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European Reference Materials

CERTIFICATION REPORT

The certification of the mass fractions of hexachlorobenzene (HCB) and hexachlorobutadiene (HCBD) in fish tissue: ERM®-CE100

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Abstract
This report describes the production of ERM®-CE100, a fish (biota) material certified for the mass fractions of hexachlorobenzene and hexachlorobutadiene. This material was produced following ISO Guide 34:2009 and is certified in accordance to ISO Guide 35:2006.
The starting material was wild Wels catfish (Silurus glanis) caught in the area of the Flix reservoir at the Ebro River, Spain. The filleted fish was cut and cryogenically milled before mixing. Pre-cooking followed and after further mixing, the material was filled in jars, sterilised in an autoclave and finally labelled as ERM-CE100.
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 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 quality control charts or validation studies. The CRM is available in glass jars closed with twist-off lids containing at least 40 g of sterilised fish paste. The minimum amount of sample to be used is 1 g (wet mass).
The CRM was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

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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®-CE100, a fish (biota) material certified for the mass fractions of hexachlorobenzene and hexachlorobutadiene. This material was produced following ISO Guide 34:2009 [1] and is certified in accordance to ISO Guide 35:2006 [2].

The starting material was wild Wels catfish (*Silurus glanis*) caught in the area of the Flix reservoir at the Ebro River, Spain. The filleted fish was cut and cryogenically milled before mixing. Pre-cooking followed and after further mixing, the material was filled in jars, sterilised in an autoclave and finally labelled as ERM-CE100.

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 material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025 [3]. 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 quality control charts or validation studies. The CRM is available in glass jars closed with twist-off lids containing at least 40 g of sterilised fish paste. The minimum amount of sample to be used is 1 g (wet mass).

The CRM was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

The following values were assigned:

	Mass fraction (relative to wet weight)	
	Certified value [2] [µg/kg]	Uncertainty [3] [µg/kg]
Hexachlorobenzene [1]	120	8
Hexachlorobutadiene [1]	36	4

1) as obtained by using gas chromatography

2) 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 values and their uncertainties are traceable to the International System of units (SI).

3) The uncertainty of the certified value is the expanded uncertainty 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
ASE	Accelerated solvent extraction
c	Mass concentration $c = m / V$ (mass / volume)
C-QC	Calibration quality control
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CRM	Certified reference material
EC	European Commission
EI	Electron ionisation
EQS	Environmental Quality Standards
ERM®	Trademark of European Reference Materials
EU	European Union
GC-ECD	Gas chromatography-electron capture detection
GC-(HR)MS	Gas chromatography-(high resolution)mass spectrometry
GC-MS/MS	Gas chromatography- tandem mass spectrometry
GC-IDMS	Gas chromatography isotope dilution-mass spectrometry
GPC	Gel permeation chromatography
GUM	Guide to the Expression of Uncertainty in Measurements
HCB	Hexachlorobenzene
HCBD	Hexachlorobutadiene
IEC	International Electrotechnical Commission
IRMM	Institute for Reference Materials and Measurements of the JRC
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
$MS_{between}$	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
M-QC	Method quality control
n	Number of replicates per unit
n.c.	Not calculated
NIST	National Institute of Standards and Technology (US)
PS	Priority substances

QA	Quality assurance
QC	Quality control
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
RSD	Relative standard deviation
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
SPE	Solid phase extraction
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 elapsed at time point i of the short-term stability study (for $u_{sts,rel}$) or long-term stability study (for $u_{lts,rel}$)
$t_{\alpha, df}$	Critical t-value for a t-test, with a level of confidence of 1-α and df degrees of freedom
t_{sl}	Proposed shelf life
t_{tt}	Transport time chosen
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_c	Combined standard uncertainty; an additional index "rel" is added as appropriate
u_{cal}	Standard uncertainty of calibration
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_{Δ}	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
WFD	Water Framework Directive
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

Hexachlorobenzene (HCB) and hexachlorobutadiene (HCBD) are two substances which are considered as global environmental pollutants, especially of aquatic ecosystems. They are used in agricultural and industrial applications [5-6] and are listed as Persistent Organic Pollutants (POP) of the Stockholm convention. During the last decades both pollutants were detected in surface waters and related matrices (sediments, biota) [7]. Bioaccumulation and biomagnification effects have been shown for both chemicals, most pronounced for HCB [8-9].

Since 2000, Directive 2000/60/EC, known as the Water Framework Directive (WFD) [10] is in force in the European Union (EU) for developing a pollution control strategy of all EU water bodies. Subsequently, Directive 2008/105/EC [11] set Environmental Quality Standards (EQS) for the established list of Priority Substances (PS). HCB and HCBD are among the PS that Member States are expected to assess, monitor and control in EU water resources. As foreseen in Article 3 of this Directive, EU Member States that opt to apply EQS for sediment and biota, shall apply an EQS of 10 µg/kg for HCB and 55 µg/kg for HCBD. The most recent Directive 2013/39/EU [12] re-evaluated the EQS for some of the PS and biota EQS were introduced for more PS, with the specification that, wherever a biota EQS is given, biota shall be the "default" monitoring matrix.

To ensure the quality and comparability of analytical results reported by the Member States, the EU issued Directive 2009/90/EC [13] in 2009, setting minimum analytical performance criteria for monitoring water quality. The competence of EU laboratories selected for this task must be guaranteed by the use of certified reference materials (CRMs) of appropriate matrix and with corresponding levels to the established EQS.

1.2 Choice of the material

In support of the EU legislation, ERM-CE100 was developed as a fresh-like biota matrix CRM, addressing the legislative trend of the Directive 2013/39/EU which introduced biota EQS for more PS, and declared that, for those substances, biota shall be the "default" monitoring matrix. The fish selected for the production of ERM-CE100 was wild Wels catfish (*Silurus glanis*). The choice of the species *Silurus glanis* for the production of a biota CRM for the analysis of HCB and HCBD was made for several reasons. This fish can reach very large sizes and is a predator, i.e. positioned high in the trophic chain, which potentially leads to bioaccumulation and biomagnification of organic pollutants. The presence of large specimens should lead to a reduced variation of pollutant levels between pooled individuals, introducing a clear advantage to the CRM preparation. The overall purpose of the project was to develop a naturally rather than artificially contaminated (spiked) fresh-like biota matrix material.

1.3 Design of the project

The CRM consists of units containing approximately 40 g of sterilised fish paste. The material was not freeze-dried in order to resemble the routine environmental samples as close as possible and in consideration of the fact that EQS are expressed as mass fraction relative to wet weight. Homogeneity and stability were assessed for both analytes in the final CRM. The certification was performed by interlaboratory comparison using analytical methods based on different analytical principles to reduce bias in the analytical result.

The HCB and HCBD levels in the designed CRM were targeted to be as close as possible to the EQS of these pollutants [12]. On the other hand, the natural level of the analytes of interest in the individual fish collected had to be taken into consideration [7] and determined the final certified value obtained.

2 Participants

2.1 Project management and evaluation

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

2.3 Homogeneity study

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM; measurements under the scope of ISO/IEC 17025 accreditation BELAC No. 268-TEST)

Helmholtz Zentrum München, Deutsches Forschungzentrum für Gesundheit und Umwelt (GmbH), Neuherberg, DE
(measurements under the scope of ISO/IEC 17025 accreditation DAkkS; D-PL-14138-02-00)

2.4 Stability study

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM; measurements under the scope of ISO/IEC 17025 accreditation BELAC No. 268-TEST)

2.5 Characterisation

BAM, Bundesanstalt für Materialforschung und –prüfung, Berlin, DE
(measurements partially under the scope of ISO/IEC 17025 accreditation DAkkS; D-PL-11075-14-00)

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM; measurements under the scope of ISO/IEC 17025 accreditation BELAC No. 268-TEST)

GBA, Gesellschaft für Bioanalytik mbH, Pinneberg, DE
(measurements under the scope of ISO/IEC 17025 accreditation DAkkS; D-PL-14170-01-00)

Helmholtz Zentrum München, Deutsches Forschungzentrum für Gesundheit und Umwelt (GmbH), Neuherberg, DE
(measurements under the scope of ISO/IEC 17025 accreditation DAkkS; D-PL-14138-02-00)

IDAEA-CSIC, Institut de Diagnosi Ambiental i Estudis de l'Aigua, Consejo Superior de Investigaciones Científicas, Departamento de Química Ambiental, Barcelona, ES

IMARES, Institute for Marine Resources and Ecosystem Studies, Wageningen UR, IJmuiden, NL
(measurements partially under the scope of ISO/IEC 17025 accreditation Raad voor Accreditatie/Dutch Accreditation Council; L097)

Instituto Hidrográfico, Divisão de Química e Poluição do Meio Marinho, Lisboa, PT

IVM, Institute for Environmental Studies, Vrije Universiteit, Amsterdam, NL

LABERCA – ONIRIS, Laboratoire d' Etude des Résidus et Contaminants dans les Aliments – École Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes Atlantique, Nantes, FR
(measurements under the scope of ISO/IEC 17025 accreditation COFRAC; 1-0549)

RIKILT, Institute of Food Safety, Wageningen UR, Wageningen, NL
(measurements under the scope of ISO/IEC 17025 accreditation Raad voor Accreditatie/Dutch Accreditation Council; L014)

University of Barcelona, Department of Analytical Chemistry, Group of Chromatography, Capillary Electrophoresis and Mass Spectrometry, Barcelona, ES

3 Material processing and process control

3.1 Origin of the starting material

The starting material used for the production of ERM-CE100 was approximately 110 kg of filleted Wels catfish (*Silurus glanis*) originating from the area of the Flix reservoir at the Ebro River, Spain. Twenty-nine specimens were caught by angling during the period December 2010 – June 2011. The fish was filleted (excluding skin, inner part and bones but including the fatty part of the fish tissue), separated into head and tail, sealed into plastic bags and labelled to unambiguously identify the origin. The material was transported frozen to IRMM and kept at -20 °C until further treatment.

3.2 Processing

Flowchart and scheme of the complete production process are presented below in Figures 1 and 3.

Aliquotes were analysed from all heads and tails to screen the content of HCB and HCBD. Sampling was performed using a steel drill on the still frozen parts.

