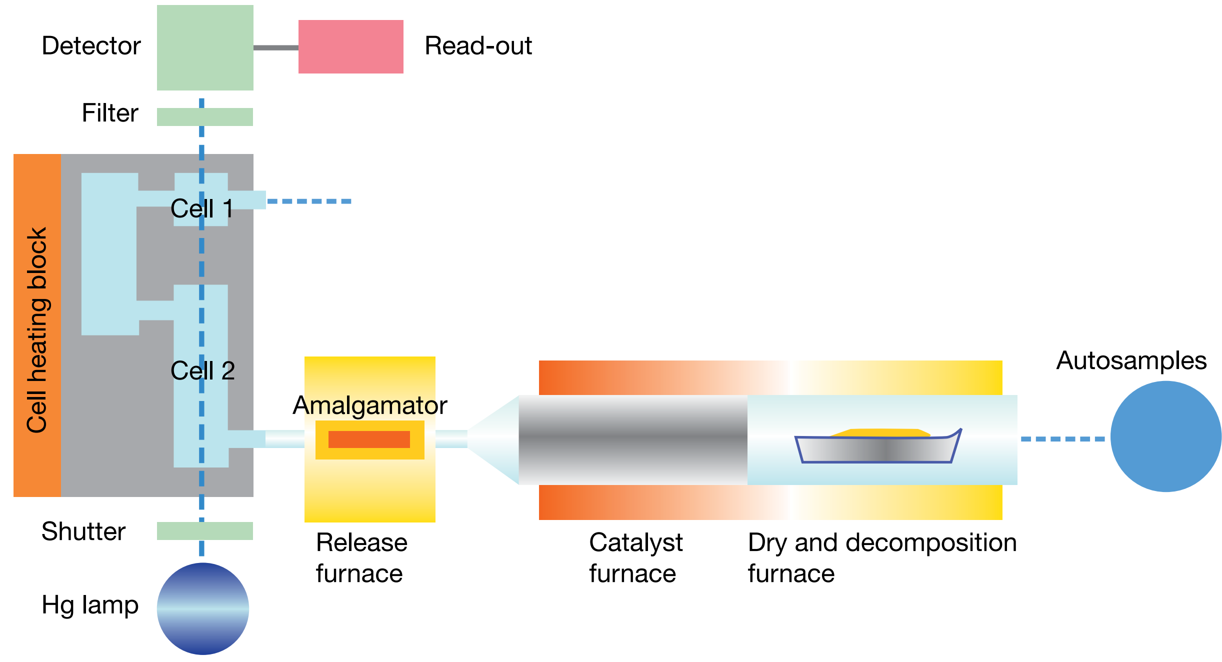
In this SOP, mercury in hair is determined by thermal decomposition-gold amalgamation-atomic absorption spectroscopy, a very sensitive and selective analytical technique that is highly suitable for trace-level analysis. This technique is commonly used in biomonitoring studies of long-term exposures for the detection of very low concentrations of mercury in non-invasive human samples.

Hair samples are weighed and introduced into the sample boat without any pre-treatment. The sample is then introduced into the direct mercury analyser (Fig. 2), where it is initially dried and then thermally decomposed in a continuous flow of oxygen. The combustion products are carried off and further decomposed over a hot catalyst bed. Mercury vapours are trapped on a gold amalgamator and subsequently desorbed for quantification. The mercury content is determined by atomic absorption spectrophotometry at 254 nm.

The quantitative determination of mercury is achieved using a calibration curve obtained from human hair reference materials analysed in the same way as the hair samples.

The direct mercury analyser can be configured in various different ways depending on the type and model used. For the procedure described here, a standard version equipped with two measuring cells of different path flow lengths was used. The guidance values for the working ranges of the two measuring cells are 0–20 ng mercury (low range) and 20–1000 ng mercury (high range).

Fig. 2. Direct mercury analyser



Hg = mercury.
Note: the standard version of the Milestone DMA-80 (illustrated here) is equipped with two measuring cells, a mercury lamp and mercury detector.
Source: Milestone *(13)*.

2.3. Safety precautions

The following safety precautions should be taken when analysing total mercury in human hair.

- No special safety precautions for biological hazards need be taken when working with hair.
- Gloves, a laboratory coat and safety glasses should be worn when handling all solutions.
- Special care should be taken with concentrated hydrochloric acid since it is a caustic chemical that can cause severe eye and skin damage.
- The possible hazards of equipment use include exposure to ultraviolet radiation, high voltages and high temperatures.

2.4. Equipment, materials and solutions

2.4.1. Equipment

The following equipment is required for analysing total mercury in human hair:

- direct mercury analyser (e.g. Milestone DMA-80).

2.4.2. Materials

The following materials are required for analysing total mercury in human hair:

- analytical balance (readability: 0.01 mg)
- micropipette, adjustable between 100 and 1000 µL

- scissors
- spatula
- nickel boats, 0.5 mL
- quartz boats, 1.5 mL
- antistatic tweezers
- sample tray conveyor
- 100 mL volumetric flask
- talc-free gloves.

2.4.3. Reagents, chemicals and gases

The following reagents, chemicals and gases are required for analysing total mercury in human hair:

- oxygen gas (99.995% purity)
- 70% ethanol (pro analysis)
- 37% hydrochloric acid (pro analysis)
- purified water (bidistilled water).

2.4.4. Solutions

The following solution is required for analysing total mercury in human hair:

- 0.37% hydrochloric acid (Pipette 1 mL of 37% hydrochloric acid into a 100 mL volumetric flask then fill to the nominal volume with ultrapure water).

2.4.5. Calibration standards

Two hair reference materials containing different mercury levels are used. The standards used in this SOP are as follows:

- NIES CRM No.13 (NIES-13): 4.42±0.20 ng/mg
- Reference Material IAEA-086: 0.573 (0.534–0.612) ng/mg.

2.5. Calibration

Calibration is performed using human hair reference materials NIES-13 and IAEA-086 in the range 1–25 ng mercury.

Table 3 lists the approximate weight of reference material that should be weighed in triplicate for each calibration point.

Calibration standards are then measured under the same conditions used for the samples. The quadratic equation parameters and correlation coefficient r^2 are obtained from the resulting calibration graph. These parameters should comply with the ranges established in the validation of the method.

The calibration frequency should be established by each laboratory. As a guidance value, a new calibration should be performed every three months. A new calibration should also be performed if the quality control sample values do not fall within the established range.

Table 3. Weight of reference materials

Hg (ng)	Reference standard	Weight (mg)
0		0.00
1	IAEA 086	1.75
2.5	IAEA 086	4.36
5	IAEA 086	8.73
10	NIES 13	2.26
15	NIES 13	3.39
20	NIES 13	4.53
25	NIES 13	5.66

Hg = mercury, IAEA = International Atomic Energy Agency, NIES = National Institute for Environmental Studies.

2.6. Procedure

2.6.1. Analytical equipment conditioning

Technical data

The technical data for the analytical equipment is as follows:

- principle: atomic absorption spectrometry;
- mercury detection system: single-beam spectrophotometer with sequential flow through two measurement cells;
- light source: low pressure mercury vapour lamp;
- wavelength: 253.65 nm;
- interference filter: 254 nm, 9 nm bandwidth;
- detector: silicon ultraviolet photodetector;
- autosampler: built-in, 40 positions;
- carrier gas: oxygen, inlet gas 4 bar (60 psi), flow rate approximately 200 mL/min.

The technical data listed here were established during configuration of the instrument used in this case.

Step 1. Preparation of the direct mercury analyser

The following operations should be carried out in accordance with the user manual: opening the oxygen supply, direct mercury analyser start-up and data file creation.

Step 2. System cleaning

An empty position should be measured following the appropriate method. The measurement conditions listed here were established for the configuration of the instrument used in this case and must be optimized for other instruments in accordance with the manufacturer's instructions:

- drying time: 10 s
- drying temperature: 200 °C
- decomposition time: 240 s
- decomposition temperature: 650 °C
- purge time: 60 s.

This step is repeated until two consecutive values of absorbance below 0.003 are obtained. If the desired background level is not attained the direct mercury analyser should be cleaned by analysing a hydrochloric acid solution (0.37%) in a quartz combustion boat, and then the system cleaning step should be repeated.

Step 3. System background check

Three empty nickel combustion boats should be analysed using the previous method. The absorbance values obtained must be less than 0.003, otherwise the sample boat must be cleaned.

Step 4. Pre-measurement quality control

Two samples of certified reference material IAEA-086 containing approximately 5 ng mercury (approximately 8.7 mg of material) should be analysed with the following parameters (guidance parameters, which must be optimized for other instruments in accordance with the manufacturer's instructions):

- drying temperature: 200 °C
- drying time: 60 s
- decomposition temperature: 650 °C
- decomposition time: 150 s
- purge time: 60 s.

The concentration determined for the second reference material sample should be within the uncertainty range for this point described in the validation. If this is not the case, the measurement should be repeated until a value within that range is obtained. If such a value is not obtained after five attempts, the system should be recalibrated.

Once the previous four steps have been successfully completed, the direct mercury analyser is ready for sample analysis.

2.6.2. Analytical determination

Sample weighing

Both the combustion boats and the support used to weigh the hair samples should be handled using tweezers.

Place the combustion boat support on the balance. Place a nickel combustion boat on top of the support and set the balance to zero.

Open the flask containing the sample and transfer small portions of hair to the combustion boat, using a spatula, until a weight of 3.0–6.0 mg is reached.

Place the combustion boat containing the sample onto the sample tray and note the sample code, weight and tray position in the weighing log. Three replicates should be prepared for each sample.

The spatula should be cleaned with 70% ethanol between samples.

To ensure that the analyser is measuring correctly, a quality-control sample consisting of a weight of reference material, which will vary randomly between the points included on the calibration curve, should be weighed every three samples (nine combustion boats).

Sample analysis

The nickel combustion boats containing samples and quality controls should be placed in the direct mercury analyser autosampler in the order in which they were weighed.

The samples and quality controls should then be programmed by entering their code and weight and selecting the method and last valid human hair calibration. The parameters of the method are as follows (guidance parameters must be optimized for other instruments in accordance with the manufacturer's instructions):

- drying temperature: 200 °C
- drying time: 60 s
- decomposition temperature: 650 °C
- decomposition time: 150 s
- purge time: 60 s.

Under these conditions, the analysis time for each sample is around five minutes.

2.6.3. Calculation of the analytical results

Data are reported directly by the equipment in terms of nanograms of mercury per milligram of hair (ng Hg/mg) by interpolation of the measurement on the calibration curve.

The final value reported corresponds to the average of the three replicated measurements per sample. The standard deviation of these measurements can be calculated according to the following formula.

$$SD = \sqrt{\frac{\sum(c_i - \bar{c})^2}{n-1}}$$

SD – standard deviation

c_i – individual sample value

c – mean

n – number of measurements

The measurement uncertainty can be calculated using the formula obtained in the validation procedure.

2.6.4. Reportable results range

Mercury values are reportable in the range between the LOQ (1 ng mercury) and the highest calibration standard (25 ng mercury).

If the amount of mercury obtained in the sample is out of this range, the sample should be retested as follows.

- If the value is below 1 ng (the lowest concentration of mercury included in the calibration), on the basis of the obtained concentration, the necessary amount of hair for three new replicates should be weighed in order to obtain a new determination within the calibration range. In light of the organic content of the sample and the capacity of the nickel boats used, the maximum sample size that can be introduced into the DMA-80 direct mercury analyser is 100 mg.
- If the value is above 25 ng (highest standard of mercury included in the calibration), on the basis of the obtained concentration, the necessary amount of hair for three new replicates should be weighed in order to obtain a new determination within the calibration range. The sample weight should not be less than 1 mg.

Only those measurements obtained between two quality controls whose values lie within the established range (assigned value for the reference material ± uncertainty in that level) are considered valid. A new calibration should be performed if the values for the quality control samples do not lie within the established range.

If the concentration of one of the replicates is not within the range determined by the mean ± uncertainty, the Dixon test should be applied to determine whether the suspected value should discarded.

$$Q = \frac{X_{suspected} - X_{nearest}}{X_{highest} - X_{lowest}}$$

Q – Q value for evaluation according to Dixon Q test

X – single value (suspected value, nearest to suspected value, highest value and lowest value)

If Q is greater than or equal to 0.970, the suspected value can be rejected and the concentration of the sample calculated as the mean of the two remaining values. If it is lower, the sample should be re-analysed.

2.7. Quality control

The precision and accuracy of biomarker analyses carried out by toxicological laboratories must be continuously checked by means of quality assurance measures.

In general, quality assurance in laboratories comprises internal and external quality control (see also the Quality control programme for mercury human biomonitoring).

2.7.1. Internal quality control

Internal quality assurance serves to systematically monitor repeatability, check for random errors, and assess the accuracy of quantitative laboratory investigations.

In practice, the repeatability is monitored by using a control material (reference material), which is measured as part of each analytical series. The results of the daily or batch-wise internal quality controls are entered into control charts.

If not commercially available, the control material can be prepared by spiking a pool of native biological material (blood, urine, etc.) with a defined amount of the analyte (biomarker). Aliquots of this pool can be used for internal quality control as well as for inter-laboratory comparison programmes. These aliquots have proven to be, and to remain, homogeneous under specific storage and shipment

conditions, with the analyte concentration remaining unchanged. The control material should cover the whole concentration range (e.g. Qlow, Qmedium, Qhigh) and also include blanks.

Accuracy should preferably be tested using a certified reference material (CRM). A CRM is a material (biological material) with a certified concentration of one or more analytes. Certification is performed as part of a programme in which laboratories that are highly skilled in analysing the biomarker in question, analyse control materials.

A certified value is established for each analyte following a validation procedure that includes expert judgment as well as statistical procedures. CRMs are therefore expensive and should only be used when validating or revalidating an analytical method.

For this SOP, quality control materials are used to evaluate the accuracy and precision of the analysis process and to determine whether the analytical system produces results that are acceptably accurate and precise.

Two hair reference materials containing different levels of mercury, namely NIES CRM No.13 (4.42 ng/mg) and Reference Material IAEA-086 (0.573 ng/mg), have been used to evaluate the method.

Quality controls consisting of a weight of reference material that varies randomly among the points included in the calibration curve are included every three samples (nine measurements).

Only those measurements obtained between two quality controls whose values lie within the established range (assigned value for the reference material ± uncertainty in that level) are considered valid.

Two blind hair samples are measured each year as part of the internal quality control programme.

2.7.2. External quality control

External quality control is a means of improving the comparability and accuracy of analytical results. Comparability is the pre-state of accuracy and ensures that analytical results can be compared between laboratories and with the corresponding limit values. Comparable and accurate results in HBM are necessary to achieve equal health prevention irrespective of the laboratory that analyses the biological sample.

An inter-laboratory comparability investigation (ICI) is a means of harmonizing analytical methods and their application, thereby improving the comparability of analytical results. Control materials (reference materials) can be used for this purpose. ICIs are even necessary when laboratories use the same analytical SOP.

An external quality assessment scheme (EQUAS) is a means of improving the accuracy of analytical results. For this purpose, a control material is usually analysed in reference laboratories that have been shown to be highly skilled in analysing a specific biomarker. The results obtained by the reference laboratories form the basis on which the assigned values and tolerance ranges for each of the biomarkers tested are determined. Those laboratories that participate in an EQUAS are certified for those results that fall within the tolerance ranges.

External quality control is realized by participation in round-robin experiments (three times a year). As an example, it is recommended to participate regularly in the Quebec Multielement External Quality Assessment Scheme (QMEQAS) organized by the Centre de Toxicologie du Quebec – Institut National de Santé Publique, Canada.

2.8. Evaluation of the method

2.8.1. Response function

The relationship between the response of an analytical instrument and the concentration or amount of an analyte introduced into the instrument is referred to as the "calibration curve".

For this SOP, the response of the method has been tested for the range 0–25 ng mercury and a quadratic regression model has been established for the calibration curve.

The data obtained are analysed statistically to calculate the regression curve, and determination coefficient.

A curve with a determination coefficient higher than 0.997 should be obtained.

2.8.2. Precision

This is a measure of the degree to which the analytical results are scattered due to random errors.

Precision is described statistically by means of the standard deviation or the confidence interval. We can distinguish between the following:

- precision under repeated conditions (repeatability)
- precision under comparable conditions (reproducibility).

The materials used when performing these measurements, and the calculation methods used, should be defined.

The different levels of mercury included in the calibration (see Section 2.5) were measured in triplicate on 16 different days, by two different analysts, to establish the precision for each level, which can be found in tables 4–6.

Table 4. Maximum standard deviation allowed

Concentration (ng Hg)	RSDrepro	RSDrepet
1	4.9	6.4
2.5	4.1	4.9
5	3.4	3.6
10	1.2	2.3
15	0.8	1.4
20	0.5	0.9
25	0.3	1.1

Hg = mercury; ng = nanogram; RSDrepet = relative standard deviation for repeatability;
RSDrepro = relative standard deviation for reproducibility.

2.8.3. Accuracy

This is a measure of the deviation of the measured value from the correct ("true") value due to a systematic error. The following approaches can be used to test the accuracy of a method:

- performance of recovery tests (spiking procedures);
- participation in inter-laboratory comparability investigations in which the theoretical value is ascertained by authorized reference laboratories;
- comparison of the analytical procedure to be validated with a reference procedure certified for determination of the parameter in the relevant sample matrix;
- comparison of the analytical results for a CRM with the certified reference value.

In our case, two hair reference materials containing different mercury levels, namely NIES CRM No.13 (4.42 42 ng/mg) and Reference Material IAEA-086 (0.573 573 ng/mg), have been used to determine the accuracy of the method.

The different levels of mercury included in the calibration (see Section 2.5) were measured to establish the accuracy for each level. The relative recovery rates are summarized in the Table 5.

Table 5. Mercury concentrations and recovery rates

Concentration (ng Hg)	Recovery (%)	Range (%)
1	101.7	83.2–131.0
2.5	99.5	88.5–126.2
5	100.9	94.5–135.7
10	98.5	88.2–102.7
15	100.6	97.7–106.7
20	100.4	97.8–103.1
25	99.7	97.1–130.2

Hg = mercury; ng = nanogram.

The recovery rates, taking into account the measurement uncertainty, must include 100%. If this is not the case, the initial concentration point of the calibration curve should be re-evaluated according to the LOQ obtained for the method.

2.8.4. Uncertainty

This is defined as the overall confidence interval or prognostic range of the measured results after taking possible errors into account. The standard measurement uncertainty is equivalent to the standard deviation of a measurement series. The combined standard measurement uncertainty includes all the working steps, interference factors and influencing factors as well as their mutual influence. The extended measurement uncertainty includes the function of a confidence interval.

The uncertainty for each of the mercury levels evaluated is listed in Table 6.

Table 6. Mercury concentrations and uncertainty level

Concentration (ng Hg)	Uncertainty %)
1	18.0
2.5	11.3
5	10.0
10	5.5
15	4.9
20	4.7
25	4.6

Hg = mercury; ng = nanogram.

The uncertainty has been calculated in accordance with the *EA guidelines on the expression of uncertainty in quantitative testing (EA-4/16) (14) and the Guide to the expression of uncertainty in measurement (15).*

2.8.5. Limit of quantification

The lower LOQ indicates the lowest possible analyte concentration that can be determined with a pre-defined uncertainty (usually 33%). The upper LOQ indicates the highest possible analyte concentration that can be determined.

The LOQ must be included in the calibration curve and can be calculated using various different methods.

Determination of the signal/background noise ratio

The background noise is determined as follows.

- The intensity of the background noise (s0) is determined in relation to the analyte.
- The LOD is calculated as three times the mean intensity of the background noise signal (LOD = 3 x s0).
- The LOQ is calculated as nine times the mean intensity of the background noise signal (LOQ = 9 x s0).

Other procedures

It should be noted that blank values in native samples have an influence on the choice of method and the approach used:

- standard deviation procedure (according to EURACHEM)
- blank value procedure (according to DIN 32 645)
- calibration curve procedure (according to DIN 32 645).

In this SOP, the LOQ has been calculated using the calibration curve procedure and the result obtained is below the lowest value of the calibration curve, namely 1 ng mercury, so this will be the LOQ applied.

If a maximum sample weight of 100 mg is considered, the LOQ in terms of concentration is 0.01 ng mercury/mg hair.

3. Data interpretation

The toxicity of methylmercury is a major public health concern, as the general population is exposed via their diet. This is of particular concern in the case of fetuses, very young children, pregnant women and those of childbearing age, due to the ability of methylmercury to cross the placenta and blood–brain barrier, thus resulting in serious effects on the developing nervous system. Although the neurological effects of methylmercury have been well known for many years, the complexity of assessing the adverse effects resulting from chronic exposure to levels present in the environment makes it difficult to establish a health-based value. This is particularly so as the range and magnitude of the neurological effects of methylmercury varies with the time window in which exposure takes place. It has been observed that effects in adults are localized in certain regions of the brain, whereas exposure during the developmental phase results in more extended and widespread effects. In this case, neuronal division and migration processes are affected and the cytoarchitecture of the developing brain is altered *(16–18)*. As a result of this difference in damage, the clinical manifestations are also different, as could clearly be observed in Minamata after the large-scale poisoning suffered by its population. Thus, adults showed sensory disorders in the limbs, ataxia, hearing and vision problems, loss of balance, slurred speech and, in severe cases, loss of consciousness and death. By contrast, the effects in children born after the incident were even more serious, with a range of widespread effects including mental retardation, poor reflexes, impaired cerebellar functions, growth and nutritional disorders, dysarthria and limb deformity, and, in 75–95% of cases, hyperkinesia, hyper-salivation, strabismus, and pyramidal system and paroxysmal disorders *(19)*.

Although much was learned about the effects of methylmercury in humans from the Minamata incident, the situation as regards environmental exposure to methylmercury is quite different. The levels at which the general population are exposed via fish consumption are significantly lower than those present in fish after the Minamata spill, thus making the assessment of adverse effects a highly complex task. This complexity arises due to the difficulty in identifying and estimating the neurological effects, which can be as subtle and nonspecific as a reduced intellectual coefficient. There may also be an interaction between the adverse effects of methylmercury and nutrients present in fish. Fish is a high-quality food that provides polyunsaturated acids and other nutrients that are essential for correct development of the nervous system and can counteract the adverse effects of methylmercury *(20,21)*. This is one of the hypotheses that have been proposed to explain the disparities observed in the Faroe Islands, Seychelles and New Zealand studies. This uncertainty concerning the effects resulting from low-level exposure also applies to other adverse effects that have been linked to methylmercury exposure (e.g. cardiovascular and immunological effects) *(22)*.

In light of the above, the interpretation of mercury concentration in hair is difficult, as reflected by the absence of an accepted health-based value to support data interpretation.

The interpretation of mercury concentrations in hair requires the collection of basic data about mercury exposure. This information can be collected by including some specific questions in the epidemiological questionnaire. As diet is an important source of exposure to environmental mercury and some nutrients affect its absorption, the questionnaire should contain sections dealing with characterization of the diet *(12,22–25)*. The concentration of methylmercury in fish depends on the species, size and region in which they were caught *(26–29)*, therefore subjects should be asked about the frequency of consumption and the type of fish consumed.

