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CERTIFICATION REPORT

The certification of the mass fractions of cadmium copper, manganese and nickel in dark chocolate: ERM®- BD512

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Abstract

This report describes the production of ERM-BD512, which is a matrix material certified for the mass fraction of cadmium, copper, manganese and nickel. This material was produced following ISO Guide 34:2009 and is certified in accordance with ISO Guide 35:2006.
The CRM was produced from commercially available dark chocolate produced in Peru. About 15 kg of chocolate bars were melted, the melt was homogenised and cast into moulds to produce pellets of about 0.5 g.
Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006. Within-unit homogeneity was quantified to determine the minimum sample intake.
The certified mass fraction of Cd was obtained by measurement, using the technique of isotope dilution ICP MS. The mass fractions of Cu, Mn and Ni were obtained by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2005. Technically invalid results were removed but no outliers were 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 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 packages of 6 glass vials, each containing a single pellet of about 0.5 g, which were sealed under an atmosphere of argon. The minimum amount of sample to be used is 250 mg.

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The certification of the mass fractions of cadmium copper, manganese and nickel in dark chocolate: ERM®- BD512

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Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Summary

This report describes the production of ERM-BD512, which is a matrix material certified for the mass fraction of cadmium, copper, manganese and nickel. This material was produced following ISO Guide 34:2009 [1] and is certified in accordance with ISO Guide 35:2006 [2].

The CRM was produced from commercially available dark chocolate produced in Peru. About 15 kg of chocolate bars were melted, the melt was homogenised and cast into moulds to produce pellets of about 0.5 g.

Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [2]. Within-unit homogeneity was quantified to determine the minimum sample intake.

The certified mass fraction of Cd was obtained by measurement, using the technique of isotope dilution ICP-MS. The mass fractions of Cu, Mn and Ni were obtained by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2005 [3]. Technically invalid results were removed but no outliers were 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 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 packages of 6 glass vials, each containing a single pellet of about 0.5 g, which were sealed under an atmosphere of argon. The minimum amount of sample to be used is 250 mg.

The following values were assigned:

	Mass Fraction	
	Certified value [1,2) [mg/kg]	Uncertainty [3) [mg/kg]
Cd	0.302	0.013
Cu	14.3	0.7
Mn	15.7	0.6
Ni	3.01	0.23

1) Cd value measured by isotope dilution inductively coupled plasma mass spectrometry.

2) Cu, Mn and Ni values are unweighted mean values 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 values and their 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

AAS	Atomic absorption spectrometry
ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
c	Mass concentration c = m / V (mass / volume)
CC	Collision cell
CI	Confidence interval
CRM	Certified reference material
EC	European Commission
EN	European norm (standard)
ERM®	Trademark of European Reference Materials
EU	European Union
GUM	Guide to the Expression of Uncertainty in Measurements [ISO/IEC Guide 98-3:2008]
ICP	Inductively coupled plasma
ICP-MS	Inductively coupled plasma-mass spectrometry
ICP-QMS	ICP-Quadrupole mass spectrometry
ICP-SFMS	ICP-Sector field mass spectrometry
IDMS	isotope dilution mass spectrometry
ILC	Interlaboratory comparison
ISO	International Organization for Standardization
IU	International units
IUPAC	International Union of Pure and Applied Chemistry
JCGM	Joint Committee for Guides on Metrology
JRC	Joint Research Centre of the European Commission
k	Coverage factor
k_0-NAA	k0-Neutron activation analysis
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar mass
MRL	Maximum residue limit
MS	Mass spectrometry
MS_{between}	Mean of squares between-unit from an ANOVA
MSDS	Material safety data sheet
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
n.d.	Not detectable
NIST	National Institute of Standards and Technology (USA)
PT	Proficiency testing
QA	Quality assurance
QC	Quality control
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
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
se	Standard error
SI	International System of Units
s_{meas}	Standard deviation of measurement data; an additional index "rel" is added as appropriate
s_{ns}	Standard deviation of results of normal stock samples
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
t_{sl}	Proposed shelf life
u	standard uncertainty
U	expanded uncertainty
u_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability/intermediate precision; an additional index "rel" is added as appropriate

u_c	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_cal	combined standard uncertainty; an additional index "rel" is added as appropriate
u_char	Standard uncertainty of calibration
u_CRM	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_Δ	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_lts	Combined standard uncertainty of measurement result and certified value
u_meas	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
U_meas	Standard measurement uncertainty
u_rec	Expanded measurement uncertainty
u_sts	Standard uncertainty related to possible between-unit inhomogeneity modelled as rectangular distribution; an additional index "rel" is added as appropriate
u_t	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
u_bb	Standard uncertainty of trueness
UV	Ultraviolet
VIM	International Vocabulary of Metrology – Basic and General Concepts and Associated Terms *[ISO/IEC Guide 99:2007]*
$\overline{x}$	Arithmetic mean
$\overline{x}_{ns}$	Arithmetic mean of all results of normal stock samples
$\overline{x}_{ref}$	Arithmetic mean of results of reference samples
α	significance level
Δ_{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

In 2009 the Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) of the European Food Safety Authority (EFSA) adopted new opinion on cadmium (Cd) in food. [5] EFSA established a tolerable weekly intake (TWI) of 2.5 µg/kg body weight for Cd. According to the scientific opinion on Cd in food of the CONTAM Panel, chocolate and cocoa products are one of the food groups that contribute to the major part of the dietary Cd exposure. Chocolate as such and other sweetened cocoa products used in cocoa beverages are frequently consumed by children and adolescents and represent one of the main sources of Cd exposure in this age group. The amount of Cd in chocolate and cocoa products depends on the Cd content of the soil where the cocoa beans were produced and because some regions have naturally high Cd in soil this is a cause for concern. To address these concerns a new amendment (EC) No 488/2014 to the Commission regulation (EC) No 1881/2006 was passed to establish among others new maximum levels for Cd in chocolate and cocoa products.

In light of the new regulation, the JRC decided to produce a CRM for chocolate to assist national reference, official control and other test laboratories in the validation and control of their test methods.

1.2 Choice of the material

Several distinct varieties of chocolate from local supermarkets were tested to find a material with mass fractions of Cd in the range below but near the regulatory limit for Cd. Initial testing indicated that a "Peru Noir" dark chocolate was the most suitable material for the production of the CRM, as it contained the highest amount of Cd out of the samples tested.

1.3 Design of the CRM project

The elements intended to be certified were chosen to assist measurements made in support of legislation on food safety, and to support studies on food safety and nutrition.

Cd – for measurements in support of the new regulation

Cu and Mn – Cocoa beans contain high amounts of these nutrients relative to other foods, and could provide significant contribution to the dietary intake even with modest consumption.

Ni – Cocoa beans can also contain high amounts of Ni, which risks causing skin sensitisation and allergic reactions in consumers. In addition, high levels could be a marker for fraudulent addition of non-cocoa vegetable fats that have been hydrogenated over a Rainey catalyst [6].

Pb – a ubiquitous contaminant. Chocolate is a favourite food of children, who are particularly susceptible to developmental problems caused by Pb intake [7].

The reference values for the Cd and Pb mass fractions were to be measured in-house, using ID-ICP-MS as a primary reference method. Full uncertainty budgets were made to identify and quantify all potential influences on the results. The results were verified by comparison with results from expert laboratories participating in the EURL-HM-20 PT exercise [8], which made use of the same chocolate material. The results are traceable to the SI through the use of isotopic reference materials that are themselves SI-traceable.

For Cu, Mn and Ni, an inter-laboratory comparison was made, using results from independent laboratories selected for their expertise in measurement of elements in food. Certified mass fractions are 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).

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, 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, Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

Callebaut PLC, Wieze, BE

2.3 Homogeneity study

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(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

ALS Scandinavia AB, Luleå, SE

The Food and Environment Research Agency, York, UK

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

2.4 Stability study

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ALS Scandinavia AB, Luleå, SE

2.5 Characterisation

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(Measurements performed under ISO/IEC 17025 accreditation; UKAS 0342)

The Food and Environment Research Agency, York, UK

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

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SCK-CEN, Mol, BE

(Measurements performed under ISO/IEC 17025 accreditation; BELAC 015-TEST)

Umweltbundesamt GmbH, Wien, AT

(Measurements performed under ISO/IEC 17025 accreditation; AA 0200)

VITO NV, Mol, BE

3 Material processing and process control

3.1 Origin of the starting material

ERM-BD512 was produced from commercially available bars of a dark chocolate, which contained a minimum of 64 % cocoa and is therefore categorised by Commission Regulation (EU) No 488/2014 as being subject to a maximum limit of 0.8 mg(Cd)/kg. Chocolate with a clear geographical designation was preferred to ensure consistency between bars. From initial testing, a chocolate from Peru with Cd content of about 0.3 mg/kg was found to be most suitable.

Seventeen kg of the Peruvian dark chocolate bars were purchased, and were taken to the research laboratory of Callebaut PLC (Wieze, BE).

3.2 Processing

With the assistance of experts in handling chocolate, 14 kg were melted in a JKV-30 tempering machine (Gilze, NL). The JKV-30 was left running over night recirculating the chocolate at 5 L/min to ensure a homogeneous mixture. The temperature was set to 45 °C. The following day the temperature of the JKV-30 was lowered to 32 °C and an additional 3 kg of fresh chocolate was melted in a microwave oven and manually manipulated on a marble table until the temperature reached 32 °C. The 3 kg of manipulated chocolate was thereafter added to the chocolate in the JKV-30 equipment and mixed for another 30 minutes.

