



CERTIFICATION REPORT

**The certification of Deuterium-to-Hydrogen (D/H)
amount-of-substance ratio in tetramethylurea (TMU):
ERM®-AE003**



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Abstract

This report describes the production of ERM®-AE003, which is a tetramethylurea (TMU) reference material certified for its deuterium-to-hydrogen (D/H) amount-of-substance ratio. This material was produced and certified in accordance with ISO 17034:2016 and ISO Guide 35:2017. ERM-AE003 was produced within the scope of ISO 17034 accreditation. TMU with a sufficiently high D/H amount-of-substance ratio ($>120 \times 10^{-6}$) was purchased, checked for sufficient chemical purity ($>99.0\%$) and filled into amber glass bottles of 100 mL under argon atmosphere. Each bottle was individually placed in a multilayer pouch. Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2017. The minimum sample size for one measurement using Nuclear Magnetic Resonance (NMR) tubes of 10 mm diameter is 1.3 mL (according to the official SNIF-NMR® method OIV-MA-AS311-05). The certified value was calculated as the arithmetic mean of 18 individual results obtained by three laboratories of demonstrated competence and adhering to ISO/IEC 17025:2017. The uncertainty of the certified value was calculated in accordance with ISO 17034:2016 and ISO Guide 35:2017 and includes uncertainties related to the purity of the starting material, possible inhomogeneity, instability and characterisation.

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The material is intended for the use as internal standard in site-specific natural isotope fractionation – nuclear magnetic resonance (SNIF-NMR) spectroscopy measurements for determining the D/H amount-of-substance ratio of distilled ethanol (method OIV-MA-AS311-05, International Organisation of Vine and Wine), an important measure in wine authenticity testing. Its use as internal standard is mentioned in the SNIF-NMR methods of other food commodities (vanillin, vinegar) and for ethanol of spirit drinks and distilled from fermented fruit juices and syrups. Before release of ERM-AE003, the certification project was subjected to peer-review involving both internal and external experts.



CERTIFICATION REPORT

The certification of Deuterium-to-Hydrogen (D/H) amount-of-substance ratio in tetramethylurea (TMU): ERM®-AE003

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Summary

This report describes the production of ERM®-AE003, which is a tetramethylurea (TMU) reference material certified for its deuterium-to-hydrogen (D/H) amount-of-substance ratio. This material was produced and certified in accordance with ISO 17034:2016 [1] and ISO Guide 35:2017 [2]. ERM-AE003 was produced within the scope of ISO 17034 accreditation.

TMU with a sufficiently high D/H amount-of-substance ratio ($>120 \times 10^{-6}$) was purchased, checked for sufficient chemical purity ($> 99.0 \%$) and filled into amber glass bottles of 100 mL under argon atmosphere. Each bottle was individually placed in a multilayer pouch.

Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2017 [2]. The minimum sample size for one measurement using Nuclear Magnetic Resonance (NMR) tubes of 10 mm diameter is 1.3 mL (according to the official SNIF-NMR® method OIV-MA-AS311-05 [3]).

The certified value was calculated as the arithmetic mean of 18 individual results obtained by three laboratories of demonstrated competence and adhering to ISO/IEC 17025:2017 [4].

The uncertainty of the certified value was calculated in accordance with ISO 17034:2016 [1] and ISO Guide 35:2017 [2] and includes uncertainties related to the purity of the starting material, possible inhomogeneity, instability and characterisation.

The material is intended for the use as internal standard in site-specific natural isotope fractionation – nuclear magnetic resonance (SNIF-NMR) spectroscopy measurements for determining the D/H amount-of-substance ratio of distilled ethanol (method OIV-MA-AS311-05, International Organisation of Vine and Wine [3]), an important measure in wine authenticity testing. Its use as internal standard is mentioned in the SNIF-NMR methods of other food commodities (vanillin, vinegar) and for ethanol of spirit drinks and distilled from fermented fruit juices and syrups.

Before release of ERM-AE003, the certification project was subjected to peer-review involving both internal and external experts.

The following value was assigned:

	Amount-of-substance ratio	
	Certified value ²⁾ [mol/mol]	Uncertainty ³⁾ [mol/mol]
Deuterium-to-hydrogen (D/H) ratio ¹⁾	123.0×10^{-6}	0.7×10^{-6}
¹⁾ As obtained by strictly adhering to the method OIV-MA-AS311-05 (SNIF-NMR, site-specific natural isotope fractionation – nuclear magnetic resonance spectroscopy).		
²⁾ The value corresponds to the arithmetic mean of 18 individual results, computed after comparison to the certified value of IRMM-425. Measurements were performed under repeatability conditions in three different laboratories employing SNIF-NMR. The value is traceable to the value of VSMOW (Vienna Standard Mean Ocean Water) via the master batch IRMM-425 and by applying the SNIF-NMR method OIV-MA-AS311-05. For VSMOW, a value of 155.76×10^{-6} was used [R. Hagemann, G. Nief, E. Roth, Tellus XXII (1970) 712-715].		
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 17034:2016 and ISO Guide 35:2017.		

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Glossary

$^1\text{H-NMR}$	Proton Nuclear Magnetic Resonance
ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
BCR [®]	Trademark owned by the European Commission; used by the JRC for reference materials
CI	Confidence interval
C-KFT	Coulometric Karl Fischer titration
CRM	Certified reference material
D/H	Deuterium (^2H) to hydrogen (^1H , protium) ratio
EC	European Commission
EI	Electron ionisation
ERC-CWS	European Reference Centre for Control in the Wine Sector
ERM [®]	Trademark owned by the European Commission; used by the JRC for reference materials
EU	European Union
GC-FID	Gas chromatography-flame ionisation detection
GC-MS	Gas chromatography-mass spectrometry
GUM	Guide to the Expression of Uncertainty in Measurement
IEC	International Electrotechnical Commission
IRMS	Isotope Ratio Mass Spectrometry
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
KFT	Karl Fischer titration
m/m	% mass/mass
MB	Master batch of tetramethylurea
MS	Mass spectrometry
MS_{between}	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
n	Number of replicate analysis per unit
N	Number of independent samples analysed
n.a.	Not applicable
OIV	Organisation Internationale de la vigne et du vin (International Organisation of Vine and Wine)
p	Number of technically valid datasets

ppm	Parts per million
QA	Quality assurance
QC	Quality control
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
RSD	Relative standard deviation
RSE	Relative standard error ($=RSD/\sqrt{n}$)
s	Standard deviation
SB	Secondary batch of tetramethylurea
s_{bb}	Between-unit standard deviation; an additional index "rel" is added when appropriate; this parameter is linked to the homogeneity of the material
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
SI	International System of Units
SNIF-NMR	Site-specific natural isotope fractionation-nuclear magnetic resonance
s_{meas}	Standard deviation of measurement data; an additional index "rel" is added as appropriate
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation; this parameter is linked to the homogeneity of the material
T	Temperature
t	Time
t_i	Time point for each replicate
TMU	Tetramethylurea, 1,1,3,3-tetramethylurea, N,N,N',N'-tetramethylurea, tetramethylcarbamide, N,N-tetramethylurea
t_{sl}	Proposed shelf life
t_{tt}	Proposed transport time
u	standard uncertainty
U	expanded uncertainty
u_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{lts}	Standard uncertainty of the long-term stability; 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
v/v	% volume/volume
VIM	International Vocabulary of Metrology – Basic and General Concepts and Associated Terms
VSMOW	Vienna Standard Mean Ocean Water
\bar{X}	Arithmetic mean
α	significance level
ν_{MSwithin}	Degrees of freedom of MS_{within}

1 Introduction

1.1 Background

Food fraud in Europe is estimated to have a financial impact of several millions euro and one of the agricultural products most affected by counterfeiting is wine [5]. The main types of wine adulteration are addition of water, alcohol or non-authorized addition of sugars (i.e. not coming from grape), but also undeclared mixing of wines from different vintages, geographic areas or countries. To check if a wine is authentic, isotopic analysis is carried out on its distilled ethanol and water.