In total, 60 kg of head parts were thereafter thawed and manually cut into pieces. After flash-cooling in liquid nitrogen the manually cut pieces were cryogenically milled in a vibrating cryogenic mill, Palla VM-KT (Janke Kunkel, Köln, DE). Thereafter 50 kg of fish tails were cryogenically milled and processed separately in the same way. Each specimen was processed separately and could be traced back to the original part and fish (part being head or tail). The cryogenic mill was cleaned with about 5 kg of crushed ice between the milling events to prevent carryover from one piece of fish to another. Each piece of milled fish was thereafter homogenised in a Stephan UM12 mixer (Hameln, DE) and samples were taken for additional checks of the contaminants' levels. The milled fish was stored at -20 °C until the analysis results for HCB and HCBD became available.

According to the analysis results, different head and tail parts were selected in a mixing scheme to target the EQS levels of HCB and HCBD as close as possible (Batch 4, Figure 3). For the mixing to obtain the different batches (Figure 3) a Stephan UM12 mixer (Hameln, DE) and a larger Stephan UM80 mixer were used. During the in-between batches analysis, the paste material was stored at 4 °C overnight. The final control analysis on batch 4 confirmed values close to the targeted mass fraction levels for the two analytes. The paste was thereafter transferred into a Firex cucimix mixer (Sedico, IT) with a 70 L capacity that was used to gradually increase the temperature of the fish paste from 19 to 82 °C in one hour under constant mixing and to finally maintain it for two minutes.

For rapid cool-down the still warm material was thereafter transferred to trays and placed in an Martin Christ Epsilon 2-100D freeze dryer (Osterode, DE) where the shelves were kept at 1 °C. The cooled fish paste was thereafter placed at 4 °C overnight and the following day transferred to the Stephan UM 80 mixer where it was mixed 3 x 3 minutes to achieve a homogeneous viscous paste, see Figure 2.

Approximately 40 g of the fish paste was filled into 65 mL glass jars using a PSV Villa SP25 piston filling machine (Genainville, FR), see Figure 2. Twist-off lids of 66 mm diameter were placed on the jars using a Lenssen twist-off machine (Sevenum, NL).

The filled jars were placed according to fill order in a basket and sterilised at 121 °C (peak temperature maintained for about 10 minutes) using a JBTC autoclave (Sint-Niklaas, BE). The temperature inside the material during autoclavation was monitored online with a probe and additionally using autoclavation tape.

Labelling was performed manually according to the filling order. A label was placed both on the lid and at the underside of each glass jar. A total of 1023 CRM units were produced.

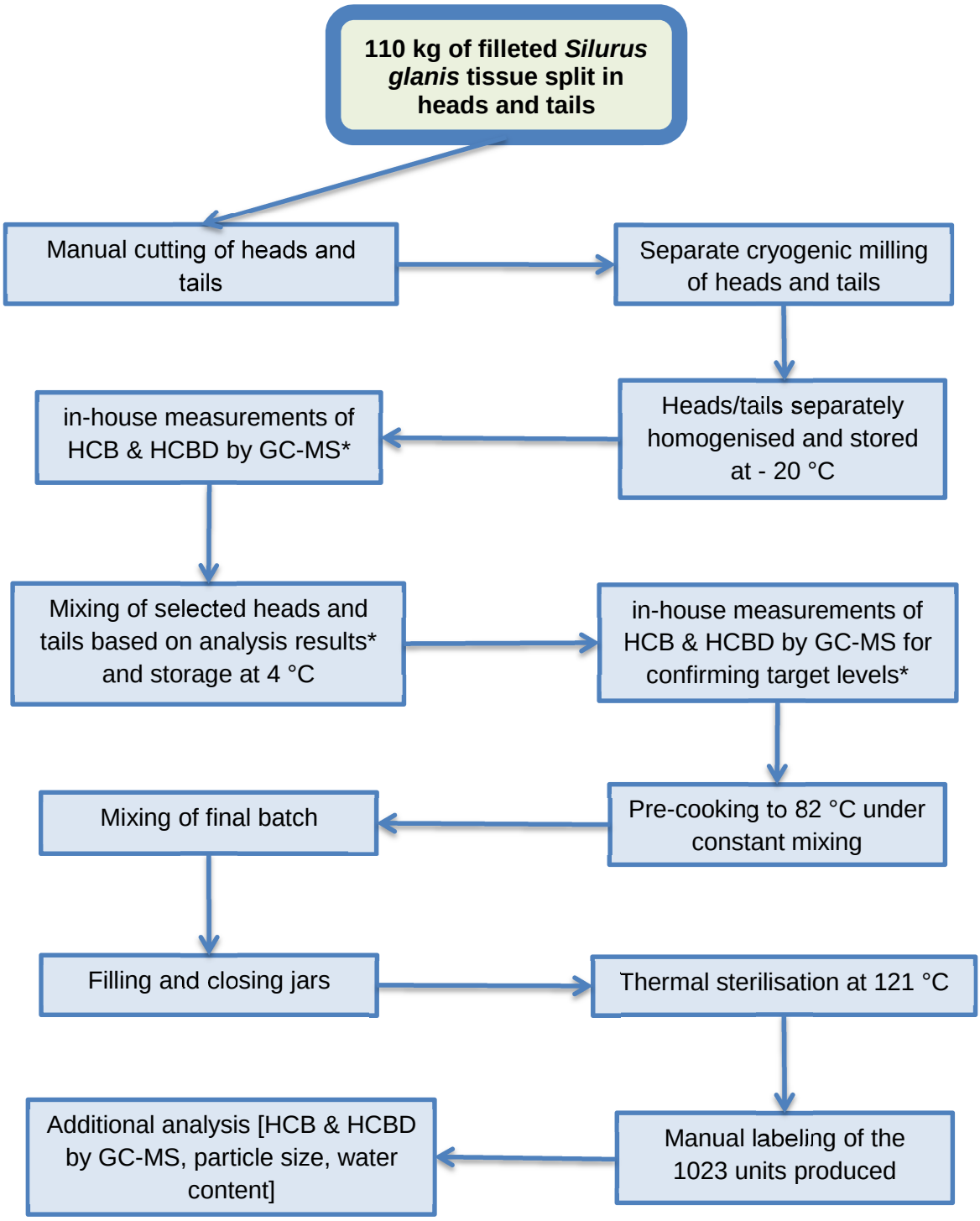

Figure 1: Flow chart of main steps in the preparation of ERM-CE100.

* the steps concerning the in-house checks of the analytes' levels and the mixing are reported in more details in Figure 3.

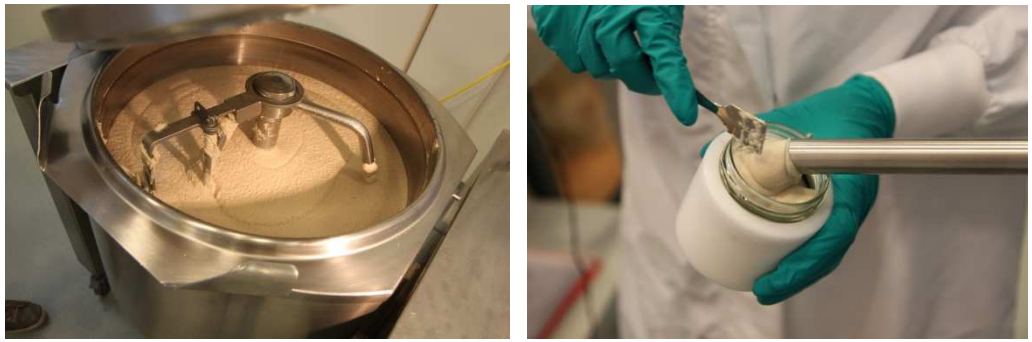

Figure 2: Pre-cooking and filling of ERM-CE100

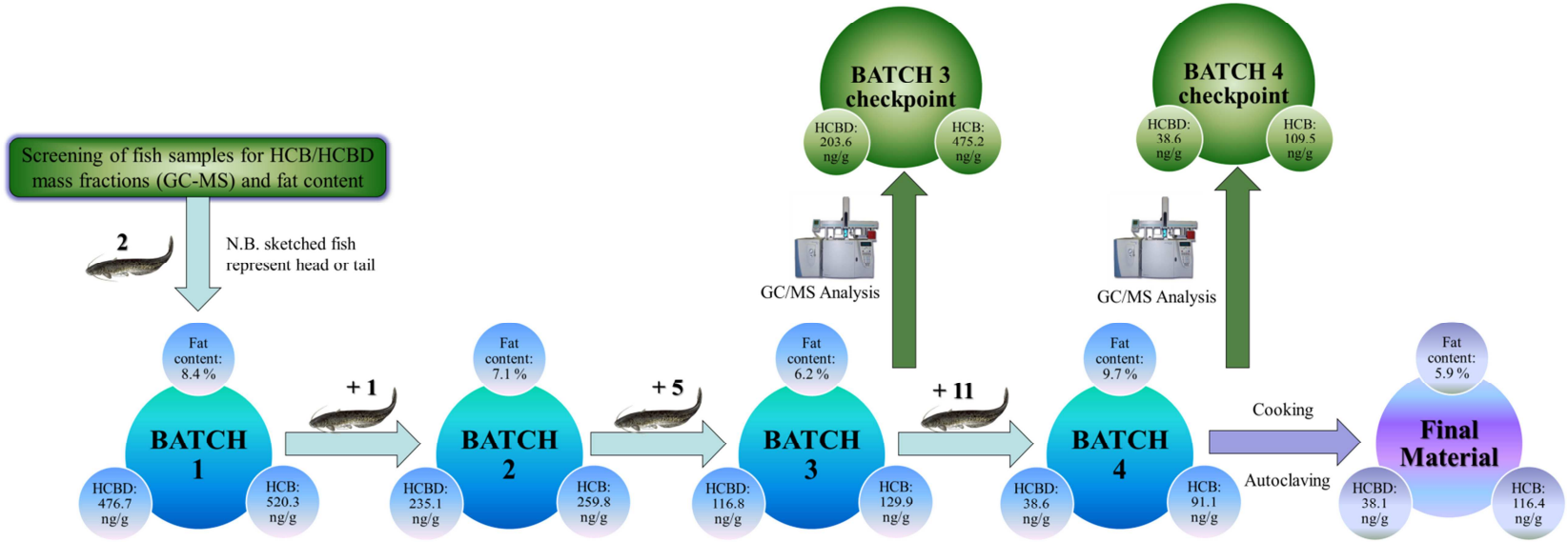

Figure 3: Processing of ERM-CE100: "mix and match" scheme (in blue the theoretical fat content [7,14] and mass fractions of HCB and HCBD, in green the mass fractions measured in-house, in purple the fat content and mass fractions of HCB and HCBD measured in-house on the finally produced material; mass fractions are expressed relative to wet weight)

An example of a final unit produced can be seen in Figure 4 below:

Figure 4: Example of ERM-CE100 unit

3.3 Process control

3.3.1 Particle size analysis

The average particle size distribution of ERM-CE100 is displayed in Fig. 5 and relevant data are reported in Table 1. Five units were measured in duplicate using a Sympatec Helos laser light diffraction instrument (Clausthal Zellerfeld, DE).