This process is called tempering and has several advantages related to the quality of the end-product. The tempering results in a pre-crystallisation of the cocoa butter in the chocolate, which is directly related to the working temperature. During tempering, the cocoa butter in the chocolate changes into a stable crystalline form (β-crystals).[9] This structure ensures the hardness and gloss of the finished product. By using this process, the physical and chemical properties of ERM-BD512 should be as similar as possible to commercial chocolate samples, with regard to their analytical behaviour and stability during storage. [10]

The melted chocolate was poured into 56 polycarbonate moulds. The moulds were machined at JRC Geel, and featured 88 pits that can each contain about 0.5 g of liquid chocolate. The moulds, the vials, the lyo-stoppers and other equipment that would be in contact with the chocolate were acid washed with 10 % nitric acid (v/v) and thereafter rinsed three times with Milli-Q purified water and dried in a Terra Universal clean cell (Fullerton, USA). Blank levels of each analyte in the vials after cleaning were confirmed to be several orders of magnitude below that of the chocolate.

After the moulds had cooled to ambient temperature, the resulting 0.5 g pellets were emptied in zip-loc plastic bags while keeping track of fill order (per 88 units). Filling and cooling of the 56 moulds was repeated three times to end up with more than 14000 chocolate pellets, needed for the preparation of ERM-BD512. The pellets were then transported to the JRC Geel site.

Filling of the chocolate pellets was done in a walk-in Terra Universal clean cell providing a low particle background in the surrounding atmosphere. One by one the chocolate pellets were placed in an acid-washed 10-mL vial using acid-washed Teflon tweezers. Thereafter the acid-washed lyo-stoppers were placed half way down the vial neck. The vials were placed into a Martin-Christ freeze dryer model Epsilon 2-100D (Osterode, DE) where the chamber was subsequently evacuated to approximately 1 mbar and then filled again with argon to about 970 mbar. All lyo-inserts were then pressed down simultaneously by lowering the shelves of the freeze dryer. Filling of chocolate pellets and argon filling was repeated until all 14,000 pellets were filled into vials.

Capping and labelling took place in a capping and labelling assembly from Bausch & Ströbel and BBK, respectively (Ilshofen and Beerfelden, DE). The capped and labelled vials were placed in aluminized sachets in sets of six vials per set. The sachet was thermally sealed and labelled on the outside.

4 Homogeneity

A key requirement for any reference material aliquoted 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 vials of the material, within the stated uncertainties.

While measurements are made on individual vials containing a chocolate pellet, the vials are distributed in sachets containing 6 vials. For the study, the number of sachets selected corresponds to approximately the cube root of the total number of sachets produced. Two sets of 8 sachets were selected using a random stratified sampling scheme covering the whole batch for the between-vial homogeneity tests. For this, the batch was divided into 8 groups (with a similar number of sachets) and one sachet was selected randomly from each group. From each sachet, 3 vials were selected. For measurement of Cd, Cu, Mn and Ni mass fractions, two independent samples were taken from each selected vial by halving the pellet, and were analysed by ICP-MS after acid digestion. For Pb, entire pellets were sampled and analysed by ICP-MS, as the natural Pb content was close to the technique's LOQ. 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 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. No trends in the filling sequence were observed at a 95 % confidence level. Some significant (95 % confidence level) trends in the analytical sequence were visible, pointing at a changing parameter, e.g. a signal drift in the analytical system. The correction of biases, even if they are statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value [11]. Correction of trends is therefore expected to improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. As the analytical sequence and the unit numbers were not correlated, trends significant on at least a 95 % confidence level were corrected as shown below:

$$x_{i\_corr} = x_i - b \cdot i$$ **Equation 1**

b **=** slope of the linear regression

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

The trend-corrected dataset was 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 Pb, and one for Ni. Since no technical reason for the outliers could be found, all the data were retained for statistical analysis.

For Cd, Cu, Mn and Ni, 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 vial. As whole pellets were measured for Pb, separation of within-unit variation was not possible: In this case, the inhomogeneity was estimated from the measurement repeatability.

Evaluation by ANOVA requires mean values per vial, which follow at least a unimodal distribution and results for each vial that follow unimodal distributions with approximately the same standard deviations. The distribution of the mean values per vial was visually tested using histograms and normal probability plots. Too few data, 2 measurements per vial, are available for the unit means to make a clear statement of the distribution. Therefore, it was checked visually whether all individual data follow a unimodal distribution 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

Mass fraction	Trends (before correction)*		Outliers**		Distribution	
	Analytical sequence	Filling sequence	Individual results	Unit means	Individual results	Unit means
Cd	yes	No	none	none	unimodal	normal
Cu	yes	No	none	none	unimodal	normal
Mn	yes	No	none	none	unimodal	normal
Ni	yes	No	1–statistical reason (retained)	none	Unimodal	normal
Pb	yes	No	n/a	1–statistical reason (retained)	n.a.	bimodal

* 95 % confidence level

** 99 % confidence level

It should be noted that $s_{\mathrm{bb,rel}}$ and $s_{\mathrm{wb,rel}}$ 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.* [12]. u^*_{bb} is comparable to the LOD of a measurement procedure, 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:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\overline{y}} \qquad \textbf{Equation 2}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\overline{y}} \qquad \textbf{Equation 3}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}}\sqrt[4]{\frac{2}{\nu_{MSwithin}}}}{\overline{y}} \qquad \textbf{Equation 4}$$

MS_{within}	mean of squares within-unit from an ANOVA
$MS_{between}$	mean of squares between-unit from an ANOVA
$\overline{y}$	mean of all results of the homogeneity study
n	mean number of replicates per unit
$\nu_{MSwithin}$	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. For Cd and Cu, the uncertainty contribution for homogeneity was determined by the method repeatability.

Table 2: Results of the homogeneity studies

Mass fraction	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,rel}$ [%]
Cd	1.5	n.c.	0.56	0.56
Cu	2.6	n.c.	0.97	0.97
Mn	2.5	0.78	0.96	0.96
Ni	4.6	1.23	1.75	1.75
Pb	n.a.	6.4	n.a.	6.4

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

2) n.a.: not applicable

For Cd, Cu, Mn and Ni, 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. For Pb, one outlying measurement was found. As whole pellets were measured, it is not possible with this study to distinguish outlying measurements from sample inhomogeneity.

To investigate whether the outlying result for Pb was caused by measurement error or sample inhomogeneity, the results from the 2 stability studies, short- and long-term, were combined with those of the homogeneity study. For the sake of the homogeneity study it was assumed that the temperatures at which the vials were tested had no effect on the Pb content.

Correction of biases, even if they are statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value [11]. As the 3 studies to be combined were performed on different measurement occasions, ANOVA was calculated to evaluate potential significant difference between the measurement series. No significant (95 % confidence level) difference between the studies was detected, and results were combined without normalisation.

The combined study included 136 measurements. From the plot of results given in Annex A, it is apparent that there are a number of outliers, all above the mean. A Hampel test found 6 outliers at the 99 % confidence level, which corresponds to 4.4 % of samples measured. This indicates that these samples were contaminated with additional lead. Furthermore, it is not possible to exclude the possibility that the contamination occurred on preparation of the CRM, and thus other units may be similarly contaminated. Therefore it was not possible to reliably estimate the $u_{\mathrm{bb,rel}}$.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. 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.

Homogeneity experiments were performed using a 250 mg sample intake for Cd, Cu, Mn and Ni, and using a 500 mg intake for Pb. For Cd, Cu, Mn and Ni, this sample intake gives a repeatability such that the within-unit inhomogeneity no longer contributes to analytical variation at this sample intake. For Pb inhomogeneity was found even when measuring whole 500 mg pellets.

The overall minimum sample intake for this material is set to 250 mg for Cd, Cu, Mn and Ni.

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 brown glass vials which reduces light exposure. In addition, materials are stored in the dark and dispatched in aluminised sachets, thus removing any possibility of degradation by light. 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 samples are to be transported without any additional cooling.

The stability studies were carried out using an isochronous design [13]. In this approach, samples were stored for a particular length of time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). 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, samples were stored at -40 ºC, 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to 4 °C. Two sachets per storage time were selected using a random stratified sampling scheme. From each sachet, four samples were measured by ICP-MS. 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 data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %. Some outlying individual results were found (Table). As no technical reason for the outliers could be found all data were 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 of the mass fraction due to shipping conditions. The slopes of the regression lines were tested for statistical significance. One of the trends for Cd was statistically significant at a 95 % confidence level for the 60 ºC temperature test.

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

Mass fraction	Number of individual outlying results*			Significance of the trend **		
	-40 ºC	18 ºC	60 ºC	-40 ºC	18 ºC	60 ºC
Cd	2 statistical – retained	none	none	no	no	yes
Cu	2 statistical – retained	none	none	no	no	no
Mn	2 statistical – retained	none	none	no	no	no
Ni	None	none	none	no	no	no
Pb	1 statistical – retained	1 statistical - retained	1 statistical - retained	no	no	no

* 99 % confidence level

** 95 % confidence level

Statistical outliers were detected for Cd, Cu, Mn and Pb, and these were retained for the estimation of u_{STS}.

