World Health Organization
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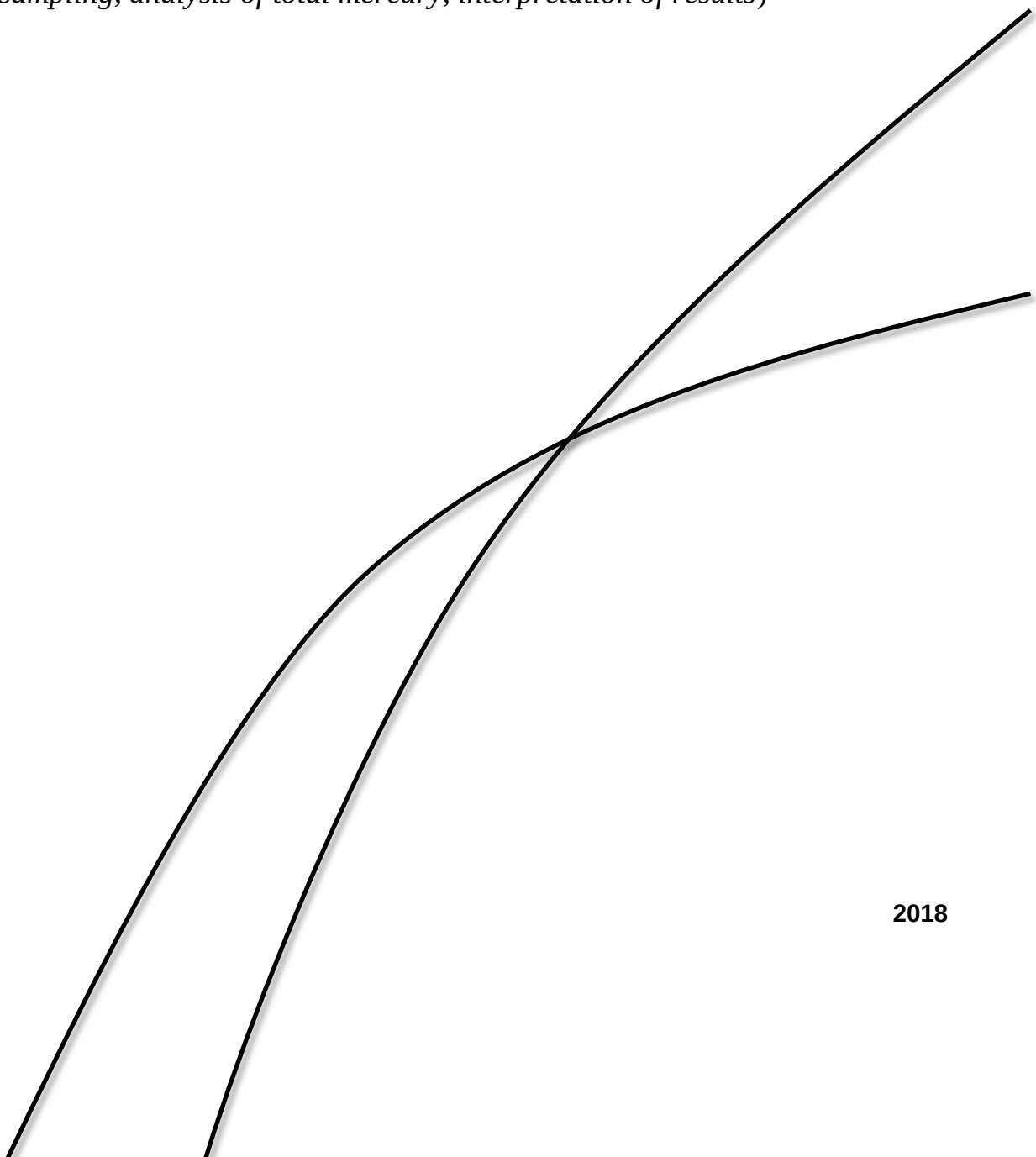
Standard operating procedure

for assessment of mercury in human scalp hair
(*sampling, analysis of total mercury, interpretation of results*)



2018

Abstract
This standard operating procedure (SOP) describes the process of assessing pre-natal 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.

Key words
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 several 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:[1]

- 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.

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

- 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.

1.3. 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).