Assuming that hair grows at a rate of 1 cm per month, the length of the segment of hair analyzed will give information about exposure at different times. As diet can vary seasonally, and therefore mercury levels in hair can also vary, it may be advisable to include questions about diet at different times (e.g. frequency on a regular basis and during the last three months).

3.1. Values for interpretation

The definition of reference values from HBM studies allows a comparison between populations. These values represent the chemical concentration in a particular population (or subgroup) as a consequence of exposure in a specific timeframe, and are derived from analysis of the concentration in hair, blood, urine or other biological matrices. Reference values are usually based on the 90th or 95th percentile and the corresponding 95% confidence interval *(30,31)* and can be representative of the general population or only of specific groups. However, reference values must be revised and updated as they describe a particular population at a given time and can be influenced by several factors, such as age, geographic region, habits and lifestyles, genetic polymorphisms and even by an improvement in analytical techniques *(32)*.

Reference values are a statistical description of the typical range of concentrations in the reference populations but are not health based *(31)*. To interpret the levels of a compound in the body from a toxicological point of view, it is necessary to define health-based guidance values. Although HBM values defined by the German Human Biomonitoring Commission should be the preferred option, these values have been defined for only a few compounds. These HBM values give a clear scale for interpreting the individual results and the actions to be taken, depending on whether they are above or below the HBM I or HBM II value.

Other health-based guidance values useful for interpreting HBM data are the so-called "biomonitoring equivalents". These are defined as the concentration of a chemical (or metabolite) in hair, blood, urine or some other tissue consistent with exposure guidance values, such as tolerable daily intake (TDI), reference dose (RfD), reference concentration (RfC) or risk-specific doses *(26)*. However, biomonitoring equivalents do not give a cut-off value to distinguish between safe and unsafe exposure and do not predict adverse effects once this value has been exceeded. As such, they should not be used to interpret individual data for predicting the potential for adverse effects *(33)*.

The particular case of mercury in hair has no defined HBM value. However, the HBM value for mercury in blood defined by the German Human Biomonitoring Commission was derived from a concentration of mercury in hair of 5 mg/kg *(34)* and could therefore be used to interpret mercury levels in hair. Table 7 shows values from different agencies that are usually employed when interpreting mercury levels in hair. However, it should be noted that these values are defined for vulnerable groups (children, women of childbearing potential and pregnant women) rather than for the general population.

In addition to the values from Table 7, the data obtained can be compared with reference values (95th) obtained in other studies; however, given the above comments concerning reference values, the population should be as comparable as possible (i.e. should cover the same age range, similar lifestyles, close in time, etc.).

Table 7. Mercury concentrations and uncertainty level

Agency	Hair levels	Reference
United States Environmental Protection Agency (US EPA)	1.0 µg/g	(35)
European Food Safety Authority (EFSA)	1.9 µg/g	(29)
German Environment Agency (UBA)	5.0 µg/g	(34)

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Annex 1. Registry for collected hair samples

ORIGIN:

CENTRE:
ADDRESS:
CITY/COUNTRY:
CONTACT (NAME AND PHONE):

ID code	Sampling date (dd/mm/yy)	Sampling questionnaire attached(Y/N)	Comments

Shipment date:

Name and signature of fieldworker:

Annex 2. Questionnaire for hair sampling

ID code:
Date of interview:
Sampling location:
Fieldworker:

1. Sample collected

☐ Yes ☐ No Reasons: ....

2. Sampling date (dd/mm/yyyy): ____/____/________

3. Natural hair colour:

☐ Black
☐ Dark brown
☐ Brown
☐ Blonde
☐ Redhead
☐ Grey
☐ White

4. Natural hair structure:

☐ Straight
☐ Wavy
☐ Curly

5. Has the hair been dyed/tinted within the previous 6 months?

☐ No ☐ Yes Months ago …………………
Weeks ago …………………..

6. Has the hair been treated within the last year, for example a perm or with a hair straightener?

☐ No ☐ Yes Months ago …………………
Weeks ago …………………..

7. Last washing of the hair:

☐ Days ago *Specify................*
☐ Yesterday
☐ Today

8. Length of sampled hair (from the scalp): ______ cm

9. Sample labelling:

☐ Yes ☐ No Reasons: ....

10. Comments:

Note. This questionnaire only collects basic information regarding the hair sample. Information related to mercury exposure is not included.

Annex 3. Registry of sample reception

1. ORIGIN OF THE SAMPLE:

Centre:
City/Country:
Date of sampling:

2. SAMPLE RECEIVED: **Signature of reviewer:**

☐ Hair

3. SAMPLE RECEPTION: Date (dd/mm/yy) Time (hh:mm)

A) PACKAGING

☐ NO PROBLEMS DETECTED
☐ PROBLEMS DETECTED:

- ☐ Packaging damaged
- ☐ Cooling agents defrosted
- ☐ Others:______________________

B) SAMPLES

☐ NO PROBLEMS DETECTED
☐ PROBLEMS DETECTED:

- ☐ Insufficient amount/volume (*specify the matrix*)___
- ☐ Inconsistency in the ID codes
- ☐ Others:______________________

C) DOCUMENTS

☐ NO PROBLEMS DETECTED
☐ PROBLEMS DETECTED:

- ☐ Absence of the registry of collected samples
- ☐ Absence of the hair sampling questionnaire
- ☐ Absence of the study questionnaire
- ☐ Inconsistency in the ID codes
- ☐ Others:______________________

4. DATE OF STORAGE/BIOBANKING:

5. COMMENTS:

ID CODES FOR RELATED SAMPLES

Cord blood	Urine
☐	☐

Annex 4. Pre-sampling checklist

1. **Are the sampling materials prepared for the fieldwork?**

- [ ] Alcohol and cotton
- [ ] Latex gloves
- [ ] Scissors
- [ ] ID labels
- [ ] Permanent marker pen
- [ ] Adhesive tape
- [ ] Paper bags
- [ ] Zip-lock plastic bags

2. **Are all documents related to the sampling ready?**

- [ ] Registry for collected samples
- [ ] Hair sampling questionnaires
- [ ] Informed consent form

3. **Observation**..

....

....

Annex 5. Post-sampling check-list

1. **Are all samples correctly labelled and recorded in the registry for collected samples?**

☐ Yes ☐ No

Please describe any problem detected and the solution:.......................................

....

....

2. **Are all the informed consent forms signed and labelled?**

☐ Yes ☐ No

Please describe any problem detected and the solution:.......................................

....

....

3. **Are all sampling questionnaires correctly filled in and labelled?**

☐ Yes ☐ No

Please describe any problem detected and the solution:.......................................

....

....

4. **Is there a correlation between the ID codes of the samples and the documents?**

☐ Yes ☐ No

Please describe any problem detected and the solution:.......................................

....

....

Standard operating procedure for assessment of mercury in cord blood

(sampling, analysis of total mercury, interpretation of results)

Abstract

This standard operating procedure describes the process of assessing exposure to mercury through human biomonitoring using cord blood as a biological matrix. Sampling of cord blood, analysis of total mercury and interpretation of results are detailed in this document.

Keywords

Mercury – analysis
Methylmercury Compounds – analysis
Biomarkers – analysis
Fetal Blood – chemistry
Umbilical Cord – chemistry
Maternal Exposure
Maternal-Fetal Exchange
Infant, Newborn
Environmental Exposure

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Abbreviations

Hg	mercury
ID	identity
LOD	limit of detection
LOQ	limit of quantification
SOP	standard operating procedure

Introduction: cord blood as a matrix for mercury human biomonitoring

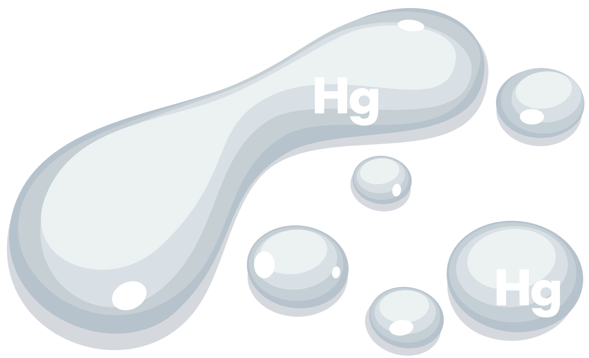
Mercury (Hg) is a toxic and persistent pollutant that bioaccumulates and biomagnifies through food webs *(1,2)*. People are exposed to methylmercury mainly through their diet, especially through the consumption of freshwater and marine fish *(3)*. They may also be exposed to elemental or inorganic mercury through inhalation during occupational activities and via dental amalgams *(4)*. Exposure to elemental or inorganic mercury can also occur due to the use of some skin lightening creams and soaps, the presence of mercury in some traditional medicines, the use of mercury in cultural practices, and accidental mercury spills in homes, schools or other locations *(5)*.

Although the general population is exposed to only low levels of mercury, the occurrence and severity of its adverse health effects depend on its chemical form, dose, the age or developmental stage of those exposed (the fetus is considered to be the most susceptible), and the duration and route of exposure *(1,6)*.

The primary targets for mercury toxicity are the nervous system, kidneys and cardiovascular system, with developing organ systems (such as the fetal nervous system) being the most sensitive to its toxic effects. Nervous system effects are the most sensitive toxicological endpoint observed following exposure to elemental mercury and methylmercury, whereas kidney damage is the key endpoint in exposure to inorganic mercury compounds *(1)*.

The selection of biological media to assess human exposure depends on mercury compounds, exposure patterns (e.g. chronic, acute) and time of sampling after the exposure *(7)*. The presence of mercury in blood represents short-term exposure to organic and inorganic mercury, and does not provide information on long-term exposure and its variations *(7–9)*. Mercury levels in cord blood and hair are suitable biomarkers of prenatal low-level methylmercury exposure due to its selective transfer through biological barriers such as blood or hair and placenta, while inorganic mercury does not have this property. Levels in cord blood are proportional to maternal blood, but with slightly higher levels *(10,11)*. As a biomarker of prenatal exposure, mercury in cord blood is preferable, as it provides information on both the exposure of mothers and prenatal exposures of their children *(12)*.

Mercury in cord blood shows a better association with mercury-related neurobehavioural deficit in the child compared to mercury determined in maternal hair *(13)*. Hair mercury concentrations can be affected by several factors, including hair colour and variable growth rates, which limit its usefulness as an indicator of mercury concentrations in the body *(14)*. Cord blood is a non-invasive matrix, but should be collected by a nurse after birth.



1. Sampling of cord blood

1.1. Scope of the method

Collection of cord blood should be done immediately after delivery in a delivery room. Two basic methods can be used for collecting cord blood.

- Collection of cord blood after the baby is born, but before delivery of the placenta; this is referred to as "in-utero collection", and is usually performed by a physician or a midwife.
- Collection of cord blood after the placenta is delivered and the umbilical cord is clamped; this is referred as "ex-utero collection". This method can take place in a separate area and can be performed by nursing and/or research staff.

WHO recommends that only ex-utero methods be used for cord blood sampling to prevent any negative effects on the mother and child.

1.2. Safety precautions

All precautionary measures necessary for working with blood specimens are applicable for sampling of cord blood.

- Use products that are specifically designed for cord blood collection; if needle and syringe are used, use a safe needle that can be separated from the syringe barrel.
- Gloves should be worn at all times when sampling cord blood.
- If gloves become punctured or grossly contaminated during use, they should be removed and disposed of, hands should be washed and clean gloves put on.
- On completion of handling samples, gloves should always be removed and discarded, and hands should be washed.
- Disinfectants should be used if necessary.

1.3. Materials required

The materials required for sampling cord blood are as follows:

- registration sheets for samples;
- sampling materials:
  - needles and syringes;
  - tube B1: polypropylene tube 50 mL with 0.5 mL ethylenediaminetetraacetic acid (EDTA);
  - tube B2: 10 mL polypropylene metal-free tube.

The maternity hospital should get prepared boxes with labelled collection tubes from a research laboratory in advance.

Instructions should be provided for contacting research staff and for collection, storage and transport.

1.4. Preparation/pre-treatment of the sampling material

All tubes should be washed with 10% nitric acid in purified water solution to eliminate background contamination. The details of this procedure are described below.

1. Prepare a 10% nitric acid solution from nitric acid (65% extra pure) and purified water.
2. Put the solution in a tank.
3. Open the tubes and put the tubes and lids into the tank. Ensure that all items are completely immersed.
4. Tubes should be immersed in this tank for at least three hours (preferably overnight).
5. Take out the tubes from the acid tank and put them in a tank with purified water. Shake them for 2–3 minutes. Then, move the tubes and lids to a second tank of purified water. Shake them again for 2–3 minutes.
6. Take out the tubes and lids and put them face down on clean filter paper to dry them.
7. Once the drying is finished, screw on the lids of the resulting nitric acid pre-treated tubes. Make a mark on tube B1 indicating the minimum amount of cord blood required (10 mL). Put the pre-treated vessel into a zip-lock plastic bag.

The acid solution can be re-used up to one month after its preparation. All of the procedures must be done in a chemical fume hood, using suitable personal protective equipment.

To check for contaminants, after the cleaning procedure, 5% of all tubes should be randomly selected and analysed for the mercury contamination. For this purpose, the tubes should be filled with purified water and shaken for 10 minutes. An aliquot should be analysed for the biomarkers in question (total mercury).

1.5. Sampling procedure

The cord blood collection procedure (ex-utero) is as follows.

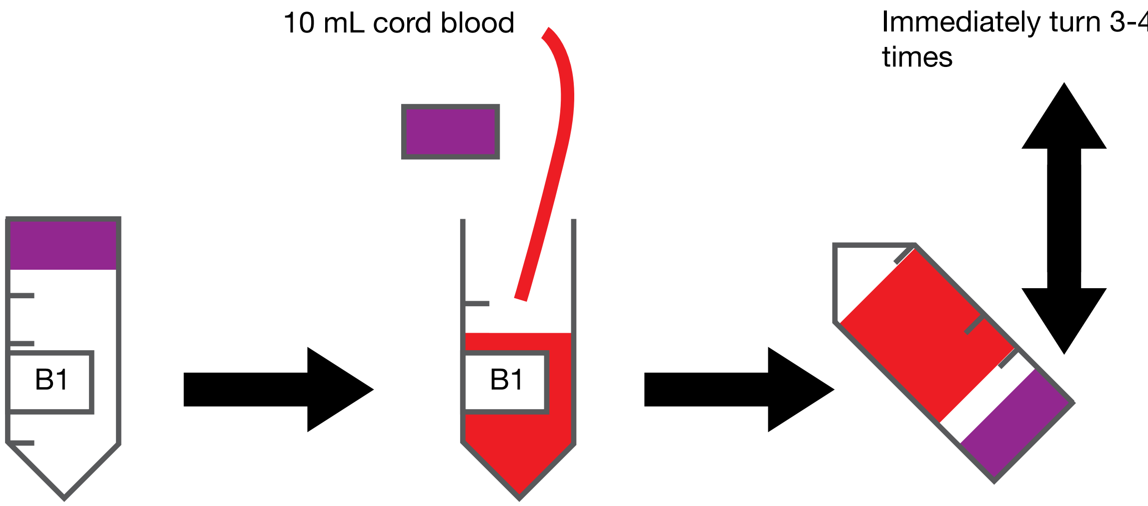
1. Following the clamping of the umbilical cord and separation of the infant and placenta, wipe any maternal blood off the umbilical cord using gauze soaked in alcohol or an iodine-based antiseptic liquid for at least 30 seconds at the venipuncture site (vein). Sterilization of the cord at the puncture site is very important as it will prevent any contamination of the cord blood.
2. Allow the venipuncture site to dry.
3. Remove the needle covers and keep them nearby as you will need to recap the needles at the end of the collection procedure.
4. Puncture the vein of the umbilical cord at the sterilized site and allow the blood to flow out into the syringe.
5. When the syringe is full, change to a narrower needle and insert it into the vacutainer stopper so that the blood drains into the tube.
6. If blood flow stops, please proceed to the sterilization of another site closer to the placenta and use a second needle for further blood collection.
7. At the end of the collection procedure, the needles must be recapped using the saved covers to prevent needle accidents.
8. After collection, wait for 10 minutes. Then the tube should be gently turned to thoroughly mix the blood sample.

The cord blood sample should be collected and labelled as follows (see Fig. 1).

1. Collect the cord blood in tube B1 (minimum 10 mL).
2. Tube B1: turn three to four times for the blood to mix with the EDTA.

3. Put tube B1 into the zip lock bag and bring it to the laboratory.
4. Fill out the sampling form (Annex 1).

Fig. 1. Collecting the cord blood



Register the participant's information on the registration form (Annex 2). The following details should be documented:

- participant's name
- sample identity (ID) code
- date and time of child birth
- start and end times of the collection of cord blood
- collected volume of cord blood.

The form should be submitted to the survey coordination centre or the survey coordinator.

1.6. Labelling

The plastic bag, questionnaires and all collection tubes should be labelled with the identification code of the participant.

1.7. Transport and conservation of the sample

The samples must be sent to the local hospital laboratory or another special storage place in the hospital within two hours of sampling. Samples should be kept in a refrigerator or in a cold box during transportation, at below 4 °C.

1.8. Sample reception

The criteria for accepting or rejecting a sample should be defined in advance and applied during sample reception. These criteria should focus on transportation conditions, attached documentation, integrity of the packaging, correct identification, and the amount of the sample (sufficient for analysis and biobanking if samples will be stored for other research purposes).

The following points should be checked upon receipt of the cord blood samples.

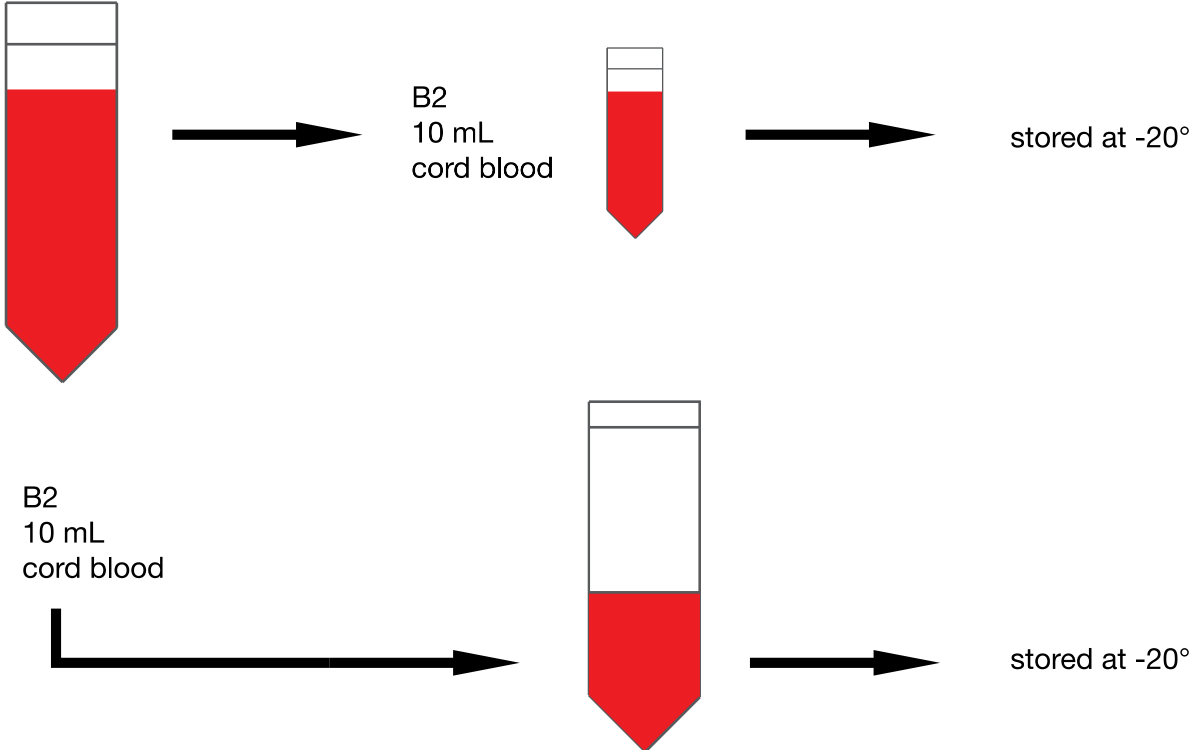
- Amount and quality of the samples: an unacceptable specimen is of low volume (< 0.25 mL).
- Suspect contamination: due to improper collection procedures or collection devices.
- Integrity of the packaging: must be correctly sealed and must not have been manipulated (Note: a security seal can be placed on the package at the sampling site).
- Attached documents: all samples listed in the registry of collected samples should be contained in the package; they must be accompanied by the corresponding documents (questionnaires, etc.).
- Correct identification: samples and documents received must be properly identified with the corresponding ID code.

1.9. Sample aliquoting and preparation

Aliquoting can be done in a hospital laboratory in a mercury free atmosphere. The hospital laboratory should prepare in advance boxes with labelled aliquoting tubes. For mercury measurements the optimal amount of specimen is 1–2 mL (the minimum is 0.5 mL). If larger quantities are intended for storage, it is advisable to store aliquots of 1–2 mL in separate vials, rather than larger volumes in one sampling vial. Frequent thawing of blood may result in mercury losses.

Fig. 2 illustrates the process of aliquoting and storage. In Tube B2, an aliquot of 2 mL of cord blood is stored at -20 °C for analysis of mercury. A corning tube (B1) with the remaining blood is stored for possible duplicate analysis of mercury later on or analysis of other pollutants (if samples are planned to be stored for other research purposes).

Fig. 2. Aliquoting and storage of the sample



1.10. Storage and conservation

All aliquoted samples have to be stored in a freezer at below -20 °C until analysis. Specimen stability has been demonstrated for several months at -20 °C, or for several years at -70 °C.

1.11. Quality control: traceability

Traceability of the sample throughout the study is crucial, therefore this aspect should be guaranteed. The cord blood sample must be labelled with the ID code. As noted above, correct labelling of the samples and related documents is essential, but it is also necessary to be able to link the sample with the information provided by the volunteer. To this end, all documents related to the samples (questionnaires, registries, etc.) must be labelled with the same sample ID code immediately.

2. Analysis of total mercury in cord blood

The determination of total mercury in cord blood requires sensitive analytical methods performed under good quality-control conditions. Numerous analytical methods are available for analysis of total mercury in human blood, some of which are automated. In principle, two approaches exist: *(1)* methods based on acid digestion followed by cold vapour atomic absorption spectroscopy (CVAAS), cold vapour atomic fluorescence spectroscopy (CVAFS) and/or inductively coupled plasma mass spectrometry (ICPMS); and *(2)* methods based on thermal decomposition and CVAAS. The method described in this standard operating procedure (SOP) is based on the second principle combining combustion, gold amalgamation of mercury and detection by atomic absorption spectroscopy. It permits the reliable and accurate determination of total mercury in blood samples at the typical concentration ranges for environmental and occupational exposure. In order to perform these measurements a dedicated instrument needs to be provided as described later in the procedure. When such an instrument is not available, laboratories can use a procedure described in the SOP for assessment of total mercury in urine or similar *(15)*.