A positive trend was observed for Cd at 60 °C. As the analyte cannot be created in the sample, a positive trend could only be due to degradation of the matrix. This, however, should be seen for all measurands, which is not the case. The observed trend was therefore regarded as statistical artefact. However, at 60 ºC the chocolate melts, and sticks to the side of the vial when it solidifies at room temperature. As this makes sampling difficult, temperatures high enough to melt the chocolate should be avoided during transport.

At -40 ºC ice crystals may form in the chocolate, which could affect its homogeneity once thawed. While the test conducted on whole pellets did not find any significant difference in mass fractions between samples whether they had been frozen or not, freezing of samples on transport should be avoided in case users use sub-samples smaller than the whole pellet for the analysis.

The material shall be shipped under cooled conditions.

5.2 Long-term stability study

For the long-term stability study, samples were stored at 18 °C for 0, 8, 12 and 18 months (at each temperature). The reference temperature was set to 4 °C. Two sachets per storage time were selected using a random stratified sampling scheme. From each sachet, four samples were measured by ICP-MS. 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.

Significant (95 % confidence level) trends in the analytical sequences for Cd, Cu and Mn were visible, pointing at a changing parameter, e.g. a signal drift in the analytical system. The correction of biases, even if they are statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value [11]. Correction of trends is therefore expected to improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. As the analytical sequence and the unit numbers were not correlated, trends significant on at least a 95 % confidence level were corrected as shown below:

$$x_{i\_corr} = x_i - b \cdot i \qquad \textbf{Equation 5}$$

b **=** slope of the linear regression

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

The long-term stability data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. One statistical outlying individual result was found for Pb (Table 4). In addition, a second individual result was found to be significantly greater than the mean value plus measurement uncertainty. Both of these results were considered to have come from contaminated pellets, as explained in Section 4.1, and they were excluded from statistical analysis.

In addition, the data were plotted against storage time and linear regression lines of mass fraction versus time were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected for all 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

Mass fraction	Number of individual outlying results*	Significance of the trend**
Cd	None	no
Cu	None	no
Mn	None	no
Ni	None	no
Pb	1 statistical and 1 technical - removed	no

* 99 % confidence level

** 95 % confidence level

None of the trends were statistically significant on a 99 % confidence level for any of the temperatures. 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, as described in [14] for each element. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated, as shown in equations 6 and 7. For the STS study on Cd, the uncertainty was based on the magnitude of the observed slope, and its standard error, as shown in equation 8. 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:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{tt}$$ **Equation 6**

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{sl}$$ **Equation 7**

$$u_{sts,rel} = \sqrt{\left(\frac{b}{\sqrt{3}}\right)^2 + se_b{}^2}$$ **Equation 8**

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 °C)
t_{sl}	chosen shelf life (24 months at 18 °C)
b	observed slope; change of mass fraction with time

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

Mass fraction	$u_{sts\ ,rel}$ [%]	$u_{lts,rel}$ [%]
Cd	0.36*	1.83
Cu	0.18	0.89
Mn	0.24	0.88
Ni	0.25	1.11
Pb	2.1	4.3

*including degradation

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

6 Characterisation

The material characterisation is the process of determining the property values of a reference material. For ERM-BD512, different approaches were used for the establishment of certified values for Cu, Mn and Ni and for Cd and Pb.

Cu, Mn and Ni

Values were 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.

Cd and Pb

The material characterisation was based on a primary method of measurement, confirmed by an independent method. A primary method of measurement (also called "primary reference method" in the International Vocabulary of Metrology (VIM) [15]) is a method that does not require calibration with a measurement standard of the same measurand and does not depend on a chemical reaction. Such methods are of highest metrological order and often yield results with low uncertainties. However, it is nevertheless prudent to demonstrate absence of bias or gross errors by use of an independent method of lower metrological order.

6.1 Selection of participants

Cu, Mn and Ni

Nine laboratories 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 food matrices. 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 of each laboratory is stated in the list of participants (Section 2).

Cd and Pb

The ID-ICP-MS measurements were made in-house. They were verified by comparing results with those provided by the two external laboratories that were contracted for measurements for the homogeneity and stability studies. The external laboratories also fulfilled the requirement to operate a quality system.

6.2 Study setup

Cu, Mn and Ni

Each laboratory received 2 sachets of ERM-BD512 and was requested to provide 6 independent results, 3 per sachet. The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations and measurements had to be spread over at least two days to ensure intermediate precision conditions. An independent calibration was performed for each result.

Each participant received a sample of NIST SRM 2384 Baking Chocolate as a blinded 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.

Cd and Pb

For each element, 3 independent measurements were made on vials from each of 2 sachets, and measurements were made on 2 separate days.

6.3 Measurement Methods used

Cu, Mn and Ni

A variety of measurement methods with different measurement principles (ICP-OES, ICP-MS) as well as methods without sample preparation (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. L01) is a random number and does not correspond to the order of laboratories in Section 2. The lab-method code consists of a number assigned to each laboratory (e.g. L01) and abbreviation of the measurement method used, (e.g. ICP-MS).

Cd and Pb

Measurements by the method of direct isotope dilution by ICP-MS were used to provide the values for Cd and Pb. Complete uncertainty budgets were established for each measurement result that included all factors that could potentially influence the result.

Amongst others, this included correction factors for instrumental background, detector dead time and instrumental mass discrimination, procedural blanks, uncertainties of isotope ratios and uncertainties associated to the isotopic composition of the elements, weighing of sample and spike, spike concentration and its isotopic composition. In addition, for Cd measurement a correction was applied for the isobaric overlap of MoO^+ ions on the Cd isotopes used for the ID determination, and for Pb, correction was made for the isobaric overlap of ^{204}Hg on ^{204}Pb measurements to establish the isotopic composition of Pb in the sample.

Cadmium mass fractions were measured by direct ID-ICP-MS by blending solid samples gravimetrically with an isotopic CRM solution, IRMM-622, enriched in ^{111}Cd. Pellets were weighed into PTFE tubes with isotopically enriched spike solutions by a method of substitution weighing. About 5 ml of concentrated nitric acid (62 % Merck Suprapur®) was added and the tubes were left to stand overnight. The tubes were transferred to a pressurised microwave digestion system (Milestone Ultraclave). Digests were diluted with high purity water (18 $M\Omega.cm^{-1}$) to give an acid concentration of 2 – 5 %. The m/z 111/113 intensity ratio was measured in the digests by ICP-MS without collision cell operation using an Agilent 7500ce ICP-MS (Agilent Technologies Inc., Tokyo (JP)). Samples were assumed to have natural isotopic composition as tabulated by IUPAC. [16] Unspiked samples were measured in a sequence bracketing blends for instrumental mass discrimination correction.

Pb mass fractions were measured by direct ID-ICP-MS by blending samples gravimetrically with an isotopic CRM solution from Inorganic Ventures (Christiansburg, VA (USA)), enriched in ^{206}Pb and measurement of the m/z 206/208 ratio. Sample isotopic composition was established by measuring all Pb isotopes in an unspiked sample to which an isotopic CRM of Tl, IRMM-649, was added. Instrumental mass discrimination was corrected by measuring the ^{203}Tl/^{205}Tl ratio and using a linear correction model.

Further details of the measurements are given in an IRMM internal report.[17]

6.4 Evaluation of results

Cu, Mn and Ni

The characterisation study resulted in between 8 and 11 datasets per element. All individual results of the participants, grouped per element are displayed in tabular and 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:

- appropriate validation of the measurement procedure
- compliance with the analysis protocol: sample preparations and measurements performed on two days.
- absence of values given as below limit of detection or below limit of quantification
- method performance, i.e. for Cu and Mn, agreement of the measurement results with the assigned value of the QC sample within the combined uncertainties of the assigned value and the measurement.

Based on the above criteria, the following datasets were rejected as not technically valid: L03 reported that the Ni content was below their detection limit. In addition, one laboratory (L02) reported results for the 3 elements that were significantly higher that the means of all laboratories. After re-checking their data, they discovered a calibration error, and withdrew the results.

For Cu, the QC result from L08 was 11 % above the assigned value, which is slightly higher than the combined uncertainties of measurement (reported to be 8 %, k=2) and QC (4 %). It was therefore considered to reject the result. However, laboratories' approaches to uncertainty estimation differ, and L08 reported lower uncertainties than most other participants despite using a similar technique (the median of this exercise was 13 %). Therefore, it was decided that the L08 result was of acceptable quality in comparison to results from the other participants and should not be excluded.

6.4.2 Statistical evaluation

Cu, Mn and Ni

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 6.

Table 6: Statistical evaluation of the technically accepted datasets for ERM-BD512. *p*: number of technically valid datasets

Mass fraction ERM-BD512	*p*	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Mean [mg/kg]	*s* [mg/kg]	s_{between} [mg/kg]	s_{within} [mg/kg]
Cu	11	none	none	yes	14.31	0.94	0.93	0.44
Mn	11	none	none	yes	15.71	0.50	0.46	0.45
Ni	8	none	none	yes	3.01	0.27	0.26	0.16

The laboratory means follow normal distributions. None of the data contains outlying means and variances. The datasets are therefore consistent and the mean of laboratory means is a good estimate of the true value. Standard deviations between laboratories are considerably larger than the standard deviation within laboratories, showing that confidence intervals of replicate measurements are unsuitable as estimate of measurement uncertainty.

