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REPORT

Laboratory Procedure Book
ANALYSIS OF TRACE METALS
IN BIOLOGICAL AND SEDIMENT SAMPLES

IAEA/NAEL

Marine Environmental Studies Laboratory in co-operation with

UNEP/MAP MED POL

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Laboratory Procedure Book

ANALYSIS OF TRACE METALS IN BIOLOGICAL AND SEDIMENT SAMPLES

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TABLE OF CONTENTS

I. MICROWAVE-OVEN DIGESTION PROCEDURES

I-1. DIGESTION OF SEDIMENT FOR THE DETERMINATION OF TRACE METAL

Principle:

The sediment samples are treated in closed Teflon vessels with hydrofluoric acid (HF) in combination with nitric acid, in order to decompose the samples. The use of HF is essential as it is the only acid that completely dissolves the silicate lattices and releases all the metals.

Reagents:

- HNO_3 (65%, Suprapur, Merck).
- HF (48%, analytical grade ISO, Merck).
- H_2O_2 (analytical grade), to be kept in the fridge after opening.
- Boric acid crystals, H_3BO_3 (analytical grade ISO, Merck).
- Milli-Q deionised water (> 18MΩ cm, Millipore).

Procedure:

1. Shake the sample bottles for about 2 min. for homogenization.
2. Wait a few minutes before opening the bottles.
3. Weigh accurately about 0.2 g of dry sample in labeled Teflon reactor (CEM)
4. Slowly add 5 ml of HNO_3 and 2 ml of concentrated hydrofluoric acid (HF). If the samples are strongly reactive, leave them at room temperature for at least 1 hour.
5. After room temperature digestion add 2 ml of H_2O_2
6. Close the reactor and put them in a microwave oven.
7. Set up the correct program:

Step	Power (W)	% Power	Ramp time (min sec)	PSI	°C	Hold time (min sec)
1	1200	100	10.00	600	190	12.00

8. Allow for the samples to cool to room temperature, release pressure carefully by opening the valve, and then open the reactor.
9. Weigh 0.8 g of boric acid into a polyethylene weighing boat, transfer it to the reactor, then add about 15 ml of Milli-Q water

10. Close the reactor and put them in a microwave oven.
11. Set up the correct program:

Step	Power (W)	% Power	Ramp time (min sec)	PSI	°C	Hold time (min sec)
1	1200	100	10.00	600	170	12.00

12. Allow for the samples to cool to room temperature, release pressure carefully by opening the valve, and then open the reactor.
13. Label some polyethylene 50 ml tubes and record the weight of the empty tubes.
14. Transfer the samples into 50 ml polypropylene graduated tubes. Rinse the Teflon reactor with Milli-Q water 3 times.
15. Shake the tubes.
16. Allow them to cool and then dilute to the mark (50 ml) with Milli-Q water, weigh each tube and record the weight.

Reagent blanks:

At least 2 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 vessels.

Reference materials:

At least one certified reference material should be used and prepared in duplicate for each digestion batch. These digestions are prepared in a similar manner as the samples. A reference material of similar composition and concentration range should be used.

I-2. DIGESTION OF BIOTA FOR THE DETERMINATION OF TRACE METAL

Principle:

The biological samples are treated with concentrated nitric acid in order to decompose the samples and solubilize all metals.

Reagents:

- HNO_3 (65%, Suprapur, Merck).
- H_2O_2 (analytical grade) to be kept in the fridge after opening.
- Milli-Q deionised water (> 18MΩ cm, Millipore).

Procedure:

1. Shake the samples bottles for about 2 min. for homogenization.
2. Wait a few minutes before opening the bottles.
3. Weigh accurately about 0.2 g of dry sample in labeled Teflon reactor (CEM)
4. Add 5 ml of concentrated Nitric acid (HNO_3). Leave the samples at room temperature for at least 1 hour.
5. Add 2 ml of H_2O_2.
6. Close the reactor and place them in a microwave oven.
7. Run the appropriate program:

Step	Power (W)	% Power	Ramp time (min sec)	PSI	°C	Hold time (min sec)
1	1200	100	5 00	600	50	5 00
2	1200	100	5 00	600	100	5 00
3	1200	100	10 00	600	200	8 00

8. Allow for the samples to cool to room temperature, release pressure carefully by opening the valve, and then open the reactor.
9. Label some polyethylene 50 ml tubes and record the weight of the empty tubes.
10. Transfer samples into the labeled 50 ml polypropylene graduated tubes. Rinse the Teflon tubes with Milli-Q water 3 times.
11. Dilute to the mark (50 ml) with Milli-Q water and shake.

12. Allow them to cool and then dilute to the mark (50 ml) with Milli-Q water, weigh each tube and record the weight.

Reagent blanks:

At least 2 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 vessels.

Reference materials:

At least one certified reference material should be used and prepared in duplicate for each digestion batch. These digestions are prepared in a similar manner as the samples. A reference material of similar composition and concentration range should be used.

II. HOT PLATE DIGESTION PROCEDURES

II-1. DIGESTION OF SEDIMENT FOR THE DETERMINATION OF TRACE METAL

Principle:

The sediment samples are treated in closed Teflon vessels with hydrofluoric acid (HF) in combination with aqua regia, in order to decompose the samples. The use of HF is essential as it is the only acid that completely dissolves the silicate lattices and releases all the metals.

Reagents:

- HNO_3 (65%, Suprapur, Merck).
- HF (48%, analytical grade ISO, Merck).
- HCl (30%, Suprapur, Merck).
- Boric acid crystals, H_3BO_3 (analytical grade ISO, Merck).
- Milli-Q deionised water (> 18MΩ cm, Millipore).

Procedure:

1. Shake the sample bottles for about 2 min. for homogenization.
2. Wait a few minutes before opening the bottles.
3. Weigh accurately about 0.2 g of dry sample in labeled Teflon tubes (FEP, 50 ml, Nalgene)
4. Slowly add 1 ml of aqua regia (HNO_3: HCl, 1:3 v/v) and 6 ml of concentrated hydrofluoric acid (HF). Leave the samples at room temperature for at least 1 hour.
5. Close the tubes and place them in an aluminum block on a hot plate at 120°C for 2hrs 30min.
6. Weigh 2.70 g of boric acid into the labeled 50 ml polypropylene graduated tubes or volumetric flask, then add about 20 ml of Milli-Q water and shake.
7. Allow samples to cool to room temperature then open the tubes.
8. Transfer the samples into the 50 ml polypropylene graduated tubes (containing the boric acid). Rinse the Teflon tubes with Milli-Q water 3 times.
9. Put in ultrasonic bath (at 60°C) for at least 30 minutes, until all the boric acid is dissolved.

10. Allow them to cool to room temperature and then dilute to the mark (50 ml) with Milli-Q water If using glass transfer the solution in plastic container. Allow particles to settle before analysis.

Reagent blanks:

At least 2 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 vials.

Reference materials:

At least one certified reference material should be used and prepared in duplicate for each digestion batch. These digestions are prepared in a similar manner as the samples. A reference material of similar composition and concentration range should be used.

II-2. DIGESTION OF BIOTA FOR THE DETERMINATION OF TRACE METAL

Principle:

The biological samples are treated with concentrated nitric acid, in order to decompose the samples and solubilize all metals.

Reagents:

- HNO_3 (65%, Suprapur, Merck).
- Milli-Q deionised water (> 18MΩ cm, Millipore).

Procedure:

1. Shake the samples bottles for about 2 min. for homogenization.
2. Wait a few minutes before opening the bottles.
3. Weigh accurately about 0.2 g of dry sample in labeled Teflon tubes (FEP, 50 ml, Nalgene)
4. Add 5 ml of concentrated Nitric acid (HNO_3). Leave samples at room temperature for at least 1 hour.
5. Close the tubes and place them in an aluminum block on a hot plate at 90°C for 3hrs.
6. Allow the samples to cool to room temperature then open the tubes carefully.
7. Transfer the samples in the labeled 50 ml polypropylene graduated tubes or volumetric flask. Rinse the Teflon tubes with Milli-Q water 3 times.
8. Dilute to the mark (50 ml) with Milli-Q water and shake. If using glass transfer the solution in plastic container.

Reagent blanks:

At least 2 blanks should be prepared for each batch of analysis. They are prepared in a similar manner as samples, except that no sample is added to the digestion vials.

Reference materials:

At least one certified reference material should be used and prepared in duplicate for each digestion batch. These digestions are prepared in a similar manner as the samples. A reference material of similar composition and concentration range should be used.

II-3. DIGESTION OF BIOTA OR SEDIMENT FOR THE DETERMINATION OF TOTAL MERCURY BY Cold vapour-AAS

Principle:

The biological or sediment samples are treated with concentrated nitric acid, in order to decompose the samples and solubilize all metals.

Reagents:

- HNO_3 (65%, analytical grade, low in mercury, Merck).
- Milli-Q deionised water (> 18MΩ cm, Millipore).
- 10% $K_2Cr_2O_7$ (w/v) solution (e.g. 10 g $K_2Cr_2O_7$ diluted into 100 ml with Milli-Q water).

Procedure:

1. Shake the samples bottles for about 2 min. for homogenization.
2. Wait a few minutes before opening the bottles.
3. Number the Teflon tubes.
4. Weigh accurately about 0.2 g to 1.5 g of dry sample in Teflon tubes (FEP, 50 ml, Nalgene) depending of the expected concentration.
5. If processing plants or high weight of bivalve (> 1g), add 40 mg of V_2O_5 to each tube (including blanks).
6. Add 5 ml of concentrated Nitric acid (HNO_3). If large amount of sample is used add more acid until the mixture becomes liquid.
7. Leave the samples at room temperature for at least 1 hour.
8. Close the tubes and place them in an aluminum block on a hot plate at 90°C for 3hrs.
9. Allow for the samples to cool to room temperature then open the tubes carefully.
10. Add about 20 ml of Milli-Q water
11. Add 1 ml of $K_2Cr_2O_7$ solution (*NOTE*: final concentration should be 2% v/v).
12. Dilute to 50 ml preferably in Teflon, but glass is also good.

Reagent blanks:

At least 2 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 vials.

Reference materials:

At least one certified reference material should be used and prepared in duplicate for each digestion batch. These digestions are prepared in a similar manner as the samples. A reference material of similar composition and concentration range should be used.

III. INSTRUMENTAL TECHNIQUES

III.1. PREPARATION OF METAL STANDARD SOLUTIONS FOR THE CALIBRATION CURVE

Principle:

The calibration curve must be made by at least 3 points (standard solutions of different concentration) plus a zero calibration. The concentration of the standard solutions must be calculated so that they bracket the concentrations of the samples and the Reference Materials.

If the concentration of the samples is unknown, the calibration curve will be centered on the Reference Materials. If the concentration of the samples exceeds the limit of the calibration curve, either the samples must be diluted to the appropriate concentration, or the calibration curve must be extended with a higher concentration standard. If, on the contrary, the concentration of the samples is lower than the lowest calibration curve's point, a new calibration curve must be prepared.

Reagent:

- Milli-Q deionised water (> 18MΩ cm, Millipore).
- Commercial standard solution 1000 µg ml^{-1}: Use a certified reference material solution; this solution should be accompanied by a certificate stipulating at the minimum the traceability of the certified concentration as well as the expiration date. The density of the solution or the certified content in mg kg^{-1} should also be defined.
- Acid solutions used for sample preparation.

Standards preparation:

1. Put approximately 10 ml of Milli-Q water into clean polypropylene tubes (50 ml)
2. MATRIX MATCH the standards: add reagents in order to obtain a similar matrix as in the samples. Ex: for BIOTA: 5 ml of concentrated nitric acid and 2 ml of H_2O_2. For SEDIMENTS (hot plate digestion): 2.7 g Boric acid, 1 ml of aqua regia, 6 ml of HF.
3. Add the appropriate quantity of standard solution with a micropipette.
4. Dilute to the mark (50 ml) with Milli-Q water.
5. Shake well.

External Calibration Verification (ECV):

In order to check the accuracy of the prepared curve an independent standard is prepared. The concentration of this ECV should be in the calibration curve. This solution is prepared as describe above but using a second source of stock standard solution.

NOTE:

Some standard producers are selling specific multi-element solution for ECV purpose.

III-2. DETERMINATION OF TRACE METALS IN SEDIMENT AND BIOLOGICAL MATERIALS BY GF-AAS

Principle:

The samples are digested with strong acids (see Digestion Procedures).

For graphite furnace (GF) AAS, an aliquot of sample solution (10-50 µl) is introduced into a graphite tube of the GF-AAS and atomized by rapid heating at high temperature. A light beam is directed through the graphite tube, into a monochromator, and onto a detector that measures the amount of light absorbed by the atomized element in the tube. Each metal has its own characteristic wavelength, so a source hollow cathode lamp composed of that element is used. The amount of energy absorbed at the characteristic wavelength is proportional to the concentration of the element in the sample.

Reagents:

- Argon.
- Standard solution of the element of interest 1000 mg l^{-1} (Merck).
- Milli-Q deionized water (>18 MΩ cm, Millipore).

Materials:

- Volumetric material, polypropylene tubes with caps (50 ml) cleaned according to Cleaning Procedures or glass volumetric flask and plastic container (for transferring).
- Atomic Absorption Spectrometer.
- Micropipettes.
- Polypropylene cups for automatic sampler.

Reagent solutions:

Metal standard solutions for the calibration curve: (See procedure III.1)

1. Put approximately 10 ml of Milli-Q water into clean polypropylene tubes (50 ml) or in volumetric flasks.
2. Add reagents in order to obtain a similar matrix as the sample (e.g. if sample is in 10% nitric acid add 5 ml of nitric acid).

3. Add the appropriate quantity of stock standard solution (1000 mg l^{-1} or an intermediate stock standard) with a micropipette.
4. Dilute to the mark (50 ml) with Milli-Q water.
5. Shake well.
6. If glass is used then transfer the solution into a polypropylene container.

These solutions can be kept for a few days if stored in the refrigerator (+4°C).

Depending on the element, it may be necessary to make an intermediate stock standard solution. This intermediate stock is prepared as described above in a 2% HNO_3 matrix.

Before analysis:

Analytical conditions may change for each element, so it is necessary to first carefully read the relevant manufacturer manual before starting. Nowadays instrument software have integrated cookbook and already develop program to be used as starting point. Example of working conditions is given in table 1.

Determine the calibration curve according to the expected concentrations of the samples and the linearity of the AAS response for the element considered, software will usually provide recommended working range.

ANALYSIS BY GF-AAS

General operation:

1. Switch on the instrument (make sure the lamp of interest is on).
2. Make sure the rinsing the bottle is filled with fresh Milli-Q water (as this bottle is under argon pressure it should be disconnected before opening the gas).
3. Switch on argon and cooling system.
4. Open the furnace and take out the graphite tube.
5. Clean the inside, outside and quartz window with alcohol.
6. Install an appropriate graphite tube and close the furnace.
7. Optimize the lamp position and record the gain in the instrument logbook.
8. Install the auto sampler.
9. Make sure there is no air inside the syringe system.
10. Set up the capillary position (including length).
11. Run a "tube clean" cycle.

Operation when using a develop program:

Calibration curve:

The automatic sampler can make the calibration curve by mixing an appropriate volume of standard and zero calibration solutions, so only one standard solution needs to be prepared. It can be the highest standard solution of the calibration curve, or a solution more concentrated in case of standard additions. The solution must be chosen so that the volumes pipetted by the automatic sampler to make the standards are not lower than 2 µl. The calibration curve can also be prepared manually.

Sequence:

At least one blank, one reference material and one check standard (ECV, See procedure III.1) are measured before the samples, so it is possible to check that the system is under control before allowing the instrument to work automatically.

A reslope should be measured every 5 to 10 samples, as well as either a reference material or the ECV or a sample. This allows checking the accuracy of the reslope and the precision of the instrument over the run.

The instrument is recalibrated regularly (every 10-20 samples) to correct for instrumental drift and graphite tube efficiency.

Running a sequence:

1. Fill the carousel with samples, standard, zero calibration and matrix modifier if needed.
2. Select the program needed and carefully check all parameters (type of measurements, matrix modifier, the temperature program, reslope standard and rate, type of calibration, etc….)
3. Check that the number of fires from the graphite tube in use is low enough to allow for the full sequence to be run.
4. Program the auto sampler and the sequence.
5. Make an instrument zero.
6. Measure the zero calibration as a sample and record the absorbance in the logbook. It should be low or comparable with previous data.
7. Inject a known volume one standard solution, calculate the M_0 (quantity in pg to get a signal of 0.0044ABS) and record it in the logbook. Compare with previous records. Check the peak shape and the RSD of the reading (should be <5%).

$$M_{0(pg)} = \frac{Cstandard\ (\text{ng ml}^{-1}) \times Qut\ standard\ injected\ (\mu l)}{ABS\ standard} \times 0.0044$$

8. Inject a reference material solution and check if the concentration is correct. Check the peak shape and the RSD of the reading (should be <5%).
9. Run the sequence.
10. Even if the instrument is all automatic, stay around to check the beginning of the sequence (calibration curve, procedure blank, reference material and check std ECV), and ideally return regularly to check the reslope, so that the sequence can be stopped if needed.

Minimum quality control checks

The ECV should be within 10% of the true value, in case of failure any results obtained after the last acceptable ECV should be rejected. The samples can be measured again after the ECV is under acceptable limit again (i.e. changing graphite tube, verifying calibration curve...)

The Zero calibration blanks measured during the run stay under acceptable limit (to be defined during the method validation), in case of failure the calibration should be redone and all results obtained after last acceptable blank should be re-measured.

The sample blanks measured during the run stay under acceptable limit (to be defined during the method validation), in case of failure all samples prepared along the failing blanks should be redone (prepared again).

The Certified Reference Material: At least one certified reference material of a representative matrix will be prepared with each batch of sample; the calculated result should fall in the value of the certificate and within the coverage uncertainty, to show evidence of unbias result. The results for the CRM should be recorded for quality control purpose and plotted on a control chart

Verify the RSD of reading (<5%).

Check that all samples were within the concentration limits of the calibration curve. If not, take the appropriate action (dilution or new calibration curve) and restart the sequence.

Developing a program:

The AAS software generally gives typical electrothermal programs for each element for 10 µl of sample in diluted HNO_3 (0.1%) and indications concerning maximum ashing and atomization temperatures. More specific information may also be found in the literature, such as recommendations regarding matrix modifiers and the use of partition tubes or tubes with platform. Some examples of working conditions are listed in table 1.

When a program is optimized for the determination of an element in a specific matrix, all information should be reported in the logbook of methods of the laboratory, with all needed information such as:

- Matrix
- Type of tube
- Volume of injection
- Type of calibration (direct or standard addition)
- Matrix modifier used and quantity
- Examples of a typical sample and standard peak
- Maximum number of fires

A program is ideally optimized when:

The sensitivity is correct (comparable to the one in the literature)

The background is minimal

The peak shape is correct and comparable in the standard and the sample

It is possible to have a reference material of the same matrix and the same concentration as the sample, and the concentration found in the reference material is acceptable.

*NOTE***:**

The optimization is done first on the sample solution (reference material can be a good one to start with).

Some software has the option of automatic program optimization where ashing and atomization temperature can be varied automatically, it is highly recommended to use those options with each new matrix or new element.

Optimization of drying stage:

The drop of sample should be dry before beginning the ashing stage to avoid boiling, which would spread the sample through the entire graphite tube.

A typical drying stage would bring the solution close to 100°C slowly, and then just above 100°C.

The drying is correct when no noise can be heard when ashing stage starts.

The signal can be measured from the beginning of the temperature program; if the drying stage is correctly set, no perturbations should be seen before ashing stage.

Optimization of ashing stage:

The ashing temperature should be set so that no element is lost.

This stage is separated into three steps: ramping (time to optimize), staying (time to optimize) and staying without gas (generally 2 seconds).

To find this optimal temperature, fix the atomization T° at the recommended T° and increase ashing T° by increments of 50°C until the absorbance decreases.

When the optimum T° is found, the time can be optimized the same way: increase the aching time (ramp and stay) until the ratio between Abs and Background is maximum.

Matrix modifier:

For some elements and some matrices, the results obtained are still not satisfactory (e.g. maximum ashing T° is not sufficient to eliminate the background), this procedure should be

redone with the addition of a matrix modifier. Different matrix modifiers could be tried before finding the best solution.

Often the matrix modifier solution is added to the injection (e.g. 2 or 4 µl for 20 µl injection). If the total volume of injection changes, it is necessary to check that the drying stage is still correct.

The absence of analyte of interest in the matrix modifier should be checked.

The main matrix modifiers are listed in section III.3.

The ashing temperature optimization protocol will be repeated with the addition of matrix modifier, to define the optimum temperature using a specific matrix modifier.

Optimization of atomization stage:

Before the atomization stage, the argon must be stopped. There are two steps in the atomization stage: ramping and staying. The read command should be on during these two-steps.

WARNING: if the Zeeman correction is on, the reading time cannot exceed 4s.

The T° of ashing is fixed at the T° found in the optimization procedure, and the atomization T° is increased. The best T° is the lowest one that gives the best signal.

The ramping should also be optimized.

Cleaning stage:

Add a cleaning stage after the atomization, by increasing the T° to 100-200°C and opening the argon. To increase the lifetime of the graphite tube, it is recommended to do this gradually, in two steps. First open the argon at 0.5 ml/min, and second open argon at maximum gas flow (3 ml/min).

Cooling stage:

It is highly recommended to impose the cooling stage to increase the lifetime of the graphite furnace. It can also be helpful to add a last step at injection T° for 2 or 3 second to stabilize the T° before the next injection.

Check for matrix effect:

When developing a program for a new matrix it is necessary to evaluate the accuracy of the method.

Each unknown type of samples should be spike to check for potential matrix effect.

This spike is considered as a single point standard addition, and should be performed with a minimum dilution factor. The recovery for spike calculated using equation 2 should be 85-115%. If this test fails, it is recommended to run analyses with standard addition method.

Spike solution: mix a fixed volume (V1) of the sample solution with a known volume (V2) of a standard solution of a known concentration (Cstandard).

Unspike solution: mix the same fixed volume (V1) of the sample solution with the same volume (V2) of reagent water.

Measure concentration C (mg l^{-1}) in both solutions on the calibration curve, and calculate recovery as:

Equation 1 $$Cspike = \frac{Cstandard \times V2}{(V1+V2)}$$

Equation 2 $$R = \frac{C\ Spike\ Solution - C\ Unspike\ solution}{Cspike} \times 100$$

To be valid, the concentrations of spike and unspike solutions should be in the linearity range of the calibration curve and Spike concentration (equation 1) should be in the range of 50-150% of the concentration of unspike solution.

When the program is ready, save all information and run it as described in the previous section.

Standard addition:

Main points to check before standard addition run:

Determine the linearity of the instrument.

Make sure the last point of the curve is in the linearity range (quantity of analyte in sample + quantity of analyte in last addition).

The zero addition should be above the DL. Generally the quantity of sample injected is smaller to permit the addition.

The curve should contain at least 3 points plus zero addition, adequately chosen. Best results will be obtained using additions representing 50, 100, 150 and 200% of the expected concentration of sample.

The standard addition curve should be done for each matrix; a fish should not be quantified on a mussel calibration curve!

Switching off the instrument

Print and save the results.

Verify that all needed information is recorded in the logbook.

Switch off the gas, cooling system and instrument.

Empty the carousel and the waste bottle.

Calculation:

The software can calculate the final concentrations. Alternatively, it can be done by hand using the following formula. If the same volume is always injected

$$C(\mu g / g) = \frac{(C_d - C_b) x V x F}{W}$$

Where:

C = Concentration of element in original sample (µg g^{-1} dry weight);

C_d = Concentration of element in sample solution (µg ml^{-1});

C_b = Mean concentration of element in reagent blanks (µg ml^{-1});

V = Volume of dilution of digested solution (ml);

W = Dry weight of sample;

F = Dilution factor, if needed (=1 in case of no additional dilution other than that resulting from digestion procedure).

III-3. MATRIX MODIFIERS

1) AMMONIUM PHOSPHATE AND MAGNESIUM NITRATE

Make the following 2 solutions in ultra pure deionized water:

- $NH_4H_2PO_4$ (Suprapure, Merck) 25 g/l
- $Mg(NO_3)_2$ (Suprapure, Merck) 10 g/l

In a polyethylene cup (for AAS autosampler) make a solution with:

1000 µl $NH_4H_2PO_4$ solution

+ 50 µl $Mg(NO_3)_2$ solution

Add about 4 µl of modifier solution for 20 µl of sample.

2) PALLADIUM NITRATE AND MAGNESIUM NITRATE

SOLUTION (A): $Pd(NO_3)$ (0.2%)

$Pd(NO_3)$ pure (1g)

- In a Teflon beaker, dissolve 1 g of $Pd(NO_3)$ in aqua regia on a hot plate using a minimum amount of acid.
- Transfer into a 100 ml volumetric flask and complete to 100 ml with ultrapure deionized water. Keep this solution (1%) in the refrigerator (+4 °C).
- Dilute the $Pd(NO_3)$ solution (1%) with ultrapure deionized water to make a 0.2% solution:

Add 20 ml of solution in a 100 ml volumetric flask and complete to the volume.

- This 0.2% solution can be kept in the refrigerator (+4°C) for 6 months.

SOLUTION (B): $Mg(NO_3)$ $6H_2O$ (1%)

$Mg(NO_3)$ $6H_2O$ Suprapure, Merck

Make a 10 g/l solution in ultra pure deionized water.

SOLUTION A+B:

In a polyethylene cup (for AAS autosampler) make the following mixture every day of analysis:

800 µl $Pd(NO_3)$ (0.2 %) + 200 µl $Mg(NO_3)$ $6H_2O$ (1%)

Use about 4 µl of this solution for 20 µl sample.

3) PALLADIUM NITRATE, MAGNESIUM NITRATE AND AMMONIUM PHOSPHATE:

Make the following 2 solutions in ultrapure deionized water:

- $NH_4H_2PO_4$ (Suprapure, Merck) 25 g/l
- $Mg(NO_3)_2$ (Suprapure, Merck) 10 g/l

And a palladium nitrate solution (1%) as described in 2)

In a plastic container make the folowing mixture every day:

2 ml $Pd(NO_3)$ + 1 ml $Mg(NO_3)$ $6H_2O$ + 400 µl $NH_4H_2PO_4$ + 6.6 ml of Milli-Q water.

Use about 4 µl of this solution for 20 µl sample.

4) Permanent modification with Iridium:

Use commercial solution of iridium 1000 µg ml^{-1}

- Inject 50 µl of the solution and run the temperature program below
- Repeat this 3 times
- The coating is stable for about 200 injections and can be repeated

Step	Temperature (°C)	Ramp Time (s)	Hold Time (s)
1	100	5	30
2	1200	20	5
3	100	5	2
4	2500	2	10

TABLE 1. EXAMPLES OF GRAPHITE FURNACE CONDITIONS

Element	Cu	Cu	Cd	Cd	Pb	Pb	As	As	Cr	Cr
Sample type	Sediment	Biota	Sediment	Biota	Sediment	Biota	Sediment	Biota	Sediment	Biota
Wavelength (nm)	327.4	327.4	228.8	228.8	283.3	283.3	193.7	193.7	357.9	357.9
Lamp current (mA)	4	4	4	4	5	5	10	10	7	7
Slit	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5R	0.5R
Graphite tube	Partition Tube	Partition Tube	platform	platform	platform	platform	platform	platform	Partition Tube	Partition Tube
Matrix Modifier	none	none	none	Pd, Mg, Amonium Phosphate	none	Pd, Mg, Amonium Phosphate	Pd, Mg	Pd, Mg	none	none
Peak Measurement	area	area	area	area	area	area	area	area	area	area
M_0(pg/0.0044 UA) on standard	13	13	1	1	16	16	15	15	2.5	2.5
Ashing T° (C°)	700	700	300	700	400	925	1400	1400	1100	1100
Atomisation T° (C°)	2300	2300	1800	1900	2100	2200	2600	2600	2600	2600
Remark							Data for Ultra Lamp only!! Number of Fire is critical	Data for Ultra Lamp only!! Standard Addition often required. Number of fire is critical	Use peak Height for lower concentration (peak shape)	Standard Addition often required. Use peak Height for lower concentration (peak shape)

III-4. DETERMINATION OF TRACE METALS IN SEDIMENT AND BIOLOGICAL MATERIALS BY FLAME-AAS

Principle:

The samples are digested with strong acids (see procedure). Atomic absorption spectrometry resembles emission flame photometry in the fact that the sample solution is aspirated into a flame and atomized. In case of flame-AAS, a light beam is directed through the flame, into a monochromator, and onto a detector that measures the amount of light absorbed by the element in the flame. Each metal has its own characteristic wavelength so a source hallow cathode lamp composed of that element is used. The amount of energy absorbed at the characteristic wavelength is proportional to the concentration of the element in the sample.

In case of flame emission, the amount of light emitted at the characteristic wavelength for the element analyzed is measured.

Reagents:

- Acetylene (pure quality).
- Air (pure quality).
- Standard solution of the element of interest 1000 mg l^{-1} (Merck).
- Milli-Q deionised water (>18 MΩ cm, Millipore).

Material:

- Volumetric material, polypropylene tubes with caps (50 ml, Sarstedt), cleaned according to Cleaning Procedures or glass volumetric flask and plastic container (for transferring).
- AAS Varian Spectra-AA10.
- Micropipettes (Finnpipette).
- 1 polyethylene bottle (500 ml) for Milli-Q water.

Reagent solutions:

Metal standard solutions for the calibration curve (See procedure III-1):

1. Put about 10 ml of Milli-Q water into clean polypropylene tubes (50 ml) or in volumetric flask.

2. Add reagents in order to obtain a similar matrix as in the sample (e.g. if sample is in 10% nitric acid add 5 ml of nitric acid).
3. Add the appropriate quantity of stock standard solution (1000 mg l^{-1} or an intermediate stock standard) with a micropipette.
4. Dilute to the mark (50 ml) with Milli-Q water.
5. Shake well.
6. If glass is used then transfer the solution into a polypropylene container.

These solutions can be kept for a few days if stored in the refrigerator (+4°C).

Depending on the element, it may be necessary to make an intermediate stock standard solution. This intermediate stock is prepared as described above in a 2% HNO_3 matrix.

Before analysis:

Analytical conditions may change for each element, so it is necessary to first carefully read the analytical methods book of the AAS before starting an analysis.

Determine the calibration curve according to the expected concentrations of the samples, and the linearity of the AAS response for the element considered (absorbance versus concentration curve given in the analytical methods book).

If ionization or interferences are likely, choose the right option according to the analytical method book, e.g. use of correction for non atomic absorption by using deuterium lamp background corrector, use of oxidizing air-acetylene flame; use of nitrous oxide-acetylene flame; addition of a releasing agent or ionization suppressant.

Prepare a standard solution in 2% HNO_3 for optimization and sensitivity check. The concentration is given in the method book (Concentration for 0.2 abs).

ANALYSIS BY FLAME-AAS:

Calibration curve:

Prepare standards with at least three concentrations plus zero. The zero calibration solution is prepared as other standard solutions without adding analyte.

If the samples are not within the calibration range, dilute them in the same matrix, or prepare a new calibration curve.

General operation:

1. Switch on the instrument and the gas.
2. Make sure the rinsing bottle is filled with fresh water.
3. Make sure the lamp of interest is on.
4. Before beginning optimization, wait approximately 15 minutes so that the lamp is stable.
5. Optimize the lamp position in order to get maximum energy. Record the gain in the logbook.
6. Use a card to optimize the burner position.
7. Switch the flame on.
8. Make instrument zero with **no solution.**
9. Aspirate the sensitivity standard solution.
10. Adjust the burner position slightly in order to get the maximum signal.

WARNING: make sure that the burner is not in the light !! The signal should be zero when no solution is aspirated.

11. Adjust flame composition in order to get the maximum signal.
12. Put the capillary back in the rinsing solution.

Running a sequence:

1. Make an instrument zero while aspirating **NO SOLUTION**.
2. **MEASURE THE ZERO CALIBRATION AS A SAMPLE** and record the absorbance in the logbook. It should be low or comparable with previous data. This should be done before calibration, because while the zero calibration is set up, the instrument automatically

subtracts it from all measurements! If the absorbance of the zero solution is high, it is necessary to check for the source of contamination before beginning an analysis.

3. Run a calibration curve.
4. At least one blank, one reference material and one check standard (ECV See procedure III-1) are measured before any samples, so that it is possible to verify that the system is under control before running the samples.
5. Run the samples, a zero calibration and reslope should be measured every 5 to 10 samples, as well as either a reference material or the ECV or a sample. This allows to check the accuracy of the reslope and the precision of the instrument over the run, as well as to see if the instrument is still under control.
6. During the run verify that the RSD between reading (abs) is below 5%, if it increases the nebulizer should be checked.

Switching off:

1. Save and print out results.
2. Rinse the flame with at least 500 ml of Milli-Q water (by aspirating)
3. Switch off the flame, the instrument and the computer
4. Empty the waste bottle
5. Switch off the gas

Calculation:

The software can calculate the final concentration. Alternatively, it can be done by hand using the following formula. If the same volume is always injected

$$C(\mu g / g) = \frac{(C_d - C_b) x V x F}{W}$$

Where:

C = Concentration of element in original sample (µg g^{-1} dry weight);

C_d = Concentration of element in sample solution (µg ml^{-1});

C_b = Mean concentration of element in reagent blanks (µg ml^{-1});

V = Volume of dilution of digested solution (ml);

W = Dry weight of sample;

F = Dilution factor, if needed (=1 in case of no additional dilution other than that resulting from digestion procedure).

III-5. DETERMINATION OF TOTAL MERCURY IN SEDIMENT AND BIOLOGICAL SAMPLES BY VGA-AAS

Principle and application:

The sediment or biological samples are mineralized with strong acids. The inorganic mercury is reduced to its elemental form with stannous chloride. The cold mercury vapor is then passed through the quartz absorption cell of an AAS where its concentration is measured. The light beam of Hg hallow cathode lamp is directed through the quartz cell, into a monochromator and onto a detector that measures the amount of light absorbed by the atomized vapor in the cell. The amount of energy absorbed at the characteristic wavelength is proportional to the concentration of the element in the sample.

Reagents:

- HNO_3 (65%, analytical grade, low in Hg, Merck).
- $K_2Cr_2O_7$ (analytical grade, low in Hg, Merck).
- $SnCl_2$ (analytical grade, Merck).
- HCl (30%, Suprapur, Merck)
- $HgCl_2$ (salt, Merck) or standard Hg solution (1000 mg l^{-1}, Merck).
- Milli-Q deionised water (>18 MΩ cm, Millipore).
- Argon (pure quality).

Material:

- AAS Varian-Spectra AA-10 and VGA-76.
- Glass volumetric flasks from 50 to 1000 ml (Class A),
- Micropipettes (Finnpipette).

Reagent solutions:

<u>20% w/v $SnCl_2$ in 20 % v/v HCl (200 ml)</u>:

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 Milli-Q 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. If $SnCl_2$ is found to be contaminated, it should be purged with nitrogen for 30 minutes before use.

This solution should be made fresh for each day of analysis.

NOTE:

All glassware used for preparation of $SnCl_2$ solution should be kept separately from remaining laboratory ware in order to avoid cross contamination of ware for trace element determination.

Nitric acid 10% v/v (500 ml):

1. Put about 400 ml of Milli-Q water into a 500 ml volumetric flask.
2. Add carefully 50 ml of concentrated nitric acid.
3. Make up to the mark with Milli-Q water.
4. Shake well.
5. This solution can be stored if kept in a tightly closed flask.

$K_2Cr_2O_7$ 10% (w/v) in Milli-Q water:

1. Weigh 50 g of $K_2Cr_2O_7$ into a clean 500 ml glass volumetric flask.
2. Add about 250 ml of Milli-Q water and shake until $K_2Cr_2O_7$ is dissolved.
3. Make up to the mark with Milli-Q water.

Mercury standards

Preferably use a commercial stock of Hg

Solution stock 1: 1 mg ml^{-1} Hg in 10% nitric acid

1. Weigh exactly 1.354 g of $HgCl_2$ into a 1 liter glass volumetric flask.

2. Add about 500 ml of Milli-Q water.
3. Add 10 ml of concentrated nitric acid (low in Hg).
4. Complete to the mark with Milli-Q water
5. Shake well until complete dissolution is achieved.
6. Transfer into a 1 liter Teflon bottle.

Closed tightly with a torque wrench and keep in the refrigerator (+4º C).

Calibration curve (at least 3 standards and zero calibration) (See procedure III-1):

1. Put about 10 ml of Milli-Q water into a clean 50 ml glass volumetric (or plastic tube).
2. Add reagents as in the digested samples.
3. Add the appropriate quantity of stock standard solution (stock 1 or stock 2 depending on the samples concentrations) with a micropipette.
4. 1 ml of $K_2Cr_2O_7$ solution.
5. Dilute to the mark (50 ml) with Milli-Q water.
6. Shake well.

These solutions should be done fresh every day of analysis.

Sample digestion procedure:

It is strongly recommended to use the digestion procedure for Hg.

In case you use the digestion prepared by microwave oven for trace metal determination, it is strongly recommended that an aliquot of the solution be treated with 2% v/v $K_2Cr_2O_7$ solution as a preservative. Or that Hg is measured in the day following the digestion.

For sediment, the blank as to be checked as generally boric acid is not clean enough! It might be better to use Suprapur boric acid if mercury has to be measured in the sediment digestion solution.

ANALYSIS BY CV-AAS:

Calibration curve:

Prepare standard solutions with at least three standard concentrations plus one zero. The zero calibration is prepared as standard solutions without adding the mercury standard.

If the samples are not within the calibration curve, dilute them in the same matrix, or prepare a new calibration curve.

General operation:

1. Switch on the instrument.
2. Make sure the mercury lamp is on.
3. Before beginning optimization, wait approximately 15 minutes so that the lamp is stable.
4. Optimize the lamp position **without the cell** in order to get maximum energy. Record the gain in the logbook.
5. Optimize the burner position with the cell, the maximum energy should be read.
6. Make instrument zero**.**

Operation of the VGA:

1. Switch on the argon.
2. Put each of the 3 Teflon capillary tubes into the appropriate solutions:
    a) $SnCl_2$ solution
    b) Milli-Q water
    c) 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 over tighten as this will shorten the life of the pump tubes).
4. Check that there are no leaks.

5. Let the system running for about 10 min. 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).

Running a sequence:

1. Make an instrument zero without connecting the VGA to the cell.
2. Connect the VGA to the cell.
3. Set up the delay time (about 50s for VGA Varian), this can be optimized under the optimized signal, aspirate a standard solution and measure the time needed to reach the maximum (stable) signal.

NOTE: this is for online determination system.

4. Measure **AS SAMPLE** the signal, obtained when only SnCl2 and Milli-Q water are aspirating. It should be zero.
5. Measure **AS SAMPLE** the signal, obtained when all three solution are measured, it should be zero, so the next instrument zero can be done on that.
6. **MEASURE THE ZERO CALIBRATION AS A SAMPLE** and record the absorbance in the logbook. It should be low or comparable with previous data. This should be done before calibration, because while the zero calibration is set up, the instrument automatically subtracts it from all measurements! If the absorbance of the zero solution is high, it is necessary to check for the source of contamination before beginning an analysis.
7. Run a calibration curve.
8. At least one blank, one reference material and one check standard (ECV See procedure III-1) are measured before any samples, so that it is possible to verify that the system is under control before running the samples.
9. Run the samples, a zero calibration and reslope should be measured every 5 to 10 samples, as well as either a reference material or the ECV or a sample. This allows to check the accuracy of the reslope and the precision of the instrument over the run, as well as to see if the instrument is still under control.

Shutdown procedure:

1. Rinse all tubing with Milli-Q water for about 20 min. (make sure to keep separate the tube for the $SnCl_2$ solution from the other tubes).
2. Turn off the VGA system.
3. Release the tension from the tubing.
4. Turn off the gas and instrument.
5. Empty the waste bottle.

