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# CERTIFICATION REPORT

# The certification of the mass fractions of elements in bovine liver: ERM®-BB185

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European Commission
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Directorate F – Health, Consumers and Reference Materials

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Abstract

This report describes the production of ERM-BB185, which is a bovine liver material certified for the mass fraction of As, Cd, Cu, Mn, Pb, Se and Zn. This material was produced following ISO Guide 34:2009 and is certified in accordance with ISO Guide 35:2006. This CRM is produced and certified to replace the CRM BCR-185R.
The CRM was produced from raw livers from cattle slaughtered in Belgium. The raw livers were cut into cubes and freeze dried. The freeze dried cubes were milled and sieved to obtain a fine powder. The powder was homogenised and afterwards checked for water content and particle size distribution. The homogenised powder was put in vials and packed in aluminised pouches.
Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006.
The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.
Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties related to possible inhomogeneity, instability and characterisation.
The material is intended for the quality control and assessment of method performance. As with any reference material, it can be used for establishing control charts or validation studies. The CRM is available in amber glass vials containing at least 10 g of dried powder which were sealed under an atmosphere of argon and packed in aluminised pouches. The minimum amount of sample to be used is 400 mg.

# CERTIFICATION REPORT

# The certification of the mass fractions of elements in bovine liver: ERM®-BB185

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# Summary

This report describes the production of ERM-BB185, which is a bovine liver material certified for the mass fraction of As, Cd, Cu, Mn, Pb, Se and Zn. This material was produced following ISO Guide 34:2009 [1] and is certified in accordance with ISO Guide 35:2006 [2]. This CRM is produced and certified to replace the CRM BCR-185R [3].

The CRM was produced from raw livers from cattle slaughtered in Belgium. The raw livers were cut into cubes and freeze dried. The freeze dried cubes were milled and sieved to obtain a fine powder. The powder was homogenised and afterwards checked for water content and particle size distribution. The homogenised powder was put in vials and packed in aluminised pouches.

Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006.

The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.

Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible inhomogeneity, instability and characterisation.

The material is intended for the quality control and assessment of method performance. As with any reference material, it can be used for establishing control charts or validation studies. The CRM is available in amber glass vials containing at least 10 g of dried powder which were sealed under an atmosphere of argon and packed in aluminised pouches. The minimum amount of sample to be used is 400 mg.

The following values were assigned:

| **BOVINE LIVER** | | |
|---|---|---|
| Mass fraction | | |
| | Certified value [2] [mg/kg] | Uncertainty [3] [mg/kg] |
| As[1] | 0.0177 | 0.0021 |
| Cd[1] | 0.280 | 0.014 |
| Cu | 598 | 22 |
| Mn | 13.1 | 0.5 |
| Pb[1] | 0.0326 | 0.0021 |
| Se | 2.99 | 0.18 |
| Zn | 143 | 5 |

[1] As, Cd and Pb value as measured by inductively coupled plasma mass spectrometry (ICP-MS) and/or inductively coupled plasma sector-field mass spectrometry (ICP-SFMS) and/or isotope-dilution inductively coupled plasma sector-field mass spectrometry (ID-ICP-SFMS) only.

[2] Certified values are values that fulfil the highest standards of accuracy. The given values represent the unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory and/or with a different method of determination. The certified value and its uncertainty are traceable to the International System of Units (SI).

[3] The uncertainty is the expanded uncertainty of the certified value with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

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# Glossary

| | |
|---|---|
| ANOVA | Analysis of variance |
| *b* | Slope in the equation of linear regression $y = a + bx$ |
| BCR® | One of the trademarks of CRMs owned by the European Commission; formerly Community Bureau of Reference |
| CRM | Certified reference material |
| EC | European Commission |
| ERM® | Trademark of European Reference Materials |
| EU | European Union |
| GUM | Guide to the Expression of Uncertainty in Measurements *[ISO/IEC Guide 98-3:2008]* |
| HEPA | High-efficiency particulate air |
| ICP | Inductively coupled plasma |
| ICP-MS | Inductively coupled plasma-mass spectrometry |
| ICP-OES | Inductively coupled plasma-optical emission spectrometry |
| ICP-SFMS | ICP-sector-field mass spectrometry |
| ID | Isotope dilution |
| ID-ICP-SFMS | isotope dilution – inductively coupled plasma sector-field mass spectrometry |
| ILC | Interlaboratory comparison |
| (I)NAA | (Instrumental) neutron activation analysis |
| IRMM | Institute for Reference Materials and Measurements of the JRC |
| ISO | International Organization for Standardization |
| IU | International units |
| JRC | Joint Research Centre of the European Commission |
| *k* | Coverage factor |
| $k_0$-NAA | $k_0$- Neutron Activation Analysis |
| KFT | Karl Fischer titration |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| MS | Mass spectrometry |
| $MS_{between}$ | Mean of squares between-unit from an ANOVA |
| $MS_{within}$ | Mean of squares within-unit from an ANOVA |
| *n* | Number of replicates per unit |
| *N* | Number of samples (units) analysed |
| n.a. | Not applicable |
| n.c. | Not calculated |

| | |
|---|---|
| OES | Optical emission spectrometry |
| PE | Polyethylene |
| PSA | Particle size analysis |
| PT | Proficiency testing |
| QA | Quality assurance |
| QC | Quality control |
| rel | Index denoting relative figures (uncertainties etc.) |
| RM | Reference material |
| RMP | Reference material producer |
| RM Unit | Reference Materials Unit of Directorate F |
| RSD | Relative standard deviation |
| RSE | Relative standard error (=RSD/$\sqrt{n}$) |
| RT | Room temperature |
| $r^2$ | Coefficient of determination of the linear regression |
| $s$ | Standard deviation |
| $s_{bb}$ | Between-unit standard deviation; an additional index "rel" is added when appropriate |
| $s_{between}$ | Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate |
| SI | International System of Units |
| $s_{meas}$ | Standard deviation of measurement data; an additional index "rel" is added as appropriate |
| $s_{within}$ | Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate |
| $s_{wb}$ | Within-unit standard deviation |
| T | Temperature |
| $t$ | Time |
| $t_i$ | Time point for each replicate |
| $t_{\alpha, df}$ | Critical $t$-value for a $t$-test, with a level of confidence of 1-$\alpha$ and df degrees of freedom |
| $u$ | Standard uncertainty |
| $U$ | Expanded uncertainty |
| $u^*_{bb}$ | Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability an additional index "rel" is added as appropriate |
| $u_{bb}$ | Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate |
| $u_{char}$ | Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate |
| $u_{CRM}$ | Combined standard uncertainty of the certified value; an additional index |

| | |
|---|---|
| | "rel" is added as appropriate |
| $U_{CRM}$ | Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate |
| $u_{\Delta}$ | Combined standard uncertainty of measurement result and certified value |
| $u_{lts}$ | Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate |
| $u_{meas}$ | Standard measurement uncertainty |
| $U_{meas}$ | Expanded measurement uncertainty |
| $u_{rec}$ | Standard uncertainty related to possible between-unit inhomogeneity modelled as rectangular distribution; an additional index "rel" is added as appropriate |
| $u_{sts}$ | Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate |
| $u_{t}$ | Standard uncertainty of trueness |
| $\bar{x}$ | Arithmetic mean |
| $\bar{x}_{ns}$ | Arithmetic mean of all results of normal stock samples |
| $\bar{x}_{ref}$ | Arithmetic mean of results of reference samples |
| $\alpha$ | Significance level |
| $\Delta_{meas}$ | Absolute difference between mean measured value and the certified value |
| $\nu_{s,meas}$ | Degrees of freedom for the determination of the standard deviation $s_{meas}$ |
| $\nu_{MSwithin}$ | Degrees of freedom of $MS_{within}$ |

# 1 Introduction

## 1.1 Background

This report describes the preparation and certification of a reference material to replace the CRM BCR-185R (bovine liver).

The target parameters for certification were the mass fractions of As, Cd, Cu, Mn, Pb, Se and Zn in liver. The desired mass fractions were those at natural levels, similar to BCR-185R, and below regulatory limits for food contaminants (EC466/2001 and amendments 1881/2006 and 488/2014 of Cd < 0.5 mg/kg and Pb < 0.5 mg/kg wet weight).
Values are traceable to the SI, expressed as mass fraction of the dry mass.

The purpose of the reference material is for validation and quality control of measurement methods for trace element mass fractions in bovine liver.

Throughout this report, results are expressed as mass fraction on a dry mass basis. For practical purposes, the dry mass is established by determining the "loss of mass on drying" under conditions defined in Section 8.5. It should be noted that determination of the dry mass correction factor under conditions other than specified in this report might lead to results that differ from the certified values.

## 1.2 Choice of the material

The raw material used for the preparation of ERM-BB185 was liver from cattle slaughtered in Belgium. About 90 kg of whole livers, packed in vacuum plastic bags, were delivered. The livers came from animals older than 30 months.

## 1.3 Design of the CRM project

This certified reference material was prepared to replace the CRM BCR-185R (elements in bovine liver). Therefore the same elements (As, Cd, Cu, Mn, Pb, Se and Zn) were chosen to be certified. The certification of the mass fractions of As, Cd, Cu, Mn, Pb, Se and Zn was performed by interlaboratory comparison.

# 2 Participants

## 2.1 Project management and evaluation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

## 2.2 Processing

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

## 2.3 Homogeneity study

ALS Scandinavia AB, Luleå, SE

(measurements performed under the scope of ISO/IEC 17025 accreditation; SWEDAC-2030)

## 2.4 Stability study

ALS Scandinavia AB, Luleå, SE

(measurements performed under the scope of ISO/IEC 17025 accreditation; SWEDAC-2030)

## 2.5 Characterisation

ALS Scandinavia AB, Luleå, SE

(measurements performed under the scope of ISO/IEC 17025 accreditation; SWEDAC-2030)

Campden BRI (Chipping Campden) Ltd, UK

(measurements performed under the scope of ISO/IEC 17025 accreditation; UKAS 1079)

Public Health Laboratory of Alicante (LSPA), ES

(measurements performed under the scope of ISO/IEC 17025 accreditation; ENAC 148/LE370)

Sciensano, Tervuren, BE

(measurements performed under the scope of ISO/IEC 17025 accreditation; BELAC 172-TEST)

The Food and Environment Research Agency, York, UK

(measurements performed under ISO/IEC 17025 accreditation; UKAS 1642)

Institut "Jozef Stefan", Ljubljana, SI

(measurements performed under ISO/IEC 17025 accreditation; Slovenska Akreditacija LP-090)

National Food Institute (DTU Food), Søborg, DK

(measurements performed under the scope of ISO/IEC 17025 accreditation; DANAK No 350)

SCK-CEN, Mol, BE

(measurements performed under the scope of ISO/IEC 17025 accreditation; BELAC 015-TEST)

Solvias, Kaiseraugst, CH

Umweltbundesamt GmbH, Wien, AT

(measurements under the scope of ISO/IEC 17025 accreditation Akkreditierung Austria ID 200)

VITO, Vlaams Instituut voor Technologische Ontwikkeling, Mol, BE

(measurements performed under the scope of ISO/IEC 17025 accreditation; BELAC 045-TEST)

# 3 Material processing and process control

## 3.1 Origin of the starting material

The raw material used for the preparation of ERM-BB185 was livers from cattle slaughtered in Belgium. These livers were purchased from a slaughter house and were fit for human consumption.

## 3.2 Processing

Upon arrival at JRC Geel, the bovine livers were immediately stored at 4°C. The ceramic knives, cutting boards, boxes, nylon sieve, spoons, vials and any other equipment that were used and in contact with the liver, had initially been washed with a solution of 6 % $HNO_3$ (Merck, Darmstadt, Germany), and carefully rinsed with ultrapure water of Type 1 (Millipore) and dried in a clean cell with HEPA filtered air to prevent contamination.

The raw livers were cut into cubes of about 1 $cm^3$ using ceramic knives and low density polyethylene cutting boards. The cutting took place in an area that was curtained off and supplied from above with HEPA-filtered air. Cubes were placed on PE-foil covered trays, weighed and loaded into an Epsilon 2-100D freeze-dryer (Martin Christ, Osterode, Germany). The material was freeze dried until a water content of less than 3 % (m/m) was reached.

The dried cubes of bovine liver were placed in stainless-steel drums that were immersed in liquid nitrogen overnight. Drums were removed from the liquid nitrogen one-by-one and the contents were transferred to a cryogenic vibrating Palla-mill (KDH Humboldt Wedag, Köln, Germany) that was pre-cooled with liquid nitrogen to - 196°C. The vibrating Palla-mill has a titanium inner surface and Ti-milling rods to avoid contamination from elements normally present in stainless steel equipment.

After milling, the resulting powder was sieved through a 250 µm nylon sieve, with an ultra-sonic aid (Russel Finex, London, United Kingdom). The coarse fraction (>250 µm) was remilled in the cryogenic vibrating Palla-mill and re-sieved over the 250 µm sieve.

The powder was homogenised in a three-dimensional mixer, Dyna-MIX CM200 mixer (WAB, Basel, Switzerland) for 1 hour.

The material was then checked for again for its water content and particle size distribution. The water content was below 3 % (m/m), and no particles were larger in size than 250 µm. The material was therefore considered suitable to be filled in vials.

A batch of 100 mL amber glass vials were acid washed with 6 % $HNO_3$ (Merck, Darmstadt, Germany), carefully rinsed with ultrapure water of Type 1 (Millipore) and dried in a drying cabinet. Filling 10 g of the bovine liver powder into 100 mL amber glass vials was performed using an MCPI fine dosing filling machine (MCPI, Meythet, France). Acid-washed lyo inserts were manually placed in each vial. The vials were closed in the Epsilon 2-100D freeze-dryer (Martin Christ, Osterode, Germany) to fill the head space above the material with argon.

The vials were then capped with aluminium caps and labelled. The labelled vials were placed in aluminised pouches which were thermally sealed and labelled. Vials for general distributions were stored at 18 °C.

## 3.3 Process control

After processing, 5 vials were selected at random and 2 replicate water measurements and particle size distribution measurements were made on each vial.

KFT results showed an average water content of 2.62 % (m/m) with s = 0.03 %,in the samples. The material was therefore considered to be sufficiently dry to preserve the matrix over the desired shelf-life.

Particle size analysis (PSA) technique was performed with laser diffraction spectrometry using a Helos laser light scattering instrument (Sympatec GmbH System-Partikel-Technik, Clausthal, Zellerfeld, Germany). Five independent subsamples of bovine liver powder were measured in duplicate. Results showed that 90 % of particles were below a value of 152 µm with s = 28 µm. This shows that the target particle size (< 250 µm) was achieved.

# 4 Homogeneity

A key requirement for any reference material aliquotted into units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, but it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 [1] requires RM producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample intake is respected, but determines the minimum size of an aliquot that is representative for the whole unit. Quantification of within-unit inhomogeneity is therefore necessary to determine the minimum sample intake.

## 4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRM are valid for all units of the material, within the stated uncertainty. In the case of ERM-BB185, 'unit' is defined as an individual glass vial of the material.

The number of selected units corresponds to approximately the cube root of the total number of units produced. Thirteen units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch was divided into groups (with a similar number of units) and one unit was selected randomly from each group. From each of the thirteen vials, four independent samples were taken and analysed by ICP-SFMS.

For the between-unit homogeneity, the measurements were performed under repeatability conditions, and in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence. The results were corrected for the water content determined in each unit (Section 8.5). The results are shown as graphs in Annex A.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. For the filling sequence, no trends were visible at the 95% confidence level. Significant trends in the analytical sequence at 95% confidence level were visible for Cd, Cu, Mn, Pb and Zn indicating a drift of the analytical system. As the analytical sequence and the unit numbers were not correlated, trends in the analytical sequence were corrected if the trend was significant on at least 95% confidence level as shown below:

*Corrected result = measured result-b.i*

*b= slope of the linear regression*

*i= position of the result in the analytical sequence*

All datasets were assessed for consistency using Grubbs outlier tests at a confidence level of 99 % on the individual results and on the unit means. One outlying individual result was detected for Zn. Since no technical reason for the outlier could be found, all the data were retained for statistical analysis.

Quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation ($s_{bb}$) from the within-unit variation ($s_{wb}$). The latter is equivalent to the method repeatability, if the individual samples were representative for the whole unit.

Evaluation by ANOVA requires mean values per unit, which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. The distribution of the mean values per unit was visually inspected using histograms and normal probability plots. Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviations. The results of all statistical evaluations are given in Table 1.