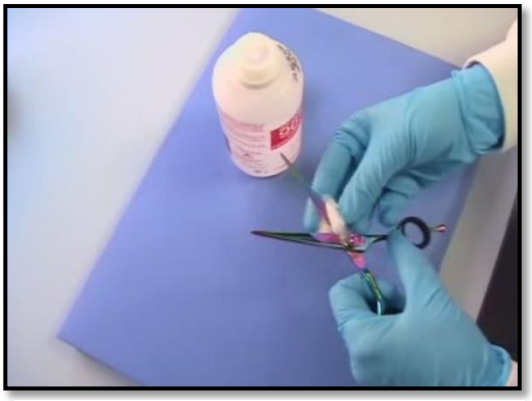

Photo 1. Cleaning the scissors
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1.5. Sampling procedure

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]

[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)*.

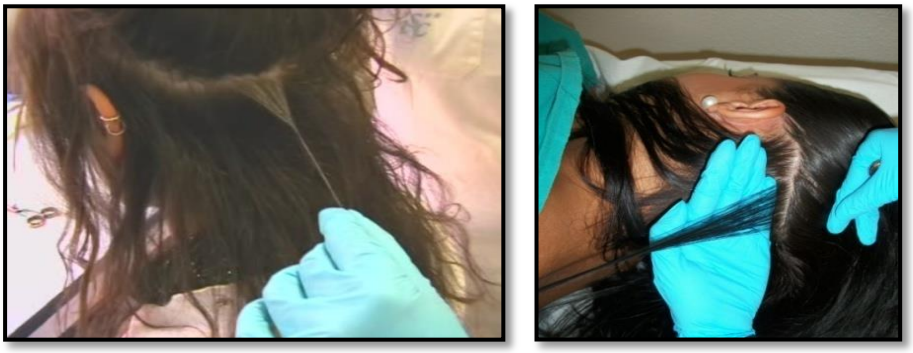
1.5.1. Hair longer than 5 cm (1.97 in)

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).

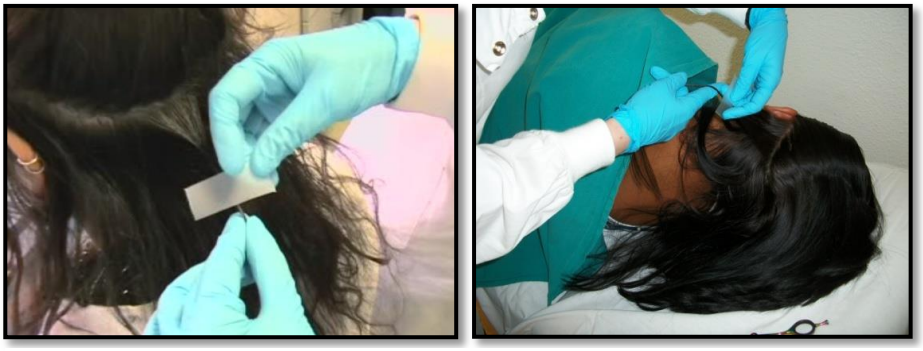
Photos 2a and b. Grasping the hair; (a) sitting, (b) lying

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



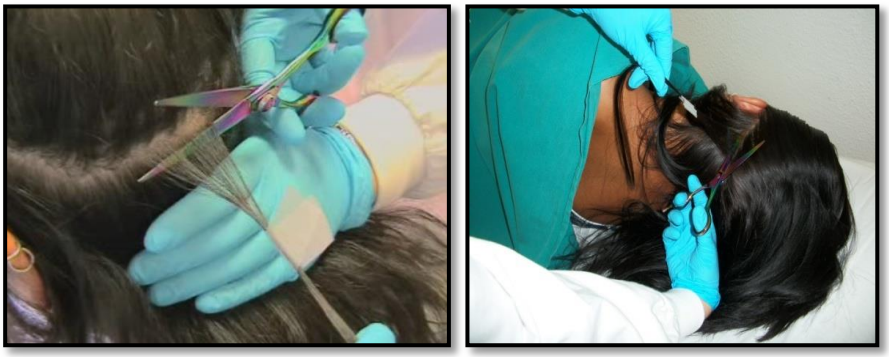
Photos 3a and b. Forming a lock; (a) sitting, (b) lying

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

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
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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)
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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).