Calculation:

$$C(\mu g / g) = \frac{(C_d - C_b) x V x F}{W}$$

Where:

C = Concentration of total mercury in dry sample (µg g^{-1} dry);

Cd = Concentration of mercury in sample solution (µg ml^{-1});

Cb = Mean concentration of mercury in reagent blanks (µg ml^{-1});

V = Volume of dilution of digested samples (ml)=57.5 ml;

W = Dry weight of sample (g);

F = Dilution factor, if needed (=1 in case of no additional dilution other than that resulting from digestion procedure).

IV. CLEANING PROCEDURES

IV-1. CLEANING GENERAL LABWARE FOR THE DETERMINATION OF TRACE ELEMENTS

Reagents:

- HNO_3 (65% analytical grade, ISO, Merck).
- Special laboratory detergent (Micro).
- Milli-Q deionised water (>18 MΩ cm, Millipore).

Procedure:

1. Allow the vessels to soak overnight in a plastic container in a soap solution (Micro solution 2% in tap water).
2. Rinse thoroughly first with tap water then with Milli-Q water.
3. Leave the vessels to stand in 10% (v/v) concentrated HNO_3 solution at room temperature for at least 6 days.
4. Rinse thoroughly with Milli-Q water (at least 4 times).
5. Allow the vessels to dry under a laminar flow hood.
6. Store the vessels in closed plastic polyethylene bags to prevent the risk of contamination prior to use.

This procedure should be used for all plastic ware use in the laboratory as tips, cup for autosampler, plastic container……

IV-2. CLEANING OF DIGESTION TEFLON VESSELS FOR THE DETERMINATION OF TRACE ELEMENTS

Reagents:

- HNO_3 (65% analytical grade, ISO, Merck).
- HCl (25% analytical grade, Merck).
- Special laboratory detergent (Micro).
- Milli-Q deionised water (>18 MΩ cm, Millipore).

Procedure:

1. Soak the vessels (Teflon reactors, CEM) and their caps overnight in a detergent solution (Micro solution 2% in tap water) in a plastic container.
2. Rinse thoroughly first with tap water then with Milli-Q water.
3. Fill the Teflon reactor with 5 ml of HNO_3 (conc), close the reactor and put them in the microwave oven.
4. Set up the correct program:

Step	Power (W)	% Power	Ramp time (min sec)	PSI	°C	Hold time (min sec)
2	1200	100	10.00	600	100	5 00
3	1200	100	10 00	600	200	10.00

5. Allow the samples to cool to room temperature, release pressure carefully by opening the valve, and then open the reactor.
6. Empty the reactor (acid can be kept for some run of cleaning) and rinse them carefully with Milli-Q water.
7. Put them to dry under a laminar flow hood.
8. Once dry, the vessels should be closed and put into polyethylene bags to prevent the risk of contamination prior to use.

IV-3. CLEANING TEFLON LABWARE FOR THE DETERMINATION OF MERCURY AND METHYL MERCURY

Reagents:

- HNO_3 (65% analytical grade, ISO, Merck).
- HCl (25% analytical grade, Merck).
- HCl (30%, Suprapur, Merck).
- Special laboratory detergent (Micro).
- Milli-Q deionised water (>18 MΩ cm, Millipore).

Procedure:

1. Allow the vessels to soak overnight in a plastic container in a soap solution (Micro solution 2% in tap water).
2. Rinse thoroughly first with tap water then with Milli-Q water.
3. Put the vessels in 50% (v/v) concentrated HNO_3 solution and heat at 60º C for 2 days.
4. Rinse thoroughly with Milli-Q water (at least 4 times).
5. Transfer the vessels into 10% (v/v) concentrated HCl solution for a further 3 days (at least) at room temperature.
6. Rinse thoroughly with Milli-Q water (at least 4 times).
7. Allow the vessels to dry in a laminar flow hood.
8. All vessels are stored in polyethylene plastic bags. When possible (especially for Teflon bottles), the vessels are filled with 1% HCl (Suprapur, Merck) heated on a hot plate for one night and hermetically closed with a torque wrench.

IV-4. CLEANING LABWARE FOR THE DETERMINATION OF MERCURY BY VGA-CV-AAS; SIMPLIFIED PROCEDURE FOR TEFLON AND GLASSWARE

Reagents:

- HNO_3 (65% analytical grade, ISO, Merck).
- Special laboratory detergent (Micro).
- Milli-Q deionised water (>18 MΩ cm, Millipore).

Procedure:

1. Allow the vessels to soak overnight in a plastic container in a soap solution (Micro solution 2% in tap water).
2. Rinse thoroughly first with tap water then with Milli-Q water.
3. Fill the glass or Teflon vessels with 10% (v/v) concentrated HNO_3 solution.
4. Heat at 60ºC for 2 days. In case of volumetric flasks, let stand for 6 days at room temperature.
5. Rinse thoroughly with Milli-Q water (at least 4 times).
6. Allow the vessels to dry in a laminar flow hood.
7. All vessels are stored in polyethylene plastic bags. Clean volumetric flasks are filled with Milli-Q water.

NOTE:

For contaminated labware, a precleaning step with 50% (v/v) concentrated HNO_3 solution should be used. In this case, steps 3) to 5) should be done twice: first with 50% acid solution, then with 10% acid solution.

REPORT

Recommended Method on

MICROWAVE DIGESTION OF MARINE SAMPLES FOR THE DETERMINATION OF TRACE ELEMENT CONTENT

IAEA/NAEL

Marine Environmental Studies Laboratory in co-operation with

UNEP/MAP MED POL

November 2011

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Recommended Method on
MICROWAVE DIGESTION OF MARINE SAMPLES
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TABLE OF CONTENTS

NOTE: This method is not intended to be an analytical training manual. Therefore, the method is written with the assumption that it will be performed by formally trained analytical chemist. Several stages of this procedure are potentially hazardous, especially stages with HF; users should be familiar with the necessary safety precautions.

In addition, the IAEA's recommended methods are intended to be guidance methods that can be used by laboratories as a starting point for generating their own standard operating procedure. If performance data are included in the method, they shall not be used as absolute QC acceptance criteria.

1. SCOPE

The method here below describes the protocol for dissolution of samples from marine origin. Digests are suitable for analyses of total content of trace element in sediment and biological material.

The goal of this method is the total sample decomposition with the judicious choice of acid combinations this is achievable for most matrices. The selection of reagents which give the highest recoveries for the target analytes is considered the optimum method condition.

The recommended protocol is mainly based on the EPA 3052 method; users are encouraged to consult this document (EPA, 1996).

2. PRINCIPLE

The grinded and dried samples are solubilized in an acid mixture using microwave oven apparatus.

The use of hydrofluoric acid allows the decomposition of silicates by reaction of F with Si to form the volatile SiF4. The excess of hydrofluoric acid is either neutralized by boric acid, or digests are evaporated to dryness depending on the method used to analyze samples.

3. SAMPLE PRE-TREATMENT

Sediment samples are prepared following the recommendations of UNEP (2005).

Marine organisms are prepared following the recommendations of UNEP (1984, 1994).

4. REAGENTS

The reagents used shall meet the purity requirement of the subsequent analyses

4.1. ULTRAPUR WATER (type MilliQ).
4.2. NITRIC ACID 65%.
4.3. HYDROFLUORIC ACID.
4.4. HYDROCHLORIC ACID.
4.5. BORIC ACID.
4.6. HYDROGEN PEROXIDE.

5. MATERIAL

5.1. MICROWAVE APPARATUS

The microwave decomposition system should be temperature controlled. The temperature sensor should be accurate at ±2.5°C. The calibration of the temperature sensor should be done at least once a year, preferably by the maintenance service of the manufacturer.

The microwave unit should be corrosion resistant.

The unit cavity should be well ventilated and connected to fume cleaner or special neutralizing system.

The method requires microwave transparent and acid resistant material (i.e. PFA, TFM) to be used as reactor. The minimal volume of the vessels should be 45 ml and it should be able to work under the pressure of 800PSI. the reactor system should be equipped with a pressure relief system.

5.2. ANALYTICAL BALANCE with 0.001 g precision at least.
5.3. FUME HOOD.
5.4. LAMINAR FLOW HOOD.
5.5. VOLUMETRIC CONTAINERS of 50 ml or 100 ml in polypropylene.
5.6. WEIGHING CUP in polyethylene.
5.7. PLASTIC SPATULAS.

6. PROCEDURE

6.1. All PLASTIC MATERIAL (i.e. volumetric, weighing cup…) should be acid cleaned by soaking in laboratory soap (or 10% alcohol) for at least 24h, followed by 24h of soaking in 10% nitric acid. Stronger acid cleaning protocol could be applied depending on the requirement of the subsequent analyses.
6.2. MICROWAVE VESSELS should be at least cleaned after each use by running the same microwave program used for samples with 5 ml of HNO_3. If the risk of cross contamination is high (i.e. running sandy sediment after organic rich sediment) and/or in the case of long storage, the vessels should be cleaned twice. If available, an acid cleaner

(using acid vapors) can be used as a final cleaning stage. After cleaning, the vessels should be carefully rinsed with water and dried under a laminar flow hood. If a laminar flow hood is not available, vessels should be kept locked in double plastic bag; date of storage should be mentioned on the second bag.

6.3. Accurately weigh 0.1 to 0.5 g of well mixed sample in the microwave vessel.

6.4. In a fume hood, add 5 ml of nitric acid and 2 ml of hydrofluoric acid, close vessels with caps, then it is recommended to let samples react for at least 1 hour (or more if possible). Protect vessels by covering them with plastic bags or place them in a laminar flow hood compatible with acid fume. The quantity of hydrofluoric acid depends on the expected content of silicon dioxide, samples with low concentrations of silicon dioxide (< 10% like plant material to 0% like biological sample) may require less hydrofluoric acid (0.5 ml to 0 ml). Examples of acid quantities for different matrix are listed in table below.

	HF (ml)	HNO_3 (ml)	HCl (ml)	H_2O_2 (ml)	Boric (g)
Sediment	2	5	2 or 0	2	0.8
Fish	0	5	2 or 0	2	0
Sea plant	0.5	5	2 or 0	2	0

6.5. After room temperature pre-digestion, add 2 ml of hydrogen peroxide and close the reactors as recommended by the microwave manufacturer.

NOTE: The quantity and ratio of reagent can be adapted on a performance based judgment (i.e. visual total digestion, certified reference material results).

- In case of a sample containing high calcium carbonate, the hydrofluoric acid content can be set to 0 to avoid precipitation of insoluble CaF.
- A two stage digestion, using half of the hydrofluoric acid at the first stage and half at the second, could increase recovery and help achieving total decomposition.
- Additional reagent can be added depending on the sample composition to achieve complete dissolution. For example, 2±2 ml of HCl can be added to help the stabilization of As, Sb, Hg, Fe and Al at high level; however HCl might increase analytical difficulties for some techniques (i.e. ICP-MS) (Kingston 1997)
- Only one acid mixture or quantity should be used in a single batch, in the microwave, to insure consistent reaction conditions between all vessels and monitored conditions. This limitation is due to the current practice of monitoring a representative vessel, and applying a uniform microwave field to reproduce these reaction conditions within a group of vessels being simultaneously heated.

6.6. Place the closed reactor in the microwave apparatus, connect temperature and pressure control as specified by the manufacturer. The samples should be heated at 180°C

(minimum) in about 6 minutes and the temperature maintained for at least 10 minutes. The total decomposition is primarily controlled by maintaining samples at 180°C for 10 minutes. The ramping profile can be adapted, especially for safety purpose when very reactive samples are decomposed (i.e. biological material). In that case, it is recommended to increase the ramping time to 10 or 15 minutes. If possible, record temperature and pressure profile. In most samples matrices, pressure should peak between 5 and 15 minutes; profiles can be used to optimize temperature program.

6.7. At the end of the temperature profile, let the sample cool until the inside temperature goes down to 60°C, then remove the reactors from the microwave and place them in a ventilated fume hood. The pressure is carefully released following the manufacturer's instruction and reactors are opened.

6.8. In the case of removal of hydrofluoric acid excess with boric acid, 0.8 g of boric acid and 15 ml of water are added in the vessel. The quantity of boric acid is proportional to the quantity of hydrofluoric acid (usually 0.4 g for 1 ml should be sufficient). The vessels are closed again and run in the microwave with a program that heat samples at 170°C in 10 minutes and maintain this temperature for 10 minutes.

6.9. At the end of the temperature profile, let the sample cool until inside the temperature goes down to 60°C, then remove the reactors from the microwave and place them in a ventilated fume hood. The pressure is carefully released following the manufacturer's instruction and reactors are opened. Transfer the samples in a volumetric container and dilute them to a known volume (or a known weight, this requires to record the tare of each container before).

NOTE: An excess of boric acid will produce cloudy solutions, this might cause problem with sample introduction system of ICP. The use of boric acid will prevent measurement of boron, and possible bias introduced should be carefully investigated.

- If the use of boric acid is not possible, or if it is necessary to reduce the concentration of acid in final solutions, digest can be evaporated to incipient dryness on a hot plate at about 140°C. This stage should be performed in a controlled environment to avoid contamination and acid vapour should be treated. Some microwave oven apparatus can perform evaporation. The residue is then diluted to a known volume in nitric or hydrochloric diluted solution (usually 2% v/v) depending on the subsequent analytical method used.
- In case of insoluble precipitate or residue some extra steps can be performed like the addition of 2 ml of perchloric acid to the solution before evaporation, but this requires doing the evaporation under a specific hood for safety reason. Another option is the addition of 2 ml of concentrated hydrochloric acid, evaporation to near dryness, addition of concentrated nitric acid, evaporation to near dryness and dilution in known volume in 2% nitric acid solution.

Most samples will be totally dissolved by this method with the judicious choice of the acid combinations. A few refractory sample matrix compounds, such as TiO2, alumina, and other oxides may not be totally dissolved, and in some cases may sequester target analyte elements.

7. QUALITY CONTROL

7.1. Each microwave batch should contain at the minimum one certified reference material of representative matrix.

7.2. A duplicate or triplicate sample should be processed on a routine basis. A duplicate sample should be processed with each analytical batch or every 10 samples. A duplicate sample should be prepared for each matrix type (i.e. sediment, sea plant, etc.).

7.3. A spiked sample should also be included whenever a new sample matrix is being analyzed, especially if no certified reference material is available for that matrix.

7.4. Blank samples should be prepared using the same reagents and quantities used in sample preparation, placed in vessels of the same type, and processed with the samples. Each microwave batch should contain at least two blank samples.

8. REFERENCES

EPA (1996) U.S. Environmental Protection Agency, EPA method 3052, Microwave assisted acid digestion of siliceous and organically based matrices Rev 0, December 2007, (http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3052.pdf).

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REPORT

Recommended Method for the EXTRACTION OF ORGANIC MERCURY IN MARINE SAMPLES BEFORE THERMAL DECOMPOSITION, AMALGAMATION AND ATOMIC ABSORPTION SPECTROMETRY

IAEA/NAEL

Marine Environmental Studies Laboratory in co-operation with

UNEP/MAP MED POL

January 2014

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Recommended Method for the EXTRACTION OF ORGANIC MERCURY IN MARINE SAMPLES BEFORE THERMAL DECOMPOSITION, AMALGAMATION AND ATOMIC ABSORPTION SPECTROMETRY

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1. SCOPE:

This method here below describes the protocol for extraction of organic mercury in biological material of marine origin in the range of 0.01 to 5 mg kg^{-1} orgHg as mercury.
The final aqueous phase should then be directly analyzed following recommended method for determination of mercury by elemental mercury analyzer (IAEA, 2012).

2. PRINCIPLE:

The organic mercury species is released from the matrix by acid leaching, extracted in solvent and back extracted in sodium thiosulfate. The final aqueous solution can be measured directly in the elemental mercury analyzer.

3. SAMPLE PRE-TREATMENT:

Marine organisms are prepared following recommendation of UNEP/IOC/IAEA 1984, UNEP/IOC/IAEA 1994

4. REAGENTS:

The reagents used shall meet the purity requirement of the subsequent analyses

4.1. Ultrapur Water (type MilliQ)

4.2. Hydrochloric Acid 25% (v/v): In 500ml bottle adds about 250ml of ultrapure water, add 125ml of HCl and dilute to 500ml with ultrapure water.

4.3. Sodium thiosulfate: Prepare a solution fresh each day at 0.002mmol.l^{-1} dissolving 0.248g of Na2S2O5.5H2O in 500 ml ultra-pure water

4.4. Toluene

5. MATERIAL:

5.1. Analytical balance with 0.001g precision at least

5.2. Disposable **c**entrifuge tube in polypropylene 50ml

5.3. Glass or Plastic vials (about 5ml)

5.4. Vortex Agitator

5.5. Centrifuge

6. PROCEDURE:

(Schematic of the extraction protocol is shown in fig.1)
Each batch of extraction should contain at least: 2 method blank (extraction without sample) and 1 certified reference material of representative matrix and level.

6.1. Weight between 0.1-0.8g of sample in 50ml plastic tube (Tube 1)

6.2. Add 5 ml of 25% (v/v) HCl

6.3. Record the weight of Tube 1 as W1

6.4. Vortex for 30s (make sure sample is "wet")

6.5. Add 10ml of Toluene

6.6. Record the weight of Tube 1 as W2

6.7. Vortex for 3 minutes

6.8. Centrifuge 5000 rpm 15 minutes

6.9. If the separation is not clear repeat step 6.9

6.10. Collect about 70-80% of toluene upper phase in Tube 1 (without interface) and transfer it in another centrifuge tube (tube 2).

6.11. Record the weight of Tube 1 (W3) to determine ratio of solvent transferred

6.12. For sample with expected organic Hg around 0.2 mg kg^{-1} and below, add 5ml of sodium thiosulfate solution in tube 2. For sample with higher concentration add 10ml of sodium thiosulfate solution in tube 2.

6.13. Vortex Tube 2 for 3 minutes and centrifuge for 5-15 minutes at 5000 rpm

6.14. Recuperate the lower aqueous phase in 5ml vials, make sure no solvent is pipetted, if needed centrifuge the collected thiosulfate solution and keep only aqueous phase. Injection of toluene in the mercury analyzer is DANGEROUS

6.15. Solutions are ready to be injected in the analyzer.

6.16. Use 50 to 400µl of solution depending on expected concentration

6.17. Same injection volume should be used for sample and method blank

6.18. Thiosulfate solutions are stable for 2 days if kept at 4°C ± 2°C

NOTE: Memory effect during analysis with solid mercury analyzer has be found to be more significant than with solid or acidic solutions, so it is recommended to keep injected quantities below 10 ng

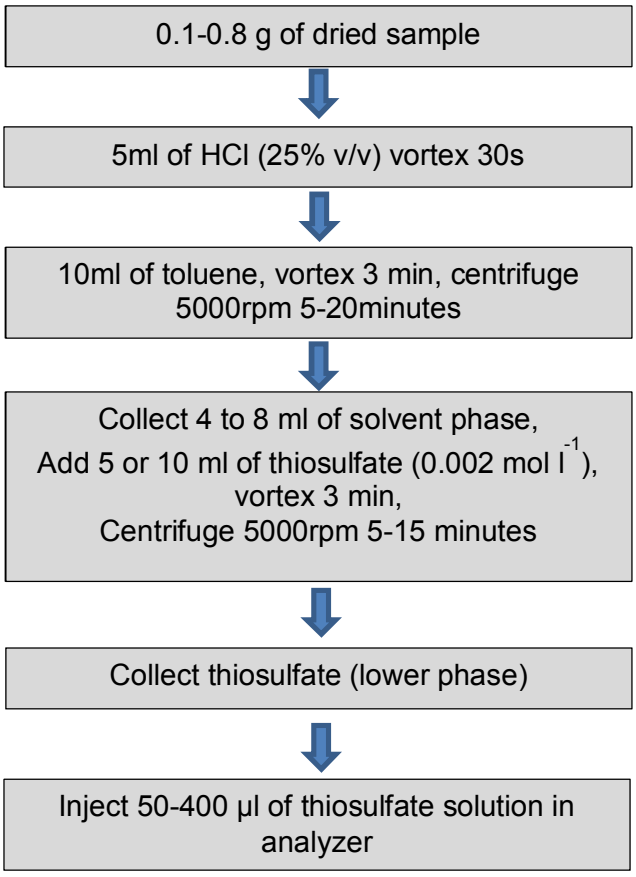

Fig 1 : Schematic of extraction protocol

7. WASTE MANAGEMENT:

Waste produced during the extraction is considered as hazardous waste and should be collected in a glass container (i.e. 5 liter bottle). The laboratory is responsible for complying with local regulations for waste management, particularly hazardous waste identification rules and land disposal restrictions, and for protecting the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations.

8. QUALITY CONTROL:

8.1. Method blank: prepare at least two (preferably 3) method blanks per batch of extraction following procedure (6.) without sample. Maximum allowed blank concentration should be well documented and if blank solution exceeds this value all samples prepared along the contaminated blank should be prepared again and reanalysed.

8.2. Certified Reference Material: At least one certified reference material of a representative matrix should be measured with each batch of sample, the calculated result should fall in the value of the certificate within the coverage uncertainty (Linsinger, 2010), to show evidence of unbiased result. Results of CRM should be recorded for quality control purpose and plot in control chart (UNEP/IOC/IAEA 1994).

8.3.A spiked sample should also be included in each batch. Measure a spike sample by adding a known volume of standard solution in sample before extraction.

9. CALCULATION OF RESULTS:

$$W_{OrgHg} = \frac{\rho 1 - \rho 0}{V_{inj} \times W \times Ratio} \times V_{thio} \times R$$

Note: ρ1 and ρ0 are calculated using calibration curve equation (usually done by software).

Where:
w(Hg) is the mass fraction of mercury in the sample, expressed in mg kg^{-1};
ρ1 is the quantity of mercury, expressed in ng as measured in the sample solution;
ρ0 is the quantity of mercury expressed in ng as measured in the blank solution
R is the recovery calculated using the CRM (see 8.1) or spike (see 8.3)
Vinj is the injected volume (should be the same in sample and blank solution) in ml
W is the amount of sample in g
Vthio is the volume of thiosulfate solution in L
Ratio: is the ratio of solvent calculated as:

$$Ratio = \frac{W2 - W3}{W2 - W1}$$

See section 6. for description of W1, W2 and W3

10. EXPRESSION OF RESULTS

The rounding of values will depend on the uncertainty reported with the result; in general for this method two or three significant figures should be reported.
Uncertainty component should be reported with all results. (ISO 2005, Nordtest 2004)
Example: w(MeHg) = 0.512 ± 0.065 mg kg^{-1}.

11. REFRENCES:

- **ISO (1995).** Guide to the expression of uncertainty of measurements International Organisation for Standardization: Geneva
- **Linsinger, T. (2010),** European Commission - Joint Research Centre, Institute for Reference Materials and Measurements Application note 1 (www.erm-crm.org)
- **Nordtest (2004).** Handbook For Calculation Of Measurement Uncertainty In Environmental Laboratories Edition 2
- **UNEP/IOC/IAEA (1984)** reference method 7 rev2: Sampling of selected marine organisms and sample preparation for trace metal
- **UNEP/IOC/IAEA (1994)** reference method 57: Quality assurance and good laboratory practice, UNEP, 1994

REPORT

Recommended Method for the DETERMINATION OF ORGANOTIN COMPOUNDS IN BIOLOGICAL SAMPLES

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November 2012

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TABLE OF CONTENTS

NOTE: This method is not intended to be an analytical training manual. Therefore, the method is written with the assumption that it will be used by formally trained analytical chemists. Several stages of this procedure are potentially hazardous; users should be familiar with the necessary safety precautions.

In addition, the IAEA recommended methods are intended to be guidance methods that can be used by laboratories as a starting point for generating their own standard operating procedure. If performance data are included in the method they must not be used as absolute QC acceptance criteria.

1 GENERAL DISCUSSION

1.1 PRINCIPLE

Butyl- and phenyltin compounds are used worldwide mainly as antifouling agents and biocides. The large-scale application of these compounds to submerged marine structures, commercial vessels and pleasure crafts has resulted in dispersion to every marine environmental compartment. These compounds, especially tributyltin (TBT), exhibit broad spectrum of biocidal properties and so have elicited considerable research attention. Due to the deleterious effects on non-target organisms, the use of organotin compounds as antifouling agents on the boat hulls of small vessels has been widely prohibited.

Many analytical procedures based on chromatographic separation coupled to various detection techniques have been developed. Organotin compounds must be extracted from the matrix and derivatized into suitable forms for gas chromatographic analyses. The most common derivatization procedures include hydride generation in the aqueous phase using sodium borohydride, or extraction of organotin complexes with tropolone or diethyldithiocarbamate, followed by Grignard derivatization. The instability and volatility of butyltin hydrides can lead to losses of compounds and consequently to an underestimation of contamination. The Grignard derivatization requires scrupulous dry conditions and is rather time consuming.

To avoid such difficulties, an organotin derivatization procedure directly applied to the aqueous phase using sodium tetraethylborate ($NaBEt_4$) was developed, whereby the derivatization and extraction of organotin compounds can be carried out simultaneously. This technique allows the determination of several butyl- and phenyltin compounds.

The method for the determination of organotin compounds presented here is based on the ethylation with sodium tetraethylborate. The simultaneous derivatization and extraction with

sodium tetraethylborate converts butyltin and phenyltin ions into their corresponding volatile derivatives in the organic phase:

$$R_nSn^{(4-n)+} + (4-n)\ NaBEt_4 \longrightarrow R_nSnEt_{(4-n)} + (4-n)\ Na^+ + (4-n)\ BEt_3$$

This derivatization reaction is the most critical step of the protocol. For optimal derivatization efficiency, the solution of sodium tetraethylborate should be either freshly prepared, i.e. just prior to sample processing, or stored frozen (-20°C) for no longer than two weeks. The optimal conditions for the sodium tetraethylborate comprise a 30 min. reaction time at a pH between 5 and 6.

It should be appreciated that sodium tetraethylborate is extremely air-sensitive and must be handled with care to keep its chemical integrity.

1.2 ANALYTICAL STRATEGY

Many of the internal standards and the target compounds in the samples need to be derivatised to be detectable by GC techniques. Two different approaches are possible in order to overcome this problem.

- The first would be to purchase calibration standard solutions and internal standard solutions of ***ethylated organotin compounds***. These solutions contain tetra-substituted organotin species which are detectable by gas chromatography without further treatments. The calibration solutions contain certified concentrations of target compounds and can be injected directly into the GC (at previous suitable dilution). The ethylated internal standards are spiked in both the samples to be analysed and the calibration solutions. This approach eliminates any errors due to the performance of the derivatization reaction in the calibration standards. Unfortunately, the costs of ethylated standard solutions are fairly high and their use in routine analyses is not cost effective.
- The second approach available would be to use a ***matrix matched calibration curve***. In this instance, the calibration standards which are not derivatized are spiked into organotin-free environmental matrices (same matrix as the samples to be analysed). These “calibration samples” are then handled and analysed as if they were unknown samples, and thus, they undergo all the different steps of the analytical method including the derivatization step. This approach allows the normalization of some systematic errors and, more importantly, a matrix-matched calibration curve that will take into account the variability linked to the use of sodium tetraethylborate. In case of a low efficiency of the derivatization reaction, for example, both the samples and the calibration curve will be affected, normalizing thus, the results.

1.3 DESCRIPTION AND USE OF ORGANOTIN CALIBRATION STANDARDS

The calibration standards are multi-compound solutions of *Mono*-, *D*i- and *Tributyltin*, *Mono*- , *Di*- and *Triphenyltin*. Ethyl-substituted compounds can be analysed directly, or the "chloride" forms of these compounds can be spiked in uncontaminated environmental samples (calibration samples), to create a matrix-matched calibration curve (see section 2.2).

The calibration curve must be made by at least 3 points (standard solutions at different concentrations). The concentration of the standard solutions must be calculated so that they bracket the concentrations of the samples and the Reference Materials (RMs).

If the concentrations of the target compounds in the samples are unknown, the calibration curve should be centred on the reference materials. If the concentrations of the target compounds exceed the limit of the calibration curve, either the samples must be brought to the appropriate dilution (extracting less material), or the calibration curve must be extended with a higher concentration standard. If, on the contrary, the concentration of the samples is lower than the lowest calibration curve's point, a new calibration curve must be prepared.

Example:

The reference material NIES-11 (fish tissue) contains 1.3 µg/g of TBT as chloride.

0.5 g of NIES 11 in a 0.5 mL solution (assuming 0.5 mL as the final dilution of the vial prior to GC injection) means a concentration of:

$$\frac{(0.5\ \text{g} \times 1.3\ \mu\text{g/g})}{0.5\ \text{mL}} = 1.3\ \mu\text{g/mL} = 1.3\ \text{ng/µl}$$

A calibration curve centred on this value would be:

0.5 1.25 2.5 ng/µl

Assuming an organotins mixture working solution concentration of 50 ng/µl and a final vial dilution of 1mL, the calibration curve can be created by spiking 10, 25 and 50 µl of organotins mixture into 3 different uncontaminated matrices.

1.4 DESCRIPTION AND USE OF ORGANOTIN INTERNAL STANDARDS

Internal standards are non-interfering compounds added to a sample with a known concentration, in order to eliminate the need for the measurement of the final sample dilution in quantitative analyses, and for correction of instrumental variation. The internal standards must be selected based on their characteristics, and thus on the information they have to provide. Internal standards can be added to the samples at the very beginning of the procedure, to check for the overall performance of the method, or at the end, just prior to GC

injection, to check mainly for GC performance. A combination of several internal standards with different properties allows a better understanding and control of an analytical protocol.

The method proposed here is based on the use of three internal standards:

- *Tripropyltin chloride* used as the main internal standard to indicate the derivatization reaction efficiency. All the chromatographic peak areas are normalized to those of Tripropyltin.
- *Tetrapropyltin* used as secondary internal standard to check the overall recovery of the protocol. It is already tetra-substituted and thus, it is not concerned by the derivatization reaction.
- *Tetrabutyltin* spiked in all samples prior to GC injection is used as a GC-internal standard to quantify recovery of both internal standards

2 APPARATUS AND REAGENTS

2.1 APPARATUS

- Glass jars and aluminium foil, stainless steel knives, scoops, forceps, labels, marking pens, log book;
- Insulated plastic boxes for transporting samples, ice or dry ice;
- Deep freezer (-18 to -20°C) for sample preservation;
- Rotary evaporator;
- Glassware including boiling flasks, ground glass stoppers, beakers, Erlenmeyer flasks, separatory funnels, centrifuge tubes, weighing bottles, pipettes, tissue grinders;
- Drying oven (temperature range up to at least 450°C) for determining the sample's dry weight, baking of contaminant residues from glassware and reagents;
- Centrifuge and tubes;
- Freeze-dryer and porcelain mortar and pestle;
- Analytical balance with a precision of 0.1 mg and an electro-balance with a precision of at least 1 µg;
- Stainless steel tweezers and spatulas;
- Dessicator - completely cleaned and with no grease applied to sealing edges;
- Supply of clean dry nitrogen;
- Vacuum pump (water-jet air pump);
- Ultrasonic bath;

- Litmus papers;
- Solid Phase Extraction columns (Supelclean™ LC-Florisil® SPE Tubes 6 mL, 1 g);
- 12 or 24 ports Glass Vacuum manifold for simultaneous use of multiple SPE columns. The manifold must be fitted with flow control valves to allow for a fine adjustment of flow through the SPE column.

2.2 LIST OF REAGENTS

- Acetic acid, 96%;
- Tetra methyl ammonium hydroxide 25% solution in water
- Potassium acetate / acetic acid buffer (CH_3COOH/ CH_3COO^-, K^+);
- Organotin-free sea water;
- 1% $NaBEt_4$ in 2% KOH solution;
- Na_2SO_4, anhydrous granular;
- Florisil cartridges (Supelclean™ LC-Florisil® SPE Tubes 6 mL, 1 g);
- Isooctane, pesticide grade or equivalent purity;
- *n*-hexane, pesticide grade or equivalent purity;
- Nitrogen, 99.8% purity.

2.3 STANDARDS

- Internal standard: Tripropyltin choride (ca. 500 ng);
- Secondary Internal Standard: Tetrapropyltin (ca. 1000 ng);
- GC-Internal Standard: Tetrabutyltin (ca. 100 ng).

The GC vials containing Tetrapropyltin and Tetrabutyltin are added to the injection sequence just before GC injection. These standards are used to quantify recovery of Tetrapropyltin in samples.

The working solutions from the stock reference solutions are prepared on a regular basis, and stored in clean glass volumetric flasks tightly capped with non-contaminating materials such as Teflon or glass. Extreme care must be taken to ensure that standards have not changed their concentrations through solvent evaporation.

3 PROCEDURES

3.1 GLASSWARE CLEANING AND HANDLING

The glassware for the analyses of organotin compounds requires special precautions and specific equipment.

3.1.1 Reagents

- Detergent for glassware;
- *n*-Hexane, pesticide grade or equivalent purity;
- Acetone, pesticide grade or equivalent purity.

3.1.2 Procedure

The glassware must be cleaned with detergent, rinsed first with tap water, then with high purity acetone and finally with *n*-hexane. Any contact with plastics of any kind, as well as silicone and/or grease or any other material must be avoided in order to minimize the risk of contamination. Only Teflon, aluminium, glass or stainless steel (previously cleaned with acetone and *n*-hexane) should be in contact with the samples.

As an alternative, glassware can be pyrolitically cleaned using a muffle furnace at a temperature of 450°C for 4 hours. Even for low level determinations, it is still recommended to rinse the glassware with small amount of *n*-hexane.

NOTES:

The contamination of laboratory apparatus with organotin compounds arises from the use of diorganotin stabilizing compounds in many types of plastic, e.g. PVC, and the use of such materials should be avoided.

Organotins are readily adsorbed onto glassware and losses may occur through the use of glassware. We recommend using the minimum of glassware, and wherever possible rinsing glassware at least three times with appropriate solvent when transferring the sample solutions.

3.2 SODIUM SULFATE (Na_2SO_4) CLEANING

3.2.1 Principle

The sodium sulfate is pyrolitically cleaned using a muffle furnace at a temperature of 450°C for 4 hours.

3.2.2 Reagents

- Sodium sulfate, anhydrous granular.

3.2.3 Procedure

Transfer an adequate amount of sodium sulfate in a glass container (the glass must be resistant to high temperature).

Put the container in the muffle furnace at a temperature of 450°C for 4 hours.

3.3 SEA WATER CLEANING

3.3.1 Principle

Organotin-free sea water is used to achieve a better phase separation after the derivatization reaction.

3.3.2 Reagents

- Sea water;
- *n*-hexane, pesticide grade or equivalent purity.

3.3.3 Procedure

- Fill a 1 L separating funnel with sea water;
- Add 30 mL of *n*-hexane;
- Shake vigorously for 1-2 minutes;
- Discard the organic phase;
- Add another 30 ml of *n*-hexane;
- Shake vigorously for 1-2 minutes;
- Discard the organic phase;
- Store the sea water in a pre-cleaned glass bottle.

NOTE:

A solution of 25% NaCl in deionized water could be used instead of sea water and cleaned following the same procedure.

3.4 PREPARATION OF BUFFER SOLUTION (CH_3COOH/ CH_3COO, K^+)

3.4.1 Principle

A potassium acetate/acetic acid buffer solution is used to adjust the pH between 5 and 6 prior to derivatization reaction.

3.4.2 Reagents

- Acetic acid (96%, d=1.06);
- Potassium acetate (CH_3COOK);
- Milli-Q water (organotin-free deionized water).

3.4.3 Procedure

- Weigh 125.08 g (or 118 mL) of acetic acid;
- Weigh 192 g of potassium acetate;
- Dissolve the potassium acetate in Milli-Q water;
- Add the acetic acid and make 1L up with Milli-Q water;
- Store in a pre-cleaned glass bottle.

3.5 PREPARATION OF 2% KOH SOLUTION

3.5.1 Principle

A 2% KOH solution is used for the preparation of the sodium tetraethylborate ($NaBEt_4$) solution.

3.5.2 Reagents

- KOH;
- Milli-Q water.

3.5.3 Procedure

- Dissolve 2.3 g of KOH in Milli-Q water and make it 100 mL;
- Store the solution in a pre-cleaned Teflon bottle and keep in the freezer until incipient frost formation.

3.6 PREPARATION OF 1% $NaBEt_4$ SOLUTION IN 2% KOH

3.6.1 Principle

The method for the determination of organotin compounds presented here is based on the ethylation with sodium tetraethylborate.

It should be appreciated that sodium tetraethylborate is extremely air-sensitive and must be handled with care to keep its chemical integrity.

3.6.2 Reagents

- 2% KOH solution, 100 mL;
- $NaBEt_4$, 1 g sealed bottle;
- Argon or nitrogen gas.

3.6.3 Procedure

- Operate when possible in a glove box, or at least in a fume-hood using a stream of argon or nitrogen to minimize contact between $NaBEt_4$ and the air;
- Carefully open the $NaBEt_4$ bottle and fill it with KOH solution;
- Close the bottle and shake manually;
- Pour the content into the KOH solution bottle;
- Rinse the $NaBEt_4$ bottle with KOH;
- Prepare the Teflon bottles of appropriate volume and fill them with argon or nitrogen;
- Fill up the Teflon bottles with the $NaBEt_4$ solution;
- Fill the Teflon bottles with argon or nitrogen;
- Put the Teflon bottle in single zip-lock bags filled up with argon or nitrogen;
- Store at -20°C.

3.7 PREPARATION OF STANDARD SOLUTION

3.7.1 Principle

The individual stock solutions of organotin compounds are prepared by dissolving approximately 60-80 mg of the original standard compound in 100 mL *n*-hexane (for tri- and tetra- substituted organotin species), and in acetone (for mono- and di-substituted organotin species).

3.7.2 Reagents

- Organotin standards;
- *n*-hexane, pesticide grade or equivalent purity;
- Acetone, pesticide grade or equivalent purity;
- Isooctane, pesticide grade or equivalent purity.

3.7.3 Procedure

- Using a pre-cleaned glass weighing boat, weigh 60-80 mg of the standard;
- Transfer into a 100 mL glass volumetric flask;
- Rinse the weighing boat with the appropriate solvent into the volumetric flask;

- Bring to volume with the appropriate amount of solvent;
- Store at -20°C.

NOTE:

Individual working solutions of Internal and GC-Internal Standards are prepared in the appropriate solvent:

- Acetone for Internal Standards of tripropyltin (TPrT) and tetrapropyltin (TePrT);

- Acetone for the multi-compound mixture used for the calibration curve (organotins mixture, OTs mix);

- Isooctane for GC-Internal Standards, tetrabutyltin (TeBT) and tetrapropyltin (TePrT).

The concentration of working solutions usually ranges between 10 and 40 ng/µl which correspond to a 25-50 times dilution of the stock solution.

4 ANALYTICAL PROCEDURE ON THE DETERMINATION OF ORGANOTIN COMPOUNDS IN BIOLOGICAL SAMPLES

About 0.5 grams of freeze-dried biological samples are dissolved (alkaline digestion) by sonication in an ultrasonic bath with tetra methyl ammonium hydroxide, TMAH, (25% solution in water). This step is very important and the samples must be completely dissolved before the derivatization reaction. Using buffer and acetic acid, PH is stabilized between 5 and 6. The organotin compounds are simultaneously derivatized and extracted using sodium Tetrahetylborate (NaBEt4) and n-hexane. Florisil cartridges are used to clean up the samples. The purified samples are concentrated to about 0.5 mL (prior to solvent change from *n*-hexane to isooctane) and analysed by GC.

1- Internal standards spiking:

- Spike appropriate amounts of TrPrT and TePrT in each sample.

2- Alkaline Digestion:

- Dried biological samples are weighed into 50 mL screw cap Teflon tubes;
- Add 10 mL of TMAH to each Teflon tube;
- Mechanically shake the Teflon tubes for 30 minutes;
- dissolve samples in an ultrasonic bath for 1 hour at least or until complete dissolution;

3- pH Adjustment:

- Add 5 mL buffer solution to each Teflon tube;
- Add 5 mL of Acetic acid
- Add 7 ml of organotin-free sea water

4- Derivatization/Extraction:

- Add 1 mL of $NaBEt_4$ solution to each Teflon tube;
- Immediately add 5 mL of *n*-hexane to each Teflon tube;
- Shake Teflon tubes manually and mechanically for 30 minutes;
- Centrifuge the Teflon tubes at 5000 rpm for 5 minutes;
- Recover organic phase in 15 mL glass tube;
- Add 5 mL of *n*-hexane to the water phase and repeat the extraction;
- Shake manually vigorously and mechanically for 10 minutes;
- Centrifuge the Teflon tubes at 5000 rpm for 5 minutes;
- Recover organic phase and combine;
- Add ca. 1-2 g of pre-cleaned Na_2SO_4 to each glass tube to dry organic phase;

5- Evaporation:

- The extract is reduced to ~1 mL under a gentle stream of N_2.