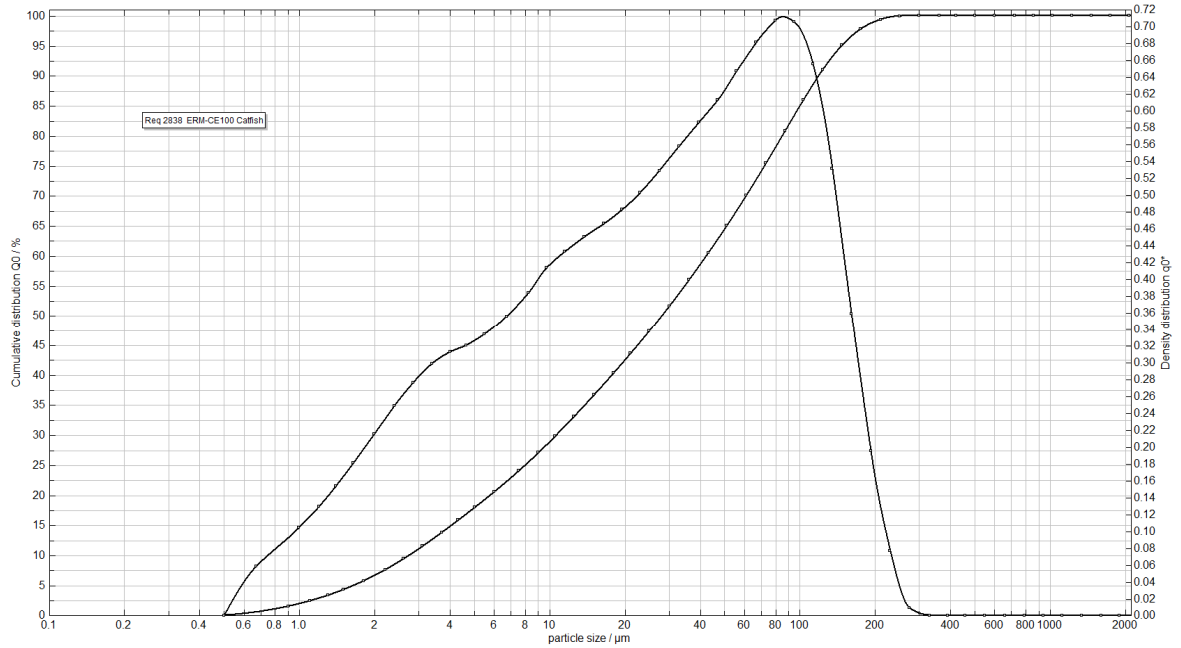

Figure 5: Average particle size distribution in ERM-CE100 using water as dispersant

Table 1: Particle size data for ERM-CE100 (average of ten measurements)

Upper band limit	Average particle size (µm)	s (µm)	RSD (%)
X_{10}	2.76	0.21	4.78
X_{50}	28.14	1.17	3.09
X_{90}	118.97	3.47	1.52

As an overall assessment of comparability of the particle size distribution between the different units, the average of the deviation for X_{10}, X_{50} and X_{90} from their respective average values is calculated. Results with an average deviation for X_{10}, X_{50} and X_{90} below 20 % are considered as acceptable.

For the ten replicates, the average deviations are all below 10 %. Consequently the material is homogeneous and uniform over the whole batch with respect to particle size distribution.

3.3.2 Water content by oven drying method

A ventilated oven method at 102 °C provided the best option for measuring the high water content in ERM-CE100, typical of a fresh tissue material. Volatile components are also lost but these are assumed to constitute a very small fraction of the total mass loss. Five units randomly selected over the whole batch were analysed in duplicate. An analytical balance was used to record the mass loss after one and two hours, respectively. Two hours were deemed sufficient to obtain reliable and relatively uniform mass loss data. The sample amounts placed in the drying oven varied between 4 to 12 g of wet material and the recorded mass loss was found to be independent from the sample mass. Based on the variability in the data from the same unit, no trend over the filling sequence could be observed for the water content in this material. An average water content of 74.4 % (m/m) was measured.

4 Homogeneity

A key requirement for any reference material distributed into units is the 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 uncertainties.

The number of units selected corresponds to approximately the cube root of the total number of units produced. Twelve 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 twelve groups (with a similar number of units) and one unit was selected randomly from each group. Two independent samples of 2 g each (wet mass) were taken from each selected unit and in-house analysed by accelerated solvent extraction (ASE), solid-phase extraction (SPE) clean-up and GC-IDMS [7,14]. For each independent replicate, sample preparation and analytical determination were carried out within one day. Due to the high number of samples (twenty-four), the measurements were performed under intermediate precision conditions (spread over four days). Consequently, day-to-day effects could occur that could mask the between-bottle variation. Therefore, it was first checked if there was a significant difference between the day means using ANOVA. Indeed, significant day-to-day effect was present for HCBD content and a correction was applied by normalising i.e., dividing the data points by the respective day mean. 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 or the analytical sequence were observed at a 95 % confidence level.

The 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 result was detected for HCBD. Since no technical reason for the outliers could be found, all 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 of 2 g 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 tested using histograms and normal probability plots. Too few data 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 2.

Table 2: Results of the statistical evaluation of the homogeneity studies

Analyte	Trends (before correction)*		Outliers**		Distribution	
	Analytical sequence	Filling sequence	Individual results	Unit means	Individual results	Unit means
HCB	No	No	None	None	normal/ unimodal	normal/ unimodal
HCBD	No	No	1-statistical reason (retained)	None	normal/ unimodal	normal/ 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.* [15]. 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:

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

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

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

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 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 3. For HCBD, the uncertainty contribution was determined by the method repeatability.

Table 3: Outcome of the homogeneity study

Analyte	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,rel}$ [%]
HCB	2.6	2.0	1.2	2.0
HCBD	3.0	n.c. [1)]	1.3	1.3

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

The homogeneity study showed no outlying unit means or trend in the filling sequence for both analytes. 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 can be used in an analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty.

Study of decreasing sample intakes

To estimate the minimum sample intake, a series of measurements with decreasing amounts of sample on six randomly selected units were performed. The following sample intakes were tested: 0.5, 1 and 2 g (wet mass). For each sample intake, 2 units were measured in triplicate each by ASE, silica column clean-up and GC-MS quantification under repeatability conditions (for the 0.5 and 1 g; the 2 g sample intake measurements were carried out for the scope of the characterisation campaign few days before), and in a randomised manner. The measurement method was robust over the whole range of the sample intake tested and its repeatability was in the same range or better than the repeatability achieved during the material characterisation (Section 6).

The obtained datasets (for the sample intakes 0.5 and 1 g taken together) were first tested whether they follow a normal, or at least unimodal distribution. This was done by visual inspection of normal probability plots and histograms (if the data do not follow at least a unimodal distribution, the calculation of standard deviations is doubtful or impossible). All results were normally and unimodally distributed.

Furthermore, the results (corresponding to the sample intakes 0.5 and 1 g taken together) were scrutinised for outliers using the single Grubbs-test at a 99 % confidence level. No outliers were detected.

The minimum sample intake was established by comparison of the variances obtained for 0.5 and 1 g sample intakes with the variance obtained for 2 g sample intake. It was done using the F-test for equality of two samples for variances with 5 degrees of freedom and a confidence level of 95 %.

The study results are presented in Annex B and the minimum sample intakes are summarised in Table 4.

Table 4: Outcome of the minimum sample intake determination

Analyte	Minimum sample intake (wet mass) [g]
HCB	1
HBCD	0.5

As shown above, the minimum sample intake representative for both analytes is 1 g (wet mass).

5 Stability

Time and temperature were regarded as the most relevant influences on stability of the material. Additionally the material was sterilised by heat treatment (in an autoclave) to eliminate microbial growth. 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 [16]. 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 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to 4 °C. Two units per storage time were selected using a random stratified sampling scheme. From each unit, two samples of 2 g each (wet mass) were in-house measured by ASE, SPE clean-up and GC-IDMS [7,14]. The measurements were performed under intermediate precision conditions (different days) and a randomised sequence was applied to differentiate any potential analytical drift from a trend over storage time. It was first checked if there was a significant difference between the day means using ANOVA (measurements spread over three days). Significant day-to-day effect was present for HCB content and a correction was applied by normalising i.e. dividing the data points by the respective day mean.

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 %. No outliers were found (Table 5).

In addition, the data were evaluated against storage time, and regression lines of mass fraction versus time were calculated, to test for potential increases/decreases of the measurands due to shipping conditions. The slopes of the regression lines were tested for statistical significance. None of the trends was statistically significant at a 95 % confidence level for any of the temperatures for HCB, while a significant trend at a 95 % confidence level was evidenced for HCBD at 60 °C.

The results of the measurements are shown in Annex C. The results of the statistical evaluation of the short-term stability are summarised in Table 5.

Table 5: Results of the statistical evaluation of the short-term stability tests

Analyte	Number of individual outlying results*		Significance of the trend**	
	18 ºC	60 ºC	18 ºC	60 ºC
HCB	None	None	No	No
HCBD	None	None	No	Yes

* 99 % confidence level
** 95 % confidence level

Neither technically unexplained nor statistical outliers were detected for both analytes. A significant trend at 60 °C was found for HCBD, but the material appeared to be stable at 18 °C.

The material shall be shipped under cooled conditions.