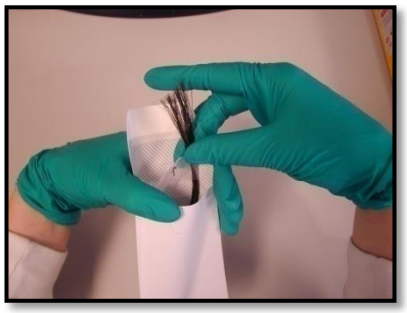

Photo 7. Placing the hair in a paper envelope
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7. Repeat this process with a second lock from the other side of the back of the head.
8. Place the paper envelope in the zip-lock plastic bag (Photo 8).

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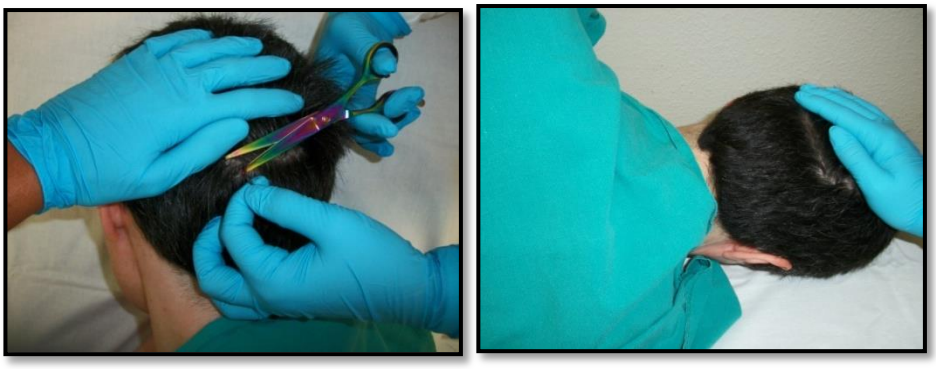
Note. To ensure that the required amount is collected, the locks should have approximately 250 strands. However the weight of the sample can change depending on the sort and length of hair. The minimum amount required for the analysis must be checked with the laboratory that will analyse the sample.

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
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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).

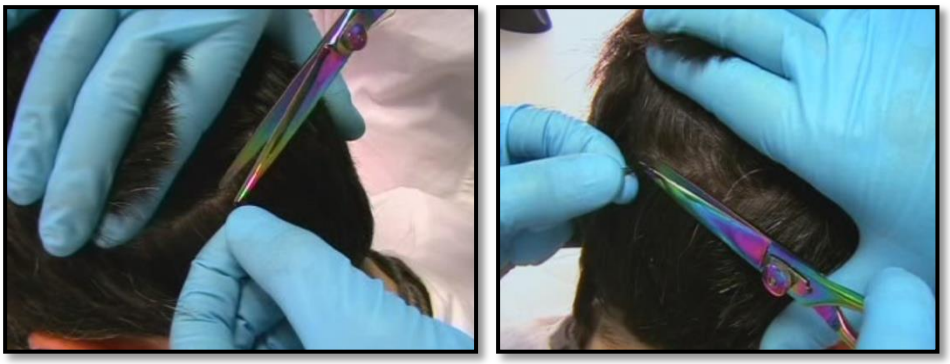

Photo 10a and b. Repeating the cutting of strands of hair; (a) sitting, (b) lying
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4. Place the paper envelope in the zip-lock plastic bag (Photo 8).

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).

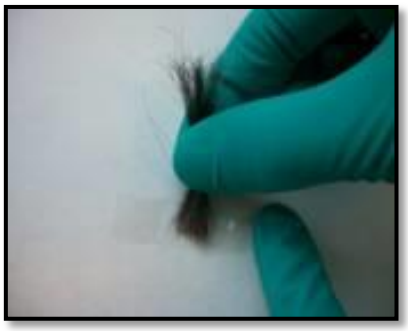

Photo 12. Placing the lock in the tape
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c. Place another piece of adhesive tape over the first piece.

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).

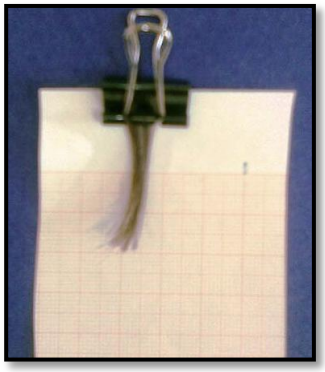

Photo 13. Holding the lock with a binder clip
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b. Place the hair sample in a paper envelope and label it with the sample ID code.
c. Repeat the process with a second lock from the other side of the back of the head.
d. Place the paper envelope in the 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

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).