**Table 1:** Results of the statistical evaluation of the homogeneity studies

| Element | Trends (before correction)* | | Outliers** | | Distribution | |
|---|---|---|---|---|---|---|
| | Analytical sequence | Filling sequence | Individual results | Unit means | Individual results | Unit means |
| As | no | no | none | none | unimodal | unimodal |
| Cd | yes | no | none | none | unimodal | unimodal |
| Cu | yes | no | none | none | unimodal | unimodal |
| Mn | yes | no | none | none | unimodal | unimodal |
| Pb | yes | no | none | none | unimodal | unimodal |
| Se | no | no | none | none | unimodal | unimodal |
| Zn | yes | no | 1 (retained) | none | unimodal | unimodal |

* 95 % confidence level

** 99 % confidence level

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups ($MS_{within}$), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, $u^*_{bb}$, the maximum inhomogeneity that could be hidden by method repeatability, was calculated as described by Linsinger *et al.* [5]. $u^*_{bb}$ is comparable to the LOD of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

Method repeatability ($s_{wb,rel}$), between–unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$ **Equation 1**

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$$ **Equation 2**

$$u^{*}_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}\sqrt[4]{\frac{2}{v_{MSwithin}}}}}{\bar{y}}$$ **Equation 3**

$MS_{within}$ mean of squares within-unit from an ANOVA

$MS_{between}$ mean of squares between-unit from an ANOVA

$\bar{y}$ mean of all results of the homogeneity study

$n$ mean number of replicates per unit

$v_{MSwithin}$ degrees of freedom of $MS_{within}$

The results of the evaluation of the between-unit variation are summarised in Table 2. The resulting values from the above equations were converted into relative uncertainties. In more than half of the cases, the uncertainty contribution for homogeneity was determined by the method repeatability.

**Table 2:** Results of the homogeneity studies

| Element | $s_{wb,rel}$ [%] | $s_{bb,rel}$ [%] | $u^{*}_{bb,rel}$ [%] | $u_{bb,rel}$ [%] |
|---|---|---|---|---|
| As | 11.20 | n.c. | 2.67 | 2.67 |
| Cd | 1.52 | 0.24 | 0.36 | 0.36 |
| Cu | 1.85 | n.c. | 0.44 | 0.44 |
| Mn | 1.78 | 0.64 | 0.42 | 0.64 |
| Pb | 4.89 | n.c. | 1.16 | 1.16 |
| Se | 4.22 | 0.45 | 1.01 | 1.01 |
| Zn | 2.09 | n.c. | 0.50 | 0.50 |

[1)] n.c.: cannot be calculated as $MS_{between} < MS_{within}$

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore the between-unit standard deviation can be used as estimate of $u_{bb}$. . As $u^{*}_{bb}$ sets the limits of the study to detect inhomogeneity, the larger value of $s_{bb}$ and $u^{*}_{bb}$ is adopted as uncertainty contribution to account for potential inhomogeneity.

## 4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. Due to this correlation, individual aliquots of a material will not contain the same amount of analyte. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used in an analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty. The between-unit homogeneity experiments were performed using a 400 mg sample intake for all elements. This sample intake gives acceptable repeatability, demonstrating that the within-unit inhomogeneity no longer contributes to analytical variation at this sample intake. In the characterisation, it was also prescribed to use 400 mg as minimum sample intake, which gave an acceptable repeatability. One lab used 250 mg, due to limitations on their method. Even with a lower minimum sample intake, it gives an acceptable repeatability.

The overall minimum sample intake is set to 400 mg.

# 5 Stability

Time, temperature, light (including ultraviolet radiation) and water content were regarded as the most relevant influences on the stability of the materials. The influence of ultraviolet or visible light was minimised by storing the material in amber glass vials, packed in aluminised pouches which eliminates incoming light. In addition, materials are stored in the dark and dispatched in boxes, thus removing any possibility of degradation by light. The water content was adjusted to an optimum during processing. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the materials to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C can be reached and stability under these conditions must be demonstrated, if the units are to be transported without any additional cooling.

The stability studies were carried out using an isochronous design. In this approach, units were stored for a particular length of time at different temperature conditions. Afterwards, the units were moved to conditions where further degradation can be assumed to be negligible ("reference conditions"), effectively "freezing" the degradation status of the materials. At the end of the isochronous storage, the samples were analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

## 5.1 Short-term stability study

For the short-term stability study, units were stored at 60 °C for 0, 1, 2 and 4 weeks. The reference temperature was set to -20 °C. Two units per storage time were selected using a random stratified sampling scheme. From each unit, 4 samples were measured by ICP-SFMS. The measurements were performed under repeatability conditions and a randomised sequence was used to differentiate any potential analytical drift from a trend over storage time. The results were corrected for the water content determined once in each unit.

The data were evaluated individually for 60 °C. The results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %. One outlying individual result (Pb) was found (Table 3). As no technical reason for the outlier could be found this result was retained for statistical analysis.

In addition, the data were evaluated against storage time, and regression lines of mass fraction versus time were calculated, to test for potential increases/decrease due to shipping conditions. The slopes of the regression lines were tested for statistical significance. For all elements, the slopes of the regression lines were not significantly different from 0 (on 95% confidence level) at 60°C.

The results of the measurements are shown in Annex B. The results of the statistical evaluation of the short-term stability are summarised in Table 3.

**Table 3:** Results of the short-term stability tests

| Element | Number of individual outlying results on a 99% confidence level | Significance of trend on a 95% confidence level |
|---|---|---|
| As | none | no |
| Cd | none | no |
| Cu | none | no |
| Mn | none | no |
| Pb | 1 | no |
| Se | none | no |
| Zn | none | no |

There was no technical reason to exclude the outlier of Pb, and this was retained for the calculation of the uncertainty for short term stability. None of the trends was statistically significant on a 95 % confidence level for any of the temperatures.

No significant degradation during dispatch even at 60 °C was observed. Therefore, the material can be transported at ambient conditions without special precautions.

## 5.2 Long-term stability study

For the long-term stability study, samples were stored at 18 °C for 0, 8, 16 and 24 months. The reference temperature was set to -20 °C. Two units per storage time were selected for analyses, thus a total number of eight units was analysed. From each unit, 3 aliquots were measured by ICP-SFMS. The measurements were performed under repeatability conditions, in a random sequence to be able to separate any potential analytical drift from a trend over storage time. The results were corrected for the water content determined once in each unit.

The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. There were no outlying individual results found (Table 4).

In addition, the data were plotted against storage time and linear regression lines of mass fraction versus time were calculated. The slope of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected for any of the analytes at a 95 % confidence level.

The results of the long-term stability measurements are shown in Annex C. The results of the statistical evaluation of the long-term stability study are summarised in Table 4.

**Table 4:** Results of the long-term stability tests

| Element | Number of individual outlying results on a 99% confidence level | Significance of trend on a 95% confidence level |
|---|---|---|
| As | none | no |
| Cd | none | no |
| Cu | none | no |
| Mn | none | no |
| Pb | none | no |
| Se | none | no |
| Zn | none | no |

No technically unexplained outliers were observed on a 99% confidence level and none of the trends was statistically significant on a 95 % confidence level at 18 °C. The material can therefore be stored at 18 °C.

## 5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can entirely rule out degradation of materials, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method repeatability, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation within an uncertainty to be estimated.

The uncertainties of stability during dispatch and storage were estimated for each analyte. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions $u_{sts}$ and $u_{lts}$ were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{tt} \qquad \textbf{Equation 4}$$

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \textbf{Equation 5}$$

$s_{rel}$ relative standard deviation of all results of the stability study

$t_i$ time elapsed at time point *i*

$\bar{t}$ mean of all $t_i$

$t_{tt}$ chosen transport time (1 week at 60 ºC)

$t_{sl}$ chosen shelf life (24 months at 18 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 60 °C studies. The uncertainty describes the possible change during a dispatch at 60 °C lasting for one week.

- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the 18 °C studies. The uncertainty contribution describes the possible degradation during 24 months storage at 18 °C.

The results of these evaluations are summarised in Table 5.

**Table 5:** Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of 60 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of 18 °C and 24 months.

| Element | $u_{sts,rel}$ [%] | $u_{lts,rel}$ [%] |
|---|---|---|
| As | 1.83 | 2.96 |
| Cd | 0.12 | 0.79 |
| Cu | 0.21 | 0.95 |
| Mn | 0.17 | 1.37 |
| Pb | 1.39 | 1.49 |
| Se | 0.30 | 1.34 |
| Zn | 0.21 | 0.93 |

After the certification study, the material will be included in the JRC Geel's regular stability monitoring programme, to control its further stability.

# 6 Characterisation

The material characterisation is the process of determining the assigned property values of a reference material.

The material characterisation was based on an interlaboratory comparison of expert laboratories, i.e. the element mass fractions of the material were determined in different laboratories that applied different measurement procedures to demonstrate the absence of a measurement bias. This approach aims at randomisation of laboratory bias, which reduces the combined uncertainty.

## 6.1 Selection of participants

Eleven laboratories (of which some offered different measurement principles) were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of element measurements in relevant matrices as demonstrated by good performance in past characterisation studies organised by JRC Geel. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2).

## 6.2 Study setup

Each laboratory received 2 units of ERM-BB185 and was requested to provide 6 independent results, 3 per unit. The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparation and measurements had to be spread over at least two days to ensure intermediate precision conditions. Sample 1 of ERM-BB185 was prepared and measured on day 1, sample 2 was prepared and measured on day 2. The water content had to be determined on a separate portion from each unit per day of measurement and results are reported on dry mass basis.

Each participant received a sample of CRM BCR185R as a blind quality control (QC) sample. The results for this sample were used to support the evaluation of the characterisation results.

Laboratories were also requested to give estimations of the expanded uncertainties of the mean value of the six results. No approach for the estimation was prescribed, i.e. top-down and bottom-up were regarded as equally valid procedures.

## 6.3 Methods used

A variety of methods with different quantification steps (ICP-MS, ICP-OES, ICP-SFMS, ID-ICP-SFMS) as well as methods without sample preparation (k0-NAA) were used to characterise the material. The combination of results from methods based on completely different principles mitigates undetected method bias.

All methods used during the characterisation study are summarised in Annex D. The laboratory code (e.g. L1) is a random number and does not correspond to the order of laboratories in Section 2.

For all measurements carried out during certification (homogeneity, stability and characterisation studies) the following protocol for dry mass determination was applied:

The samples' water content was measured for each of the vials, at the time of sample preparation on a separate portion. The method used was (atmospheric pressure) oven drying of a portion of at least 0.2 g at 103 ± 2 ºC until constant mass was attained.

The water content determined by the laboratories was in the range of 23 g/kg to 95 g/kg. (median is 58 g/kg).

## 6.4 Evaluation of results

The characterisation study resulted in between 7 and 12 datasets per element. All individual results of the participants, grouped per element are displayed in tabular and/or graphical form in Annex E.

### 6.4.1 Technical evaluation

The obtained data were first checked for compliance with the requested analysis protocol and for their validity based on technical reasons. The following criteria were considered during the evaluation:

- compliance with the analysis protocol: sample preparations and measurements performed on two days, and the analytical sequence and water content determination.
- absence of values given as below limit of detection or below limit of quantification
- method performance, i.e. agreement of the measurement results with the assigned value of the QC sample

Based on the above criteria, a total of 11 element datasets were rejected as not technically valid (Table 6).

**Table 6:** Datasets that showed non-compliances with the analysis protocol and technical specifications, and action taken

| Element | Lab-method code | Description of problem | Action taken |
|---|---|---|---|
| As | 2 | Results for the QCM differ significantly from the certified value | Data not used for evaluation |
| | 3 | Measurements reported as < LOQ | Data not used for evaluation |
| | 11 | Results for the QCM differ from the certified value | Data not used for evaluation |
| | 13 | Measurements reported as < LOQ | Data not used for evaluation |
| Cd | 9 | Results for the QCM differ from the certified value | Data not used for evaluation |
| Cu | 5 | Results for the QCM differ from the certified value | Data not used for evaluation |
| Mn | 7 | Results for the QCM differ from the certified value | Data not used for evaluation |
| Pb | 2 | Results for the QCM differ from the certified value, but under-estimation of the uncertainties | Data retained for evaluation |
| | 12 | Results for ERM-BB185 were 4 times higher than the other data sets. After repeating the measurements and not changing the parameters, the results are 2 times higher than the other data sets. This inconsistency of results is indicating a method problem. | Data not used for evaluation |
| Se | 2 | Results for the QCM differ from the certified value, but under-estimation of the uncertainties | Data retained for evaluation |
| Zn | 7 | Results for the QCM differ from the certified value | Data not used for evaluation |

For Pb, the QC result from L2 was respectively 7 % below the assigned value, which is higher than the combined uncertainties of measurement (reported to be 1 %, k=2) and QC (2,61 %). For Se, the QC result from L2 was respectively 11 % below the assigned value, which is higher than the combined uncertainties of measurement (reported to be 5 %, k=2) and QC (4,17 %). It was therefore considered to reject both results. However, laboratories'

approaches to uncertainty estimation differ, and L2 reported lower uncertainties than most other participants despite using a similar technique (the median of this exercise for Pb was 12 %, and for Se the median was 13%). Therefore, it was decided that the L2 result for Pb and Se were of acceptable quality in comparison to results from the other participants and should not be excluded.

One laboratory (L12) reported results for Pb in ERM-BB185 that were four times higher than the means of all laboratories. After repeating the measurements for Pb, the results were still two times higher than the means of all laboratories. As this inconsistency of results indicates a method problem, the results were not used for value assignment.

### 6.4.2 Statistical evaluation

The datasets accepted based on technical reasons were tested for normality of dataset means using kurtosis/skewness tests and normal probability plots and were tested for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations, (both at a 99 % confidence level). Standard deviations within ($s_{within}$) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Table 7.

**Table 7:** Statistical evaluation of the technically accepted datasets for ERM-BB185. *p*: number of technically valid datasets

| Element | *p* | Outliers | | Normally distributed | Statistical parameters | | | |
|---|---|---|---|---|---|---|---|---|
| | | Means | Variances | | Mean [mg/kg] | *s* [mg/kg] | $s_{between}$ [mg/kg] | $s_{within}$ [mg/kg] |
| As | 7 | 0 | 1 | yes | 0.0177 | 0.0018 | 0.0017 | 0.0014 |
| Cd | 9 | 0 | 0 | yes | 0.280 | 0.018 | 0.018 | 0.007 |
| Cu | 12 | 0 | 1 | yes | 598 | 30 | 28 | 22 |
| Mn | 11 | 0 | 1 | yes | 13.1 | 0.3 | 0.2 | 0.5 |
| Pb | 10 | 0 | 0 | yes | 0.0326 | 0.0022 | 0.0019 | 0.0027 |
| Se | 12 | 0 | 2 | yes | 2.99 | 0.24 | 0.23 | 0.11 |
| Zn | 12 | 0 | 3 | yes | 143 | 5 | 5 | 7 |

The laboratory means follow normal distributions. None of the data contains outlying means. The datasets are therefore consistent and the mean of laboratory means is a good estimate of the true value. As the standard deviations of the means is comparable to the standard deviations between the laboratories, the uncertainty related to the characterisation is estimated as the standard error of the mean of the laboratory means.

The statistical evaluation flags various laboratories as having outlying variances for some datasets. This merely reflects the fact that different measurement principles have different intrinsic variability. As all measurement methods were found technically sound, all results were retained. The coefficient of variation is also below or just above the expected coefficient of variation between labs (10 %). L1 shows a large variance for As, confirmed as outlier by the Cochran test, which is associated to a day to day variation of the results. Since none of the laboratories indicated technical issues related to the specific measurements, the values are retained for certified value assignment.

In addition to the statistical tests, the agreement of individual laboratories' results with the dataset means was tested according to ERM Application Note 1 [6]. For Cu, Se and Zn one result did not agree with the dataset within the combined $U_{CRM}$ and reported measurement uncertainty, $U_m$. Because approaches to uncertainty estimation differ between laboratories, it is possible that not all sources of uncertainty are included in the budgets. For these labs, $U_m$ were assigned based on the $U_m$ reported by the other laboratories (18%), representing an acceptable level of $U_m$ for inclusion of the dataset.

The uncertainty related to the characterisation ($u_{char}$) is estimated as the standard error of the mean of laboratory means. The estimated uncertainties are listed by element, before rounding, in Table 8.

It should be borne in mind that the measurement procedure used in the characterisation are methods routinely applied for measuring elements in bovine liver. The agreement of results from different measurement procedures demonstrates that the processing did not affect any properties relevant for these methods and that ERM-BB185 behaves like a real sample.