6- S.P.E. Clean Up:

- The concentrated extract is purified using Florisil cartridges;
- The sample and solvent rinses are added to the top of Florisil cartridge and eluted with 5 mL of *n*-hexane twice (2x5 mL). The eluent is collected in a 15 mL glass tube.

7- Evaporation:

- The eluent is reduced to ~0.5 mL under a gentle stream of N_2.

8- Transfer into Vials/Solvent Exchange:

- Transfer the concentrated eluents into auto-injector vials. Rinse the glass tube with isooctane and add to the vial.

9- Evaporation:

- Reduce to appropriate volume under a gentle stream of N_2.

10- Standard Spiking:

- Add GC-Internal Standard (TeBT) to each sample vial;

- Prepare vials of GC-Internal Standard.

11- GC Analyses**:**

- Inject 1 uL of sample to GC-FPD and/or GC-MS.

5 GAS CHROMATOGRAPHY CONDITIONS

5.1 GC-FPD CONDITIONS FOR THE QUANTIFICATION OF ORGANOTIN COMPOUNDS

- Column: 30 × 0.25 mm × 0.25 μm HP-5 capillary column (5% phenyl methyl silicone) or equivalent (Supelco);
- Inlet: 250°C, splitless;
- Detector: FPD at 270°C, 610nm Sn filter. Hydrogen rich flame (air flow: 90 ml/min.; hydrogen flow: 130 ml/min.);
- Carrier gas: Helium, at a flow rate of 1 mL min^{-1};
- Oven program: 60°C for 2 min, then 60-270°C at 6°C min^{-1} and 270°C for 20 min.

5.2 GC-MS-SIM CONDITIONS FOR THE QUANTIFICATION OF ORGANOTIN COMPOUNDS

- Column: 30 × 0.32 mm × 0.25 μm HP-5 capillary column (5% phenyl methyl silicone);
- Detector: Electron Impact-MS in SIM mode. Details of the acquisition are provided in Table 1;
- Ion energy of the MS: 70 eV;
- Ion source temperature: 240°C;
- Interface: 280°C;
- Inlet: 250°C, splitless;
- Carrier gas: Helium, at a flow rate of 2 mL min^{-1};
- Oven program: 60°C for 1 min, then from 60 to 100°C at 10°C min^{-1} and from 100°C to 280 at °C at 4°C min^{-1}.

TABLE 1. TARGET AND CONFIRMATION IONS FOR GC/MS ANALYSES OF ORGANOTIN COMPOUNDS

Group	Time (min)	Compounds (m/z monitored)[a]	Cycles (s^{-1})	Ions (m/z)
1	6–10	MBT (121, 179, <u>235</u>) TrPT (121, 193, 235, <u>249</u>)	3	179, 235, 121, 193, 249
2	10–16.5	TePrT (121,165,249, <u>207</u>) DBT (121, 207, 235, <u>263</u>) MPhT (197, <u>255</u>) TBT (204, 235, 263, <u>291</u>)	2.18	263, 207, 291, 235, 121, 255, 197
3	16.5–20	TEBT (179, 235, <u>291</u>)	4.83	291, 179, 235
4	20–26	DPhT (120, 197, 275, <u>303</u>)	3.70	303, 275, 197, 120
5	26–40	TPhT (120, 197, 349, <u>351</u>)	3.7	351, 197, 120, 349

[a] The m/z used in quantification is underlined.

6 RESULTS QUANTIFICATION

This method is based on the use of an ***internal standard,*** which is defined as a non-interfering compound, added to a sample with known concentrations in order to eliminate the need to measure the final extract volume in quantitative analysis and for correction of instrumental variation.

In this method, the internal standard is added to each sample at the beginning of the analytical protocol. The ratio of the areas of the internal standard and analyte are then used to construct the calibration curve.

In a multiple point internal calibration each analyses contains the internal standard whose total amount is kept constant, and the analyte of interest whose amount covers the range of concentrations expected.

A multiple points relative response factor (RRF) calibration curve is established, for analytes of interest in each working batch. A RRF is determined, for each analyte, at each calibration level using the following equation:

$$RRF(X) = \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{\text{Qty (IS)}}{\text{Qty (X)}}$$

Where:

Area (X) = the area of the analyte to be measured (target compound);

Area (IS) = the area of the specific internal standard;

Qty (X) = the known quantity of the analyte in the calibration solution;

Qty (IS) = the known quantity of the internal standard in the calibration solution.

The relative response factors determined for each calibration level are averaged to produce a mean relative response factor (mRRF) for each analyte. The percent relative standard deviation (%RSD) for the 3 response factor must be less than or equal to 15%, for each analyte.

$$\%RSD = \frac{\text{Standard deviation of the RRFs}}{\text{Average of the RFs}} \text{ x } 100$$

The sample analyte concentrations are calculated based on the quantity and response of the internal standard.

The following equation gives the amount of analyte in the solution analysed.

$$Qty\,(\text{X}) = \text{Qty (IS)} \text{ x } \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{1}{\text{mRR}F(X)}$$

Where:

Qty (X) = the unknown quantity of the analyte in the sample;

Qty (IS) = the known quantity of the internal standard added to the sample;

Area (X) = the area of the analyte;

Area (IS) = the area of the internal standard

mRRF (X) = the average response factor of the analyte.

The sample analyte concentrations are then calculated by dividing the amount found (Qty) by the grams of the samples extracted.

6.1 REPORTING CONCENTRATIONS

The organotin compounds are purchased "as chloride". The standard solution prepared and the results obtained after the calculation are expressed "as chloride". The certified values of the reference materials and its literature usually report organotin concentration "as Sn". The conversion between the two forms is given by the ratio between the atomic mass of Sn and the molecular mass of the chloride form.

Example:

For MBT = Sn $(C_4H_9)Cl_3$

Atomic masses: Sn = 118.71; C = 12; H = 1; Cl = 35.45.

Molecular mass of Sn $(C_4H_9)Cl_3$ = 282.06

The ratio between atomic mass of Sn and molecular mass of MBT gives the conversion factor: Sn/chloride = 0.421

10 ng/g MBT as chloride correspond to 10 x 0.421 = 4.21 ng/g as Sn

6.2 QUALITY ASSURANCE/QUALITY CONTROL

The goal in analytical chemistry is to obtain accurate compositional information about an unknown sample. The analyst introduces a processed sample into an instrument that will measure the parameter(s) of interest, and a result is obtained. But how accurate is the result? There are a myriad of factors - including contamination, loss of analyte, analyst error, instrument problems, to name only a few - that could occur at any time throughout the analytical process and potentially impact the accuracy of the final result. Sometimes, the impact of one or more of these factors is evident; often it is not.

Quality assurance (QA) is a comprehensive system of activities to ensure that the quality of the data produced meets pre-defined standards, within a stated level of confidence. A laboratory must design its QA program to meet its specific needs, so that the data produced is acceptable for its intended use; i.e. 'fit for purpose.' A good laboratory QA program will

include both technical and administrative components to achieve this objective. Required component systems of a QA program generally include:

- Staff training;
- Procedures and procedure validation;
- Records and document control;
- Procurement and verification of item quality before use;
- Control of items (e.g. sample receipt, chains of custody, etc.);
- Periodic assessment of the QA program and its components;
- Corrective action management;
- Instrument maintenance and calibration.

Having a fully implemented QA program provides the necessary ingredients for producing a reliable and defensible product. Due to the fact that many of the factors that could impact data quality are transient, the analytical process must be tested and evaluated throughout the process, to verify that the results produced are accurate and reproducible, and have not been impacted. The Quality Control (QC) is a system of activities to ensure that the analytical process is controlled, and verifies that quality parameters are within required limits. QC is often separated into method (or process) QC and analytical (or instrument) QC. Method QC is generally introduced at the beginning of the analytical process, to ensure that all the steps comprising the process (e.g. digestion, separation, concentration, etc., and including analysis) have not impacted the final result. Method QC may include the following QC samples:

- Method blank to monitor the contamination resulting from the process;
- Laboratory control sample (e.g. representative reference material) to monitor accuracy;
- Laboratory control sample duplicate to monitor method reproducibility.

The analytical QC ensures that the instrument is controlled throughout the analysis, and is usually comprised of the following QC samples:

- Reagent blank to monitor the contamination resulting from the reagents and/or instrument;
- Independent calibration verification (ICV) to verify that the calibration standard is acceptable;
- Continuing calibration verification (CCV) throughout the analysis, and at the end of the analysis to verify that the instrument remains under control.

The last two points are more difficult to apply in analytical chemistry, since the analyses as well as the samples handling can take a lot of time. In this case, an alternative could be the diligent use of internal standards and the evaluation of their recoveries.

Other QC samples may be introduced as well. Interference check standards often contain high concentrations of matrix components that are known to potentially interfere with the analyte(s) of interest. Sample (or matrix) spikes (i.e. samples spiked with analyte(s) of interest) may be introduced either before or after sample processing to verify that the results are not impacted by the sample matrix. The samples may also be analysed at 2 or more dilutions to verify that the results are not impacted by the matrix. Other QC samples are possible, depending on the potential concern. The analyst should be familiar with the analytical process and the parameters that could potentially impact the results, and introduce appropriate QC samples accordingly.

The effective QC protocol also includes monitoring of the process over time by charting the parameters of interest and concern. The control charts provide a graphical representation of historic data that can be helpful in identifying trends. Regular control chart evaluation is an effective means of *preventive action*, i.e. a proactive approach to preventing problems before they occur.

The QC protocol should also include regular participation in external proficiency testing (also known as performance evaluation) campaigns, as well as inter-laboratory comparison exercises. In a proficiency testing campaign, the laboratory is sent a relevant reference material standard as a 'blind', i.e. an unknown to the laboratory. The laboratory must process and analyse the material and report the final results as it would for any similar sample received. The campaign organizers then provide feedback to the laboratory on performance in terms of accuracy and precision. The inter-laboratory comparisons are similar, but the subject sample material is often an unknown. The objective of such an exercise is generally to characterize the subject material. As a result, the analytical proficiency cannot be unequivocally determined. Even so, the information obtained from such an exercise generally includes consensus values and ranges of data, which can be an indication of analytical performance.

6.3 ANALYTICAL QUALITY CONTROL CHARTS (AQCCS)

The reference materials should be used by laboratories to establish their precision and accuracy. These materials should be analysed periodically to provide a check on the quality of the analytical data. The simplest way to assess the results of these analyses is to examine them at the end of the analytical period, and to decide whether or not they are satisfactory, and thus whether or not the data generated for the samples is acceptable.

It is recommended for the results of the analyses of the reference materials to be plotted on a simple chart, which contains guidelines, to objectively assess their quality. This chart is

known as an “analytical quality control chart”. The figure 1 shows an example of a blank control chart.

Analyst should only use methods that have proved to provide good quality results. Assuming that such methods are used, an AQQC should be constructed along the following lines:

1. Select an appropriate reference material to be analysed along with the samples;
2. Analyse the chosen reference material at least 10 times. These analyses should not be carried out on the same day, but spread out over a period of time, in an attempt to ensure that the full range of random errors within and between batch analyses are covered.
3. Calculate the mean value (X), and the standard deviation (S) and then plot the following values on a blank control chart:

   X, **X+2S** (UWL**)** **X+3S** (UCL) **X-2S** (LWL) and **X-3S** (LCL).

   Where:

   UWL = upper warning limit

   UCL = upper control limit

   LWL = lower warning limit

   LCL = lower control limit

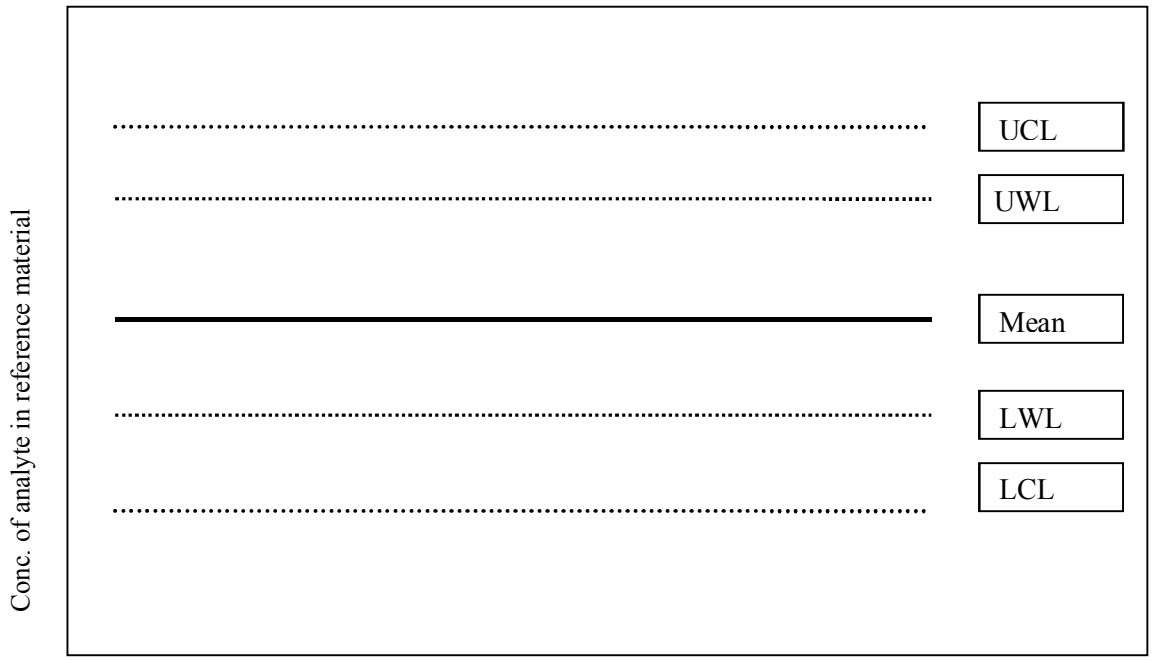

FIG. 1. Blank control chart

Assuming that the analytical measurements for the RM(s) follow a normal distribution, 95% of them should fall within the area between UWL and LWL. Similarly 99.7% of the results should fall within the area between UCL and LCL.

The analyst should plot the results for the analyses of the RM(s) after each batch of analyses to check whether the data lies within the limits.

The following guidelines can be used to assess whether the data for the RM(s), and consequently, the data for the samples are acceptable or not.

1. The mere fact that one result falls outside the warning limits doesn't mean that the analyses has to be rejected provided that the following results falls within the warning limits;
2. If the results fall outside the warning limits too frequently, particularly, if the same warning limit has been crossed more than once on consecutive results, then the analyst needs to assess the source of this systematic error;
3. If the results on more than 10 consecutive occasion fall on the same side of the X line (either between X and UWL or X and LWL), then the analyst needs to check the analytical procedure to determine the cause of this error;
4. If the results fall outside the UCL or LCL lines, the analyst should check the analytical procedure to determine the cause of this error.

If any of the above cases occur, the analyst should reject the results of the analyses for the particular batch of samples, and should not carry out any further analyses until the sources of the errors have been identified and eliminated.

Guidelines on the QA/QC requirements for analyses of sediments and marine organisms are detailed in the Reference Method No 57, "Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice".

7 BIBLIOGRAPHY

Reference Method No 57, UNEP/IOC/IAEA/FAO: Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice. UNEP, 1990.

ANNEX I

GC-FPD CHROMATOGRAMS

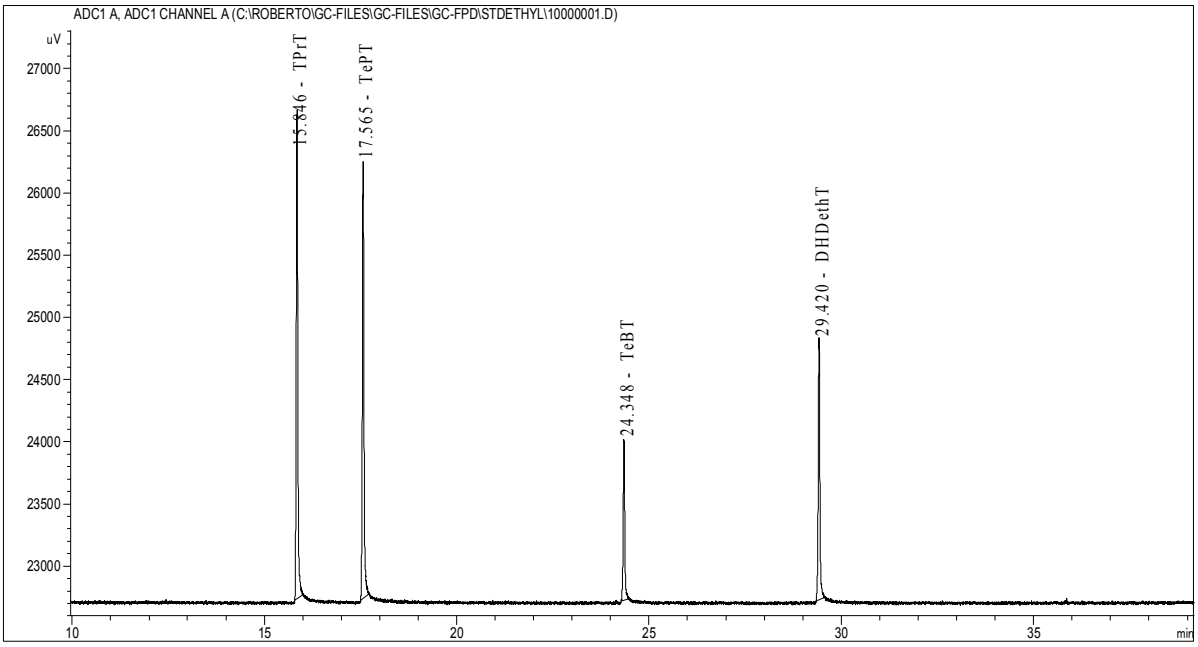

FIG. I.1. Chromatogram of an already ethylated Internal STDs (ca. 4 ng/ul)

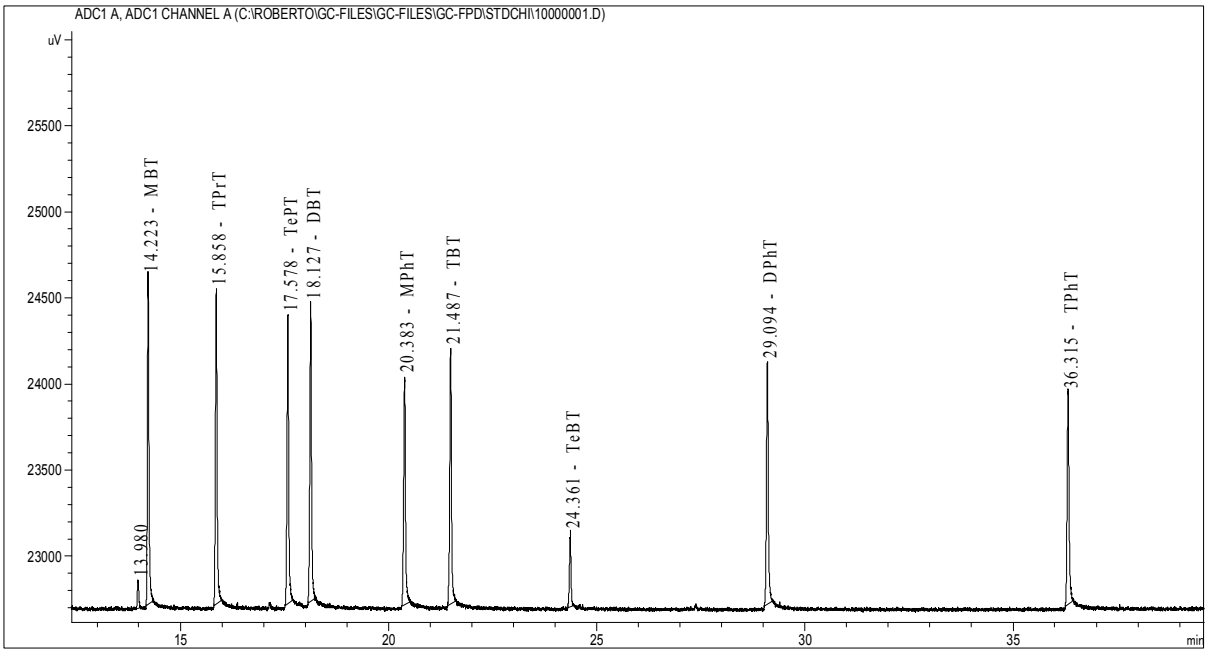

FIG. I.2. Chromatogram of Calibration STDs (ca. 2.8 ng/ul)

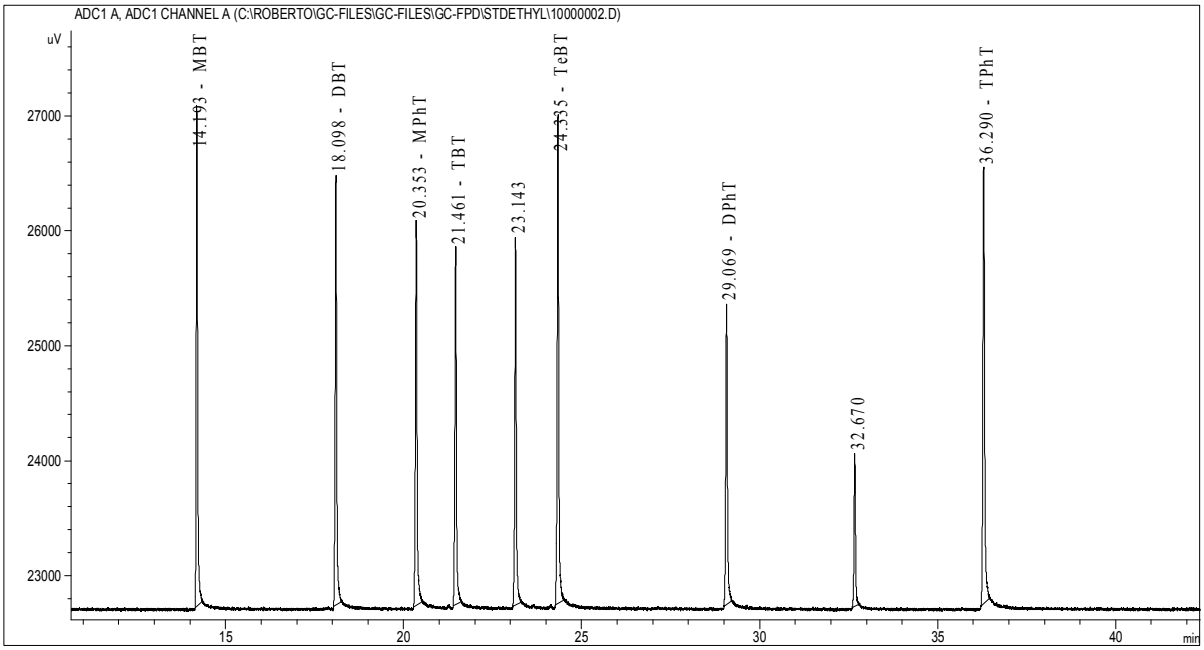

FIG. I.3. Chromatogram of an already ethylated Calibration STDs (ca. 4 ng/ul)

ANNEX II

GC-MS CHROMATOGRAMS

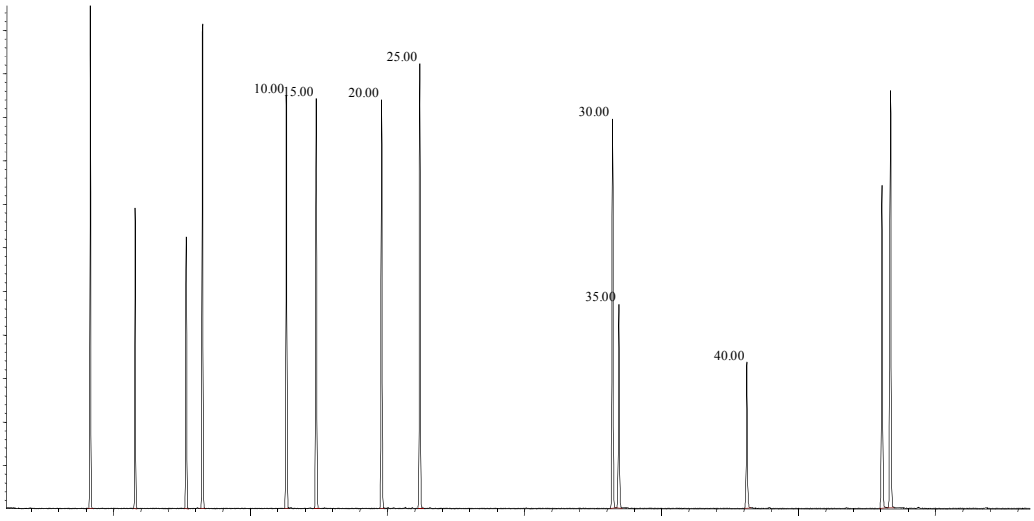

FIG. II.1. GC-MS chromatogram of already ethylated STDs by EI in total scan.

Monobutyltin (MBT), tripropyltin (TrPT), tetrapropyltin (TePrT), dibutyltin (DBT), monophenyltin (MPhT), tributyltin (TBT), monooctyltin (MOcT), tetrabutyltin (TeBT), diphenyltin (DPhT), diheptyltin (DHT), dioctyltin (DOcT), tricyclohexyltin (TCyHT), triphenyltin (TPhT).

- Column: DB-XLBMSD, 30m x 0.25 μm x 0.25 mm.;
- Oven program: 600°C for 1 min, then from 60 to 100 at 10°C min and from 100 to 280 at 4°C min;
- Inlet: Pulsed splitless at 270°C
- Carrier gas: helium at a flow rate of 1.6 ml/min.;
- Ion energy of the MS: 70 eV;
- Ion source temperature: 230°C.

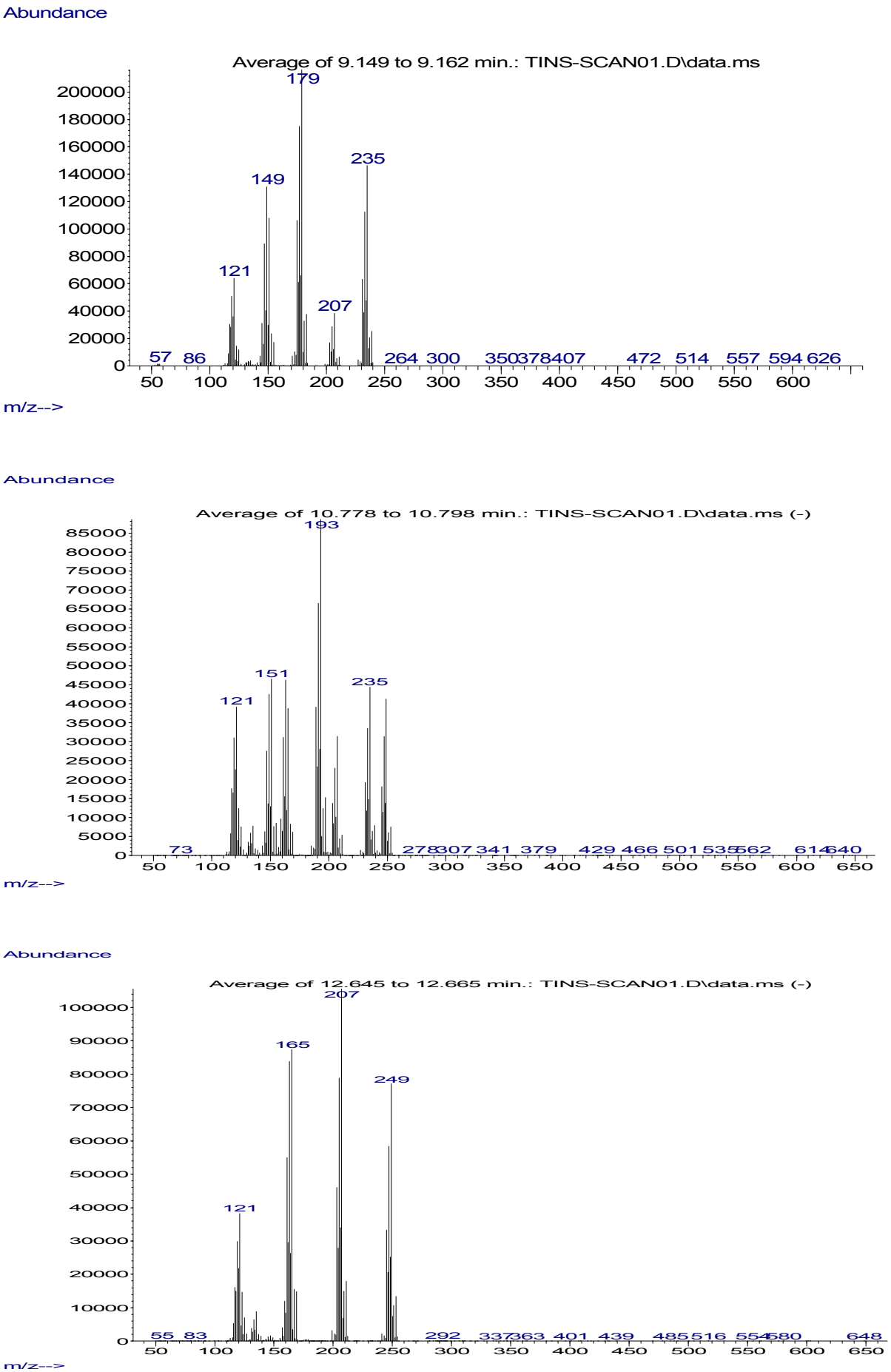

FIG. II.2. MS spectra of different Organotin species

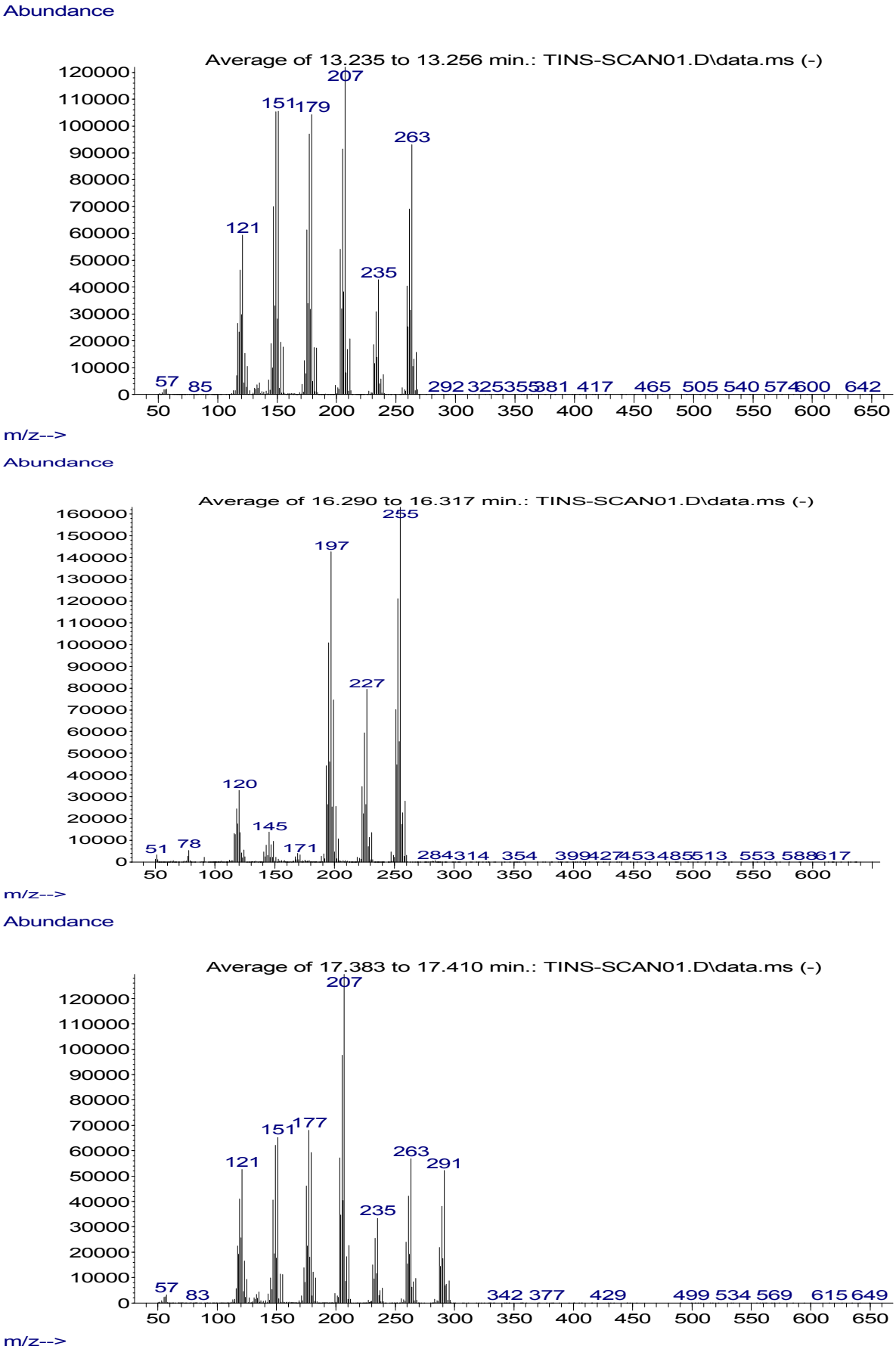

FIG. II.3. MS spectra of different Organotin species

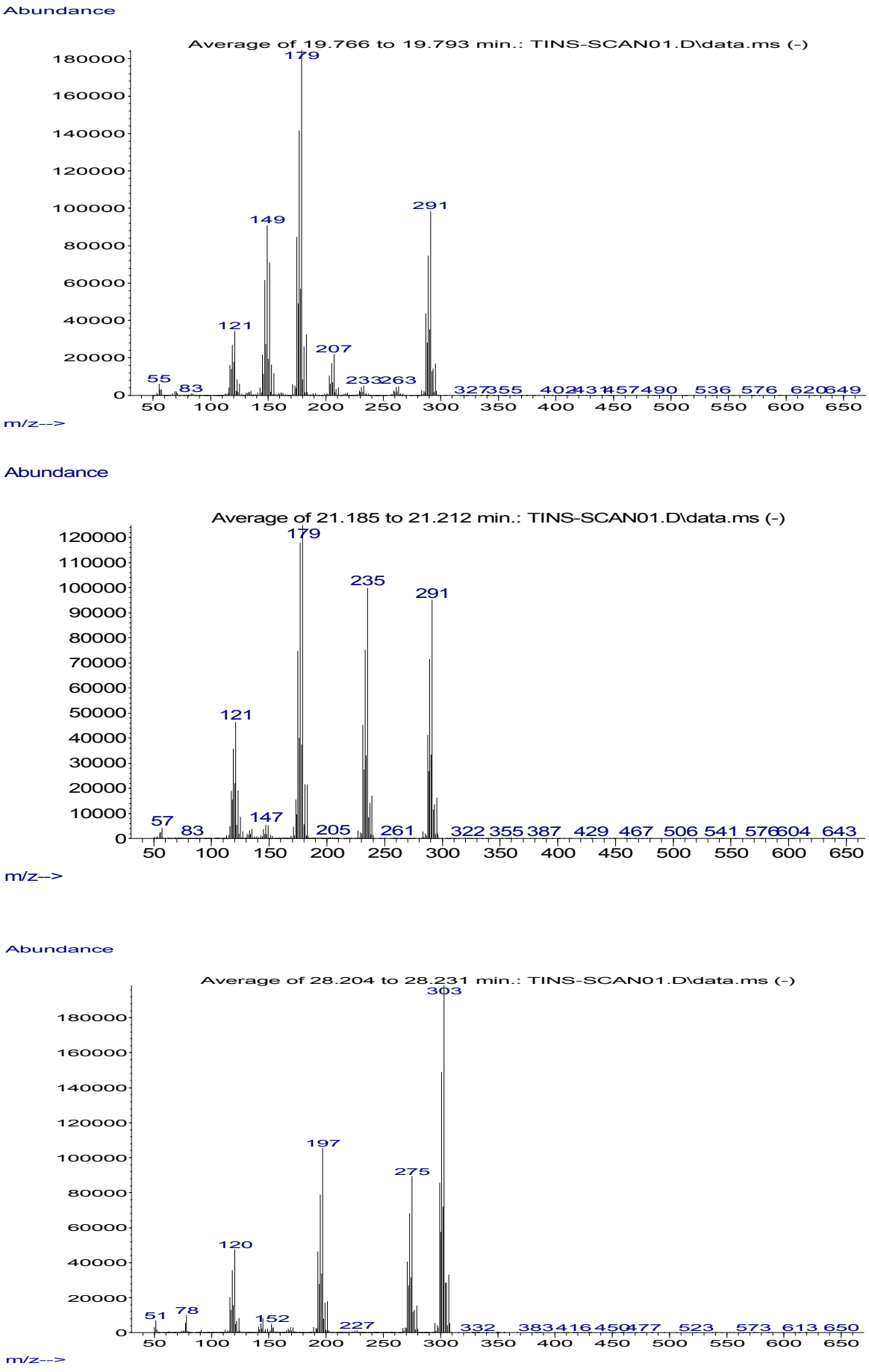

FIG. II.4. MS spectra of different Organotin species

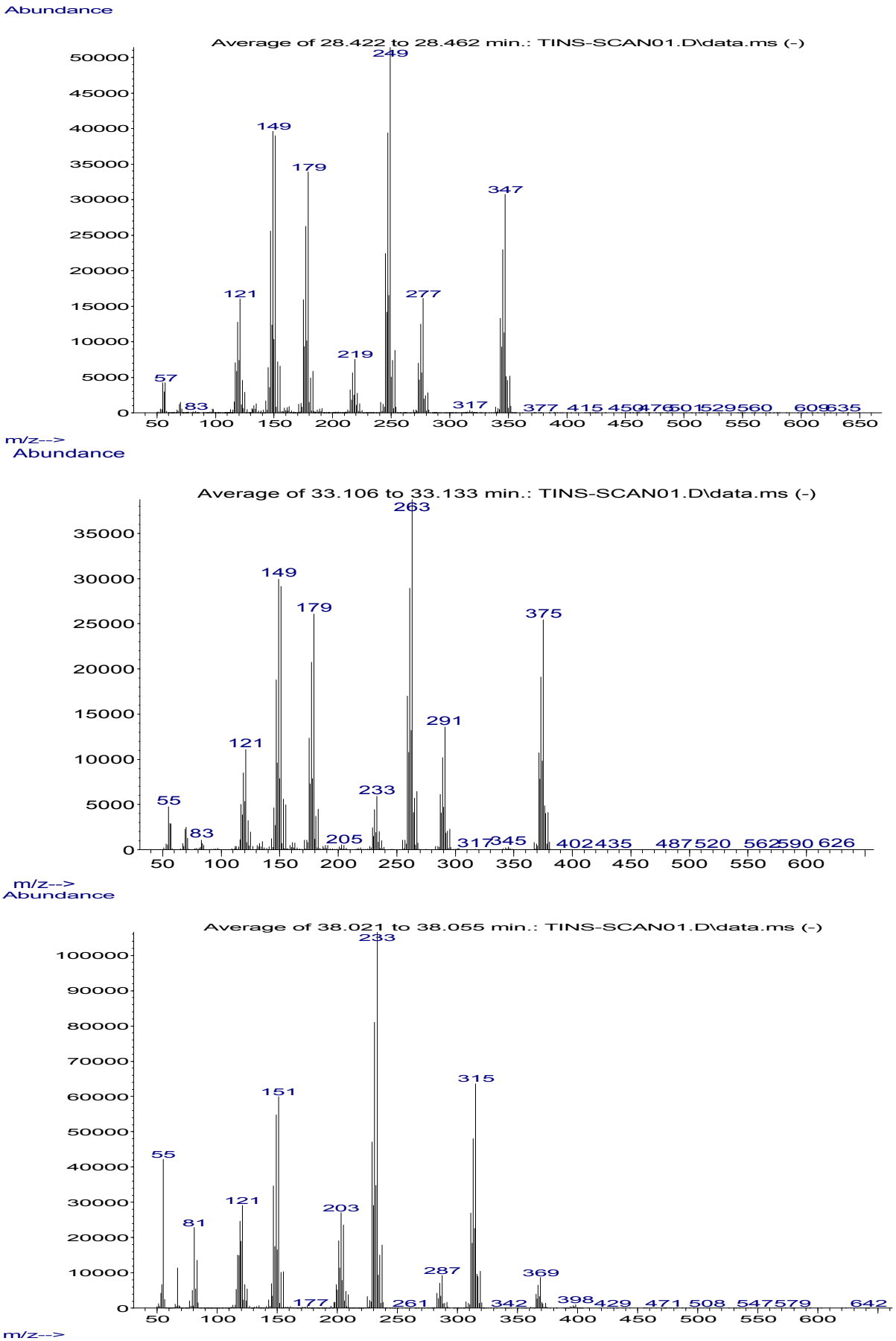

FIG. II.5. MS spectra of different Organotin species

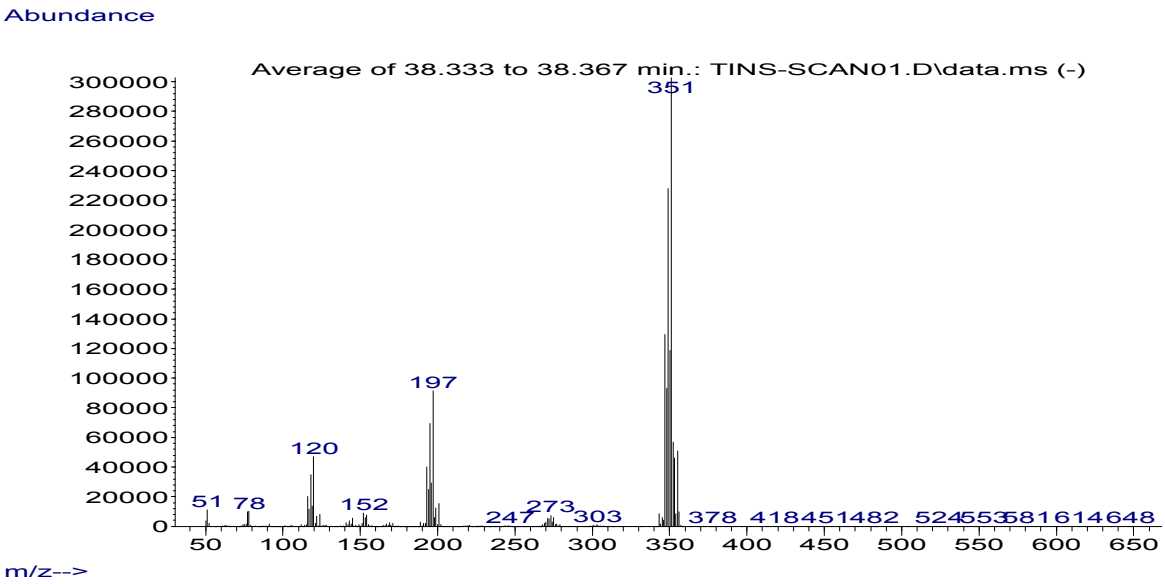

FIG. II.6. MS spectra of different Organotin species

REPORT

Recommended Method for the DETERMINATION OF ORGANOTIN COMPOUNDS IN SEDIMENT SAMPLES

IAEA/NAEL

Marine Environmental Studies Laboratory in co-operation with

UNEP/MAP MED POL

November 2012

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Recommended Method for the DETERMINATION OF ORGANOTIN COMPOUNDS IN SEDIMENT SAMPLES

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TABLE OF CONTENTS

NOTE: This method is not intended to be an analytical training manual. Therefore, the method is written with the assumption that it will be used by formally trained analytical chemists. Several stages of this procedure are potentially hazardous; users should be familiar with the necessary safety precautions.