Numerous laboratories also use a technique proposed in the guidelines prepared by the National Institute for Minamata Disease, Japan *(15)*. This method is proposed in the SOP for assessment of total mercury in urine and can also be used for blood. The method is simple, sensitive, efficient, and most of all low cost as it requires simple equipment with atmospheric air as a carrier gas.

The method described in this SOP does not require any sample pre-treatment or extraction, very little chemical waste is expected and the likelihood of contamination is minimal.

If laboratories have other equipment for the detection of mercury in acid digested samples, it is advisable to follow the instructions provided by the instrument producers. The instructions for sampling and sample handling provided in this SOP are fit for purpose regardless of the instrumentation used for mercury detection. The limit of detection (LOD) and limit of quantification (LOQ) should be checked to assess whether they are suitable for blood samples.

2.1. Scope of the method

The method described in this SOP is intended for the determination of total mercury in whole cord blood. For a sample of 200 mg the LOQ is 0.2 ng/mL. Concentrations of total mercury in cord blood of non-fish eaters are normally in the range of 0.5–5.0 ng/mL. In cases of higher fish consumption, values higher than 10 ng/mL frequently occur. The method described in this SOP can cover all the ranges normally reported.

2.2. Technical principle

In this SOP, mercury in blood is determined by thermal decomposition-gold amalgamation-atomic absorption spectroscopy, a very sensitive and selective analytical technique that is highly suitable for trace level analysis. Blood samples are weighed and introduced into the sample boat without any pre-treatment. The sample is then inserted into the direct mercury analyser (Fig. 3), where it is initially dried and then thermally decomposed in a continuous flow of oxygen. The combustion products are carried to and further decomposed over a hot catalyst bed. Mercury vapours are trapped on a gold amalgamator and subsequently heated, which releases all mercury vapours to the absorption cell of the atomic absorption spectrophotometer. Mercury content is determined using atomic absorption spectrometry at 253.7 nm.

Fig. 3. Direct mercury analyser

Hg = mercury.
Note: the standard version of the Milestone DMA-80 (illustrated here) is equipped with two measuring cells, a mercury lamp and mercury detector.
Source: Milestone *(13)*.

The quantitative determination of mercury is achieved using a calibration curve obtained with mercury standard solutions. The direct mercury analyser can be configured in various different ways depending on the type of model used. For the procedure described here, a measuring cell covering a working range of 0–20 ng mercury (low range) was used.

2.3. Safety precautions

The following safety precautions should be taken when analysing total mercury in cord blood.

- Place disposable plastic, glass and paper (e.g. pipette tips, auto sampler tubes and gloves) that come in contact with human biological fluids, such as blood, in a biohazard autoclave bag. Keep these bags in appropriate containers until they are sealed and autoclaved.
- Gloves, a laboratory coat and safety glasses should be worn when handling all solutions.
- Special care should be taken with concentrated hydrochloric acid since it is a caustic chemical that can cause severe eye and skin damage.
- The possible hazards of equipment use include exposure to ultraviolet radiation, high voltages and high temperatures.
- When work is finished, wipe down all work surfaces where human biological fluid was handled, with a 10% sodium hypochlorite solution or equivalent. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis, according to guidelines for disposal of hazardous waste.

2.4. Equipment, materials and solutions

2.4.1. Equipment

The following equipment is required for analysing total mercury in cord blood:

- direct mercury analyser (e.g. Milestone DMA-80).

2.4.2. Materials

The following materials are required for analysing total mercury in cord blood:

- analytical balance (readability: 0.1 mg)
- microlitre pipette for 100 µL
- microlitre pipette, adjustable between 20 and 200 µL
- microlitre pipette, adjustable between 100 and 1000 µL
- tube for aliquots of blood samples – Cryovial; 2 mL
- laboratory vortex shaker
- quartz boats (1.5 mL)
- sample tray conveyor
- talc-free gloves.

2.4.3. Reagents, chemicals and gases

The following reagents, chemicals and gases are required for analysing total mercury in cord blood:

- oxygen gas (99.995% purity)
- 70% ethanol (pro analysis)
- 37% hydrochloric acid (pro analysis)
- purified water (bidistilled water).

2.4.4 Standard solutions

Stock standard solution

A primary standard solution of mercury (stock solution) with a concentration of 1 mg/mL is prepared by weighing 0.2500 g of elemental liquid mercury (Hg^{o}) in a 250 mL Pyrex glass flask. To this, 2 mL of nitric acid is added, which is diluted with bidistilled water to 250 mL. The solution must be stored in a refrigerator.

Intermediate standard solution

An intermediate standard solution of mercury with a concentration of 5 µg/mL is prepared in 5% nitric acid by appropriate dilution with bidistilled water. Calibration standards are preferably prepared in glass flasks and are stable for one year if kept in a refrigerator. Before dilutions of working standard solutions are prepared the intermediate standard solutions should reach room temperature.

Working standard solutions

Working standard solutions of mercury, at two different concentrations (2 ng/mL and 10 ng/mL) are prepared in 5% hydrochloric acid by appropriate dilution. These are preferably kept in glass flasks (Teflon is suitable as well). Working standard solutions should be stored in a refrigerator when not in use. Working standard solutions should be removed from the refrigerator about two hours before use so they can reach room temperature. Working standard solutions are prepared weekly, but users are advised to check the stability under their laboratory conditions.

2.4.5 Reference materials

Reference materials certified for total mercury in blood should be used. For example, Seronorm Trace Elements Whole Blood L-1, with a reference value of 2.2 ng/g (2.0–2.4 ng/g), has been used for the validation and regular quality control of this SOP. Table 1 lists other reference materials which are available for measuring total mercury in human blood.

Table 1. Reference materials for total mercury in blood

Reference material	Reference values (ng/mL)
NIST 955c Lead in caprine blood	17.8 ± 1.6
NIST-966 Toxic metals in blood	31.4 ± 1.7
SERO210105 Trace elements in whole blood, level 1	1.97 ± 0.2
SERO210205 Trace elements in whole blood, level 2	15.2 ± 1.6
SERO210305 Trace elements in whole blood, level 3	31.4 ± 3.4

Note: new reference materials are continuously produced and replace the obsolete ones, therefore users are advised to regularly check on the availability of appropriate reference materials.

2.5. Calibration

Calibration is performed using working standard solutions described in 2.4.4.

In order to cover the appropriate measurement range, a known volume of calibration standard is pipetted. Normally a range of 0–20 ng needs to be covered (typical amounts covered are 0.05, 0.1, 0.2, 0.4, 0.5, 1.0, 2.0, 5.0, 10.0 ng). The volume of the calibration standard should not exceed 0.2

mL. The new calibration curve is done weekly or even less frequently, because calibrations are very stable. The instrument software allows for automated readings from calibration graphs stored in the system. However, working calibration standards covering the range of mercury concentrations in the sample (typically 0.2, 1.0 and 10.0 ng) should be used daily to verify the correctness of the calibration graphs stored in the system.

At the beginning of the measurement run it is necessary to check the adequacy of the calibration curve. Standard solutions 2 and 10 ng/mL are used. If the standards of mercury are not within the required range then it is necessary to repeat the calibration curve.

2.6. Procedure

2.6.1. Analytical equipment conditioning

Technical data

The technical data for the analytical equipment is as follows:

- principle: atomic absorption spectrometry;
- mercury detection system: single-beam spectrophotometer with sequential flow through two measurement cells;
- light source: low pressure mercury vapour lamp;
- wavelength: 253.65 nm;
- interference filter: 254 nm, 9 nm bandwidth;
- detector: silicon ultraviolet photodetector;
- working ranges:
  - low range: 0–7.5 ng (absorbance limit of cell 1: 0.45);
  - LOD: 0.005 ng;
- autosampler: built-in, 40 positions;
- carrier gas: oxygen, inlet gas 4 bar (60 psi), flow rate approximately 200 mL/min.

The technical data listed here were established during configuration of the instrument used in this case.

Step 1. Preparation of the direct mercury analyser

The following operations should be carried out in accordance with the user manual: opening the oxygen supply, direct mercury analyser start-up and data file creation.

Step 2. System cleaning

Before the measurements are taken, the system needs to be cleaned. Detergent is first pipetted into two quartz boats and then the empty position. An empty position should be measured with the appropriate programme. An optimum is suggested below, but users are advised to check it in their own configuration:

- drying time: 0 s (with using detergent the time is prolonged to 60 s)
- drying temperature: 200 °C
- decomposition time: 150 s

- decomposition temperature: 650 °C
- purge time: 60 s.

Step 3. System background check

Three empty quartz combustion boats should be analysed using the previous method to check that the absorbance (measured in terms of peak height) of the final samples is less than 0.0030. The acceptable system background should be established by the laboratory in accordance with the manufacturer's instructions. If the absorbance is above 0.0030, further quartz combustion boats should be analysed until the target value is obtained. If the desired background level is not attained after five quartz combustion boats have been analysed, the system should be cleaned by analysing a solution of detergent in a quartz combustion boat, followed by the procedure described above.

Step 4. Control of working standard solution

Two replicates of each standard solution should be measured (2 ng/mL and 10 ng/mL) containing approximately 0.2 ng and 1 ng mercury (100 µL of working standard solution) in order to check cell 1 (low range). The standard mercury solution is measured as follows:

- drying temperature: 200 °C
- drying time: 60 s
- decomposition temperature: 650 °C
- decomposition time: 150 s
- purge time: 60 s.

The concentration determined for the mercury working standard solution is compared to the one established by the calibration curve. If the targeted value differs more than 10%, the measurement should be repeated until a value within the targeted range is obtained. If such a value is not obtained after five attempts, a fresh standard should be prepared. If it still does not achieve the desired value, the system should be recalibrated with a set of newly prepared working calibration standards.

Step 5. Pre-measurement quality control

Two samples of certified reference material (e.g. Seronorm Whole Blood L-1) containing approximately 0.2 ng mercury (approximately 100 mg of material) should be measured in order to check cell 1 (low range). The reference material is measured as follows:

- drying temperature: 200 °C
- drying time: 120 s
- decomposition temperature: 650 °C
- decomposition time: 180 s
- purge time: 60 s.

The concentration determined for the reference material sample should be within the uncertainty range of the certified value. If this is not the case, the measurement should be repeated until a value within this range is obtained. If such a value is not obtained after five attempts, the system should be recalibrated.

Once the previous five steps have been successfully completed, the direct mercury analyser is ready for sample analysis.

2.6.2. Analytical determination

Sample weighing

Both the combustion boats and the support used to weigh the blood samples should be handled using tweezers.

Place the combustion boat support on the balance. Place a quartz combustion boat on top of the support and set the balance to zero.

Open the flask containing the sample, measure out around 200 µL of blood into the combustion boat and weigh the sample. Place the combustion boat containing the sample onto the sample tray and note the sample code, weight and tray position in the weighing log. Two replicates should be prepared for each sample. Between each sample it is necessary to measure one blank. Special pipette tips with a filter must be used for each different sample.

Sample analysis

The quartz combustion boats containing samples and quality controls should be placed in the direct mercury analyser autosampler in the order in which they were weighed.

The samples should then be programmed by entering their code and weight, and selecting the method. The parameters of the method are as follows:

- drying temperature: 200 °C
- drying time: 200 s
- decomposition temperature: 650 °C
- decomposition time: 180 s
- purge time: 60 s.

Note. Guidance parameters must be optimized for each instrument in accordance with the manufacturer's instructions.

An example of sample sequences is provided below:

- detergent
- detergent
- blank (8x)
- working standard 2 ng/mL
- working standard 2 ng/mL
- blank
- working standard 10 ng/mL
- working standard 10 ng/mL
- blank
- reference material
- reference material
- blank
- blank

- sample 1
- sample 1
- blank
- sample 2
- sample 2
- blank
- working standard 2 ng/mL
- working standard 10 ng/mL
- blank
- sample 5
- sample 5
- blank
- sample 8
- sample 8
- working standard 2 ng/mL
- working standard 10 ng/mL
- blank.

2.6.3. Calculation of the analytical results

Data are reported directly by the equipment in terms of nanograms of mercury per gram of blood by interpolation of the measurement on the calibration curve.

The final value reported corresponds to the average of two independent measurements. If the values differ by more than 10%, the sample is to be re-measured, and the mean values of two similar results are reported.

2.6.4. Reportable results range

Mercury values are reportable when the results are obtained from a range defined by a calibration curve. If the amount of mercury obtained in the sample is outside this range, the sample should be retested as follows.

- If the value is below the lowest concentration of mercury included in the calibration, the necessary amount of blood for two new replicates should be weighed in order to obtain a new determination within the calibration range. The maximum amount of blood taken for analysis should not exceed 250 mg.
- If the value is above the highest calibration point, lower amounts of the sample should be weighed in order to obtain readings within the calibration range.

2.7. Quality control

The precision and accuracy of biomarker analyses carried out by laboratories must be continuously checked by means of quality assurance measures.

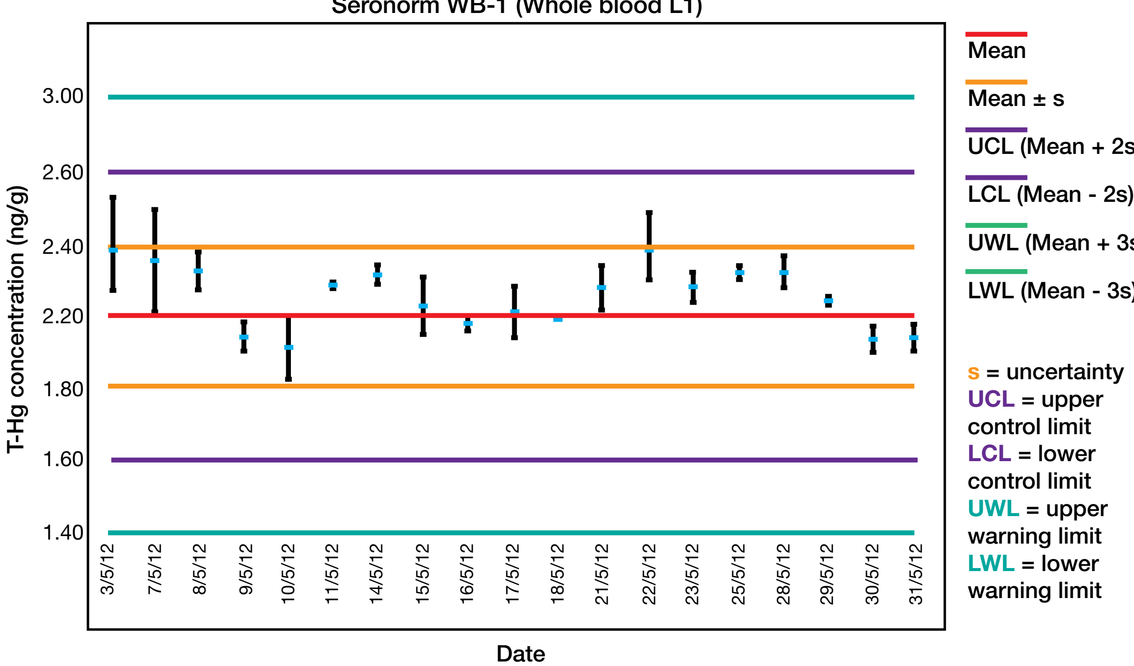
In general, quality assurance in laboratories comprises internal and external quality control (see also the *Quality control programme for mercury human biomonitoring*), as described below.

- Internal quality control is a set of procedures used by the staff of a laboratory to continuously assess results as they are produced in order to determine whether they are reliable enough to be released.
- External quality assessment is a system for objectively checking laboratory performance using an external quality control system. External quality control can be achieved by participation in suitable inter-laboratory comparisons, if available.

In this SOP, quality control materials are used to evaluate accuracy and precision. Fig. 4 provides an example of a quality control chart for blood reference material. Seronorm Whole Blood L-1 (2.2±0.2 ng/mL) has been used for quality control in this SOP.

Laboratories are advised to carefully control the performance of the analytical method on a regular basis as described in International Organization for Standardization/ International Electrotechnical Commission (ISO/IEC) 17 025:2005 *(17)*.

Fig. 4. An example quality control chart



T-Hg = total mercury.

2.8. Evaluation of the method

Each laboratory should comply with the standard "ISO/IEC 17025:2005 General requirements for the competence of testing and calibration laboratories" *(17)*. The method should be validated for its performance criteria (sensitivity, linearity, recovery, robustness, precision, accuracy, LOD, etc.) and should be accompanied by measurement uncertainty estimation, as the latter is a fundamental property of a result and a requirement of the standard ISO/IEC 17025:2005. It is advisable to consult freely available guides from EURACHEM *(18)*, particularly those dealing with validation protocols and uncertainty assessment. The concentration levels of mercury in the blood can be very low and the LOQ should be below 0.1 ng/mL to be able to measure concentrations in the general population. Those using the methodology outlined in this SOP are highly recommended to follow the glossary available in *Terminology in analytical measurement: Introduction to VIM 3 (19)*.

For the method described in this SOP, the performance criteria and measurement uncertainty estimation are specified below.

2.8.1. Limit of detection and limit of quantification

The LOQ was determined from the lowest point of the calibration curve, which was 0.05 ng. Taking into account the mass of a sample measured (0.2 g), the LOQ was 0.25 ng/g.

The LOD was determined as the LOQ/3, which was 0.8 ng/g.

2.8.2. Precision

As a measure of the degree of reproducibility of the described analytical method, routine analysis of cord blood samples over the course of a longer time period (e.g. one year) is used. For the purpose of demonstration, the results of one measurement series (n=15) of total mercury in cord blood are shown in Table 2. Each sample was analysed in two replicates.

Table 2. Results of duplicate measurements of total mercury in cord blood samples and their relative differences

Sample	Result D1 (ng/g)	Result D2 (ng/g)	Mean value (D1+D2)/2	Difference (D1-D2)	Relative difference (D1-D2/mean)
Cord blood 1	0.87	0.86	0.87	0.01	0.012
Cord blood 2	3.24	3.25	3.25	-0.01	-0.003
Cord blood 3	5.45	5.68	5.57	-0.23	-0.041
Cord blood 4	1.22	1.22	1.22	0.00	0.000
Cord blood 5	1.28	1.40	1.34	-0.12	-0.090
Cord blood 6	4.67	4.55	4.61	0.12	0.026
Cord blood 7	1.34	1.32	1.33	0.02	0.015
Cord blood 8	2.92	2.92	2.92	0.00	0.000
Cord blood 9	1.16	1.21	1.19	-0.05	-0.042
Cord blood 10	1.85	1.58	1.72	0.27	0.157
Cord blood 11	3.67	3.73	3.70	-0.06	-0.016
Cord blood 12	1.42	1.36	1.39	0.06	0.043
Cord blood 13	2.83	2.81	2.82	0.02	0.007
Cord blood 14	1.94	1.98	2.00	-0.04	-0.020
Cord blood 15	1.19	1.24	1.22	-0.05	-0.041

D1 = measurement 1; D2 = measurement 2.

To assess reproducibility or repeatability, standard deviation of replicate measurements is calculated using the following equation.

$$RSD_d = \frac{s_d}{\sqrt{n}}$$

RSDd – relative standard deviation of duplicate measurements

Sd – standard deviation of relative differences ((D1-D2)/mean)

n – number of replicates (n=2)

The repeatability calculated for the given set of measurements was 3.9%.

2.8.3. Trueness

The trueness of our results was estimated using the reference material Seronorm WB-1 (Whole blood L1). As a measure of trueness of our results, recovery *(R)* was calculated based on measurements of the reference material over a course of one month. The observed levels were compared against the reference value using the following equation.

$$R = \frac{observed\ value}{reference\ value}$$

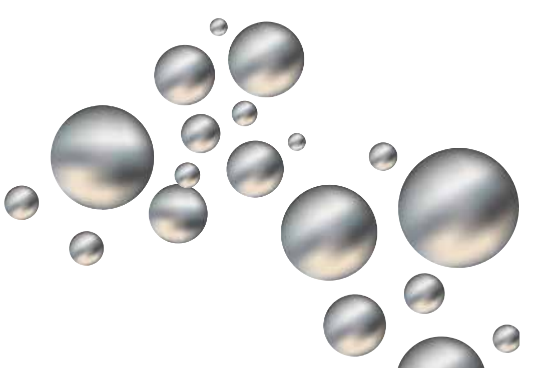
R – recovery

An example of measurements of total mercury in the reference material is given in Table 3.

Table 3. Measurements of total mercury in Seronorm WB-1 (Whole blood L1)

Measurement	Measured value (ng/g)	True value (ng/g)	Recovery (%)
Day 1	2.39	2.2	109
Day 2	2.35	2.2	107
Day 3	2.32	2.2	105
Day 4	2.14	2.2	97
Day 5	2.11	2.2	96
Day 6	2.28	2.2	104
Day 7	2.31	2.2	105
Day 8	2.23	2.2	101
Day 9	2.17	2.2	99
Day 10	2.21	2.2	100
Day 11	2.19	2.2	99
Day 12	2.27	2.2	103
Day 13	2.39	2.2	109
Day 14	2.28	2.2	104
Day 15	2.32	2.2	105
Day 16	2.32	2.2	105
Day 17	2.24	2.2	102
Day 18	2.13	2.2	97
Day 19	2.14	2.2	97

Based on the measurements given in Table 3, the recovery calculated was 102%.



2.8.4. Measurement uncertainty

Measurement uncertainty for total mercury in cord blood by thermal desorption and CVAAS was estimated based on ISO 21748:2010 “Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation”. For this purpose, reproducibility (repeatability) and recovery data from our validation study was used.

Uncertainty of repeatability (u_{rep}) was 3.9% (Section 2.8.2), while uncertainty of recovery ($u(R_m)$ or u_{rec}) was 1.7 % and was calculated using the following equation.

$$u(\overline{R}_m) = \overline{R}_m \times \sqrt{\frac{s_{obs}^2}{n \cdot \overline{C}_{obs}^2} + \left(\frac{u(C_{ref})}{C_{ref}}\right)^2}$$

R_m – recovery
s_{obs} – standard deviation of the observed data
C_{obs} – mean value of the observed data
C_{ref} – reference value
$u(C_{ref})$ – uncertainty of reference value

In the final step, Step 4, combined uncertainty was calculated. Before combination, all uncertainty contributions must be expressed as standard uncertainties (standard deviations). The combined uncertainty (u_c) was calculated using the following equation.

$$u_c = \sqrt{u_{rep}^2 + u_{rec}^2}$$

u_c – combined uncertainty
u_{rep} – error due to reproducibility
u_{rec} – error due to recovery

Expanded uncertainty (*U*) was expressed by multiplying u_c with the factor *k*. The choice of the factor *k* is based on the level of confidence desired. For an approximate level of confidence of 95%, *k* is 2.

The estimated measurement uncertainty for the determination of total mercury in cord blood by thermal decomposition and CVAAS is 4.3%, expanded uncertainty (k=2) is 8.4%. The estimation is valid for a “normal” exposure range, that is below 5.8 ng/g.