**Table 8:** Uncertainty of characterisation for ERM-BB185

| Element | *p* | Mean [mg/kg] | *s* [mg/kg] | $u_{char}$ [mg/kg] |
|---|---|---|---|---|
| As | 7 | 0.0177 | 0.0018 | 0.0007 |
| Cd | 9 | 0.280 | 0.0183 | 0.0061 |
| Cu | 12 | 598 | 29.5043 | 8.5172 |
| Mn | 11 | 13.1 | 0.3090 | 0.0932 |
| Pb | 10 | 0.0326 | 0.0022 | 0.0007 |
| Se | 12 | 2.99 | 0.2372 | 0.0685 |
| Zn | 12 | 143 | 5.3612 | 1.5477 |

## 6.5 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 7 was assigned as certified value for each parameter.

The assigned uncertainty consists of uncertainties relating to characterisation, $u_{char}$ (Section 6), potential between-unit inhomogeneity, $u_{bb}$ (Section 4.1), and potential degradation during transport, $u_{sts}$, and long-term storage, $u_{lts}$ (Section 5). These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,\,rel}$) with a coverage factor *k* given as:

$$U_{CRM,rel} = k \cdot \sqrt{u_{bb,rel}^2 + u_{sts,rel}^2 + u_{lts,rel}^2 + u_{char,rel}^2} \qquad \textbf{Equation 6}$$

- $u_{char}$ was estimated as described in Section 6
- $u_{bb}$ was estimated as described in Section 4.1.
- $u_{sts}$ and $u_{lts}$ were estimated as described in section 5.3

Because of the sufficient numbers of the degrees of freedom of the different uncertainty contributions, a coverage factor *k* of 2 was applied, to obtain the expanded uncertainties. The certified values and their uncertainties are summarised in Table 9.

**Table 9:** Certified values and their uncertainties for ERM-BB185

| Element | Certified value[1)] [mg/kg] | $u_{char}$ [%] | $u_{bb}$ [%] | $u_{sts}$ [%] | $u_{lts,}$ [%] | $U_{CRM}$ [2)] [mg/kg] |
|---|---|---|---|---|---|---|
| As | 0.0177 | 3.81 | 2.67 | 1.83 | 2.96 | 0.0021 |
| Cd | 0.280 | 2.17 | 0.36 | 0.12 | 0.79 | 0.014 |
| Cu | 598 | 1.42 | 0.44 | 0.21 | 0.95 | 22 |
| Mn | 13.1 | 0.71 | 0.64 | 0.17 | 1.37 | 0.5 |
| Pb | 0.0326 | 2.16 | 1.16 | 1.39 | 1.49 | 0.0021 |
| Se | 2.99 | 2.29 | 1.01 | 0.30 | 1.34 | 0.18 |
| Zn | 143 | 1.08 | 0.50 | 0.21 | 0.93 | 5 |

[1)]: reported on dry mass basis (section 8.5)

[2)] Expanded ($k$ = 2) and rounded uncertainty.

# 7 Metrological traceability and commutability

## 7.1 Metrological traceability

**Identity**

The measurands Cu, Mn, Se and Zn are clearly defined as total element mass fractions. The participants used different measurement principles and procedures for the sample preparation as well as for the final determination, demonstrating absence of measurement bias. The measurands are therefore structurally defined and independent of the measurement method.

As, Cd and were only measured by inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma sector field mass spectrometry (ICP-SFMS). Pb was besides ICP-MS and ICP-SFMS, also measured by isotope dilution inductively coupled plasma sector field mass spectrometry (ID-ICP-SFMS)

Only validated methods were used for the determination of the assigned values. Different calibrants of specified traceability of their assigned values were used and all relevant input parameters were calibrated. The individual results are therefore traceable to the SI. As the assigned values are combinations of agreeing results individually traceable to the International System of units (SI), the assigned quantity values themselves are traceable to the SI as well.

## 7.2 Commutability

Many measurement procedures include one or more steps which select specific (or specific groups of) analytes from the sample for the subsequent whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions that define this concept. For instance, the CLSI Guideline C53-A [7] recommends the use of the following definition for the term *commutability*:

"The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and is therefore a crucial characteristic when applying different measurement methods. When the commutability of a CRM is not established, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrant.

ERM-BB185 was produced from a raw bovine liver by freeze drying, milling and mixing. The analytical behaviour will be the same as for a routine sample of bovine liver. The methods used in the characterisation are methods routinely applied for measuring elements in bovine liver. The agreement of results from different methods demonstrates that the processing did not affect any properties relevant for these methods and that ERM-BB185 behaves like a real sample.

# 8 Instructions for use

## 8.1 Safety information

The usual laboratory safety measures apply.

## 8.2 Storage conditions

The materials should be stored at 18 ± 5 °C in the dark. Care should be taken to avoid any change of the moisture content once the units are open, as the material is hygroscopic. The user should close any vials immediately after taking a sample.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially for opened vials.

## 8.3 Preparation and use of the material/Reconstitution

The units shall be shaken by turning upside down for at least 2 min before opening to ensure material re-homogenisation.

## 8.4 Minimum sample intake

The minimum sample intake representative for all elements is 400 mg.

## 8.5 Dry mass correction

Dry mass determination shall be carried out on a separate portion of at least 1 g, by drying in an oven at 103 $^{o}$C ± 2 $^{o}$C until constant mass (separate weighing should not differ by more than 5 mg) is attained. Weighing of the samples for dry mass determination and weighing for the analysis shall be done at the same time to avoid differences due to possible take up of moisture by the material.

## 8.6 Use of the certified value

The main purpose of these materials is to assess method performance, i.e. for checking accuracy of analytical results/calibration. As any reference material, it can be used for establishing control charts or validation studies.

Use as a calibrant

It is not recommended to use this matrix material as calibrant. If used nevertheless, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, www.erm-crm.org [6].

When assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value ($\Delta_{meas}$).
- Combine the measurement uncertainty ($u_{meas}$) with the uncertainty of the certified value ($u_{CRM}$): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty ($U_{\Delta}$) from the combined uncertainty ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %
- If $\Delta_{meas} \leq U_{\Delta}$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

Use in quality control charts

The materials can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

# 9 Acknowledgments

The authors would like to acknowledge the support received from Håkan Emteborg and John Seghers from JRC Geel relating to the processing of this CRM and from Maria Contreras Lopez concerning the set-up of the required isochronous studies.

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# 10 References


1 ISO Guide 34:2009, General requirements for the competence of reference materials producers, International Organization for Standardization, Geneva, Switzerland

2 ISO Guide 35:2006, Reference materials – General and statistical principles for certification, International Organization for Standardization, Geneva, Switzerland

3 A. Lamberty, G.N. Kramer, The certification of the mass fraction of As, Cd, Cu, Mn, Pb, Se and Zn in bovine liver, CRM 185R, EUR 18841 – European Union Luxembourg-1998- ISBN 92-828-6060-4

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# Annexes

Annex A: Results of the homogeneity measurements

Annex B: Results of the short-term stability measurements

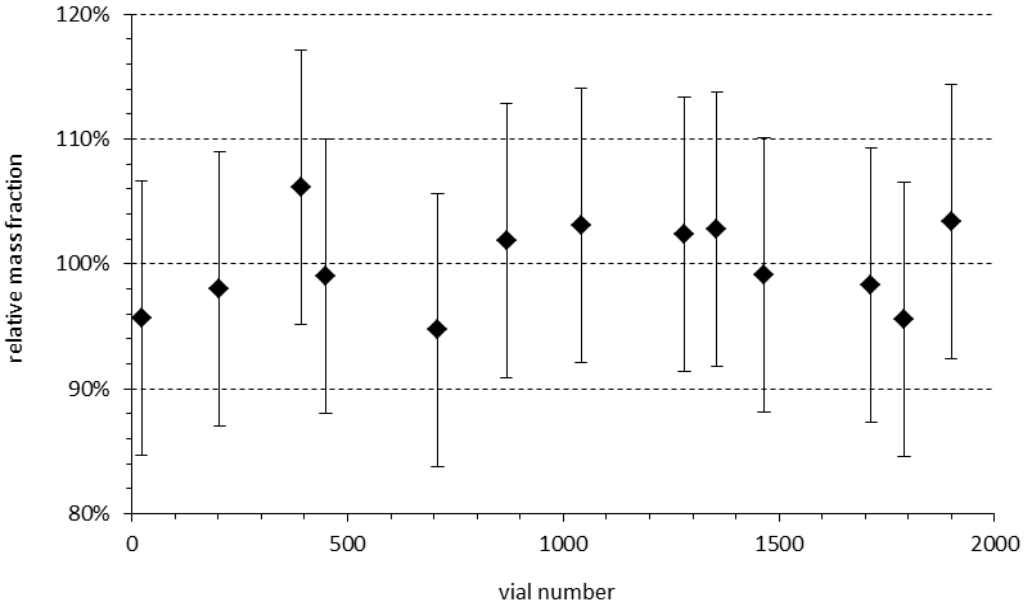
Annex C: Results of the long-term stability measurements

Annex D: Summary of methods used in the characterisation study of As, Cd, Cu, Mn, Pb, Se and Zn as reported by the laboratories

## Annex A: Results of the homogeneity measurements

Graphs present mass fractions of vial means relative to the grand mean, against vial number, and individual measurement replicates, against sequence number. Vertical bars are a confidence interval of 95 % derived from $s_{wb}$ of the homogeneity study.

**Figure A1:** As



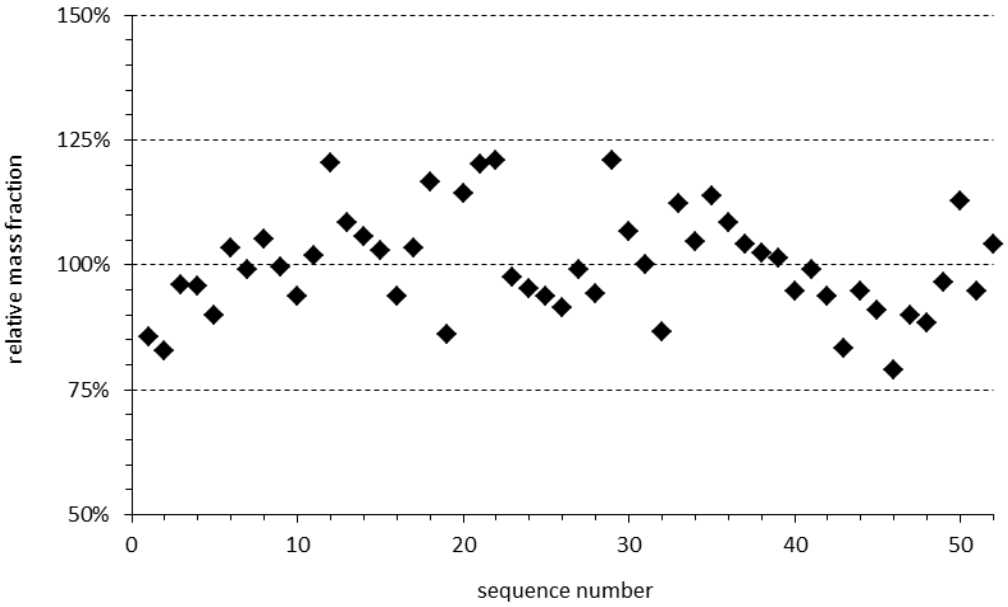
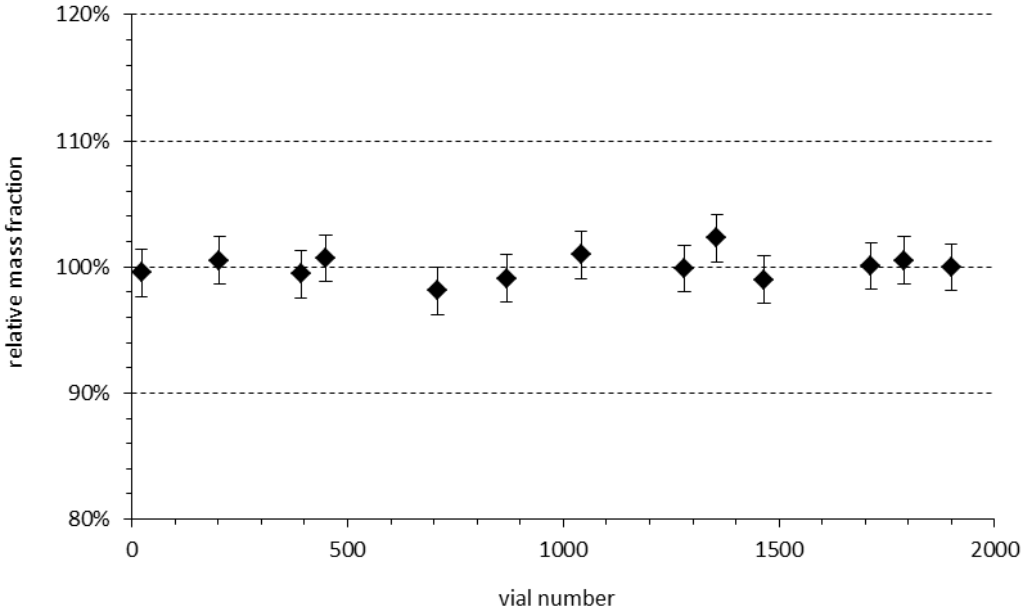



**Figure A2:** Cd


120%
110%
100%
90%
80%
relative mass fraction
0
500
1000
1500
2000
vial number

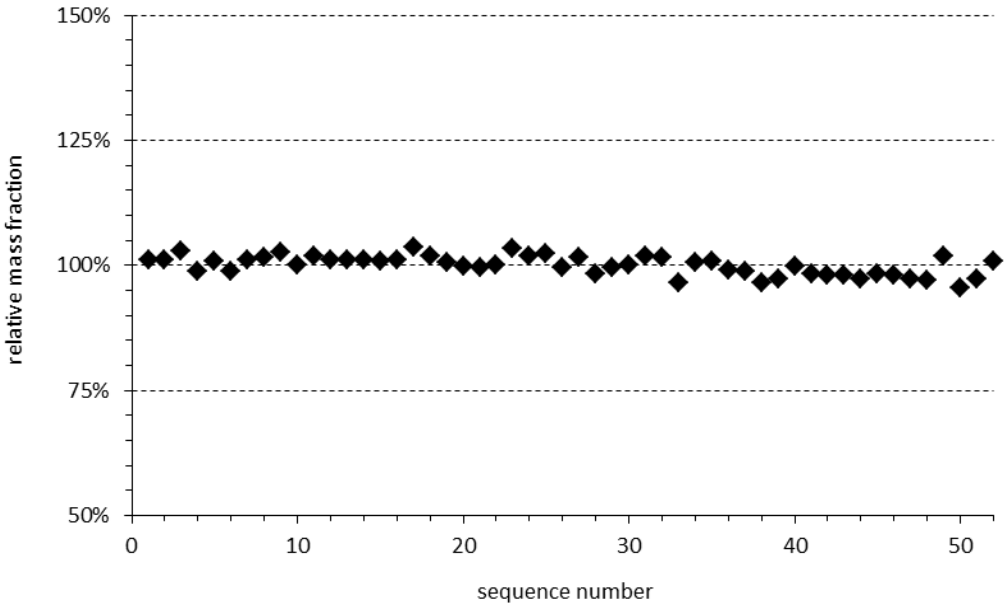
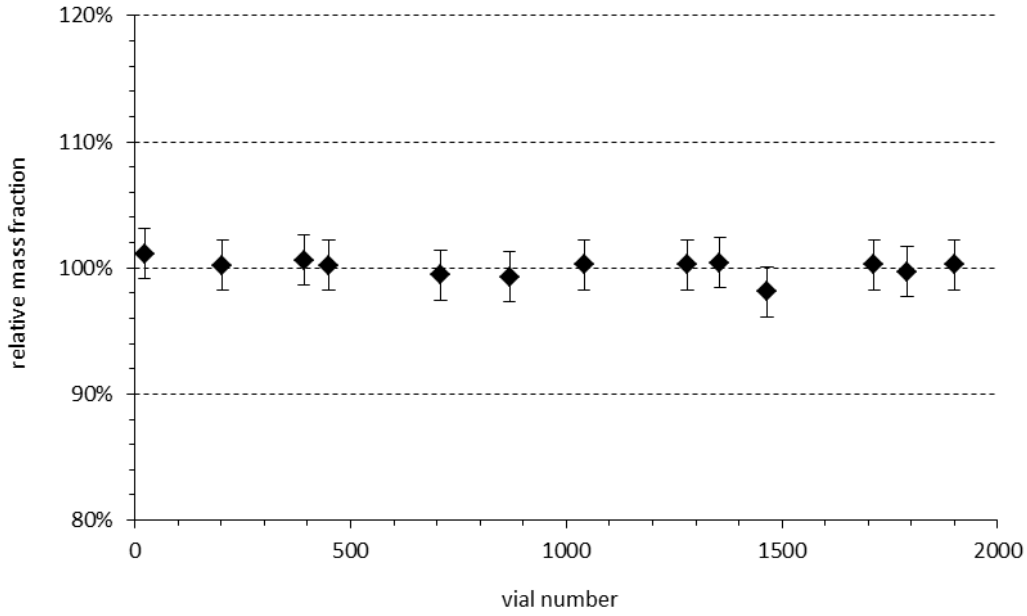