5.2 Long-term stability study

For the long-term stability study, data from two isochronous studies have been combined to assess the stability of the CRM.

For the first isochronous study, four units were stored at 4 °C for 0, 4, 8 and 12 months. For the second isochronous study, four units were stored at 4 °C for 0, 8, 16 and 24 months. The reference temperature was set to -20 °C in both studies. Two units per storage time were selected using a random stratified sampling scheme. From each unit within each study, two samples of 2 g each (wet mass) were measured in-house by ASE, SPE clean-up and GC-IDMS [7,14]. The measurements were performed under intermediate precision conditions (different days) in a random sequence to be able to separate any potential analytical drift from a trend over storage time. It was first checked if there was a significant difference between the day means using a t-test at a 95 % confidence level (measurements spread over two days). Significant day-to-day effect was present for HCBD content in the second isochronous study and a correction was applied by normalising i.e. dividing the data points by the respective day mean.

To combine the two studies, the data of the first isochronous study was also normalised by dividing each data point by the mean of the study.

The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. One outlying individual result for HCBD was found. As no technical reason for this outlier could be found all data were retained for statistical analysis. No outlying individual results were found for HCB (Table 6).

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 both analytes at a 95 % confidence level.

The results of the long term stability measurements are shown in Annex D. The results of the statistical evaluation of the long-term stability study are summarised in Table 6.

Table 6: Results of the statistical evaluation of the long-term stability tests (4 ºC)

Analyte	Number of individual outlying results*	Significance of the trend**
HCB	None	No
HCBD	One	No

* 99 % confidence level
** 95 % confidence level

One statistical outlier was observed for HCBD and none for HCB. None of the trends was statistically significant on a 95 % confidence level. The material can therefore be stored at 4 °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 [17] for HCB and HCBD. 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:

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

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

RSD_{sts} relative standard deviation of all results of the short-term stability study

RSD_{lts} relative standard deviation of all results of the long-term stability study

t_i time elapsed at time point *i* of the short-term stability study (for $u_{sts,rel}$) or long-term stability study (for $u_{lts,rel}$)

$\bar{t}$ mean of all t_i of the short-term stability study (for $u_{sts,rel}$) or long-term stability study ($u_{lts,rel}$)

t_{tt} chosen transport time (1 week at 18 ºC)

t_{sl} chosen shelf life (36 months at 4 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 18 °C studies. The uncertainty describes the possible change during a dispatch at 18 °C lasting for one week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the 4 °C studies. The uncertainty contribution describes the possible degradation during 36 months storage at 4 °C.

The results of these evaluations are summarised in Table 7.

Table 7: Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of 18 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of 4 °C and 36 months

Measurand	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
	18 °C	4 °C
HCB	0.4	0.9
HCBD	0.5	2.6

The material showed significant degradation at 60 °C but no significant degradation was observed for transport at or below 18 °C. Cooled shipment is therefore necessary.

No trend was statistically significant on a 95 % confidence level. The material can therefore be stored at 4 °C.

After the certification study, the material will be included in the IRMM's 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.

The material characterisation was based on an interlaboratory comparison of expert laboratories, i.e. the analytes in 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 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 HCB and HCBD measurements in biota matrices by submitting results for intercomparison exercises or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 [3] was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 0).

6.2 Study setup

Each laboratory received two units of ERM-CE100 and was requested to provide six independent results reported relative to wet weight, three per unit. The units for the material characterisation were selected using a random stratified sampling scheme and covered the whole batch. For each unit, 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 day. Results are reported as µg/kg wet weight.

Each participant received a sample of NIST SRM1947 Lake Michigan Fish Tissue, certified for HCB mass fraction, as blind method quality control (M-QC) and an *iso*-octane solution containing both HCB and HCBD as blind calibration quality control (C-QC). The C-QC was gravimetrically prepared in-house targeting concentrations of HCB and HCBD lying approximately in the middle of the envisaged calibration curve. The necessity of having also the C-QC was due to the absence of matrix reference materials certified for HCBD. The results for these samples 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 extraction [ASE, Soxhlet, ultrasonic extraction after freeze drying, liquid liquid extraction (LLE), organic solvent extraction] and clean-up methods [Florisil column, silica gel column, gel permeation chromatography (GPC)] with different quantification techniques (GC-ECD, GC-MS, GC-HRMS, GC-MS/MS) were used to characterise the material. The combination of results from methods based on completely different principles mitigates undetected method bias. Two of the expert laboratories offered two different analytical methods and these were handled as separate data sets.

All methods used during the characterisation study are summarised in Annex E. The laboratory code (e.g. L01) is a random number and does not correspond to the order of laboratories in Section 0. For the laboratories offering two different analytical methods, the letter A and B was added to the laboratory code. The lab-method code consists of a number assigned to each laboratory and abbreviation of the measurement method used (e.g. L01-GC-MS).

6.4 Evaluation of results

The characterisation campaign resulted in thirteen datasets per analyte. All individual results of the participants, grouped per analyte, are displayed in tabular form in Annex F.

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, as well as analytical sequence determination performed on two days.
- absence of values given as below limit of detection or below limit of quantification
- method performance, i.e.

  agreement of the measurement result with the assigned value of the C-QC sample. Values reported were expected to remain within a 15 % offset from the assigned value (median value of measurement results from all laboratories)

  and

  agreement of the measurement result with the certified value of HCB in SRM 1947 (7.44 ± 0.66 µg/kg), according to ERM Application Note 1 [18].

Based on the above criteria, the following datasets were rejected as not technically valid (Table 8).

L04: the results for both HCB and HCBD were rejected because the measurement result of the C-QC sample was deviating more than 15 % from the assigned value; in addition the measurement result for the M-QC sample did not agree with the certified value.

L05: the results for HCB was rejected because the measurement result of the C-QC sample was deviating more than 15 % from the assigned value.

L07: the results for HCBD were rejected because the measurement result of the C-QC sample was deviating more than 15 % from the assigned value.

L09: the results for both HCB and HCBD were rejected because the measurement result of the C-QC sample was deviating more than 15 % from the assigned value.

L10: the results for both HCB and HCBD were rejected because the measurement result of the C-QC sample was deviating more than 15 % from the assigned value; in addition the measurement result for the M-QC sample did not agree with the certified value.

Table 8: Datasets that showed non-compliances with the analysis' protocol and technical specifications, and action taken

Analyte	Lab code	Description of problem	Action taken
HCB	L04, L05, L09, L10	Failure to measure C-QC sample (and M-QC sample for L04 and L10)	not used for evaluation
HCBD	L04, L07, L09, L10	Failure to measure C-QC sample	not used for evaluation

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 (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 9.

Table 9: Statistical evaluation of the technically accepted datasets for ERM-CE100. *p*: number of technically valid datasets

Analyte	*p*	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Mean [µg/kg]	*s* [µg/kg]	$s_{between}$ [µg/kg]	s_{within} [µg/kg]
HCB	9	No	Yes (L03B)	Yes	120.211	8.546	8.249	5.469
HCBD	9	No	No	Yes	36.365	4.378	4.229	2.777

The laboratory means follow normal distributions (at a 99 % confidence level).

The statistical evaluation flags laboratory L03B as outlying variance for HCB. This merely reflects the fact that different methods have different intrinsic variability. As all measurement methods were found technically sound, all results were retained.

The uncertainty related to the characterisation is estimated as the standard error of the mean of laboratory means (Table 10).

Table 10: Uncertainty of characterisation for ERM-CE100

Analyte	*p*	Mean [µg/kg]	*s* [µg/kg]	u_{char} [µg/kg]	$u_{char,rel}$ [%]
HCB	9	120.211	8.546	2.844	2.4
HCBD	9	36.365	4.378	1.459	4.0

7 Value Assignment

Certified values were assigned.

Certified values are values that fulfil the highest standards of accuracy. Procedures at IRMM require generally pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the ISO/IEC Guide 98-3 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 10 was assigned as certified value for each parameter.

The assigned uncertainty consists of uncertainties related to characterisation, u_{char} (Section 0), potential between-unit inhomogeneity, u_{bb} (Section 4.1) and potential degradation during transport (u_{sts}) and long-term storage, u_{lts} (Section 0). The uncertainty related to degradation during transport (u_{sts}) was found to be negligible. These different contributions were combined to estimate the expanded, relative uncertainty of the certified value ($U_{CRM, rel}$) with a coverage factor k as:

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

- u_{char} was estimated as described in Section 6
- u_{bb} was estimated as described in Section 4.1.
- u_{lts} was estimated as described in Section 5.3.

Because of the sufficient number 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 11.

Table 11: Certified values and their uncertainties for ERM-CE100

Analyte	Certified value [1] [µg/kg]	$u_{char, rel}$ [%]	$u_{bb, rel}$ [%]	$u_{lts, rel}$ [%]	$U_{CRM, rel}$ [%]	U_{CRM} [2] [µg/kg]
HCB	120	2.4	2.0	0.9	6.5	8
HCBD	36	4.0	1.3	2.6	9.9	4

[1] The certified values and their uncertainties are expressed as mass fractions relative to wet weight
[2] Expanded (k = 2) and rounded uncertainty

7.2 Additional material information

The data provided in this section should be regarded as informative only on the general composition of the material and cannot be, in any case, used as certified or indicative value.

An additional material information value was assigned for the fat content of ERM-CE100. Three CRM units were randomly selected over the whole batch and for each unit a sample of 1 g was processed in-house according to the following procedure [7,14]: after ASE, the extract was concentrated under nitrogen stream and finally placed in an oven at 105 °C until constant mass was reached. In Table 12, the extractable fat content is expressed as the mean of the three replicates and given in mass fraction % (equivalent to g/100 g) relative to wet weight of ERM-CE100.

Table 12: Fat content of ERM-CA100 (as additional material information)

	Value [% m/m]
Extractable fat	5.9

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

HCB and HCBD are chemically clearly defined analytes. The participants used different methods for the sample preparation as well as for the final detection, demonstrating absence of measurement bias. All participants used GC-based analytical methods, therefore the measurands are operationally defined by GC.

Quantity value

Only validated methods were used for the determination of the assigned values. Different 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 International System of units (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 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 are selecting 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 the 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 C-53A [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.