The uncertainty related to the characterisation is estimated as the standard error of the mean of laboratory means. (Table 7).

Table 7: Uncertainty of characterisation for ERM-BD512

Mass fraction ERM-BD512	*p*	Mean [mg/kg]	*s* [mg/kg]	u_{char} [mg/kg]
Cu	11	14.31	0.94	0.29
Mn	11	15.71	0.50	0.15
Ni	8	3.01	0.27	0.09

Cd and Pb

For Cd, the 95 % confidence intervals of each individual result overlapped with each other and the mean value. This indicates that the uncertainty associated to the value is reliably estimated. For Pb, not all results overlapped with the mean value. However, the difference between measurements was far less than the estimated uncertainty due to homogeneity, as presented in section 4.1.

For each mean value, the uncertainty was estimated by making uncertainty budgets that covered all parameters that might influence the result. The means and their uncertainties are given in Table 8, together with the standard deviation of the 6 measurements. The individual results and the summarised relative contributions to the uncertainty budgets of the means are tabulated in Annex F.

Table 8: Uncertainty of characterisation for ERM-BD512

Mass fraction ERM-BD512	p	Mean [mg/kg]	s [mg/kg]	u_{char} [mg/kg]
Cd	6	0.3017	0.0011	0.0023
Pb	6	0.02391	0.00065	0.00016

For Cd, the major uncertainty contribution comes from the natural isotopic abundances of Cd as tabulated by IUPAC [16]. Contributions from isotope ratio measurement were relatively low, and those from sample preparation were insignificant. The isotopic composition of Cd within the sample was not investigated further as the u_{char}, estimate was as low as the uncertainty estimates for stability and homogeneity of the sample (u_{lts} and u_{bb}). As such, reducing the u_{char} estimate further would not reduce U_{CRM}.

For Pb, uncertainty in the spike mass fraction accounted for just over half of the measurement uncertainty. Other significant contributions included the measurement of blend and K-factor ratios, and the measurement of the Pb isotopic composition in the sample. Background correction of ICP-MS signals also made a significant contribution to the uncertainty budget, which is due to the Pb content of the sample being close to environmental background levels. The balanced mixture of uncertainty contributions in the budget showed that measurement parameters were optimised within their performance limits.

To demonstrate absence of bias or gross errors in the measurement of Cd and Pb, results were compared with the mean measured values made by 5 other expert laboratories. Chocolate samples identical to ERM-BD512 were measured by the expert laboratories as well as the JRC to assign the reference value for the PT scheme EURL-HM-20. [18] Based on their results, the reference values were set as 0.303 ± 0.021 mg/kg for Cd and 0.0270 ± 0.0030 for PB. The JRC measured values overlap with the reference value for the PT, which confirms the reliability of the IDMS values.

7 Value Assignment

Certified values were assigned.

Certified values are values that fulfil the highest standards of accuracy. Procedures at the JRC Directorate F require generally pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

7.1 Certified values and their uncertainties

For Cu, Mn and Ni, the unweighted mean of the means of the accepted datasets as shown in Table was assigned as certified value for each parameter. For Cd, the mean value determined by ID-ICP-MS as shown in Table 9 was assigned as certified value. For Pb, no value was assigned as inhomogeneity was found between pellets, as discussed in section 4.1.

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). The uncertainty related to inhomogeneity/degradation during transport/long-term storage was found to be negligible. These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{\mathrm{CRM,\ rel}}$) with a coverage factor k given as:

$$U_{\mathrm{CRM,rel}} = k \cdot \sqrt{u_{\mathrm{bb}}^2 + u_{\mathrm{lts}}^2 + u_{\mathrm{char}}^2} \qquad \textbf{Equation 9}$$

- 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-BD512

	Certified value [mg/kg]	u_{char} [mg/kg]	u_{bb} [mg/kg]	u_{lts} [mg/kg]	U_{CRM} [1] [mg/kg]
Cd	0.302	0.0023	0.0017	0.0055	0.013
Cu	14.3	0.28	0.14	0.13	0.7
Mn	15.7	0.15	0.15	0.14	0.6
Ni	3.01	0.09	0.05	0.03	0.23

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

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

Certified values are element mass fractions of clearly defined analytes. For those certified by laboratory intercomparison, the participants used different methods for the sample preparation as well as for the final determination, demonstrating absence of measurement bias. For those certified by ID-ICP-MS, measurements were made by a process that is completely understood. The measurands are therefore structurally defined and independent of the measurement method.

Quantity value

Only validated methods were used for the determination of the assigned values. Different calibrants/calibrants of (known purity and) 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 it is also confirmed by the agreement among the technically accepted datasets. 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.

8.2 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 [19] 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-BD512 was produced by melting, mixing and recasting chocolate by a typically used commercial process. The analytical behaviour will be the same as for a routine sample of chocolate. For samples other than chocolate the commutability has to be assessed.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

9.2 Storage conditions

The materials should be stored at (18 ± 5) °C) in the dark.

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.

9.3 Minimum sample intake

The minimum sample intake is 250 mg.

9.4 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 [20].

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 (Δ_{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_{Δ}) from the combined uncertainty ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %
- If $\Delta_{\text{meas}} \leq U_{\Delta}$ 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.

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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

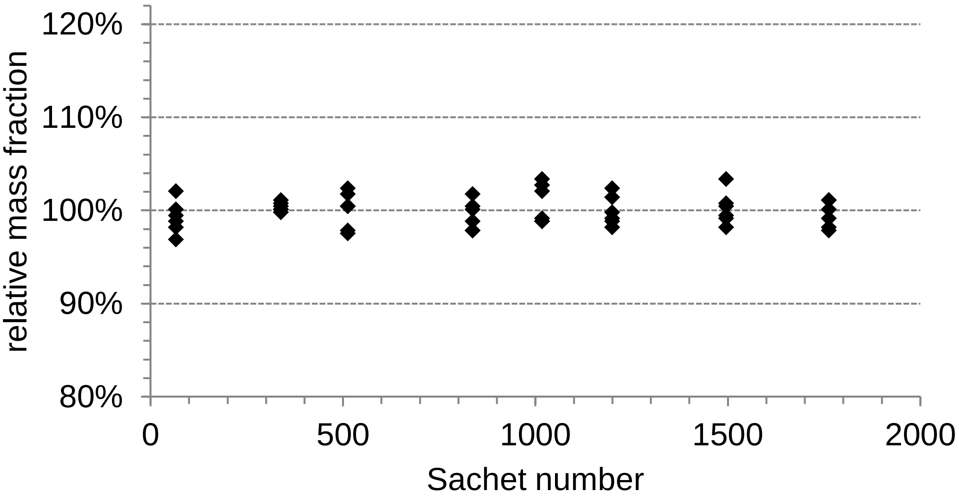
Annex E: Results of the characterisation measurements

Annex F: Summarised IDMS Uncertainty budgets

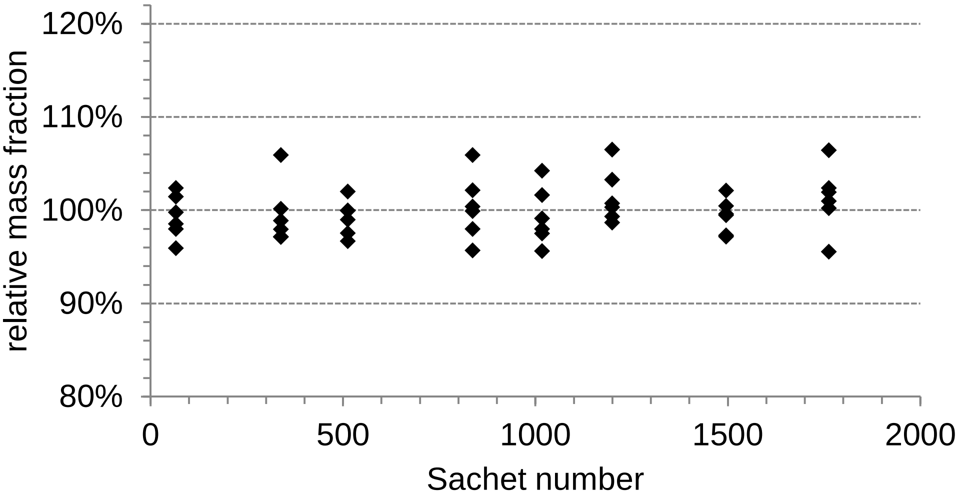
Annex A: Homogeneity study

Data points are individual measurement results, presented as mass fractions of elements relative to the mean of all measurements, against sachet number.