The SNIF-NMR (site-specific natural isotope fractionation measured by nuclear magnetic resonance) method OIV-MA-AS311-05 is one of the official methods adopted by the International Organisation of Vine and Wine (OIV) [3] and the European Union for wine analysis [6].

Briefly, the deuterium from sugar and water molecules is transferred differently during alcoholic fermentation into the methyl and the methylene positions of the ethanol molecule. Approximately, 85 % of deuterium in the sugar molecule is transferred into the methyl group of ethanol and 75 % of deuterium of the grape water is transferred into the methylene group. Thus the resulting isotopic amount-of-substance ratio of deuterium to hydrogen (D/H) of the methyl group, which is defined as (D/H)_i, represents the botanical origin of the fermented sugar whereas that of the methylene group (D/H)_{ii} is typical for the deuterium content of the grape water and reflects the climatic conditions related to the geographical origin and the year of vintage [7]. This type of measurements can be used (and it was originally developed) for distinguishing alcohols fermented from different sugar sources, allowing detecting illegal practices such as unauthorized chaptalisation of wines.

The isotopic ratios measured on wine samples are then compared to reference data, being the isotopic ratios of the respective authentic micro-vinified wines, contained in the European Union (EU) Wine Databank. This database is managed by the European Reference Centre for Control in the Wine Sector (ERC-CWS), hosted by the Joint Research Centre (JRC) of the European Commission (EC). The EU Wine Databank (which online version was officially released in July 2016) is continuously updated with data of authentic wines provided by the wine-growing countries of the EU.

1.2 Choice of the material

The official method OIV-MA-AS311-05, *Determination of the deuterium distribution in ethanol derived from fermentation of grape musts, concentrated grape musts, grape sugar (rectified concentrated grape musts) and wines by application of nuclear magnetic resonance (NMR)*, specifies that tetramethylurea (TMU), with a known D/H amount-of-substance ratio, available as certified reference material (CRM) from the EC-JRC, must be used as internal standard in the SNIF-NMR measurements of the alcohol distillate.

ERM-AE003 is the "standard TMU with a calibrated isotope ratio D/H" to be used as stipulated in sub-clause 5.2.2 of clause 5. *Reagents and materials* of the OIV-MA-AS311-05 method. Its use as internal standard is additionally mentioned in the SNIF-NMR methods OIV-MA-BS-23 (*Determination of the distribution of deuterium in ethanol of spirit drinks of vitivinicultural origin by application of nuclear magnetic resonance of deuterium*) and OENO 71/2000 (*Determination of the distribution of deuterium in acetic acid extracted from wine vinegar using nuclear magnetic resonance (NMR)*) [3]. The application of certified reference standard TMU available from the JRC is further stated in the following SNIF-NMR methods: AOAC Official Method 995.17 (*Beet sugar in fruit juices*), AOAC Official Method 2000.19 (*Beet or cane sugar in maple syrup*) and AOAC Official Method 2006.05 (*Site-Specific Deuterium/Hydrogen (D/H) Ratios in Vanillin*) [8].

TMU (also referred as standard N,N-tetramethyl urea in OIV-MA-AS311-05, molar mass 116.16 g/mol, Figure 1) possesses appropriate physico-chemical properties, such as a low volatility and high miscibility with ethanol. Furthermore, it exhibits a convenient NMR chemical shift to avoid interference with ethanol peaks and it yields a signal width similar to that of the ethanol methyl signal.

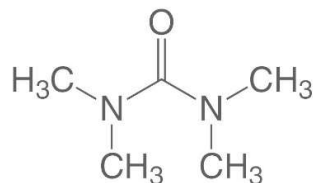


Figure 1: tetramethylurea, C₅H₁₂N₂O (CAS Registry Number 632-22-4)

A commercially available TMU with a sufficiently high D/H amount-of-substance ratio ($>120 \times 10^{-6}$) and fulfilling defined specifications was selected as raw material for the production of ERM-AE003. The specifications were as follows:

- Purity > 99 %;
- Water content < 0.15 % (1.5 g/kg);
- Appearance: clear colourless liquid.

1.3 Outline of the CRM project

The production of a CRM as defined in ISO 17034 [1] is a project comprising planning, processing of the material, homogeneity and stability testing, characterisation, assignment of property values, distribution and post-certification monitoring to control stability.

The first TMU reference material was certified by measuring mixtures of TMU with VSMOW (Vienna - Standard Mean Ocean Water [9, 10]) by SNIF-NMR [11]. The subsequent TMU batches, however, were certified using reference ethanols, which themselves were calibrated against the most recent TMU batch. This approach was not ideal because the newly assigned certified value was linked to the certified value of the previous batch, resulting in a constant increase of the uncertainty of the certified value and in an elongated metrological traceability chain from batch to batch.

For this reason, the certification strategy was revised and in 2008 a TMU master batch (MB) was produced (IRMM-425), to which all future secondary batches (SB) shall be linked. At this point in time, Isotope Ratio Mass Spectrometry (IRMS) was evaluated as an alternative methodology for value assignment, but was found not suitable due to a systematic bias between IRMS and NMR results [12] and the certification was carried out by means of an international laboratory intercomparison based on SNIF-NMR measurements [13].

The first TMU secondary batch with a certified value linked to the TMU master batch IRMM-425 was STA-003k (consisting of the very same TMU material as the master batch). Value assignment for this and future secondary batches is accomplished by comparing the mean value of a number of samples of the MB, tested together with SB samples under intra-laboratory repeatability conditions, with the certified value of the MB.

The second SB was produced and certified in 2013 as STA-003m. ERM-AE003 is the third TMU SB. The change of the CRM code to ERM was made for harmonisation purposes to the codes employed by the JRC for the production of its CRMs.

The format of ERM-AE003 consists of an amber glass bottle of 100 mL and differs to the previous SB, which were produced in units of 500 mL. This size change was implemented to prevent stability problems of TMU bottles opened for too long and to insert flexibility in the use of this CRM by the

laboratories. TMU is hygroscopic and not all laboratories have a rapid consumption of TMU, leading to an increased risk of moisture uptake for the CRM provided in larger volumes.

The isotopic amount-of-substance ratio of deuterium to hydrogen (D/H) certified value and its corresponding uncertainty are expressed with the measurement unit of mol/mol. However, SNIF-NMR results are commonly expressed in parts per million (ppm).