150%
125%
100%
75%
50%
relative mass fraction
0
10
20
30
40
50
sequence number

**Figure A3:** Cu


120%
110%
100%
90%
80%
relative mass fraction
0
500
1000
1500
2000
vial number

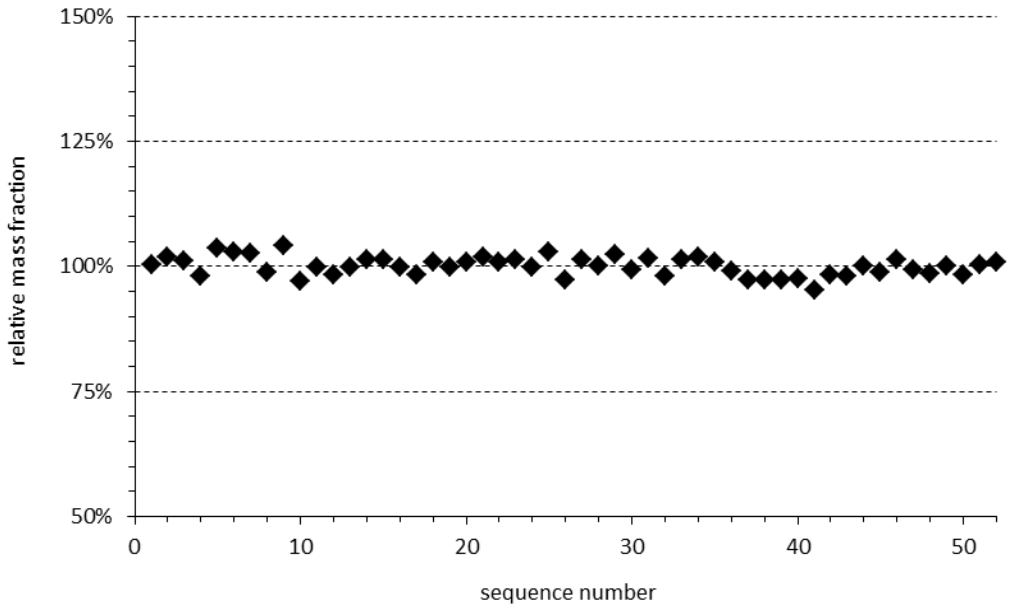
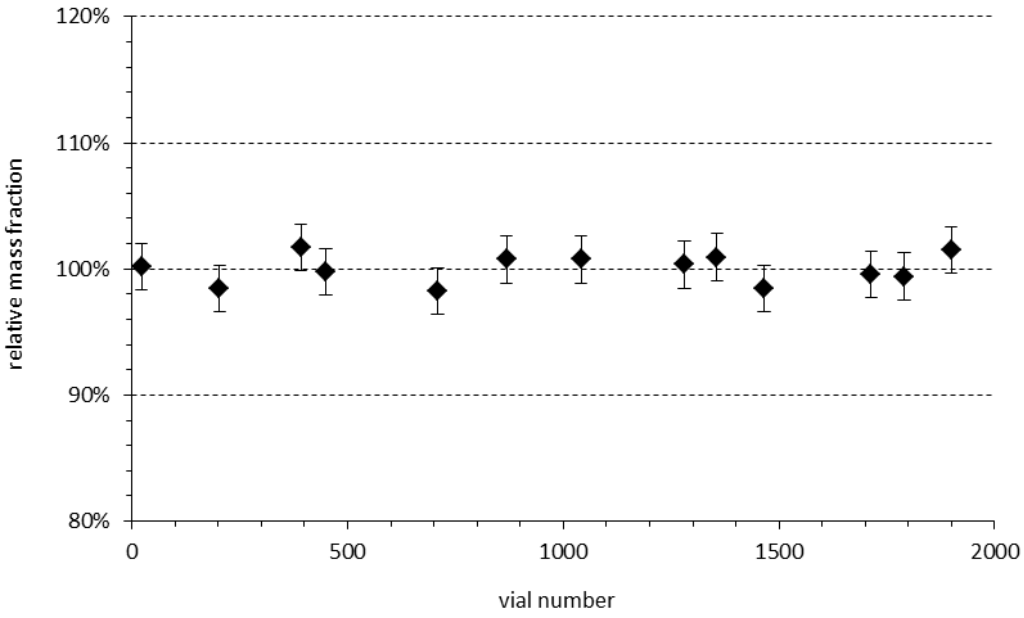

150%
125%
100%
75%
50%
relative mass fraction
0
10
20
30
40
50
sequence number

**Figure A4:** Mn


120%
110%
100%
90%
80%
relative mass fraction
0
500
1000
1500
2000
vial number

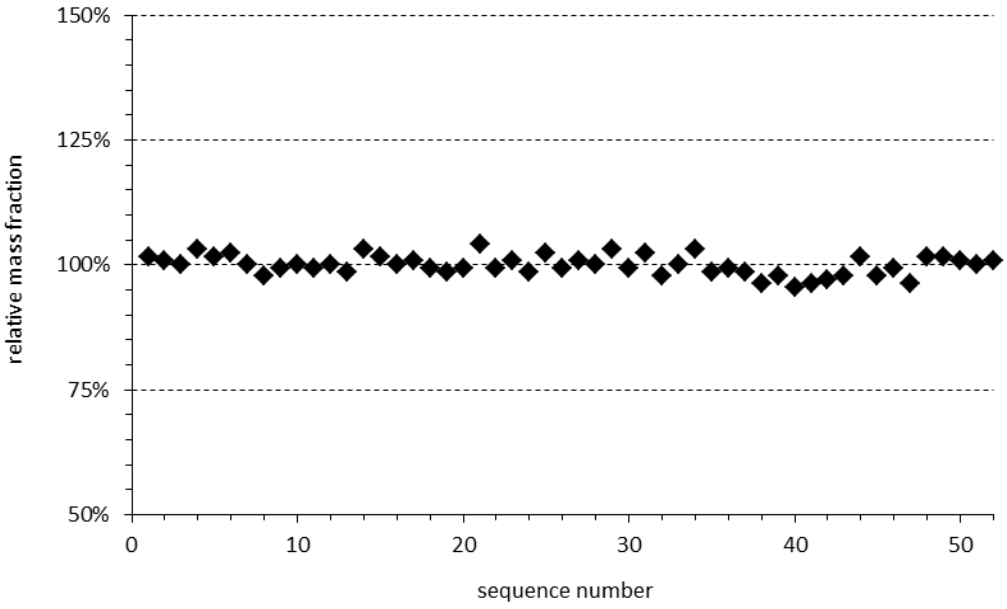
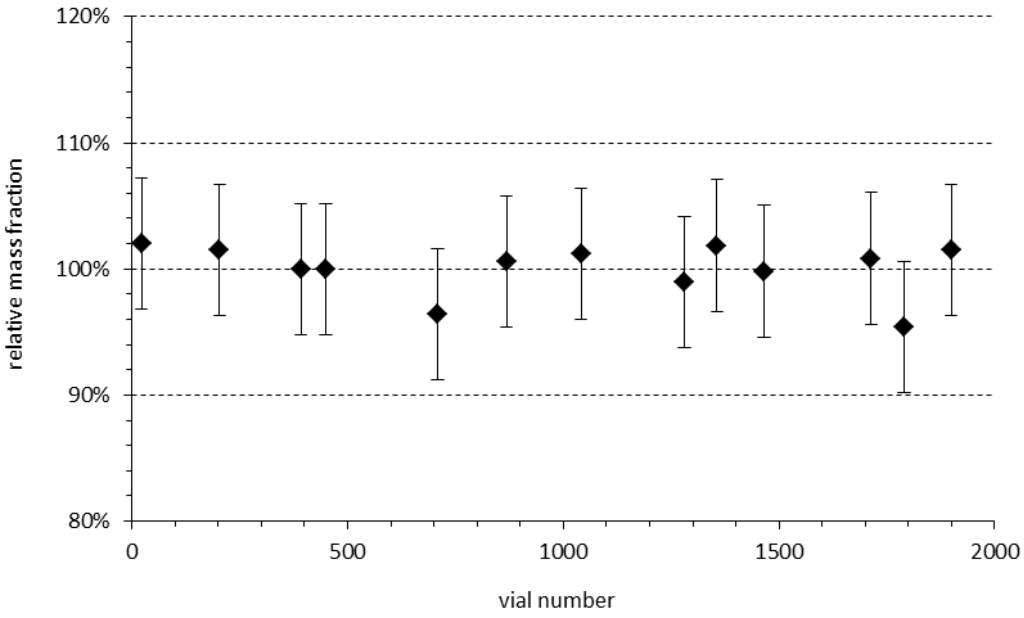

150%
125%
100%
75%
50%
relative mass fraction
0
10
20
30
40
50
sequence number

**Figure A5:** Pb


120%
110%
100%
90%
80%
relative mass fraction
0
500
1000
1500
2000
vial number

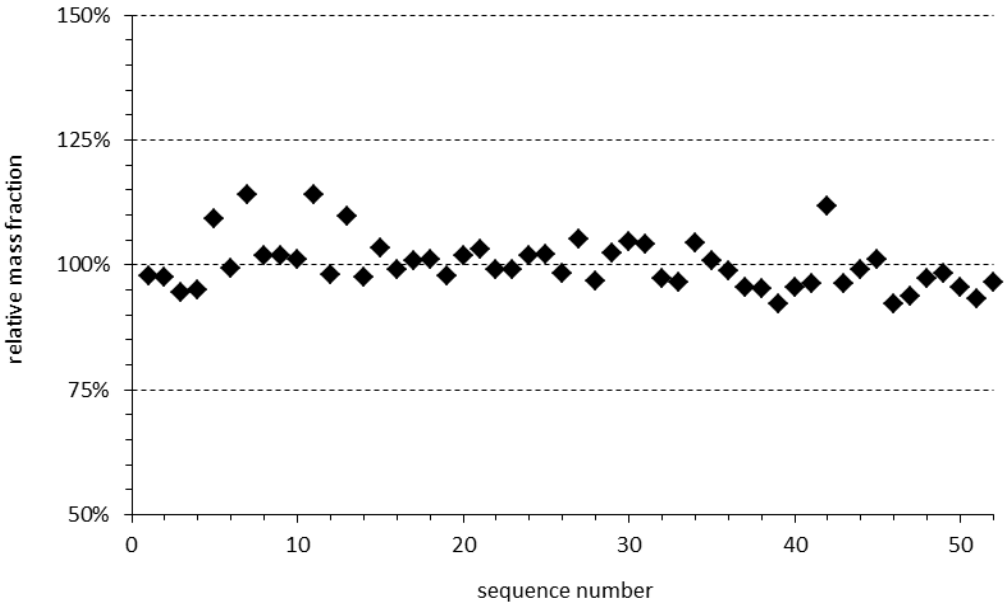
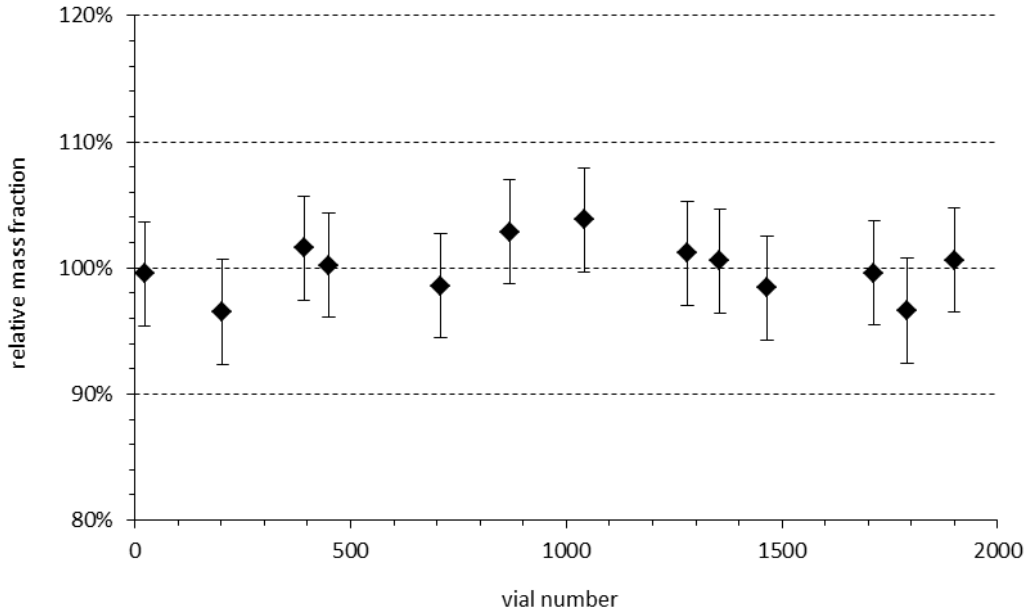

150%
125%
100%
75%
50%
relative mass fraction
0
10
20
30
40
50
sequence number

**Figure A6:** Se


120%
110%
100%
90%
80%
relative mass fraction
0
500
1000
1500
2000
vial number

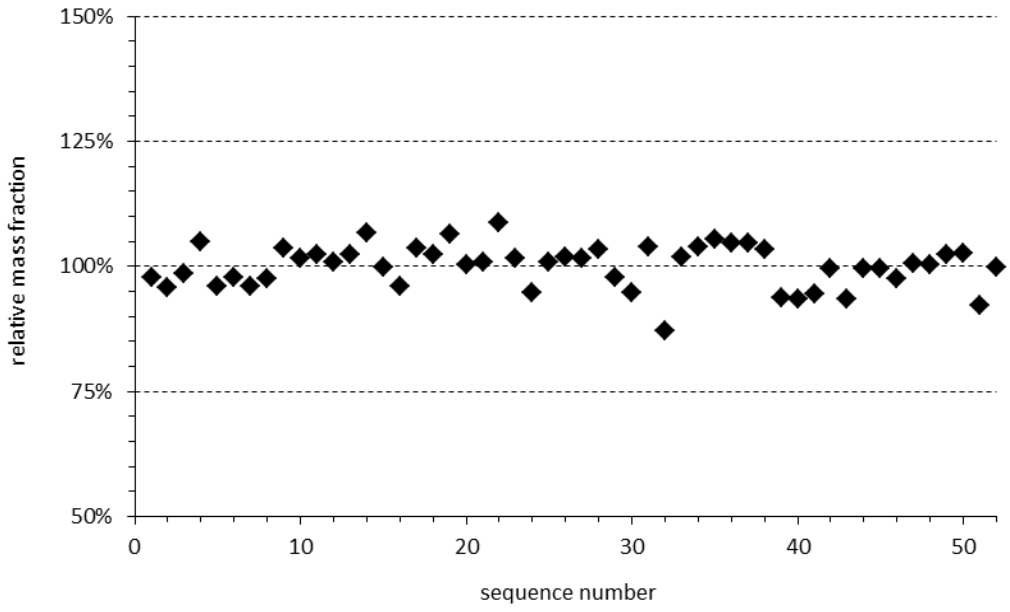
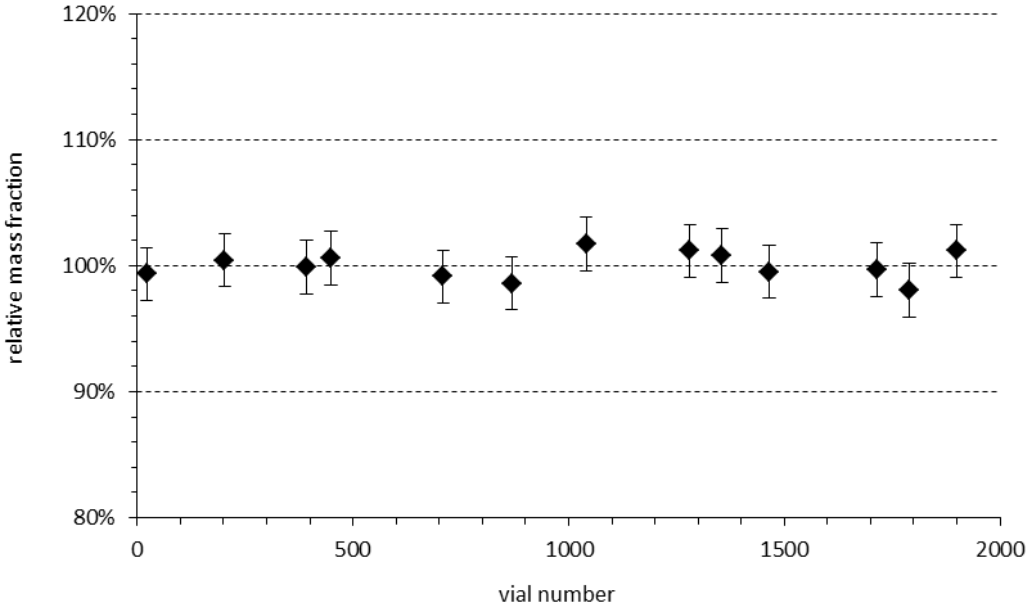
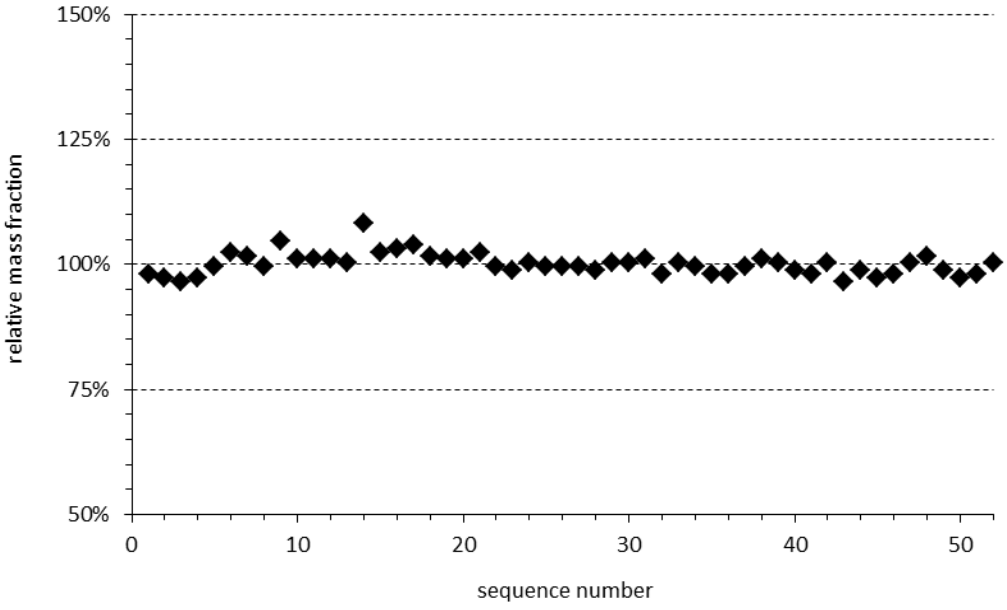