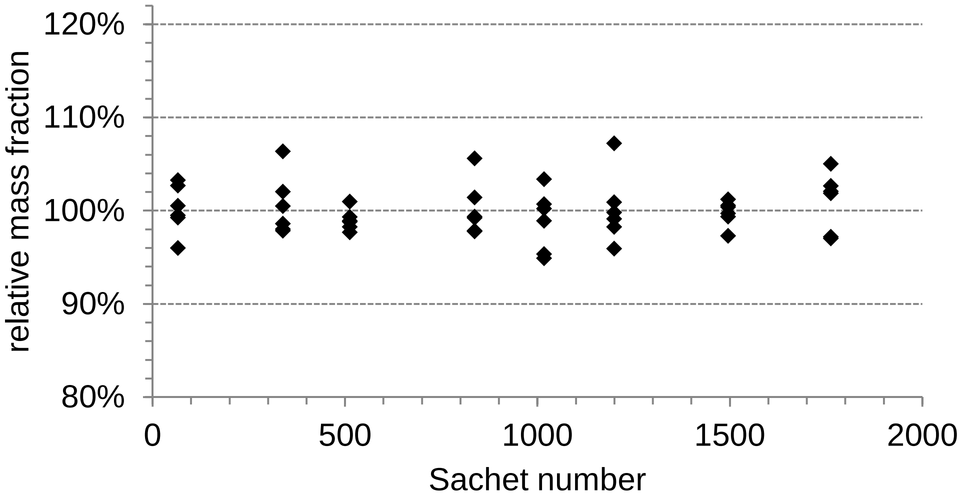
A1: Cd



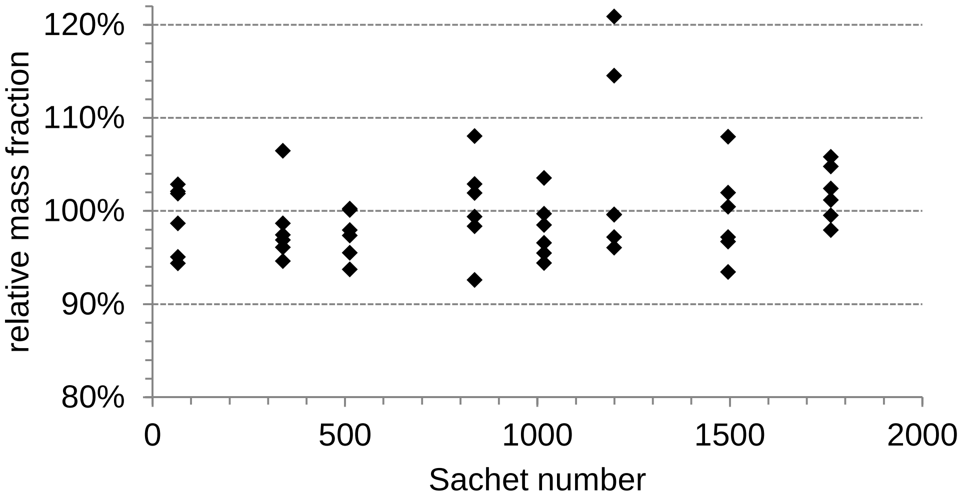
A2: Cu



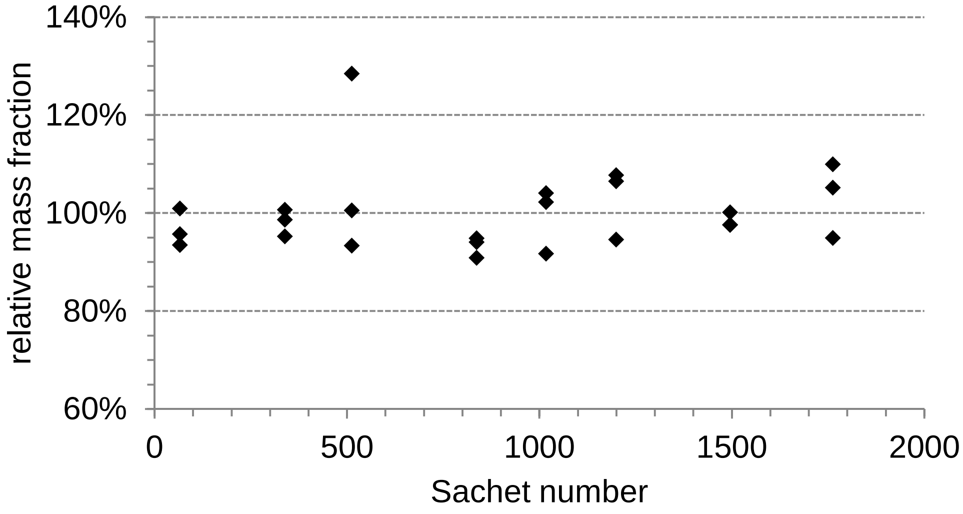
A3: Mn


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

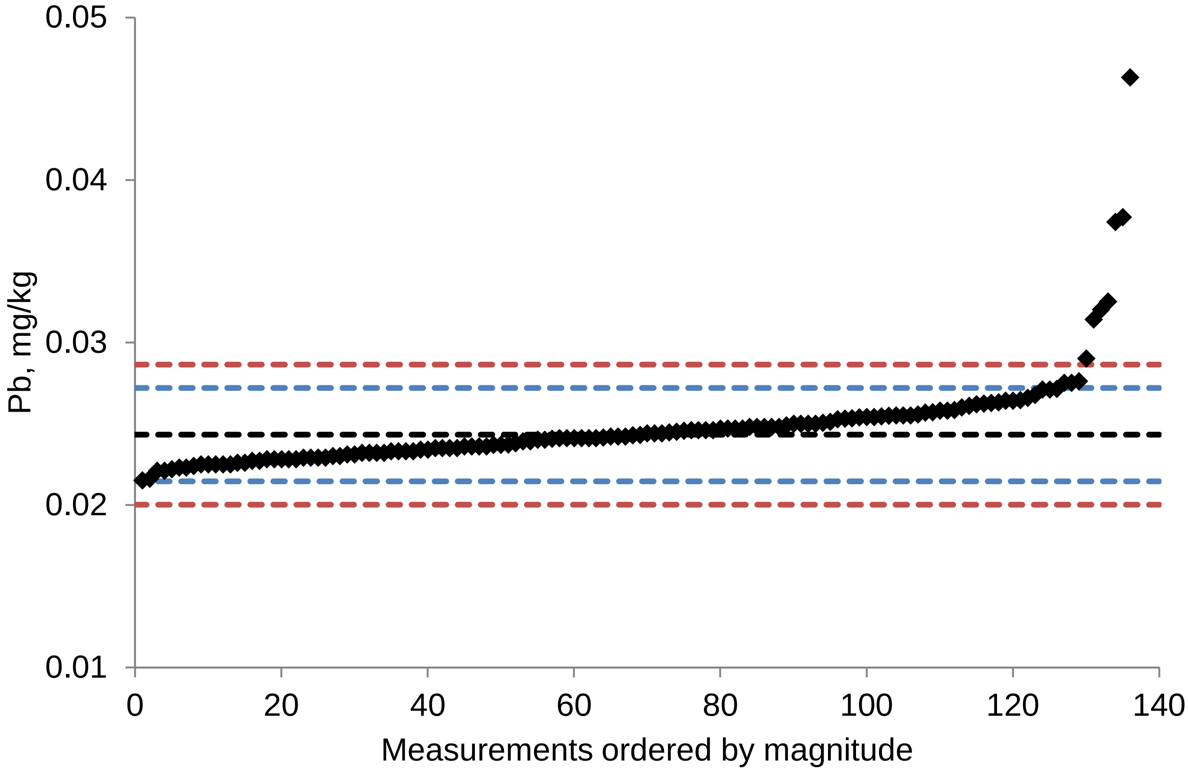
A4: Ni


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

A5: Pb



A6: Pb combined study

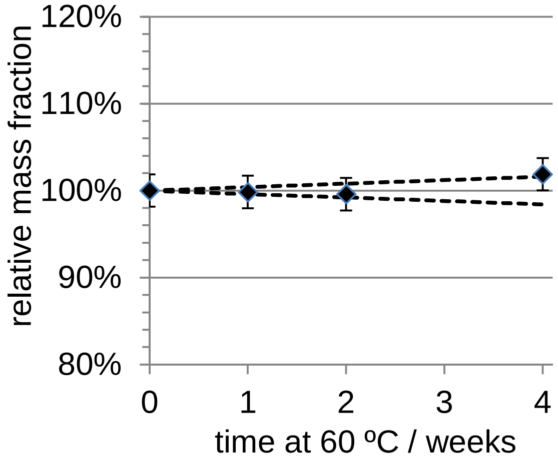


Combined results of homogeneity, short- and long-term stability studies ordered by magnitude. Dotted lines represent mean, 2s and 3s of all results after exclusion of outliers significant at 99 % by the Hampel test.

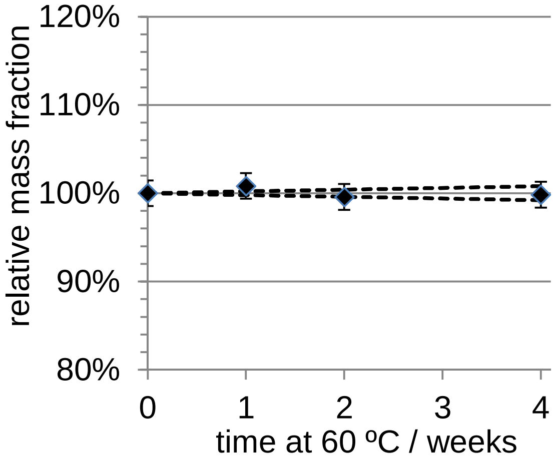
Annex B: Results of the short term stability study at 60 ºC

The graphs show mean measured mass fractions per time point relative to the mean at time zero, with vertical bars representing the 95 % confidence interval. Dotted lines represent u_{STS}.