The uncertainty of certified value was estimated in compliance with ISO 17034 [1], which implements the basic principles of ISO/IEC Guide 98 (GUM) [14].

The CRM project, including the certification approach and the evaluation of the obtained measurement data, was subjected to peer-review involving both internal and external experts.

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Unit F.6 (Reference Materials Unit), Geel, BE
(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

2.2 Processing

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Unit F.6 (Reference Materials Unit), Geel, BE
(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

Sigma-Aldrich Production GmbH, Buchs, CH
(accredited to ISO 17034:2016 for production of certified reference materials, SRMS 0001)

2.3 Homogeneity study

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(measurements under the scope of ISO/IEC 17025:2017 accreditation, BELAC No. 268-TEST)

2.4 Stability study

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(measurements under the scope of ISO/IEC 17025:2017 accreditation, BELAC No. 268-TEST)

2.5 Characterisation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(measurements under the scope of ISO/IEC 17025:2017 accreditation, BELAC No. 268-TEST)

Fondazione Edmund Mach - Istituto Agrario di San Michele all'Adige, Dipartimento Qualità Alimentare e Nutrizione, Piattaforma Isotopi Stabili e Tracciabilità, San Michele all'Adige (TN), IT
(measurements under the scope of ISO/IEC 17025:2017 accreditation, ACCREDIA 0193L)

Service Commun des Laboratoires (SCL) Laboratoire de Bordeaux, Pessac, FR
(measurements under the scope of ISO/IEC 17025:2017 accreditation, COFRAC N° 1-0152)

All laboratories are identified by a code (e.g. L01). The numbering is not in the alphabetical order presented above.

3 Material processing and process control

3.1 Origin and purity of the starting material

A batch of about 380 L of TMU with a sufficiently high D/H amount-of-substance ratio (> 120 ppm) was acquired from HONGRUI FINE CHEMICAL CO by Sigma-Aldrich Production GmbH.

After the TMU synthesis step, the excess of water was removed by azeotropic distillation (to comply with the technical specifications of water content < 0.15 %).

The material identity confirmation and purity investigations were carried out at Sigma-Aldrich Production GmbH in Buchs (CH) applying different analytical methods. The identity of ERM-AE003 was confirmed against a sample of STA-003m by GC-FID (identical retention time), ^1H -NMR and ^{13}C -NMR (identical chemical shifts; ^1H -NMR: s, 2.76 ppm, 12H, CH_3 ; ^{13}C -NMR: 165.8 ppm, CO and 38.7 ppm, CH_3) and by GC-MS [same ion fragment signal pattern in the electron ionisation (EI) spectrum, main signals at m/z 116, 72 and 56]. Additionally, the identity was confirmed comparing the EI mass spectrum with the TMU record in the NIST Chemistry WebBook database [15].

Purity assessment was accomplished employing GC-FID, GC-MS (EI), ^1H -NMR and ^{13}C -NMR. The GC-FID purity of ERM-AE003 was calculated as 99.87 % (relative chromatographic area) against 99.86 % of STA-003m. The chromatogram of ERM-AE003 showed the presence of two unidentified impurities vs the presence of six unidentified impurities for STA-003m (each of the impurities with a relative chromatographic area < 0.10 %). The ^1H -NMR and the ^{13}C -NMR spectra of ERM-AE003 (as well as of STA-003m) showed no detectable impurities, therefore supporting the evidence that no residual organic solvents were present. The water content was quantified in-house by a validated coulometric Karl Fischer titration (C-KFT) method yielding the value of 0.38 ± 0.02 mg/g (mean \pm expanded uncertainty, $k=2$; $N = 4$).

Taking into account these data, it was concluded that no major impurities remained undetected. Considering that ERM-AE003 is not certified for its chemical purity, a more comprehensive assessment was not needed and the purity value of ERM-AE003 was estimated as 99.83 % by applying a mass balance method via relative chromatographic response according to the formula below:

$$w_{\text{TMU}} = (1000 - \text{imp}_{\text{Grel}}) \times [1000 - (w_{\text{W}}/1000)] \text{ mg/g} \quad \text{Equation 1}$$

w_{TMU} = mass fraction of TMU

imp_{Grel} = ratio of total peak area of GC-FID structure-related impurities to combined peak area of TMU and structure-related impurities per mille

w_{W} = mass fraction of water content

Note: using the GC-FID relative chromatographic areas assumes equivalent relative response factors on mass fraction basis for the structure-related impurities and TMU

The uncertainty of the material purity (u_{pur}), was estimated as 0.08 %, by combining the standard uncertainty of the C-KFT measurements with the standard uncertainty of the GC-FID measurements (expressed as rectangular distribution around the total impurity value of 0.13 %).

3.2 Processing

The procurement of the TMU starting material was divided in two phases and entirely sub-contracted to Sigma-Aldrich Production GmbH: 1) preparation of a “trial” batch (~ 100 units, 100 mL each) and 2) preparation of the “final” batch (~ 3700 units, 100 mL each). This approach was

chosen to ensure as much as possible that the characteristics of the TMU starting material respected the required technical specifications, especially with regard to the D/H amount-of-substance ratio, that was measured in-house by site-specific natural isotope fractionation - nuclear magnetic resonance (SNIF-NMR) spectroscopy. The technical specifications stated that the trial and final batch had to originate from the same lot of starting material.

The trial batch respected the technical specifications with regard to purity and water content values and material appearance (clear colourless liquid), and the D/H ratio was preliminarily measured in-house as 121.5 ppm. This D/H ratio was deemed as sufficiently high and fit-for-purpose for the certification based on the careful inspection of the NMR spectra with regard to height of the peaks (see Figure 2), the positive feedback received from the expert laboratories belonging to the Working Group Isotopic Measurements of the ERC-CWS and the comparison with the certified value of previous TMU CRMs.