3. Interpretation of results

Blood mercury levels reflect exposure through ingestion of contaminated fish or drinking water, inhalation of elemental mercury vapour in ambient air, and exposure through dental amalgams and medical treatments. The presence of mercury in blood indicates recent or current exposure to mercury. There is a direct relationship between mercury concentrations in human blood and consumption of fish contaminated with methylmercury. Usually blood methylmercury concentration reaches a maximum within 4–14 hours and undergoes clearance from the blood to other body tissues after 20–30 hours *(6)*.

At the initial stage of data analysis (descriptive statistics), basic statistical values are calculated for each biomarker: minimum and maximum values, percentage of subjects having the biomarker value above the LOQ or above the LOD, and geometric mean. Percentile values, the values of a variable below which a certain percentage of observations fall, may also be calculated: 50th percentile (P50; median), 90th percentile (P90) and 95th percentile (P95). Percentages of results exceeding reference values or health-based values may also be reported *(20)*.

Human biomonitoring data can be interpreted via comparing the measured biomarker levels to health-relevant biomonitoring reference values. In this context, the German Human Biomonitoring Commission has derived reference values for several compounds *(21)*. These values have been determined based on either exposure-effect relationships (e.g. for cadmium, lead, mercury and pentachlorophenol) or derived from tolerable daily intake values *(20)*.

The blood mercury geometric means in most national surveys in Europe were below or around 1 μg/L. However, in some subpopulations exposure levels exceeded the health-based value of 5 μg/L *(20)*.

WHO considers the normal mean concentration of total mercury in blood to be 5–10 μg/L in individuals with no consumption of contaminated fish (6). The United States National Research Committee identifies 2 μg/L as the normal mean concentration for populations with little or no fish consumption in the United States *(22)*.

Estimating exposure through biomonitoring cord blood levels of about 5–6 μg/L and blood mercury concentrations of about 4–5 μg/L. This relationship is generally directly proportional.

Table 4 provides an example of blood concentrations in populations in different provinces of Canada, from an Arctic Monitoring and Assessment Programme (AMAP) study (2003) *(23)*.

Table 4. Summary of data from Canada on levels of mercury and methylmercury in maternal blood

Country/ ethnic group/region	Number of individuals sampled	Total mercury mean (μg/L)	Total mercury range (μg/L)	Methyl mercury mean (μg/L)	Methyl mercury range (μg/L)
Canada	134	0.9	nd–4.2	0.69	nd–3.6
Caucasian 1 (1994–1999)					
Metis/Dene 1994–1995)	92	1.4	nd–6.0	0.8	nd–4.0
Other (1995)	13	1.3	0.2–3.4	1.2	nd–3.0
Baffin 1(1996)	31	6.7	nd–34	6.0	nd–29
Inuvik 1 (1998–1999)	31	2.1	0.6–24	1.8	nd–21
Kitikmeot 1 (1994–1995)	63	3.4	nd–13	2.9	nd–11
Kivalliq 1 (1996–1997)	17	3.7	0.6–12	2.7	0.4–9.7
Nunavik 2 (1995–2000)	162	9.8	1.6–44	na	na

nd = not detected; na = not available.
Source: AMAP 2003 *(23)*.

The average ratios between intake (μg/kg day) and blood levels (μg/L) among a population, overtime, are expected to be generally consistent. The quantitative relationship between mercury levels in blood and daily average dose (or intake) levels of mercury (especially methylmercury) are fairly well understood.

Therefore, such dose conversions can often be made with reasonable confidence, if enough information is known about the various mercury forms and other factors. Population variability should, however, be noted in dose conversion *(24)*. For example, a daily average methylmercury intake of 0.1 μg per kilogram of body weight (0.1 μg/kg per day) by an adult woman is estimated to result in a cord blood level of about 5–6 μg/L *(24)*.

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Annex 1. Cord blood sample collection form

Name of mother	
Medical record number	
Study ID of mother	
Medical worker	Signature: Printed name:

1. Date and time of sample collection	------/-----/-------/ (day/month/year) Start: -------/------ (hour/min)
2. How many hours prior to the sample collection was your last meal?	_ _ hours
3. Volume of collected blood (approximately)	_ _ _ mL

Annex 2. Registration of cord blood samples

To be filled in by the midwife

Name of hospital:__________ Name of midwife collecting samples:__________

Patient name	**Sample ID**	**Childbirth information**	**Collection of cord blood**	**Sample information Tube 1**	**Sample information Tube 2**
		Date:______ Time:______	Time start collection Time end collection	**Tube B1 with EDTA:** volume=______mL (**min 10 mL**) stored at -20 ⁰C	**Tube B2:** volume= ______mL (**min 2 mL**) stored at -20 ⁰C
		Date:______ Time:______	Time start collection Time end collection	**Tube B1 with EDTA:** volume=______mL (**min 10 mL**) stored at -20 ⁰C	**Tube B2:** volume= ______mL (**min 2 mL**) stored at -20 ⁰C
		Date:______ Time:______	Time start collection Time end collection	**Tube B1 with EDTA:** volume=______mL (**min 10 mL**) stored at -20 ⁰C	**Tube B2:** volume= ______mL (**min 2 mL**) stored at -20 ⁰C

EDTA = Ethylenediaminetetraacetic acid.

Standard operating procedure for assessment of mercury in urine

(sampling, analysis of total mercury, interpretation of results)

Abstract

This standard operating procedure describes the process of assessing exposure to mercury through human biomonitoring using urine as a biological matrix. Sampling of urine, analysis of total mercury and interpretation of results are detailed in this document.

Keywords

Mercury – analysis
Mercury – urine
Methylmercury Compounds – analysis
Urine – chemistry
Biomarkers – analysis
Maternal Exposure
Maternal-Fetal Exchange
Infant, Newborn
Environmental Exposure

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Abbreviations

CVAAS	cold vapour atomic absorption spectrometry
HBM	human biomonitoring
Hg	mercury
ID	identity
LOD	limit of detection
LOQ	limit of quantification
SG	specific gravity
SOP	standard operating procedure

Introduction: urine as a matrix for mercury human biomonitoring

Mercury (Hg) is a naturally occurring element that is distributed throughout the environment by both natural and anthropogenic processes. It is persistent in the environment and is found in various chemical forms, namely elemental mercury, inorganic mercury (Hg2+ compounds) and organic mercury (mainly methylmercury, MeHg) *(1)*.

The primary targets for mercury toxicity are the nervous system, kidneys and cardiovascular system. Nervous system effects are the most sensitive toxicological endpoint observed following exposure to elemental mercury and methylmercury, whereas kidney damage is the key endpoint in exposure to inorganic mercury compounds *(1,2)*.

Exposure to elemental or inorganic mercury occurs due to mercury spills, dental amalgams, inhalation indoors due to broken thermometers and mercury-containing bulbs, the use of some skin-lightening creams and soaps, the presence of mercury in some traditional medicines, the use of mercury in cultural practices and occupational exposure *(3–5)*.

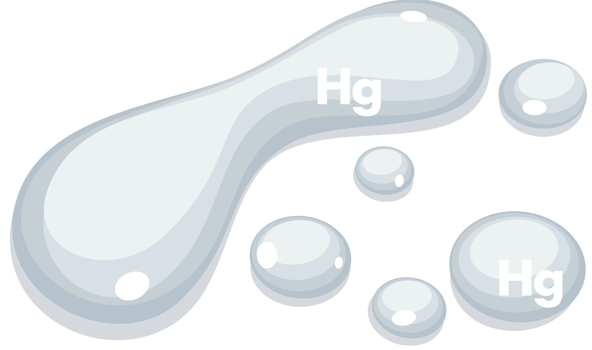
Exposure can be estimated by measuring pollutant levels in various human matrices, such as hair, blood or urine, all of which are useful tools for assessing mercury exposure in individuals and populations *(3,6–8)*.

Urinary mercury levels are usually considered to be the best measure of recent exposure to inorganic mercury or elemental mercury vapour, as urinary mercury is thought to most closely indicate the mercury levels present in the kidneys *(3)*.

Urine is easy to collect and is available in larger amounts than other biological matrices. Spot urine samples are usually employed instead of 24-hour samples as the latter are more uncomfortable to collect and are more likely to become contaminated due to continuous opening of the vessel.

The disadvantage of spot urine samples is the variability in the volume of urine produced and the fact that the concentration of endogenous and exogenous chemicals can vary significantly from void to void depending on the hydration status, time of last urination, etc. Consequently, spot samples need a dilution adjustment. Several methods, such as creatinine adjustment or specific gravity, can be used to adjust the urinary biomarker concentration *(9–11)*.

Although spot urine samples can be collected at any time of day, a first morning urine sample is recommended as otherwise the target biomarker may be below the limit of quantification (LOQ) due to sample dilution. A further possibility is to collect samples after at least five hours without urination.



1. Sampling of urine

1.1. Scope of the method

The method of collecting urine samples described in this standard operating procedure (SOP) allows analysis of mercury concentrations, and covers all pre-analytical phases of mercury human biomonitoring (HBM) using urine. Following the sampling procedure detailed in this SOP enables field technicians to properly collect and handle the biological samples before they are analysed in the laboratory.

1.2. Safety precautions

Urine samples will be collected by the recruited women themselves. However, when working with urine (aliquoting or making other manipulations) universal precautions for working with biological materials should be followed.

- Wear gloves, a laboratory coat and safety glasses while handling human bodily fluids or tissue.
- Place disposable plastic, glass and paper (e.g. pipette tips, autosampler tubes and gloves) that come in contact with human biological fluids, such as urine, in a biohazard autoclave bag.
- Keep these bags in appropriate containers until they are sealed and autoclaved.

1.3. Materials required

Table 1 shows the materials required for sampling and for pre-treatment of the sampling material.

Table 1. Materials for urine sampling

Material	Rationale	Alternative
Extra pure 65% nitric acid	Used to clean the vessels in order to eliminate background metal contamination.	
Purified water	Used in the cleaning process.	Bidistilled water
Containers	3 different tanks for the cleaning process; 1 for the acid solution and the other 2 for water.	
Urine vessels (see below)	Vessels that can be closed reliably. The volume of the vessel depends on the amount of urine required for analysis and biobanking (if planned).	
Acid-resistant gloves	A safety measure.	
Labels	Samples must be unequivocally identified.	Writing the ID code directly on the vessel with a permanent marker pen
Permanent marker pen	Not essential but very useful to mark the minimum amount of sample that should be collected.	Any other writing material which ensures that the mark remains clearly legible
Filter paper	Used during vessel washing.	
Zip-lock plastic bags	Used to further isolate the vessel.	Any other type of bag

contd. Table 1. Materials for urine sampling

Material	Rationale	Alternative
Isothermal packaging	Urine samples have to be kept at 4 °C until arrival at the laboratory.	
Freeze-safe labels for sample identification	Used for labelling of samples.	
2M sulfamic acid	For the purpose of preventing loss of mercury from the urine before analysis.	

ID = identity.

For mercury analysis, 2M sulfamic acid should be added prior to urine sampling in the proportion of 10 µL of preservative solution per 1 mL of urine (e.g. for a tube containing 50 µL of preservative, up to 5 mL of urine can be added for urine mercury analysis).

1.4. Preparation/pre-treatment of the sampling material

The vessels employed for urine sampling must be pre-cleaned to eliminate the background metal contamination. All vessels and their lids should be washed with nitric acid solution according to the following procedure. Note that pre-washing of the vessels should be performed in a chemical fume hood according to good laboratory practice, following the laboratory's safety guidelines and whilst wearing protective equipment.

1. Mark the different containers according to the solution contained in them: 10% nitric acid; rinse tank 1; rinse tank 2.
2. Place them in the chemical fume hood.
3. Prepare the dilute acid solution from extra pure 65% nitric acid and purified water. (Note: 18 L of acid solution (2.8 L 65% nitric acid and 15.2 L of bidistilled water) is required to clean approximately 240 100 mL vessels. The acid solution can be used for up to one month after preparation).
4. Fill the tanks with the corresponding solution.
5. Open the vessels and place them in the tank containing the acid solution together with their lids (overnight or for at least for three hours). Ensure that the vessels and lids are completely immersed (Photo 1).

Photo 1. Placing the vessels and lids in the acid solution.
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6. Remove the vessels from the acid tank and place them in the first tank of purified water, shaking for 2–3 minutes. Then move the vessels and lids to the second tank, again shaking for 2–3 minutes (Photo 2).

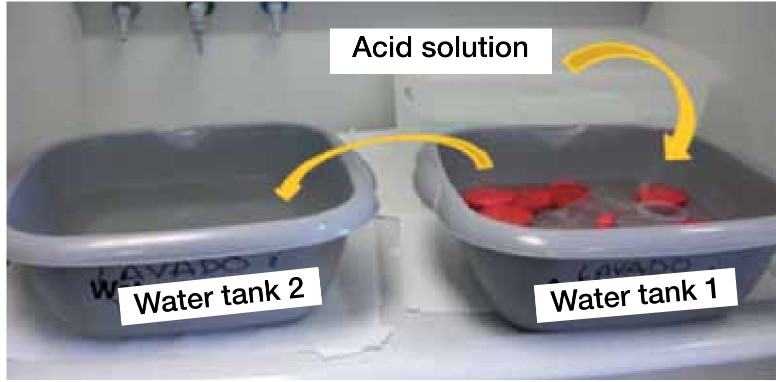

Photo 2. Rinsing the vessels in purified water tanks.
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7. Remove the vessels and lids from the second water tank and place them face down on a clean sheet of filter paper inside the chemical fume hood to dry (Photo 3).

Photo 3. Drying the vessels and lids in the fume hood.
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8. Replace the lids on the vessels, then mark the vessels to indicate the minimum volume required (Note: this step is optional but is very useful to avoid the collection of samples with insufficient volume). Place each washed vessel into a zip-lock plastic bag (photos 4a and b).

Photos 4a and b. Marking the minimum volume (a) and placing the vessels in a zip-lock bag.
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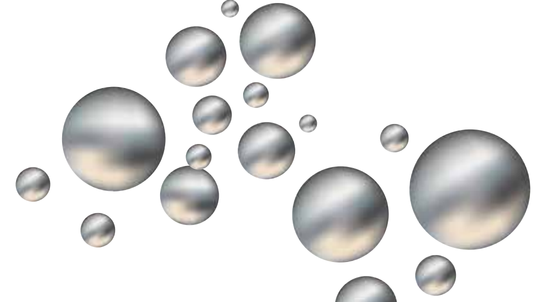


The presence of contamination after the washing procedure should be checked by selecting 5% of the washed vessels at random and filling them with purified water. After shaking for 10 minutes, one aliquot should be taken from each vessel to determine the mercury concentration.

Finally, if the vessels come from different batches, information regarding the batch of vessels sent to each sampling centre should be recorded.

1.5. Sampling procedure

Ideally, urine vessels should be provided to the volunteers by the fieldworkers in advance in order to allow them to collect the first morning urine. (Alternatively, urine samples can be collected from the mother at admission to the maternity ward, prior to child birth.) Each vessel should be accompanied by detailed written instructions on how to collect the urine sample (see an example in Annex 1). In addition to these written instructions, fieldworkers should explain personally to the volunteers how to collect the sample and clarify any questions and doubts. Questionnaires for urine samples (Annex 2) should be administered at the time of sampling.

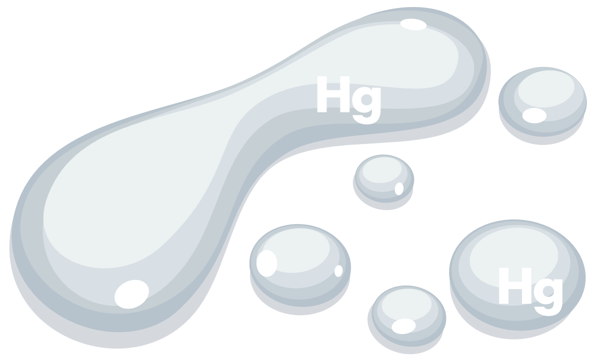
Urine samples collected should be kept at 4 °C until their arrival at the laboratory. Alternatively, urine samples can be aliquoted in the maternity unit and frozen at -20 ºC. In this case, samples have to be kept frozen during transportation to the laboratory.

Note. Control blank samples should be used regularly (at least one blank sample in each maternity unit). Containers for blank samples should be opened in the maternity ward and manipulated exactly like containers for regular samples but without collecting the sample. This allows assessment of potential sample contamination at the sampling location.

1.6. Labelling

Containers for urine samples can be labelled in two different ways.

- In advance, after the washing procedure: a label with the identity (ID) code and a space to note the sampling date is affixed.
- After sample collection: immediately after the volunteer delivers the sample to the fieldworker, the container should be labelled with the ID code and sampling date.



1.7. Transportation and conservation of the sample

Urine samples must be kept at 4 °C until their arrival at the laboratory, where they will be aliquoted and analysed or stored until analysis. (Alternatively, the samples can be aliquoted and frozen in the maternity unit.) Furthermore, urine samples must be transported in compliance with the relevant shipping regulations for biological material. Photo 5 illustrates appropriate isothermal packaging for sample transport.

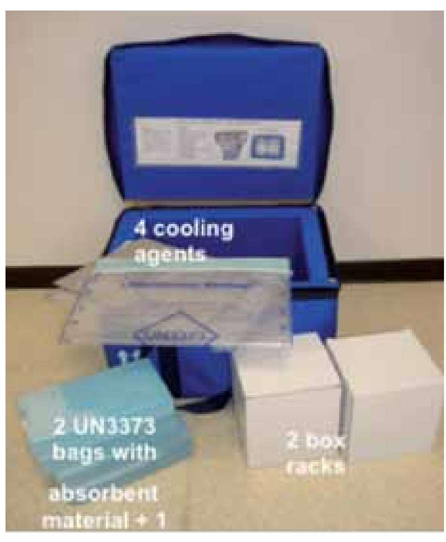


Photo 5. Example of isothermal packaging.
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1.8. Sample reception

The following points should be checked upon receipt of urine samples.

- Condition of sample transportation and storage (samples transported and stored at high temperature cannot be accepted).
- Use of preservatives during sampling (containers with 2M sulfamic acid should be used for urine sampling for mercury analysis).
- The package must be correctly sealed and must not have been manipulated (Note: a security seal can be placed on the package at the sampling site).
- All samples listed in the registry of collected samples should be contained in the package.
- All samples must be accompanied by the corresponding documents (questionnaires, etc.).
- All samples and documents received must be properly identified with the corresponding ID code.
- The samples must have been collected properly (sufficient volume).
- The transportation container should not be contaminated.

Annex 3 contains a sample reception sheet for urine samples. The items in this document may vary according to the requirements for sample conservation or analyte stability/conservation.

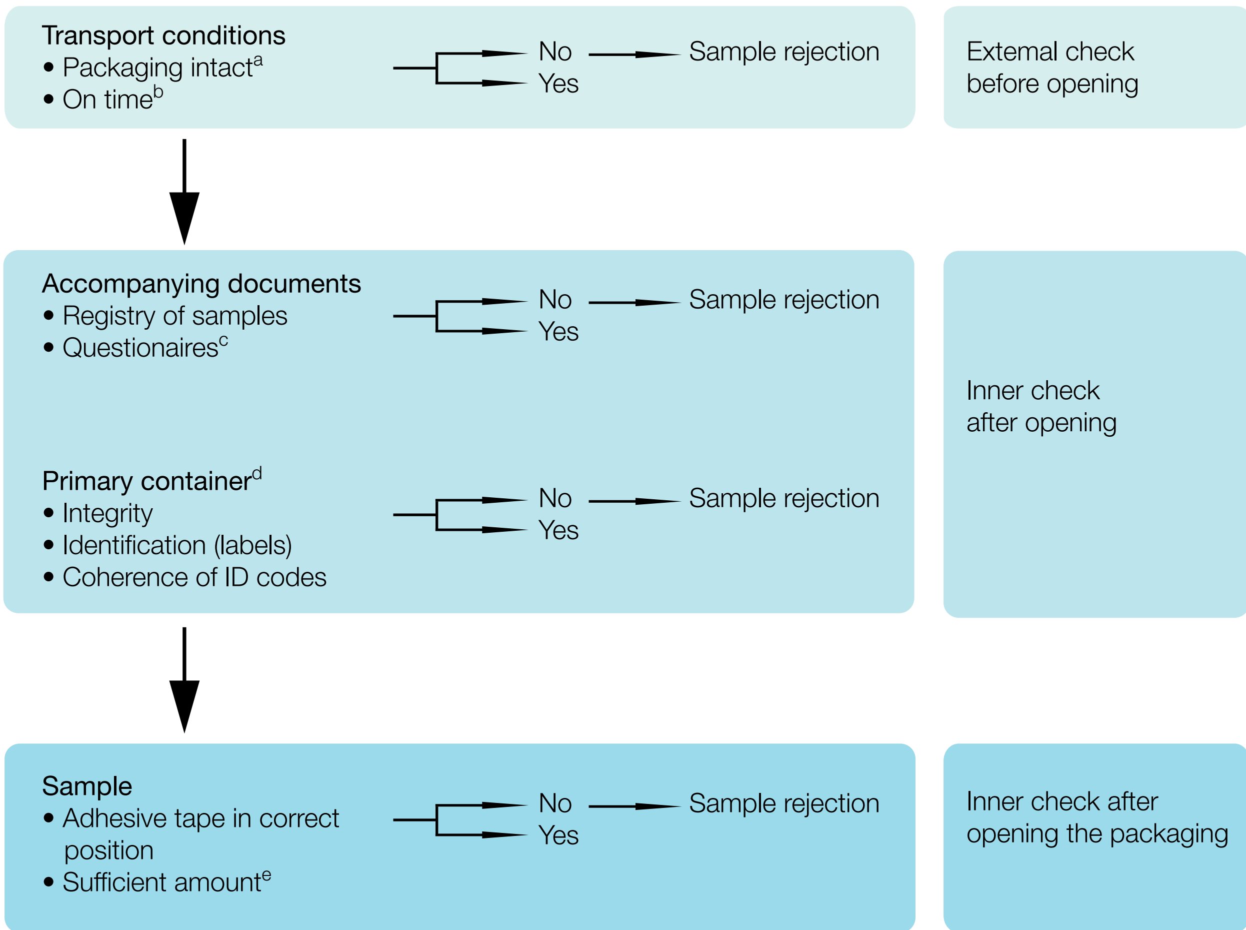
Note. If field blanks (for example, vessels with purified water) have been employed, they should be checked and registered in the same manner as the other samples.

1.8.1. Sample acceptance/rejection criteria

The criteria for accepting or rejecting a sample should be defined in advance and applied during sample reception. These criteria should focus on transport conditions, attached documentation, integrity of

the packaging, correct identification, amount of sample (sufficient for analysis and biobanking if samples will be stored for other research purposes). In order to follow a unique procedure and apply the same criteria to all samples received, the plan illustrated in Fig. 1 can be applied.