In addition, the IAEA recommended methods are intended to be guidance methods that can be used by laboratories as a starting point for generating their own standard operating procedure. If performance data are included in the method they must not be used as absolute QC acceptance criteria.

1 SAMPLING

The detailed guidelines for collecting sediment samples are available in UNEP(DEC)/MEDWG.282/Inf.5/Rev.1.

2 GENERAL DISCUSSION

2.1 PRINCIPLE

Butyl- and phenyltin compounds are used worldwide mainly as antifouling agents and biocides. The large-scale application of these compounds to submerged marine structures, commercial vessels and pleasure crafts has resulted in dispersion to every marine environmental compartment. These compounds, especially tributyltin (TBT), exhibit broad spectrum of biocidal properties and so have elicited considerable research attention. Due to the deleterious effects on non-target organisms, the use of organotin compounds as antifouling agents on the boat hulls of small vessels has been widely prohibited.

Many analytical procedures based on chromatographic separation coupled to various detection techniques have been developed. Organotin compounds must be extracted from the matrix and derivatized into suitable forms for gas chromatographic analyses. The most common derivatization procedures include hydride generation in the aqueous phase using sodium borohydride, or extraction of organotin complexes with tropolone or diethyldithiocarbamate, followed by Grignard derivatization. The instability and volatility of butyltin hydrides can lead to losses of compounds and consequently to an underestimation of contamination. The Grignard derivatization requires scrupulous dry conditions and is rather time consuming.

To avoid such difficulties, an organotin derivatization procedure directly applied to the aqueous phase using sodium tetraethylborate ($NaBEt_4$) was developed, whereby the

derivatization and extraction of organotin compounds can be carried out simultaneously. This technique allows the determination of several butyl- and phenyltin compounds.

The method for the determination of organotin compounds presented here is based on the ethylation with sodium tetraethylborate. The simultaneous derivatization and extraction with sodium tetraethylborate converts butyltin and phenyltin ions into their corresponding volatile derivatives in the organic phase:

$$R_nSn^{(4-n)+} + (4-n)\ NaBEt_4 \longrightarrow R_nSnEt_{(4-n)} + (4-n)\ Na^+ + (4-n)\ BEt_3$$

This derivatization reaction is the most critical step of the protocol. For optimal derivatization efficiency, the solution of sodium tetraethylborate should be either freshly prepared, i.e. just prior to sample processing, or stored frozen (-20°C) for no longer than two weeks. The optimal conditions for the sodium tetraethylborate comprise a 30 min. reaction time at a pH between 5 and 6.

It should be appreciated that sodium tetraethylborate is extremely air-sensitive and must be handled with care to keep its chemical integrity.

2.2 ANALYTICAL STRATEGY

Many of the internal standards and the target compounds in the samples need to be derivatized to be detectable by GC techniques. Two different approaches are possible in order to overcome this problem.

- The first would be to purchase calibration standard solutions and internal standard solutions of ***ethylated organotin compounds***. These solutions contain tetra-substituted organotin species which are detectable by gas chromatography without further treatments. The calibration solutions contain certified concentrations of target compounds and can be injected directly into the GC (at previous suitable dilution). The ethylated internal standards are spiked in both the samples to be analysed and the calibration solutions. This approach eliminates any errors due to the performance of the derivatization reaction in the calibration standards. Unfortunately, the costs of ethylated standard solutions are fairly high and their use in routine analyses is not cost effective.
- The second approach available would be to use a ***matrix matched calibration curve***. In this instance, the calibration standards which are not derivatized are spiked into organotin-free environmental matrices (same matrix as the samples to be analysed). These “calibration samples” are then handled and analysed as if they were unknown samples, and thus, they undergo all the different steps of the analytical method including the derivatization step. This approach allows the normalization of some systematic errors and, more importantly, a matrix-matched calibration curve that will take into account the variability linked to the use of sodium tetraethylborate. In case of

a low efficiency of the derivatization reaction, for example, both the samples and the calibration curve will be affected, normalizing thus, the results.

2.3 DESCRIPTION AND USE OF ORGANOTIN CALIBRATION STANDARDS

The calibration standards are multi-compound solutions of *Mono*-, *D*i- and *Tributyltin*, *Mono*- , *Di*- and *Triphenyltin*. Ethyl-substituted compounds can be analysed directly, or the "chloride" forms of these compounds can be spiked in uncontaminated environmental samples (calibration samples), to create a matrix-matched calibration curve (see section 2.2).

The calibration curve must be made by at least 3 points (standard solutions at different concentrations). The concentration of the standard solutions must be calculated so that they bracket the concentrations of the samples and the Reference Materials (RMs).

If the concentrations of the target compounds in the samples are unknown, the calibration curve should be centred on the reference materials. If the concentrations of the target compounds exceed the limit of the calibration curve, either the samples must be brought to the appropriate dilution (extracting less material), or the calibration curve must be extended with a higher concentration standard. If, on the contrary, the concentration of the samples is lower than the lowest calibration curve's point, a new calibration curve must be prepared.

Example:

The reference material NIES-11 (fish tissue) contains 1.3 µg/g of TBT as chloride.

0.5 g of NIES 11 in a 0.5 mL solution (assuming 0.5 mL as the final dilution of the vial prior to GC injection) means a concentration of:

$$\frac{(0.5\ \text{g} \times 1.3\ \mu\text{g/g})}{0.5\ \text{mL}} = 1.3\ \mu\text{g/mL} = 1.3\ \text{ng/µl}$$

A calibration curve centred on this value would be:

0.5 1.25 2.5 ng/µl

Assuming an organotins mixture working solution concentration of 50 ng/µl and a final vial dilution of 1mL, the calibration curve can be created by spiking 10, 25 and 50 µl of organotins mixture into 3 different uncontaminated matrices.

2.4 DESCRIPTION AND USE OF ORGANOTIN INTERNAL STANDARDS

Internal standards are non-interfering compounds added to a sample with a known concentration, in order to eliminate the need for the measurement of the final sample dilution in quantitative analyses, and for correction of instrumental variation. The internal standards must be selected based on their characteristics, and thus on the information they have to provide. Internal standards can be added to the samples at the very beginning of the procedure, to check for the overall performance of the method, or at the end, just prior to GC

injection, to check mainly for GC performance. A combination of several internal standards with different properties allows a better understanding and control of an analytical protocol.

The method proposed here is based on the use of three internal standards:

- *Tripropyltin chloride* used as the main internal standard to indicate the derivatization reaction efficiency. All the chromatographic peak areas are normalized to those of Tripropyltin.
- *Tetrapropyltin* used as secondary internal standard to check the overall recovery of the protocol. It is already tetra-substituted and thus, it is not concerned by the derivatization reaction.
- *Tetrabutyltin* spiked in all samples prior to GC injection is used as a GC-internal standard to quantify recovery of both internal standards

3 APPARATUS AND REAGENTS

3.1 APPARATUS

- A coring device with liners and plunger or a grab sampler;
- Glass jars and aluminium foil, stainless steel knives, scoops, forceps, labels, marking pens, log book;
- Insulated plastic boxes for transporting samples, ice or dry ice;
- Deep freezer (-18 to -20°C) for sample preservation;
- Rotary evaporator;
- Glassware including boiling flasks, ground glass stoppers, beakers, Erlenmeyer flasks, separatory funnels, centrifuge tubes, weighing bottles, pipettes, tissue grinders;
- Drying oven (temperature range up to at least 450°C) for determining the sample's dry weight, baking of contaminant residues from glassware and reagents;
- Centrifuge and tubes;
- Freeze-dryer and porcelain mortar and pestle;
- Analytical balance with a precision of 0.1 mg and an electro-balance with a precision of at least 1 µg;
- Stainless steel tweezers and spatulas;
- Dessicator - completely cleaned and with no grease applied to sealing edges;
- Supply of clean dry nitrogen;
- Vacuum pump (water-jet air pump);

- Ultrasonic bath;
- Litmus papers;
- Solid Phase Extraction columns (Supelclean™ LC-Florisil® SPE Tubes 6 mL, 1 g);
- 12 or 24 ports Glass Vacuum manifold for simultaneous use of multiple SPE columns. The manifold must be fitted with flow control valves to allow for a fine adjustment of flow through the SPE column.

3.2 LIST OF REAGENTS

- Acetic acid, 96%;
- Potassium acetate / acetic acid buffer (CH_3COOH/ CH_3COO^-, K^+);
- 25% Ammonia solution;
- Organotin-free sea water;
- 1% $NaBEt_4$ in 2% KOH solution;
- Elemental mercury;
- Na_2SO_4, anhydrous granular;
- Florisil cartridges (Supelclean™ LC-Florisil® SPE Tubes 6 mL, 1 g);
- Isooctane, pesticide grade or equivalent purity;
- *n*-hexane, pesticide grade or equivalent purity;
- Nitrogen, 99.8% purity.

3.3 STANDARDS

- Internal standard: Tripropyltin choride (ca. 500 ng);
- Secondary Internal Standard: Tetrapropyltin (ca. 1000 ng);
- GC-Internal Standard: Tetrabutyltin (ca. 100 ng).

The GC vials containing Tetrapropyltin and Tetrabutyltin are added to the injection sequence just before GC injection. These standards are used to quantify recovery of Tetrapropyltin in samples.

The working solutions from the stock reference solutions are prepared on a regular basis, and stored in clean glass volumetric flasks tightly capped with non-contaminating materials such as Teflon or glass. Extreme care must be taken to ensure that standards have not changed their concentrations through solvent evaporation.

4 PROCEDURES

4.1 GLASSWARE CLEANING AND HANDLING

The glassware for the analyses of organotin compounds requires special precautions and specific equipment.

4.1.1 Reagents

- Detergent for glassware;
- *n*-Hexane, pesticide grade or equivalent purity;
- Acetone, pesticide grade or equivalent purity.

4.1.2 Procedure

The glassware must be cleaned with detergent, rinsed first with tap water, then with high purity acetone and finally with *n*-hexane. Any contact with plastics of any kind, as well as silicone and/or grease or any other material must be avoided in order to minimize the risk of contamination. Only Teflon, aluminium, glass or stainless steel (previously cleaned with acetone and *n*-hexane) should be in contact with the samples.

As an alternative, glassware can be pyrolitically cleaned using a muffle furnace at a temperature of 450°C for 4 hours. Even for low level determinations, it is still recommended to rinse the glassware with small amount of *n*-hexane.

NOTES:

The contamination of laboratory apparatus with organotin compounds arises from the use of diorganotin stabilizing compounds in many types of plastic, e.g. PVC, and the use of such materials should be avoided.

Organotins are readily adsorbed onto glassware and losses may occur through the use of glassware. We recommend using the minimum of glassware, and wherever possible rinsing glassware at least three times with appropriate solvent when transferring the sample solutions.

4.2 SODIUM SULFATE (Na_2SO_4) CLEANING

4.2.1 Principle

The sodium sulfate is pyrolitically cleaned using a muffle furnace at a temperature of 450°C for 4 hours.

4.2.2 Reagents

- Sodium sulfate, anhydrous granular.

4.2.3 Procedure

Transfer an adequate amount of sodium sulfate in a glass container (the glass must be resistant to high temperature).

Put the container in the muffle furnace at a temperature of 450°C for 4 hours.

4.3 ELEMENTAL MERCURY (Hg) CLEANING

4.3.1 Principle

Elemental mercury is used to remove elemental sulfur and sulfur compounds which can interfere with gas chromatograph separation.

4.3.2 Reagents

- Elemental Hg;
- Methanol, pesticide grade or equivalent purity.

4.3.3 Procedure

Mercury is sucked up though a capillary tube, such as a Pasteur pipette, connected to a guard-flask and then to a vacuum pump. Mercury is cleaned by going through the Pasteur pipette. The clean mercury is then collected and cleaned with methanol in the guard-flask. Elemental mercury is then transferred into a thick wall glass bottle with a ground glass stopper. The mercury is covered with a layer of *n*-hexane to protect it from oxidation.

4.4 SEA WATER CLEANING

4.4.1 Principle

Organotin-free sea water is used to achieve a better phase separation after the derivatization reaction.

4.4.2 Reagents

- Sea water;
- *n*-hexane, pesticide grade or equivalent purity.

4.4.3 Procedure

- Fill a 1 L separating funnel with sea water;
- Add 30 mL of *n*-hexane;
- Shake vigorously for 1-2 minutes;
- Discard the organic phase;
- Add another 30 ml of *n*-hexane;
- Shake vigorously for 1-2 minutes;
- Discard the organic phase;
- Store the sea water in a pre-cleaned glass bottle.

NOTE:

A solution of 25% NaCl in deionized water could be used instead of sea water and cleaned following the same procedure.

4.5 PREPARATION OF BUFFER SOLUTION (CH_3COOH/ CH_3COO, K^+)

4.5.1 Principle

A potassium acetate/acetic acid buffer solution is used to adjust the pH between 5 and 6 prior to derivatization reaction.

4.5.2 Reagents

- Acetic acid (96%, d=1.06);
- Potassium acetate (CH_3COOK);
- Milli-Q water (organotin-free deionized water).

4.5.3 Procedure

- Weigh 125.08 g (or 118 mL) of acetic acid;
- Weigh 192 g of potassium acetate;
- Dissolve the potassium acetate in Milli-Q water;
- Add the acetic acid and make 1L up with Milli-Q water;
- Store in a pre-cleaned glass bottle.

4.6 PREPARATION OF 2% KOH SOLUTION

4.6.1 Principle

A 2% KOH solution is used for the preparation of the sodium tetraethylborate ($NaBEt_4$) solution.

4.6.2 Reagents

- KOH;
- Milli-Q water.

4.6.3 Procedure

- Dissolve 2.3 g of KOH in Milli-Q water and make it 100 mL;
- Store the solution in a pre-cleaned Teflon bottle and keep in the freezer until incipient frost formation.

4.7 PREPARATION OF 1% $NaBEt_4$ SOLUTION IN 2% KOH

4.7.1 Principle

The method for the determination of organotin compounds presented here is based on the ethylation with sodium tetraethylborate.

It should be appreciated that sodium tetraethylborate is extremely air-sensitive and must be handled with care to keep its chemical integrity.

4.7.2 Reagents

- 2% KOH solution, 100 mL;
- $NaBEt_4$, 1 g sealed bottle;
- Argon or nitrogen gas.

4.7.3 Procedure

- Operate when possible in a glove box, or at least in a fume-hood using a stream of argon or nitrogen to minimize contact between $NaBEt_4$ and the air;
- Carefully open the $NaBEt_4$ bottle and fill it with KOH solution;
- Close the bottle and shake manually;
- Pour the content into the KOH solution bottle;
- Rinse the $NaBEt_4$ bottle with KOH;
- Prepare the Teflon bottles of appropriate volume and fill them with argon or nitrogen;
- Fill up the Teflon bottles with the $NaBEt_4$ solution;
- Fill the Teflon bottles with argon or nitrogen;
- Put the Teflon bottle in single zip-lock bags filled up with argon or nitrogen;
- Store at -20°C.

4.8 PREPARATION OF STANDARD SOLUTION

4.8.1 Principle

The individual stock solutions of organotin compounds are prepared by dissolving approximately 60-80 mg of the original standard compound in 100 mL *n*-hexane (for tri- and tetra- substituted organotin species), and in acetone (for mono- and di-substituted organotin species).

4.8.2 Reagents

- Organotin standards;
- *n*-hexane, pesticide grade or equivalent purity;
- Acetone, pesticide grade or equivalent purity;

- Isooctane, pesticide grade or equivalent purity.

4.8.3 Procedure

- Using a pre-cleaned glass weighing boat, weigh 60-80 mg of the standard;
- Transfer into a 100 mL glass volumetric flask;
- Rinse the weighing boat with the appropriate solvent into the volumetric flask;
- Bring to volume with the appropriate amount of solvent;
- Store at -20°C.

NOTE:

Individual working solutions of Internal and GC-Internal Standards are prepared in the appropriate solvent:

- Acetone for Internal Standards of tripropyltin (TPrT) and tetrapropyltin (TePrT);

- Acetone for the multi-compound mixture used for the calibration curve (organotins mixture, OTs mix);

- Isooctane for GC-Internal Standards, tetrabutyltin (TeBT) and tetrapropyltin (TePrT).

The concentration of working solutions usually ranges between 10 and 40 ng/µl which correspond to a 25-50 times dilution of the stock solution.

5 ANALYTICAL PROCEDURE ON THE DETERMINATION OF ORGANOTIN COMPOUNDS IN SEDIMENT SAMPLES

About 3 to 5 grams of dried sediment samples are leached by shaking with acetic acid, centrifuged and filtered. Using buffer solution and ammonia, the pH is stabilized between 5 and 6. The organotin compounds are simultaneously derivatized and extracted using sodium tetraethylborate ($NaBEt_4$) and *n*-hexane. Elemental Hg is used to eliminate sulfur, and Florisil cartridges are used to clean up the samples. The purified samples are concentrated to about 0.5 mL (prior to solvent change from *n*-hexane to isooctane) and analysed by GC.

1- Internal standards spiking:

- Spike appropriate amounts of TrPrT and TePrT in each samples.

2- Leaching:

- Dried sediment samples are weighed into 50 mL screw cap Teflon tubes;
- Add 10 mL of acetic acid to each Teflon tube;
- Mechanically shake the Teflon tubes for 30 minutes;

- Centrifuge the Teflon tubes at 4000 rpm for 5 minutes.

3- Filtration:

- The supernatant is decanted into a 50 mL Teflon tube through 9 cm Whatman filter paper;
- Rinse the filter paper with uncontaminated sea water (2x5 mL).

4- pH Adjustment:

- Add 10 mL buffer solution to each Teflon tube;
- Add 4 mL of ammonia solution to each Teflon tube;
- the pH must be between 5 and 6.

5- Derivatization/Extraction:

- Add 1 mL of $NaBEt_4$ solution to each Teflon tube;
- Immediately add 5 mL of *n*-hexane to each Teflon tube;
- Shake Teflon tubes manually and mechanically for 30 minutes;
- Centrifuge the Teflon tubes at 4000 rpm for 5 minutes;
- Recover organic phase in 15 mL glass tube;
- Add 5 mL of *n*-hexane to the water phase and repeat the extraction;
- Shake manually vigorously and mechanically for 10 minutes;
- Centrifuge the Teflon tubes at 4000 rpm for 5 minutes;
- Recover organic phase and combine;
- Add ca. 1-2 g of pre-cleaned Na_2SO_4 to each glass tube to dry organic phase;
- Recover and transfer the organic phase in a new 15 mL glass tube.

6- Evaporation:

- The extract is reduced to ~5 mL under a gentle stream of N_2.

7- Sulfur Removal:

- Add a small quantity of elemental Hg in each glass tube;
- Shake the glass tubes manually;
- Recover and transfer the organic phase in a new 15 mL glass tube;
- Add more Hg. Repeat the shaking and recovering until the Hg stops reacting with the sulfur (dark color).

8- Evaporation:

- The extract is reduced to ~1 mL under a gentle stream of N_2.

9- S.P.E. Clean Up:

- The concentrated extract is purified using Florisil cartridges;
- The sample and solvent rinses are added to the top of Florisil cartridge and elute with 5 mL of *n*-hexane twice (2x5 mL). The eluent is collected in a 15 mL glass tube.

10- Evaporation:

- The eluent is reduced to ~0.5 mL under a gentle stream of N_2.

11- Transfer into Vials/Solvent Exchange:

- Transfer the concentrated eluents into auto-injector vials. Rinse the glass tube with isooctane and add to the vial.

12- Evaporation:

- Reduce to appropriate volume under a gentle stream of N_2.

13- Standard Spiking:

- Add GC-Internal Standard (TeBT) to each sample vial;
- Prepare vials of GC-Internal Standard.

14- GC Analyses**:**

- Inject 1 uL of sample to GC-FPD and/or GC-MS.

6 GAS CHROMATOGRAPHY CONDITIONS

6.1 GC-FPD CONDITIONS FOR THE QUANTIFICATION OF ORGANOTIN COMPOUNDS

- Column: 30 × 0.25 mm × 0.25 μm HP-5 capillary column (5% phenyl methyl silicone) or equivalent (Supelco);
- Inlet: 250°C, splitless;
- Detector: FPD at 270°C, 610nm Sn filter. Hydrogen rich flame (air flow: 90 ml/min.; hydrogen flow: 130 ml/min.);
- Carrier gas: Helium, at a flow rate of 1 mL min^{-1};
- Oven program: 60°C for 2 min, then 60-270°C at 6°C min^{-1} and 270°C for 20 min.

6.2 GC-MS-SIM CONDITIONS FOR THE QUANTIFICATION OF ORGANOTIN COMPOUNDS

- Column: 30 × 0.32 mm × 0.25 μm HP-5 capillary column (5% phenyl methyl silicone);

- Detector: Electron Impact-MS in SIM mode. Details of the acquisition are provided in Table 1;
- Ion energy of the MS: 70 eV;
- Ion source temperature: 240°C;
- Interface: 280°C;
- Inlet: 250°C, splitless;
- Carrier gas: Helium, at a flow rate of 2 mL min^{-1};
- Oven program: 60°C for 1 min, then from 60 to 100°C at 10°C min^{-1} and from 100°C to 280 at °C at 4°C min^{-1}.

TABLE 1. TARGET AND CONFIRMATION IONS FOR GC/MS ANALYSES OF ORGANOTIN COMPOUNDS

Group	Time (min)	Compounds (m/z monitored)[a]	Cycles (s^{-1})	Ions (m/z)
1	6–10	MBT (121, 179, <u>235</u>) TrPT (121, 193, 235, <u>249</u>)	3	179, 235, 121, 193, 249
2	10–16.5	TePrT (121,165,249, <u>207</u>) DBT (121, 207, 235, <u>263</u>) MPhT (197, <u>255</u>) TBT (204, 235, 263, <u>291</u>)	2.18	263, 207, 291, 235, 121, 255, 197
3	16.5–20	TEBT (179, 235, <u>291</u>)	4.83	291, 179, 235
4	20–26	DPhT (120, 197, 275, <u>303</u>)	3.70	303, 275, 197, 120
5	26–40	TPhT (120, 197, 349, <u>351</u>)	3.7	351, 197, 120, 349

[a] The m/z used in quantification is underlined.

7 RESULTS QUANTIFICATION

This method is based on the use of an ***internal standard,*** which is defined as a non-interfering compound, added to a sample with known concentrations in order to eliminate the need to measure the final extract volume in quantitative analysis and for correction of instrumental variation.

In this method, the internal standard is added to each sample at the beginning of the analytical protocol. The ratio of the areas of the internal standard and analyte are then used to construct the calibration curve.

In a multiple point internal calibration each analyses contains the internal standard whose total amount is kept constant, and the analyte of interest whose amount covers the range of concentrations expected.

A multiple points relative response factor (RRF) calibration curve is established, for analytes of interest in each working batch. A RRF is determined, for each analyte, at each calibration level using the following equation:

$$RRF(X) = \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{\text{Qty (IS)}}{\text{Qty (X)}}$$

Where:

Area (X) = the area of the analyte to be measured (target compound);

Area (IS) = the area of the specific internal standard;

Qty (X) = the known quantity of the analyte in the calibration solution;

Qty (IS) = the known quantity of the internal standard in the calibration solution.

The relative response factors determined for each calibration level are averaged to produce a mean relative response factor (mRRF) for each analyte. The per cent relative standard deviation (%RSD) for the 3 response factor must be less than or equal to 15%, for each analyte.

$$\%RSD = \frac{\text{Standard deviation of the RRFs}}{\text{Average of the RFs}} \text{ x } 100$$

The sample analyte concentrations are calculated based on the quantity and response of the internal standard.

The following equation gives the amount of analyte in the solution analyzed.

$$Qty\,(\text{X}) = \text{Qty (IS)} \text{ x } \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{1}{\text{mRR}F(X)}$$

Where:

Qty (X) = the unknown quantity of the analyte in the sample;

Qty (IS) = the known quantity of the internal standard added to the sample;

Area (X) = the area of the analyte;

Area (IS) = the area of the internal standard

mRRF (X) = the average response factor of the analyte.

The sample analyte concentrations are then calculated by dividing the amount found (Qty) by the grams of the samples extracted.

7.1 REPORTING CONCENTRATIONS

The organotin compounds are purchased “as chloride”. The standard solution prepared and the results obtained after the calculation are expressed “as chloride”. The certified values of the reference materials and its literature usually report organotin concentration “as Sn”. The conversion between the two forms is given by the ratio between the atomic mass of Sn and the molecular mass of the chloride form.

Example:

For MBT = Sn $(C_4H_9)Cl_3$

Atomic masses: Sn = 118.71; C = 12; H = 1; Cl = 35.45.

Molecular mass of Sn $(C_4H_9)Cl_3$ = 282.06

The ratio between atomic mass of Sn and molecular mass of MBT gives the conversion factor: Sn/chloride = 0.421

10 ng/g MBT as chloride correspond to 10 x 0.421 = 4.21 ng/g as Sn

7.2 QUALITY ASSURANCE/QUALITY CONTROL

The goal in analytical chemistry is to obtain accurate compositional information about an unknown sample. The analyst introduces a processed sample into an instrument that will measure the parameter(s) of interest, and a result is obtained. But how accurate is the result? There are a myriad of factors - including contamination, loss of analyte, analyst error, instrument problems, to name only a few - that could occur at any time throughout the analytical process and potentially impact the accuracy of the final result. Sometimes, the impact of one or more of these factors is evident; often it is not.

Quality assurance (QA) is a comprehensive system of activities to ensure that the quality of the data produced meets pre-defined standards, within a stated level of confidence. A laboratory must design its QA program to meet its specific needs, so that the data produced is acceptable for its intended use; i.e. 'fit for purpose.' A good laboratory QA program will include both technical and administrative components to achieve this objective. Required component systems of a QA program generally include:

- Staff training;
- Procedures and procedure validation;
- Records and document control;
- Procurement and verification of item quality before use;
- Control of items (e.g. sample receipt, chains of custody, etc.);
- Periodic assessment of the QA program and its components;
- Corrective action management;
- Instrument maintenance and calibration.

Having a fully implemented QA program provides the necessary ingredients for producing a reliable and defensible product. Due to the fact that many of the factors that could impact data quality are transient, the analytical process must be tested and evaluated throughout the process, to verify that the results produced are accurate and reproducible, and have not been impacted. The Quality Control (QC) is a system of activities to ensure that the analytical process is controlled, and verifies that quality parameters are within required limits. QC is often separated into method (or process) QC and analytical (or instrument) QC. Method QC is

generally introduced at the beginning of the analytical process, to ensure that all the steps comprising the process (e.g. digestion, separation, concentration, etc., and including analysis) have not impacted the final result. Method QC may include the following QC samples:

- Method blank to monitor the contamination resulting from the process;
- Laboratory control sample (e.g. representative reference material) to monitor accuracy;
- Laboratory control sample duplicate to monitor method reproducibility.

The analytical QC ensures that the instrument is controlled throughout the analysis, and is usually comprised of the following QC samples:

- Reagent blank to monitor the contamination resulting from the reagents and/or instrument;
- Independent calibration verification (ICV) to verify that the calibration standard is acceptable;
- Continuing calibration verification (CCV) throughout the analysis, and at the end of the analysis to verify that the instrument remains under control.

The last two points are more difficult to apply in analytical chemistry, since the analyses as well as the samples handling can take a lot of time. In this case, an alternative could be the diligent use of internal standards and the evaluation of their recoveries.

Other QC samples may be introduced as well. Interference check standards often contain high concentrations of matrix components that are known to potentially interfere with the analyte(s) of interest. Sample (or matrix) spikes (i.e. samples spiked with analyte(s) of interest) may be introduced either before or after sample processing to verify that the results are not impacted by the sample matrix. The samples may also be analysed at 2 or more dilutions to verify that the results are not impacted by the matrix. Other QC samples are possible, depending on the potential concern. The analyst should be familiar with the analytical process and the parameters that could potentially impact the results, and introduce appropriate QC samples accordingly.

The effective QC protocol also includes monitoring of the process over time by charting the parameters of interest and concern. The control charts provide a graphical representation of historic data that can be helpful in identifying trends. Regular control chart evaluation is an effective means of *preventive action*, i.e. a proactive approach to preventing problems before they occur.

The QC protocol should also include regular participation in external proficiency testing (also known as performance evaluation) campaigns, as well as inter-laboratory comparison exercises. In a proficiency testing campaign, the laboratory is sent a relevant reference material standard as a ‘blind’, i.e. an unknown to the laboratory. The laboratory must process

and analyse the material and report the final results as it would for any similar sample received. The campaign organizers then provide feedback to the laboratory on performance in terms of accuracy and precision. The inter-laboratory comparisons are similar, but the subject sample material is often an unknown. The objective of such an exercise is generally to characterize the subject material. As a result, the analytical proficiency cannot be unequivocally determined. Even so, the information obtained from such an exercise generally includes consensus values and ranges of data, which can be an indication of analytical performance.

7.3 ANALYTICAL QUALITY CONTROL CHARTS (AQCCS)

The reference materials should be used by laboratories to establish their precision and accuracy. These materials should be analysed periodically to provide a check on the quality of the analytical data. The simplest way to assess the results of these analyses is to examine them at the end of the analytical period, and to decide whether or not they are satisfactory, and thus whether or not the data generated for the samples is acceptable.

It is recommended for the results of the analyses of the reference materials to be plotted on a simple chart, which contains guidelines, to objectively assess their quality. This chart is known as an "analytical quality control chart". The figure 1 shows an example of a blank control chart.

Analyst should only use methods that have proved to provide good quality results. Assuming that such methods are used, an AQQC should be constructed along the following lines:

1. Select an appropriate reference material to be analysed along with the samples;
2. Analyse the chosen reference material at least 10 times. These analyses should not be carried out on the same day, but spread out over a period of time, in an attempt to ensure that the full range of random errors within and between batch analyses are covered.
3. Calculate the mean value (X), and the standard deviation (S) and then plot the following values on a blank control chart:

   X, **X+2S** (UWL**)** **X+3S** (UCL) **X-2S** (LWL) and **X-3S** (LCL).

   Where:

   UWL = upper warning limit

   UCL = upper control limit

   LWL = lower warning limit

   LCL = lower control limit

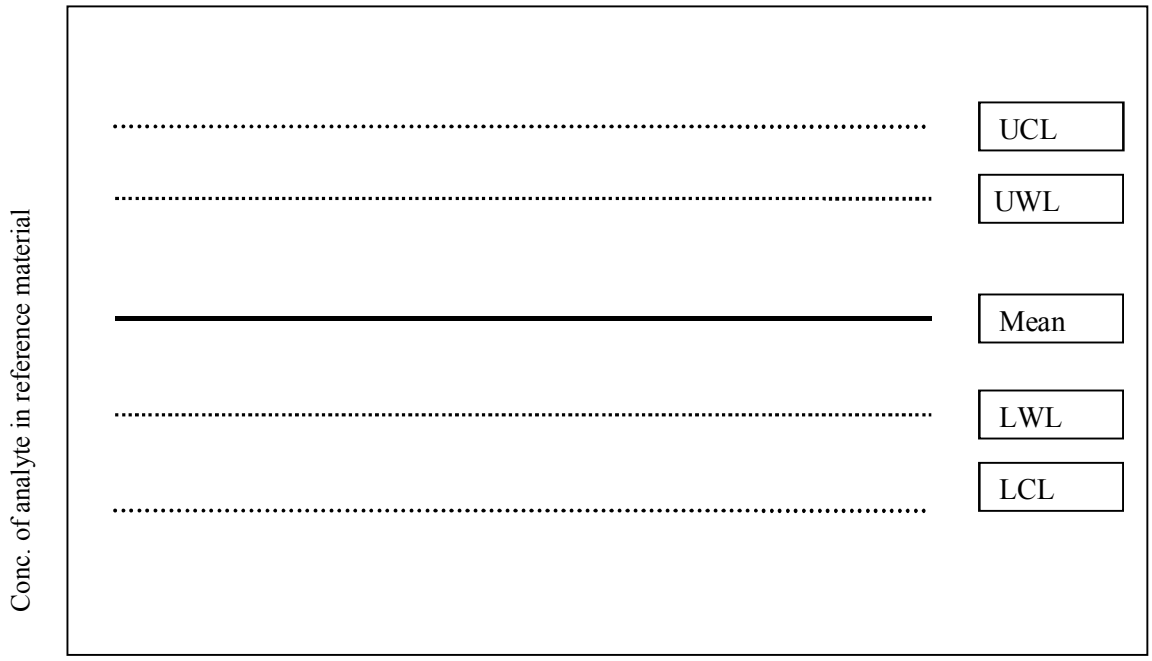

FIG. 1. Blank control chart

Assuming that the analytical measurements for the RM(s) follow a normal distribution, 95% of them should fall within the area between UWL and LWL. Similarly 99.7% of the results should fall within the area between UCL and LCL.

The analyst should plot the results for the analyses of the RM(s) after each batch of analyses to check whether the data lies within the limits.

The following guidelines can be used to assess whether the data for the RM(s), and consequently, the data for the samples are acceptable or not.

1. The mere fact that one result falls outside the warning limits doesn't mean that the analyses has to be rejected provided that the following results falls within the warning limits;
2. If the results fall outside the warning limits too frequently, particularly, if the same warning limit has been crossed more than once on consecutive results, then the analyst needs to assess the source of this systematic error;
3. If the results on more than 10 consecutive occasion fall on the same side of the X line (either between X and UWL or X and LWL), then the analyst needs to check the analytical procedure to determine the cause of this error;
4. If the results fall outside the UCL or LCL lines, the analyst should check the analytical procedure to determine the cause of this error.

If any of the above cases occur, the analyst should reject the results of the analyses for the particular batch of samples, and should not carry out any further analyses until the sources of the errors have been identified and eliminated.

Guidelines on the QA/QC requirements for analyses of sediments and marine organisms are detailed in the Reference Method No 57, "Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice".

8 BIBLIOGRAPHY

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Reference Method No 57, UNEP/IOC/IAEA/FAO: Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice. UNEP, 1990.