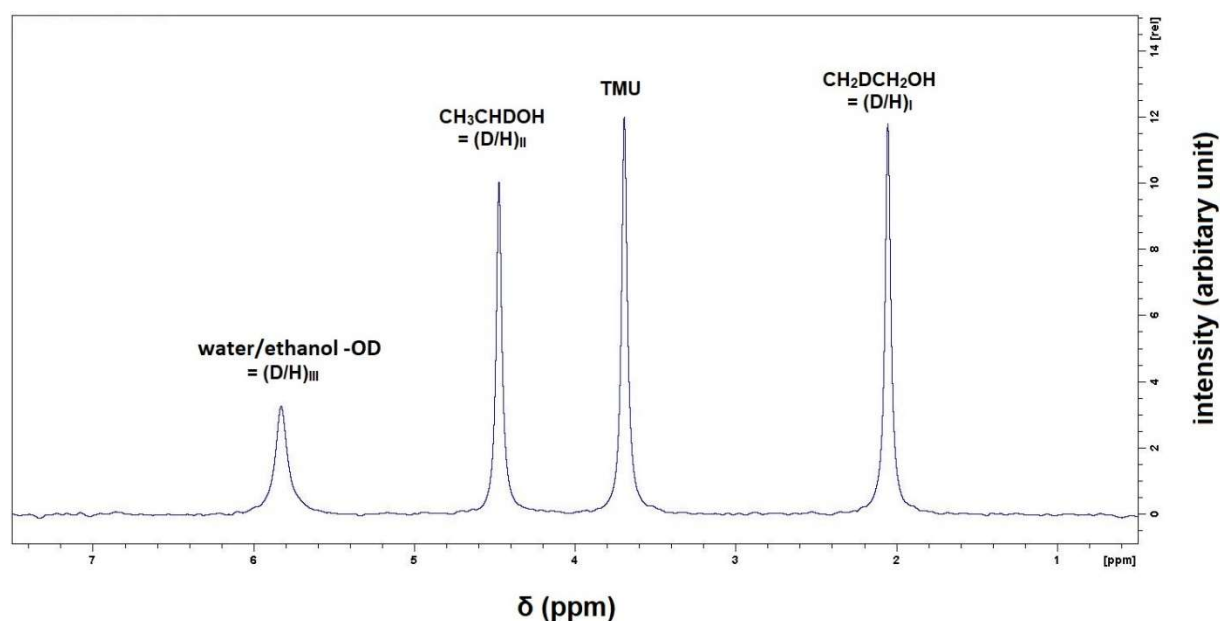


Figure 2: Representative ^2H spectrum of an ethanol/TMU mixture acquired during the in-house check of the D/H ratio. An in-house ethanol with assigned values for D/H and ethanolic strength by mass were used in the measurements.

An additional check of the D/H ratio of the remaining bulk TMU to be bottled as the final batch was carried out in-house and estimated as 123.3 ppm, thus slightly higher compared to the D/H ratio of the trial batch.

ERM-AE003 was aliquoted into 100 mL portions (unit size) under argon atmosphere in leak-proof screw-cup amber glass bottles with break ring and labelled at the Sigma-Aldrich Production GmbH premises in Buchs. The bottles were transported to JRC Geel where they were individually placed in labelled polyethylene terephthalate/aluminium/nylon/low density polyethylene pouches (as additional measure to prevent light exposure and moisture uptake) which were thermo-sealed using a DAKLA sealing machine (Daklapak, Kortrijk, BE). A total of 3745 units of ERM-AE003 was produced.

For the purpose of this report, the term 'unit' refers to one bottle of ERM-AE003.

One unit of ERM-AE003 is shown in Figure 3.



Figure 3: ERM-AE003: bottle and its protective pouch

3.3 Process control

3.3.1 Water content

The water content was measured by C-KFT on five units randomly selected over the whole batch and analysed in duplicate. No trend over the filling sequence was observed for the moisture content in the candidate CRM (Figure 4). Each data point is displayed as the normalised unit mean \pm confidence interval (95 % confidence level, calculated from s_{wb} from ANOVA for all units).

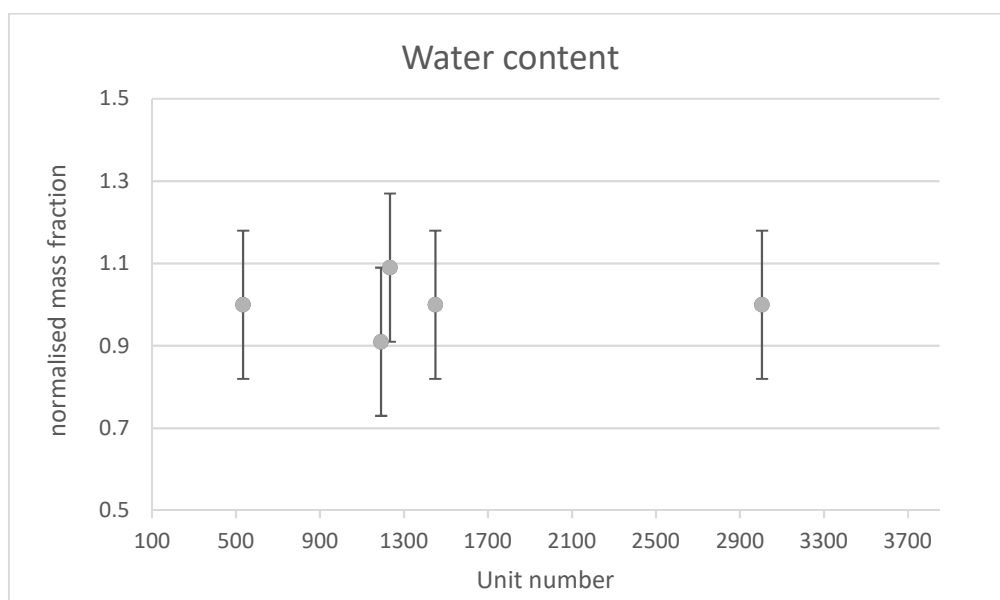


Figure 4: Water content of ERM-AE003 by coulometric Karl Fischer titration

4 Homogeneity

A key requirement for any reference material produced as a batch of 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 is significant compared to the variation of measurement results. Consequently, ISO 17034 [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 size is respected, but determines the minimum size of sample that is representative for the whole unit.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified value of the CRM is valid for all units of the material, within the stated uncertainty.

The number of units selected corresponds to approximately the cube root of the total number of units produced. Sixteen units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. Random stratified sampling involves dividing the batch into sixteen groups (with a similar number of units in each group) and randomly selecting one unit from each group. Two independent samples were taken from each selected unit, and analysed in-house for the deuterium-to-hydrogen ratio by SNIF-NMR. The guidelines for measurements prescribed several QA/QC measures, see Section 6 for details.

The sample preparations were performed under intermediate precision conditions (two days), while the measurements were performed within a single sequence, 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 graph in Annex A.

Given the intermediate precision conditions of the sample preparations, day-to-day effects can occur that could mask the between-unit variation. Significant differences between the day means were checked using a *t*-test at a 95 % confidence level. No significant day-to-day effects were found for the deuterium-to-hydrogen ratio.

Regression analyses were performed to evaluate potential trends in the measurement sequence as well as trends in the filling sequence. No trends in the filling sequence or in the measurement sequence were observed at a 95 % confidence level.

The dataset was assessed for consistency using Grubbs outlier tests at a confidence level of 99 % on the individual results and on the unit means. Neither outlying individual results nor outlying unit means were detected.

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

Evaluation by ANOVA requires mean values per unit which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. The distribution of the mean values per unit was visually 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 1.

Table 1: Results of the statistical evaluation of the homogeneity study

Parameter	Trends ¹⁾		Outliers ²⁾		Distribution	
	Measurement sequence	Filling sequence	Individual results	Unit means	Individual results	Unit means
D/H ratio	no	no	none	none	normal	normal

¹⁾ 95 % confidence level

²⁾ 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 a negative number under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be less 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.* [16]. u_{bb}^* is comparable to the limit of detection of a measurement method, yielding the maximum degree of inhomogeneity that might be undetected by the given study setup.