Fig. 1. Plan for receipt of samples



[a] The package must be correctly sealed and must not have been manipulated

[b] The maximum time between sample collection and its arrival at the laboratory should be defined beforehand.

[c] If one or more of the questions in the questionnaires are crucial for results interpretation or are an inclusion/exclusion criterion, this should be verified.

[d] The conditions of the zip-lock plastic bag should be checked. All samples must be properly identified and the consistency between sample ID codes and questionnaires should be checked.

[e] The amount of sample is a critical point. If the amount of sample is insufficient to perform the chemical analysis, the sample should be rejected.

1.9. Sample aliquoting/preparation

Sample aliquoting can be conducted in the hospital or in the laboratory after transportation from the hospital. Urine samples should be aliquoted in accordance with good laboratory practice, following the laboratory's safety guidelines and whilst wearing protective equipment. The number and volume of aliquots required should be estimated in order to avoid freeze-thaw cycles. The laboratory performing the analysis should be consulted to establish the aliquot volume required and the minimum volume required for analysis.

The full list of tubes for aliquoting is as follows.

1. Tube U1 (mercury)

a. 5 mL urine: pour (no pipetting!) into a pre-treated plastic metal-free urine container with sulfamic acid added prior to sampling. Mix the urine well after adding it to the vial.

b. Freezing and storage at -20 °C.

2. Tube U2 (creatinine)

a. 5 mL urine: pour into a 15 mL polypropylene tube.

b. Freezing and storage at -20°C.

3. Additional tubes: the rest of the urine can be poured into separate tubes for additional analyses. It is recommended to also store polypropylene tube(s) in fractions of 10 mL or 40 mL in the biobank at -80 °C.

Note. Ensure that the aliquots are homogeneous by shaking the original sample between aliquots.

1.10. Storage and conservation

Samples to be stored for more than one month should be frozen. Since urine contains many inorganic salts, even fresh urine may generate precipitate. Thus, the sample must be homogenized by shaking before analysis. A method also exists where the solubility of the salts is increased by lowering the pH of the urine sample by adding a small amount of hydrochloric acid. Take steps to ensure that microorganisms do not proliferate, as they may cause inorganic mercury to reduce to mercury vapour, which will escape and be lost. It is believed that the average mercury level in the urine of the general population in a region without any particular mercury exposure is less than 10 ng/mL. Mercury stability has been demonstrated for one year at -20 °C.

In general, urine specimens are transported and stored at -20 °C. Sample storage procedures should be established in order to control the sample location, number of aliquots remaining, etc. Upon receipt, freeze the specimens at -20 °C until the time for analysis. The analyst must put the remaining samples in the freezer after analytical aliquots are taken and refreeze them at -20 °C. Samples that are thawed and refrozen several times will not be compromised even if preservatives were added for storage.

1.11. Quality control

1.11.1. Traceability

Traceability of the sample throughout the study is crucial, therefore this aspect should be guaranteed. As noted above, correct labelling of the samples and related documents is essential, but it is also necessary to be able to link the sample with the information provided by the volunteer. It is strongly recommended that a log of samples be maintained (Annex 3). To this end, a database should be designed where this information can be stored. Access to this file or document must be restricted whenever it contains confidential personal information.

If more than one ID code is associated with a sample, for example aliquots from the samples can have different codes, or if an internal code has to be assigned when samples arrive at the laboratory, all these codes should be recorded in the database.

2. Analysis of total mercury in urine

The method described in this SOP is suitable for the determination of total mercury in human urine in a general population with low exposure to mercury and for occupationally exposed humans. The method is based on acid digestion, reduction and measurement by cold vapour atomic absorption spectrometry (CVAAS). The method is simple and sensitive. It is designed to be suitable for an instrument that requires simple maintenance and has been promoted by the National Institute for Minamata Disease (Japan) *(12)*.

Where laboratories have other equipment for the detection of mercury in acid digested samples it is advisable to follow the instructions provided by the instrument producers. The instructions for sampling and sample handling provided in this SOP are fit for purpose regardless of the instrumentation used for mercury detection. The limit of detection (LOD) and limit of quantification (LOQ) should be checked to ensure that they are suitable for human urine samples.

2.1. Scope of the method

The described procedure refers to the treatment and processing of the sample after sub-aliquots are taken for mercury analysis. Concentrations of total mercury in urine of a non-exposed population are normally in the range of 0.1–5 ng/mL. In cases of exposure to inorganic and elemental mercury, values up to 10 ng/mL have been reported; however, at workplaces levels higher than 50 ng/mL frequently occur. The described method can cover all the ranges normally reported in general populations, as well as in occupational exposure settings.

2.2. Technical principle

Urine samples are digested by acids and mercury is detected by CVAAS. This process is based on the reduction of ionic mercury in the solution to its elemental state and its subsequent transfer into the absorption cell of the mercury analyser for measurement at 253.7 nm. The measurement process is based on the open air flow system, which requires clean ambient air as a carrier gas, making the apparatus easy to operate.

Many detectors are available today for the measurement of mercury and are based either on atomic absorption or atomic fluorescence spectrometry. The procedures used by the laboratories must comply with the instructions provided by instrument producers *(13,14)*. See also the *Standard operating procedures for the determination of total mercury in hair, blood and urine by the alternative method*.

2.3. Safety precautions

Follow universal precautions: wear gloves, a laboratory coat and safety glasses while handling human bodily fluid or tissue. Place disposable plastic, glass and paper (e.g. pipette tips, autosampler tubes and gloves) that come into contact with human biological fluids, such as urine, in a biohazard autoclave bag. Keep these bags in appropriate containers until they are sealed and autoclaved.

When the work is finished, wipe down all work surfaces where human biological fluid was handled using a 10% (v/v) sodium hypochlorite solution or equivalent. The use of the foot pedal on the Micromedic Digiflex is recommended because it reduces analyst contact with work surfaces and also keeps the hands free to hold specimen cups and autosampler tubes. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis in accordance with guidelines for disposal of hazardous waste.

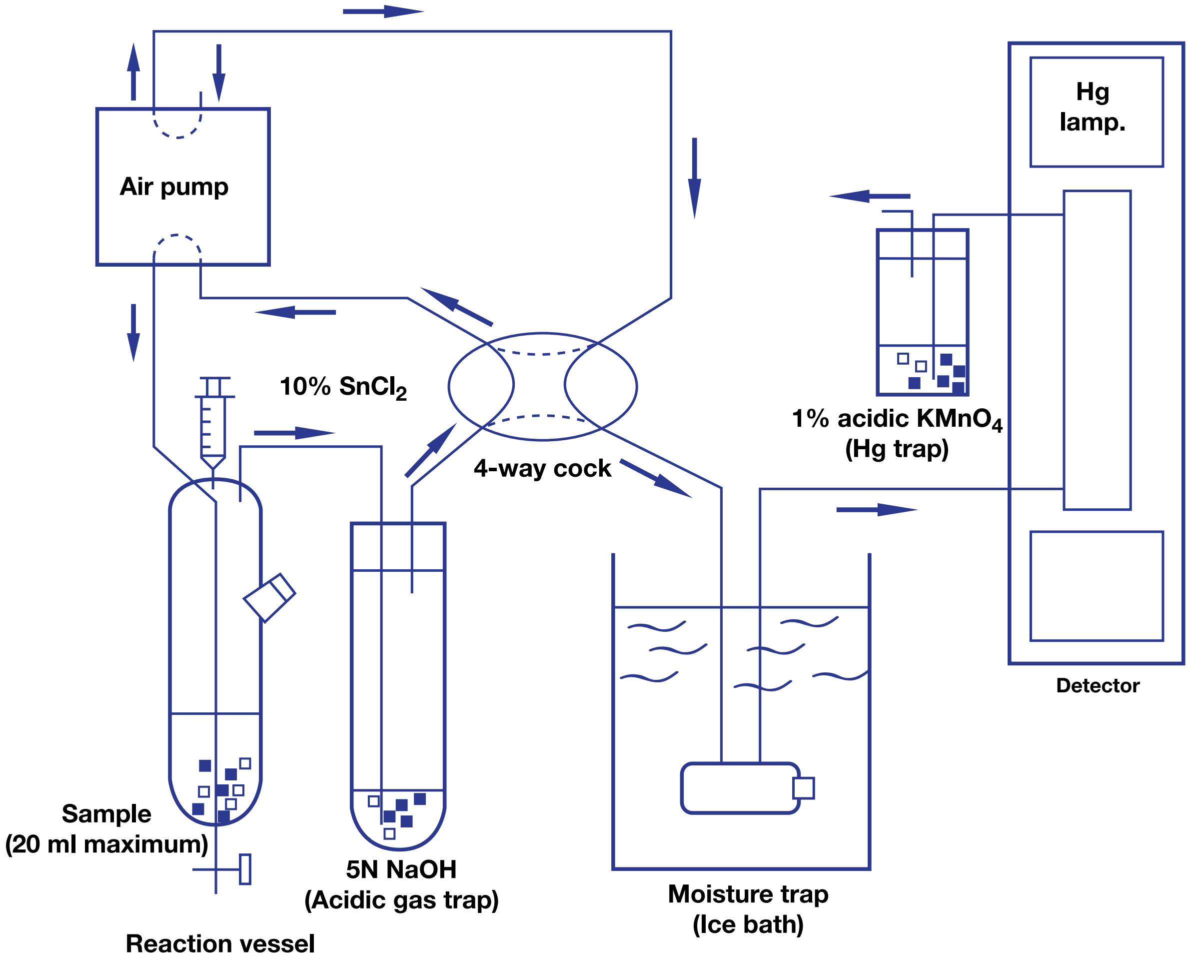
2.4. Equipment, materials and solutions

2.4.1. Equipment

The method described in this SOP includes the following: reduction of inorganic mercury ions in the sample test solution with stannous chloride to generate elemental mercury vapour; and the introduction of mercury vapour into the photo-absorption cell of the mercury analyser for the measurement of absorbance at 253.7 nm. This method uses a circulation-open air flow system as shown in Fig. 2. The apparatus constitutes a closed system and comprises a diaphragm pump, reaction vessel, acid gas trap, moisture trap (ice bath) and a 4-way cock.

During its operation, the elemental vapour generated by the addition of stannous chloride is circulated via the 4-way cock at a flow rate of 1–1.5 L/min for 30 seconds to allow the mercury vapour to come to an equilibrium between gaseous and aqueous phases. The 4-way cock is then rotated by 90° to introduce the gas phase into the photo-absorption cell all at once. The measurement is completed within one minute per sample with this apparatus, which can measure even 0.1 ng of mercury with high accuracy and precision.

Fig. 2. Schematic diagram of reduction/cold vapour atomic absorption spectrometry (circulation-open air flow system)



Source: Akagi 1997 *(12).*

2.4.2. Materials

The following materials are required for analysing total mercury in urine:

- mercury analyser: Model Hg-201 Semi-Automated Mercury Analyser;
- hot plate: capable of attaining a surface temperature of 250 °C;
- sample digestion flask: 50 mL thick-walled volumetric flask made of Pyrex (150 mm total height, 13 mm inlet diameter);
- volumetric flasks: 10, 100 and 1000 mL;
- measuring pipettes: 0.2, 0.5, 1.5 and 10 mL; automatic pipettes can also be used (range 0.1–10 mL);
- centrifuge;
- multi-flow meter: V4-type flow meter multi-kit.

2.4.3. Reagents and chemicals

The following reagents and chemicals are required for analysing total mercury in urine.

- Nitric acid-perchloric acid (1+1): mix 100 mL of perchloric acid (for measurement of toxic metals) into 100 mL of nitric acid (for measurement of toxic metals). Store in a cool dark place.
- Sulfuric acid (for measurement of toxic metals).
- Distilled water: distil deionized water and store in a clean glass container.
- Hydrochloric acid (analytical grade).
- 2M sulfamic acid: partially fill a pre-screened or pre-acid-washed 50 mL polypropylene centrifuge tube with bidistilled water. Add 10 g of sulfamic acid. Fill to the 50 mL mark with bidistilled water. Dissolve the sulfamic acid by mixing well (use of a vortexer, or warm water bath is helpful in this process). Store at room temperature. Expiration is one year from preparation.
- 10% tin (II) chloride solution: dissolve 10 g of tin (II) chloride dihydrate (analytical grade), in 9 mL of hydrochloric acid and dilute to 100 mL with distilled water. Aerate with nitrogen gas (100 mL/min, 20–30 minutes) to expel any mercury from the solution.
- 5M sodium hydroxide: dissolve 20 g of sodium hydroxide (analytical grade) in distilled water to make a final volume of 100 mL.
- 0.1M sodium hydroxide: dilute 5N sodium hydroxide 50-fold with distilled water.
- 2M sulfuric acid: gradually add 30 mL of sulfuric acid (for measurement of toxic metals) to distilled water to make a final volume of 1000 mL.
- 0.5% potassium permanganate solution: dissolve 0.5 g of potassium permanganate (analytical grade) in distilled water to make a final volume of 100 mL. This is used for cleaning the glass ware.

2.4.4. Calibration standards

Inorganic mercury standard solution

Weigh out 13.5 mg of mercury (II) chloride (standard) in a 100 mL volumetric flask, dissolve in 4 mL of nitric acid-perchloric acid (1+1) and 10 mL of sulfuric acid added in turn, and top up to the mark with distilled water to make a stock mercury solution (1 mL of the stock mercury solution = 100 µg mercury). The stock mercury solution obtained in such way will be stable for several years if sealed and stored in a cool dark place. At every use, the stock solution is diluted 10 000 times with the above blank test solution to make a mercury standard solution (1 mL of this solution = 0.010 µg mercury). This should be done in two consecutive steps. Dilutions should be made at an ambient temperature of 20–23 ºC.

Mercury standard solution

A mercury standard solution (SRM 3177) prepared from high-purity mercury (II) chloride is available from the National Institute of Standards and Technology (NIST), for calibration purposes. A unit of this material consists of five borosilicate glass ampoules, with each ampoule containing approximately 10 mL of solution. A certified value is assigned for mercury, with a nominal mass fraction of 1 mg/g. Working standard solutions are prepared by appropriate dilutions by a factor of 10 000. The working calibration solution is prepared in two steps, to obtain a concentration of 0.010 µg/mL.

2.5. Calibration

The multi-point calibration curve method is not always required because the calibration curve is linear across a wide concentration range. Therefore, a three-point calibration curve method is used. In addition to the blank solution, the most suitable concentration of the standard test solution should be chosen (e.g. 0.01, 0.03 or 0.05 µg mercury/50 mL) for a total mercury measurement with a peak height close to that of the sample test solution. In this case, the same volume of both the standard test solution and sample test solution should be used during the measurements. This will facilitate quantification.

2.6. Procedure

2.6.1. Acid digestion

Fig. 3 illustrates the procedure for determination of total mercury in urine. Put 2 mL of nitric acid-perchloric acid (1+1) and 5 mL of sulfuric acid into a sample digestion flask beforehand. Gradually add a known volume (usually 2 mL) of the urine sample while stirring slowly. Follow the same steps to make a blank test solution and a standard test solution.

Each sample should be prepared in duplicates. Blank solution is prepared in the same way as the sample, except no sample is added to the blank flasks. Standard test solutions are also prepared in the same way as the sample, except that instead of urine samples, standard solution is added to the flasks. At least three calibration points are needed, normally covering the range of 0.5–5 ng/mL.

The sample flasks should be heated for 30 minutes on a hot plate at 200–230 °C. Once the flasks have cooled, add distilled water to make a fixed volume of 50 mL, mix well and use the resulting solutions as the sample test solutions.

Fig. 3. Determination of total mercury in urine

Sample digestion flask

| HNO_3-$HClO_4$ (1+1), 2 mL
| H_2SO_4, 5 mL

Urine samples, 2 mL

| Add dropwise while swirling slowly
| Heat at 200–230 ^{0}C for 30 min

Digested samples

| Cool
| Top up to 50 mL with distilled water

Test solution, a fixed volume (usually 5 L)

| 10% $SnCl_2$ solution, 1 mL

CVAAS

CVAAS = cold vapour atomic absorption spectrometry;
H_2SO_4 = sulfuric acid;
HNO_3-$HClO_4$ = nitric acid-perchloric acid;
$SnCl_2$ = tin (II) chloride

2.6.2. Measurement

The automated apparatus used for this process is commercially available as a Model Hg-201 Semi-automated Mercury Analyser.

Gently transfer known volumes of test solution (usually 5 mL, up to a maximum of 10 mL) to the reaction vessel of the mercury analyser, add mercury-free water up to the 20 mL mark and apply the stopper. Add 1 mL of 10% tin (II) chloride in 1N hydrochloric acid solution with the accessory dispenser and push the start button. The diaphragm pump will run and the generated elemental mercury vapour will be circulated through the 4-way cock between the reaction vessel and the acidic gas trap for 30 seconds until equilibrium between gaseous and aqueous phases is reached. Acidic gas generated from the sample test solution is collected in the alkaline solution. After 30 seconds, the 4-way cock will turn automatically by 90°, allowing the introduction of mercury vapour into the photo-absorption cell through an ice bath for measurement of the absorbance. The readings of the recorder will increase sharply and decrease with a sharp peak. When the recorder reading begins to decrease, open the cock on the lower part of the reaction vessel to discard the solution inside, close it again, and allow it to aerate until it returns to the baseline. Push the reset button to start the next measurement. Each of the blank test solutions should be measured first. After that standard test solutions should follow. If the calibration curve is acceptable, sample test solutions can be measured.

Note. The equilibrium concentration between the aqueous phase and the gas phase of reduced and vaporized mercury vapour may differ depending on the acid concentration and volume of the sample test solution at measurement. Therefore, the blank test solution is used for dilution of the sample test solution, and both the sample test solution and the standard test solution are measured under the same conditions in every respect (acid concentration and volume).

2.7. Calculation of the analytical results

The peak heights (mm) obtained after measurement of known volumes of the blank, the standard and the sample test solutions (or their diluted solutions) are labelled P_{blank} (blank), P_{std} (standard) and P_{sample} (sample), respectively. The total mercury concentration in the sample is calculated according to the following formula.

$$c_{sample} = \left(\frac{P_{sample} - P_{blank}}{P_{std} - P_{blank}}\right) \cdot F \cdot \frac{c_{std}}{m_{sample}}$$

c_{sample} – concentration of mercury in the sample (ng/mL or ng/g)

c_{std} – concentration of mercury in the standard (ng/mL); for example 10 ng/mL

P_{sample} – peak height in mm for the digested sample (for 5 mL taken from the 50 mL of the digested sample)

P_{std} – peak height in mm for the standard solution (1 mL of standard solution of 10 ng/mL was prepared in the same way as the sample and 5 mL out of 50 mL of that sample was taken for the measurement)

P_{blank} – peak height in mm of the blank test solution

F – dilution factor of the standard (for the case study above the dilution factor was 0.1; 1 mL of the standard solution 10 ng/mL was diluted to 50 mL and 5 mL was taken for the measurement)

m_{sample} – mass of the sample in g or mL

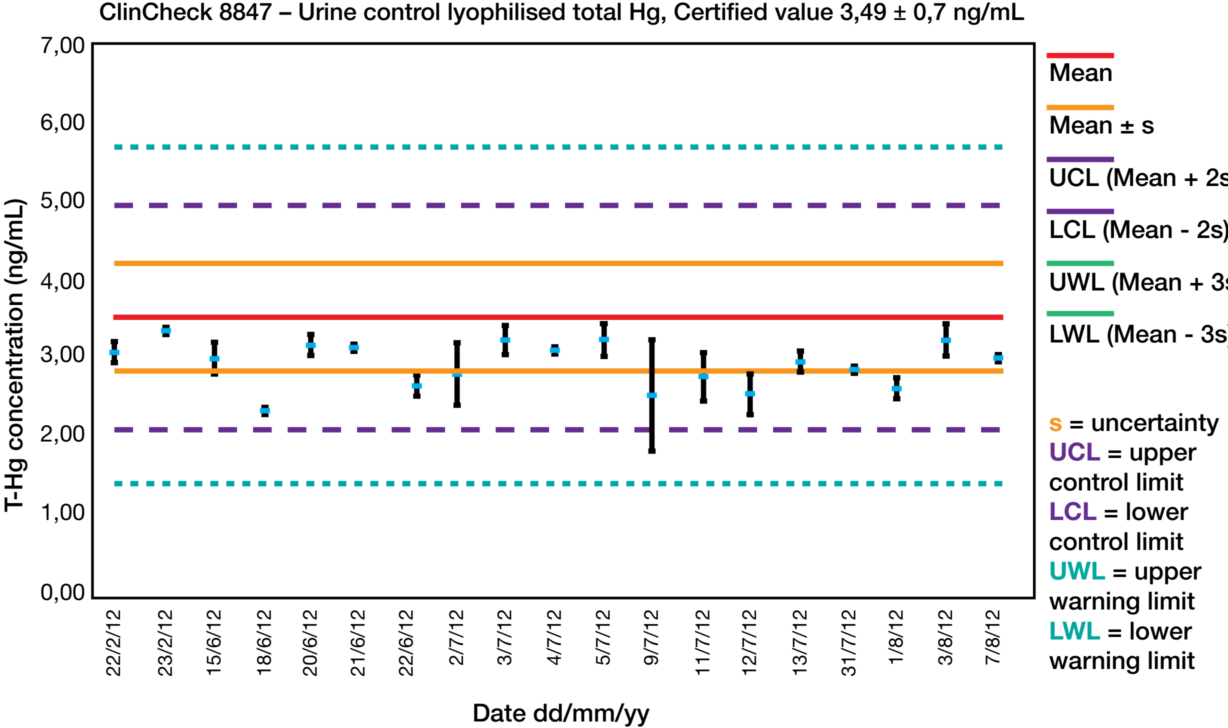
2.8. Quality control

Two reference materials were used in developing this SOP: Clin Chek 8847 (Recipe, Germany) and Seronorm trace elements urine – blank (Sero As, Norway). The analysts must obtain reference materials certified for mercury in urine at the concentrations typical for the concentration range measured in the sample.

Each sample should be analysed in duplicates. If the parallel analyses differ by more than 10%, the sample needs to be reanalysed.

In each set of the analysis, the three blanks and duplicates of the quality control material (preferably reference material) need to be analysed and the quality control charts prepared (Fig. 4).

Fig. 4. Quality Control Chart (ClinChek 8847)



2.9. Evaluation of the method

Each laboratory should comply with the standard “ISO/IEC 17025:2005 General requirements for the competence of testing and calibration laboratories” *(15)*. The method should be validated for its performance criteria (sensitivity, linearity, recovery, robustness, precision, accuracy, LOD, etc.) and should be accompanied by measurement uncertainty estimation, as the latter is a fundamental property of a result and a requirement of the standard ISO/IEC 17025:2005. The concentration levels of mercury in urine are low and the LOD of the method should be at least 0.05 ng/mL, and the LOQ at least 0.1 ng/mL, to be able to measure concentrations in the general population.

For the method described in this SOP, the performance criteria and measurement uncertainty estimation are specified below.