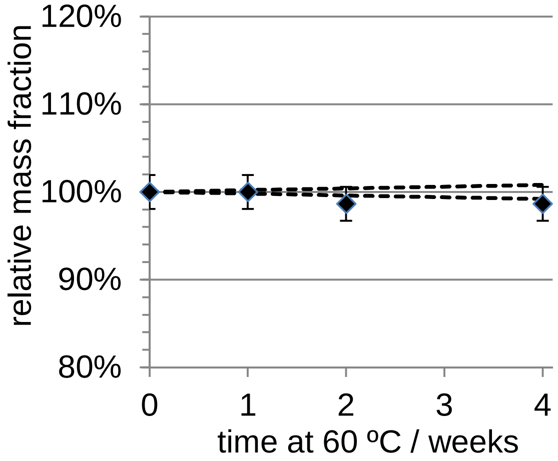
B1: Cd



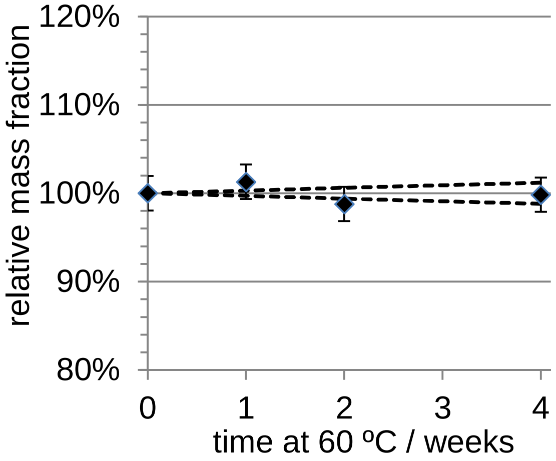
B2: Cu



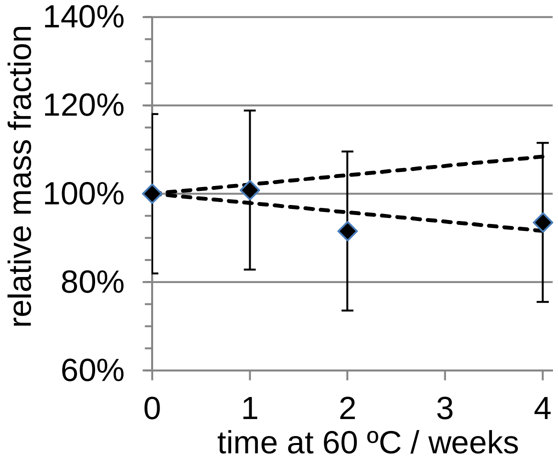
B3: Mn



B4: Ni



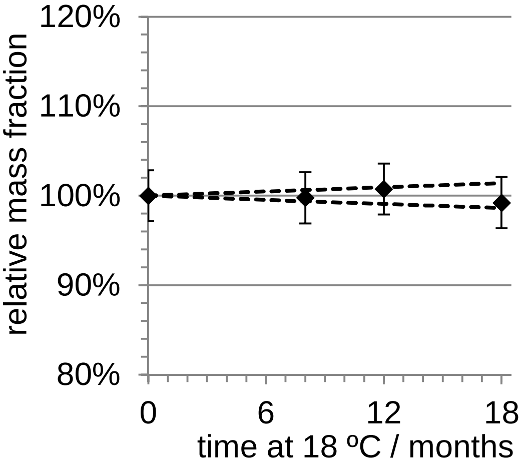
B5: Pb



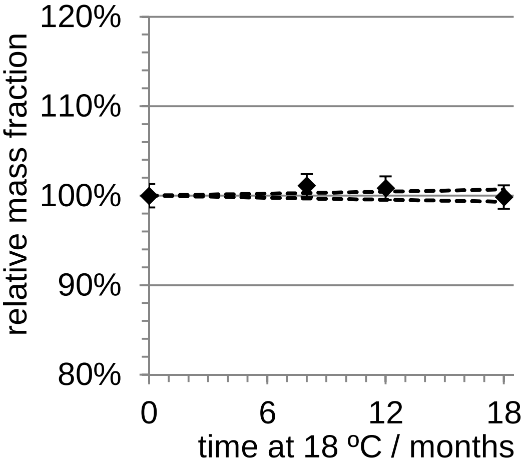
Annex C: Results of the long-term stability study at 18 ºC

The graphs show mean measured mass fractions per time point relative to the mean at time zero, with vertical bars representing the 95 % confidence interval. Dotted lines represent u_{LTS}.

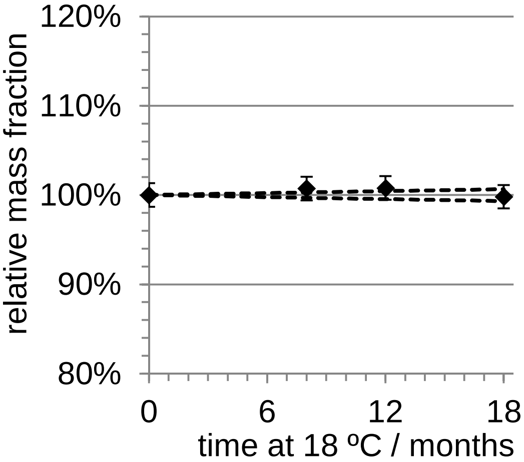
C1: Cd



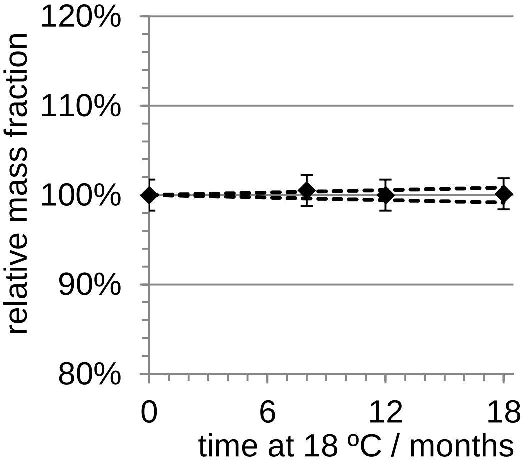
C2: Cu



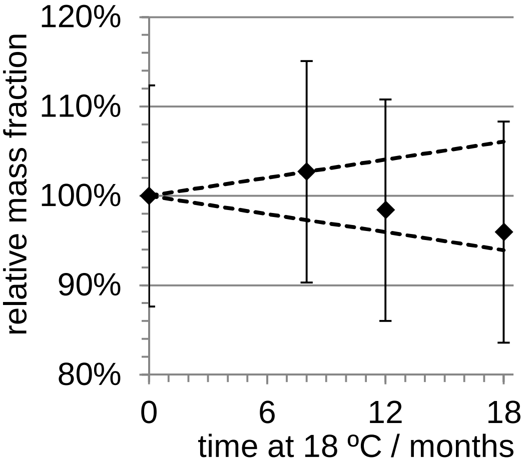
C3: Mn



C4: Ni



C5: Pb



Annex D: Summary of analytical techniques used in the characterisation of Cu, Mn and Ni in ERM-CA403 as reported by the laboratories

Lab-method code	Sample pretreatment	Analytical method	Calibrant	Instrument
L01	None	k0-NAA	IRMM-530	GA, TRIGA Mark II reactor; Canberra, HPGe detector
L02	To approx. 0.5 g of sample 8 mL of HNO_3 (Suprapur®) and 2 mL of H_2O_2 (Suprapur) were added into Teflon vessel. Closed vessel microwave assisted digestion was then performed.	Digests were measured by ICP-MS, collision cell operation with He	Merck, traceable to NIST SRM 3114, 3132, 3136	Agilent 7700x ICPMS
L03	*Laboratory did not report results*			
L04	Samples (chocolate pellets) were digested in HNO_3 + HCl using a Milestone UltraWave digestion system, achieving 240 ºC to ensure complete mineralisation	Digests were measured by ICP-MS, collision cell operation with He	All VWR, traceable to NIST SRMs	Agilent 7700x ICPMS
L05	Microwave digestion	Digests were measured by ICP-MS with collision cell operation	Cu: Fluka, Mn and Ni: Spex Certiprep®	Agilent 7500
L06	Microwave digestion	ICP-OES, quadratic calibration	Cu: Fluka, Mn: Spex Certiprep	Agilent 700
L07	SRC (Single reaction chamber) UltraClave 0.5 g sample dissolved with HNO_3 and HF. After digestion samples were diluted and measured on ICP-SFMS against standards prepared in the same concentration of acids as in the samples.	ICP-SFMS	Ultragrade Solutions from Ultra Scientific, traceable to NIST SRM 3114, 3132, 3136	Thermo Scientific Element XR ICP-SFMS
L08	In a closed microwave (8 positions) 0.5 g of chocolate was digested with 6 ml of concentrated nitric acid and 2 ml of hydrogen peroxide. 20 minutes at 800 Watt (max pressure 40 bar) After ramp of 20 minutes to 800 Watt	ICP-SFMS	Spex Certiprep multi-element, traceable to NIST SRM 3114, 3132, 3136	Thermo