Method repeatability ($s_{wb,rel}$) (equivalent to the within-unit standard deviation), between-unit standard deviation ($s_{bb,rel}$) and $u_{bb,rel}^*$ were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \quad \text{Equation 2}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \quad \text{Equation 3}$$

$$u_{bb,rel}^* = \frac{\sqrt{\frac{MS_{within}}{n}} \sqrt{\frac{2}{\nu_{MS_{within}}}}}{\bar{y}} \quad \text{Equation 4}$$

MS_{within} mean of squares within-unit from ANOVA
 $MS_{between}$ mean of squares between-unit from ANOVA
 \bar{y} mean of all results of the homogeneity study
 N mean number of replicate analysis per unit
 $\nu_{MS_{within}}$ 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.

Table 2: Results of the homogeneity study

Parameter	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u_{bb,rel}^*$ [%]	$u_{bb,rel}$ [%]
D/H ratio	0.136	0.142	0.057	0.142

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 limit 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 size. The minimum sample size is the minimum amount of sample that is, for a given measurand, representative of the whole unit and thus should be used in an analysis. Using sample sizes equal or above the minimum sample size guarantees the certified value within its stated uncertainty.

In the case of ERM-AE003, this parameter was not assessed, as the SNIF-NMR method applied for the characterisation (OIV-MA-AS311-05) predefines the sample size: 1.3 mL must be used for NMR tubes of 10 mm diameter.

In any case, TMU is a liquid and is not expected to have any relevant inhomogeneity. This assumption was confirmed by the homogeneity, stability and characterisation studies, where method predefined sample sizes of 1.3 mL were found to give acceptable repeatability, demonstrating that there is no intrinsic inhomogeneity or contamination at this sample size.

5 Stability

Time, temperature, light (including ultraviolet radiation) and water content are regarded as the most relevant influences on the stability of materials. The influence of ultraviolet or visible light was minimised by storing ERM-AE003 in amber glass bottles individually placed in a multilayer pouch as additional measure to prevent light exposure and moisture uptake. In addition, materials are stored in the dark and dispatched in boxes, thus removing any possibility of degradation by light. The water content was checked before and after the bottling to be within specifications (see Section 3.3). 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 short-term stability study was carried out using an isochronous design [17]. In this approach, units were stored for a particular length of time in different temperature conditions. Afterwards, the units were moved to conditions where further degradation can be assumed 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.

Evidence for the long-term stability of TMU was available from the previously certified SB TMU STA-003k, and it was confirmed by the evaluation of stability monitoring results of the SB TMU STA-003m.

5.1 Short-term stability study

A previously performed short-term stability study on a similar material, master batch IRMM-425, showed that no detectable degradation was observed, even at storage conditions of 60 °C for 4 weeks. The uncertainty of short-term stability (u_{sts}) was estimated to be 0.034 % (1 week, 60 °C). A dedicated short-term stability study was nonetheless carried out on ERM-AE003.

In the short-term stability study the conditions for dispatch of the material to the customers were established. To this end, units were stored at 60 °C for 0, 1, 2 and 4 weeks. 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 were measured in-house by SNIF-NMR. The guidelines for measurements prescribed several QA/QC measures, see Section 6 for details. The measurements were performed under repeatability conditions and a randomised sequence was used to differentiate any potential drift in the measurement results from a trend over storage time.

The results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %.

In addition, the data were evaluated against storage time, and regression lines of D/H amount-of-substance ratio versus time were calculated, to test for potential increases or decreases of the measurand due to shipping conditions. The slope of the regression line was tested for statistical significance.

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 test

Parameter	Number of individual outlying results ¹⁾	Significance of the trend ²⁾
D/H ratio	none	no

¹⁾ 99 % confidence level²⁾ 95 % confidence level

No statistical outliers were detected. The trend at 60 °C was statistically not significant on a 95 % confidence level.

The material can be dispatched without further precautions under ambient conditions.

5.2 Long-term stability study

Long-term storage conditions and shelf life guaranteeing the stability of the material and the certified value were established.

Data from the post-certification stability monitoring programme for STA-003m were available. Previously released SB TMU STA-003m was analysed for the D/H amount-of-substance ratio by SNIF-NMR after the certification on two occasions over a period of 56 months. At each time point measurements were performed on units stored at the normal storage temperature of 18 °C. These data can be evaluated similarly to an isochronous study.

The D/H ratio results taken all together were screened for outliers using the single and double Grubbs test at a confidence level of 99 %.

In addition, the data were plotted against storage time and a linear regression line of D/H amount-of-substance ratio versus time was calculated. The slope of the regression line was tested for statistical significance (loss/increase due to storage).

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 evaluation

Parameter	Number of individual outlying results ¹⁾	Significance of the trend ²⁾
D/H ratio	none	no

¹⁾ 99 % confidence level²⁾ 95 % confidence level

No outlying individual results were found. All data were retained for statistical analysis. The trend at 18 °C was statistically not significant on a 95 % confidence level. 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 [18]. In this approach, the uncertainty of the linear regression line with a slope of zero is calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts, rel} = \frac{s_{rel}}{\sqrt{\sum (t_i - \bar{t})^2}} \cdot t_{tt} \quad \text{Equation 5}$$

$$u_{lts, rel} = \frac{s_{rel}}{\sqrt{\sum (t_i - \bar{t})^2}} \cdot t_{sl} \quad \text{Equation 6}$$

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 (60 months at 18 °C)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of stability during dispatch. This was estimated from the 60 °C study. The uncertainty describes the possible change during a dispatch at 60 °C lasting for one week.
- $u_{lts,rel}$, the uncertainty of stability during storage. This uncertainty contribution was estimated from the post-certification stability monitoring data of STA-003m. The uncertainty contribution describes the possible degradation during storage for 60 months 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 60 months

Parameter	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
D/H ratio	0.026	0.072

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

The material can be stored at 18 °C.

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

5.4 Repeated use study

For ERM-AE003 the impact of repeated use has been investigated. The study aimed at assessing the stability of the CRM when the containment is repeatedly opened and closed during several weeks. This is the typical scenario of use of ERM-AE003, that is employed as internal standard in

the SNIF-NMR measurements. The rate of consumption of TMU depends on the laboratory's needs, with an average amount estimated as approximately 100 mL per month.

For the purpose of this study, 2 units were stored at 18 °C in a desiccator and analysed at time points 0, 4, 9 and 13 weeks for the water content by coulometric Karl Fischer titration and at time points 0, 5 and 12 weeks for the D/H ratio by SNIF-NMR, respectively. After opening and taking aliquots for analysis, the units were immediately tightly closed and stored at the chosen conditions. One-way ANOVA was performed to identify potential significant differences between the means of the different time points.

No significant difference was found between the means of time points 0, 4, 9 and 13 weeks for the C-KFT measurements and between the means of time points 0, 8 and 12 for the SNIF-NMR measurements. Therefore, it can be concluded that the certified value is still valid within 12 weeks from the opening of a CRM unit, provided it is carefully closed and stored at 18 °C in a desiccator.

Additional ^1H -NMR measurements were performed on ERM-AE003 samples stored in the same conditions as above (18 °C in a desiccator) for 0, 10 and 14 weeks: the spectra were compared visually for the development of extra-peaks over time, possibly indicating degradation of the TMU. This qualitative evaluation did not show any change in the appearance of the TMU ^1H -NMR spectrum, adding evidence to the material's stability during the studied period.