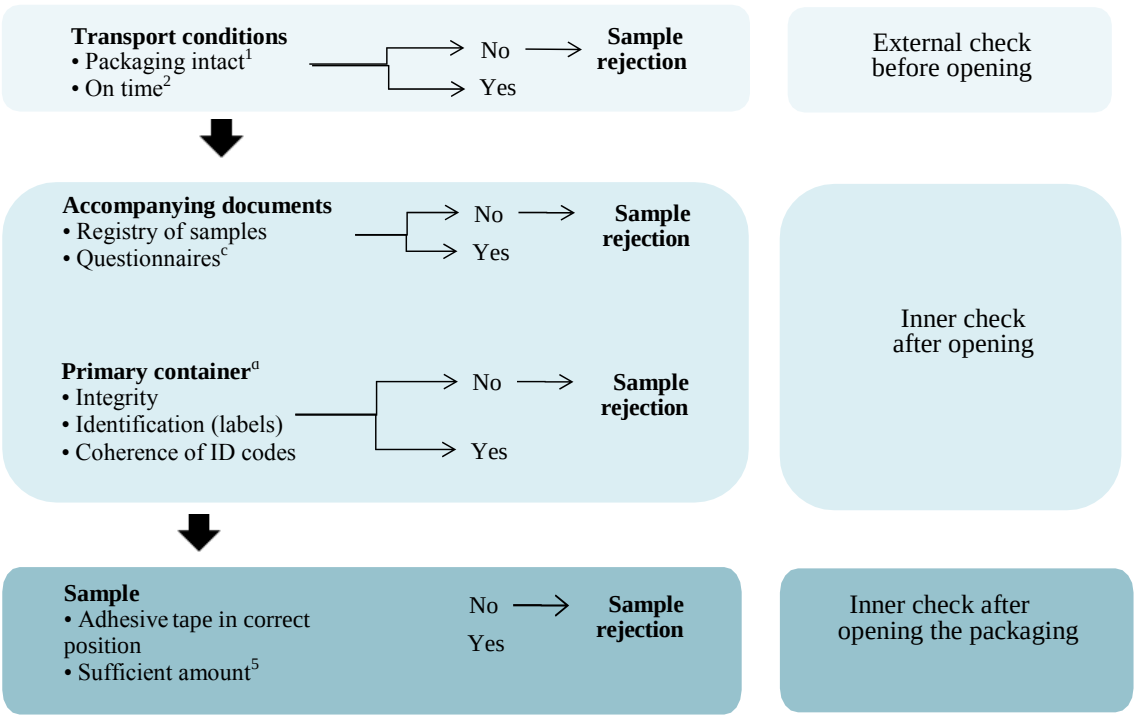
The following points should be checked upon receipt of hair samples.

- 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



[1] The package must be correctly sealed and must not have been manipulated
[2] The maximum time between sample collection and its arrival at the laboratory should be defined beforehand.
[3] 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.
[4] 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.
[5] 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 check-lists 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.

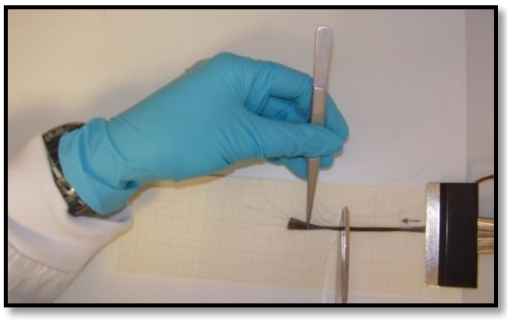


Photo 15. Immobilizing the strand with a pin clip
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3. Cut the first 3 cm (or the defined length for the analysis) closest to the scalp with the help of the laboratory tweezers.
4. Place the segment into the vessel labelled with the sample code. The 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).