It should be borne in mind that the methods used in the characterisation of ERM-CE100 are methods routinely applied for measuring HCB and HCBD in biota matrices. The agreement of results from different methods demonstrates that ERM-CE100 behaves like a real sample.

ERM-CE100 was produced from naturally contaminated wild fish specimens by cryogenic milling, mixing and cooking to produce a sterilised paste, enhancing the commutability of the material by avoiding any freeze drying process. The analytical behaviour should match as close as possible a routine biota sample.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

9.2 Storage conditions

The material should be stored at 4 °C ± 3 °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 of opened units.

9.3 Preparation and use of the material

Before analysis, ERM-CE100 units should be left to equilibrate to room temperature.

To make it ready for use and before sub-sampling, the material has to be thoroughly and manually re-homogenised with the help of a spatula. In case that a small quantity of oxidised material is observed attached to the lid, it is advisable not to include it.

9.4 Minimum sample intake

The minimum sample intake representative for both analytes is 1 g (wet mass).

9.5 Use of the certified value

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

Use as a calibrant

It is not recommended to use this matrix material as calibrant.

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 [19].

When assessing the method performance, the measured values of the CRM 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 $\Delta_{meas} \leq U_{\Delta}$ no significant difference exists between the measurement result and the certified value, at a confidence level of about 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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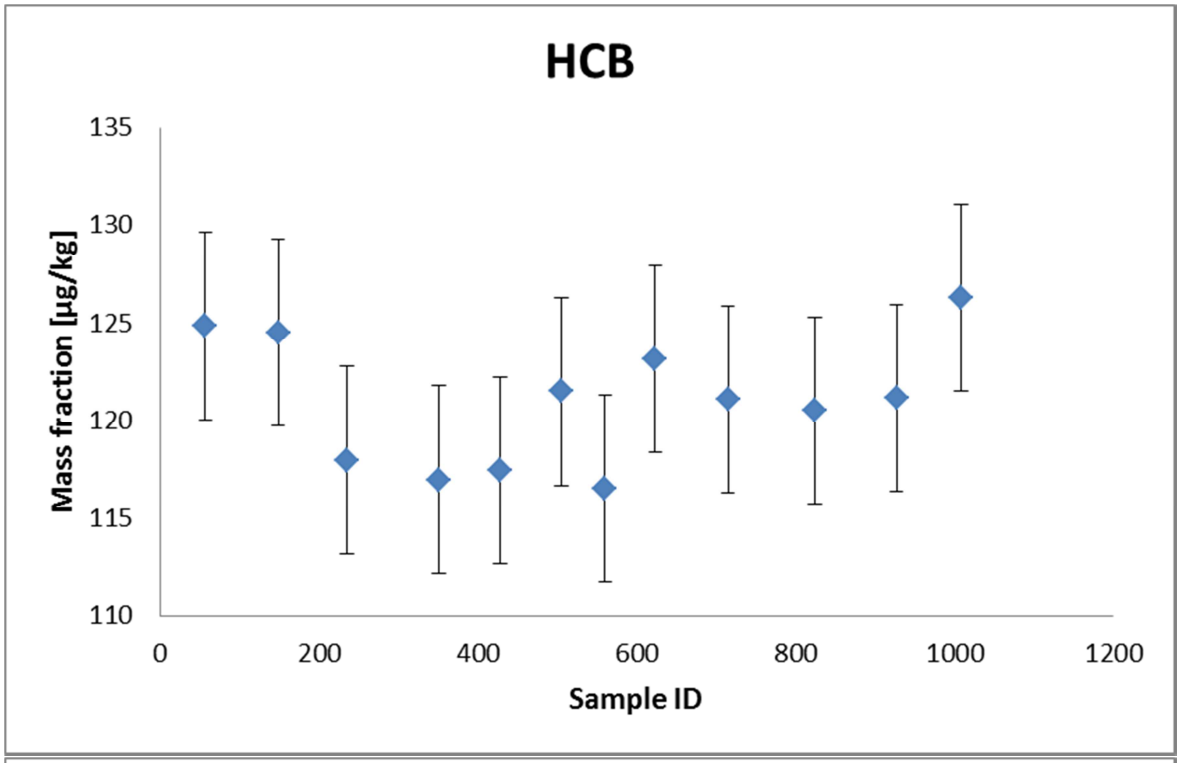
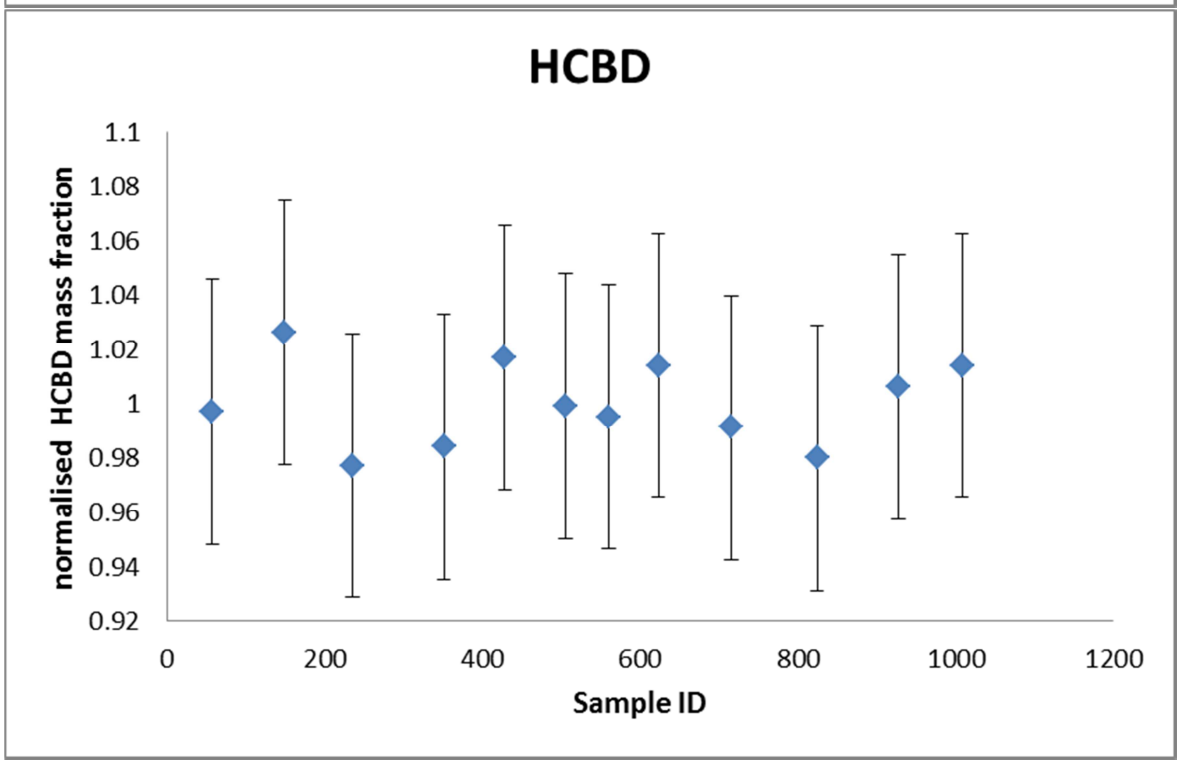
Annexes

Annex A: Results of the homogeneity measurements

- The graphs report mass fraction unit means (normalised for HCBD) ± 95 % confidence interval (CI) of the means.





Annex B: Measurement results of the minimum sample intake study

Hexachlorobenzene (HCB) µg/kg (relative to wet weight)

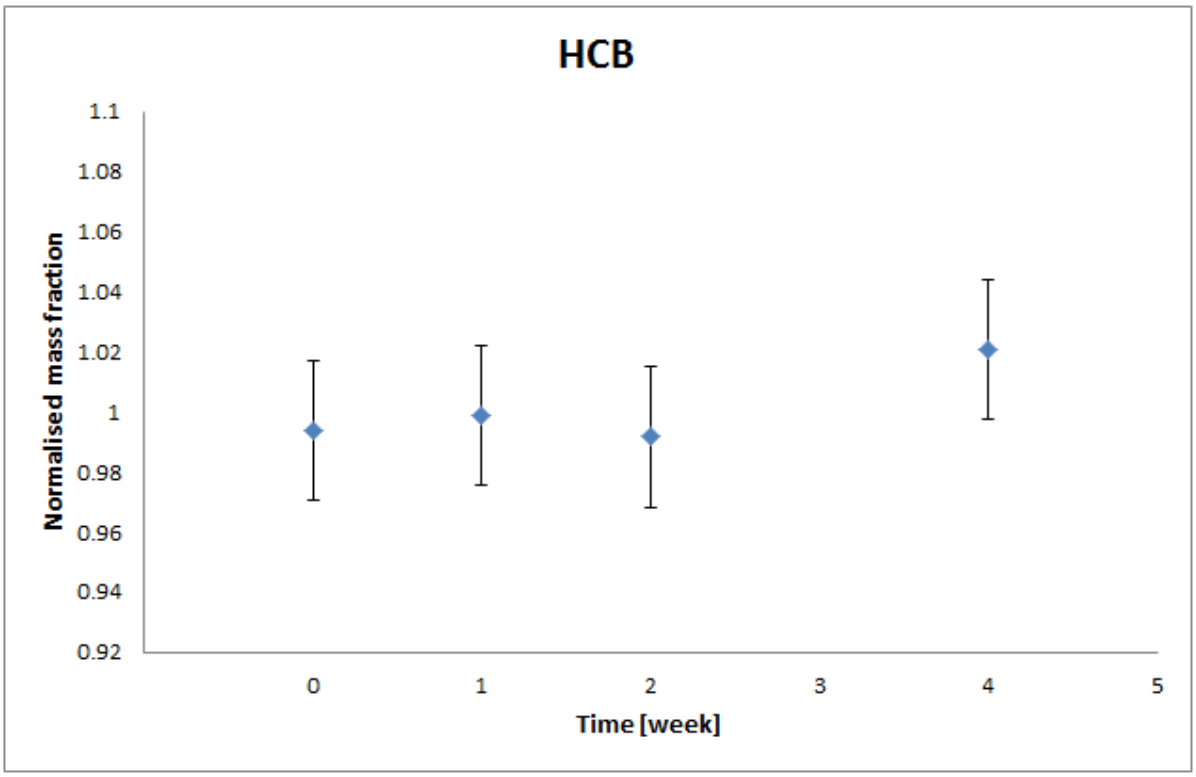
	2 g	**1 g**	**0.5 g**
	Unit 282	*Unit 287*	*Unit 851*
	118.00	114.00	105.00
	117.00	94.00	116.00
	124.00	84.10	98.50
	Unit 959	*Unit 100*	*Unit 667*
	115.00	99.90	82.80
	127.00	104.00	87.00
	124.00	97.00	99.80
RSD	4.0 %	10.1 %	12.3 %