The results of the repeated use studies for water content and D/H ratio are shown in Figure D1 and D2, respectively, of Annex D.

6 Characterisation

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

This was based on an interlaboratory comparison of three expert laboratories i.e. the D/H amount-of-substance ratio of the material was determined by applying the official SNIF-NMR method OIV-MA-AS311-05. This approach converts the systematic bias of each laboratory into a random variable, the combined effect of which is reduced by averaging over several laboratories.

6.1 Selection of participants

Three laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participating laboratory was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the application of the SNIF-NMR method OIV-MA-AS311-05 by submitting results for interlaboratory comparison exercises. All three laboratories are ISO/IEC 17025 accredited for the SNIF-NMR method and the accreditation number is stated in the list of participants (Section 2).

6.2 Study setup

Laboratories received two units of ERM-AE003 and two units of master batch IRMM-425 and were requested to provide six independent results, three per each unit received of the SB and MB, respectively. The in-house characterisation measurements consisted of duplicate measurements of three units of ERM-AE003 that were acquired during the homogeneity study. The units distributed were selected using a random stratified sampling scheme and covered the whole batch.

Batch characterisation was accomplished by measuring ERM-AE003 and MB IRMM-425 and calculating the ERM-AE003 values by comparing the mean measured value of the MB with the certified value of the MB.

The sample preparations of ERM-AE003 and the MB IRMM-425 had to be done under repeatability conditions (within one day) and the measurements were to be included in one single measurement sequence.

Prescribed QA/QC measures included the measurement of BCR[®]-123 (reference ethanol for SNIF-NMR [19]) and of an in-house ethanol (as internal standard) with assigned (D/H)_i value and ethanolic strength by mass.

Each independent measurement consisted of a separate sample preparation and ten recorded spectra, which average was reported as the measurement result.

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 [14] were regarded as equally valid procedures.

6.3 Methods used

All laboratories used the official SNIF-NMR method OIV-MA-AS311-05 for the deuterium-to-hydrogen amount-of-substance ratio, which is briefly described hereunder.

In-house ethanol distilled from wine with assigned values for (D/H)_i value and ethanolic strength by mass (details are given in Table E1, Annex E) were used in the measurements of both ERM-AE003 and IRMM-425. The influence of the employed ethanol cancels therefore out, as the initially determined D/H ratios of ERM-AE003 are re-calculated by comparison of the mean measured value

of IRMM-425 with the certified value of IRMM-425 (D/H ratios determined using the same ethanol), see Equation 10.

For the NMR measurements, two of the laboratories used an Avance III 400 MHz spectrometer and the third one used an Ascend 400 MHz spectrometer (all from Bruker). Ten-mm diameter NMR tubes were used. Sample preparation was done by accurately weighing in approximately 3.2 mL of the in-house ethanol and 1.3 mL of TMU; finally, 150 µL of field-frequency stabilisation substance (mixture of 10 % v/v of trifluoroacetic acid in hexafluorobenzene) was added. All exact weights and sample numbers were recorded and reported.

SNIF-NMR instrument performance was verified by analysing BCR-123. Data processing was accomplished by Fourier transformation, line broadening and automated baseline correction. Peak heights were determined by the respective software used (details are given in Table E.1, Annex E). The formula depicted below and stipulated in the method OIV-MA-AS311-05 is used to calculate the ethanol D/H_i value using the certified TMU D/H value.

$$(D / H)_{I A} = \frac{M_A}{M_{TMU}} \cdot \frac{P_{TMU}}{P_I} \cdot T_I \cdot \frac{m_{TMU}}{m_A} \cdot \frac{(D / H)_{TMU}}{t_m^D} \quad \text{Equation 7}$$

(D/H) _{I A}	calculated (D/H) _i value of ethanol
M _A	molecular mass of ethanol
M _{TMU}	molecular mass of TMU
P _{TMU}	number of hydrogen atoms in TMU molecule
P _I	number of hydrogen atoms at site I of the ethanol molecule
T _I	height of signal (D/H) _i (i.e. CH ₂ DCH ₂ OH) divided by height of TMU signal
m _{TMU}	mass of TMU to nearest 0.1 mg
m _A	mass of ethanol to nearest 0.1 mg
(D/H) _{TMU}	certified value of TMU
t _m ^D	strength by mass of the ethanol in % (m/m), expressed by

$$t_m^D = \frac{p - p'}{p} \cdot 100 \quad \text{Equation 8}$$

p	mass of wine ethanol
p'	mass of water in the wine ethanol determined by Karl Fischer titration

In this exercise, the formula was reversed to calculate the TMU (ERM-AE003) D/H value using the assigned values of D/H_i and ethanolic strength by mass t_m^D of the in-house ethanol used:

$$(D / H)_{TMU} = \frac{P_I}{P_{TMU}} \cdot \frac{M_{TMU}}{M_A} \cdot \frac{1}{T_I} \cdot \frac{m_A}{m_{TMU}} \cdot t_m^D \cdot (D / H)_{I A} \quad \text{Equation 9}$$

The D/H_I value of the ethanol (D/H_{IA} in Equation 7 and 9) is used for the calculation of the ERM-AE003 certified value, due to the favourable stoichiometric factors: 3 H in the case of D/H_I (CH₂D-CH₂-OH) signal to 12 H in TMU, compared to 2 H in the case of D/H_{II} (CH₃-CHD-OH) signal to 12 H in TMU. An investigation comparing different methods of data evaluation revealed that the official method [3] tends to slightly overestimating the D/H_{II} ratio due to field distortion effects [20]. This underpins the decision to use solely the assigned value of D/H_I of the ethanol for calculating the D/H amount-of-substance ratio of the TMU batch.

6.4 Evaluation of results

The characterisation study resulted in three datasets for the D/H value of ERM-AE003, for a total of 18 independent measurements. All individual results of the participants (including the measurements results of IRMM-425) are displayed in tabular form in Annex F (Table F1).

6.4.1 Technical evaluation

The obtained data were first checked for compliance with the official SNIF-NMR method OIV-MA-AS311-05 and with the requested instructions (prescribing sample preparations and measurements performed under repeatability conditions) and for their validity based on technical reasons. The evaluation included:

- agreement of the BCR-123 measurement results with the certified values
- within-laboratory repeatability ≤ 0.25 %

6.4.2 Statistical evaluation

The three datasets were accepted based on the technical evaluation. They were further tested for normality of dataset means, using kurtosis/skewness tests and normal probability plots and for outliers using the Grubbs test (at a 99 % confidence level). No outliers were found.

Subsequently, each individual measurement of ERM-AE003 was computed applying the following formula:

$$x_{calcERM-AE003} = \frac{cert.value_{IRMM-425}}{\bar{x}_{IRMM-425}} \cdot x_{measERM-AE003} \quad \text{Equation 10}$$

$x_{calcERM-AE003}$	individual result for ERM-AE003 computed after comparison to MB IRMM-425 certified value
$cert.value_{IRMM-425}$	certified value of the MB (141.9×10^{-6} according to the certificate)
$\bar{x}_{IRMM-425}$	mean value of six MB measurements performed in each laboratory
$x_{measERM-AE003}$	measured individual result for ERM-AE003

Table F2 and Figure F1 of Annex F show all the individual results of the participants, computed after comparison of the mean measured value to the certified value of the MB IRMM-425. Pooling of all 18 individual measurements for calculating the certified value was considered appropriate based on the fact that one-way ANOVA at a 95 % confidence level indicated no significant difference among laboratories means and that all individual data followed a normal distribution, showing that they belong to a single population (supporting the evidence of no laboratory bias).