150%
125%
100%
75%
50%
relative mass fraction
0
10
20
30
40
50
sequence number

**Figure A7:** Zn


120%
110%
100%
90%
80%
relative mass fraction
0
500
1000
1500
2000
vial number


150%
125%
100%
75%
50%
relative mass fraction
0
10
20
30
40
50
sequence number

## Annex B: Results of the short-term stability measurements

Graphs present the mean mass fractions measured at each time-point relative to the grand mean, against the time that the samples were held at 60 ºC. Vertical bars represent the 95 % confidence interval of the measurements, based on the variance of measurements for each time-point calculated by ANOVA.

**Figure B1: As**

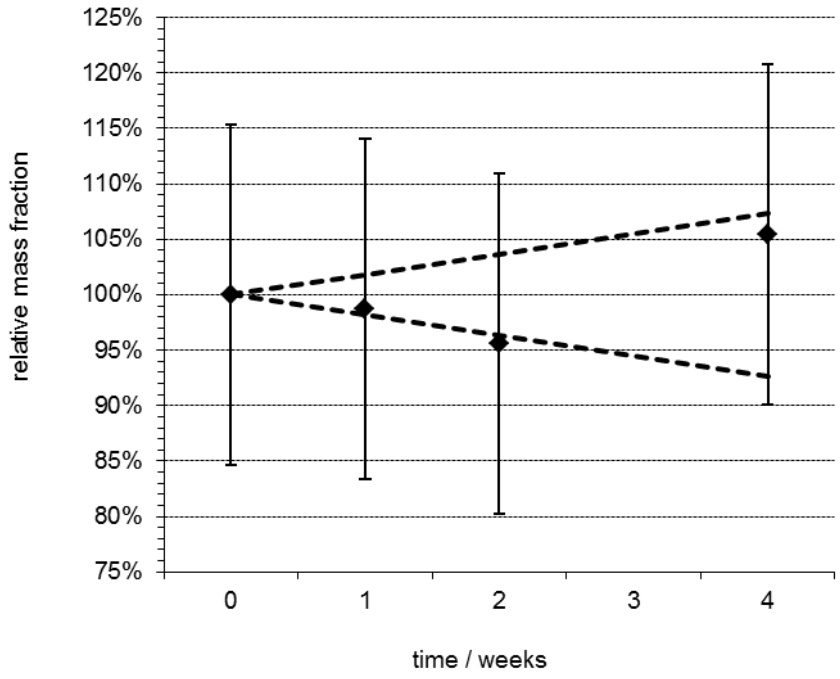



**Figure B2: Cd**

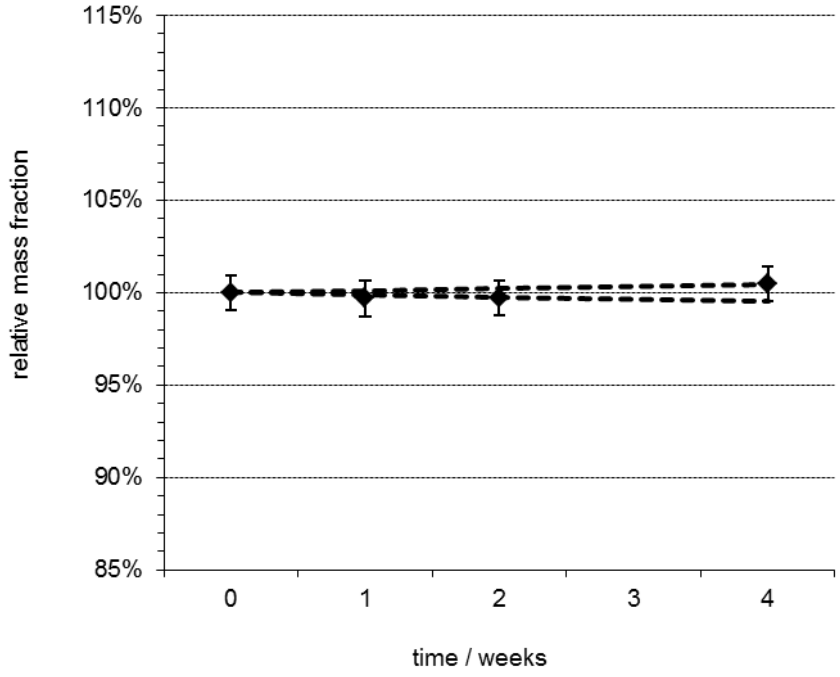


**Figure B3: Cu**

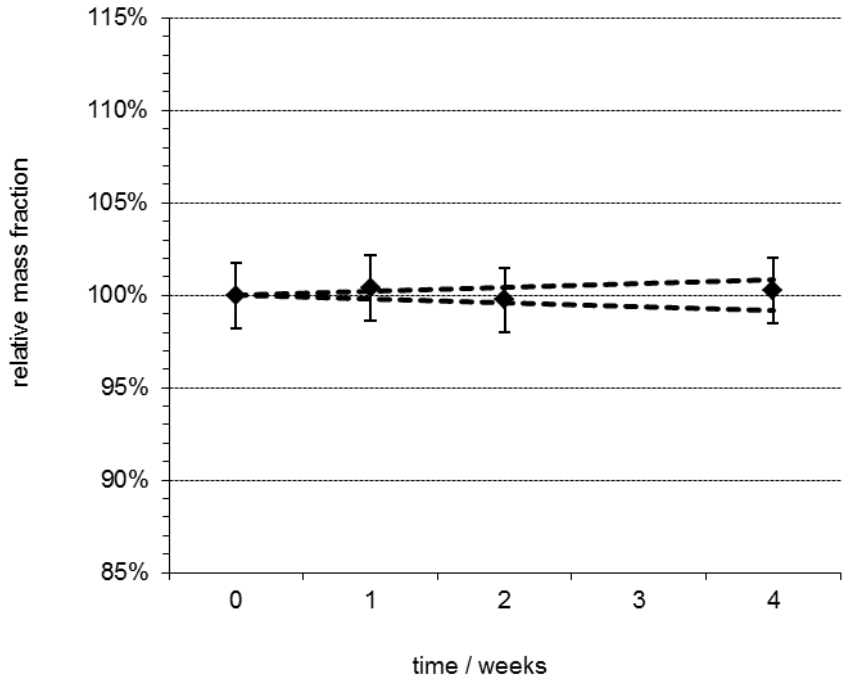



**Figure B4: Mn**

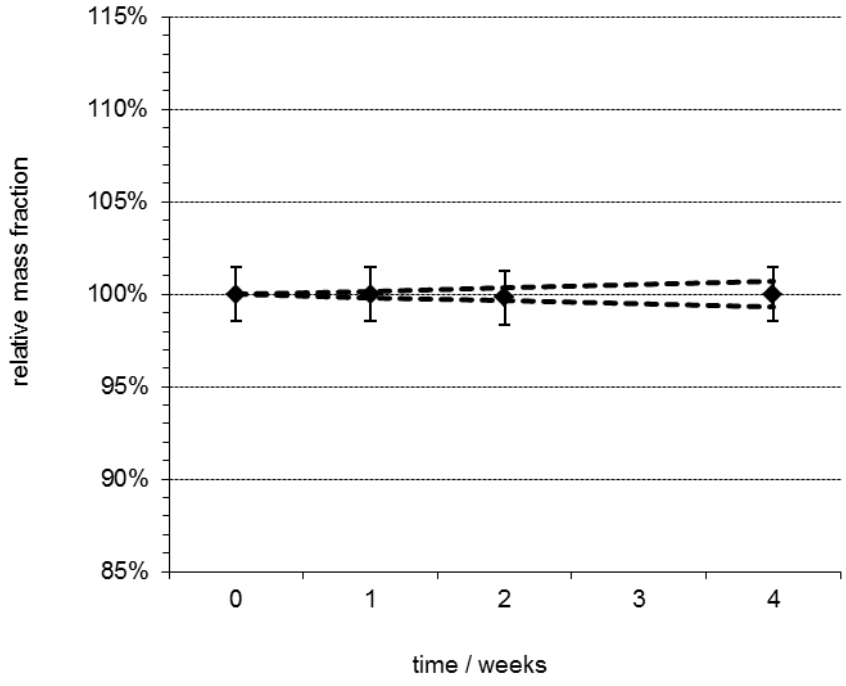


**Figure B5: Pb**

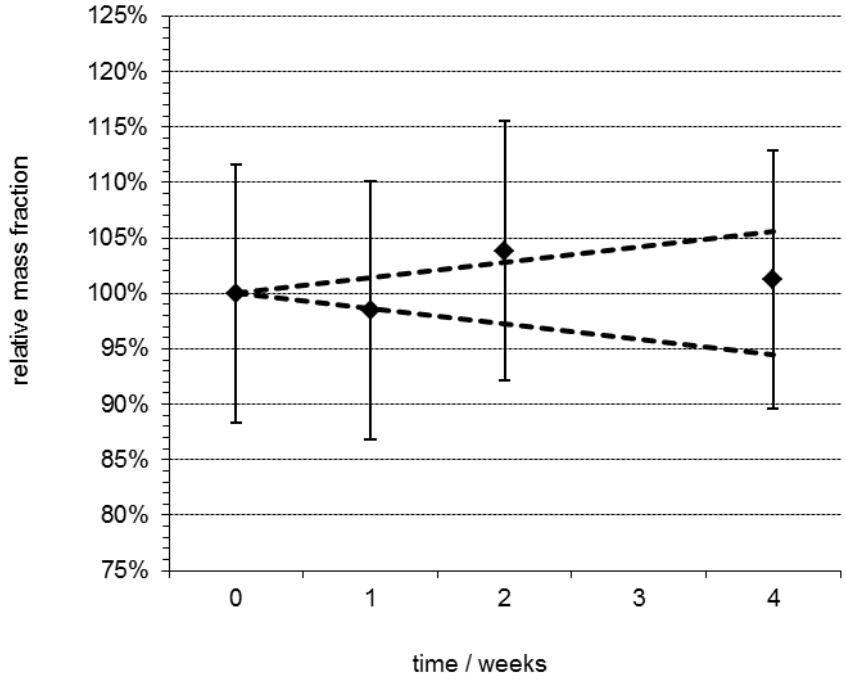



**Figure B6: Se**

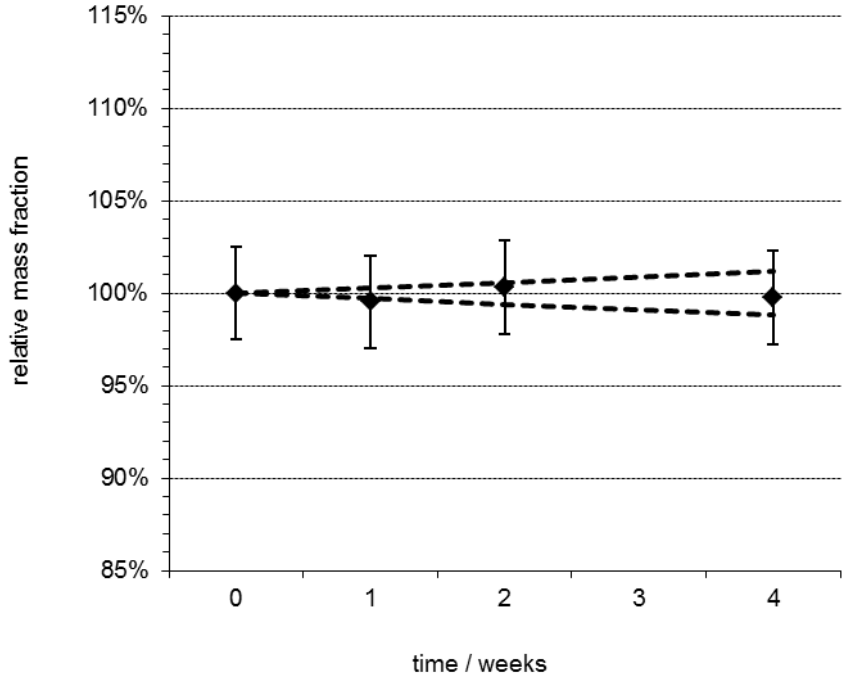
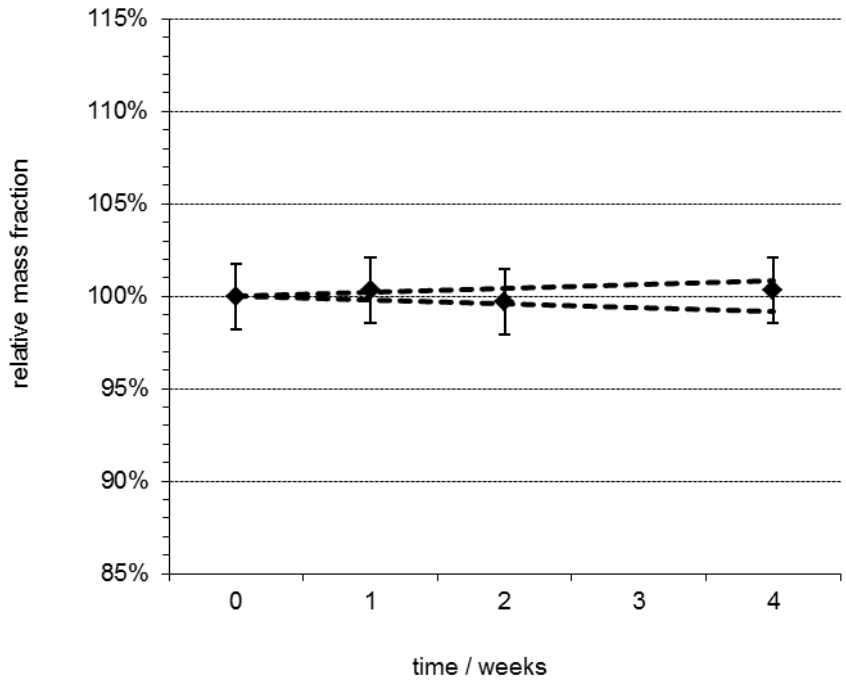


**Figure B7: Zn**


115%
110%
105%
100%
95%
90%
85%
relative mass fraction
0
1
2
3
4
time / weeks

## Annex C: Results of the long-term stability measurements

Graphs present the mean mass fractions measured at each time-point relative to the grand mean, against the time that the samples were held at 18 ºC. Vertical bars represent the 95 % confidence interval of the measurements, based on the variance of measurements for each time-point calculated by ANOVA.