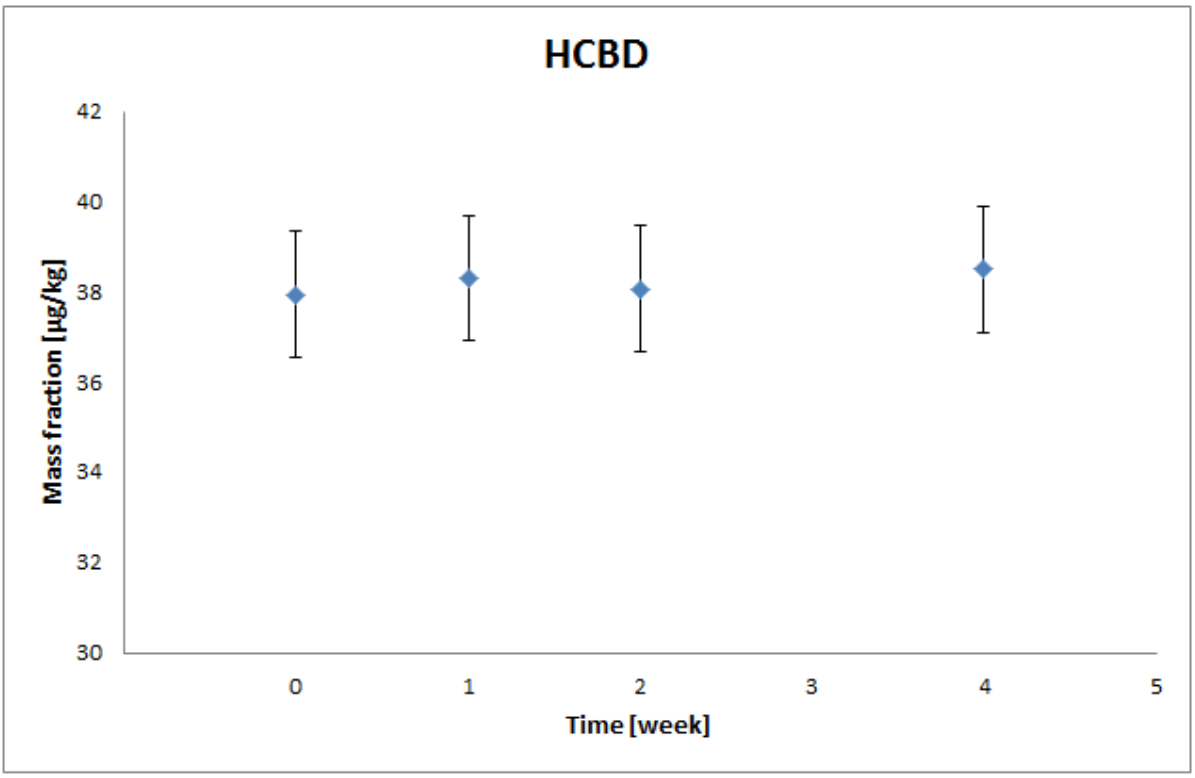
Hexachlorobutadiene (HCBD) µg/kg (relative to wet weight)

	2 g	**1 g**	**0.5 g**
	Unit 282	*Unit 287*	*Unit 851*
	36.90	34.20	35.80
	38.80	33.50	39.40
	40.00	31.50	34.00
	Unit 959	*Unit 100*	*Unit 667*
	37.20	35.10	35.20
	38.10	34.90	33.60
	41.50	35.80	38.80
RSD	4.5 %	4.5 %	6.7 %

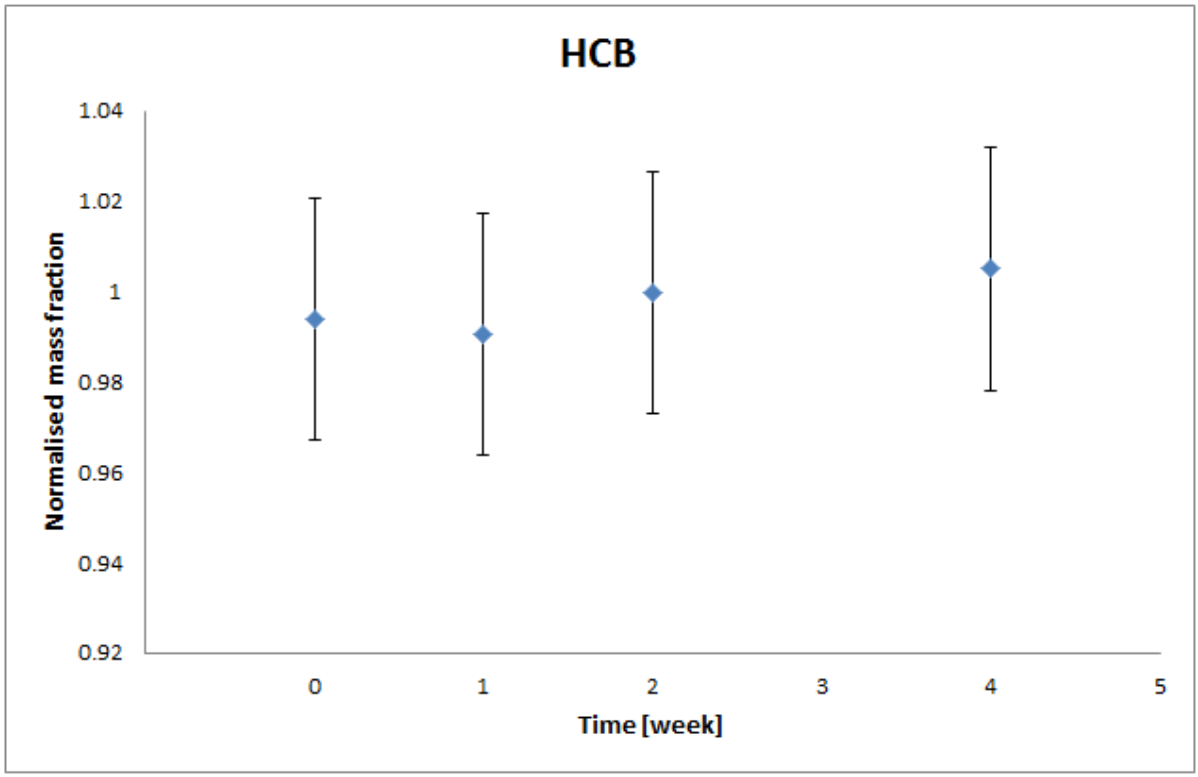
Annex C: Results of the short-term stability measurements

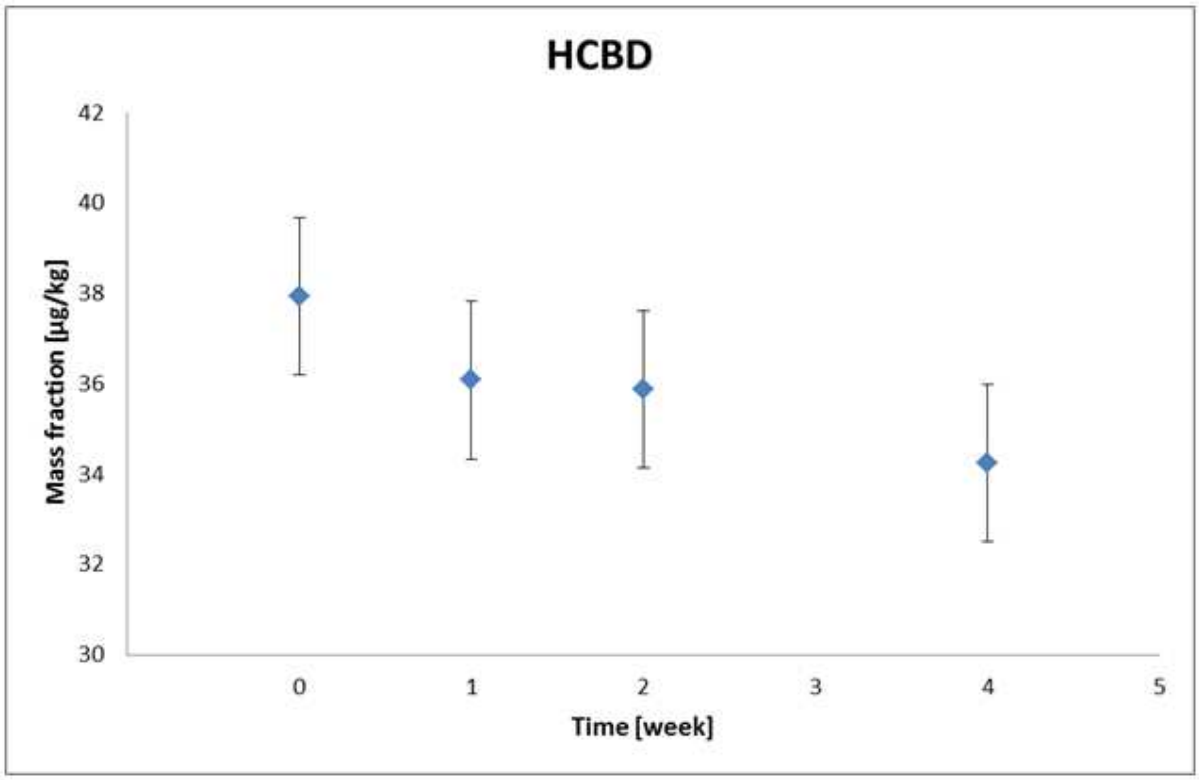
- Data for the short-term stability study at 18 °C. The graphs report mass fraction means per time point (normalised for HCB) ± 95 % CI of the means.