Table 6: Statistical evaluation of the measurement results for ERM-AE003.
N: number of individual measurements

Parameter	N	Outliers	Normally distributed	Mean of all samples [ppm]	s [ppm]
D/H ratio ¹⁾	18	none	yes	123.037	0.215

¹⁾ D/H ratio with measurement unit of mol/mol is commonly expressed in parts per million (ppm)

The uncertainty related to the characterisation is estimated by combining the standard uncertainty of the certified value of MB IRMM-425 with the standard uncertainty related to the characterisation measurements of ERM-AE003 (estimated as the standard error of all measurements $\frac{S_{ERM-AE003}}{\sqrt{N}}$) as follows:

$$u_{char,rel} = \sqrt{\left(\frac{u_{MB}}{cert.value_{MB}}\right)^2 + \left(\frac{\frac{S_{ERM-AE003}}{\sqrt{N}}}{\bar{X}_{ERM-AE003}}\right)^2} \quad \text{Equation 11}$$

u_{MB} standard uncertainty of the certified value of MB IRMM-425 (Table 7, retrievable from the certification report)

$S_{ERM-AE003}$ standard deviation of all measurements of ERM-AE003 (N=18)

$\bar{X}_{ERM-AE003}$ mean value of all measurements of ERM-AE003 (N=18) (computed after comparison to MB IRMM-425)

Table 7: Uncertainty budget for the characterisation of ERM-AE003

	Uncertainty contribution	Value [ppm]	Standard uncertainty [ppm]	Probability distribution	u_{rel} [%]	Degrees of freedom (ν)
$cert.value_{IRMM-425}$	Certification of MB	141.9	0.311 ¹⁾	Normal	0.220	10
$\bar{X}_{ERM-AE003}$	Characterisation measurements of SB	123.037	0.051	Normal	0.041	17
$u_{char,rel}$					0.223	10 ²⁾

¹⁾ unrounded u

²⁾ effective number of degrees of freedom (ν_{eff}) obtained using the Welch-Satterthwaite equation [14]

Table 8: Uncertainty of characterisation for ERM-AE003

Parameter	Mean of all measurements [ppm]	u_{char} [ppm]
D/H ratio	123.037	0.275

7 Value Assignment

A certified value for the deuterium-to-hydrogen (D/H) amount-of-substance ratio was assigned.

Certified values are values that fulfil the highest standards of accuracy. Full uncertainty budget in accordance with ISO 17034 [1] and ISO Guide 35 [2] was established.

7.1 Certified values and their uncertainties

The certified value of ERM-AE003 was calculated as arithmetic mean of 18 individual results as shown in Table 8, after comparing the mean value obtained for IRMM-425, measured in repeatability conditions with ERM-AE003, with the certified value of IRMM-425 (Table F2, Annex F).

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

$$U_{\text{CRM,rel}} = k \cdot \sqrt{u_{\text{pur,rel}}^2 + u_{\text{bb,rel}}^2 + u_{\text{sts,rel}}^2 + u_{\text{lts,rel}}^2 + u_{\text{char,rel}}^2} \quad \text{Equation 12}$$

- u_{char} was estimated as described in Section 6
- u_{pur} was estimated as described in Section 3.1
- u_{bb} was estimated as described in Section 4.1
- u_{sts} and u_{lts} were estimated as described in Section 5.3

The choice of the coverage (k) factor was based on the number of effective degrees of freedom as calculated using the Welch-Satterthwaite equation [14]. Applying this equation, 22 effective degrees of freedom were obtained. The JRC's procedures for assigning uncertainties to certified values stipulate that for more than five effective degrees of freedom, a coverage factor of $k = 2$ can be applied to obtain the expanded uncertainty.

The certified value and its uncertainty are presented in Table 9.

Table 9: Certified value and uncertainty for ERM-AE003

	Certified value ¹⁾ [mol/mol]	$u_{\text{pur,rel}}$ [%]	$u_{\text{char,rel}}$ [%]	$u_{\text{bb,rel}}$ [%]	$u_{\text{sts,rel}}$ [%]	$u_{\text{lts,rel}}$ [%]	$U_{\text{CRM,rel}}$ [%]	U_{CRM} ¹⁾ [mol/mol]
D/H ratio	123.0×10^{-6}	0.075	0.223	0.142	0.026	0.072	0.571	0.7×10^{-6}

¹⁾ Expanded ($k = 2$) and rounded uncertainty; uncertainties are always rounded up [21] and in a way that the rounding error corresponds to 3 % to 30 % of the uncertainty

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

Deuterium-to-hydrogen amount-of-substance ratio is an operationally defined measurand and can only be obtained by following the measurement procedure specified in the official method OIV-MA-AS311-05 *Determination of the deuterium distribution in ethanol derived from fermentation of grape musts, concentrated grape musts, grape sugar (rectified concentrated grape musts) and wines by application of nuclear magnetic resonance (SNIF-NMR)*. An attempt to demonstrate the independence of the D/H ratio of the SNIF-NMR measurement method was conducted in the past [12], but a systematic bias between IRMS and NMR results was observed. The measurand is therefore operationally defined by the SNIF-NMR method.

Quantity value

Traceability of the obtained results is based on the traceability of all relevant input factors. Investigation of the method and measurement details of the individual results show that all relevant input parameters of each technically accepted dataset have been properly calibrated.

All technically accepted datasets are traceable to the same reference, namely the value of VSMOW (Vienna Standard Mean Ocean Water) via the master batch IRMM-425 and by strictly adhering to the official SNIF-NMR method OIV-MA-AS311-05 [3]. IRMM-425 was certified by an interlaboratory comparison via the measurement of TMU-reference ethanol mixtures by SNIF-NMR. The reference ethanol used was BCR-656 with $(D/H)_I$ and $(D/H)_{II}$ certified values traceable, through a series of comparisons, to the first TMU certified reference material. The first TMU reference material was certified by measuring mixtures of TMU with VSMOW by SNIF-NMR [11]. The traceability to the same reference is also confirmed by the agreement of results within their respective uncertainties. As the assigned value comes from the combination of agreeing results individually traceable to the value of VSMOW, the assigned quantity value itself is traceable to the value of VSMOW as well.

For VSMOW, a value of 155.76×10^6 for the D/H amount-of-substance ratio was used [9].

9 Instructions for use

9.1 Safety information



The following hazards and precautionary statements apply:

H302	Harmful if swallowed
H360d	Suspected of damaging the unborn child
P202	Do not handle until all safety precautions have been read and understood
P264	Wash hands thoroughly after handling
P281	Use personal protective equipment as required
P301+P312+P330	IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell. Rinse mouth
P308+P313	IF exposed or concerned: Get medical advice/attention

9.2 Storage conditions

ERM-AE003 should be stored at 18 ± 5 °C in the dark. After prolonged storage, TMU might turn slightly yellow. This colour change does not significantly alter the isotopic content.