ANNEX I

GC-FPD CHROMATOGRAMS

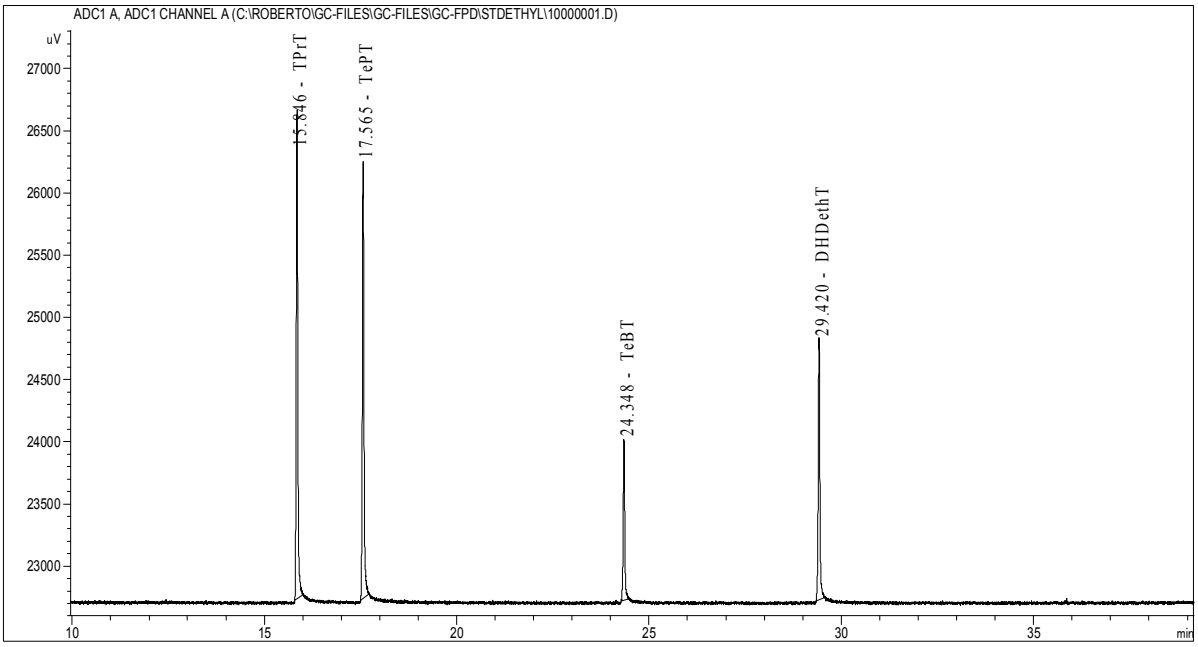

FIG. I.1. Chromatogram of an already ethylated Internal STDs (ca. 4 ng/ul)

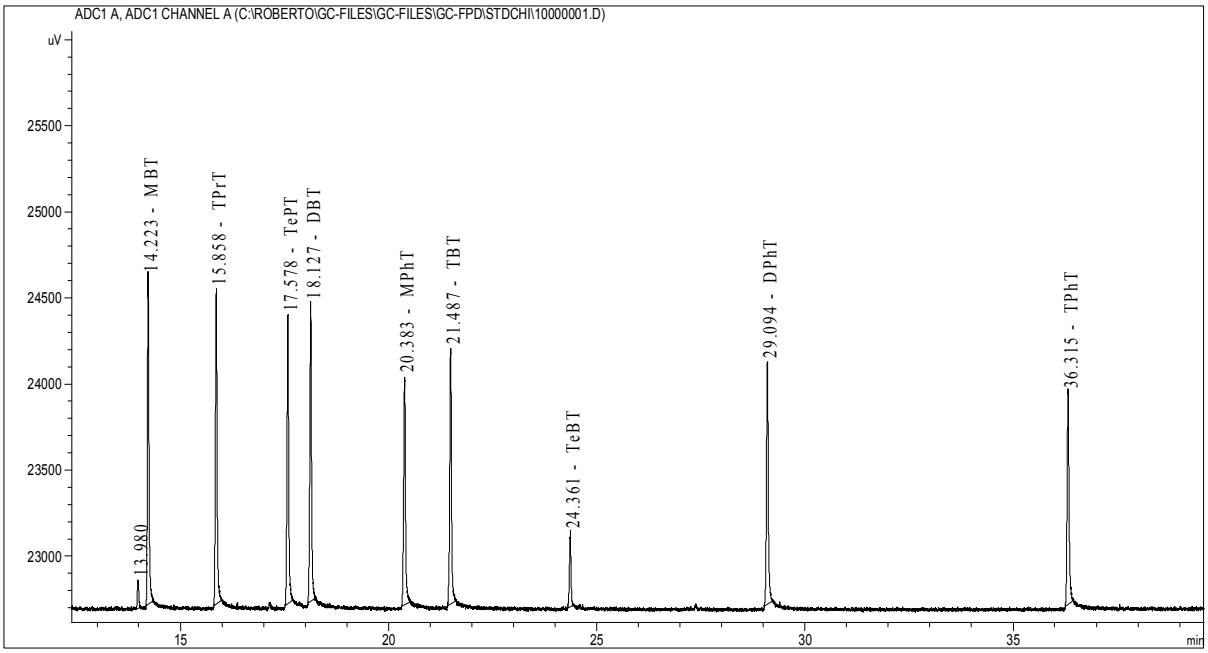

FIG. I.2. Chromatogram of Calibration STDs (ca. 2.8 ng/ul)

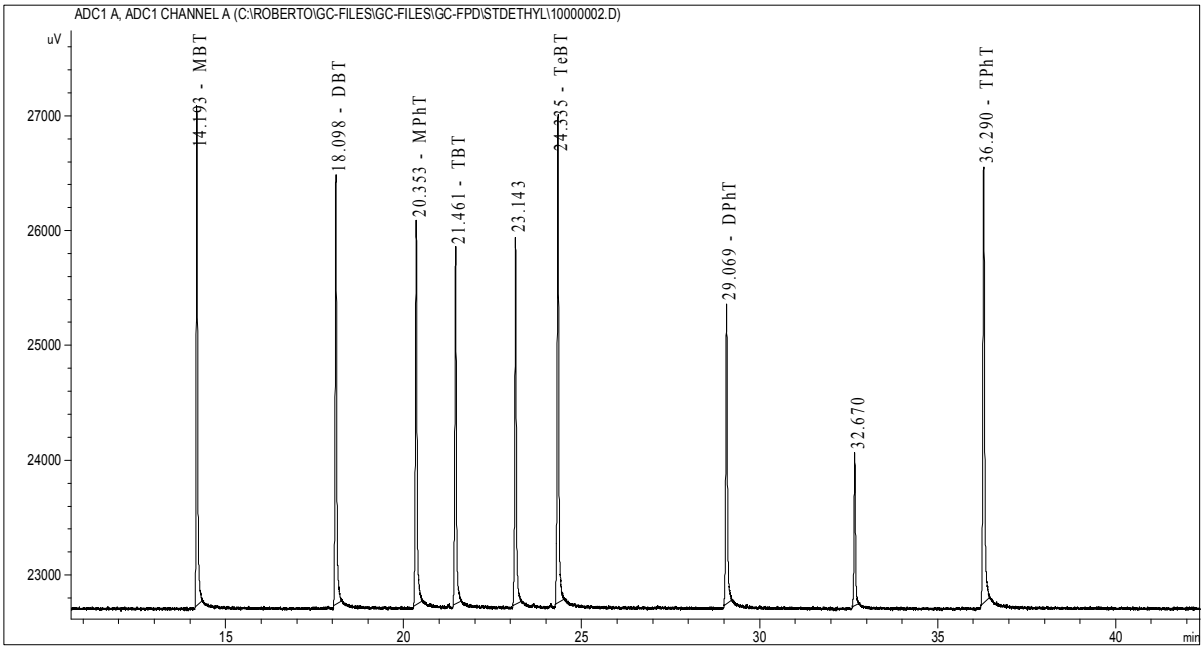

FIG. I.3. Chromatogram of an already ethylated Calibration STDs (ca. 4 ng/ul)

ANNEX II

GC-MS CHROMATOGRAMS

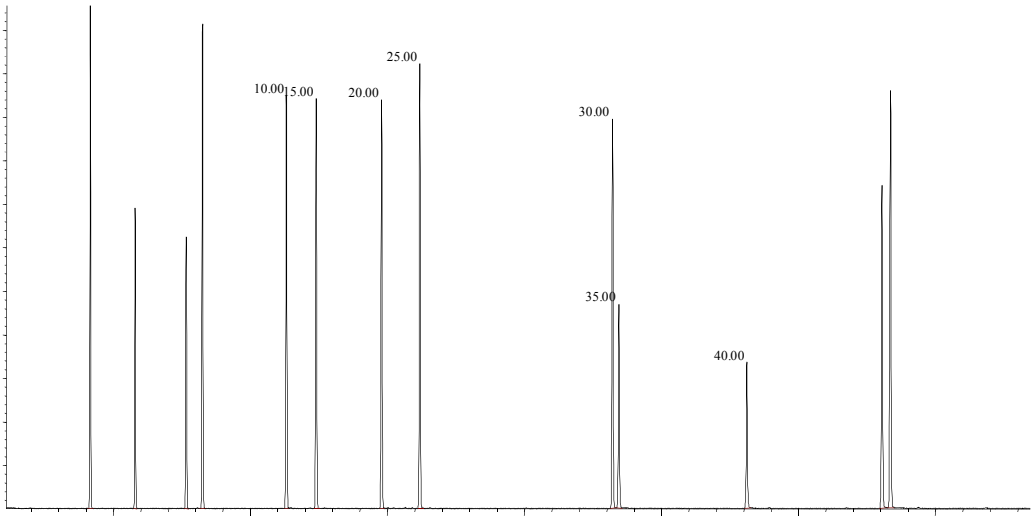

FIG. II.1. GC-MS chromatogram of already ethylated STDs by EI in total scan.

Monobutyltin (MBT), tripropyltin (TrPT), tetrapropyltin (TePrT), dibutyltin (DBT), monophenyltin (MPhT), tributyltin (TBT), monooctyltin (MOcT), tetrabutyltin (TeBT), diphenyltin (DPhT), diheptyltin (DHT), dioctyltin (DOcT), tricyclohexyltin (TCyHT), triphenyltin (TPhT).

- Column: DB-XLBMSD, 30m x 0.25 μm x 0.25 mm.;
- Oven program: 600°C for 1 min, then from 60 to 100 at 10°C min and from 100 to 280 at 4°C min;
- Inlet: Pulsed splitless at 270°C
- Carrier gas: helium at a flow rate of 1.6 ml/min.;
- Ion energy of the MS: 70 eV;
- Ion source temperature: 230°C.

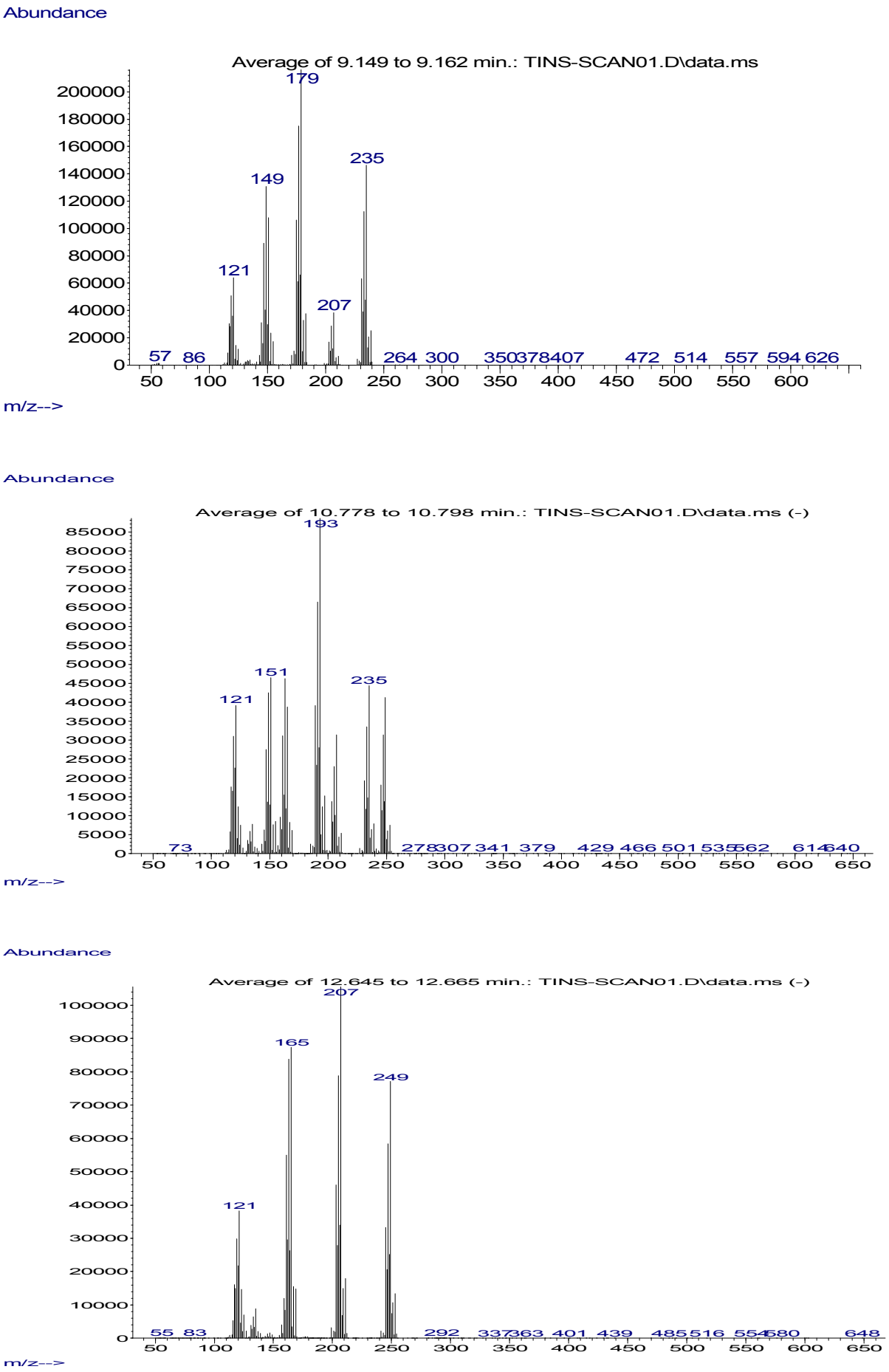

FIG. II.2. MS spectra of different Organotin species

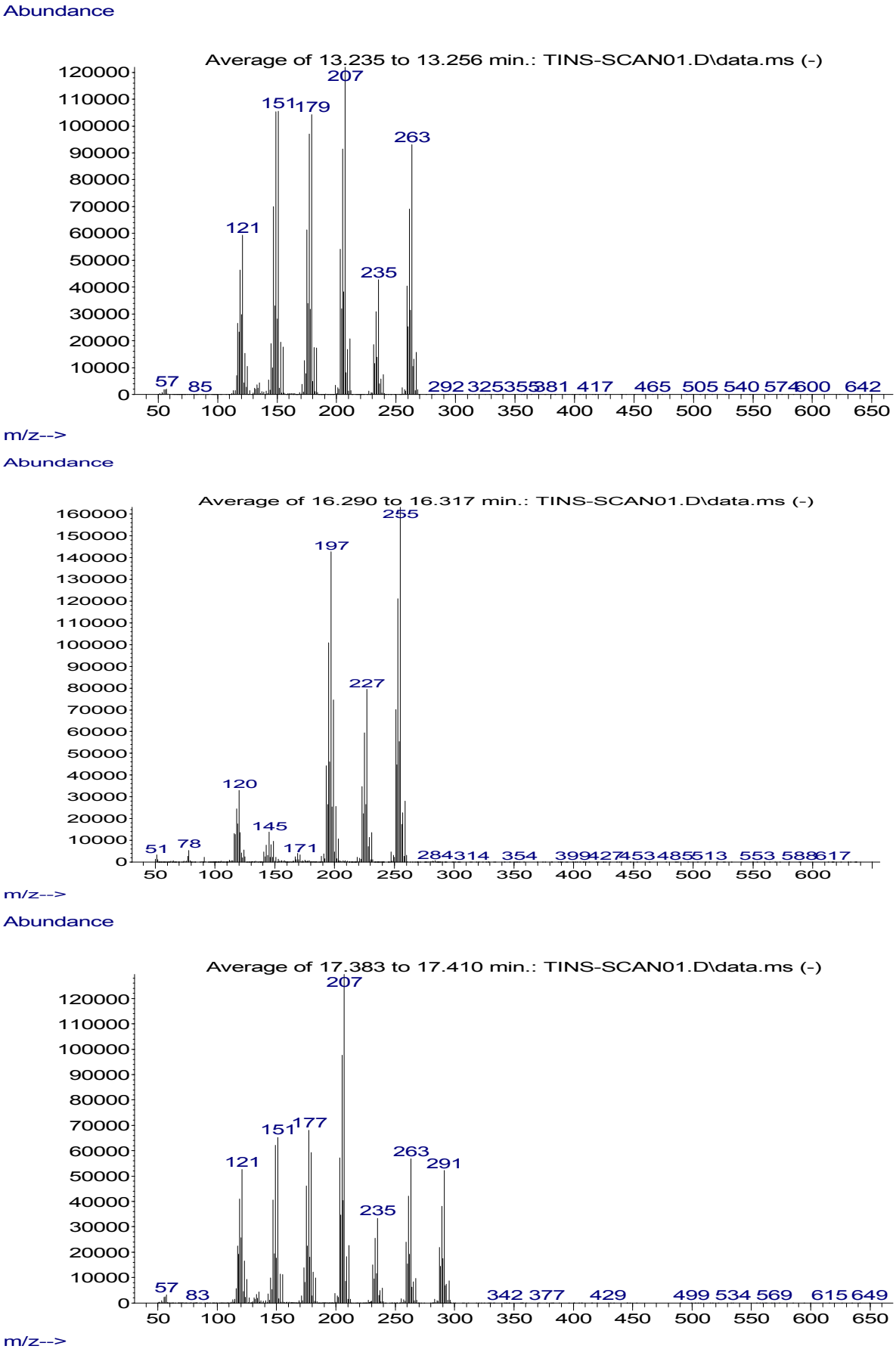

FIG. II.3. MS spectra of different Organotin species

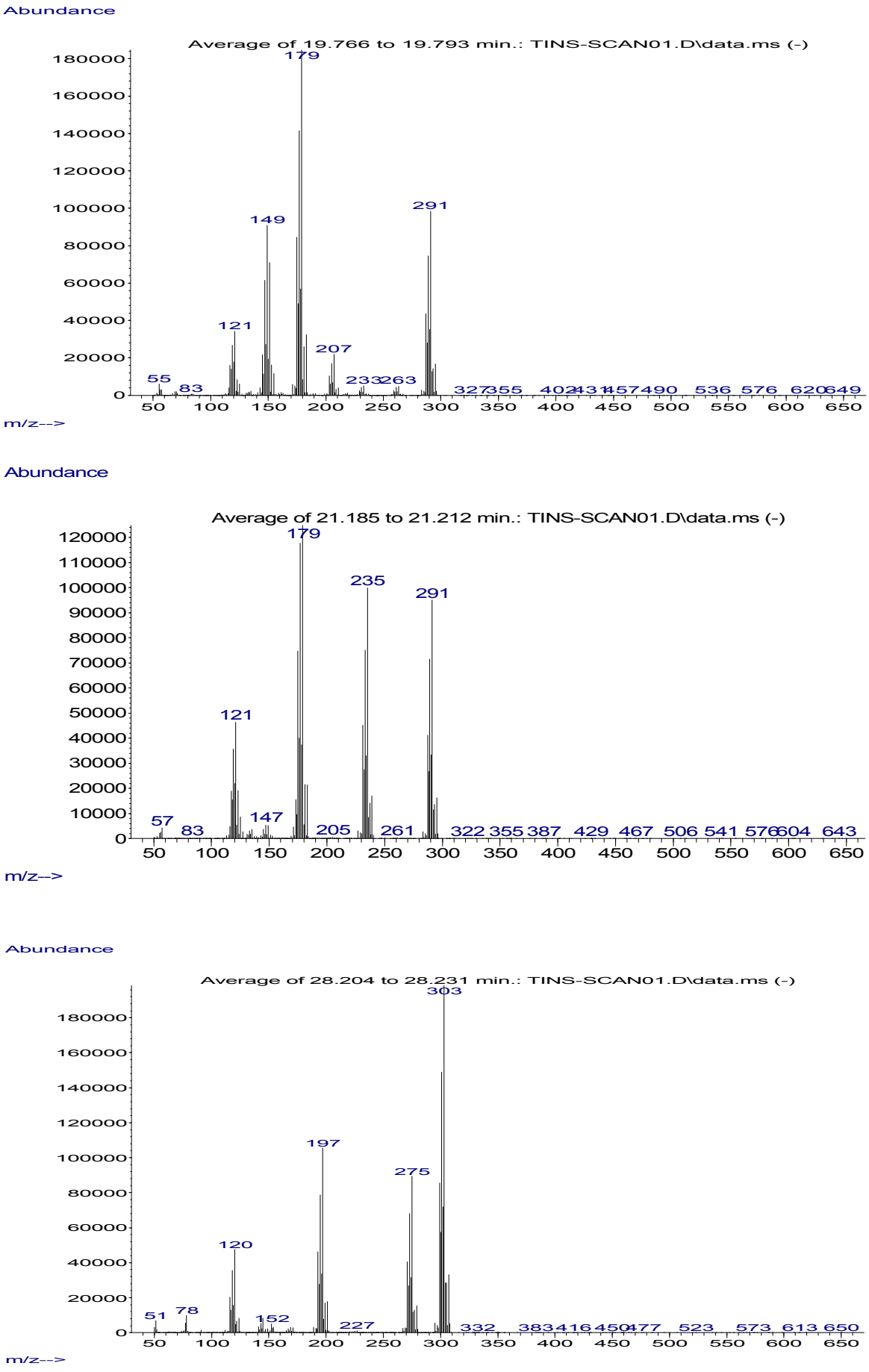

FIG. II.4. MS spectra of different Organotin species

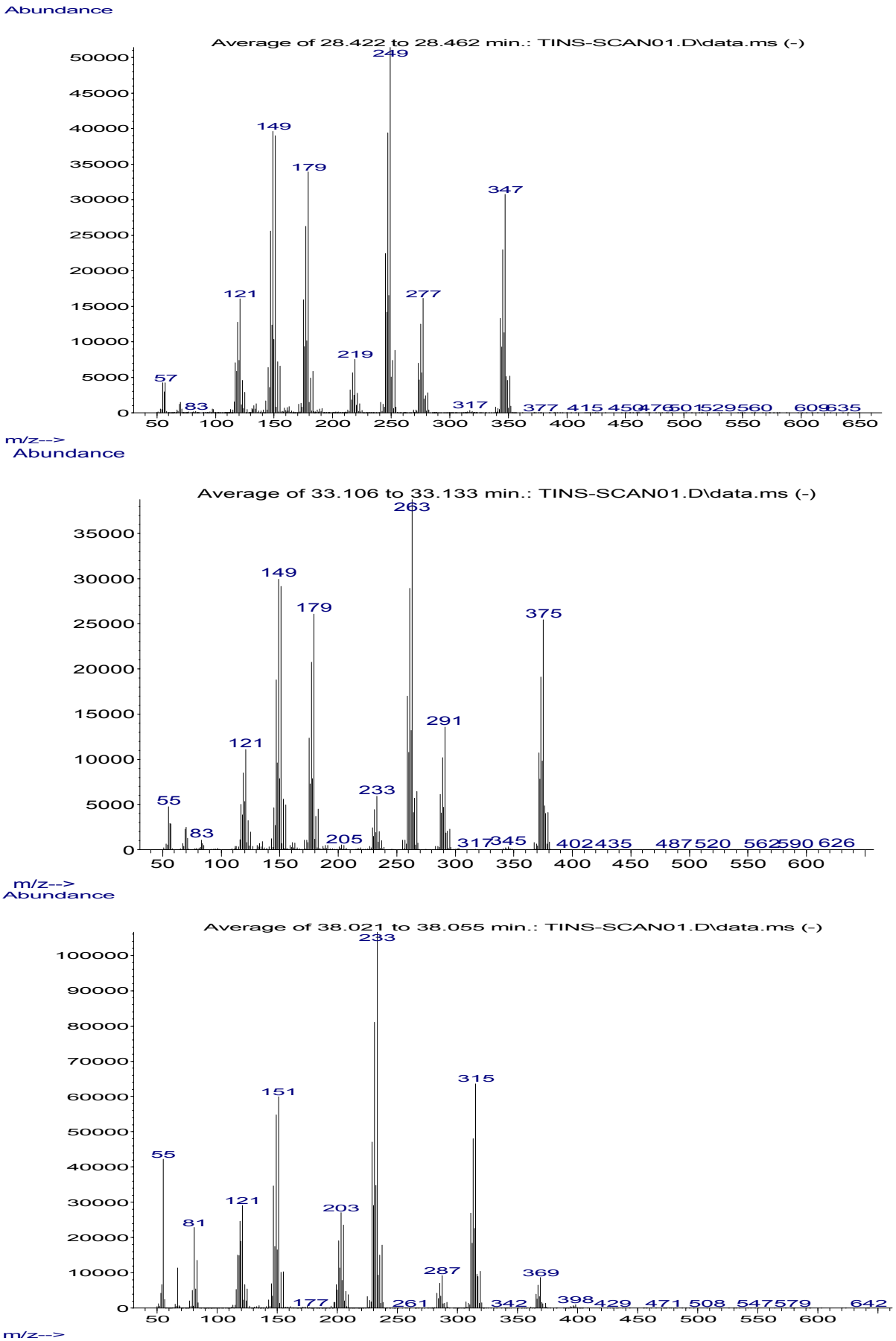

FIG. II.5. MS spectra of different Organotin species

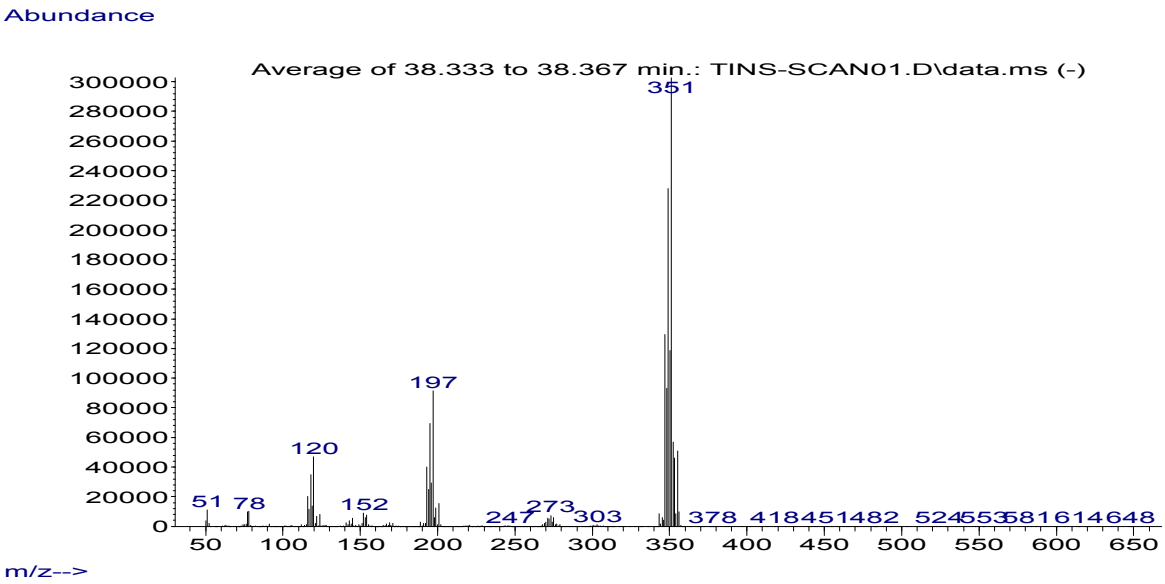

FIG. II.6. MS spectra of different Organotin species

REPORT

Recommended Method for the
DETERMINATION OF ORGANOTIN COMPOUNDS IN WATER SAMPLES

IAEA/NAEL

Marine Environmental Studies Laboratory in co-operation with

UNEP/MAP MED POL

November 2012

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Recommended Method for the DETERMINATION OF ORGANOTIN COMPOUNDS IN WATER SAMPLES

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TABLE OF CONTENTS

NOTE: This method is not intended to be an analytical training manual. Therefore, the method is written with the assumption that it will be used by formally trained analytical chemists. Several stages of this procedure are potentially hazardous; users should be familiar with the necessary safety precautions.

In addition, the IAEA recommended methods are intended to be guidance methods that can be used by laboratories as a starting point for generating their own standard operating procedure. If performance data are included in the method they must not be used as absolute QC acceptance criteria.

1 GENERAL DISCUSSION

1.1 PRINCIPLE

Butyl- and phenyltin compounds are used worldwide mainly as antifouling agents and biocides. The large-scale application of these compounds to submerged marine structures, commercial vessels and pleasure crafts has resulted in dispersion to every marine environmental compartment. These compounds, especially tributyltin (TBT), exhibit broad spectrum of biocidal properties and so have elicited considerable research attention. Due to the deleterious effects on non-target organisms, the use of organotin compounds as antifouling agents on the boat hulls of small vessels has been widely prohibited.

Many analytical procedures based on chromatographic separation coupled to various detection techniques have been developed. Organotin compounds must be extracted from the matrix and derivatized into suitable forms for gas chromatographic analyses. The most common derivatization procedures include hydride generation in the aqueous phase using sodium borohydride, or extraction of organotin complexes with tropolone or diethyldithiocarbamate, followed by Grignard derivatization. The instability and volatility of butyltin hydrides can lead to losses of compounds and consequently to an underestimation of contamination. The Grignard derivatization requires scrupulous dry conditions and is rather time consuming.

To avoid such difficulties, an organotin derivatization procedure directly applied to the aqueous phase using sodium tetraethylborate ($NaBEt_4$) was developed, whereby the derivatization and extraction of organotin compounds can be carried out simultaneously. This technique allows the determination of several butyl- and phenyltin compounds.

The method for the determination of organotin compounds presented here is based on the ethylation with sodium tetraethylborate. The simultaneous derivatization and extraction with

sodium tetraethylborate converts butyltin and phenyltin ions into their corresponding volatile derivatives in the organic phase:

$$R_nSn^{(4-n)+} + (4-n)\ NaBEt_4 \longrightarrow R_nSnEt_{(4-n)} + (4-n)\ Na^+ + (4-n)\ BEt_3$$

This derivatization reaction is the most critical step of the protocol. For optimal derivatization efficiency, the solution of sodium tetraethylborate should be either freshly prepared, i.e. just prior to sample processing, or stored frozen (-20°C) for no longer than two weeks. The optimal conditions for the sodium tetraethylborate comprise a 30 min. reaction time at a pH between 5 and 6.

It should be appreciated that sodium tetraethylborate is extremely air-sensitive and must be handled with care to keep its chemical integrity.

1.2 ANALYTICAL STRATEGY

Many of the internal standards and the target compounds in the samples need to be derivatized to be detectable by GC techniques. Two different approaches are possible in order to overcome this problem.

- The first would be to purchase calibration standard solutions and internal standard solutions of ***ethylated organotin compounds***. These solutions contain tetra-substituted organotin species which are detectable by gas chromatography without further treatments. The calibration solutions contain certified concentrations of target compounds and can be injected directly into the GC (at previous suitable dilution). The ethylated internal standards are spiked in both the samples to be analysed and the calibration solutions. This approach eliminates any errors due to the performance of the derivatization reaction in the calibration standards. Unfortunately, the costs of ethylated standard solutions are fairly high and their use in routine analyses is not cost effective.
- The second approach available would be to use a ***matrix matched calibration curve***. In this instance, the calibration standards which are not derivatized are spiked into organotin-free environmental matrices (same matrix as the samples to be analysed). These "calibration samples" are then handled and analysed as if they were unknown samples, and thus, they undergo all the different steps of the analytical method including the derivatization step. This approach allows the normalization of some systematic errors and, more importantly, a matrix-matched calibration curve that will take into account the variability linked to the use of sodium tetraethylborate. In case of a low efficiency of the derivatization reaction, for example, both the samples and the calibration curve will be affected, normalizing thus, the results.

1.3 DESCRIPTION AND USE OF ORGANOTIN CALIBRATION STANDARDS

The calibration standards are multi-compound solutions of *Mono*-, *D*i- and *Tributyltin*, *Mono*-, *Di*- and *Triphenyltin*. Ethyl-substituted compounds can be analysed directly, or the

"chloride" forms of these compounds can be spiked in uncontaminated environmental samples (calibration samples), to create a matrix-matched calibration curve (see section 2.2).

The calibration curve must be made by at least 3 points (standard solutions at different concentrations). The concentration of the standard solutions must be calculated so that they bracket the concentrations of the samples and the Reference Materials (RMs).

If the concentrations of the target compounds in the samples are unknown, the calibration curve should be centred on the reference materials. If the concentrations of the target compounds exceed the limit of the calibration curve, either the samples must be brought to the appropriate dilution (extracting less material), or the calibration curve must be extended with a higher concentration standard. If, on the contrary, the concentration of the samples is lower than the lowest calibration curve's point, a new calibration curve must be prepared.

Example:

The reference material NIES-11 (fish tissue) contains 1.3 µg/g of TBT as chloride.

0.5 g of NIES 11 in a 0.5 mL solution (assuming 0.5 mL as the final dilution of the vial prior to GC injection) means a concentration of:

$$\frac{(0.5\ \text{g} \times 1.3\ \mu\text{g/g})}{0.5\ \text{mL}} = 1.3\ \mu\text{g/mL} = 1.3\ \text{ng/µl}$$

A calibration curve centred on this value would be:

0.5 1.25 2.5 ng/µl

Assuming an organotins mixture working solution concentration of 50 ng/µl and a final vial dilution of 1mL, the calibration curve can be created by spiking 10, 25 and 50 µl of organotins mixture into 3 different uncontaminated matrices.

1.4 DESCRIPTION AND USE OF ORGANOTIN INTERNAL STANDARDS

Internal standards are non-interfering compounds added to a sample with a known concentration, in order to eliminate the need for the measurement of the final sample dilution in quantitative analyses, and for correction of instrumental variation. The internal standards must be selected based on their characteristics, and thus on the information they have to provide. Internal standards can be added to the samples at the very beginning of the procedure, to check for the overall performance of the method, or at the end, just prior to GC injection, to check mainly for GC performance. A combination of several internal standards with different properties allows a better understanding and control of an analytical protocol.

The method proposed here is based on the use of three internal standards:

- *Tripropyltin chloride* used as the main internal standard to indicate the derivatization reaction efficiency. All the chromatographic peak areas are normalized to those of Tripropyltin.
- *Tetrapropyltin* used as secondary internal standard to check the overall recovery of the protocol. It is already tetra-substituted and thus, it is not concerned by the derivatization reaction.
- *Tetrabutyltin* spiked in all samples prior to GC injection is used as a GC-internal standard to quantify recovery of both internal standards

2 APPARATUS AND REAGENTS

2.1 APPARATUS

- Glass or Teflon bottle of appropriate volume;
- Glass or Teflon separatory funnels of appropriate volume;
- Insulated plastic boxes for transporting samples, ice or dry ice;
- Refrigerator (4°C) for sample preservation;
- Rotary evaporator;
- Glassware including boiling flasks, ground glass stoppers, beakers, Erlenmeyer flasks, centrifuge tubes, weighing bottles, pipettes, tissue grinders;
- Drying oven (temperature range up to at least 450°C) for baking of contaminant residues from glassware and reagents;
- Centrifuge and tubes;
- Analytical balance with a precision of 0.1 mg and an electro-balance with a precision of at least 1 µg;
- Stainless steel tweezers and spatulas;
- Dessicator - completely cleaned and with no grease applied to sealing edges;
- Supply of clean dry nitrogen;
- Vacuum pump (water-jet air pump);
- Ultrasonic bath;
- Litmus papers;
- Solid Phase Extraction columns (Supelclean™ LC-Florisil® SPE Tubes 6 mL, 1 g);

- 12 or 24 ports Glass Vacuum manifold for simultaneous use of multiple SPE columns. The manifold must be fitted with flow control valves to allow for a fine adjustment of flow through the SPE column.

2.2 LIST OF REAGENTS

- Potassium acetate / acetic acid buffer (CH_3COOH/ CH_3COO^-, K^+);
- 1% $NaBEt_4$ in 2% KOH solution;
- Na_2SO_4, anhydrous granular;
- Florisil cartridges (Supelclean™ LC-Florisil® SPE Tubes 6 mL, 1 g);
- Isooctane, pesticide grade or equivalent purity;
- *n*-hexane, pesticide grade or equivalent purity;
- Dichloromethane, pesticide grade or equivalent purity
- Nitrogen, 99.8% purity.

2.3 STANDARDS

- Internal standard: Tripropyltin choride (ca. 500 ng);
- Secondary Internal Standard: Tetrapropyltin (ca. 1000 ng);
- GC-Internal Standard: Tetrabutyltin (ca. 100 ng).

The GC vials containing Tetrapropyltin and Tetrabutyltin are added to the injection sequence just before GC injection. These standards are used to quantify recovery of Tetrapropyltin in samples.

The working solutions from the stock reference solutions are prepared on a regular basis, and stored in clean glass volumetric flasks tightly capped with non-contaminating materials such as Teflon or glass. Extreme care must be taken to ensure that standards have not changed their concentrations through solvent evaporation.

3 PROCEDURES

3.1 GLASSWARE CLEANING AND HANDLING

The glassware for the analyses of organotin compounds requires special precautions and specific equipment.

3.1.1 Reagents

- Detergent for glassware;
- *n*-Hexane, pesticide grade or equivalent purity;
- Acetone, pesticide grade or equivalent purity.

3.1.2 Procedure

The glassware must be cleaned with detergent, rinsed first with tap water, then with high purity acetone and finally with *n*-hexane. Any contact with plastics of any kind, as well as silicone and/or grease or any other material must be avoided in order to minimize the risk of contamination. Only Teflon, aluminium, glass or stainless steel (previously cleaned with acetone and *n*-hexane) should be in contact with the samples.

As an alternative, glassware can be pyrolitically cleaned using a muffle furnace at a temperature of 450°C for 4 hours. Even for low level determinations, it is still recommended to rinse the glassware with small amount of *n*-hexane.

NOTES:

The contamination of laboratory apparatus with organotin compounds arises from the use of diorganotin stabilizing compounds in many types of plastic, e.g. PVC, and the use of such materials should be avoided.

Organotins are readily adsorbed onto glassware and losses may occur through the use of glassware. We recommend using the minimum of glassware, and wherever possible rinsing glassware at least three times with appropriate solvent when transferring the sample solutions.

3.2 SODIUM SULFATE (Na_2SO_4) CLEANING

3.2.1 Principle

The sodium sulfate is pyrolitically cleaned using a muffle furnace at a temperature of 450°C for 4 hours.

3.2.2 Reagents

- Sodium sulfate, anhydrous granular.

3.2.3 Procedure

Transfer an adequate amount of sodium sulfate in a glass container (the glass must be resistant to high temperature).

Put the container in the muffle furnace at a temperature of 450°C for 4 hours.

3.3

3.4 PREPARATION OF BUFFER SOLUTION (CH_3COOH/ CH_3COO, K^+)

3.4.1 Principle

A potassium acetate/acetic acid buffer solution is used to adjust the pH between 5 and 6 prior to derivatization reaction.

3.4.2 Reagents

- Acetic acid (96%, d=1.06);
- Potassium acetate (CH_3COOK);
- Milli-Q water (organotin-free deionized water).

3.4.3 Procedure

- Weigh 125.08 g (or 118 mL) of acetic acid;
- Weigh 192 g of potassium acetate;
- Dissolve the potassium acetate in Milli-Q water;
- Add the acetic acid and make 1L up with Milli-Q water;
- Store in a pre-cleaned glass bottle.

3.5 PREPARATION OF 2% KOH SOLUTION

3.5.1 Principle

A 2% KOH solution is used for the preparation of the sodium tetraethylborate ($NaBEt_4$) solution.

3.5.2 Reagents

- KOH;
- Milli-Q water.

3.5.3 Procedure

- Dissolve 2.3 g of KOH in Milli-Q water and make it 100 mL;
- Store the solution in a pre-cleaned Teflon bottle and keep in the freezer until incipient frost formation.

3.6 PREPARATION OF 1% $NaBEt_4$ SOLUTION IN 2% KOH

3.6.1 Principle

The method for the determination of organotin compounds presented here is based on the ethylation with sodium tetraethylborate.

It should be appreciated that sodium tetraethylborate is extremely air-sensitive and must be handled with care to keep its chemical integrity.

3.6.2 Reagents

- 2% KOH solution, 100 mL;
- $NaBEt_4$, 1 g sealed bottle;
- Argon or nitrogen gas.

3.6.3 Procedure

- Operate when possible in a glove box, or at least in a fume-hood using a stream of argon or nitrogen to minimize contact between $NaBEt_4$ and the air;
- Carefully open the $NaBEt_4$ bottle and fill it with KOH solution;
- Close the bottle and shake manually;
- Pour the content into the KOH solution bottle;
- Rinse the $NaBEt_4$ bottle with KOH;
- Prepare the Teflon bottles of appropriate volume and fill them with argon or nitrogen;
- Fill up the Teflon bottles with the $NaBEt_4$ solution;
- Fill the Teflon bottles with argon or nitrogen;

- Put the Teflon bottle in single zip-lock bags filled up with argon or nitrogen;
- Store at -20°C.

3.7 PREPARATION OF STANDARD SOLUTION

3.7.1 Principle

The individual stock solutions of organotin compounds are prepared by dissolving approximately 60-80 mg of the original standard compound in 100 mL *n*-hexane (for tri- and tetra- substituted organotin species), and in acetone (for mono- and di-substituted organotin species).

3.7.2 Reagents

- Organotin standards;
- *n*-hexane, pesticide grade or equivalent purity;
- Acetone, pesticide grade or equivalent purity;
- Isooctane, pesticide grade or equivalent purity.