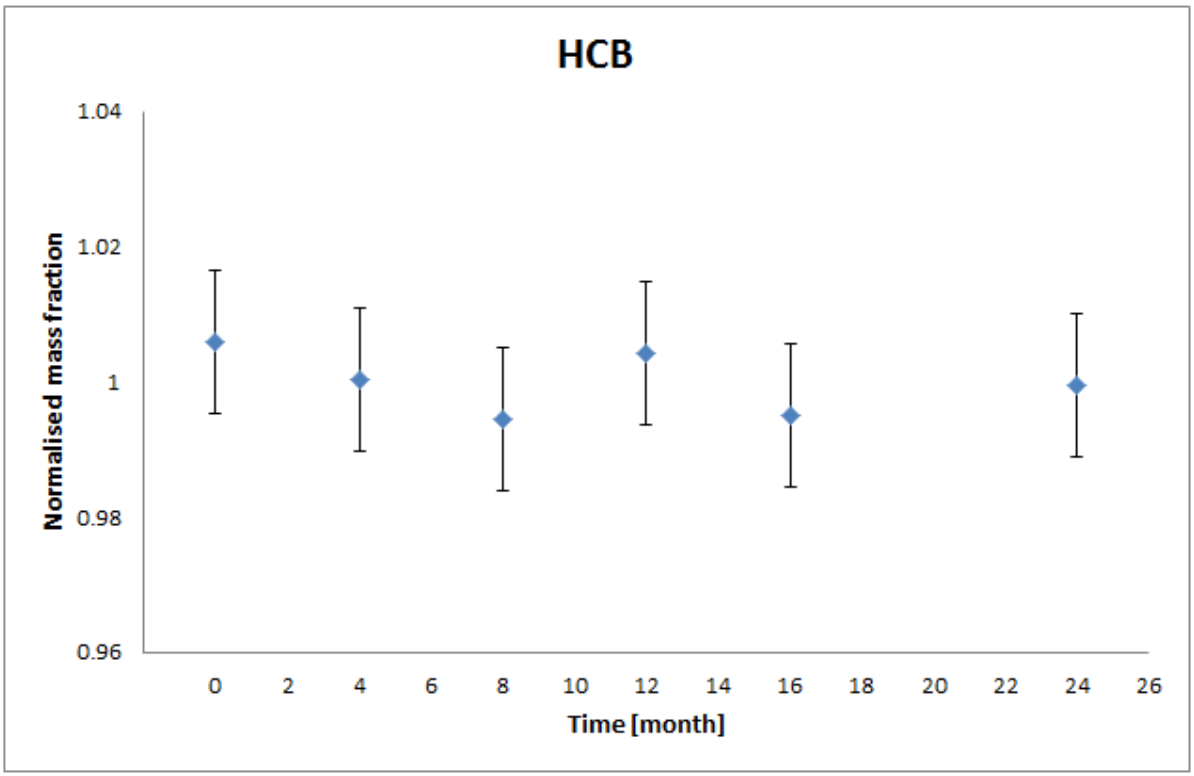
- Data for the short-term stability study at 60 °C. The graphs report mass fraction means per time point (normalised for HCB) ± 95 % CI of the means.

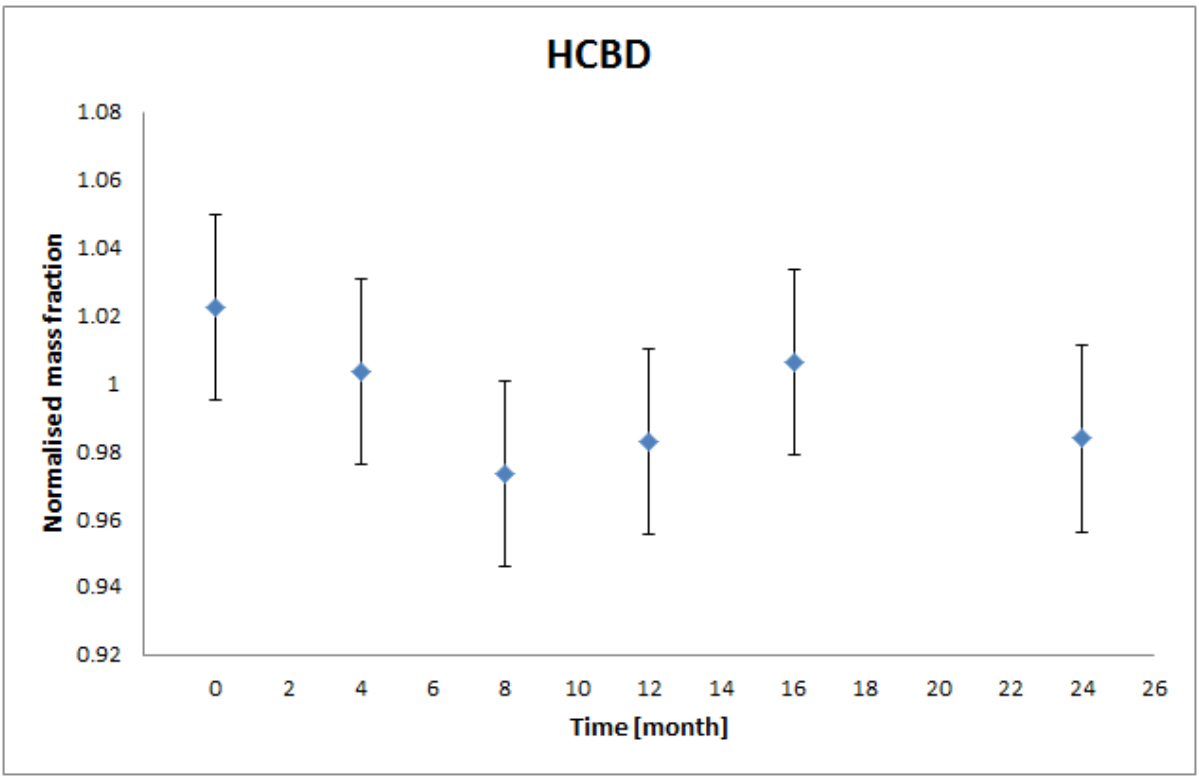




Annex D: Results of the long-term stability measurements

- Data for the long-term stability study at 4 °C. The graphs report normalised mass fraction means per time point ± 95 % CI of the means.





Annex E: Summary of methods used in the characterisation study

- Method information is reported as given by laboratories

Laboratory code–method	Sample pre-treatment	Detection method	Type of calibration Calibrants' details (supplier and purity)	LOQ [µg/kg wet weight]
L00-GC-MS	accelerated solvent extraction (ASE), clean-up by SPE (solid phase extraction) column	GC-IDMS $^{13}C_6$-HCB and $^{13}C_4$-HCBD as internal standards	5 points internal standard calibration HCB (purity 99.5 %) and HCBD (purity 99 %) by Dr. Ehrenstorfer.	HCB: 3.7 HCBD:15.7
L01A-GC-ECD	Soxhlet extraction, clean-up with alumina and silica columns	GC-ECD PCB 112 as internal standard	Quadratic calibration curve Pesticide standard in *iso*-octane by Accustandard, Inc. Purity: HCBD 98.1 %, HCB: 100 %	HCB: 1.35 HCBD:1.35
L01B-GC-MS	ASE (inline fat removal with Florisil), clean-up with alumina and silica columns	GC-MS PCB 112 as internal standard	Quadratic calibration curve Pesticide standard in *iso*-octane by Accustandard, Inc. Purity: HCBD 98.1 %, HCB: 100 %	HCB: 1.6 HCBD: 1.6
L02-GC-HRMS	ASE, clean-up with 1) silica and alumina column, 2) C_{18}-modified silica column	GC-ID-HRMS $^{13}C_6$-HCB and $^{13}C_4$-HCBD as internal standards	Single point HCB solution by Wellington Laboratories, > 98 % HCBD solution by Dr. Ehrenstorfer, 99 %.	HCB: 0.02 HCBD:0.1
L03A-GC-ECD	ASE, clean-up with alumina	GC-ECD PCB 103 as internal standard (used for recovery)	8 points external standard calibration curve Pesticide standard in *iso*-octane by Accustandard, Inc. Purity: HCBD 98.1 %, HCB: 100 %	HCB: 0.7 HCBD:0.7
L03B-GC-MS	ASE, clean-up with alumina	GC-MS $^{13}C_6$-HCB and $^{13}C_4$-HCBD as internal standards (used for recovery)	8 points external standard calibration curve Pesticide standard in *iso*-octane by Accustandard, Inc. Purity: HCBD 98.1 %, HCB: 100 %	HCB: 0.3 (LOD) HCBD:0.3 (LOD)
L05-GC-MS/MS	Extraction with ethylacetate and centrifugation, clean-up by gel permeation chromatography (GPC)	GC-MS/MS PCB 137 as internal standard	6 points calibration curve HCB by Dr. Ehrenstorfer, purity 99.5 % HCBD by Sigma-Aldrich, purity 97.7 %	HCB: 7 HCBD: 7

L06-GC-MS	ASE, GPC clean-up	GC-IDMS $^{13}C_6$-HCB and $^{13}C_4$-HCBD as internal standards	6-7 points internal standard calibration HCB (purity 99.5 %) and HCBD (purity 99.0 %) by Dr. Ehrenstorfer	HCB: 1.4 HCBD: 3.7
L07-GC-HRMS	Acid digestion, liquid liquid extraction, clean-up with Florisil	GC-ID-HRMS $^{13}C_6$-HCB and $^{13}C_4$-HCBD as internal standards	7 points internal standard calibration HCB and HCBD from Cambridge Isotope Laboratories, ≥ 98 %	HCB: 0.004 HCBD: 0.015
L08-GC-MS/MS	Extraction with organic solvent, clean-up with Kieselguhr/H_2SO_4	GC- MS/MS $^{13}C_6$-HCB and $^{13}C_4$-HCBD as internal standards	5 points external standard calibration curve HCB and HCBD solutions from Neochema, 99 %	HCB: 1 HCBD: 1
Not used in certification				
L04-GC-MS	Freeze-drying of the sample; ultrasonic extraction and clean-up with Florisil SPE	GC-MS $^{13}C_6$-HCB as internal standard for both analytes	8 points internal standard calibration HCB from Supelco, 99.6 % HCBD solution from Supelco, 99 %	HCB: 0.01 HCBD: 0.02
L09-GC-ECD	Soxhlet, clean-up with Florisil column	GC-ECD no internal standard	5 points external calibration curve Mix organochlorine pesticides solution from ULTRA SCIENTIFIC (purity not specified)	HCB: 0.02 HCBD: 0.12
L10-GC-MS	Pressurised liquid extraction, clean-up with Florisil column	GC-MS $^{13}C_6$-HCB as internal standard for both analytes	7 points internal standard calibration HCB from Fluka/ Riedel-de-Haën, > 99.6 % HCBD solution from Supelco (purity not specified)	HCB: 0.4 HCBD: 1.5