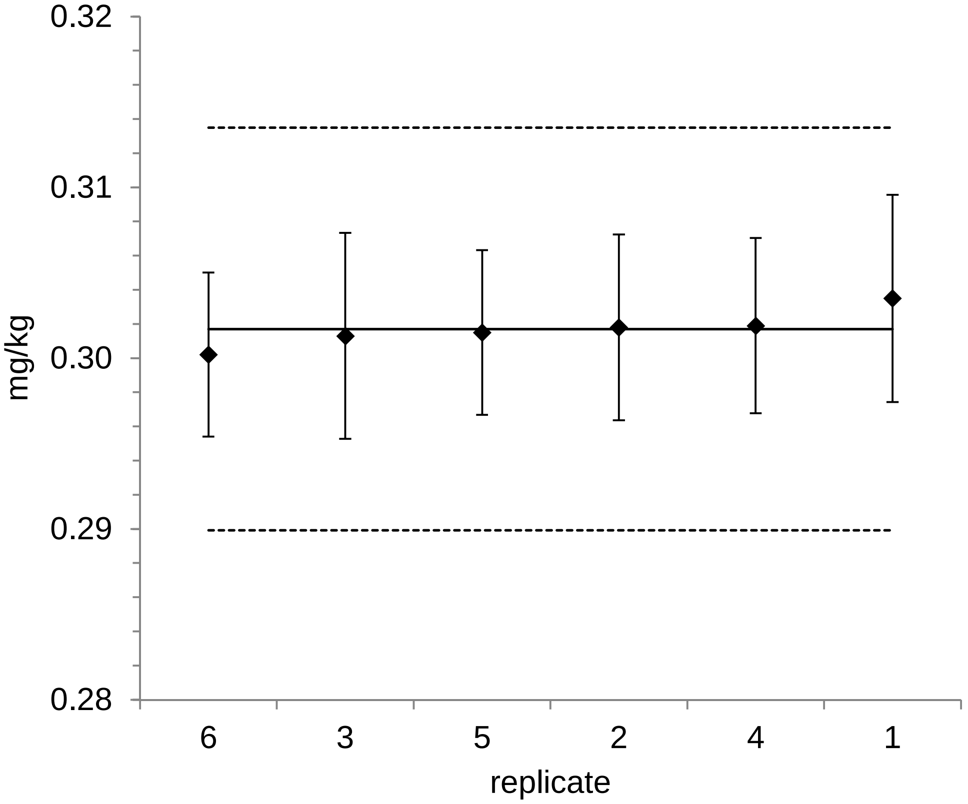
L09	Microwave assisted acid digestion with nitric acid and hydrogen peroxide	ICP-MS	Baker Instra Analyzed, Ultra Scientific, traceable to NIST SRM 3114, 3132, 3136	Perkin Elmer Elan DRC II
L10	Microwave assisted acid digestion with nitric acid and hydrogen peroxide	ICP-OES (Cu, Mn) ETAAS (Ni)	Baker Instra Analyzed, Ultra Scientific, traceable to NIST SRM 3114, 3132, 3136	Perkin Elmer Optima 7300 for Cu, Mn Perkin Elmer AA800 for Ni
L11	Samples are weighted in Teflon vessel for microwave digestion. We add 6 ml concentrated HNO_3 and 1 ml concentrated H_2O_2. The sample is then digested at high pressure, in closed vessel. One blank per batch of digestion is included. Once digested, the sample is transferred to volume. If dilution is needed, we dilute with HNO_3 1 M	ICP-OES	Fluka TraceCE RT®, 1000 mg/L single element standards in nitric acid	Perkin Elmer Optima 7300DV
L12	Pellets were cut up and about 0.25 g transferred to PE vials prior to irradiation	k0-NAA	IRMM-530	Gamma ray spectrometers (built in-house).
L13	Closed microwave digestion with HNO_3:H_2O_2 (7 ml:1 ml) controlled temperature program (10 min to 200°C, 20 min at 200°C) ~ 0.5 g of sample; end volume 25 ml	ICP-MS	Merck, ICP multi-element standard VI	Perkin-Elmer SCIEX ELAN DRC-e

Annex E, Part 1: Results of the characterisation study for Cd and Pb

Individual measurement results by ID-ICP-MS. Vertical bars are the uncertainties of the individual results (k=2) while the solid line represents the certified value (the mean of the measurements), and the broken lines represent the expanded uncertainty of the certified value.

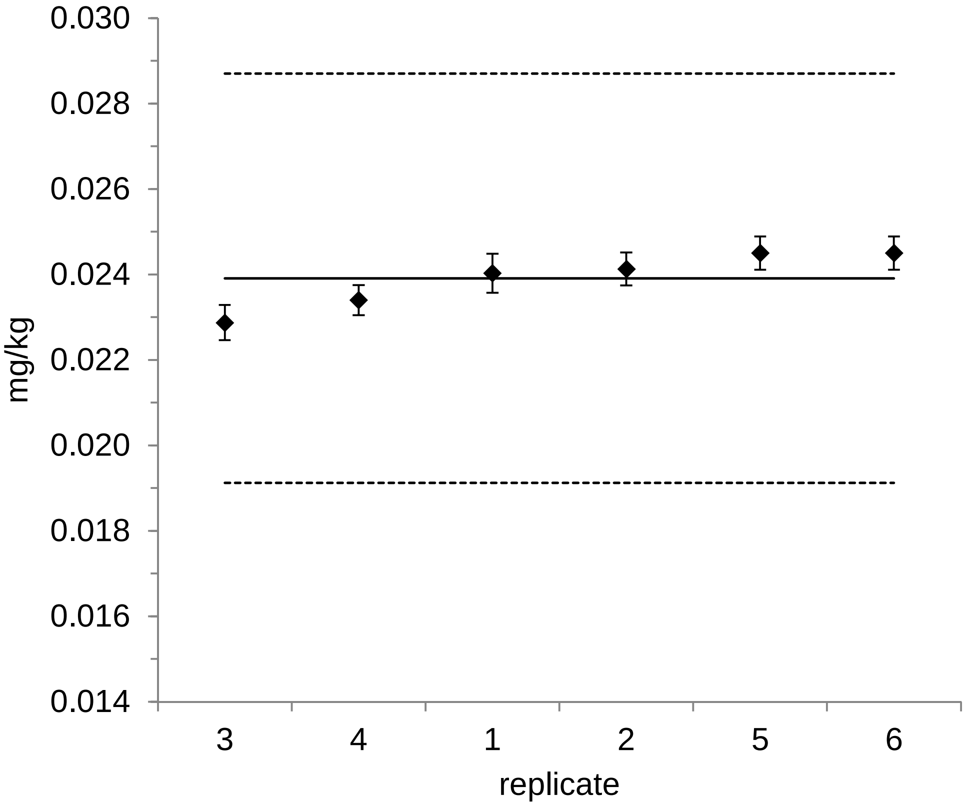
E.1 Cd mass fraction [mg/kg]

Sample	**mass fraction**	**U**
1	0.3035	0.0061
2	0.3018	0.0054
3	0.3013	0.0060
4	0.3019	0.0051
5	0.3015	0.0048
6	0.3002	0.0048
Mean	0.3017	0.0045



E.2 Pb mass fraction, not certified [mg/kg]

Sample	mass fraction	U
1	0.02403	0.00046
2	0.02413	0.00039
3	0.02287	0.00041
4	0.02340	0.00035
5	0.02450	0.00039
6	0.02450	0.00039
Mean	0.02391	0.00031



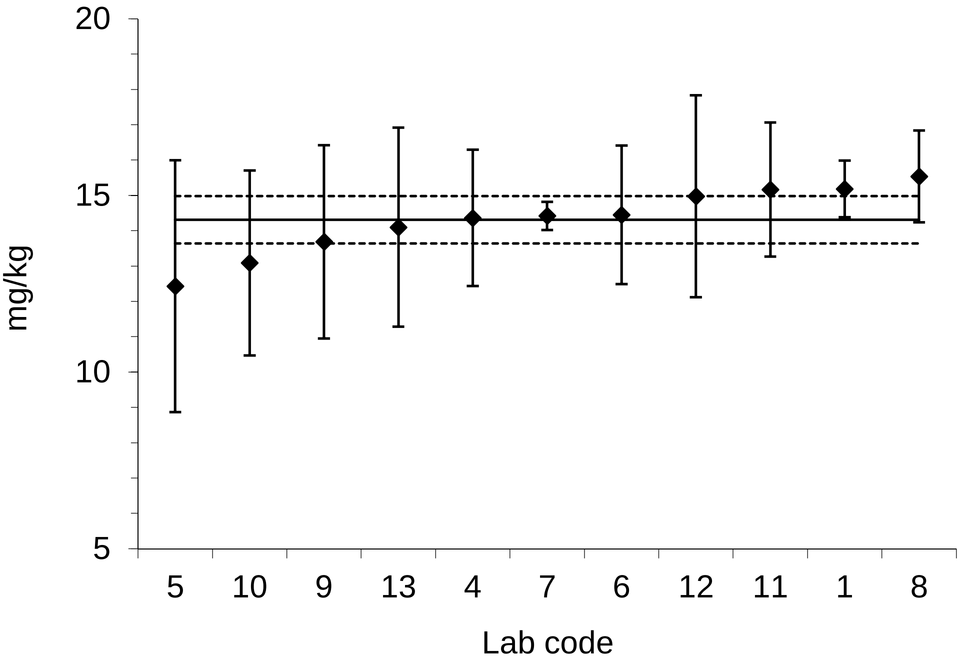
Annex E, Part 2: Results of the characterisation study for Cu, Mn and Ni

Individual measurement results from each laboratory participating in the intercomparison. Vertical bars represent expanded uncertainties as reported by participating laboratories. The solid line represents the certified values (the mean of the laboratory means), while the broken lines represent the expanded uncertainty of the certified value.