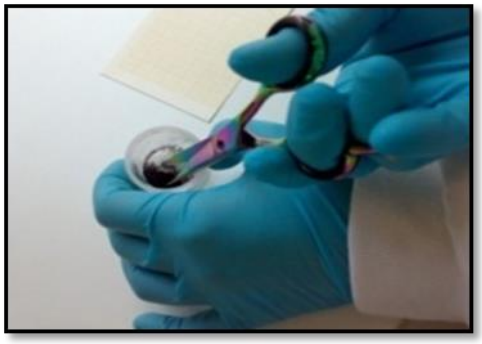

Photo 16. Chopping the sample into small pieces
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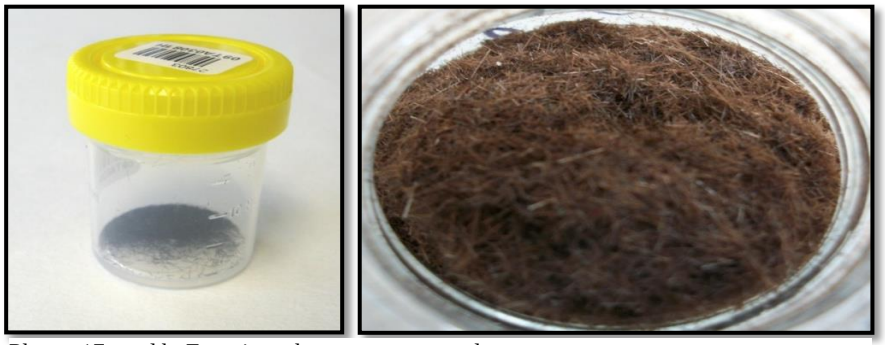
6. Ensure that the final sample is homogeneous (Photos 17a and b).



Photos 17a and b. Ensuring a homogenous sample
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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 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

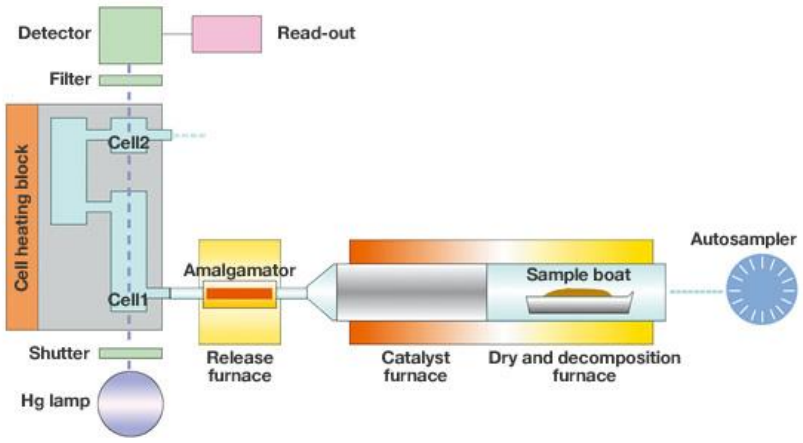
The method described in this SOP allows rapid and accurate quantification of mercury in human scalp hair. The assay range is 1–25 ng total mercury.

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.

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)*.

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).

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; e.g. Mettler XP205)
- micropipette, adjustable between 100 and 1000 µL (e.g. from Gilson)
- 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.

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.

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.

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 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 heath 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)	RSD_{repro}	RSD_{repet}
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; RSD_{repet} = relative standard deviation for repeatability; RSD_{repro} = 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 ng	101.7	83.2–131.0
2.5 ng	99.5	88.5–126.2
5 ng	100.9	94.5–135.7
10 ng	98.5	88.2–102.7
15 ng	100.6	97.7–106.7
20 ng	100.4	97.8–103.1
25 ng	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. Reference levels for interpreting mercury levels in hair

Agency	Hair levels	Reference
United States Environmental Protection Agency (US EPA)	1.0 µg/g	*(35)*
Joint FAO/WHO Expert Committee on Food Additives (JECFA)	2.3 µg/g	*(36)*
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	☐	Redhead	☐
Dark brown	☐	Grey	☐
Brown	☐	White	☐
Blonde	☐		

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

ID code ☐

1. Origin of the sample:

Centre:
City/country:
Date of sampling:

2. Sample received:

☐ Urine
☐ Hair

Signature of reviewer:

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 study questionnaire
- ☐ Inconsistency in the ID codes
- ☐ Others:_____

4. Date of storage/biobanking:

5. Comments:

ID codes for related samples

Urine	**Hair**

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. Observations:……

……

……

……

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: ……
……
……

5. Are all samples and documents in the shipment packaging?

☐ Yes ☐ No

6. Are the address of the laboratory and the contact person details in the delivery note?

☐ Yes ☐ No