3.7.3 Procedure

- Using a pre-cleaned glass weighing boat, weigh 60-80 mg of the standard;
- Transfer into a 100 mL glass volumetric flask;
- Rinse the weighing boat with the appropriate solvent into the volumetric flask;
- Bring to volume with the appropriate amount of solvent;
- Store at -20°C.

NOTE:

Individual working solutions of Internal and GC-Internal Standards are prepared in the appropriate solvent:

- Acetone for Internal Standards of tripropyltin (TPrT) and tetrapropyltin (TePrT);

- Acetone for the multi-compound mixture used for the calibration curve (organotins mixture, OTs mix);

- Isooctane for GC-Internal Standards, tetrabutyltin (TeBT) and tetrapropyltin (TePrT).

The concentration of working solutions usually ranges between 10 and 40 ng/µl which correspond to a 25-50 times dilution of the stock solution.

4 ANALYTICAL PROCEDURE ON THE DETERMINATION OF ORGANOTIN COMPOUNDS IN WATER SAMPLES

1 L samples are extracted in Teflon or glass separating funnels with dichloromethane and *n*-hexane. Buffer is used to stabilize the pH between 5 and 6 prior the derivatization reaction. The organotin compounds are simultaneously derivatized and extracted using sodium Tetraethylborate (NaBEt4). Samples are concentrated to about 0.5mL (prior a solvent exchange to isooctane) and injected into GC.

1- Internal standards spiking:

- Spike appropriate amounts of TrPrT and TePrT in each sample.

2- pH Adjustment:

- Add 10 mL buffer solution to each Teflon tube;

3- Derivatization/Extraction:

- Add 1 mL of $NaBEt_4$ solution to each sample;
- Immediately add 30 mL of dichloromethane to each sample;
- Shake manually vigorously for 1-2 minutes;
- Recover organic phase in 100 mL glass flat bottom flasks;
- Add 30 mL of *n*-hexane to each separating funnels;
- Shake manually vigorously for 1-2 minutes;
- Recover organic phase and combine;
- Add ca. 1-2 g of pre-cleaned Na_2SO_4 to each glass flaks to dry organic phase;

4- Evaporation:

- The extract is reduced to ~10 mL using a rotary evaporator;
- Transfer samples into 15 ml glass graduated tubes
- Concentrate samples to 1 mL under a gentle stream of N_2.

5- S.P.E. Clean Up:

- The concentrated extract is purified using Florisil cartridges;
- The sample and solvent rinses are added to the top of Florisil cartridge and eluted with 5 mL of *n*-hexane twice (2x5 mL). The eluent is collected in a 15 mL glass tube.

6- Evaporation:

- The eluent is reduced to ~0.5 mL under a gentle stream of N_2.

7- Transfer into Vials/Solvent Exchange:

- Transfer the concentrated eluents into auto-injector vials. Rinse the glass tube with isooctane and add to the vial.

8- Evaporation:

- Reduce to appropriate volume under a gentle stream of N_2.

9- Standard Spiking:

- Add GC-Internal Standard (TeBT) to each sample vial;
- Prepare vials of GC-Internal Standard.

10- GC Analyses**:**

- Inject 1 uL of sample to GC-FPD and/or GC-MS.

5 GAS CHROMATOGRAPHY CONDITIONS

5.1 GC-FPD CONDITIONS FOR THE QUANTIFICATION OF ORGANOTIN COMPOUNDS

- Column: 30 × 0.25 mm × 0.25 μm HP-5 capillary column (5% phenyl methyl silicone) or equivalent (Supelco);
- Inlet: 250°C, splitless;
- Detector: FPD at 270°C, 610nm Sn filter. Hydrogen rich flame (air flow: 90 ml/min.; hydrogen flow: 130 ml/min.);
- Carrier gas: Helium, at a flow rate of 1 mL min^{-1};
- Oven program: 60°C for 2 min, then 60-270°C at 6°C min^{-1} and 270°C for 20 min.

5.2 GC-MS-SIM CONDITIONS FOR THE QUANTIFICATION OF ORGANOTIN COMPOUNDS

- Column: 30 × 0.32 mm × 0.25 μm HP-5 capillary column (5% phenyl methyl silicone);
- Detector: Electron Impact-MS in SIM mode. Details of the acquisition are provided in Table 1;
- Ion energy of the MS: 70 eV;
- Ion source temperature: 240°C;
- Interface: 280°C;
- Inlet: 250°C, splitless;

- Carrier gas: Helium, at a flow rate of 2 mL min^{-1};
- Oven program: 60°C for 1 min, then from 60 to 100°C at 10°C min^{-1} and from 100°C to 280 at °C at 4°C min^{-1}.

TABLE 1. TARGET AND CONFIRMATION IONS FOR GC/MS ANALYSES OF ORGANOTIN COMPOUNDS

Group	Time (min)	Compounds (m/z monitored)[a]	Cycles (s^{-1})	Ions (m/z)
1	6–10	MBT (121, 179, <u>235</u>) TrPT (121, 193, 235, <u>249</u>)	3	179, 235, 121, 193, 249
2	10–16.5	TePrT (121,165,249, <u>207</u>) DBT (121, 207, 235, <u>263</u>) MPhT (197, <u>255</u>) TBT (204, 235, 263, <u>291</u>)	2.18	263, 207, 291, 235, 121, 255, 197
3	16.5–20	TEBT (179, 235, <u>291</u>)	4.83	291, 179, 235
4	20–26	DPhT (120, 197, 275, <u>303</u>)	3.70	303, 275, 197, 120
5	26–40	TPhT (120, 197, 349, <u>351</u>)	3.7	351, 197, 120, 349

[a] The m/z used in quantification is underlined.

6 RESULTS QUANTIFICATION

This method is based on the use of an ***internal standard,*** which is defined as a non-interfering compound, added to a sample with known concentrations in order to eliminate the need to measure the final extract volume in quantitative analysis and for correction of instrumental variation.

In this method, the internal standard is added to each sample at the beginning of the analytical protocol. The ratio of the areas of the internal standard and analyte are then used to construct the calibration curve.

In a multiple point internal calibration each analyses contains the internal standard whose total amount is kept constant, and the analyte of interest whose amount covers the range of concentrations expected.

A multiple points relative response factor (RRF) calibration curve is established, for analytes of interest in each working batch. A RRF is determined, for each analyte, at each calibration level using the following equation:

$$RRF(X) = \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{\text{Qty (IS)}}{\text{Qty (X)}}$$

Where:

Area (X) = the area of the analyte to be measured (target compound);

Area (IS) = the area of the specific internal standard;

Qty (X) = the known quantity of the analyte in the calibration solution;

Qty (IS) = the known quantity of the internal standard in the calibration solution.

The relative response factors determined for each calibration level are averaged to produce a mean relative response factor (mRRF) for each analyte. The percent relative standard deviation (%RSD) for the 3 response factor must be less than or equal to 15%, for each analyte.

$$\%RSD = \frac{\text{Standard deviation of the RRFs}}{\text{Average of the RFs}} \text{ x } 100$$

The sample analyte concentrations are calculated based on the quantity and response of the internal standard.

The following equation gives the amount of analyte in the solution analyzed.

$$Qty\ (\text{X}) = \text{Qty (IS)} \text{ x } \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{1}{\text{mRRF}(X)}$$

Where:

Qty (X) = the unknown quantity of the analyte in the sample;

Qty (IS) = the known quantity of the internal standard added to the sample;

Area (X) = the area of the analyte;

Area (IS) = the area of the internal standard

mRRF (X) = the average response factor of the analyte.

The sample analyte concentrations are then calculated by dividing the amount found (Qty) by the grams of the samples extracted.

6.1 REPORTING CONCENTRATIONS

The organotin compounds are purchased “as chloride”. The standard solution prepared and the results obtained after the calculation are expressed “as chloride”. The certified values of the reference materials and its literature usually report organotin concentration “as Sn”. The conversion between the two forms is given by the ratio between the atomic mass of Sn and the molecular mass of the chloride form.

Example:

For MBT = Sn $(C_4H_9)Cl_3$

Atomic masses: Sn = 118.71; C = 12; H = 1; Cl = 35.45.

Molecular mass of Sn $(C_4H_9)Cl_3$ = 282.06

The ratio between atomic mass of Sn and molecular mass of MBT gives the conversion factor: Sn/chloride = 0.421

10 ng/g MBT as chloride correspond to 10 x 0.421 = 4.21 ng/g as Sn

6.2 QUALITY ASSURANCE/QUALITY CONTROL

The goal in analytical chemistry is to obtain accurate compositional information about an unknown sample. The analyst introduces a processed sample into an instrument that will measure the parameter(s) of interest, and a result is obtained. But how accurate is the result? There are a myriad of factors - including contamination, loss of analyte, analyst error, instrument problems, to name only a few - that could occur at any time throughout the analytical process and potentially impact the accuracy of the final result. Sometimes, the impact of one or more of these factors is evident; often it is not.

Quality assurance (QA) is a comprehensive system of activities to ensure that the quality of the data produced meets pre-defined standards, within a stated level of confidence. A laboratory must design its QA program to meet its specific needs, so that the data produced is acceptable for its intended use; i.e. ‘fit for purpose.’ A good laboratory QA program will include both technical and administrative components to achieve this objective. Required component systems of a QA program generally include:

- Staff training;
- Procedures and procedure validation;
- Records and document control;
- Procurement and verification of item quality before use;
- Control of items (e.g. sample receipt, chains of custody, etc.);
- Periodic assessment of the QA program and its components;
- Corrective action management;

- Instrument maintenance and calibration.

Having a fully implemented QA program provides the necessary ingredients for producing a reliable and defensible product. Due to the fact that many of the factors that could impact data quality are transient, the analytical process must be tested and evaluated throughout the process, to verify that the results produced are accurate and reproducible, and have not been impacted. The Quality Control (QC) is a system of activities to ensure that the analytical process is controlled, and verifies that quality parameters are within required limits. QC is often separated into method (or process) QC and analytical (or instrument) QC. Method QC is generally introduced at the beginning of the analytical process, to ensure that all the steps comprising the process (e.g. digestion, separation, concentration, etc., and including analysis) have not impacted the final result. Method QC may include the following QC samples:

- Method blank to monitor the contamination resulting from the process;
- Laboratory control sample (e.g. representative reference material) to monitor accuracy;
- Laboratory control sample duplicate to monitor method reproducibility.

The analytical QC ensures that the instrument is controlled throughout the analysis, and is usually comprised of the following QC samples:

- Reagent blank to monitor the contamination resulting from the reagents and/or instrument;
- Independent calibration verification (ICV) to verify that the calibration standard is acceptable;
- Continuing calibration verification (CCV) throughout the analysis, and at the end of the analysis to verify that the instrument remains under control.

The last two points are more difficult to apply in analytical chemistry, since the analyses as well as the samples handling can take a lot of time. In this case, an alternative could be the diligent use of internal standards and the evaluation of their recoveries.

Other QC samples may be introduced as well. Interference check standards often contain high concentrations of matrix components that are known to potentially interfere with the analyte(s) of interest. Sample (or matrix) spikes (i.e. samples spiked with analyte(s) of interest) may be introduced either before or after sample processing to verify that the results are not impacted by the sample matrix. The samples may also be analysed at 2 or more dilutions to verify that the results are not impacted by the matrix. Other QC samples are possible, depending on the potential concern. The analyst should be familiar with the analytical process and the parameters that could potentially impact the results, and introduce appropriate QC samples accordingly.

The effective QC protocol also includes monitoring of the process over time by charting the parameters of interest and concern. The control charts provide a graphical representation of historic data that can be helpful in identifying trends. Regular control chart evaluation is an effective means of *preventive action*, i.e. a proactive approach to preventing problems before they occur.

The QC protocol should also include regular participation in external proficiency testing (also known as performance evaluation) campaigns, as well as inter-laboratory comparison exercises. In a proficiency testing campaign, the laboratory is sent a relevant reference material standard as a 'blind', i.e. an unknown to the laboratory. The laboratory must process and analyse the material and report the final results as it would for any similar sample received. The campaign organizers then provide feedback to the laboratory on performance in terms of accuracy and precision. The inter-laboratory comparisons are similar, but the subject sample material is often an unknown. The objective of such an exercise is generally to characterize the subject material. As a result, the analytical proficiency cannot be unequivocally determined. Even so, the information obtained from such an exercise generally includes consensus values and ranges of data, which can be an indication of analytical performance.

6.3 ANALYTICAL QUALITY CONTROL CHARTS (AQCCS)

The reference materials should be used by laboratories to establish their precision and accuracy. These materials should be analysed periodically to provide a check on the quality of the analytical data. The simplest way to assess the results of these analyses is to examine them at the end of the analytical period, and to decide whether or not they are satisfactory, and thus whether or not the data generated for the samples is acceptable.

It is recommended for the results of the analyses of the reference materials to be plotted on a simple chart, which contains guidelines, to objectively assess their quality. This chart is known as an "analytical quality control chart". The figure 1 shows an example of a blank control chart.

Analyst should only use methods that have proved to provide good quality results. Assuming that such methods are used, an AQQC should be constructed along the following lines:

1. Select an appropriate reference material to be analysed along with the samples;
2. Analyse the chosen reference material at least 10 times. These analyses should not be carried out on the same day, but spread out over a period of time, in an attempt to ensure that the full range of random errors within and between batch analyses are covered.
3. Calculate the mean value (X), and the standard deviation (S) and then plot the following values on a blank control chart:

   X, **X+2S** (UWL**)** **X+3S** (UCL) **X-2S** (LWL) and **X-3S** (LCL).

Where:

UWL = upper warning limit

UCL = upper control limit

LWL = lower warning limit

LCL = lower control limit

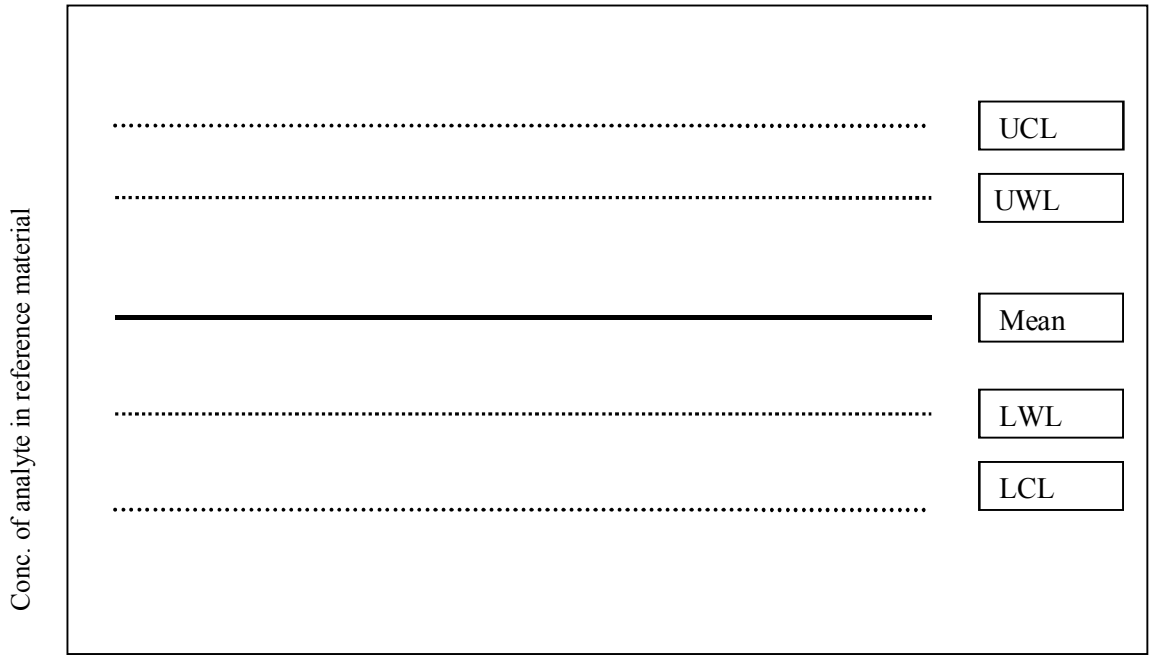

FIG. 1. Blank control chart

Assuming that the analytical measurements for the RM(s) follow a normal distribution, 95% of them should fall within the area between UWL and LWL. Similarly 99.7% of the results should fall within the area between UCL and LCL.

The analyst should plot the results for the analyses of the RM(s) after each batch of analyses to check whether the data lies within the limits.

The following guidelines can be used to assess whether the data for the RM(s), and consequently, the data for the samples are acceptable or not.

1. The mere fact that one result falls outside the warning limits doesn't mean that the analyses has to be rejected provided that the following results falls within the warning limits;

2. If the results fall outside the warning limits too frequently, particularly, if the same warning limit has been crossed more than once on consecutive results, then the analyst needs to assess the source of this systematic error;
3. If the results on more than 10 consecutive occasion fall on the same side of the X line (either between X and UWL or X and LWL), then the analyst needs to check the analytical procedure to determine the cause of this error;
4. If the results fall outside the UCL or LCL lines, the analyst should check the analytical procedure to determine the cause of this error.

If any of the above cases occur, the analyst should reject the results of the analyses for the particular batch of samples, and should not carry out any further analyses until the sources of the errors have been identified and eliminated.

Guidelines on the QA/QC requirements for analyses of sediments and marine organisms are detailed in the Reference Method No 57, "Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice".

7 BIBLIOGRAPHYY

Reference Method No 57, UNEP/IOC/IAEA/FAO: Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice. UNEP, 1990.

ANNEX I

GC-FPD CHROMATOGRAMS

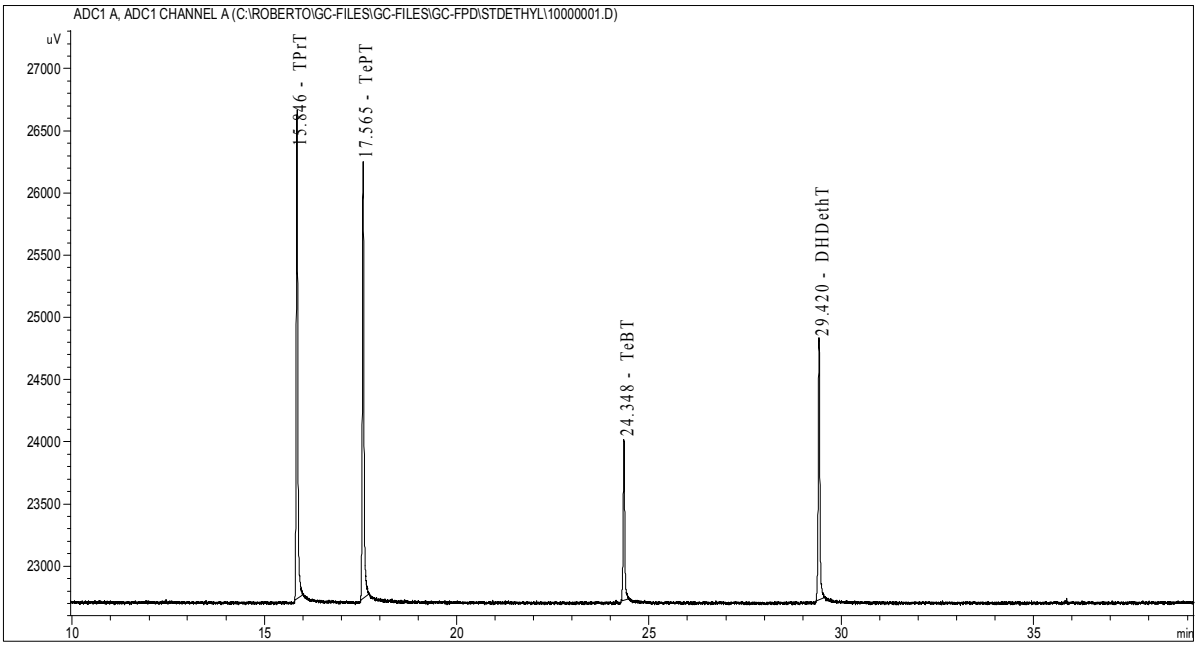

FIG. I.1. Chromatogram of an already ethylated Internal STDs (ca. 4 ng/ul)

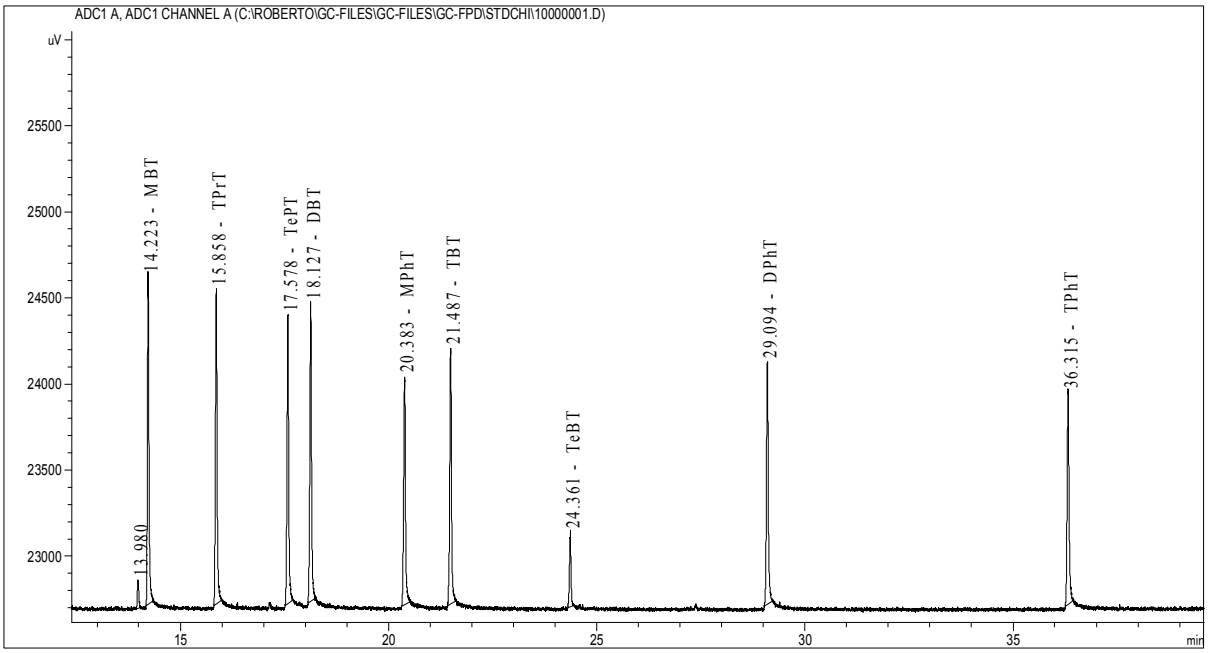

FIG. I.2. Chromatogram of Calibration STDs (ca. 2.8 ng/ul)

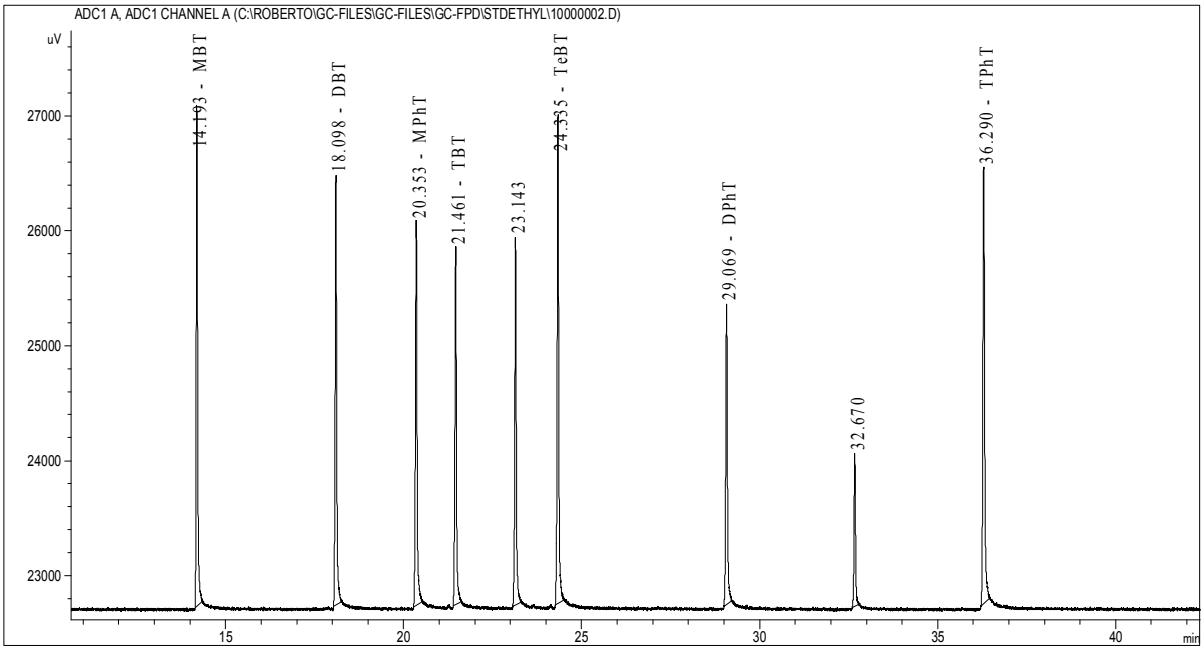

FIG. I.3. Chromatogram of an already ethylated Calibration STDs (ca. 4 ng/ul)

ANNEX II

GC-MS CHROMATOGRAMS

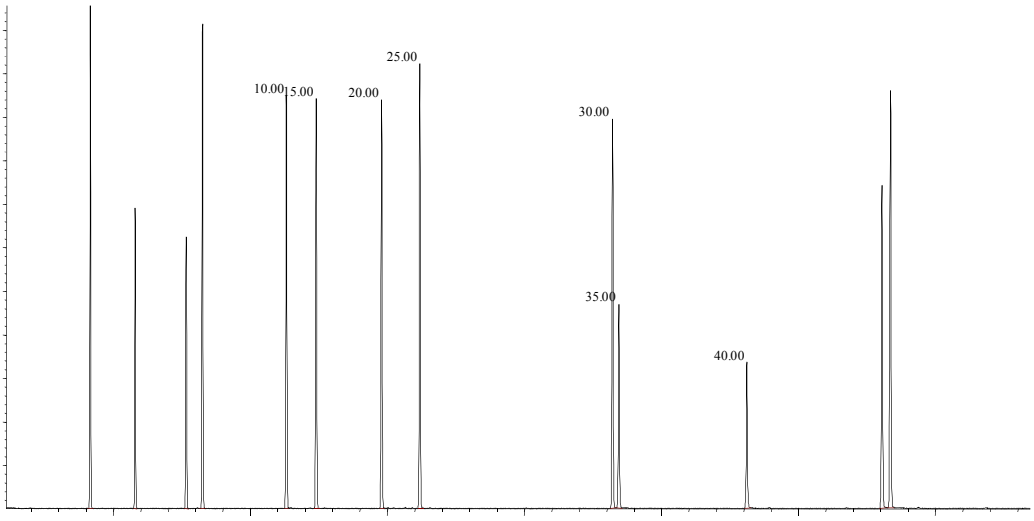

FIG. II.1. GC-MS chromatogram of already ethylated STDs by EI in total scan.

Monobutyltin (MBT), tripropyltin (TrPT), tetrapropyltin (TePrT), dibutyltin (DBT), monophenyltin (MPhT), tributyltin (TBT), monooctyltin (MOcT), tetrabutyltin (TeBT), diphenyltin (DPhT), diheptyltin (DHT), dioctyltin (DOcT), tricyclohexyltin (TCyHT), triphenyltin (TPhT).

- Column: DB-XLBMSD, 30m x 0.25 μm x 0.25 mm.;
- Oven program: 600°C for 1 min, then from 60 to 100 at 10°C min and from 100 to 280 at 4°C min;
- Inlet: Pulsed splitless at 270°C
- Carrier gas: helium at a flow rate of 1.6 ml/min.;
- Ion energy of the MS: 70 eV;
- Ion source temperature: 230°C.

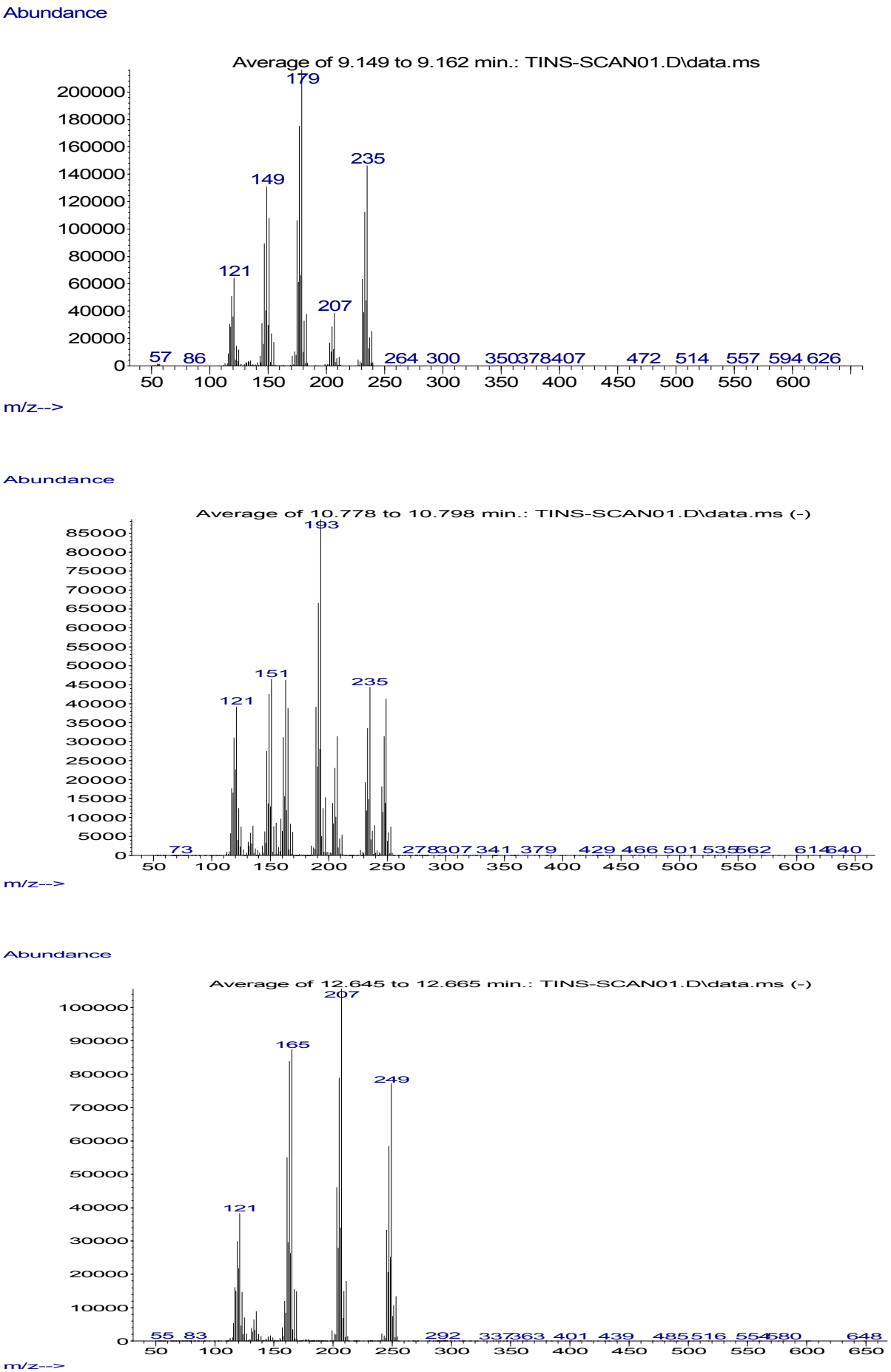

FIG. II.2. MS spectra of different Organotin species

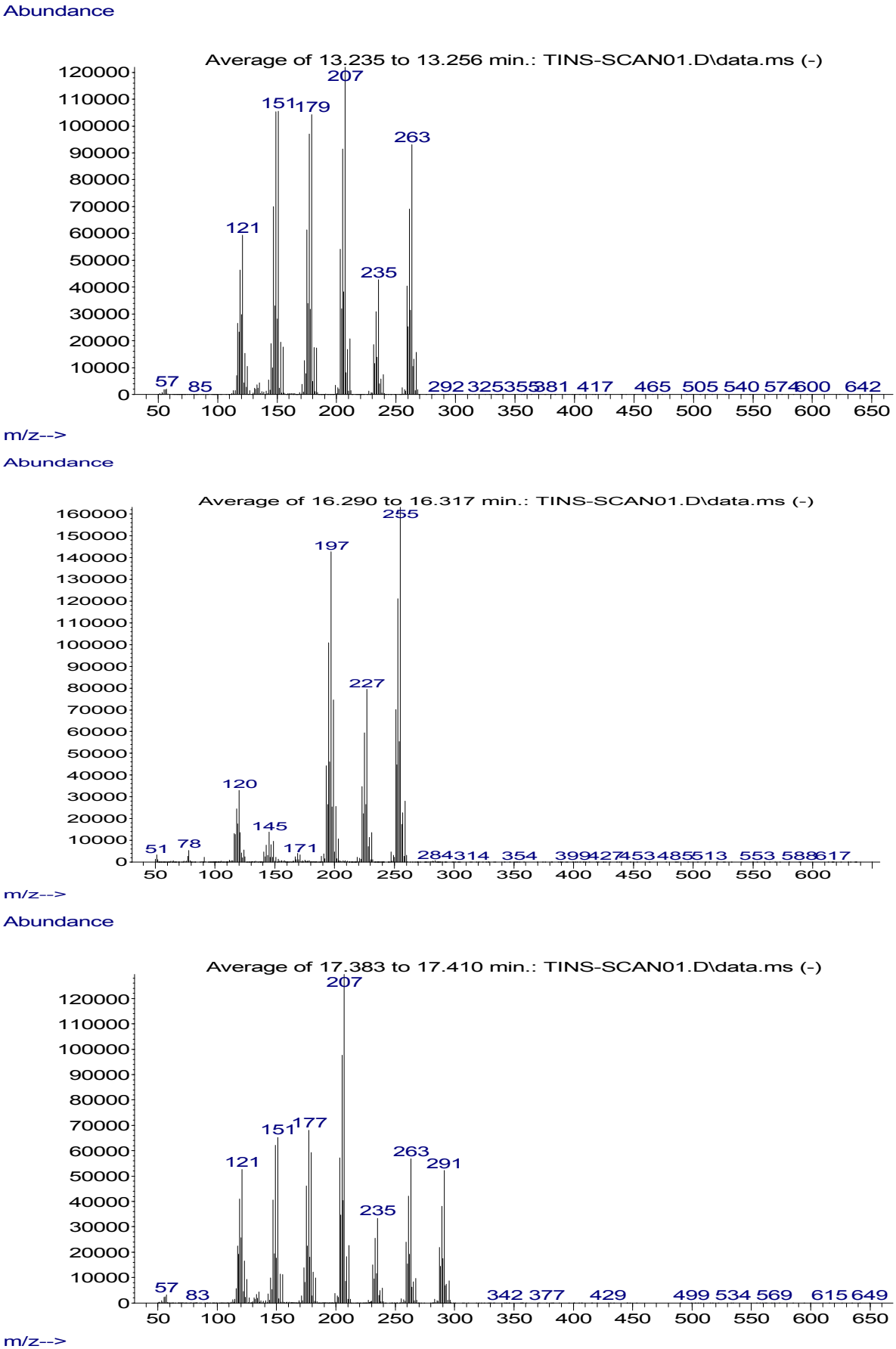

FIG. II.3. MS spectra of different Organotin species

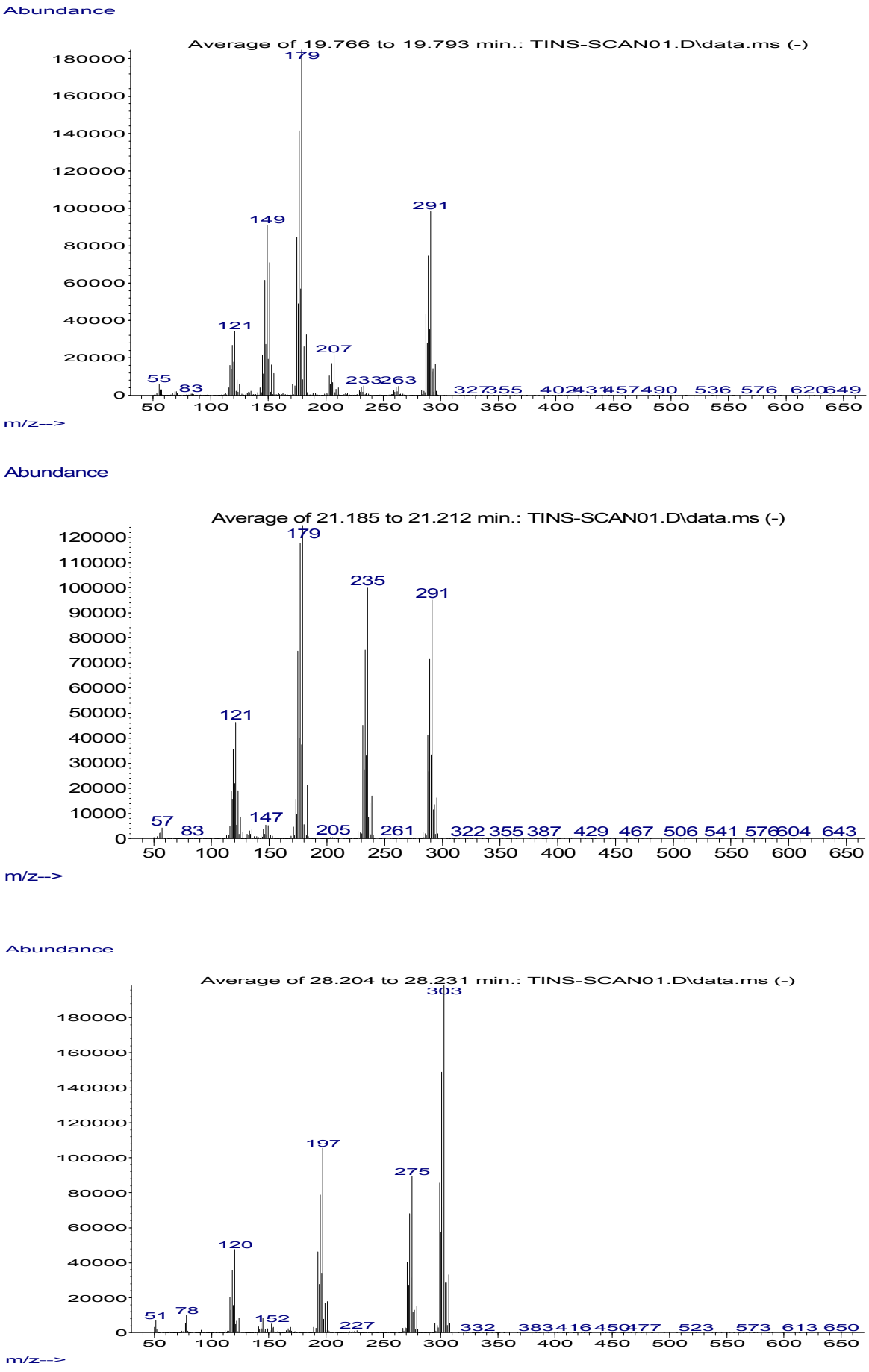

FIG. II.4. MS spectra of different Organotin species

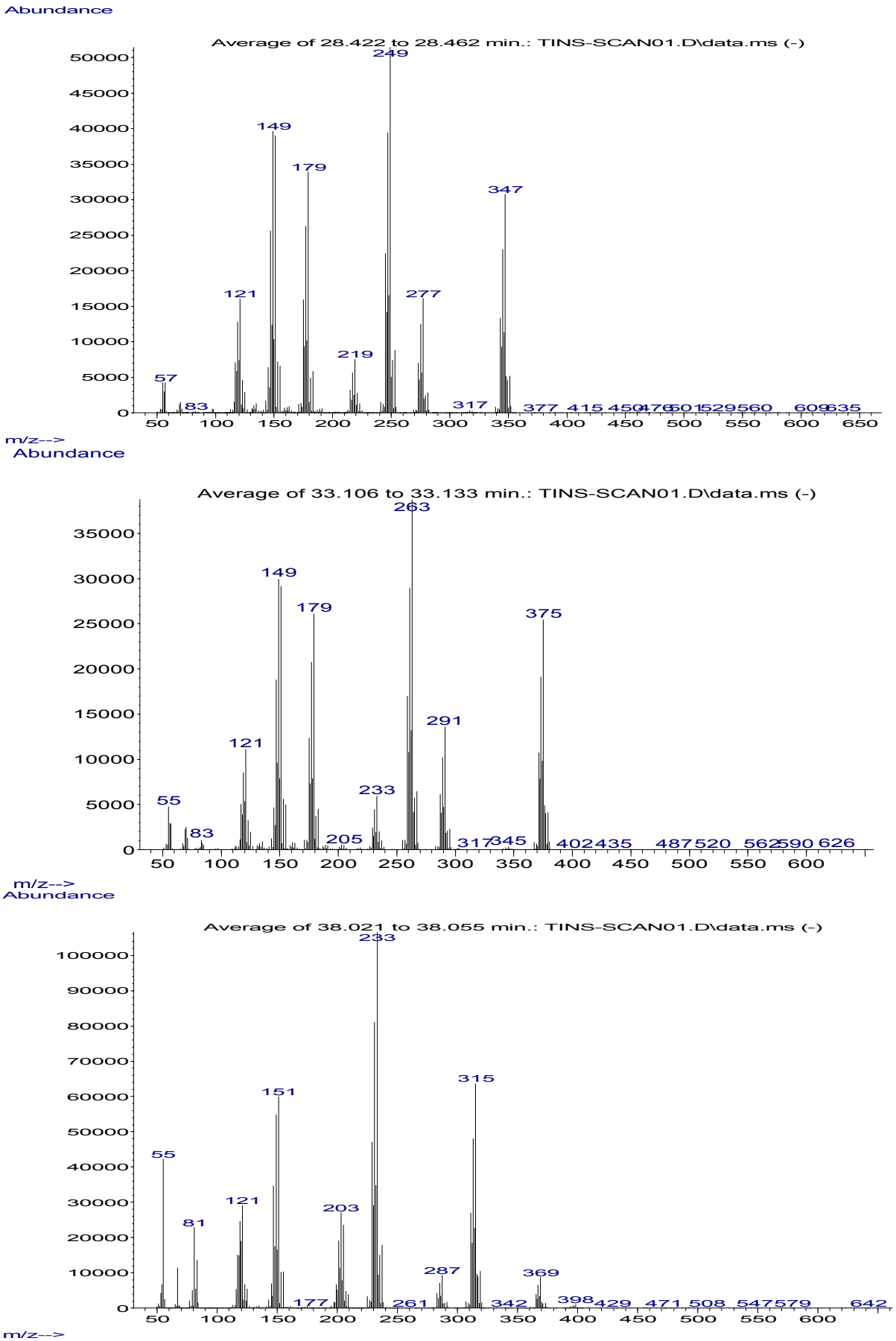

FIG. II.5. MS spectra of different Organotin species

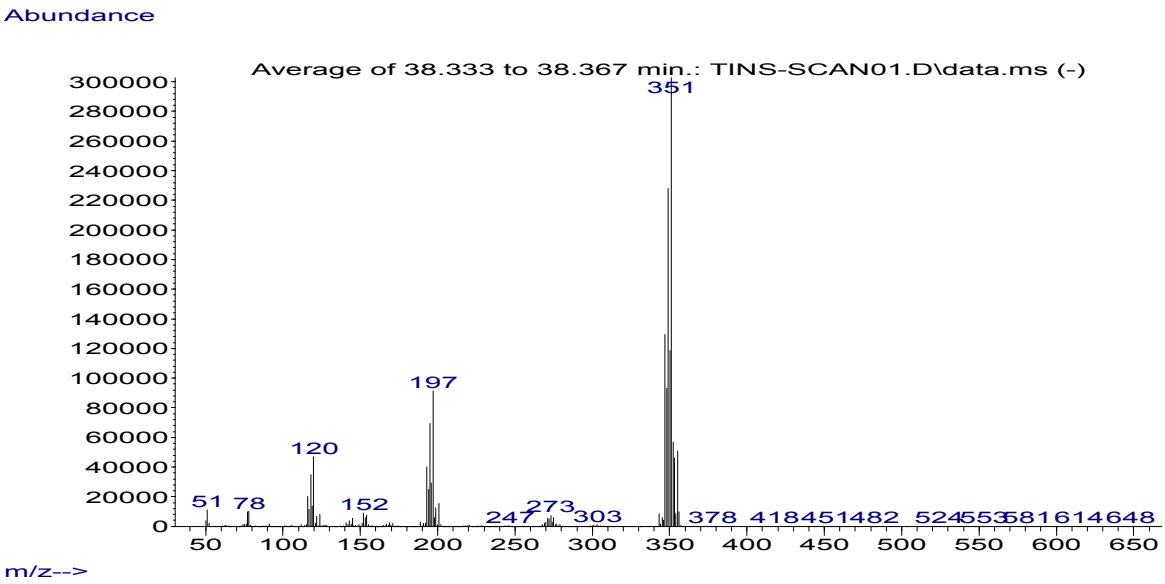

FIG. II.6. MS spectra of different Organotin species

REPORT

Recommended Method for the DETERMINATION OF PETROLEUM HYDROCARBONS IN BIOLOGICAL SAMPLES

IAEA/NAEL Marine Environmental Studies Laboratory in co-operation with UNEP/MAP MED POL

November 2013

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Recommended Method for the DETERMINATION OF PETROLEUM HYDROCARBONS IN BIOLOGICAL SAMPLES

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Table of Contents

NOTE: This method is not intended to be an analytical training manual. Therefore the method is written with the assumption that it will be used by formally trained analytical chemists. Several stages of this procedure are potentially hazardous; user should be familiar with the necessary safety precautions.