**Figure C1: As**

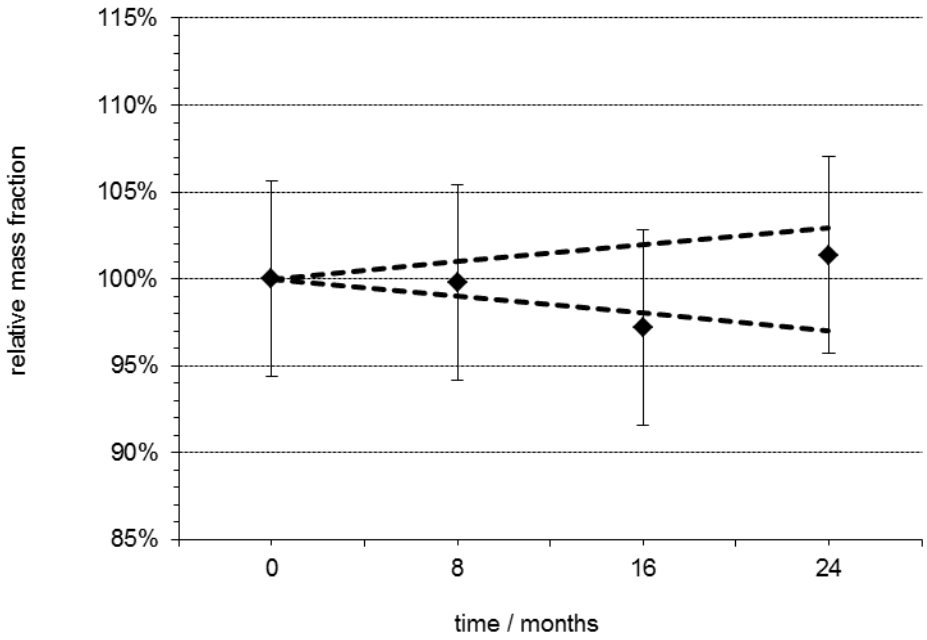



**Figure C2: Cd**

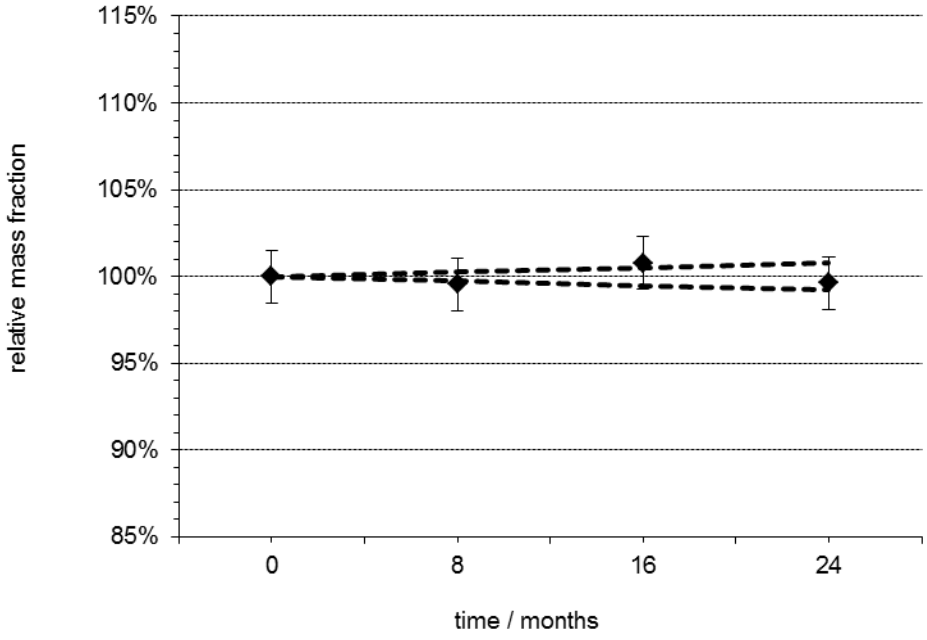


**Figure C3: Cu**

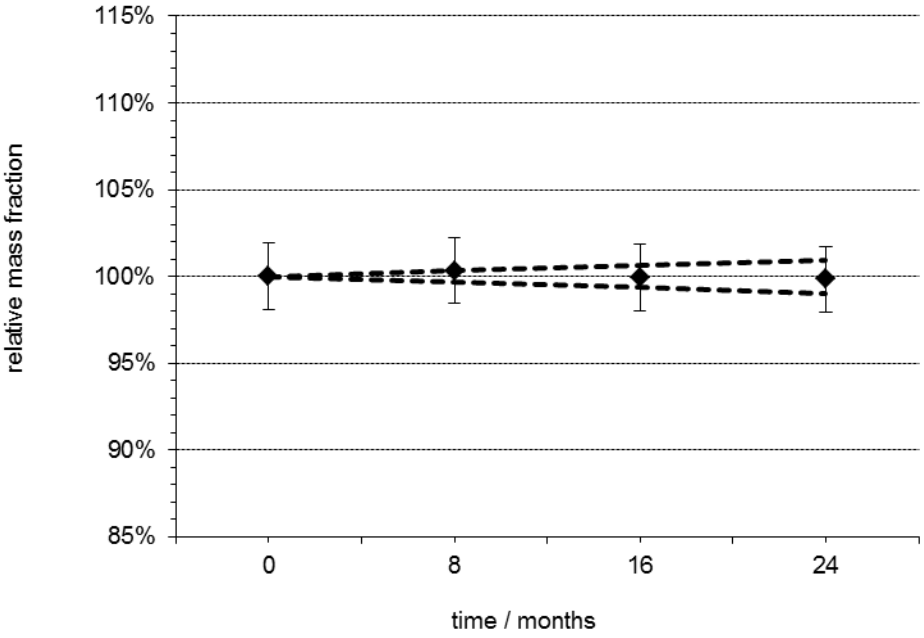



**Figure C4: Mn**

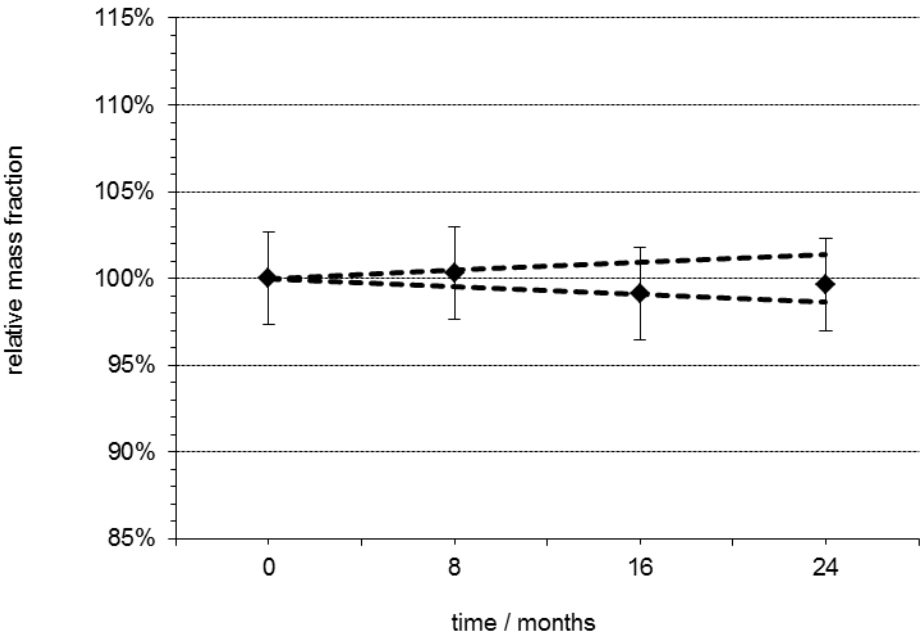


**Figure C5: Pb**

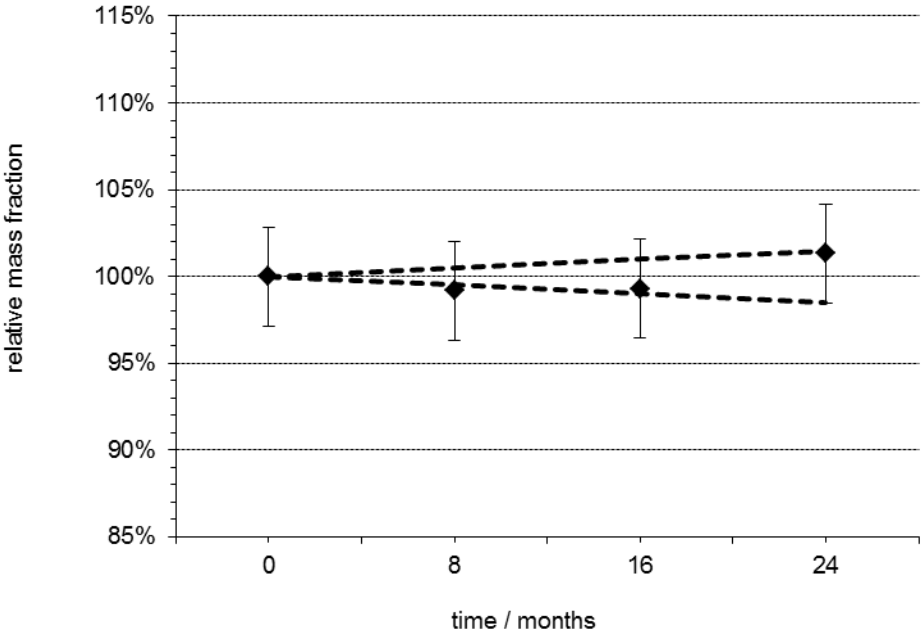



**Figure C6: Se**

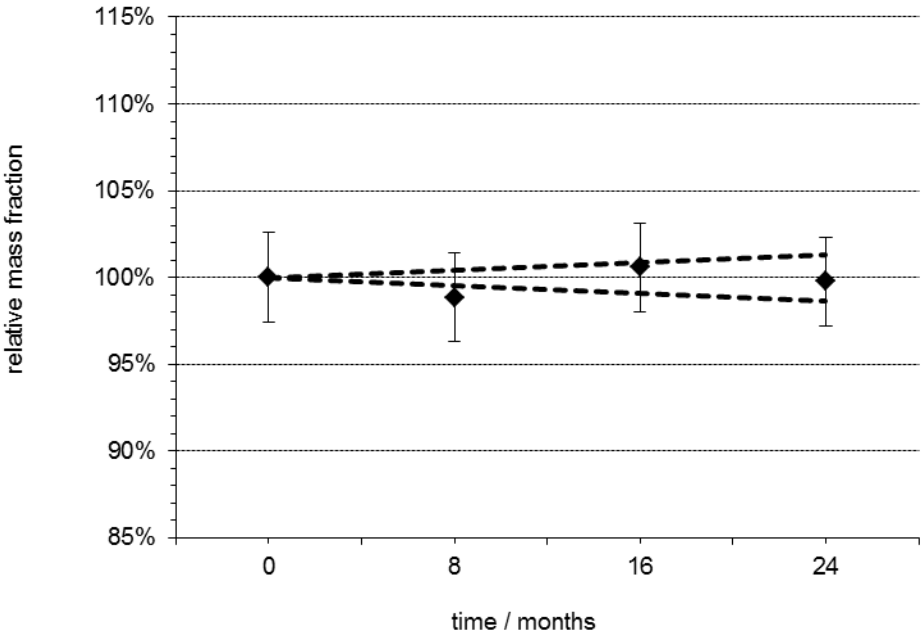
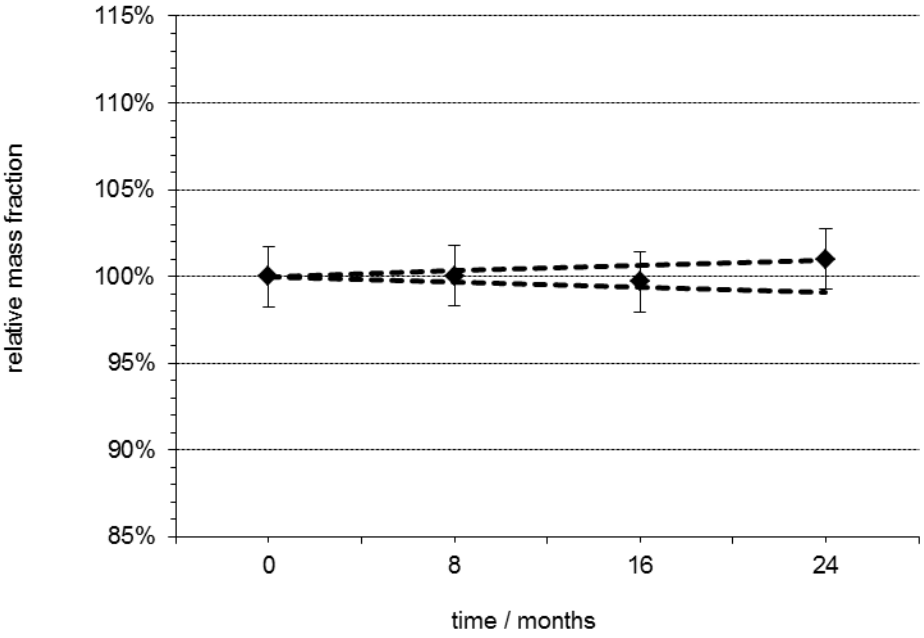


**Figure C7: Zn**


115%
110%
105%
100%
95%
90%
85%
relative mass fraction
0
8
16
24
time / months

## Annex D: Summary of methods used in the characterisation study of As, Cd, Cu, Mn, Pb, Se and Zn in ERM-BB185 as reported by the laboratories

| Lab code | Sample preparation method | Measurement technique | Calibration method | Quality Assurance |
|---|---|---|---|---|
| L0 | Microwave digestion<br>$HNO_3/H_2O_2$ | ICP-MS | External - 4% propanol for As and Se<br>External for Cd, Cu, Pb, Se and Zn | Spike, duplicate, reference material<br>Traceable to NIST SRM3103a (As), NIST SRM3108 (Cd), NIST SRM3114 (Cu), NIST SRM3132 (Mn), NIST SRM3128 (Pb), NIST SRM3149 (Se) and NIST SRM3168a (Zn) |
| L1 | Microwave-assisted digestion with concentrated nitric acid. Sample intake 0,4 gram digested with 5 mL of conc $HNO_3$ in quartz vessels in an high-pressure microwave oven from Anton Paar (Multiwave 3000). The sample preparation followed the principles in EN13805:2014. | ICP-MS | Quantification was done by standard addition calibration with internal standardisation. Certified standard stock solution at 1000 mg/L (SCP Science) were used for all elements to make the calibration | Quality assurance of the analytical results included the analysis of CRM BB185. |
| L2 | Microwave assisted high pressure digestion (nitric acid hydrochloric acid) | ICP-MS | Initially (only on day one), a calibration using 0 / 0.1 / 1 / and 10 ng/mL solutions was done for this analytical sequence. Based on the results for the test solutions, individual calibration levels for each element were prepared and measured at the end of the analytical sequence. On the second day, the individual calibration approach was directly applied. The final approach was directly applied. The final calibration range was chosen in a way, that the measured results are approximately in the middle of the calibration curve and the concentration steps are equally spaced (calibration blank and at least 5 points). | Calibration checks were performed using solutions containing 10 ng/mL for each analyte. At the end of the analytical sequence a calibration control was performed (10 ng/mL). For all calibration controls and calibration checks recoveries were between 80 and 120 % for the evaluated isotopes. As part of system suitability test (SST) the certified reference material TMDA-26.4 (low level fortified sample for trace elements reference material, National Research Council Canada), DORM-4 (fish protein reference material, National Research Council Canada) and INCT-MPH-) 2 (mixed polished herbs, INSTYTUT CHMII I TECKNIKI JADRWEJ were used. Additionally to those CRMs, an in-house prepared reference material (spiked nicotinic acid) was used. Acceptance criteria for this part of SST as a recovery of 70-130% for each analyte. |