2.9.1. Limit of detection and limit of quantification

The LOD was determined by assessment of mercury in 10 blank solutions. The concentration of mercury in 50 mL of the blank solution was 0.10 ± 0.010 ng. LOD was calculated using the following equation.

$$LOD = 3 \cdot SD_{blank}$$

SD – standard deviation

The LOD for the sample was then calculated as follows.

$$LOD = \frac{3 \cdot SD_{blank}}{V_{sample}(m_{sample})}$$

V_{sample} – volume (mL) or a mass (g) of the sample

m_{sample} – mass of the sample in g or mL

In the above case, the LOD was 0.03 ng/50 mL and the LOQ for the 2 mL of sample intake was 0.015 ng/mL.

The LOQ was calculated as five times the LOD.

$$LOQ = 5 \cdot LOD$$

The LOQ for the example above is 0.075 ng/mL.

2.9.2. Precision

As a measure of the degree of reproducibility of the described analytical method, routine analysis of urine samples over the course of a longer time period (e.g. one year) is used. For the purpose of demonstration, the results of one measurement series (n=15) of total mercury in urine are shown in Table 2. Each sample was analysed in 2 replicates.

Table 2. Results of duplicate measurements of total mercury in urine samples and their relative differences

Sample	Result D1 (ng/mL)	Result D2 (ng/mL)	Mean value (D1+D2/2)	Difference (D1-D2)	Relative difference (D1-D2/mean)
Urine 1	2.02	1.62	1.82	0.40	0.22
Urine 2	0.71	0.63	0.67	0.08	0.12
Urine 3	0.51	0.51	0.51	0.00	0.00
Urine 4	0.54	0.51	0.53	0.03	0.06
Urine 5	1.19	1.27	1.23	-0.08	-0.07
Urine 6	0.67	0.67	0.67	0.00	0.00
Urine 7	1.66	1.62	1.64	0.04	0.02
Urine 8	3.80	3.76	3.78	0.04	0.01
Urine 9	0.59	0.55	0.57	0.04	0.07
Urine 10	0.61	0.69	0.65	-0.08	-0.12
Urine 11	0.69	0.69	0.69	0.00	0.00
Urine 12	0.61	0.55	0.58	0.06	0.10
Urine 13	0.92	0.98	0.95	-0.06	-0.06
Urine 14	0.72	0.70	0.71	0.02	0.03
Urine 15	0.79	0.74	0.77	0.05	0.07

D1 = measurement 1; D2 = measurement 2.

To assess reproducibility or repeatability, standard deviation of replicate measurements is calculated using the following equation.

$$RSD_d = \frac{s_d}{\sqrt{n}}$$

RSD_d – relative standard deviation of duplicate measurements

s_d – standard deviation of relative differences ((D1-D2)/mean)

n – number of replicates (n=2)

The repeatability calculated for the given set of measurements was 5.9%.

2.9.3. Trueness

The trueness of our results was estimated using the reference material ClinChek Urine Controls (Level I). As a measure of trueness of our results, recovery *(R)* was calculated based on measurements of the reference material over the course of six months. The observed levels were compared against the reference value using the following equation.

$$R = \frac{observed\ value}{reference\ value}$$

R – recovery

An example of measurements of total mercury in the reference material is given in Table 3.

Table 3. Measurements of total mercury in ClinChek Urine Controls (Level I)

Date	Mean value (ng/mL)	Reference value (ng/mL)	Recovery (%)
date 1	2.99	3.49	86
date 2	3.27	3.49	94
date 3	2.94	3.49	84
date 4	2.28	3.49	65
date 5	3.10	3.49	89
date 6	3.05	3.49	87
date 7	2.69	3.49	77
date 8	2.73	3.49	78
date 9	3.22	3.49	92
date 10	2.99	3.49	86
date 11	3.18	3.49	91
date 12	2.72	3.49	78
date 13	2.57	3.49	74
date 14	2.86	3.49	82
date 15	2.80	3.49	80
date 16	2.62	3.49	75
date 17	3.26	3.49	93
date 18	2.97	3.49	85

Based on the measurements given in Table 3, the recovery calculated was 83%.

2.9.4. Measurement uncertainty

Measurement uncertainty for total mercury in urine by acid digestion and CVAAS was estimated based on the approach and validation data set out in the ISO *Guide to the expression of uncertainty in measurement*. The procedure is described in the EURACHEM/CITAC guide, *Quantifying uncertainty in analytical measurement (16)*.

Step 1. The measurand was specified using the quantitative expression relating the value of the measurand to the parameters on which it depends (described in Section 2.7.).

$$c_{sample} = \left(\frac{P_{sample} - P_{blank}}{P_{std} - P_{blank}}\right) \cdot F \cdot \frac{c_{std}}{m_{sample}}$$

Step 2. Based on the quantitative expression, uncertainty sources were identified. These included the parameters listed in Table 4.

Table 4. Uncertainty components for the total mercury in urine

Input parameter	Value	Standard uncertainty	Relative standard uncertainty (%)
Sample signal *(P_{sample})*	30.0 mm	0.5 mm	1.6
Sample mass *(m_{sample})*	20 mg	0.06 mg	0.29
Volume of a sample in volumetric flask (V_{tot})	50 mL	0.12 mL	0.24
Volume of a sample aliquot analysed ($V_{analysed}$)	5 mL	0.0095 mL	0.2
Concentration of standard solution *(c_{std})*	10 ng/mL	0.014 ng/mL	0.14
Volume of standard solution *(V_{std})*	0.1000 mL	0.00094 mL	0.94

Step 3. In this step, uncertainty components were quantified. All uncertainty contributions must be expressed as standard uncertainties, that is, as standard deviations.

Standard uncertainties for the components identified from the quantitative expression were obtained from experimental data (e.g. pipette volumes) or from the producer's certificate (e.g. mass balance, volumetric flask).

The estimated standard uncertainties are given in Table 4. Relative standard uncertainties that do not exceed 10% of the largest uncertainty contribution are not taken into account in measurement uncertainty estimation. Among the listed uncertainties, uncertainties arising from the peak height (u_P) and the volume of standard solution (U_{Vstd}) were identified as significant.

Additional uncertainty components were estimated using validation data. For this purpose, reproducibility (repeatability) and recovery data was used.

The uncertainty of repeatability (u_{rep}) was 5.9% (Section 2.9.2), while the uncertainty of recovery (*u(Rm)*) was 8.5% and was calculated using the following equation.

$$u(\bar{R}_m) = \bar{R}_m \times \sqrt{\frac{s_{obs}^2}{n \cdot \bar{C}_{obs}^2} + \left(\frac{u(C_{ref})}{C_{ref}}\right)^2}$$

R_m – recovery
s_{obs} – standard deviation of the observed data
C_{obs} – mean value of the observed data
C_{ref} – reference value
$u(C_{ref})$ – uncertainty of reference value

In the final step, Step 4, combined uncertainty was calculated. Before combination, all uncertainty contributions must be expressed as standard uncertainties (standard deviations). The combined uncertainty (u_c) was calculated using the following equation.

$$u_c = \sqrt{u_P^2 + u_{Vstd}^2 + u_{rep}^2 + u_{rec}^2}$$

u_c – combined uncertainty
u_P – error due to repetitions of measures
u_{Vstd} – error due to the standards
u_{rep} – error due to reproducibility
u_{rec} – error due to recovery

Expanded uncertainty *(U)* was expressed by multiplying u_c with the factor *k*. The choice of the factor *k* is based on the level of confidence desired. For an approximate level of confidence of 95%, *k* is 2.

The estimated measurement uncertainty for the determination of total mercury in urine by acid digestion and CVAAS is 11%, expanded uncertainty (k=2) is 22%. The estimation is valid for a "normal" exposure range, that is below 5 ng/mL.

3. Analysis of creatinine in urine

The concentration of mercury and other chemicals in urine can vary significantly due to the amount of dilution with water, tests for contaminants in urine are often expressed in micrograms of contaminant per gram of creatinine *(3)*. Creatinine is a by-product of protein metabolism in the muscles that is formed from creatinine phosphate. Creatinine mainly undergoes glomerular filtration in the kidney and is almost completely excreted. On average, adults of a normal body weight, aged 30–60 years excrete 1.0–1.6 g of creatinine per day.

The physiological formation of creatinine in healthy people is essentially proportional to their muscle mass, thus explaining why creatinine excretion is generally lower in women than in men. Children exhibit a daily excretion of creatinine that is quite strongly age-dependent. In addition to age and gender, creatinine excretion is also particularly influenced by the consumption of meat and the intake of certain medications, such as opiates and diuretics. Urine production can vary widely depending on the intake or loss of fluids and the consumption of coffee, alcohol or medications. In contrast,

creatinine excretion generally remains relatively constant throughout the day, with only slight diurnal fluctuations. For this reason, the creatinine concentration in urine often serves as a reference value for the analysis of materials and their metabolites in urine. Thus, diurnal variations in the dilution of urine can be compensated for when exposure to xenobiotics is assessed. However, linking the concentration of hazardous substances in urine to creatinine concentration does not make sense in every case, and the above-mentioned factors that influence creatinine excretion also have to be taken into consideration.

If xenobiotics are reabsorbed to a significant extent in the tubular region of the kidneys, their concentrations cannot be assumed to be directly proportional to that of creatinine *(17,18)*. Likewise, the use of creatinine content as a reference value for highly diluted or very concentrated urine samples also leads to invalid values for substance concentrations.

Thus, on principle, the Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area advises against calculating the concentration of hazardous substances or metabolites in urine with respect to creatinine concentration.

Despite this, the creatinine concentration should be measured in each urine sample that is to be tested for hazardous substances to help assess the results obtained. In the case of creatinine concentrations of less than 0.5 g/L or more than 2.5 g/L, the results obtained for the hazardous substances or their metabolites should not be taken into account in the reported findings *(17)*.

3.1. Scope of the method

The method described herein is based on the Jaffé reaction *(19)* and permits the determination of creatinine in urine on a miniaturized scale using a photometric microplate reader. This method is used to achieve rapid and accurate quantification of creatinine in urine. The test range of the method is > 0.004 mg creatinine/mL urine.

The main advantage of the method described herein lies in the high number of samples that can be determined in a very short time. Moreover, it is easier to keep the reaction conditions stable as all samples are analysed at the end point of the reaction, thereby minimizing the risk of temporary measurement fluctuations.

3.2. Technical principle

Urine samples that have been diluted in a 1:50 ratio are applied to a microtitre plate and picric acid and sodium hydroxide are added. After a reaction time of 30 minutes, the absorbance of the reaction product is measured at an absorbance maximum of 500 nm using a photometric microplate reader.

Calibration is carried out using aqueous creatinine standard solutions, which are treated in the same manner as the samples by adding picric acid and sodium hydroxide solution and measured by photometry.

3.3. Safety precautions

The following safety precautions should be taken when analysing creatinine in urine.

- Safety precautions for biological hazards should be taken when working with urine.
- A biological safety cabinet should be used when diluting urine samples.
- Gloves, a laboratory coat and safety glasses should be worn when handling all solutions.
- Appropriate containers should be used for waste and biological residues. Pipette tips, autosampler

tubes, gloves and other items that come into contact with urine should be placed in a biohazard autoclave bag or container.

3.4. Equipment, materials and solutions

3.4.1. Equipment

The following equipment is required for analysing creatinine in urine:

- vortex mixer used for vortexing urine specimens before removing an aliquot for analysis;
- micropipette 10–100 μL;
- micropipette 100–1000 μL;
- micropipette 1–10 mL;
- multichannel pipette 50–300 μL;
- analytical balance (readability 0.01 mL);
- centrifuge;
- microplate shaker;
- spectrophotometer;
- freezer (for long-term storage of samples and reagents);
- refrigerator (for intermediate storage of stock standards and reagents);
- water purification system (for ultrapure bidistilled water used in reagent and dilution preparation) – this equipment produces deionized water to > 18 MΩ•cm.

3.4.2. Materials

The following materials are required for analysing creatinine in urine:

- gloves (powder-free, low particulate nitrile or latex gloves)
- pipette tips (1000 μL, 100 μL and 10 mL)
- 96-well microplates
- 1, 5, 10 and 50 mL polypropylene test tubes.

3.4.3. Reagents and chemicals

The following reagents and chemicals are required for analysing creatinine in urine:

- ultrapure water
- picric acid, 1.2% solution
- sodium hydroxide (analytical grade)
- hydrochloric acid, 37%.

3.4.4. Reference materials

The following reference materials are used for analysing creatinine in urine:

- Creatinine SRM 914a (National Institute of Standards and Technology)
- Quality-control solutions URN ASY CONTROL levels 2 and 3 (Randox Laboratories).

3.4.5. Solutions

The following solutions are required for analysing creatinine in urine.

- 0.1 M hydrochloric acid: 871 µL of 37% hydrochloric acid is transferred to a 100 mL volumetric flask. The flask is subsequently filled to its nominal volume with ultrapure water.
- 0.3 M sodium hydroxide: 3 g sodium hydroxide is weighed and dissolved in approximately 100 mL ultrapure water. The solution is transferred to a 250 mL volumetric flask, which is subsequently filled to its nominal volume with ultrapure water.
- Picric acid working solution: 10 mL of 1.2% picric acid solution and 10 mL of 0.3 M sodium hydroxide are transferred to a 50 mL polypropylene tube. The working solution must be prepared freshly and protected from light.

3.4.6. Calibration standards

The following calibration standards should be used for this SOP.

- Creatinine stock solution (1g/L): 10 mg of Creatinine SRM 914a is weighed into a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with 0.1 M hydrochloric acid. The stock solution is stored at 4 °C and its shelf life is two months.
- Calibration standards: the creatinine stock solution is diluted with ultrapure water in 10 mL volumetric flasks according to the scheme set out in Table 5. The calibration standards are stored at 4 °C and their shelf life is one week.

Table 5. Volume and concentrations for preparation of calibration standards

Volume of stock solution of creatinine (µL)	Final volume of calibration standard (mL)	Concentration of calibration standard (g/L)	Equivalent concentration in the urine samples (g/L)
40	10	0.004	0.2
80	10	0.008	0.4
200	10	0.020	1.0
400	10	0.040	2.0
800	10	0.080	4.0

3.5. Sample treatment and preparation

Powder-free gloves must be worn during sample handling.

The samples to be analysed are removed from the freezer and allowed to warm to room temperature. They are then vortexed and centrifuged at 3000 rpm for two minutes. Two commercial quality urine control samples of different concentrations (Assayed Urine Chemistry Control Level 2 and Level 3) are included in each analytical series. Each vial is reconstituted with 10 mL of bidistilled water and left to stand for 30 minutes at room temperature before use. It can be aliquoted and stored at -20 °C for two weeks.

Samples and quality controls diluted at 1/50 are prepared in triplicate. Next, 20 µL of sample or quality control is pipetted into 1.5 mL tubes. Then, 980 µL of ultrapure water is added. The tubes are capped and shaken to homogenize the dilution.

Next, 25 µL of standards, diluted samples and controls is added to each well of a 96-well microplate, according to the distribution outlined in Table 6.

The plate is covered with a lid and shaken on an orbital shaker at room temperature protected from light. After 30 minutes the plate is read at 492 nm.

Table 6. Distribution of standards, samples and controls on the microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	water	water	water	P1	P1	P1	P2	P2	P2	P3	P3	P3
B	P4	P4	P4	P5	P5	P5	S1	S1	S1	S2	S2	S2
C	S3	S3	S3	S4	S4	S4	S5	S5	S5	S6	S6	S6
D	S7	S7	S7	S8	S8	S8	S9	S9	S9	S10	S10	S10
E	S11	S11	S11	S12	S12	S12	S13	S13	S13	S14	S14	S14
F	S15	S15	S15	S16	S16	S16	S17	S17	S17	S18	S18	S18
G	S19	S19	S19	S20	S20	S20	S21	S21	S21	S22	S22	S22
H	S23	S23	S23	S24	S24	S24	C1	C1	C1	C2	C2	C2

Notes:
P1: aqueous 0.004 mg/mL creatinine standard
P2: aqueous 0.008 mg/mL creatinine standard
P3: aqueous 0.2 mg/mL creatinine standard
P4: aqueous 0.4 mg/mL creatinine standard
P5: aqueous 0.8 mg/mL creatinine standard
C1: 1/50 dilution quality control URN ASY CONTROL 2
C2: 1/50 dilution quality control URN ASY CONTROL 3
S1–S24: 1/50 sample dilutions.

3.6. Procedure

3.6.1. Preparation of analytical equipment

Turn on the spectrophotometer.

The system requires approximately 15 minutes of pre-heating time before measurements can be started.

3.6.2. Sample measurement

Measure the samples prepared according to point 7.1 at 500 nm.

3.6.3. Calculation of the analytical results

Data are reported directly by the equipment in terms of mg of creatinine/mL by interpolating the reading against the calibration curve taking into account the sample dilution.

The final value reported corresponds to the average of the three replicated measurements per sample. The standard deviation of these measurements can be calculated according to the following formula.

$$SD = \sqrt{\frac{\sum(c_i - \bar{c})^2}{n-1}}$$

SD – standard deviation

c_i – individual value

c – mean

n – number of determinations

3.6.4. Reportable results range

It must be checked that the values obtained for the quality controls meet the acceptance criteria established in their certificates of analysis. If these criteria are not met, analysis should be performed again.

The relative standard deviation of the three measurements for a sample should not be higher than 5%. Otherwise, the Grubbs test should be applied to determine whether one of the values is a significant outlier.

$$Z = \frac{\text{Mean} - \text{Suspected value}}{\text{SD}}$$

Z – Z value (for evaluation according to Grubbs test)

SD – standard deviation

If Z is greater than 1.15 this value can be rejected and the concentration of the sample calculated as the mean of the two remaining values. Otherwise, the sample should be retested.

Creatinine values are reportable in the range 0.3–3 mg/mL.

3.7. Quality control

The precision and accuracy of biomarker analyses carried out by toxicological laboratories must be continuously checked by means of quality assurance procedures.

In general, quality assurance in medical laboratories comprises both internal and external quality control, which is described in detail in the *Quality control programme for mercury human biomonitoring.*

3.7.1. Internal quality control

Internal quality assurance serves to systematically monitor repeatability in order to detect random errors and ensure the accuracy of quantitative laboratory investigations.

In practice, the repeatability is controlled by using a control material (reference material), which is measured as part of each analytical series. The results of daily or batch-wise internal quality control are entered into control charts.

If not commercially available, a control material (reference material) can be prepared by spiking a pool of native biological material (blood, urine, etc.) with a defined amount of the analyte (biomarker). Aliquots from this pool can be used for internal quality control as well as for inter-laboratory comparison programmes. These aliquots have proven to be, and to remain, homogeneous under specified storage and shipment conditions, with the analyte concentration remaining unchanged. A control material should cover the whole concentration range (e.g. Qlow, Qmedium, Qhigh), as well as blanks.

Accuracy should preferably be tested using a certified reference material, which is a (biological) material containing a certified concentration of one or more analytes. Certification is performed within a programme in which laboratories that are highly skilled in analysis of the biomarker in question, analyse the control material.

A certified value is established for each analyte following a validation procedure that includes expert judgment as well as statistical procedures. Certified reference materials are therefore expensive and should only be used when validating or revalidating an analytical method.

3.7.2. External quality control

External quality control is a means of improving the comparability and accuracy of analytical results. Comparability is the pre-state of accuracy and ensures that analytical results can be compared between laboratories and with the corresponding limit values.

Comparable and accurate results in HBM are necessary to achieve equal health prevention irrespective of the laboratory that analyses the biological sample.

An interlaboratory comparability investigation (ICI) is a means of harmonizing analytical methods and their application, thereby improving the comparability of analytical results.

Control materials (reference materials) can be used for this purpose. ICIs are even necessary when laboratories use the same analytical SOP.

An external quality assessment scheme (EQUAS) is a means of improving the accuracy of analytical results. For this purpose, a control material is usually analysed in reference laboratories that have been shown to be highly skilled in analysing a specific biomarker. The results obtained by the reference laboratories form the basis on which the assigned values and tolerance ranges for each of the biomarkers tested are determined. Those laboratories that participate in an EQUAS are certified for those results that fall within the tolerance ranges.

For this SOP, quality control materials are used to evaluate the accuracy and precision of the analysis process and to determine whether the analytical system produces results that are acceptably accurate and precise.

Quality control of the analytical results is carried out using the reference materials URN ASY CONTROL 2 and 3 (Randox).

It should be checked that the values obtained for quality controls meet the acceptance criteria established in their certificates of analysis. If this criterion is not met, analysis should be performed again.

Only those measurements performed between two quality controls whose values lie within the established range (assigned value of the reference material ± uncertainty in that level) are considered valid.

External quality controls are performed by participation in round-robin experiments. As an example, it is recommended to participate regularly in a G-EQUAS test organized by the Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine of the Friedrich-Alexander-University Erlangen-Nürnberg, Germany.

3.8. Evaluation of the method

3.8.1. Linearity

The linearity of an analytical procedure is the ability (within a set range) to obtain results which are directly proportional to the concentration of analyte in the sample. This parameter is evaluated by studying increasing analyte concentrations. In this SOP, the linearity of the method has been tested in the range 0.004–0.08 mg/mL creatinine.

The data obtained are analysed statistically to obtain the regression curve, correlation coefficient, determination coefficient and coefficient of linearity. A linear curve with a determination coefficient higher than 0.999 should be obtained.

3.8.2. Precision

This is a measure of how much the analytical results are scattered due to random errors. Precision is described statistically by means of the standard deviation or confidence interval. We can distinguish between the following:

- precision under repeated conditions (repeatability)
- precision under comparable conditions (reproducibility).

Samples in the range 0.2–3 mg/mL were used to determine the precision. Table 7 shows the results obtained for repeatability and reproducibility.

Table 7. Maximum standard deviation allowed

Concentration (mg/mL)	RSD_{repet}	RSD_{reprod}
0.2	11.5	7.3
0.4	4.8	5.4
0.7	2.2	4.7
2.1	1.8	2.5
2.5	4.8	5.0

RSD_{repet} – relative standard deviation for repeatability;
$RSDr_{eprod}$ – relative standard deviation for reproducibility.

3.8.3. Accuracy

This is a measure of the deviation of the measured value from the correct ("true") value due to a systematic error. The following approaches can be used to test the accuracy of a method:

- performance of recovery tests (spiking procedures);
- participation in inter-laboratory comparability investigations in which the theoretical value is ascertained by authorized reference laboratories;
- comparison of the analytical procedure to be validated with a reference procedure certified for determination of the parameter in the relevant sample matrix;

- comparison of the analytical results for a certified reference material with the certified reference value.

The accuracy was determined by adding known amounts of creatinine to the samples used for the determination of precision. Mean recovery rates obtained were in the range 98.2–104.4%.

The lower LOQ indicates the lowest possible analyte concentration that can be determined with a pre-defined uncertainty (usually 33%). The upper limit of quantification indicates the highest possible analyte concentration that can be determined.

The LOQ must be included in the calibration curve and can be calculated using various different methods.

Determination of the signal/background noise ratio

The background noise is determined as follows.