N.B. the purity values refer to the neat substances, unless otherwise specified

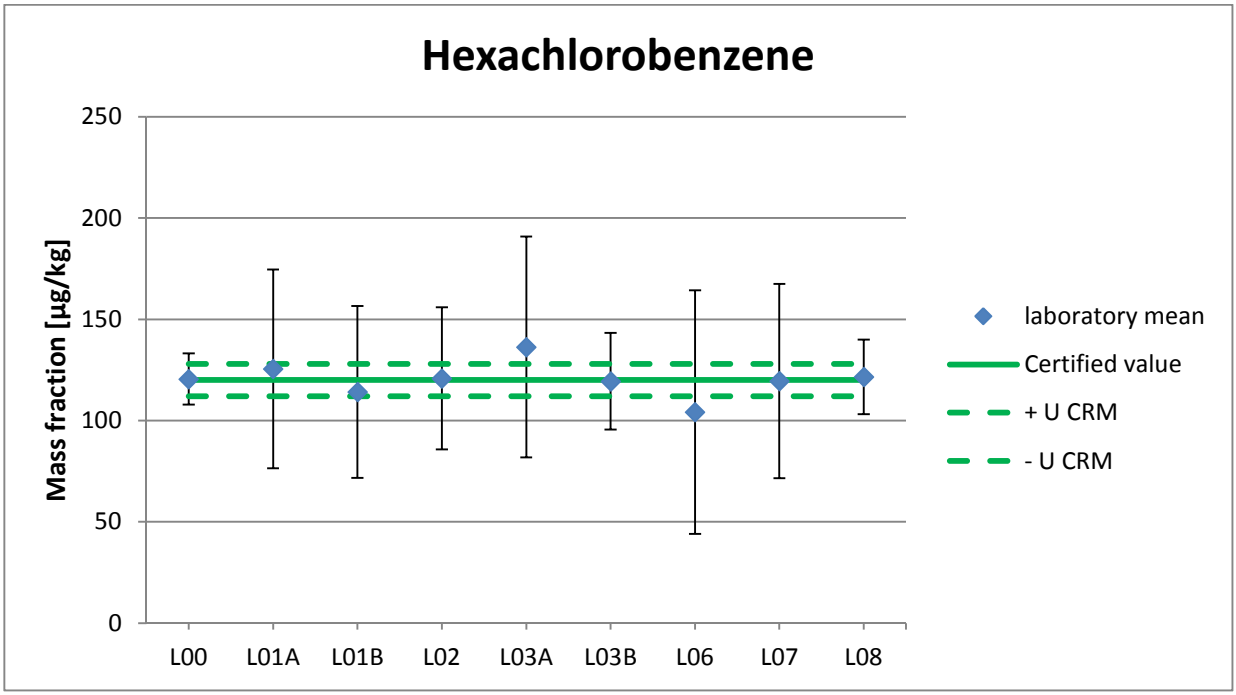
Annex F: Results of the characterisation measurements

Note: *values as reported by the laboratory and expressed relative to wet weight*

Hexachlorobenzene

Laboratory code - Method	replicate 1 [µg/kg]	replicate 2 [µg/kg]	replicate 3 [µg/kg]	replicate 4 [µg/kg]	replicate 5 [µg/kg]	replicate 6 [µg/kg]	mean [µg/kg]	Expanded uncertainty [µg/kg]
L00-GC-MS	120.84	122.10	121.02	118.73	120.04	120.17	120.48	12.65
L01A-GC-ECD	131.82	124.66	118.65	120.03	128.29	129.88	125.56	49.09
L01B-GC-MS	119.33	118.54	113.28	115.33	108.41	109.98	114.15	42.46
L02-GC-HRMS	118.00	117.00	124.00	115.00	127.00	124.00	121.00	35.00
L03A-GC-ECD	130.09	139.82	140.46	130.50	137.69	139.07	136.27	54.51
L03B-GC-MS	116.73	143.40	113.43	112.07	112.53	118.15	119.39	23.88
L06-GC-MS	105	99	104	109	106	102	104	5
L07-GC-HRMS	120.70	124.60	119.80	117.18	118.09	116.48	119.48	18.40
L08-GC-MS	119.73	121.27	125.14	117.38	125.00	121.00	121.59	21.89
Results not used for certification								
L04-GC-MS	*77.32*	*85.79*	*81.87*	*88.89*	*87.90*	*90.72*	*85.42*	*6.58**
L05-GC-MS/MS	*154.00*	*142.50*	*152.30*	*141.90*	*153.60*	*157.70*	*150.33*	*60.13*
L09-GC-ECD	*95.58*	*97.86*	*88.03*	*93.70*	*83.06*	*86.19*	*90.74*	*26.32*
L10- GC-MS	*90.46*	*82.68*	*90.65*	*79.01*	*86.16*	*78.58*	*84.59*	*23.69*

* inter-day variability



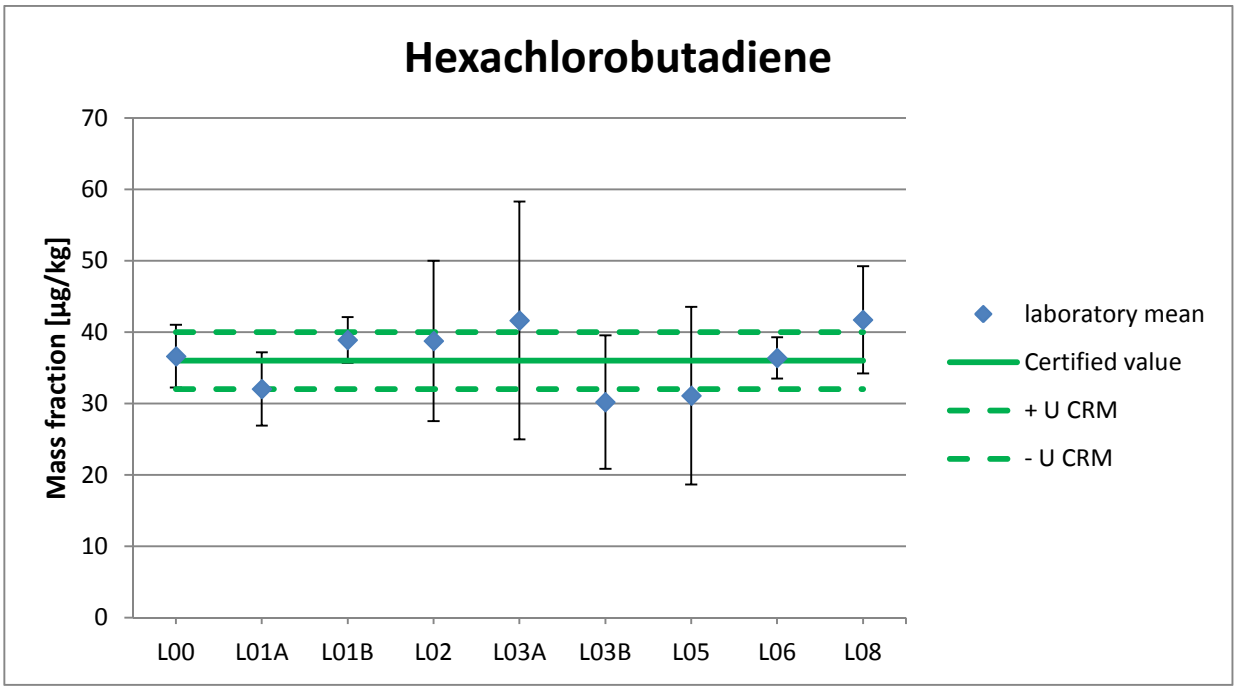
N.B. error bars represent expanded uncertainties

Hexachlorobutadiene

Laboratory code - Method	replicate 1 [µg/kg]	replicate 2 [µg/kg]	replicate 3 [µg/kg]	replicate 4 [µg/kg]	replicate 5 [µg/kg]	replicate 6 [µg/kg]	mean [µg/kg]	Expanded uncertainty [µg/kg]
L00-GC-MS	34.60	34.88	35.80	35.15	34.42	35.83	36.61	4.39
L01A-GC-ECD	31.82	29.94	32.36	32.06	33.57	32.40	32.03	4.16**
L01B- GC-MS	36.24	39.55	39.30	32.58	43.25	42.48	38.90	3.19**
L02-GC-HRMS	36.90	38.80	40.00	37.20	38.10	41.50	38.75	11.24
L03A-GC- ECD	39.68	39.75	43.38	41.42	42.65	42.89	41.63	16.65
L03B-GC-MS	33.97	33.40	34.83	27.05	23.12	28.77	30.19	9.36
L05-GC-MS/MS	32.60	31.90	32.40	31.10	25.10	33.40	31.08	12.43
L06-GC-MS	37.3	35.8	36.5	37.5	36.3	34.9	36.4	2.9
L08-GC-MS	41.06	41.75	43.79	40.61	42.65	40.41	41.71	7.51
Results not used for certification								
L04-GC-MS	*5.47*	*6.17*	*5.73*	*5.05*	*5.17*	*4.76*	*5.39*	*0.43**
L07-GC-HRMS	*82.75*	*83.37*	*84.92*	*86.30*	*86.06*	*87.09*	*85.08*	*not given*
L09-GC-ECD	*23.47*	*25.58*	*25.43*	*21.85*	*28.46*	*27.59*	*25.40*	*8.13*
L10- GC-MS	*5.38*	*5.32*	*5.48*	*6.77*	*5.88*	*6.85*	*5.95*	*1.67*

* inter-day variability

** within-laboratory reproducibility



N.B. error bars represent expanded uncertainties

European Commission

EUR 27965 EN – Joint Research Centre – Institute for Reference Materials and Measurements

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