| | | | | |
|---|---|---|---|---|
| L3 | Microwave digestion | ICP-MS | External calibration | For quality assurance the standard reference material NIST 2976 Mussle Tissue was prepared and analysed in the same manner as samples. |
| L4 | Microwave-assisted acid digestion. | ICP-SFMS;<br>LR for Cd and Pb<br>MR for Cu, Mn and Zn<br>HR for As and Se | External calibration | Preparation blanks, ERM-BB442 |
| L5 | For the determination of As, Cd, Cu, Se and Zn three samples of about 300 mg were taken from each bottle in an acclimatized room and transferred in standard high-density polyethylene (HDPE) vials. After weighing, the samples were placed in the irradiation vials together with four IRMM-530 (Al-0.1% Au alloy) neutron flux monitors, SMELS II, SMELS III and a BCR185 and ERM-BB422 validation samples. The IRMM-530 monitors are used to determine the neutron flux during irradiation, a parameter required by the $k_0$-NAA formalism. Neither additional sample treatment nor sample transfer into clean non-irradiated vials was applied. | In terms of irradiation a long irradiations X1709 - seven hours on2017-06-13 in channel Y4 of the BR1 reactor was performed. For each sample two spectra were collected on a k0-calibrated HPGe detector under repeatability conditions. The first one (for the determination of As, Cu and Cd) after a cooling time of 1-3 days, the second one (for the determination of Zn and Se) after a cooling time of 3 weeks. Measurement times were respectively 24 hours and minimum 48 hours. | | SMELS I, SMELS II, SMELS III, BCR185 and ERM-BB422 were used to validate our experimental protocols. All results yield a $\zeta < 3$ and therefore validate our analytical performance. |
| L6 | Closed microwave digestion with $HNO_3$ | ICP-MS | External linear Calibration points 0-1.0-2.0-5.0-10 µg/L<br>Analytika CZ calibrants | Analysis of certified reference materials BCR-185r, ERM-BB184 and NIST-2976. Control of calibration correctness and instrument drift by analysing and independent QC sample (5 µg/L) inbetween and at the end of the analysis series. Subtraction of procedural blank. |
| L7 | Closed microwave digestion with $HNO_3$ | ICP-OES | External linear Calibration points 0-0.01-0.05-0.10-0.50-1.0-5.0 mg/L<br>Analytika CZ calibrants | Analysis of certified reference materials BCR-185r, ERM-BB184 and NIST-2976. Control of calibration correctness and instrument drift by analysing and independent QC sample (5 mg/L) |

| | | | | |
|---|---|---|---|---|
| | | | | inbetween and at the end of the analysis series. Subtraction of procedural blank. |
| L8 | Microwave digestion: 0.25 g of sample + 5 mL of $HNO_3$:H2O 1:1 and 0.5 mL of $H_2O_2$. 25 g final weight. | ICP-MS. Three methods of analysis have been used:<br>(i) As, Cd and Pb were analysed in Standard mode.<br>(ii) Cu, Mn and Zn were analysed in Dynamic reaction cell mode using methane ($CH_4$) as a reaction gas.<br>(iii) Se was analysed also in DRC mode, but using oxygen ($O_2$) as a reaction gas for eliminating the 40Ar38Ar interference. | Matrix matched calibration standards ($HNO_3$ and $H_2O_2$) were prepared at levels ranging from 0.1 to 50 µg/kg (As, Cd, Pb and Se) and from 1 to 500 µg/kg (Cu, Mn and Zn). The calibration curve was drawn from seven points including the calibration blank and there was applied a weighted linear regression approach with internal standardization. | QCS standards (ICV and CCV) were run immediately after calibration and after every ten samples. Reagent Blank (RB) to check sample preparation contamination. Certified reference material: BCR – 185 (bovine liver) |
| L9 | Closed Microwave digestion with nitric acid and hydrogen peroxide. | Sector field ICP-MS - measurement in High resolution for As and Se<br>Sector field ICP-MS - measurement in Medium resolution for Cd, Cu, Mn, Pb and Zn | external calibration :0-1.25-2.5-12.5-25 µg/l | Spex Certiprep traceable to NIST SRM 3103a, 3108, 3114, 3128, 3132, 3149, 3168a |
| L10 | Closed Microwave digestion with nitric acid and hydrogen peroxide | Sector field ICP-MS - ID-MS measurement in low resolution | ID-MS Pb-206 | measurement of independent control sample |
| L11 | High pressure microwave digestion | collision cell ICP-MS, HEHe mode for As and Se<br>collision cell ICP-MS, He mode for Cd, Cu, Mn and Zn<br>collision cell ICP-MS, nogas mode for Pb | Reagent blanks, spikes and CRM (BCR185R) | Based on proficiency test performance |
| L12 | Microwave digestion | ICP-MS | External calibration | For the verification of the accuracy of concentrations of standard solutions used for |

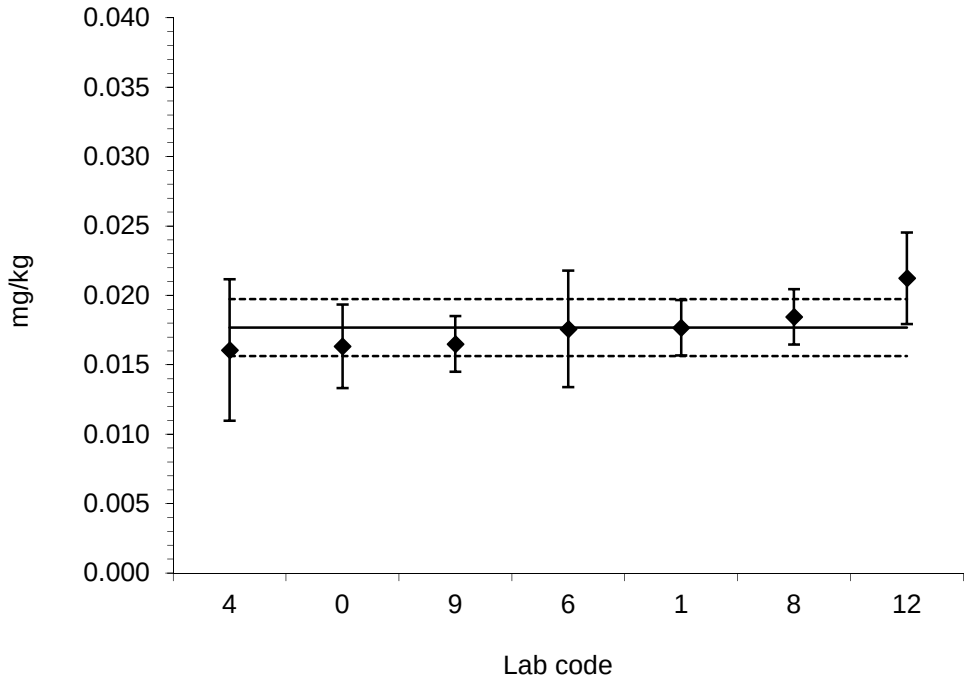
| | | | | |
|---|---|---|---|---|
| | | | | preparation of the calibration curve, Reference Material for Measurement of Elements in Surface water (SPS-SW1) was measured before sample analysis. To verify the accuracy of the analytical procedure, certified reference material DOLT-5 Dogfish Liver (NRC-CNRC, Canada) was analysed in triplicate together with the samples. |
| L13 | An aliquot prepared in form of pellet. | $k_0$-NAA | $k_0$-standardization method of Neutron Activation Analysis: Characterization of an irradiation channel, absolute calibration of an HPGe detector, k0-database with nuclear data, standard Al-0.1%Au (IRMM-530R). | NIST SRM-1547 Peach Leaves |

**Annex E: Results of the characterisation measurements**

Tables present the results of characterisation measurements (corrected to dry mass) and the estimated uncertainties of the measurements, as reported by participants. The tables in this annex also contain the data sets that were not retained. The reported values were rounded for formatting reasons. Graphs show expanded uncertainties as stated by the laboratories and the certified range, and are given for the accepted datasets only.

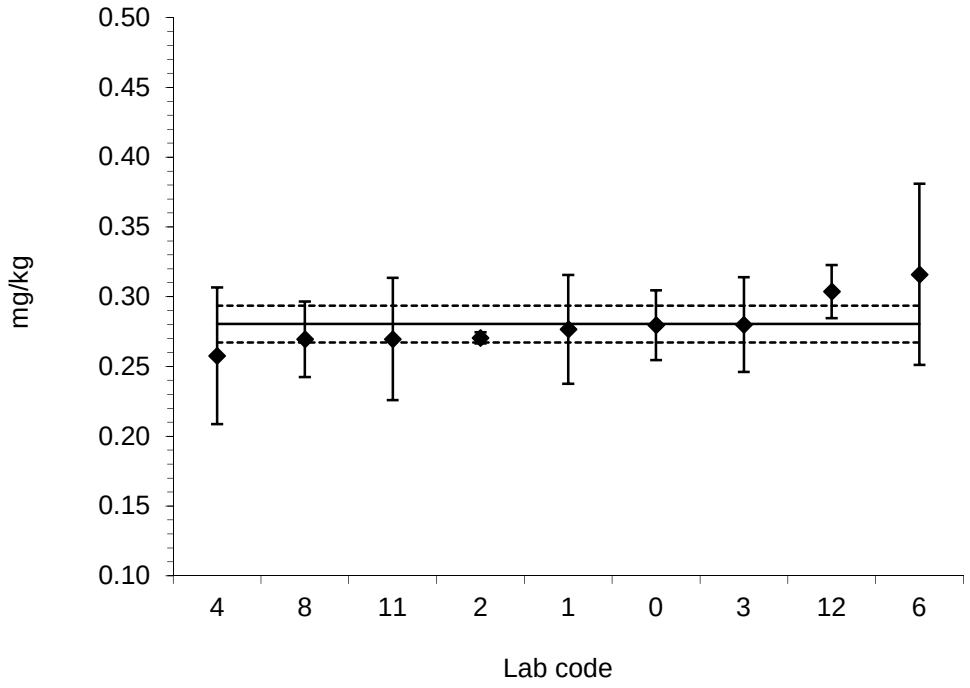
**As**

| Lab code | Technique | Replicate 1 [mg/kg] | Replicate 2 [mg/kg] | Replicate 3 [mg/kg] | Replicate 4 [mg/kg] | Replicate 5 [mg/kg] | Replicate 6 [mg/kg] | Mean [mg/kg] | Expanded uncertainty [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| 0 | ICP-MS | 0.015 | 0.015 | 0.014 | 0.018 | 0.018 | 0.018 | 0.016 | 0.003 |
| 1 | ICP-MS | 0.013 | 0.018 | 0.016 | 0.020 | 0.020 | 0.019 | 0.0177 | 0.002 |
| 4 | ICP-SFMS | 0.0149 | 0.0161 | 0.0175 | 0.0163 | 0.0154 | 0.0162 | 0.0161 | 0.0051 |
| 6 | ICP-MS | 0.0180 | 0.0174 | 0.0168 | 0.0195 | 0.0175 | 0.0163 | 0.0176 | 0.0042 |
| 8 | ICP-MS | 0.0183 | 0.0182 | 0.0168 | 0.0188 | 0.0187 | 0.0199 | 0.0185 | 0.0020 |
| 9 | ICP-SFMS | 0.017 | 0.017 | 0.017 | 0.016 | 0.016 | 0.016 | 0.017 | 0.002 |
| 12 | ICP-MS | 0.0206 | 0.0216 | 0.0205 | 0.0214 | 0.0219 | 0.0214 | 0.0212 | 0.0033 |
| Data not used in the certification | | | | | | | | | |
| 3 | ICP-MS | < 0,030 (0,030) | < 0,030 (0,029) | < 0,030 (0,030) | n.n. (0,0087) | < 0,030 (0,015) | n.n. (0,0090) | | |
| 11 | ICP-MS | <0,09 | <0,06 | <0,10 | <0,06 | <0,08 | <0,06 | | |



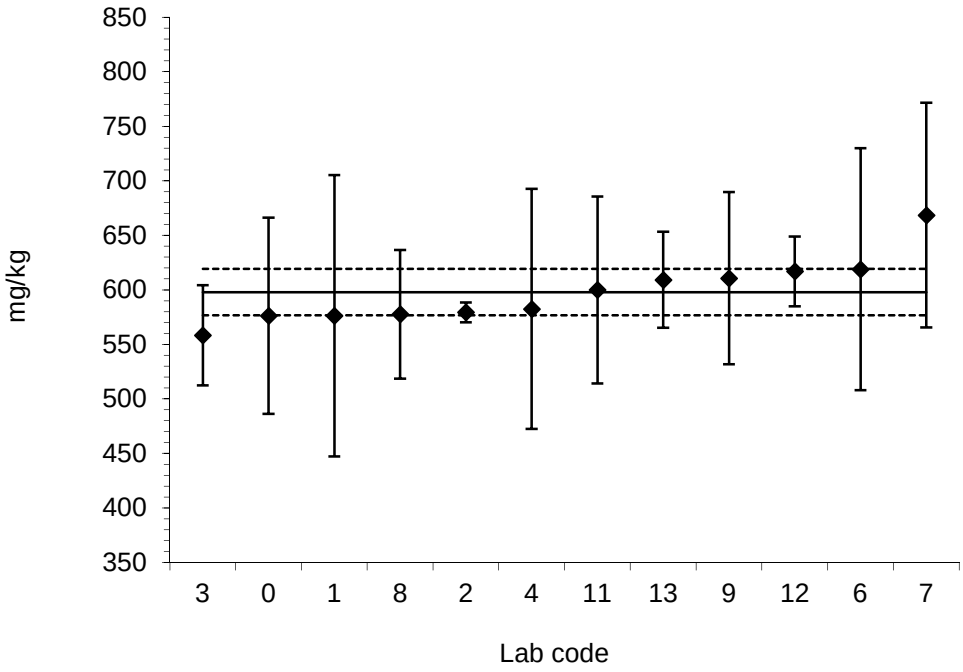
**Cd**

| Lab code | Technique | Replicate 1 [mg/kg] | Replicate 2 [mg/kg] | Replicate 3 [mg/kg] | Replicate 4 [mg/kg] | Replicate 5 [mg/kg] | Replicate 6 [mg/kg] | Mean [mg/kg] | Expanded uncertainty [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| 0 | ICP-MS | 0.268 | 0.269 | 0.269 | 0.291 | 0.289 | 0.292 | 0.280 | 0.025 |
| 1 | ICP-MS | 0.270 | 0.291 | 0.275 | 0.275 | 0.270 | 0.279 | 0.277 | 0.039 |
| 2 | ICP-MS | 0.275 | 0.273 | 0.275 | 0.267 | 0.265 | 0.268 | 0.271 | 0.004 |
| 3 | ICP-MS | 0.28 | 0.28 | 0.29 | 0.28 | 0.28 | 0.27 | 0.280 | 0.034 |
| 4 | ICP-SFMS | 0.257 | 0.261 | 0.257 | 0.253 | 0.267 | 0.251 | 0.258 | 0.049 |
| 6 | ICP-MS | 0.305 | 0.308 | 0.309 | 0.329 | 0.319 | 0.326 | 0.316 | 0.065 |
| 8 | ICP-MS | 0.273 | 0.263 | 0.271 | 0.273 | 0.267 | 0.270 | 0.270 | 0.027 |
| 11 | ICP-MS | 0.2707 | 0.2689 | 0.2709 | 0.2615 | 0.2715 | 0.2746 | 0.270 | 0.0439 |
| 12 | ICP-MS | 0.310 | 0.297 | 0.302 | 0.305 | 0.304 | 0.304 | 0.304 | 0.019 |