Approaches to number rounding differed between participants. Values presented in this annex were rounded to assist document formatting.

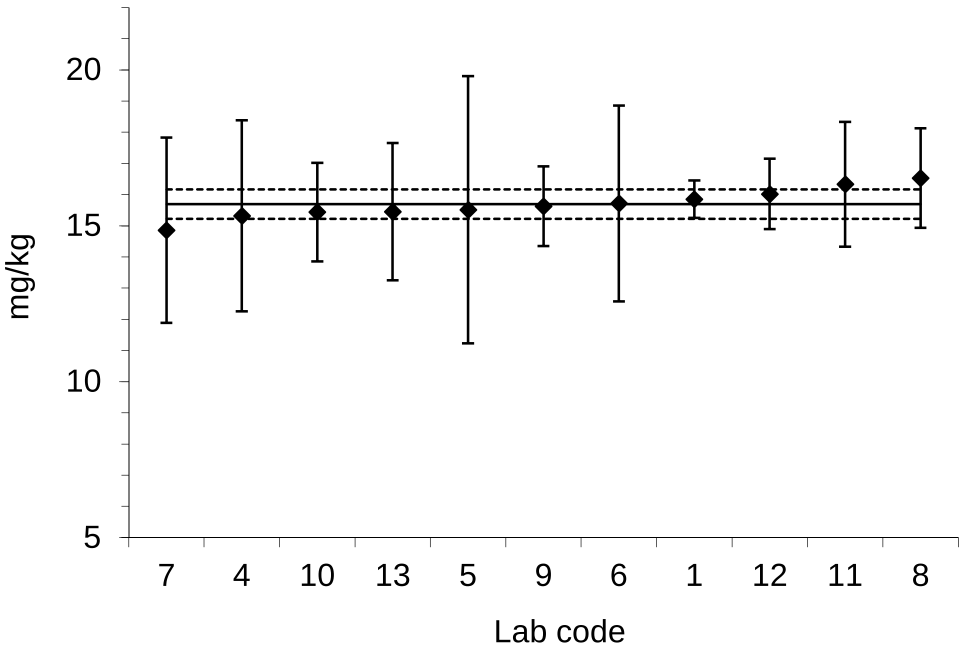
E.3 Cu mass fraction [mg/kg]

Lab code	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean	U
L01	16.1	15.4	14.3	15.5	14.2	15.6	15.183	1.600
L04	14.36	14.16	14.12	14.49	14.21	14.82	14.360	1.930
L05	11.9	12.9	13.2	12.5	12.1	12.0	12.433	3.564
L06	14.6	14.4	14.4	14.7	14.2	14.4	14.450	1.962
L07	14.322	14.515	14.567	14.348	14.110	14.657	14.420	0.398
L08	15.193	15.316	14.849	16.327	15.894	15.649	15.538	1.302
L09	13.69	13.74	13.57	13.89	13.60	13.63	13.687	2.083
L010	12.95	12.95	13.29	12.91	13.16	13.28	13.090	1.329
L011	15	16	16	14	15	15	15.167	1.900
L012	15.14	15.514	14.289	14.497	15.118	15.287	14.974	2.858
L013	14.1	14.1	14.1	14.3	13.9	14.1	14.100	1.700



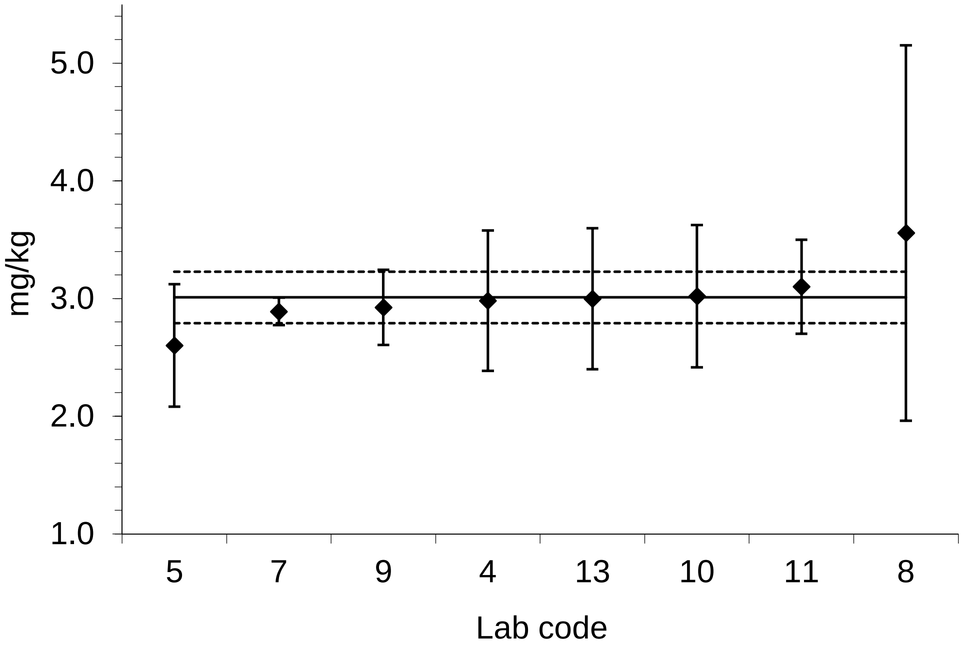
E.2 Mn mass fraction [mg/kg]

Lab code	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean	U
L01	16.1	15.8	15.6	16.0	16.0	15.6	15.850	0.600
L04	14.99	15.31	15.36	15.20	15.37	15.69	15.320	2.040
L05	14.39	14.86	15.31	16.81	16.68	15.04	15.515	4.287
L06	15.8	15.6	15.8	16.1	15.4	15.6	15.717	2.884
L07	14.725	15.493	15.430	14.616	14.305	14.584	14.859	0.975
L08	16.104	16.255	15.776	17.340	17.017	16.703	16.532	1.595
L09	15.97	15.69	15.61	15.99	15.19	15.31	15.627	1.279
L010	15.30	15.24	15.54	15.17	15.59	15.79	15.438	1.579
L011	16	17	16	16	16	17	16.333	2.000
L012	16.331	15.927	15.936	15.731	16.134	16.069	16.021	1.128
L013	15.5	15.4	15.7	15.4	15.3	15.4	15.450	2.200



E.4 Ni mass fraction [mg/kg]

Lab code	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**	**Mean**	**U**
L04	2.926	2.995	3.003	2.979	2.985	3.004	2.982	0.390
L05	2.431	2.703	2.786	2.607	2.577	2.505	2.602	1.132
L07	2.843	2.925	2.964	2.938	2.828	2.844	2.890	0.117
L08	3.338	4.179	3.303	3.643	3.438	3.440	3.432	1.595
L09	2.915	2.870	2.849	3.190	2.923	2.794	2.924	0.319
L010	2.885	2.897	3.180	2.811	3.120	3.230	3.021	0.581
L011	3.3	3.1	3.1	3.2	2.8	3.1	3.100	0.4
L013	3.01	2.99	2.98	3.01	2.99	3.01	2.998	0.910



Annex F: Summarised IDMS uncertainty budgets

F.1 Cd uncertainty budget

Parameter	***%***
Isotopic abundances, Cd [16]	84.5
K-factor ratio measurement	7.0
Blend ratio measurement	5.7
Spike mass fraction	1.4
Background correction (ICP-MS)	0.6
Deadtime correction (ICP-MS)	0.2
Isotopic abundances, IRMM-622	0.2
Procedural blank correction	0.0
Sample and spike weighings	0.0

Note that due to rounding, the table may not total 100 %.

F.2 Pb uncertainty budget

Parameter	***%***
Spike mass fraction	53.6
Background correction (ICP-MS)	12.8
Isotopic abundances, Pb	11.8
K-factor ratio measurement	11.2
Blend ratio measurement	7.1
Procedural blank correction	3.3
Deadtime correction (ICP-MS)	0.0
Isotopic abundances, Spike	0.0
Sample and spike weighings	0.0

Note that due to rounding, the table may not total 100 %.

European Commission

EUR 28936 EN – Joint Research Centre – Directorate F – Health, Consumers and Reference Materials
Title: **CERTIFICATION REPORT: The certification of the mass fractions of cadmium copper, manganese and nickel in dark chocolate: ERM®- BD512**
Author(s): James Snell, Mitja Vahcic, Hanne Leys, John Seghers and Andrea Held
Luxembourg: Publications Office of the European Union
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