In addition, the IAEA recommended methods are intended to be guidance methods that can be used by laboratories as a starting point for generating their own standard operating procedure. If performance data are included in the method they must not be used as absolute QC acceptance criteria.

1. Sampling

Detailed guidelines for collecting biological samples are available in Reference Method No 12 Rev.2, UNEP/FAO/IAEA/IOC.

2. General discussion

Following collection of biological samples using appropriate techniques, samples are stored in non-contaminating jars at -20 °C until analysis. For analysis, the samples are defrosted and prepared for solvent extraction. To achieve a satisfactory recovery of the petroleum hydrocarbons, samples are freeze-dried. Samples are then Soxhlet extracted using methanol. Following initial clean-up treatments (partial removal of lipids by saponification), extracts are fractionated using column chromatography with silica and alumina. Quantification is done by GC-FID and GC-MS. Complementary guidelines for the analytical procedures are available in the Reference Method No 20.

3. Apparatus

- Glass jars and aluminium foil, stainless steel knives, scoops, forceps, labels, marking pens, log book.
- Insulated plastic boxes for transporting samples. Ice or dry ice.
- Deep freezer (-18 to -20 °C) for sample preservation (frost free type freezers heat to above zero during frost removal cycles and they cannot be used for long term storage).
- Rotary evaporator.

- Soxhlet extraction apparatus and heaters or Microwave oven
- Glassware including boiling flasks, ground glass stoppers, beakers, Erlenmeyer flasks, separatory funnels, centrifuge tubes, weighing bottles, pipettes, tissue grinders.
- Drying oven (temperature range up to at least 300 °C) for determining sample dry weights, baking of contaminant residues from glassware and reagents.
- Centrifuge and tubes.
- Freeze-dryer and porcelain mortar and pestle.
- Analytical balance with a precision of 0.1 mg and an electro-balance with a precision of at least 1 μg.
- Stainless steel tweezers and spatulas.
- Dessicator - completely cleaned and with no grease applied to sealing edges.
- Supply of clean dry nitrogen.
- Columns for the silica/alumina chromatography.
- Mechanical blender (food mixer).
- Vacuum pump (water-jet air pump).
- Ultrasonic bath.

4. Reagents

4.1. List of reagents

- Demineralized distilled water produced by distillation over potassium permanganate (0.1 g $KMnO_4$ per liter) or equivalent quality, demonstrated free from interfering substances.
- Detergent.
- Sulfochromic cleaning solution made from concentrated sulfuric acid and potassium dichromate.
- Concentrated H_2SO_4 (d 20°C: 1.84 g/ml).
- H_2SO_4 1M
- KOH
- Potassium dichromate.

- Hexane," distilled in glass" quality.
- Dichloromethane, "distilled in glass" quality.
- Methanol, "distilled in glass" quality.
- Acetone, "distilled in glass" quality.
- Anhydrous sodium sulfate.
- Carborundum.
- Glass wool.
- pH Paper.
- Silica gel Merck Kieselgel 60 (0.04-0.063 mm, 230-400 mesh).
- Aluminium oxide neutral Merck 90 Active (0.063-0.200 mm, 70-230 mesh).
- *n*-C_{24}-d_{50}, Friedeline, Hexamethylbenzene , Naphthalene-d_8, Acenaphtene-d_{10}, Phenanthrene-d_{10}, Chrysene-d_{10}, Perylene-d_{12}, Fluorene-d_{10}, Benzo(a)pyrene-d_{12}.
- Standard solutions of aliphatic and aromatic hydrocarbons.

Working solutions from the stock reference solutions are prepared on a regular basis and stored in clean glass volumetric flasks tightly capped with non-contaminating materials such as Teflon or glass. Extreme care must be taken to ensure that standards have not changed their concentrations through solvent evaporation.

4.2. Cleaning of reagents and adsorbents

4.2.1. Cleaning of glassware

Scrub all glassware vigorously with brushes in hot water and detergent. Rinse with tap water and with distilled water. Rinse with acetone followed by hexane or alternatively bake overnight in an oven at 450 °C. All glassware should be stored in dust free cabinets and tightly sealed with pre-cleaned aluminum foil when not in use. Ideally glassware should be rinsed with the same solvent just before use.

For more detailed information, consult Reference Method No 65: UNEP/IOC/IAEA: Reagent and laboratory ware clean-up procedures for low level contaminant monitoring.

4.2.2. Cleaning of reagents

Powdered or crystalline reagents, such as anhydrous sodium sulfate (Na_2SO_4)*, glass wool* and carborundum boiling chips*, are thoroughly cleaned before use. They are extracted with hexane in a Soxhlet apparatus for 8 hours and then with methanol or dichloromethane for another 8 hours. For those indicated by an *, this will require pre-combustion in a muffle furnace at approximately 400 °C.

4.2.3. Cleaning of adsorbents

Preparation of silica and alumina: silica gel and alumina are pre-cleaned by Soxhlet extraction, first for 8 hours with methanol and then for 8 hours with hexane. They are dried at 50 °C to remove the solvent, then at 200 °C for 8 hours and then stored in amber bottle.

Before use, they are activated at 200 °C for 4 hours and partially deactivated with 5 % water.

The deactivation procedure is carried out by adding the water to the sorbent, and mixing by gentle shaking for a few minutes. The equilibration is reached overnight.

4.2.4. Cleaning of extraction thimbles

Paper extraction thimbles should be cleaned prior to sample extraction. For use in the extraction of biological samples, the extraction can be performed in the Soxhlet apparatus with 250 ml of a mixture methanol / dichloromethane (50:50) for 8 hours cycling the solvent through at a rate of 4 to 5 cycles per hour. Add into the solvent a few carborundum boiling chips to get a regular ebullition.

The use of disposable paper thimbles for the extraction procedure rather than re-usable glass fiber thimbles is recommended due to the difficulties encountered in cleaning the latter.

5. Procedure

5.1. Extraction of freeze-dried samples

A 50 to 100 g fresh weight sub-sample is selected from the sample. This sub-sample is weighed and freeze-dried. When the sub-sample appears to be dry, it is weighed again and the dry to wet ratio is calculated.

Just before starting analysis, as results have to be reported on a dry weight basis, the percent moisture or water content in a sample can be determined by weighing an aliquot, not used for analysis, of the sample before and after drying. The drying is done by heating a few grams (1-2 g) of the sample in an oven at 105°C for 24 hours to a constant weight.
The freeze-dried sub-sample is carefully pulverized in a cleaned pestle and mortar.

5 to 10 grams of freeze-dried sample are extracted with a Soxhlet extractor with 200 ml of methanol. Internal standards are added to the sample in the extraction thimble before extraction starts:

- *n*-C_{24}-d_{50} for the aliphatic hydrocarbon fraction (Friedeline as GC internal standard, spiked right before GC injection)
- Hexamethylbenzene for the unresolved compounds from the aromatic hydrocarbon fraction (Friedeline as GC internal standard, spiked right before GC injection)
- Naphthalene-d_8, Acenaphtene-d_{10}, Phenanthrene-d_{10}, Chrysene-d_{12}, Perylene-d_{12} for the aromatic hydrocarbon fraction (Fluorene-d_{10}, Benzo(a)pyrene-d_{12} as GC internal standard spiked right before GC injection)

After the extraction is completed, 20 ml of 2 M KOH are added to the flask and the extraction is continued for 2 more hours in order to saponify the lipids.

The content of the extraction flask is then transferred into a separatory funnel with 30 ml of water (distilled and extracted with hexane) and extracted with 90 ml of

hexane and re-extracted again twice with 50 ml of hexane. Then all hexane extracts are combined, filtered through glass wool and dried with anhydrous sodium sulfate.

The aqueous phase is not discarded as it is used for total lipid weight determination, it's acidified with 1 M sulfuric acid and extracted 3 times in a separatory funnel with 30 ml of hexane. Then all hexane extracts are combined, filtered through glass wool and dried with anhydrous sodium sulfate.

Alternative method:

Using the microwave oven: 3-5 g of biota is placed in a glass tube with 30 ml of methanol, the oven is set at 1200 Watts, the temperature is programmed to reach 115°C in 10 min. and then isothermal at 115°C for 20 min. (internal standards are added before extraction starts).
After cooling 5 ml of 2 M KOH are added to the glass tube and the oven is set at 1200 Watts, the temperature is programmed to reach 90°C in 5 min. and then isothermal at 90°C for 10 min. in order to saponify lipids.

The content of the glass tube is filtered through glass wool and transferred into a separatory funnel with 5 ml of water (distilled and extracted with hexane) and extracted with 20 ml of hexane and re-extracted again twice with 15 ml of hexane. Then all hexane extracts are combined, filtered through glass wool and dried with anhydrous sodium sulfate.

The aqueous phase is not discarded as it is used for total lipid weight determination, it's acidified with 2 ml of 1 M sulfuric acid and extracted 3 times in a separatory funnel with 10 ml of hexane. Then all hexane extracts are combined, filtered through glass wool and dried with anhydrous sodium sulfate.

5.2. Concentration of the extract

The hexane fraction (50 ml), containing the non-saponifiable lipids and consequently the petroleum hydrocarbons is concentrated with a rotary evaporator down to about 15 ml (maximum temperature: 30°C). Then transferred in a graduated tube and

concentrated with nitrogen down to a volume corresponding to 1 ml/ gram of freeze-dried sample extracted (this will avoid the precipitation of the lipids in the tube).

The hexane fraction (30 ml) containing the saponifiable lipids is concentrated with a rotary evaporator down to about 15 ml and then transferred in a graduated tube and concentrated with nitrogen.
The lipids are weighed with the electro-balance. The total lipid content is the sum of the lipid found in the first hexane fraction and this one. Then this fraction is discarded.

5.3. Extractable organic matter (EOM)

Solvent extractable organic matter (E.O.M.) is determined in the following manner. On the weighing pan of an electrobalance, a known volume of the extract (up to 100 µl) is evaporated and the residue weighed to about ± 1 µg. If the residue is less than 2 µg, pre-concentration of the original extract is required.

The quantity of E.O.M. is

$$\text{E.O.M. (µg/g)} = \frac{\text{Weight of residue (µg) x Volume of extract (ml) x 1000}}{\text{Volume evaporated (µl) x Quantity of sample extracted (g)}}$$

The total E.O.M. is the sum of both non-saponified and saponified lipids.

5.4. Clean-up procedure and fractionation

Especially in the case of biota samples, it is necessary to clean-up the extract before proceeding with the analysis. The clean-up should remove non-petroleum hydrocarbons material that fluoresces under certain conditions. Furthermore, materials that may cause quenching will be removed simultaneously.

5.4.1. Fractionation

The clean-up and separation are achieved by a simple column chromatographic partition as follows:

A chromatography column is prepared using 50 ml burette in which a piece of glass wool is added near the stopcock to maintain the packing material. Then, 5 g of silica are transferred into the column, then 10 g of alumina and on top 1 g of sodium sulfate is added in order to avoid the disturbance of the first layer when solvents are poured into the column.

Separation of compounds:

The sample (maximum 300 mg of non-saponified lipids) is applied on top of the column. A first fraction is obtained by eluting the sample with 20 ml of hexane (F1), this fraction will contain the saturated aliphatics. The second fraction (F2) is obtained by eluting with 30 ml of a mixture of hexane and dichloromethane (90:10), this fraction will contain the unsatured and aromatic hydrocarbons.

6. Gas Chromatography Conditions

6.1. Quantification of petroleum hydrocarbons

Gas Chromatograph	Agilent 7890
Detector	Flame Ionization Detector (FID)
Injection mode	Splitless
Carrier gas	Helium 1.2 ml min-1
Column	HP-5 (crosslinked 5% Ph Me Silicone) 30 m x 0.25 mm i.d. x 0.25 µm film thickness
Injector temperature	270°C
Detector temperature	300°C
Oven temperature program	60°C initial for 1 min., 60°C to 290°C at 4°C min^{-1}, 290°C for 40 min.

6.2. Quantification of PAHs

Gas Chromatograph	Agilent 6890 N
Detector	MSD 5975
Injection mode	Splitless
Carrier gas	Helium 1.5 ml min^{-1}
Column	DB-XLBMSD 30 m x 0.25 mm i.d. x 0.25 µm film thickness
Injection specifications	inj. press.: 13 psi, Constant flow on 13 psi at 60°C, Temp. injector 270°C
Transfer line	280°C
Ion source	240°C
Analyzer	100°C
Oven temperature program	60°C initial, 60°C to 100°C at 10°C min^{-1}, 100°C to 285°C at 4°C min^{-1}, 285°C for 20 min.

6.3. Target ions to use for quantification and confirmation ions and their relative abundance for GC/MS analyses of PAHs

Compound	Target	Confirming	% Abundance
Benzene	78		
C_1 - benzene	92		
C_2 - benzene	106		
C_3 - benzene	120		
C_4 - benzene	134		
d_8 - Naphthalene	136	134	8
Naphthalene	128	127	10
C_1 - naphthalene	142	141	80
C_2 - naphthalene	156	141	47 - 95
C_3 - naphthalene	170	155	61 - 300
C_4 - naphthalene	184	169	189
Acenaphthylene	152	151	20
d_{10} - Acenaphthene	164	162	97
Acenaphthene	154	153	86
d_{10} - Fluorene	176	174	93
Fluorene	166	165	80
C_1 - fluorene	180	165	95 - 144
C_2 - fluorene	194	179	25
C_3 - fluorene	208	193	
d_{10} - phenanthrene	188	187	22
Phenanthrene	178	179	16
Anthracene	178	176	20
C_1 - phenanthrene/anthracene	192	191	39 - 66
C_2 - phenanthrene/anthracene	206	191	16 - 150
C_3 - phenanthrene/anthracene	220	205	
C_4 - phenanthrene/anthracene	234	219, 191	73 - 297
Dibenzothiophene	184	185	14
C_1 - dibenzothiophene	198	197	53
C_2 - dibenzothiophene	212	211	

C_3 - dibenzothiophene	226	211	
C_4 - dibenzothiophene	240	211	
Fluoranthene	202	200	17
Pyrene	202	200	21
C_1 - fluoranthene/pyrene	216	215	36 - 64
Benz[a]anthracene	228	226	19
d_{12} - Chrysene	240	236	26
Chrysene	228	226	21
C_1 - benzanthracene/chrysene	242	243	20
C_2 - benzanthracene/chrysene	256	241	75 - 131
C_3 - benzanthracene/chrysene	270	255	
C_4 - benzanthracene/chrysene	284	269, 241	
d_{12} - perylene	264	260	24
Perylene	252	253	22
Benzo[b or k]fluoranthene	252	253	23
d_{12} - Benzo[a]pyrene	264	260	20
Benzo[a or e]pyrene	252	253	22
Indeno[1,2,3-c,d]pyrene	276	138	50
Dibenz[a,h]anthracene	278	279	24
Benzo[g,h,i]perylene	276	138	37

7. Quantification

The most widely used information for identification of a peak is its retention time, or its relative retention time (i.e., the adjusted retention time relative to that of a selected reference compound). Retention behavior is temperature dependent and comparison of retention times obtained at two or more temperatures may aid in determining a peak's identity. However, retention times are not specific and despite the high resolution offered by capillary columns, two compounds of interest in the same sample may have identical retention times.

In gas chromatography, results are usually quantified by either external calibration or internal calibration. Compounds identification is confirmed by GC-MS.

7.1. External Calibration

An external calibration is performed by injecting standard samples containing varying concentrations of the compound to be analyzed and creating a calibration curve (area vs. concentration). A response factor (RF) is calculated, for each target compounds, using the following equation:

$$RF = \frac{\text{Peak Area}}{\text{Sample Amount}}$$

The unknown samples are injected and the amounts of target compounds are then calculated with the following equation:

$$Amount = \frac{\text{Peak Area}}{\text{Response Factor}}$$

The method based on the external calibration doesn't take into account any variance in gas chromatograph performance and it requires the final volume of sample injected and the final volume of the extract.

7.2. Internal Calibration

This method is based on the use of an ***internal standard*** which is defined as a non-interfering compound added to a sample in known concentration in order to eliminate the need to measure the sample size in quantitative analysis and for correction of instrumental variation.

In this method, the internal standard is added to each sample and standard solution.

In a multiple point internal calibration each analyses contains the internal standard whose total amount is kept constant and the analyte of interest whose amount covers the range of concentrations expected. The ratio of the areas of the internal standard and analyte are then used to construct the calibration curve.

A multiple points relative response factor (RRF) calibration curve is established for analytes of interest for each working batch. A RRF is determined, for each analyte, for each calibration level using the following equation:

Where:

$$RRF(X) = \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{\text{Qty (IS)}}{\text{Qty (X)}}$$

Area (X) = the area of the analyte to be measured (target compound)

Area (IS) = the area of the specific internal standard

Qty (X) = the known quantity of the analyte in the calibration solution

Qty (IS) = the known quantity of the internal standard in the calibration solution

The relative response factors determined for each calibration level are averaged to produce a mean relative response factor (mRRF) for each analyte. The percent relative standard deviation (%RSD) for the 3 response factor must be less than or equal to 15%, for each analyte.

$$\%RSD = \frac{\text{Standard deviation of the RRFs}}{\text{Average of the RFs}} \text{ x } 100$$

Sample analyte concentrations are calculated based on the quantity and response of the internal standard.

The following equation gives the amount of analyte in the solution analysed.

$$Qty\,(\text{X}) = \text{Qty (IS)} \text{ x } \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{1}{\text{mRR}F(X)}$$

Where:

Qty (X) = the unknown quantity of the analyte in the sample

Qty (IS) = the known quantity of the internal standard added to the sample

Area (X) = the area of the analyte

Area (IS) = the area of the internal standard

mRRF (X) = the average response factor of the analyte

Sample analyte concentrations are then calculated by dividing the amount found (Qty) by the grams of samples extracted.

8. Quality assurance/quality control

Guidelines on the QA/QC requirements for analysis of sediments and marine organisms are detailed in Reference Method No 57, "Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice".

The precision of the method is established by the replicate analysis of samples of the appropriate matrix. The precision of the entire analytical procedure is estimated by extracting five sub-samples from the same sample after homogenization. Precision is evaluated as a matter of course during the initial implementation procedure just before initiation of sample analysis.

8.1. Accuracy

The accuracy of the methods is confirmed by analysis of a suitable RM (i.e. appropriate matrix, analytes) prior to initiation of sample analysis. Agreement between measured and certified concentrations for any individual analyte should be within 35 % and on average within 25 %. Reference Materials are introduced on a regular basis (e.g. every 10-15 samples) as a method of checking the procedure. Further description of the preparation of control charts and criteria for data acceptance is elaborated in Reference Method No 57.

8.2. Blanks

Blanks represent an opportunity to evaluate and monitor the potential introduction of contaminants into samples during processing. Contributions to the analyte signal can arise from contaminants in the reagents, those arising from passive contact between the sample and the environment (e.g. the atmosphere) and those introduced during sample handling by hands, implements or glassware. It is essential to establish a consistently low (i.e. with respect to analytes) blank prior to initiating analysis or even the

determination of the method detection limit. In addition, it is necessary to perform blank determinations on a regular basis (e.g. every batch of samples).

8.3. Recovery

Recovery reflects the ability of the analyst to fully recover surrogate compounds introduced to the sample matrix or blank at the beginning of the procedure. The primary criteria for selection of compounds to be used for testing recovery are that they: 1) have physical (i.e. chromatographic/partitioning) properties similar to and if necessary spanning those of the analytes of interest, 2) do not suffer from interferences during gas chromatographic analysis, 3) are baseline resolved from the analytes of interest.

Recovery should be tested on all samples and blanks as a routine matter. Recoveries should be within 60% - 125%. However lower recoveries might be expected for low molecular weight PAHs (d_8-Naphtalene for example) due to their higher volatility. Recoveries higher than 100 % may indicate the presence of interferences.

8.4. Archiving and reporting of results

Every sample should have an associated worksheet which follows the samples and the extracts through the various stages of the procedure and upon which the analyst notes all relevant details. An example of such a worksheet is given below. Each laboratory should construct and complete such a worksheet. Relevant chromatograms should be attached to the worksheet. Analyses should be grouped and composite or summary analysis sheets archived with each group. Final disposal of the data will depend on the reasons for which it was collected but should follow the overall plan model.

All processed samples should be archived at all steps of the procedure:

- deep frozen (in the deep-freezer as it was received).
- freeze-dried (in sealed glass container kept in a dark place).

- extracted (after injection on the GC, sample extracts should be concentrated down to 1 ml and transferred into sealed glass vials, a Pasteur pipette sealed with a butane burner is adequate and cheap).

9. Bibliography

- Reference Method No 20, UNEP/IOC/IAEA: Determination of petroleum hydrocarbons in sediments. UNEP, 1992.
- Reference Method No 65, UNEP/IOC/IAEA: Reagent and laboratory ware clean-up procedures for low-level contaminants monitoring. UNEP, 1995.
- Reference Method No 57, UNEP/IOC/IAEA/FAO: Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice. UNEP, 1990.
- Reference Method No 6, UNEP/FAO/IOC/IAEA: Guidelines for monitoring chemical contaminants in the sea using marine organisms. UNEP, 1993.
- Reference Method No 12 Rev.2, UNEP/FAO/IAEA/IOC: Sampling of selected marine organisms and sample preparation for the analysis of chlorinated hydrocarbons. UNEP, 1991.

REPORT

Recommended Method for the
DETERMINATION OF PETROLEUM HYDROCARBONS IN SEDIMENT SAMPLES

IAEA/NAEL

Marine Environmental Studies Laboratory in co-operation with

UNEP/MAP MED POL

November 2011

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Recommended Method for the DETERMINATION OF PETROLEUM HYDROCARBONS IN SEDIMENT SAMPLES

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TABLE OF CONTENTS

NOTE: This method is not intended to be an analytical training manual. Therefore the method is written with the assumption that it will be used by formally trained analytical chemists. Several stages of this procedure are potentially hazardous; users should be familiar with the necessary safety precautions.

In addition, the IAEA recommended methods are intended to be guidance methods that can be used by laboratories as a starting point for generating their own standard operating procedure. If performance data are included in the method they must not be used as absolute QC acceptance criteria.

1. SAMPLING

Detailed guidelines for collecting the sediment samples are available in UNEP(DEC)/MEDWG.282/Inf.5/Rev.1

2. GENERAL DISCUSSION

Following the collection of sediment samples using appropriate techniques, the samples are stored in non-contaminating jars at -20°C until analysis. For analysis, the samples are defrosted and prepared for solvent extraction. To achieve a satisfactory recovery of the petroleum hydrocarbons, samples are freeze-dried. Sediments are then Soxhlet extracted using hexane and dichloromethane. Following the initial clean-up treatments (removal of sulfur), extracts are fractionated using column chromatography with silica and alumina. Quantification is done by GC-FID and GC-MS.

3. APPARATUS

- A coring device with liners and plunger or a grab sampler; thoroughly cleaned with detergents and solvents before use;
- Glass jars and aluminium foil, stainless steel knives, scoops, forceps, labels, marking pens, log book;
- Insulated plastic boxes for transporting the samples. Ice or dry ice;
- Deep freezer (-18 to -20°C) for sample preservation (frost-free type freezers heat to above zero during frost removal cycles and therefore cannot be used for long term storage);
- Rotary evaporator;
- Soxhlet extraction apparatus and heaters;

- Microwave oven;
- Glassware including boiling flasks, ground glass stoppers, beakers, Erlenmeyer flasks, separatory funnels, centrifuge tubes, weighing bottles, pipettes, tissue grinders;
- Drying oven (temperature range up to at least 300°C) for determining sample dry weights, baking of contaminant residues from glassware and reagents;
- Centrifuge and tubes;
- Freeze-dryer and porcelain mortar and pestle;
- Analytical balance with a precision of 0.1 mg and an electro-balance with a precision of at least 1 µg;
- Stainless steel tweezers and spatulas;
- Dessicator - completely cleaned and with no grease applied to sealing edges;
- Supply of clean dry nitrogen;
- Columns for the silica/alumina chromatography;
- Mechanical blender (food mixer);
- Vacuum pump (water-jet air pump);
- Ultrasonic bath;
- Solid Phase Extraction glass columns: Upti-Clean SPE Glass Columns Si/CN-S (1g/0.5g)/6ml-PTFE Frits;
- 12 or 24 ports Glass Vacuum manifold for the simultaneous use of multiple SPE columns. The manifold must be fitted with flow control valves to allow a fine adjustment of flow through the SPE column.

4. REAGENTS

4.1. LIST OF REAGENTS

- Demineralized distilled water produced by distillation over potassium permanganate (0.1 g $KMnO_4$ per liter) or equivalent quality, demonstrated free from interfering substances.
- Detergent.
- Sulfochromic cleaning solution made from concentrated sulfuric acid and potassium dichromate.
- Concentrated H_2SO_4 (d 20°C: 1.84 g/ml).

- Potassium dichromate.
- HCl, 32 % (Merck).
- Hexane, “distilled in glass” quality.
- Dichloromethane, “distilled in glass” quality.
- Methanol, “distilled in glass” quality.
- Acetone, “distilled in glass” quality.
- Anhydrous sodium sulfate.
- Carborundum.
- Copper powder (Merck, <63 μm, 99 % purity).
- Glass wool.
- Silica gel Merck Kieselgel 60 (0.04-0.063 mm, 230-400 mesh).
- Aluminium oxide neutral Merck 90 Active (0.063-0.200 mm, 70-230 mesh).
- *n*-C_{14} d30, *n*-C_{19} d40, *n*-C_{32} d66.
- Hexamethylbenzene, Cadalene: 1,6-dimethyl-4-(1-methylethyl)naphthalene, Naphthalene-d8.
- Standard solutions of aliphatic and aromatic hydrocarbons.

Working solutions from the stock reference solutions are prepared on a regular basis and stored in clean glass volumetric flasks tightly capped with non-contaminating materials such as Teflon or glass. Extreme care must be taken to ensure that standards have not changed their concentrations through solvent evaporation.

4.2. CLEANING OF REAGENTS AND ADSORBENTS

4.2.1. Cleaning of reagents

Powdered or crystalline reagents, such as anhydrous sodium sulfate (Na_2SO_4)*, glass wool* and carborundum boiling chips*, must be thoroughly cleaned before use. They are extracted with hexane in a Soxhlet apparatus for 8 hours and then with methanol or dichloromethane for another 8 hours. Those indicated by an * will require pre-combustion in a muffle furnace at approximately 400°C.

4.2.2. Cleaning of adsorbents

Silica gel and Alumina are treated chemically. Reagents are first refluxed with methanol or dichloromethane in a Soxhlet apparatus for 8 hours, then with *n*-hexane for the same period. The solvent is removed in a rotary evaporator at a low speed, until the sorbent starts falling down as fine particles. Reagents are then dried in a drying oven at 120°C for 4 hours. Silica

and alumina are activated at 200°C for 4 hours. Sorbents are allowed to cool in the oven. As active sorbents attract water and contaminants from the atmosphere, controlled deactivation is carried out by adding water to the fully active sorbent (5 % by weight).

The deactivation procedure is carried out by adding water to the sorbent, and mixing by gentle shaking for a few minutes. The equilibration takes one day.

4.2.3. Cleaning of glassware

Scrub all glassware vigorously with brushes in hot water and detergent. Rinse five times with tap water and twice with distilled water. Rinse with acetone or methanol followed by hexane or petroleum ether. Bake overnight in an oven at 300°C. All glassware should be stored in dust free cabinets and tightly sealed with pre-cleaned aluminium foil when not in use. Ideally, the glassware should be cleaned just before use.

For more detailed information, consult the Reference Method No 65: UNEP/IOC/IAEA: Reagent and laboratory ware clean-up procedures for low level contaminant monitoring.

5. PROCEDURE

5.1. EXTRACTION OF FREEZE-DRIED SAMPLES

10 to 20 grams of freeze-dried sediment sample, grinded and sieved at 250 µm, are extracted in a Soxhlet extractor with a mixture of hexane and dichloromethane (50:50). Internal standards are added to the sample for recovery: 50 µl of a mixture containing: 30 ng/µl of *n*-C_{14} d30, 32.444 ng/µl of *n*-C_{19} d40, 40 ng/µl of *n*-C_{32} d66 for the first fraction and 30 ng/µl of Hexamethylbenzene and 32.688 ng/µl of Cadalene and 33.3588 ng/µl of Naphthalene-d8 for the second fraction.

The extraction is realized in the Soxhlet with 250 ml of the mixture hexane/dichloromethane (50:50), the siphon cycle is about 10 min. for 8 hours.

5.1.1. Cleaning of extraction thimbles

The paper extraction thimbles should be cleaned prior to sample extraction. For use in the extraction of sediment samples, the extraction can be performed in the Soxhlet apparatus with 250 ml of a mixture hexane/dichloromethane (50:50) for 8 hours, cycling the solvent through at a rate of 4 to 5 cycles per hour. Add into the solvent a few carborundum boiling chips to get a regular ebullition.

The use of disposable paper thimbles for the extraction procedure rather than re-usable glass fibre thimbles is recommended, mainly due to the difficulties encountered in cleaning the latter.

Alternative method:

Using a microwave oven: 10-15 g of sediment is placed in a glass tube with 40 ml of mixture hexane/methylene chloride (50:50), the oven should set at 1200 Watts, the temperature programmed to reach 115°C in 10 min. and then isothermal at 115°C for 30 min. (a combined extraction could be performed for both OC and PH if proper internal standards are added before extraction starts).

5.2. CONCENTRATION OF THE EXTRACT

When the extraction is completed, the extract is evaporated with a rotary evaporator to a volume of about 15 ml (the temperature of the water bath should not exceed 30°C).

The extract is dried with anhydrous sodium sulfate, then transferred in a graduated tube and concentrated down to 4 to 5 ml using a flow of clean nitrogen.

5.3. EXTRACTABLE ORGANIC MATTER (EOM)

The EOM is determined in the following manner: on a weighing pan of an electro-balance, a known volume of the sediment extract is evaporated (up to 100 µl) and the residue is weighted with a precision of about ±1 µg. If the residue is less than 2 µg, pre-concentration of the original extract is required. The quantity of EOM is:

$$\text{EOM (µg/g)} = \frac{\text{Weight of residue (µg) x volume of the extract (ml) x 1000}}{\text{Volume evaporated (µl) x quantity of sample extracted (g)}}$$

5.4. CLEAN-UP PROCEDURE AND FRACTIONATION

Purposes of the clean-up: removal of lipids, whenever present in significant amount; removal of elementary sulfur and sulfur compounds. Both these compound classes can interfere with the gas-chromatographic separation.

5.4.1. Sulfur and sulfur compounds removal

Preparation of Copper:

Transfer about 20 g of the copper powder in an Erlenmeyer. Add enough concentrated HCl to cover the copper powder, agitate. Sonicate for 10 min., agitate, put again in ultrasonic bath and sonicate for 10 min. Throw the used HCl, add some fresh HCl, transfer in ultrasonic bath and sonicate for 20 min., repeat that procedure four times in total. Wash with distilled water, agitate, discard, add water again, transfer in ultrasonic bath and sonicate for 15 min., discard the used water, do it again, up to pH neutral. Wash with acetone, agitate, transfer in ultrasonic bath and sonicate for 15 min., repeat that procedure four times in total. Then use the same procedure with hexane as a solvent.

Keep in hexane (use it immediately and avoid the contact of Cu with air).

Transfer 3 to 4 Pasteur pipettes per sample in the flasks containing the hexane extracts. Let the copper react all night. The presence of sulfur compounds in the sample will be detected by the tarnishing of the copper powder.

5.4.2. Fractionation

The clean-up and separation are achieved by a simple column chromatographic partition as follow:

Preparation of silica and alumina:

Silica gel and alumina are pre-cleaned by Soxhlet extraction, firstly for 8 hours with methanol and then for another 8 hours with hexane. They are dried at 60°C to remove the solvent, then at 200°C for 8 hours and then stored in amber bottle.

Before use, they are activated at 200°C for 4 hours and partially deactivated with 5% water.

A chromatography column is prepared using a 50 ml burette in which a piece of glass wool is added near the stopcock to maintain the packing material. 5 g of silica is poured into the column, followed by 10 g of alumina and on top is added 1 g of sodium sulfate in order to avoid the disturbance of the first layer when solvents are poured into the column.

Separation of compounds:

The sample (maximum 100 mg lipids for sediment) is applied on top of the column. A first fraction is obtained by eluting the sample with 20 ml of hexane (F1), this fraction will contain the saturated aliphatics. The second fraction (F2) is obtained by eluting with 30 ml of a mixture of hexane and dichloromethane (90:10), this fraction will contain the unsatured and aromatic hydrocarbons.

5.4.3. Fractionation: Alternative Method using SPE columns

An alternative method using commercially available Solid Phase Extraction cartridges have been implemented.

This method requires Solid Phase Extraction glass columns (Upti-Clean SPE Glass Columns Si/CN-S 1g/0.5g/6ml-PTFE Frits) and a 12 or 24 ports Glass Vacuum manifold for simultaneous separation on multiple SPE columns. Manifold must be fitted with flow control valves to allow a fine adjustment of flow through the SPE column and disposable Teflon liners.

Once placed onto the manifold, SPE columns are rinsed with 10 ml of *n*-Hexane.

The sample is applied on top of the Solid Phase Extraction columns. The first fraction containing aliphatics compounds is obtained by eluting with 4 ml of *n*-Hexane. The second fraction containing PAHs is obtained by eluting 5 ml of a *n*-Hexane: Dichloromethane (1:1) solution.

6. GAS CHROMATOGRAPHY CONDITIONS

6.1. QUANTIFICATION OF PETROLEUM HYDROCARBONS

Gas Chromatograph:	FISONS GC 8000
Detector:	FID
Injection Technique:	On column
Injector temperature:	30°C
Injection Volume:	1 µl
Carrier gas:	Helium
Flow rate:	1.9 ml/min.
Column used:	
Type of column:	Capillary
Length:	30 m
Diameter:	0.32 mm
Phase:	HP-5MS
Film thickness:	0.25 µm
Temperature program:	
Initial temperature:	60°C
Rate:	3.5 °C/min.
Final temperature:	300°C
Isothermal:	22 min.
Detector temperature:	310°C
Air flow:	320 ml/min.
Hydrogen flow:	27 ml/min.