- The intensity of the background noise (s0) is determined in relation to the analyte.
- The LOD is calculated as three times the mean intensity of the background noise signal (LOD = 3 x s0).
- The LOQ is calculated as nine times the mean intensity of the background noise signal (LOQ = 9 x s0).

Other procedures

It should be noted that blank values in native samples have an influence on the choice of method and the approach used:

- standard deviation procedure (according to EURACHEM)
- blank value procedure (according to DIN 32 645)
- calibration curve procedure (according to DIN 32 645).

In this SOP, the LOQ has been calculated using the calibration curve procedure and the result obtained corresponds to the lowest value of the calibration curve, 0.004 mg/mL creatinine.

3.9. Sources of error

This analytical method is based on the Jaffé colour reaction *(19)* in which the active methylene group of creatinine reacts with the C3 atom of picric acid *(20,21)* to form a coloured reaction product *(19,21)*. However, this colour reaction between picric acid and creatinine is not specific to this substance. As a general rule, reducing compounds or compounds with a methylene group activated by $-NO_2$, $-CONH_2$, $-CH_2=CH_2-$, -COOR or -N=N-, can also form coloured products. Thus, no interfering reactions are caused by glucose, fructose, maltose, hydroxylamine or ascorbic acid, whereas aminoacetone, γ-aminolevulinic acid and aminooxyacetic acid exhibit a colour reaction with picric acid *(22)*.

As the concentrations of the above-mentioned chromogens are very low in urine *(20)*, interference caused by them can be regarded as insignificant. Thus, for example, the concentration of γ-aminolevulinic acid in urine is some 100–1000 times lower than that of creatinine.

Picric acid solution is sensitive to light and should therefore be kept in the dark. This also applies to the prepared microtitre plate during incubation.

When working with microtitre plates, it is essential to ensure that no liquid splashes out of the wells while pipetting, thus leading to contamination of other samples. To prevent this, use of a freely moving hand dispenser is recommended when pipetting the picric acid working solution.

Soiling (e.g. fingerprints) on the underside of the microtitre plate may lead to considerable interference during measurement. In extreme cases this may render the plate unreadable for the measurement device. As a result, the underside of the plate must be kept clean and should be wiped with a cloth soaked in ethanol before measurement.

3.10. Alternative method: determination of specific gravity in urine samples

As an alternative to creatinine analysis, specific gravity (SG) can be determined in urine samples to normalize the urine mercury levels for inter-individual differences in urine dilution *(23)*. This method has been widely used in many HBM studies. SG is determined in a drop of urine using a refractometer. This is a simple hand-held instrument, which is very easy to operate. The following procedure applies to the PAL-10S refractometer (Atago, Japan) but any similar instrument can be used.

The equipment required is as follows:

- refractometer (e.g. PAL-10S, Atago, Japan)
- containers for urine collection (can be the same as for mercury analysis)
- pipette (0.1–1 mL)
- distilled water
- cleaning cloth or disposable tissues
- gloves.

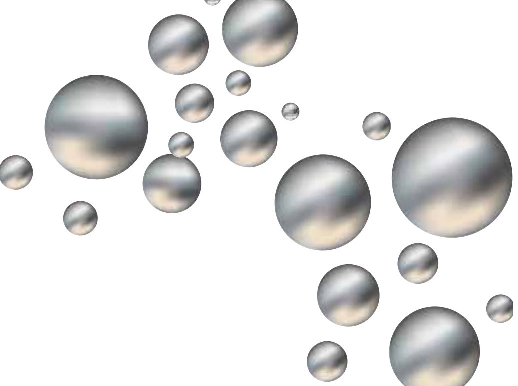
The procedure for determination of specific gravity in urine samples is as follows.

1. The temperature of the distilled water used for calibration (zero setting) and the sample should be the same as the ambient temperature.
2. Calibrate the refractometer by placing distilled water (approximately 0.3 mL) onto the prism surface and press the START key. If the display indicates “1.000”, zero setting does not need to be performed. If the indicated value is not “1.000”, press the ZERO key with the water left on the prism. After “000” is displayed, zero setting has been successfully completed. Remove the water from the prism surface using a soft non-abrasive tissue. This should be done before you begin testing, and after every 10 samples or so to ensure that the calibration remains accurate.
3. Measurement. Clean the surface with distilled water and dry with a soft non-abrasive tissue. Place a drop of urine (approximately 0.3 mL) onto the prism surface. Press the START key. The measurement value will be displayed on the screen. Remove the sample by wiping it off with a soft tissue. Use distilled water to remove any remaining sample. Dry off any excess moisture with a clean, dry tissue. To turn off the display, press and hold down the START key for approximately two seconds.
4. Calculation. As a standard in SG normalization calculations, SG means of 1.013 for females and 1.019 for males are normally used; calculation is described in the literature *(24)*.

$$U_{biomarker}/SG = U_{biomarker} * \frac{(SG_{std} - 1)}{(SG_{ob} - 1)}$$

$U_{biomarker}$ – level of a substance (e.g. mercury) measured in urine
SG_{ob} – specific gravity, observed
SG_{std} – mean specific gravity in a studied population

The SG results will normally range from 1.000 (which is equivalent to water) up to 1.035 (very dehydrated), but can also reach higher levels.



4. Interpretation of results

Urine mercury levels are usually considered the best measure of recent exposures to inorganic mercury or elemental mercury vapours because urinary mercury is thought to indicate most closely the mercury levels present in the kidneys *(25)*. However, inorganic mercury accumulates in the kidney and is slowly excreted through the urine. Therefore urine mercury levels can also represent exposures to elemental mercury and/or inorganic mercury that occurred sometime in the past *(3)*.

A strong correlation between elemental mercury levels in inhaled air and levels in urine, at medium and high concentrations, has been reported. The maximum urine mercury concentration set by WHO *(26)* is 50 µg/g creatinine. Mercury urine levels rarely exceed 5 µg/g creatinine in people who are not occupationally exposed to mercury *(3)*.

Mercury levels exceeding 20 µg/L urine have been found in urine samples from miners who frequently burn gold-mercury amalgams in open pans. Very high mercury concentrations in urine (as high as 1168 µg/L) were reported in workers of gold shops in Amazonian villages. The gold shop workers (who work in confined environments) had higher concentrations of mercury in urine than miners burning amalgam outdoors. In Alta Floresta, Mato Grosso, Brazil, the urine of employees in gold shops (where gold was melted in fume hoods with no filters) was analysed; the results showed mercury urine levels greater than 20 µg/L for at least 13 of 17 workers sampled *(27)*.

The German Human Biomonitoring Commission's reference value for adults without dental amalgam fillings is 1 µg/L in urine *(28)*. The corresponding reference value for children without amalgam fillings is 0.4 µg/L (28). The health-based HBM-I guidance value for mercury in urine is 7 µg/L or 5 µg/g creatinine. Geometric mean levels in adults in most countries are below the reference value *(8)*.

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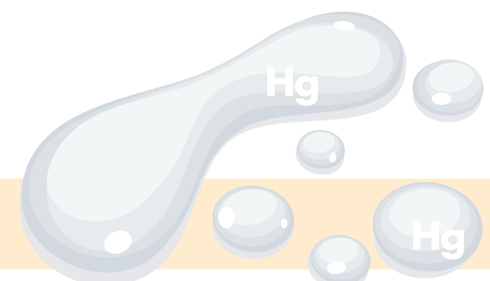

Annex 1. Urine sampling instructions for participants

Pleased read these instructions carefully before taking the first morning urine sample.

Note. At least five hours should have passed since last urination.

1. Go inside the toilet.
2. Wash your hands with soap and water and then dry them.
3. Remove the urine vessel from the zip-lock plastic bag. (Please only use the provided vessel. This vessel was pre-treated for this study.)
4. Open the urine vessel by unscrewing the lid.
5. Discharge your first morning urine into the vessel until it is filled to the pre-marked line.
6. Screw the vessel lid on tightly.
7. Place the urine vessel back into the zip-lock plastic bag.
8. Keep the sample at 4–8 ºC until you give it to the health-care staff (no longer than 24 hours).

Thank you very much for your cooperation.

Annex 2. Questionnaire for urine sample collection

Name of mother	
Medical record number	
Study ID of mother	
Medical worker	Signature: Printed name:

1. Is this a sample of urine taken in the morning?	☐ Yes ☐ No
2. Date and time of sample collection	------/-----/-------/ (day/month/year) Start: -------/------ (hour/min)
3. How many hours ago did you last urinate prior to this sample collection?	_ _ hours
4. How many hours prior to this sample collection was your last meal?	_ _ hours
5. When was the last time you had fish or other types of seafood prior to this sample collection?	☐ Today ☐ Yesterday ☐ Day before yesterday

Annex 3. Sample reception list

Volume	**Urine**			**Aliquoting urine samples**	
	U1	U2	X U		
	Mercury	Creatinine	Biobank		
	5 mL	5 mL	x 40 mL or x 10 mL		
Store t° field work and transport	Cool box	Cool box	Cool box		
Store t° laboratory	-20 °C	-20 °C	-80 °C		
Identification number				Date	hour
ID					
ID					
ID					
….					

Standard operating procedure for the determination of total mercury in hair, blood and urine by the alternative method

Abstract

The alternative standard operating procedures are described for laboratories that have access to instruments with flow injection analysis and gold amalgamation followed by either cold vapour atomic absorption spectrophotometry (CVAAS) or cold vapour atomic fluorescence (CVAFS) detection. The standard operating procedure describes the digestion procedure. Mercury present in digested samples can then be determined either by a flow injection procedure or by gold amalgamation CVAFS (or CVAAS).

Keywords

Mercury – analysis
Methylmercury compounds – analysis
Fetal blood – chemistry
Umbilical cord – chemistry
Hair – chemistry
Urine – chemistry
Biomarkers – analysis
Flow injection analysis
Spectrophotometry, atomic
Environmental exposure

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Abbreviations

BrCl	bromine chloride
CVAAS	cold vapour atomic absorption spectrophotometry
CVAFS	cold vapour atomic fluorescence
HCl	hydrochloric acid
$HgCl_2$	mercuric chloride
HNO_3	nitric acid
KBr	potassium bromide
$KBrO_3$	potassium bromate
$K_2Cr_2O_7$	potassium dichromate
KMnO4	potassium permanganate
$SnCl_2$	stannous chloride
v/v	volume/volume
V_2O_5	vanadium pentoxide
w/v	weight/volume

1. Acid digestion of the biological samples

1.1. Scope of the method

The method described is intended to determine the total mercury (Hg) in biological samples.

1.2. Technical principle

This method is applicable to all biological samples with total Hg concentrations higher than 1 ng/g. The purpose of the strong acid digestion is to decompose the samples and oxidize and convert any organic forms of Hg into inorganic Hg.

1.3. Safety precautions

Follow universal precautions. Wear gloves, a laboratory coat and safety glasses while handling human blood, plasma, serum, urine or other bodily fluids or tissues. Place disposable plastic, glass and paper items (pipette tips, autosampler tubes and gloves) that come into contact with human biological fluids (such as urine) in a biohazard autoclave bag. Keep these bags in appropriate containers until they are sealed and autoclaved.

When the work is finished, wipe down all work surfaces where human biological fluids were handled with a 10% (volume/volume (v/v)) sodium hypochlorite solution or equivalent. The use of the foot pedal on the Micromedic Digiflex™ is recommended because it reduces the analyst's contact with working surfaces that have been in contact with human biological fluids and allows the hands to be free to hold specimen cups and autosampler tubes. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis according to the guidelines for disposal of hazardous waste.

1.4. Digestion of biological material

1.4.1. Equipment

- Glass bottle, 1 litre, cleaned according to the procedure for glassware.
- Volumetric flask, 500 ml (Class A), cleaned according to the procedure for glassware.
- Teflon vials with caps (60 ml), cleaned according to the procedure for Teflon.
- Polypropylene spatulas.
- Hot plate and aluminium block.
- Precision balance.

1.4.2. Cleaning glassware

Prior to use, wash all laboratory glassware thoroughly as follows.

- Allow the Teflon and glass vessels to soak overnight in 2% Micro-90 detergent solution.
- Rinse the vessels thoroughly first with tap water then with bidistilled water.
- Rinse with 0.5% potassium permanganate ($KMnO_4$) solution.
- Rinse with water until the colour of the $KMnO_4$ solution is no longer visible.

- Fill the vessels with 1% hydrochloric acid (HCl) solution and store in Hg-free storage facilities.
- Empty vials just before using them for sample processing and allow them to dry at 60 °C in a flow hood.

1.4.3. Cleaning Teflon

- Soak the vessels overnight in a plastic container in a soap solution (Micro solution 2% in tap water).
- Rinse thoroughly first with tap water and then with bidistilled water.
- Put the vessels in 50% (v/v) concentrated nitric acid (HNO3) solution and heat at 60 °C for two days.
- Rinse thoroughly with bidistilled water (at least four times).
- Transfer the vessels into 10% (v/v) concentrated HCl solution for one day (at least) at room temperature.
- Rinse thoroughly with bidistilled water (at least four times).
- Store all vessels in polyethylene plastic bags. When possible (principally Teflon and glass bottles), fill the vessels with 1% HCl.

1.4.4. Reagents and chemicals

- HNO_3 (65%, analytical grade, low in Hg)
- HCl (30%)
- Vanadium pentoxide V_2O_5 (extra pure)
- Potassium dichromate ($K_2Cr_2O_7$)
- Potassium bromate ($KBrO_3$) (analytical grade)
- Potassium bromide (KBr) (analytical grade)
- bidistilled deionized water (>18 MΩ cm)

There are two choices for oxidizing solutions.

$K_2Cr_2O_7$ 10% (weight/volume (w/v)) in bidistilled water

1. Weigh 50 g of $K_2Cr_2O_7$ into a clean 500 ml glass volumetric flask.
2. Add about 250 ml of bidistilled water and shake until the $K_2Cr_2O_7$ is dissolved.
3. Make up to the mark with bidistilled water.

BrCl oxidizing solution

1. Weigh accurately 11 g of $KBrO_3$ and 15 g of KBr into a clean 1 litre glass bottle.
2. Add 200 ml of bidistilled water.
3. Add carefully 800 ml of concentrated HCl; the dilution must be carried out in a well-ventilated fume hood to prevent exposure to toxic fumes released during dissolution of $KBrO_3$.
4. Keep the bottle wrapped with aluminium foil.

These two solutions can be kept for an unlimited time if stored in the dark at room temperature in a tightly closed Teflon or glass bottle in an Hg-free area.

1.4.5. Procedure

1. Shake the vials containing the samples for about two minutes for homogenization.
2. Wait a few minutes before opening the vials.
3. Weigh accurately from 0.5–1 ml of blood sample, 20–100 mg of hair sample or 1–2 ml of urine sample into Teflon vials (60 ml).
4. Weigh 45 mg of V_2O_5 into these vials.
5. Add 5 ml of concentrated HNO_3 (or more if necessary: the mixture must be liquid).
6. Close the caps and leave the vials to stand for at least one hour at room temperature. If the reaction is very strong, it may be safer to leave the samples at room temperature overnight before heating.
7. Put the tubes into an aluminium block on a hot plate at 90 °C and leave for three hours.
8. Allow the samples to cool to room temperature before opening the tubes. Leave the tubes to cool in a fume hood to avoid toxic acid fumes.
9. Add about 20 ml of bidistilled water.
10. Add 1 ml of $K_2Cr_2O_7$ solution (final concentration = 2% v/v), or 0.5 ml of BrCl solution (final concentration = 1% v/v).
11. Dilute to the mark with bidistilled water (dilution volume = 57.5 ml).
12. Shake the vials and wait for sedimentation of material before analysis.

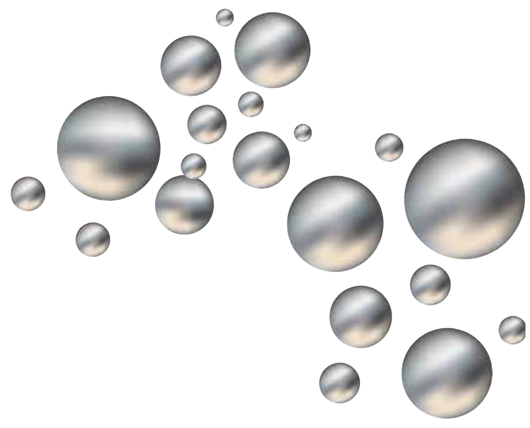
These samples can be kept for a few days before analysis if they are stored in the refrigerator (+4 °C). The maximum storage time must be determined by experience for each kind of sample.

1.4.6.Reagent blanks

At least three blanks should be prepared for each batch of analysis. They are prepared in a similar manner as the samples, except that no sample is added to the digestion tubes.

1.4.7. Reference materials

At least one certified reference material should be used and prepared in triplicate for each batch of analysis. These digestions are prepared in a similar manner as the sample. Certified reference material should be of a similar composition and concentration of Hg as the samples.



2. Determination of total Hg using flow injection analysis and CVAAS detection

2.1. Principle and application

Biological samples are mineralized with strong acids. The inorganic Hg is reduced to its elemental form with stannous chloride using a flow injection principle. The cold Hg vapour is separated from the digested samples in a gas-liquid separator and the Hg vapour is then passed through the quartz absorption cell of an AAS where its concentration is measured. The light beam of the Hg hollow cathode lamp is directed through the quartz cell into a monochromator and on to a detector that measures the amount of light absorbed by the atomized vapour in the cell. The amount of energy absorbed at the characteristic wavelength is proportionate to the concentration of the element in the sample.

2.2. Equipment, materials and solutions

2.2.1. Equipment

AAS Varian-Spectra AA-10 and VGA-76 or any equivalent system based on the flow injection principle.

2.2.2. Materials

- Micropipettes.
- Teflon bottles 125 ml, cleaned according to the procedure for Teflon.
- Precision balance.
- Glass volumetric flasks from 50 ml to 1000 ml (Class A), cleaned according to the procedure for glassware.

2.2.3. Cleaning glassware

Prior to use, thoroughly wash all laboratory glassware as follows.

- Allow the Teflon and glass vessels to soak overnight in 2% Micro-90 detergent solution.
- Rinse the vessels thoroughly first with tap water then with bidistilled water.
- Rinse with 0.5% $KMnO_4$ solution.
- Rinse with water until the colour of the $KMnO_4$ solution is no longer visible.
- Fill the vessels with 1% HCl solution and store in Hg-free storage facilities.
- Empty vials just before sample processing and allow them to dry at 60 °C in a flow hood.

2.2.4. Cleaning Teflon

- Allow the vessels to soak overnight in a plastic container in a soap solution (Micro solution 2% in tap water).
- Rinse thoroughly first with tap water and then with bidistilled water.
- Put the vessels in 50% (v/v) concentrated HNO_3 solution and heat at 60 °C for two days.
- Rinse thoroughly with bidistilled water (at least four times).

- Transfer the vessels into 10% (v/v) concentrated HCl solution for one day (at least) at room temperature.
- Rinse thoroughly with bidistilled water (at least four times).
- Store all vessels in polyethylene plastic bags. When possible (principally Teflon and glass bottles), fill the vessels with 1% HCl.

2.2.5. Reagents and chemicals

- HNO_3 (65%, analytical grade, low in Hg)
- $K_2Cr_2O_7$ (analytical grade, low in Hg)
- KBr
- $KBrO_3$
- Stannous chloride ($SnCl_2$) (analytical grade, normal or low in Hg)
- HCl (30%)
- Mercuric chloride ($HgCl_2$) (salt) or standard Hg solution (1000 mg/L)
- bidistilled deionized water (>18 MΩ cm)
- Argon (pure quality)

2.2.6. Reagent solutions

20% w/v $SnCl_2$ in 20% v/v HCl (200 ml)

1. Weigh accurately 40 g of $SnCl_2$ into a clean glass beaker using a plastic spatula (beaker and spatula are used only for $SnCl_2$).
2. Add 40 ml of concentrated HCl directly to the $SnCl_2$ and transfer to a 200 ml volumetric flask. Mix and wait for complete dissolution of $SnCl_2$.
3. Add bidistilled water to the mark (200 ml).
4. With older stock of $SnCl_2$ it may be necessary to warm up the solution on a hot plate to obtain complete dissolution of $SnCl_2$ (do not allow to boil).
5. In case of low concentration samples, if the $SnCl_2$ used is not “low in Hg”, it should be purged with nitrogen for two hours before use.
6. This solution should be made fresh for each day of analysis.

Note: all glassware used for preparation of the $SnCl_2$ solution should be kept separate from the remaining laboratory ware in order to avoid cross-contamination of ware for trace element determination.

HNO_3 10% v/v (500 ml)

1. Put about 400 ml of bidistilled water into a 500 ml volumetric flask.
2. Add carefully 50 ml of concentrated HNO_3.
3. Make up to the mark with bidistilled water.
4. Shake well.

This solution can be stored if kept in a tightly closed flask.

There are two choices for oxidizing solutions.

$K_2Cr_2O_7$ 10% (w/v) in bidistilled water

1. Weigh 50 g of $K_2Cr_2O_7$ into a clean 500 ml glass volumetric flask.
2. Add about 250 ml of bidistilled water and shake until the $K_2Cr_2O_7$ is dissolved.
3. Make up to the mark with bidistilled water.

BrCl oxidizing solution

1. Weigh accurately 11 g of $KBrO_3$ and 15 g of KBr into a clean 1 litre glass bottle.
2. Add 200 ml of bidistilled water.
3. Add carefully 800 ml of concentrated HCl; the dilution must be carried out in a well-ventilated fume hood to prevent exposure to toxic fumes released during dissolution of $KBrO_3$.
4. Keep the bottle wrapped with aluminium foil.

These two solutions can be kept for an unlimited time if stored in the dark at room temperature in a tightly closed Teflon or glass bottle in an Hg-free area.

2.2.7. Mercury standard solutions

Stock standard solution 1: 1 mg/ml Hg in 10% nitric acid

1. Weigh exactly 1.354 g of $HgCl_2$ into a 1 litre glass volumetric flask.
2. Add about 500 ml of bidistilled water.
3. Add 10 ml of concentrated HNO_3 (low in Hg).
4. Complete to the mark with bidistilled water.
5. Shake well until complete dissolution is achieved.
6. Transfer into a 1 litre Teflon bottle.
7. Close tightly with a torque wrench and keep in the refrigerator (+4 °C).

Stock standard solution 2: 1 µg/ml Hg in 4% HNO_3

1. Weigh 95 g of bidistilled water into a 125 ml Teflon bottle.
2. Add 4 ml of concentrated HNO_3 (low in Hg).
3. Add 1 ml of BrCl solution (or 2 ml of $K_2Cr_2O_7$ solution).
4. Add 100 µl of solution stock 1 (1 mg/ml Hg).
5. Shake well.
6. Close tightly with a wrench and keep in the refrigerator (+4 °C).

Calibration curve (at least three standards and zero calibration)

1. Put about 10 ml of bidistilled water into a clean 50 ml glass volumetric flask.
2. Add reagents as in the digested samples (HNO_3/H_2SO_4 2:1, or HNO_3).
3. Add an appropriate quantity of stock standard solution (stock 1or stock 2, depending on the concentrations of the samples) with a micropipette.
4. Add 1 ml of BrCl solution (or 2 ml of $K_2Cr_2O_7$ solution).
5. Dilute to the mark (50 ml) with bidistilled water.
6. Shake well.