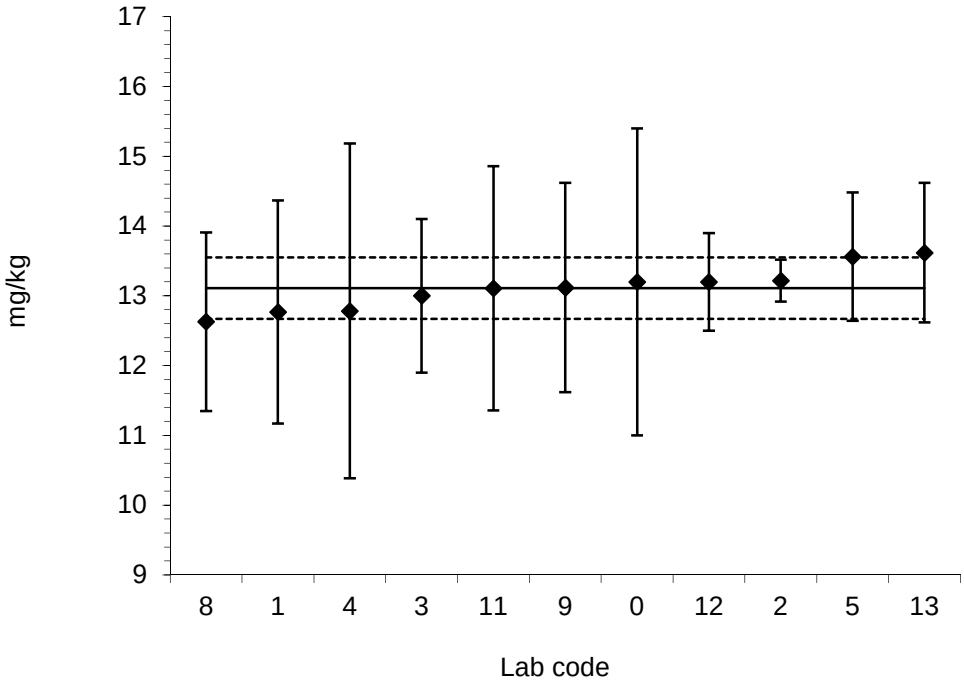
Cu

| Lab code | Technique | Replicate 1 [mg/kg] | Replicate 2 [mg/kg] | Replicate 3 [mg/kg] | Replicate 4 [mg/kg] | Replicate 5 [mg/kg] | Replicate 6 [mg/kg] | Mean [mg/kg] | Expanded uncertainty [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| 0 | ICP-MS | 553 | 561 | 562 | 593 | 593 | 595 | 576 | 90 |
| 1 | ICP-MS | 646 | 632 | 603 | 564 | 491 | 522 | 576 | 129 |
| 2 | ICP-MS | 577 | 583 | 583 | 575 | 578 | 580 | 579 | 9 |
| 3 | ICP-MS | 560 | 580 | 560 | 560 | 560 | 530 | 558 | 46 |
| 4 | ICP-SFMS | 583 | 582 | 580 | 575 | 588 | 587 | 583 | 110 |
| 6 | ICP-MS | 627 | 612 | 615 | 630 | 608 | 621 | 619 | 111 |
| 7 | ICP-OES | 652 | 646 | 656 | 686 | 690 | 681 | 669 | 103 |
| 8 | ICP-MS | 582 | 584 | 588 | 557 | 573 | 582 | 578 | 59 |
| 9 | ICP-SFMS | 642 | 631 | 611 | 587 | 601 | 592 | 611 | 79 |
| 11 | ICP-MS | 611.8 | 608.5 | 596.8 | 606.3 | 587.6 | 588.4 | 600 | 85.7 |
| 12 | ICP-MS | 597 | 633 | 621 | 618 | 604 | 629 | 617 | 32 |
| 13 | k0-NAA | 600 | 619 | 604 | 604 | 608 | 620 | 609 | 44 |



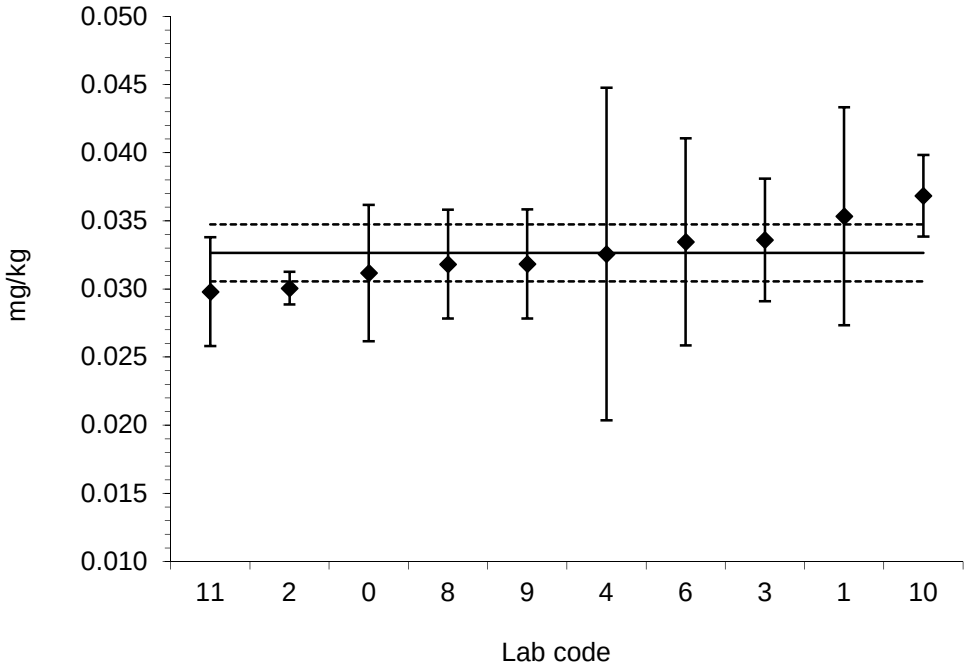
**Mn**

| Lab code | Technique | Replicate 1 [mg/kg] | Replicate 2 [mg/kg] | Replicate 3 [mg/kg] | Replicate 4 [mg/kg] | Replicate 5 [mg/kg] | Replicate 6 [mg/kg] | Mean [mg/kg] | Expanded uncertainty [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| 0 | ICP-MS | 12.4 | 12.6 | 12.5 | 13.9 | 13.8 | 14.0 | 13.2 | 2.2 |
| 1 | ICP-MS | 11.7 | 13.6 | 12.9 | 12.3 | 12.9 | 13.2 | 12.8 | 1.6 |
| 2 | ICP-MS | 13.3 | 13.5 | 13.5 | 12.9 | 13.0 | 13.1 | 13.2 | 0.3 |
| 3 | ICP-MS | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 1.1 |
| 4 | ICP-SFMS | 12.4 | 12.6 | 12.9 | 12.6 | 13 | 13.2 | 12.8 | 2.4 |
| 5 | k0-NAA | 13.471 | 13.515 | 13.660 | 13.508 | 13.563 | 13.640 | 13.560 | 0.9202 |
| 8 | ICP-MS | 12.78 | 12.85 | 12.79 | 12.31 | 12.27 | 12.77 | 12.63 | 1.28 |
| 9 | ICP-SFMS | 13.6 | 13.6 | 13.8 | 13.0 | 12.3 | 12.4 | 13.1 | 1.5 |
| 11 | ICP-MS | 12.69 | 12.16 | 11.84 | 14.61 | 13.71 | 13.63 | 13.11 | 1.75 |
| 12 | ICP-MS | 12.8 | 13.3 | 13.6 | 13.2 | 12.9 | 13.4 | 13.20 | 0.7 |
| 13 | k0-NAA | 13.7 | 13.7 | 13.5 | 13.6 | 13.6 | 13.6 | 13.62 | 1.0 |



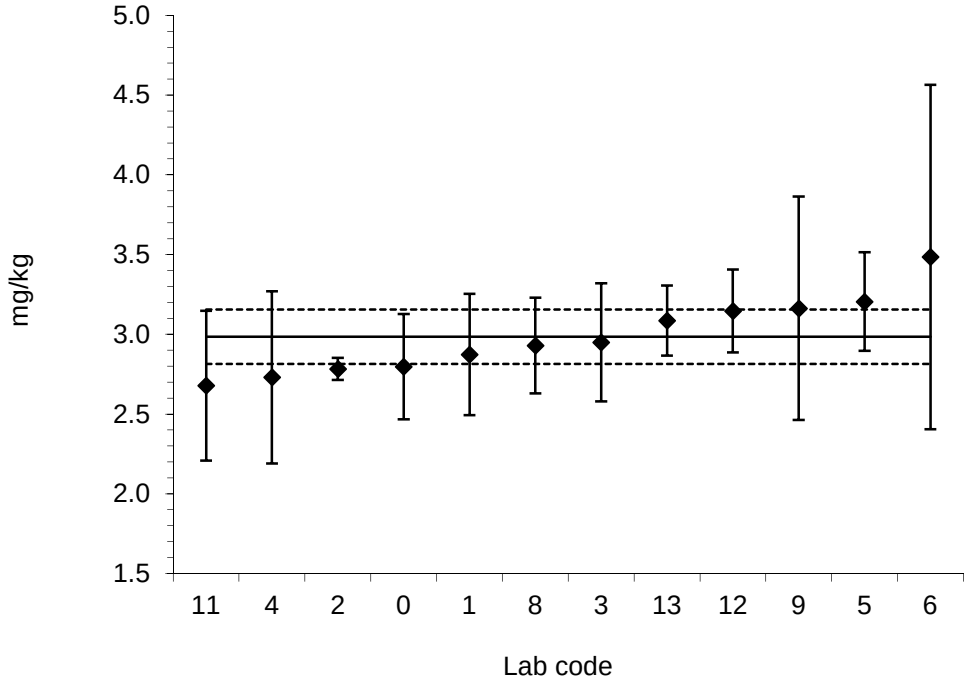
**Pb**

| Lab code | Technique | Replicate 1 [mg/kg] | Replicate 2 [mg/kg] | Replicate 3 [mg/kg] | Replicate 4 [mg/kg] | Replicate 5 [mg/kg] | Replicate 6 [mg/kg] | Mean [mg/kg] | Expanded uncertainty [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| 0 | ICP-MS | 0.033 | 0.032 | 0.031 | 0.031 | 0.030 | 0.030 | 0.031 | 0.005 |
| 1 | ICP-MS | 0.037 | 0.038 | 0.036 | 0.028 | 0.031 | 0.042 | 0.035 | 0.008 |
| 2 | ICP-MS | 0.029 | 0.031 | 0.029 | 0.0302 | 0.0312 | 0.0299 | 0.030 | 0.0012 |
| 3 | ICP-MS | 0.038 | 0.032 | 0.033 | - | 0.033 | 0.032 | 0.034 | 0.0045 |
| 4 | ICP-SFMS | 0.0337 | 0.0341 | 0.0344 | 0.0313 | 0.0312 | 0.0306 | 0.0326 | 0.0122 |
| 6 | ICP-MS | 0.0312 | 0.0327 | 0.0308 | 0.0346 | 0.0355 | 0.0359 | 0.0335 | 0.0076 |
| 8 | ICP-MS | 0.0304 | 0.0299 | 0.0316 | 0.037 | 0.031 | 0.031 | 0.032 | 0.004 |
| 9 | ICP-SFMS | 0.031 | 0.032 | 0.035 | 0.030 | 0.033 | 0.030 | 0.032 | 0.004 |
| 10 | ID-ICP-SFMS | 0.035 | 0.046 | 0.034 | 0.036 | 0.034 | 0.036 | 0.037 | 0.003 |
| 11 | ICP-MS | 0.0306 | 0.0305 | 0.0298 | 0.0278 | 0.0304 | 0.0297 | 0.0298 | 0.0040 |
| Data not used in the certification | | | | | | | | | |
| 12 | ICP-MS | 0.0665 | 0.0620 | 0.0631 | 0.0619 | 0.0704 | 0.0632 | 0.06 | 0.0070 |



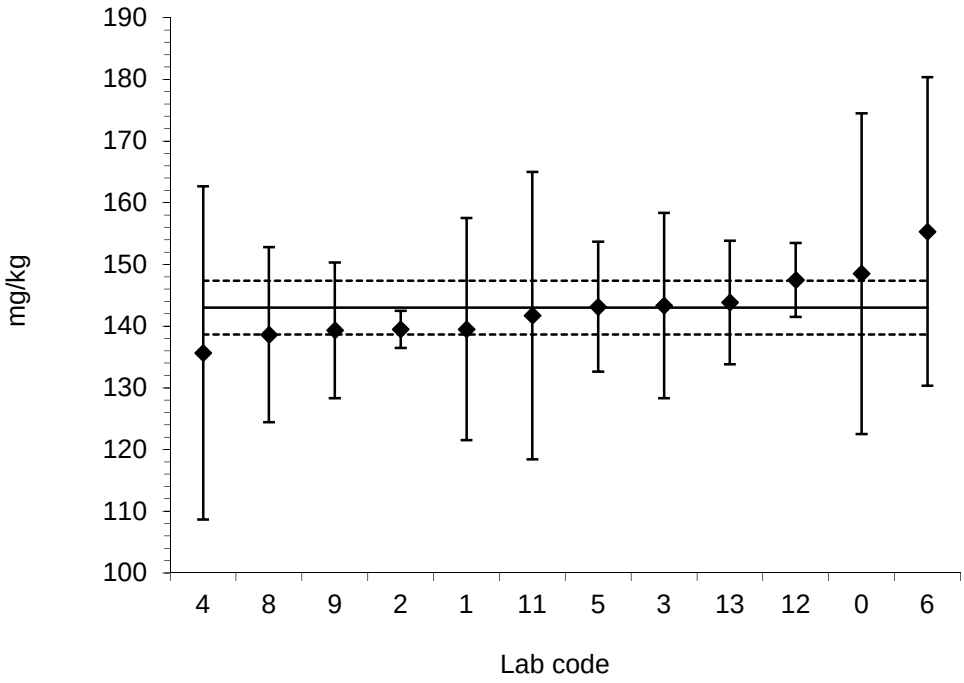
**Se**

| Lab code | Technique | Replicate 1 [mg/kg] | Replicate 2 [mg/kg] | Replicate 3 [mg/kg] | Replicate 4 [mg/kg] | Replicate 5 [mg/kg] | Replicate 6 [mg/kg] | Mean [mg/kg] | Expanded uncertainty [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| 0 | ICP-MS | 2.78 | 2.83 | 2.78 | 2.78 | 2.83 | 2.78 | 2.80 | 0.33 |
| 1 | ICP-MS | 2.50 | 2.99 | 2.73 | 3.00 | 3.15 | 2.87 | 2.87 | 0.38 |
| *2* | ICP-MS | 2.81 | 2.87 | 2.75 | 2.76 | 2.72 | 2.79 | 2.78 | 0.07 |
| 3 | ICP-MS | 2.9 | 3.1 | 2.9 | 3.0 | 3.0 | 2.8 | 3.0 | 0.37 |
| 4 | ICP-SFMS | 2.65 | 2.63 | 2.89 | 2.71 | 2.71 | 2.79 | 2.73 | 0.54 |
| 5 | k0-NAA | 3.2558 | 3.1290 | 3.2782 | 3.2818 | 3.0213 | 3.2661 | 3.2054 | 0.3084 |
| 6 | ICP-MS | 3.48 | 3.55 | 3.59 | 3.46 | 3.42 | 3.41 | 3.4850 | 1.08 |
| 8 | ICP-MS | 2.97 | 3.02 | 2.72 | 2.94 | 2.98 | 2.95 | 2.93 | 0.30 |
| 9 | ICP-SFMS | 3.31 | 3.31 | 3.36 | 3.1 | 2.9 | 3.0 | 3.16 | 0.7 |
| 11 | ICP-MS | 2.764 | 2.661 | 2.707 | 2.638 | 2.766 | 2.533 | 2.678 | 0.470 |
| 12 | ICP-MS | 3.16 | 3.16 | 3.21 | 3.08 | 3.12 | 3.15 | 3.147 | 0.26 |
| 13 | k0-NAA | 3.11 | 3.12 | 3.08 | 3.05 | 3.07 | 3.09 | 3.087 | 0.22 |



**Zn**

| Lab code | Technique | Replicate 1 [mg/kg] | Replicate 2 [mg/kg] | Replicate 3 [mg/kg] | Replicate 4 [mg/kg] | Replicate 5 [mg/kg] | Replicate 6 [mg/kg] | Mean [mg/kg] | Expanded uncertainty [mg/kg] |
|---|---|---|---|---|---|---|---|---|---|
| 0 | ICP-MS | 133 | 137 | 135 | 162 | 163 | 161 | 149 | 26 |
| 1 | ICP-MS | 154 | 145 | 140 | 136 | 127 | 135 | 140 | 18 |
| 2 | ICP-MS | 139 | 142 | 142 | 138 | 138 | 138 | 140 | 3 |
| 3 | ICP-MS | 140 | 150 | 150 | 140 | 140 | 140 | 143 | 15 |
| 4 | ICP-SFMS | 135 | 133 | 139 | 132 | 137 | 138 | 136 | 27 |
| 5 | k0-NAA | 141.852 | 140.319 | 145.85 | 146.01 | 143.91 | 140.98 | 143.154 | 10.534 |
| 6 | ICP-MS | 155 | 153 | 155 | 162 | 153 | 154 | 155 | 25 |
| 8 | ICP-MS | 135.8 | 138.9 | 138.6 | 136.9 | 139.6 | 141.8 | 138.6 | 14.2 |
| 9 | ICP-MS | 144 | 141 | 140 | 137 | 139 | 135 | 139 | 11 |
| 11 | ICP-MS | 132.8 | 130.8 | 128.1 | 155.6 | 151.9 | 151.1 | 141.7 | 23.3 |
| 12 | ICP-MS | 145 | 145 | 148 | 150 | 148 | 149 | 147.5 | 6 |
| 13 | k0-NAA | 146 | 146 | 145 | 141 | 142 | 143 | 143.8 | 10 |



European Commission

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