6.2. QUANTIFICATION OF PAHs

GC/MS:	Agilent MSD5975
Detector:	MS - SIM
Injection Technique:	Splitless
Injector temperature:	270°C
Injection Volume:	1 µl
Splitter closing time:	1 min.
Carrier gas:	Helium
Flow rate:	1.6 ml/min.
Column used:	
Type of column:	Capillary
Length:	30 m
Diameter:	0.25 mm
Phase:	DB-XLBMS
Film thickness:	0.25 µm
Temperature program:	
Initial temperature:	60°C

Isothermal:	1 min.
First rate:	10°C/min.
To:	100°C
Second rate:	3.5°C/min.
To:	290°C
Isothermal:	10 min.
Interface temperature:	290°C
Source temperature:	230°C

6.3. TARGET AND CONFIRMATION IONS FOR GC/MS ANALYSES OF PAHs

Compound	Target	Confirming	% Abundance
Benzene	78		
C_1 - benzene	92		
C_2 - benzene	106		
C_3 - benzene	120		
C_4 - benzene	134		
Naphthalene	128	127	10
C_1 - naphthalene	142	141	80
C_2 - naphthalene	156	141	47-95
C_3 - naphthalene	170	155	61-300
C_4 - naphthalene	184	169	189
d_{10} - diphenyl	164	162	32
Acenaphthylene	152	151	20
Acenaphthene	154	153	86
Fluorene	166	165	80
C_1 - fluorene	180	165	95-144
C_2 - fluorene	194	179	25
C_3 - fluorene	208	193	
d_{10} - phenanthrene	188	187	98
Phenanthrene	178	179	16
Anthracene	178	176	20
C_1 - phenanthrene/anthracene	192	191	39-66
C_2 - phenanthrene/anthracene	206	191	16-150
C_3 - phenanthrene/anthracene	220	205	
C_4 - phenanthrene/anthracene	234	219, 191	73-297
Dibenzothiophene	184	185	14
C_1 - dibenzothiophene	198	197	53
C_2 - dibenzothiophene	212	211	
C_3 - dibenzothiophene	226	211	
C_4 - dibenzothiophene	240	211	
Fluoranthene	202	200	17
Pyrene	202	200	21

C_1 - fluoranthene/pyrene	216	215	36-64
Benz[a]anthracene	228	226	19
Chrysene	228	226	21
C_1 - benzanthracene/chrysene	242	243	20
C_2 - benzanthracene/chrysene	256	241	75-131
C_3 - benzanthracene/chrysene	270	255	
C_4 - benzanthracene/chrysene	284	269, 241	
d_{12} - perylene	264	260	21
Perylene	252	253	22
Benzo[b or k]fluoranthene	252	253	23
Benzo[a or e]pyrene	252	253	22
Indeno[1,2,3-c,d]pyrene	276	138	50
Dibenz[a,h]anthracene	278	279	24
Benzo[g,h,i]perylene	276	138	37

7. QUANTIFICATION

The most widely used information for identification of a peak is its retention time, or its relative retention time (i.e. the adjusted retention time relative to that of a selected reference compound). The retention behavior is temperature dependent, and comparison of retention times obtained at two or more different temperatures may aid in determining a peak's identity. However, retention times are not specific and despite the high resolution offered by capillary columns, two compounds of interest in the same sample may have identical retention times.

In gas chromatography, results are usually quantified by either external calibration or internal calibration.

7.1. EXTERNAL CALIBRATION

An external calibration is performed by injecting standard samples containing varying concentrations of the compound to be analyzed, and creating a calibration curve (area vs. concentration). A response factor (RF) is calculated, for each target compounds, using the following equation:

$$RF = \frac{\text{Peak Area}}{\text{Sample Amount}}$$

The unknown samples are injected and the amounts of target compounds are then calculated with the following equation:

$$Amount = \frac{\text{Peak Area}}{\text{Response Factor}}$$

The method based on the external calibration do not take into account any variance in gas chromatograph performance.

7.2. INTERNAL CALIBRATION

This method is based on the use of an ***internal standard,*** which is defined as a non-interfering compound added to a sample in known concentration, in order to eliminate the need to measure the sample size in quantitative analysis, and the correction of instrumental variation.

In this method, the internal standard is added to each sample and standard solution.

In a multiple point internal calibration each analyses contains: the internal standard whose total amount is kept constant and the analyte of interest, whose amount covers the range of concentrations expected. The ratio of the areas of the internal standard and analyte are then used to construct the calibration curve.

A multiple points relative response factor (RRF) calibration curve is established for analytes of interest for each working batch. A RRF is determined, for each analyte, for each calibration level using the following equation:

$$RRF(X) = \frac{\text{Area (X)}}{\text{Area (IS)}} \text{ x } \frac{\text{Qty (IS)}}{\text{Qty (X)}}$$

Where:

Area (X) = the area of the analyte to be measured (target compound);

Area (IS) = the area of the specific internal standard;

Qty (X) = the known quantity of the analyte in the calibration solution;

Qty (IS) = the known quantity of the internal standard in the calibration solution.

The relative response factors determined for each calibration level are averaged to produce a mean relative response factor (mRRF) for each analyte. The percent relative standard deviation (%RSD) for the 3 response factor must be less than or equal to 15%, for each analyte.

$$\%RSD = \frac{\text{Standard deviation of the RRFs}}{\text{Average of the RFs}} \text{ x } 100$$

The sample analyte concentrations are calculated based on the quantity and response of the internal standard.

The following equation gives the amount of analyte in the solution analysed.

$$Qty\,(\mathrm{X}) = \mathrm{Qty\,(IS)\ x}\ \frac{\mathrm{Area\,(X)}}{\mathrm{Area\,(IS)}}\ \mathrm{x}\ \frac{1}{\mathrm{mRR}F(X)}$$

Where:

Qty (X) = the unknown quantity of the analyte in the sample;

Qty (IS) = the known quantity of the internal standard added to the sample;

Area (X) = the area of the analyte;

Area (IS) = the area of the internal standard;

mRRF (X) = the average response factor of the analyte.

The sample analyte concentrations are then calculated by dividing the amount found (Qty) by the grams of samples extracted.

8. QUALITY ASSURANCE/QUALITY CONTROL

Guidelines on the QA/QC requirements for analysis of sediments and marine organisms are detailed in the Reference Method No 57, "Contaminant monitoring programs using marine organisms: Quality assurance and good laboratory practice".

The precision of the method is established by the replicate analysis of the samples of the appropriate matrix. The precision of the entire analytical procedure is estimated by extracting five sub-samples from the same sample after homogenization. The precision is evaluated as a matter of course during the initial implementation procedure, just before initiation of sample analysis.

8.1. ACCURACY

The accuracy of the method(s) is confirmed by the analysis of a suitable RM (i.e. appropriate matrix, analytes) prior to initiation of sample analysis. Agreement between measured and certified concentrations of any individual analyte should be within 35% and on average within 25%. Reference Materials are introduced on a regular basis (e.g. every 10-15 samples) as a method of checking the procedure. Further description of the preparation of control charts and criteria for data acceptance is elaborated in the Reference Method No 57.

8.2. BLANKS

Blanks represent an opportunity to evaluate and monitor the potential introduction of contaminants into samples during processing. Contributions to the analyte signal can arise from contaminants in the reagents, from passive contact between the sample and the environment (e.g. the atmosphere) and those introduced during sample handling by hands, implements or glassware. It is essential to establish a consistently low (i.e. with respect to

analytes) blank prior to initiating analysis, or even the determination of the method detection limit. In addition, it is necessary to perform blank determinations on a regular basis (e.g. every batch of samples).

8.3. RECOVERY

Recovery reflects the ability of the analyst to fully recover surrogate compounds introduced to the sample matrix or blank at the beginning of the procedure. The primary criteria for the selection of compounds to be used in testing recovery are, that they: 1) have physical (i.e. chromatographic/partitioning) properties similar to and if necessary spanning those of the analytes of interest; 2) do not suffer from interferences during gas chromatographic analysis; 3) are baseline resolved from the analytes of interest.

Recovery should be tested on all samples and blanks as a routine matter of course. Recoveries below 70% are to be considered unacceptable. Recoveries in excess of 100% may indicate the presence of interference.

8.4. ARCHIVING AND REPORTING OF RESULTS

Every sample should have an associated worksheet which follows the samples and the extracts through the various stages of the procedure and upon which the analyst notes all relevant details. An example of such a worksheet is given below. Each laboratory should construct and complete such worksheet. Relevant chromatograms should be attached to the worksheet. Analyses should be grouped and composite or summary analysis sheets archived with each group. The final disposal of the data will depend on the reasons for which it was collected, but should follow the overall plan model.

All processed samples should be archived at all steps of the procedure:

- deep frozen (in the deep-freezer as it was received).

- freeze-dried (in sealed glass container kept in a dark place).

- extracted (after injection on the GC, sample extracts should be concentrated down to 1 ml and transferred into sealed glass vials, a Pasteur pipette sealed with a butane burner is adequate).

9. REFERENCES

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Reference Method No 65, UNEP/IOC/IAEA: Reagent and laboratory ware clean-up procedures for low-level contaminants monitoring. UNEP, 1995.

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Alzaga R., Montuori P., Ortiz L., Bayona J., Albaiges J. Journal of Chromatography A, 1025 (2004) 133-138

REPORT

Recommended Method for the

DETERMINATION OF TOTAL MERCURY IN MARINE SAMPLES BY THERMAL DECOMPOSITION AMALGAMATION AND ATOMIC ABSORPTION SPECTROPHOTOMETRY

IAEA/NAEL

Marine Environmental Studies Laboratory in co-operation with

UNEP/MAP MED POL

December 2012

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Recommended Method for the DETERMINATION OF TOTAL MERCURY IN MARINE SAMPLES BY THERMAL DECOMPOSITION AMALGAMATION AND ATOMIC ABSORPTION SPECTROPHOTOMETRY

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TABLE OF CONTENTS

NOTE: This method is not intended to be an analytical training manual. Therefore, this method is written with the assumption that it will be performed by formally trained analytical chemist.

In addition, the IAEA recommended methods are intended to be guidance methods that can be used by laboratories as a starting point for generating their own standard operating procedure. If performance data are included in the method, they shall not be used as absolute QC acceptance criteria.

1. SCOPE

The method hereinafter describes the protocol for the determination of total mercury (inorganic and organic) in sediment and biological material.

By using this method, the total mercury in solid samples can be determined without sample chemical pre-treatment.

The recommended protocol is mainly based on the EPA 7473 method; users are encouraged to consult this document (EPA, 2007).

2. PRINCIPLE

The sample is dried and then chemically decomposed under oxygen in the decomposition furnace. The decomposition products are carried out to the catalytic section of the furnace, where oxidation is completed (halogens and nitrogen/sulfur oxides are trapped). The mercury present in the remaining decomposition products is selectively trapped on an amalgamator. After flushing the system with oxygen, the mercury vapour is released by rapid heating of the amalgamator, and carried through the absorbance cell in the light path of a single wavelength atomic absorption spectrophotometer. The absorbance is measured at 253.7 nm as a function of mercury quantity (ng).

The typical working range is 0.1–500 ng. The mercury vapour is carried through a long (first) and a short path length absorbance cell. The same quantity of mercury is measured twice with different sensitivity resulting in a dynamic range that spans four orders of magnitude.

The typical detection limit is 0.01 ng of mercury.

3. SAMPLE PRE-TREATMENT

The sediment samples are prepared following the recommendations of UNEP (2005);

The marine organisms are prepared following the recommendations of UNEP (1984, 1994).

4. REAGENTS

The reagents used shall meet the purity requirement of the subsequent analysis

4.1. ULTRAPUR WATER (type MilliQ)

4.2. NITRIC ACID 65%

4.3. POTASSIUM DICHROMATE OXIDIZING SOLUTION (10% w/v)

Weight 25 g of $K_2Cr_2O_7$ in 250 ml glass bottle, fill it up to 250 ml with water, and shake until total dissolution of solids. Keep the bottle tightly closed in a double plastic bag, and in an Hg free environment (i.e. laminar flow hood). This solution is stable indefinitely and rarely becomes contaminated.

4.4. COMMERCIAL STANDARD SOLUTION 1000 µg ml^{-1} MERCURY

Use a certified reference material solution; this solution should be accompanied by a certificate stipulating at minimum the traceability of the certified concentration, as well as the expiry date. The density of the solution, or the certified content in mg kg^{-1} should also be defined, to allow for the preparation of the calibration solution by weighing. Stock solutions should be kept at 5°C.

5. MATERIAL

5.1. SOLID MERCURY ANALYZER

Optionally equipped with an auto-sampler.

5.2. ANALYTICAL BALANCE

With a 0.001 g precision at least.

5.3. VOLUMETRIC CONTAINERS

Preferably in Teflon or glass.

5.4. PIPETTES

Some microliter pipettes sized ranging from 50 to 10000 µl are needed. The accuracy and precision of the pipettes used should be checked as a routine every 6 months, and the results obtained should be compared with the individual certificates.

5.5. METAL SPATULA (inox).

5.6. SAMPLE BOAT

Metal or metal alloy. Before measurement, sample boats are cleaned by heating over a flame until constant “red” to remove mercury.

5.7. OXYGEN

It should be of high purity and free of mercury. If there is a possible mercury contamination from oxygen, install a gold mesh filter between the cylinder and the instrument to prevent any mercury from entering the instrument.

6. CALIBRATION

6.1. PRIMARY CALIBRATION. This is the calibration of the instrument working range. This calibration is performed initially (usually done by the manufacturer and stored in the instrument), and/or when any significant instrumental parameters are changed (i.e. after maintenance).

6.2. PREPARE STANDARD SOLUTIONS of appropriate concentration by dilution of a commercial standard (see 4.4). It is recommended to prepare standard solution in Teflon or glass container, in 1 or 0.5% HNO_3 (see 4.2) and 0.1% (v/v) potassium dichromate (see 4.3). Fresh mercury standard should be prepared daily. Prepare a zero calibration solution using the same quantity of acid and potassium dichromate.

6.3. START THE INSTRUMENT according to the manufacturer recommendations.

6.4. CLEAN THE SYSTEM. Inject 100 µl of water and start the measurement with the recommended parameters (see 7.1). Repeat the cleaning until the absorbance is below 0.001ABS.

6.5. SET THE INSTRUMENT PARAMETERS (see 7.1) for selected volume (usually 100 µl) and inject the zero calibration, at least three measurements should be done. The zero solution serves to correct the amount of mercury in water and reagent used for preparing the calibration curve, hence the important of keeping the injected volume equal at all points of the calibration curve. If the amount of mercury in the zero calibration is high (i.e. more than 0.01 ng), it is recommended to check for contamination sources and to prepare new standard solution with clean acid.

6.6. STANDARDS ARE MEASURED from the lowest to the highest at least twice. The maximum relative standard deviation between readings should be 3% (except for zero calibration); if higher it is recommended to carry out more measurements.

6.7. EXAMPLE OF AMOUNTS used for recalibration (primary):

First Range:

Standard (ng ml^{-1})	1	3	10	30	100	300
Volume injected (µl)	100	100	100	100	100	100
Quantity of Hg (ng)	0.1	0.3	1	3	10	30

Second Range:

Standard (µg ml^{-1})	1	2	3	4	5	6
Volume injected (µl)	100	100	100	100	100	100
Quantity of Hg (ng)	100	200	300	400	500	600

Note: The calibration of the second range might induce problems for subsequent analysis, due to the relatively high quantity of mercury introduced (especially with memory effect). It should be performed only if there is a probability of using it (i.e. measuring samples with high mercury level > 1µg g^{-1}). After the reading of the last calibration point, clean the system (see 6.4).

6.8. ALTERNATIVE CALIBRATION CURVE can be performed using a solid certified reference material. In this case, weigh accurately a CRM onto a tare sample boat, set up the instrument according to the sample type (see 7.1) and measure the absorbance. The matrix of the CRM should be as similar as possible to the sample of interest. Repeat this procedure with different weights of the CRM and/or with different CRM, to get results in the desired working range.

6.9. CONSTRUCT A CALIBRATION CURVE by plotting the absorbance against Nano grams of mercury (this could be done automatically by the software). The type of equation will depend on the levels, as the response is not linear over the entire working range.

6.10. DAILY CALIBRATION: calibration performed every day with a minimum number of standards to ensure that the primary calibration is valid. It can be performed by using either liquid standard (see 6.2) or solid certified reference material (CRM) see 6.8. It should be performed in the range of interest, with at least two standards (or matrix CRM) and the results should agree within the acceptance criteria. The acceptance criteria should be set through the use of historical data, but the maximum deviation should not exceed 10%.

7. PROCEDURE

7.1. GENERAL ANALYTICAL PARAMETERS

The analytical parameters will depend on the sample size and matrix, and are instrument specific. It is important to follow the guidelines from the instrument manufacturer. There are three time to set: drying, decomposition and waiting.

Some typical recommended conditions below:

Drying time:

Sample type	Dry (s)	Comments
Liquid	0.7 x injected Volume (µl)	
Dry inorganic	10	
Organic liquid	50–300	To be optimized[1]
Dry organic (i.e. fat)	50–200	To be optimized[1]
Wet (i.e. fresh)	0.7 x weight x % moisture	Example: 100 mg with 45% moisture 0.7 x 100 x 0.45= 31.5s (35)

[1] In the case of organic, there is a risk of explosion especially with organic liquid; to optimize set the instrument at: 300s dry/ 150s decomposition/ 45s wait, do the measurement and check for possible small explosion, note the time of the phenomenon and add to the drying time 10s more.

Decomposition time:

Sample type	Decomposition (s)	Comments
Liquid	150–400	To be optimized[1]
Solid inorganic	120 + 0.4 x sample (mg)	To be optimized[1]
Solid organic	120	

[1] Set the instrument to XX (see above) dry/ 400s decomposition/ 45s wait, run a sample and observe the results. Decrease the decomposition time by 30s and repeat measurement. Continue until you observe a significant decrease, note that time and add to the decomposition time 30s more.

Waiting time:

It is recommended to use 40–45s, except for long decomposition time (over 200s) when it is beneficial to add 10s of waiting for every 100s of decomposition.

Note: These indications above are recommended by ALTECH (AMA 254).

7.2. ANALYSIS OF A SOLID SAMPLE

Weight a sample accurately onto a tare boat, insert the boat into the instrument, set the appropriate parameters (see 7.1) and start the measurements. The results can be records on absorbance, quantity or concentration depending on the instrument software. See 9: Calculation of results.

7.3. ANALYSIS OF BLANK FOR SOLID MEASUREMENT

Analyse an empty sample boat using the same instrument settings than for the sample.

7.4. ANALYSIS OF A LIQUID SAMPLE

Dose a known volume of the sample onto a sample boat, set the appropriate parameters (see 7.1) and start the measurements. The results can be records on absorbance, quantity or concentration depending on the instrument software. See the calculation section (see 9).

7.5. ANALYSIS OF BLANK FOR LIQUID

Repeat 7.4 with the same volume of blank solution (solution that contain the same reagent and chemical than the sample).

8. QUALITY CONTROL

8.1. For every day of analysis, the CALIBRATION SHOULD BE VALIDATED by doing a daily calibration (see 6.10) before starting the measurements. The results of the daily calibration should be recorded for quality control purposes.

8.2. CERTIFIED REFERENCE MATERIAL

At least one certified reference material of a representative matrix should be measured with each batch of the sample, the calculated results should fall in the value of the certificate and within the coverage uncertainty (Linsinger, 2010), to show evidence of unbiased results. The results for the CRM should be recorded for quality control purpose and plotted in a control chart (UNEP/IOC/IAEA 1994).

8.3. A DUPLICATE OR TRIPLICATE SAMPLE should be processed on a routine basis.

A duplicate sample should be processed with each analytical batch or for every 10 samples.

8.4. A SPIKED SAMPLE should also be included, whenever a new sample matrix is being analysed, especially if no certified reference material is available for that matrix. Measure a spiked sample by adding a known volume of standard solution (prepared as in paragraph 6.2) to the sample in the boat. Keep the spike volume small enough not to overspill. The recovery of spike calculated with the equation 2 should be 85–115% (this limits should be reset after collection of historical data). If the test fails, it is recommended to check the calibration (see 6.10) and/or to revise the instrument parameters (see 7.1).

$$\text{Spike (ng)} = \text{Concentration of standard (ng/ml)} \times \text{Volume of spike (ml)} \qquad \text{Equation 1}$$

$$\text{Recovery (\%)} = \frac{\text{Spiked sample (ng)} - \text{Unspiked sample (ng)}}{\text{Spike (ng)}} \times 100 \qquad \text{Equation 2}$$

To be valid the quantity of Spike (equation 1) should be in the range of 50–150% the quantity of unspiked sample.

9. CALCULATION OF RESULTS

9.1. SOLID SAMPLE RESULTS are calculated using equation 3

$$w(Hg) = \frac{(\rho 1 - \rho 0)}{\mathrm{m}} \times R \qquad \text{Equation 3}$$

Where:

w(Hg) is the mass fraction of element m in the sample, expressed in mg kg^{-1};

ρ1 is the quantity of mercury, expressed in ng as measured in the sample;

ρ0 is the quantity of mercury expressed in ng as measured in the blank (see 7.3);

R is the recovery calculated using the CRM (see 8.2) or spike (see 8.4);

m is the amount of sample in mg.

Note: ρ1 and ρ0 are calculated using calibration curve equation (usually done by software).

9.2. LIQUID SAMPLE RESULTS are calculated using equation 4

$$w(Hg) = \frac{\frac{(\rho 1 - \rho 0)}{Vi} \times V}{\mathrm{m}} \times f \times R \qquad \text{Equation 4}$$

Where:

w(Hg) is the mass fraction of mercury in the sample, expressed in mg kg^{-1};

ρ1 is the quantity of mercury, expressed in ng as measured in the sample solution;

ρ0 is the quantity of mercury expressed in ng as measured in the blank solution (see 7.4);

R is the recovery calculated using the CRM (see 8.2) or spike (see 8.4);

Vi is the injected volume (should be the same in sample and blank solution) in ml;

m is the amount of sample in mg;

V is the volume of solution in ml;

f is the dilution factor.

Note: ρ1 and ρ0 are calculated using calibration curve equation (usually done by software).

10. EXPRESSION OF RESULTS

The rounding of values will depend on the uncertainty reported with the results; in general for this method two or three significant figures should be reported.

The uncertainty component should be reported with all results. (ISO 2005, Nordtest 2004)

Example: w(Hg) = 0.512 ± 0.065 mg kg^{-1}.

11. REFERENCES

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REPORT

Recommended Method for the

DETERMINATION OF TOTAL MERCURY IN SAMPLES OF MARINE ORIGIN BY COLD VAPOUR ATOMIC ABSORPTION SPECTROMETRY

IAEA/NAEL

Marine Environmental Studies Laboratory in co-operation with

UNEP/MAP MED POL

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TABLE OF CONTENT

NOTE: This method is not intended to be an analytical training manual. Therefore, the method is written with the assumption that it will be used by formally trained analytical chemists.

In addition, the IAEA recommended methods are intended to be guidance methods that can be used by laboratories as a starting point for generating their own standard operating procedure. If performance data are included in the method they must not be used as absolute QC acceptance criteria.

1. SCOPE

This method describes a protocol for measurement of total mercury by cold vapour atomic absorption spectrometry (CV-AAS). The method is simple, rapid and applicable to a large number of environmental samples. This method is applicable when the element content in the digested solution is above the method limit (~ 0.15 ng ml^{-1} depending on instrument). The typical working range is 0.25–100 ng ml^{-1} for direct injection of cold vapour, using "batch system"; FIAS or amalgamation accessory will give better sensitivity.

2. PRINCIPLE

The sediment or biological samples are mineralized with strong acids. The inorganic mercury is reduced to its elemental form with stannous chloride. The cold mercury vapour is then passed through the quartz absorption cell of an atomic absorption spectrometer (AAS), where its concentration is measured. The light beam of Hg hallow cathode lamp is directed through the quartz cell, into a monochromator and onto 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 proportional to the concentration of the element in the sample.

3. REAGENT

The reagents used shall meet the purity requirement of the subsequent analysis:

3.1. WATER

Reagent water (referenced also as water in the text) should be free of contamination.

3.2. NITRIC ACID 65%

3.3. HYDROCHLORIC ACID (37%)

3.4. HYDROGEN PEROXIDE

3.5. VANADIUM PENTOXIDE (V_2O_5)

3.6. SILICON ANTI-FOAMING

3.7. HYDROXYLAMINE HYDROCHLORIDE ($NH_2OH.HCl$)

Dissolve 12.0 g of $NH_2OH.HCl$ in 100 ml reagent water. This solution may be purified by the addition of 0.1 ml of $SnCl_2$ solution and purging 1 hour with Hg-free argon.

3.8. POTASSIUM DICHROMATE OXIDIZING SOLUTION (10% w/v)

Weight 25 g of $K_2Cr_2O_7$ in a 250 ml glass bottle, fill it up to 250 ml with water, and shake until total dissolution of the solid. Keep the bottle tightly closed in a double plastic bag, and in an Hg free environment (i.e. laminar flow hood). This solution is stable indefinitely and rarely becomes contaminated.

3.9. BrCl OXIDIZING SOLUTION

Weigh accurately 11 g of KBrO3 and 15 g of KBr into a clean 1 liter glass bottle. Add 200 ml of Milli-Q water; add carefully 800 ml of concentrated HCl. The dilution has to be carried out in a well-ventilated fume hood to prevent exposure to toxic fumes released during dissolution of KBrO3. Keep the bottle wrapped in aluminium foil, tightly closed in a double plastic bag, and in an Hg free environment (i.e. laminar flow hood). This solution is stable indefinitely but can become contaminated.

3.10. STANNOUS CHLORINE SOLUTION 20% (w/v) in 20% (w/v) HCl

Weigh 20 g of $SnCl_2$ in a 100 ml volumetric flask; add 20 ml of concentrated HCl; dissolve the $SnCl_2$ (if needed heat at 60°C for a few minutes on a hot plate); complete to 100 ml with water. This solution might be purified by bubbling with Hg-free argon for 15 minutes. The obtained solution should be clear and transparent, cloudy or yellow solution indicates a bad quality $SnCl_2$. This solution should be prepared fresh every day preferably, if not it should be kept in the fridge.

Note: The concentration of this solution is dependent on the type of accessory use for vapour generation, and can vary between 5 and 30%, the recommendation of the manufacturer

should be followed (i.e. the solution above is recommended for a VGA-70 from Varian). The validity of the solution (i.e. shelf-life) should be defined during method validation.

3.11. COMMERCIAL STANDARD SOLUTION 1000 µg ml^{-1}

Use a certified reference material solution; this solution should be accompanied by a certificate stipulating at minimum the traceability of the certified concentration, as well as the expiry date. The density of the solution, or the certified content in mg kg^{-1} should also be defined, to allow for the preparation of the calibration solution by weighing. Stock solutions should be kept at 5°C.

3.12. ARGON

Use of a gas purifier cartridge for removing mercury, oxygen and organic compounds is recommended.

4. MATERIAL

This section does not list the common laboratory glassware.

4.1. ATOMIC ABSORPTION SPECTROPHOTOMETER

Instrument equipped with an appropriate cold vapour generation system and a quartz or glass tube atomizer. Use a hollow cathode lamp or, preferably, an electrodeless discharge lamp (which gives a greater and more stable light intensity), operated at a current recommended for the lamp and by the instrument manufacturer. An AAS system with background correction device is recommended.

4.2. GLASSWARE

All the glassware, polypropylene, or fluorocarbon (PFA or TFM) containers, including the sample bottles, flasks and pipettes tips, should be washed in the following sequence:

- 24 hrs soaking in a laboratory soap (or 10% alcohol);
- followed by 24 hrs soaking in 10% nitric acid;
- followed by 10% soaking in water;
- final rinse in water; and
- drying under a laminar flow hood.

The cleaned items should be kept in a double sealed plastic bag. It is better to avoid storage of low level (< 5 ng ml^{-1}) solution in plastic, and for this purpose glass or Teflon is recommended.

If it can be documented, through an active analytical quality control program, using spiked samples and method blanks, then certain steps in the cleaning procedure would not be needed for routine samples, those steps may be eliminated from the procedure (i.e. for the levels

measured by flame AAS, some sterile plastic containers are sufficiently free of contamination for certain analytes).

4.3. PIPETTES

Some microliter pipettes size ranging from 50 to 10000 µl are needed. The accuracy and precision of the pipettes used should be checked as a routine every 6 months, and the results obtained should be compared with the individual certificates.

4.4. VOLUMETRIC CONTAINERS of suitable precision and accuracy.

5. INTERFERENCES

5.1. IODIDE, GOLD AND SILVER are known interferences for mercury determination by cold vapour. In samples from marine origin (biota or sediment), the levels of those elements are low, and consequently, do not interfere in the measurement process.

5.2. WATER VAPOUR (moisture) should be avoided in the measurement cell, always follow the manufacturer's protocol (e.g. use of membrane drying tube, correct position of gas separator…) and check for absence of moisture in the measurement cell.

5.3. When using GOLD AMALGAMATION, and with certain batch systems, the excess of oxidant can cause interference or damage the gold amalgamator, it is then recommended to pre-reduce the samples with hydroxylamine ammonium (see 3.7). This is important when using large amount of digested solution in "batch system".

5.4. Some samples (i.e. plants or large amount of mussels) might produce FOAM during the reduction reaction. If the amount of foam is important, it can interfere with gas liquid separation, and/or leak in the measurement cell, this phenomenon can be overcome by using silicon anti-foaming inside the gas liquid separator and/or in the "batch" system. Another option is to use vanadium pentoxide during digestion (see 6.4).

5.5. REDUCTION of inorganic mercury will induce loss, so it is important to stabilise all the solutions by using a strong oxidant as dichromate or BrCl (see 3.8 or 3.9).

6. SAMPLE PREPARATION

6.1. The sample should be prepared according to the recommended method for digestion of marine samples for the determination of trace metal (IAEA, 2011), but before diluting to the final volume (or weight) with water, add an adequate volume of potassium dichromate or BrCl to get the final concentration of 2% or 1% respectively. All the samples from marine origin (sediment or biota) can be prepared using the acid mixture recommended for fish, as Hg is not attached to silicates. For microwave digestion of sample size above 0.8 g, it is strongly recommended to do cold digestion for at least 5 hours and to use a long ramping time (i.e. 25 minutes) to avoid strong reactions in the microwave vessels.

6.2. If other trace elements have to be determined in the digested solution prepared according to the recommended method for digestion of marine samples for the determination of trace metal (IAEA, 2011), following the dilution to the final volume or weight, transfer a quantitatively sufficient amount of digested solution (i.e. at least 10 ml) into a separate container (preferably glass or Teflon), and add an oxidising solution 1% (v/v) of BrCl (see 3.9) or 2% (v/v) of potassium dichromate (see 3.8). Record the amount of oxidising solution added in order to calculate the dilution factor (i.e. dilution factor =1.01 for 0.1 ml of dichromate in 10 ml).

6.3. Alternatively, the samples can be digested using a mixture of 5 ml of HNO_3 and 2 ml of H_2O_2 at 90°C for 4 hours on a hot plate. It is recommended to leave the samples in acid at room temperature, for at least 1 hour before heating. The digestion can be performed either in a Teflon or glass closed containers. After cooling, add an adequate volume of potassium dichromate or BrCl to get the final concentration of 2% or 1% respectively, and dilute to the final volume with water (i.e. for 50 ml final volume, add 1 ml of potassium dichromate or 0.5 ml of BrCl solution). This procedure can be used with bigger sample size if needed (i.e. 2 g); in this case, the volume of nitric acid should be increased to obtain a liquid mixture.

6.4. In the case that the digested solution produces foam during the reduction process (see 5.4), 45 mg of vanadium pentoxide should be added in the digestion vessels before addition of the acid mixture, then follow either paragraph 6.1 or 6.3.

7. PROCEDURE

7.1. SAMPLE SOLUTION

Use the sample prepared with one option as described in section 6.

7.2. BLANK SOLUTION

Prepare at least two blank solutions with each batch of sample, using the same procedure than for the samples.

7.3. PREPARATION OF CALIBRATION SOLUTIONS

7.3.1. Before each batch of determination, prepare by the appropriate dilution of 1000 µg ml^{-1} stock standard solution (see 3.11), at least 4 standard solutions and one calibration blank solution, covering the appropriate range of the linear part of the curve. The calibration standards and calibration blank should be prepared using the same type of acid and oxidising solution than in the test portion (the final concentration should be similar).

7.3.2. Calibration solutions should be prepared fresh each day.

7.3.3. If the necessary intermediate stock standard solutions can be prepared in 5% nitric acid and 1% BrCl or 2% $K_2Cr_2O_7$, these solutions should be prepared monthly.

7.3.4. All volumetric material (pipettes and containers) should be of appropriate precision and accuracy, if not available standard solution can be prepared by weighing.

7.4. INSTALLATION OF VAPOUR GENERATOR ACCESSORY

7.4.1. Install the accessory according to the manufacturer's instructions. Certain systems (i.e. VGA from Varian) are designed to be used for hydride generation as well, and require in the instructions to aspirate an extra HCl solution, in the case of stannous chlorine reduction this solution is to be replaced by water. It is recommended to separate the systems used for hydride and for $SnCl_2$ (i.e. use a spare gas liquid separator and Teflon tubing).

7.4.2. Switch on the argon. For on-line system: start the pump, check the aspiration, and verify the gas liquid separator. If needed replace the pump tubing, clean the gas liquid separator by sonication in diluted detergent.

7.4.3. Clean the system by aspirating reagent and 10% nitric acid as a sample for about 10 minutes. For batch system, perform two cycles with 10% nitric acid.

7.4.4. Set up the atomic absorption spectrometer according to the manufacturer's instructions, at the appropriate wavelength, using the appropriate conditions, and with the suitable background correction system in operation.

7.4.5. Optimise the position of the measurement cell to get the maximum signal.

7.4.6. Connect the vapour generation system to the measurement cell.

7.5. CALIBRATION

7.5.1. Adjust the response of the instrument to zero absorbance whilst aspirating water.

NOTE: if the instrument zero reading is more than 0.002 ABS, the system should be clean again and reagent should be checked.

7.5.2. Aspirate the set of calibration solutions in ascending order, and as a zero member, the blank calibration solution. After the last standard, aspirate 10% nitric acid for 1 minute to rinse the system.

NOTE: The calibration curve is automatically plotted by the instrument software. The obtained curve should be linear with r>0.995.

To correct for the instrumental drift, the calibration should be performed every 20 samples or if the calibration verification has failed (see 7.8.1).

7.6. ASPIRATE SAMPLE BLANK (see 7.2) AND SAMPLE SOLUTIONS (see 7.1)

Record their concentrations as calculated by the software using the calibration curve. Rinse the system by aspirating 10% nitric acid for at least 30 s between samples.

7.7. IF THE CONCENTRATION OF THE TEST PORTION EXCEEDS THE CALIBRATION RANGE, dilute the test portion with the blank solution accordingly.

NOTE: After the measurement of high level (or over calibration) sample, measure a sample blank or water to check the absence of memory effect. If necessary, clean the system for 1 minute with 10% nitric acid.

7.8. QUALITY CONTROL SOLUTIONS

The quality control solutions as described below should be measured during the run.

7.8.1. Calibration Verification CV

After the initial calibration, the calibration curve must be verified by the use of initial calibration verification (CV) standard.

The CV standard is a standard solution made from an independent (second source) material, at/or near midrange. This solution as a calibration standard should be prepared using the same type of acid and oxidising solution than in the test portion (the final concentration should be similar).

The acceptance criteria for the CV standard must be ±10% of its true value.

If the calibration curve cannot be verified within the specified limits, the causes must be determined and the instrument recalibrated before the samples are analysed. The analysis data for the CV must be kept on file with the sample analysis.

The calibration curve must also be verified at the end of each analysis batch and/or after every 10 samples. If the calibration cannot be verified within the specified limits, the sample analysis must be discontinued, the causes must be determined and the instrument recalibrated. All samples following the last acceptable test must be reanalysed.

7.8.2. Blank solution (see 7.2)

The maximum allowed blank concentration should be well documented, and if the blank solution exceeds this value all samples prepared along the contaminated blank should be prepared again and reanalysed.

7.8.3. Post digestion spike

Each unknown type of sample should be spiked to check for potential matrix effect.

This spike is considered as a single point standard addition, and should be performed with a minimum dilution factor. The recovery of spike calculated with equation 1 should be 85-115%. If this test fails, it is recommended to run analysis with standard addition method.

Spike solution: mix a fix volume (V1) of the sample solution, and a known volume (V2) of a standard solution with known concentrations (Cstandard).

Unspike solution: mix the same fix volume (V1) of sample solution, and the same volume (V2) of reagent water.

Measure the concentration C (mg l^{-1}) in both solutions on the calibration curve (see 7.6), and calculate recovery as:

$$Cspike = \frac{Cstandard \times V2}{(V1+V2)} \qquad \text{Equation 1}$$

$$R = \frac{C\ Spike\ Solution - C\ Unspike\ solution}{Cspike} \times 100 \qquad \text{Equation 2}$$

To be valid, the concentrations of spiked and unspiked solutions should be in the linearity range of the calibration curve, and the spiked concentration (equation 1) should be in the range of 50-150% of the concentration of the unspiked solution.

7.8.4. Dilution test

If the analyte concentration is sufficiently high (minimally, a factor of 10 above the lower limit of the quantitation following dilution), an analysis of a 1:5 dilution should agree within

±10% of the original determination. If not, then a chemical or physical interference effect should be suspected, and method of standard addition is recommended.

7.8.5. Certified Reference Material

At least one certified reference material of a representative matrix should be prepared with each batch of sample, the calculated result should fall in the value of the certificate and within the coverage uncertainty (Linsinger, 2010), to show evidence of an unbiased result.

The results for the CRM should be recorded for quality control purpose and plotted on a control chart (UNEP/IOC/IAEA 1994).

An example of sequence order with recommended criteria and actions is given in table 1.

TABLE 1. EXAMPLE OF AN ANALYTICAL SEQUENCE:

Solutions Description	Performance	Action
Calibration blank	< maximum allowed calibration blank value	Stop until resolve
Standard solution 1–4	r>0.995	recalibrate in the linearity range
CV	±10% of the true value	Stop until resolve
Sample blank	< maximum allowed blank value	
CRM	Fall in the certificate value within coverage uncertainty, or fall within acceptable criteria of the QC chart	Stop until resolve, check Matrix spike and run again with standard addition method if necessary
Matrix Spike	recovery 100% ± 15%	switch to standard addition, keep records for future analysis of the same matrix
Dilution Test	sample 1 = 5x sample 1 diluted 5x within 10%	switch to standard addition, keep records for future analysis of the same matrix
Unknown Sample 1–10	should ≥ standard 1 and ≤ standard 4	report as <minimum quantification limit or dilute
CV	±10% of the true value	Stop until resolve
Unknown Sample 11–20	should ≥ standard 1 and ≤ standard 4	report as <minimum quantification limit or dilute
Calibration blank	< maximum allowed calibration blank value	Stop until resolve
Standard solution 1–4	r>0.995	recalibrate in the linearity range
CV	±10% of the true value	Stop until resolve
Etc….		

8. CALCULATION OF RESULTS

Results are calculated using equation 3

$$w(m) = \frac{(\rho 1 - \rho 0)}{\mathrm{m}} \times f \times V \times R \qquad \text{Equation 3}$$

Where:

w(m) is the mass fraction of element m in the sample, expressed in mg kg^{-1};

ρ1 is the concentration of element m, expressed in mg/l as measured in the sample solution;

ρ0 is the concentration of element m expressed in mg/l as measured in the blank solution;

F is the dilution factor calculated as follow:

$$f = \frac{final\ volume}{initial\ volume}$$

or equal to 1 if ρ1 is determined in undiluted solution;

R is the recovery calculated using the CRM (see 7.8.5) or the post digestion spike.

m is the mass of sample in g

V is the volume of solution in ml

9. EXPRESSION OF RESULTS

The rounding of values will depend on the uncertainty reported with the result; in general for this method two or three significant figures should be reported.

Uncertainty component should be reported with all results. (ISO 2005, Nordtest 2004)

Example: w(Hg) = 0.512 ± 0.065 mg kg^{-1}.

10. REFERENCES

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