These solutions should be prepared fresh for every day of analysis.

2.3. Analysis by CVAAS

2.3.1. Calibration curve

Prepare standard solutions with at least three standard concentrations plus one zero. The zero calibration is prepared as the standard solutions, but without adding the Hg standard.

If samples are not within the calibration curve, dilute them in the same matrix or prepare a new calibration curve.

2.3.2. Instrument conditions

- Wavelength: 253.7 nm
- Lamp current: 4 mA
- Slit width: 0.5 nm
- Reducing agent (20% $SnCl_2$ in 20% HCl): 1 ml/min
- Bidistilled water: 1 ml/min
- Rinse solution (10% HNO_3) or sample: 6.5 ml/min
- Inert gas: argon.

2.3.3. Optimization of the AAS

The following instructions are applicable for AAS Varian-Spectra AA-10 and VGA-76 or equivalent system based on the flow injection principle. If another instrument is used, the manufacturer's instructions should be followed.

1. Make sure the flame disk is inserted in the instrument.
2. Switch on the printer then the AAS instrument.
3. Press INDEX.
4. Select PROGRAM DIRECTORY.
5. Select mercury program number and push RECALL PROGRAM.
6. METHOD parameter must be:
   element No.: 24
   instrument mode: ABS
   calibration: must be CONCENTRATION
   measurement: must be INTEGRATION.
7. INSTRUMENT PARAMETER must be:
   lamp position: coded lamp position is automatically recognized
   lamp current: 4 mA
   sample introduction: MANUAL
   delay time (seconds): 70
   measurement time (seconds): 5.0
   replicates: 3
   background correction: ON.
8. Install the Hg lamp in the right position.
9. Go to NOTE. On this page, the concentration giving a response of 0.2 ABS is indicated.

10. Select the correct slit width (0.5) and set the monochromator to the right wavelength (253.7 nm).
11. Go to OPTIMIZATION. This step is done without the absorption cell in the light path of the AAS. On the screen there are two bars: one shows the level of energy of the Hg lamp and the other that of the deuterium lamp. Make sure the burner does not obstruct the light. Bring the lamp energy to the maximum by optimizing wavelength and lamp position successively; make these adjustments twice. If the signal bar is too large, press RESCALE. After optimization, the energy of the two lamps should be similar. If the message TOO LOW DEUTERIUM LAMP (or TOO HIGH) appears, turn on (or off) the attenuation of the deuterium lamp.
12. Check the photomultiplier value (PMV about 294 mV) and record the value in the logbook.
12. Install the absorption cell on the burner head and check that the light beam crosses the cell close to the centre.
13. Go to STANDARDS and enter the standard concentrations for the calibration curve.

2.3.4. Operation of the VGA

1. Switch on the argon. The gas flow has to be regulated to a minimum, with the orange light of the VGA off.
2. Put each of the three Teflon capillary tubes into the appropriate solutions:
   (i) $SnCl_2$ solution
   (ii) bidistilled water
   (iii) rinse solution (10% HNO_3).
3. Switch on the VGA and slowly tighten the pressure adjusting screw on the peristaltic pump until the liquids are pumped (do not overtighten the screw as this will shorten the life of the pump tubes).
4. Check that there are no leaks.
5. Leave the system running for about 10 minutes in order to clean the system. Disconnect the black tube from the quartz absorption cell if the system has not been running for a while (to prevent contamination of the cell).
6. Connect the tube between the gas-liquid separator and the absorption cell.

2.3.5. Calibration and samples measurement

At the top of the screen of the AAS, the solution that is going to be measured is indicated (blank; standard 1, 2, etc.; reslope; sample 1, 2, etc.). To choose the solution to be analysed, push SOLUTION TYPE. Always check that the solution that is going to be measured is the one asked for.

To measure a solution push READ.

The AAS and VGA should be operating at this stage.

1. Go to ANALYTICAL RESULTS.
2. Press INSTRUMENT ZERO with the rinse solution (HNO_3 10%).
3. Measure the rinse solution as a sample: this should give 0.000 ABS.
4. Measure the blank or the calibration curve as a sample. This should also be close to 0.000 ABS. If it is not, press INSTRUMENT ZERO again when aspirating the rinse solution.
5. Check the ABS value for one Hg standard (measure it as a sample). This gives the sensitivity of the instrument and should be recorded in the log-book.
6. Go to CALIBRATION.
7. Measure the calibration blank then the standards.

8. Aspirate the rinse solution for about one minute between each standard.
9. Check that the calibration curve is correct.
10. Measure first the reagent blanks then the reference materials. Calculate the concentration in µg/g of the reference material and check the accuracy of the result before continuing.
11. Run the samples.
12. Run the rinse solution for about one minute between each sample.
13. Measure a blank and reslope every four or five samples depending on the stability of the instrument.
14. Measure the same reference material at regular intervals during analysis.

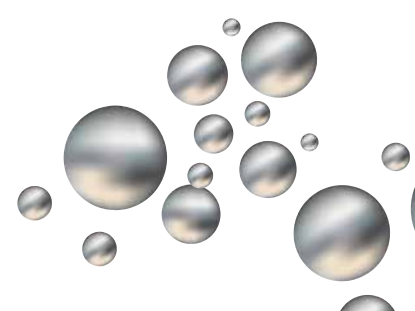
2.3.6. Shut down procedure

1. Rinse all tubing with bidistilled water for about 20 minutes (make sure to keep the tube for the $SnCl_2$ solution separate from the other tubes).
2. Turn off the VGA system.
3. Release the tension from the tubing.
4. Turn off the argon.
5. Turn off the printer and AAS instrument.

2.3.7. Calculation

$$[C]\ (mg/kg) = \frac{(Cd - Cb)\times V}{W}$$

[C] – concentration of total Hg in dry sample (µg/g dry)
Cd – concentration of Hg in sample solution (µg/ml)
Cb – mean concentration of Hg in reagent blanks (µg/ml)
V – volume of dilution of digested samples (ml) =57.5 ml
W – dry weight of sample (g).



3. Determination of total Hg using double gold amalgamation and CVAFS detection

3.1. Principle of the method

After decomposition of the samples in the presence of strong acids, Hg2+ is reduced to volatile elemental mercury Hg0 with an excess of $SnCl_2$. Elemental mercury is concentrated on a gold trap and detected after desorption at 600 °C by cold vapour atomic fluorescence at 253.7 nm.

3.2. Equipment, materials and solutions

3.2.1. Equipment

- AFS detector (Brook Rand) or any other equivalent equipment.

3.2.2. Materials

- Volumetric flask, 100, 500 and 1000 ml (Class A).
- Glass bottles 1 litre, cleaned according to the procedure for cleaning glassware.
- Teflon bubblers (60 ml) (500 ml for water samples) cleaned according to the procedure for cleaning Teflon.
- Teflon tubing cleaned according to the procedure for cleaning Teflon.
- Teflon bottles, 125 ml and 1 litre, cleaned according to the procedure for cleaning Teflon.
- Quartz wool cleaned at 500 °C.
- Gold sand.
- Quartz columns for gold traps, cleaned according to the procedure for cleaning glassware.
- Drying columns (Teflon tube or quartz tube filled with soda lime), cleaned according to the procedure for cleaning glassware or Teflon.
- Heating system for gold traps (2 VARIAC, 6A and timer; Cr/Ni wire 0.5 mm).
- Flow meters.
- Integrator.
- Precision balance.

3.2.3. Cleaning glassware

Prior to use, wash all laboratory glassware thoroughly as follows.

- Allow the Teflon and glass vessels to soak overnight in 2% Micro-90 detergent solution.
- Rinse the vessels thoroughly first with tap water then with bidistilled water.
- Rinse with 0.5% $KMnO_4$ solution.
- Rinse with water until the colour of the $KMnO_4$ solution is no longer visible.
- Fill the vessels with 1% HCl solution and store in Hg-free storage facilities.
- Empty vials just before use for sample processing and allow them to dry at 60 °C in a flow hood.

Cleaning Teflon

- Allow the vessels to soak overnight in a plastic container in a soap solution (Micro solution 2% in tap water).
- Rinse thoroughly first with tap water and then with bidistilled water.
- Put the vessels in 50% (v/v) concentrated HNO_3 solution and heat at 60 °C for two days.
- Rinse thoroughly with bidistilled water (at least four times).
- Transfer the vessels into 10% (v/v) concentrated HCl solution for one day (at least) at room temperature.

- Rinse thoroughly with bidistilled water (at least four times).
- Store all vessels in polyethylene plastic bags. When possible (principally Teflon and glass bottles), fill the vessels with 1% HCl.

Reagents and chemicals

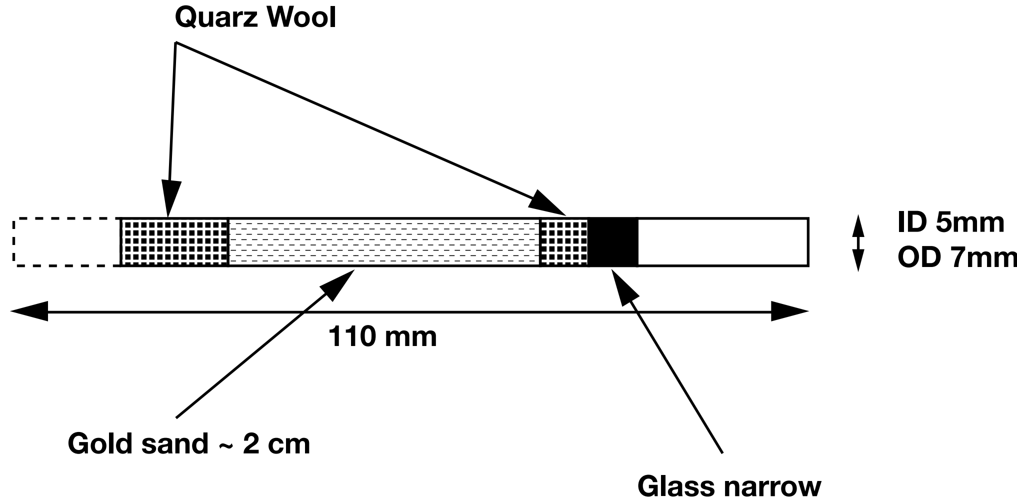
- $SnCl_2$ (analytical grade)
- $KBrO_3$
- KBr
- $HgCl_2$ (analytical grade, normal or low in Hg)
- HCl (30%)
- HNO_3 (65%)
- $K_2Cr_2O_7$ (analytical grade, low in mercury)
- Soda lime pellets (analytical grade)
- bidistilled deionized water (>18 MΩ cm)
- Argon (Hg-purified)

3.2.4. Preparation of gold trap (Fig. 1)

1. Put a small piece of quartz wool at the end of the longer part of the column. Settle it using a Pasteur pipette.
2. Insert about 2 cm of gold sand. It is better to weigh the sand put into the trap in order to obtain a better reproducibility between the traps.
3. Insert a larger piece of quartz wool using a Pasteur pipette. Try to settle all traps the same way.
4. Clean the new trap at least four times before use (see analytical procedure).

Note: when using new traps before starting with sample analysis, check the reproducibility of standard response for all traps.

Fig. 1. Gold trap



3.2.5. Reagent solutions

20% w/v $SnCl_2$ in 20% v/v HCl (100 ml)

1. Weigh accurately 20 g of $SnCl_2$ into a clean glass beaker using a plastic spatula (beaker and spatula are used only for $SnCl_2$).
2. Add 20 ml of concentrated HCl directly to the $SnCl_2$ and transfer to a 100 ml volumetric flask. Mix and wait for complete dissolution of $SnCl_2$.
3. Add bidistilled water to the mark (100 ml).
4. With older stock of $SnCl_2$ it may be necessary to warm the solution on a hot plate to obtain complete dissolution of $SnCl_2$ (do not allow to boil).
5. Purge the $SnCl_2$ solution with nitrogen for two hours in order to obtain an Hg-free solution.

There are two choices for oxidizing solutions.

$K_2Cr_2O_7$ 10% (w/v) in bidistilled water

1. Weigh 50 g of $K_2Cr_2O_7$ into a clean 500 ml glass volumetric flask.
2. Add about 250 ml of bidistilled water and shake until the $K_2Cr_2O_7$ is dissolved.
3. Make up to the mark with bidistilled water.

BrCl oxidizing solution

1. Weigh accurately 11 g of $KBrO_3$ and 15 g of KBr into a clean 1 litre glass bottle.
2. Add 200 ml of bidistilled water.
3. Add carefully 800 ml of concentrated HCl; the dilution must be carried out in a well-ventilated fume hood to prevent exposure to toxic fumes released during dissolution of $KBrO_3$.
4. Keep the bottle wrapped with aluminium foil.

These two solutions can be kept for an unlimited time if stored in the dark at room temperature in a tightly closed Teflon or glass bottle in an Hg-free area.

3.2.6. Mercury standard solutions

Standard stock solution: 1 mg/ml Hg in 10% HNO_3

1. Weigh exactly 1.354 g of $HgCl_2$ into a 1 litre glass volumetric flask.
2. Add about 500 ml of bidistilled water.
3. Add 10 ml of concentrated HNO_3 (low in Hg).
4. Complete to the mark with bidistilled water.
5. Shake well until complete dissolution is achieved.
6. Transfer into a 1 litre Teflon bottle.
7. Close tightly with a torque wrench and keep in the refrigerator (+4 °C).

Intermediate standard solution: 1 µg/ml Hg in 4% HNO_3

1. Weigh 95 g of bidistilled water into a 125 ml Teflon bottle.
2. Add 4 ml of concentrated HNO_3 (low in Hg).
3. Add 1 ml of BrCl solution (or 2 ml of $K_2Cr_2O_7$ solution).
4. Add 100 µl of solution stock (1 mg/ml Hg).
5. Shake well.

If a more dilute solution is needed, dilute intermediate standard solution by following the above procedure. The bottles of standard solutions should be closed tightly with a wrench and kept in the refrigerator (+4 °C).

3.3. Analytical procedure

1. Prepare the samples as described above.
2. Gold traps must be cleaned before use by heating to 600 °C without being connected to the AFS detector. Then check if they are free from residual Hg by measuring released Hg after heating them again.
3. Clean the bubbler (once, or several times if the system has not been used for some time) following the procedure below.
4. Measure bubbler blank by following the procedure below and verify the absence of contamination of the system. If the values of the bubbler blank are too high, continue to clean the system until the bubbler blank values are correct and stable.
5. Calibrate the system by following the "calibration curve" procedure below. This calibration must be done at least twice during the day.
6. Measure reagent blank and reference material solutions (digested at the same time as the samples) following the procedure below ("reagent blank analysis" and "sample analysis"). Verify the absence of Hg contamination and the accuracy of measurements before starting to analyse the sample.
7. Start to measure the sample as described below (sample analysis). During the run for quality control purposes, the reference material and the reagent blanks must be measured at least twice for each calibration curve.

3.3.1. Cleaning of the bubbler

1. Rinse and fill the bubbler (3/4) with bidistilled water.
2. Add 500 µl of $SnCl_2$ solution.
3. Purge with argon for 15 minutes.

3.3.2. Bubbler blank

1. Rinse and fill the bubbler (3/4) with bidistilled water.
2. Add 500 µl of $SnCl_2$ solution.
3. Fix the gold trap and purge with argon for 15 minutes.
4. Analyse the trap.

3.3.3. Calibration curve

1. Rinse and fill the bubbler (3/4) with bidistilled water.
2. Add standard solution (50–150 µL of 1 ng/ml stock solution, equivalent to 50–150 pg of Hg).
3. Add 500 µl of $SnCl_2$ solution.
4. Fix gold trap and purge with argon for 15 minutes.
5. Remove the trap and analyse it.

The calibration curve must be prepared at the level of the sample concentrations. If necessary, more concentrated standards than indicated can be used.

3.3.4. Reagent blank analysis

1. Rinse and fill the bubbler with bidistilled water (the quantity of bidistilled water depends on the volume of reagent blank to be added).
2. Add the blank solution (reagent blank). The volume must be at least equal to the volume of the sample to be used for analyses (that is, if 10 ml of sample are necessary for analysis, at least 10 ml of reagent blank must be analysed). If the level of Hg in the reagent blank is very low, larger volumes of the blank solution can be used for analysis.
3. Add 500 µl of the $SnCl_2$ solution.
4. Fix the gold trap and purge with argon for 15 minutes (Fig. 2).
5. Remove the gold trap and analyse it (Fig. 3).

Fig. 2. Bubbler system for total Hg analysis

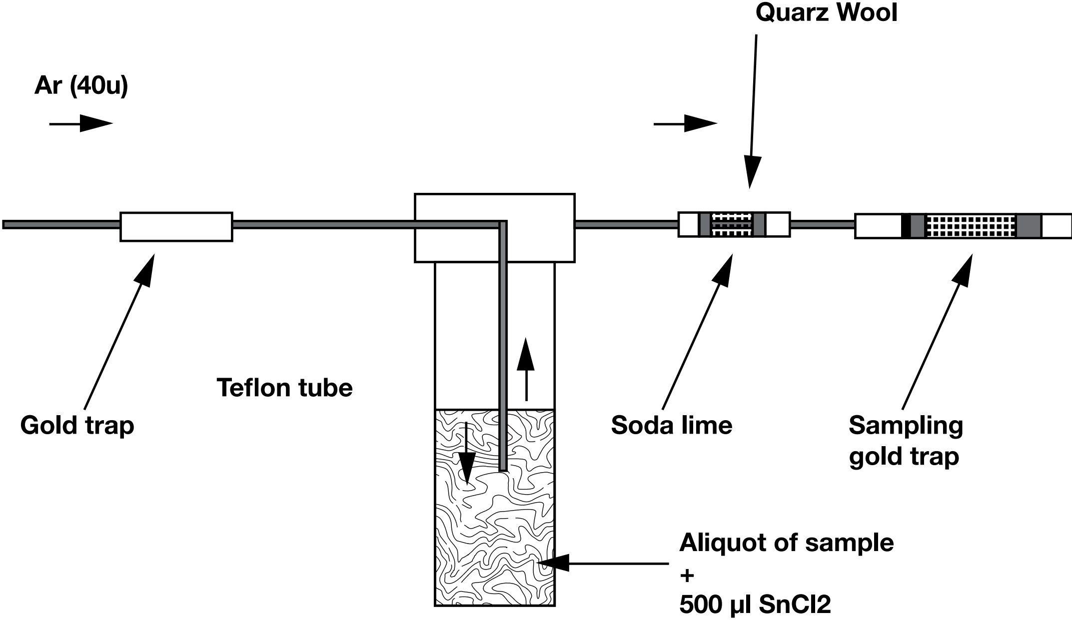
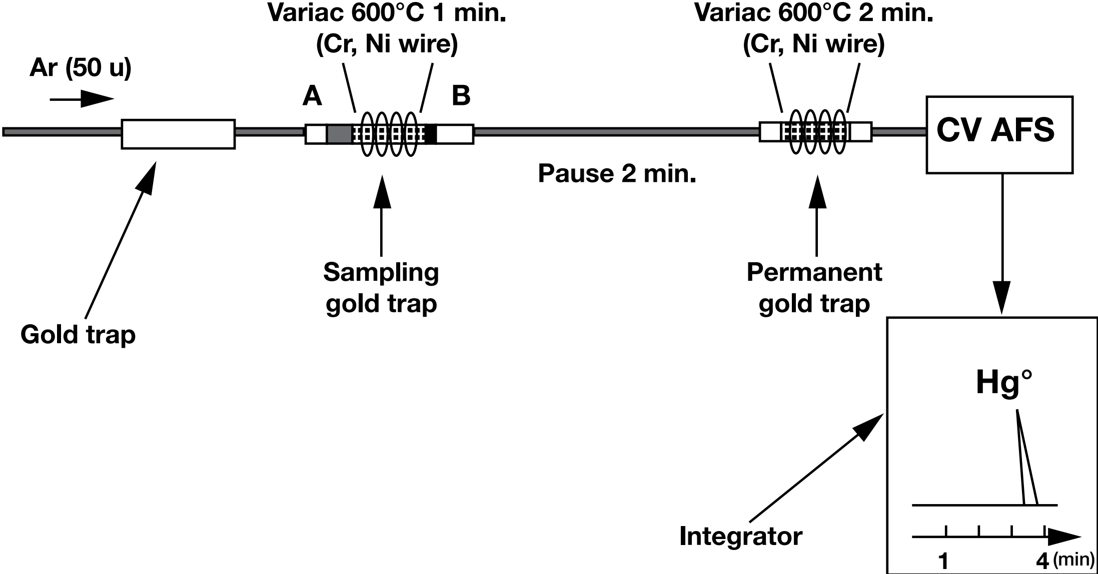


Fig. 3. Analytical system



3.3.5. Sample analysis

1. Rinse and fill the bubbler with bidistilled water (the quantity of bidistilled water depends on the volume of the sample to be added, from 0 ml for low concentration samples such as urine and blood to three quarters of the bubbler volume for higher concentration samples).
2. Add the sample solution. The volume of sample to be added depends on the concentration of the sample, from a few microlitres for hair samples to a few millilitres for blood and urine samples. The response of the sample should be within the calibration curve.
3. Add 500 µl of the $SnCl_2$ solution.
4. Fix the gold trap and purge with argon (nitrogen or air) for 15 minutes (Fig. 2).
5. Remove the gold trap and analyse it (Fig. 3).

3.3.6. Double amalgamation analysis (Fig. 3)

1. Place the sampling gold trap at measuring (analytical) system in a flow of argon.
2. Release Hg by heating the sampling gold trap at 600 °C for one minute.
3. Wait for two minutes for mercury to amalgamate on permanent gold trap.
4. Release mercury from the permanent gold trap by heating it at 600 °C for two minutes.
5. Detect by CVAFS.

3.4. Calculation

Plot the calibration curve using:

X – pg. of Hg^{2+} in added standard

Y – response of integrator (peak area in arbitrary units).

Calculate the calibration curve using linear regression of all standard points (at least three standards) and the mean of bubbler blanks (unit) for zero value:

$$y = b + ax$$

Reagent blank:

$$[B](pg/ml) = \frac{(Ab - b)}{a \times V}$$

[B] – concentration of methyl mercury in reagent blank (pg/ml)

Ab – response obtained for aliquot of reagent blank analysed (peak area in arbitrary units)

V – volume of reagent blank analysed (ml)

Samples:

$$[S](pg/g) = \frac{\left[\left[\frac{As - b}{a \times Va}\right] - [B]\right] \times Vs}{W}$$

[S] – concentration of Hg in dry sample (pg/g dry)

As – response obtained for the aliquot of sample analysed (peak area in arbitrary units)

Va – aliquot of sample analysed (ml)

Vs – total sample volume (ml)

W – dry weight of sample (g)

[B] – concentration of methyl mercury in reagent blank (pg/ml).

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