Care should be taken to avoid any change of the moisture content once the units are open, as TMU is hygroscopic. The user should tightly close any unit immediately after taking a sample and store it in a desiccator.

The stability of opened samples has been tested for a period of 12 weeks (Section 5.4). Both for the certified value (measured by SNIF-NMR) and for the water content (measured by C-KFT), no significant difference was found between the means of the considered time points. Therefore, it can be concluded that the certified value is still valid within 12 weeks from the opening of a CRM unit, provided it is immediately carefully closed and stored at 18 °C in a desiccator.

Repeated use of the material occurs anyway under the responsibility of the user. Note that the European Commission cannot be held responsible for changes that may happen during storage of the material at the customer's premises, especially of opened samples.

9.3 Minimum sample size

The minimum sample size is 1.3 mL of ERM-AE003 when using 10 mm diameter NMR tubes [3].

9.4 Use of the certified value

The intended use of ERM-AE003 is to serve as internal standard (referred to as standard TMU with a calibrated isotope ratio D/H) in the official method OIV-MA-AS311-05 *Determination of the deuterium distribution in ethanol derived from fermentation of grape musts, concentrated grape musts, grape sugar (rectified concentrated grape musts) and wines by application of nuclear*

magnetic resonance (SNIF-NMR). ERM-AE003 is mentioned as internal standard in other SNIF-NMR official OIV and AOAC methods.

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11 References

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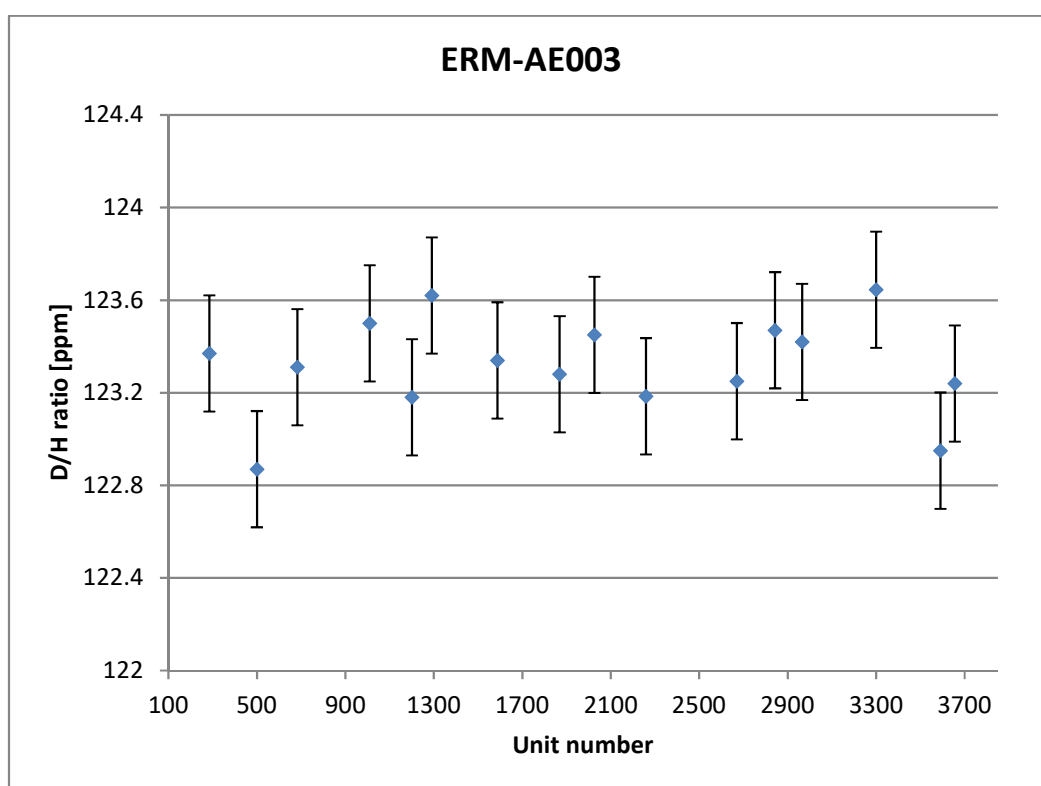
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Annexes

Measurements for the homogeneity, stability and characterisation studies were all carried out strictly adhering to the method OIV-MA-AS311-05 - *Determination of the deuterium distribution in ethanol derived from fermentation of grape musts, concentrated grape musts, grape sugar (rectified concentrated grape musts) and wines by application of nuclear magnetic resonance official (SNIF-NMR)*.

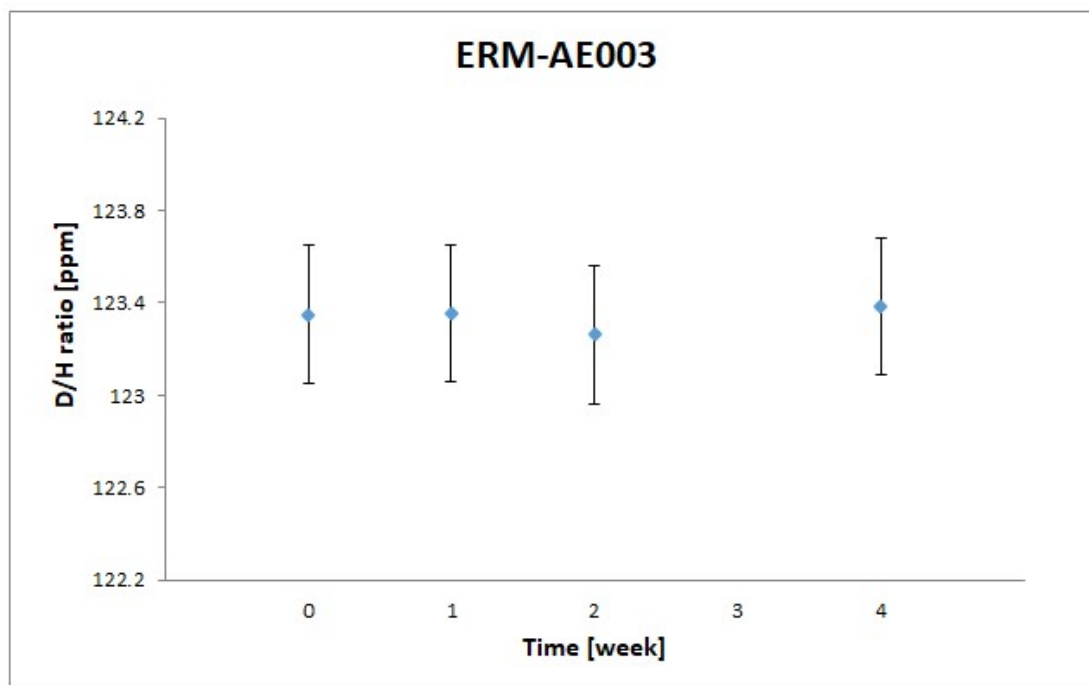
Annex A: Results of the homogeneity measurements

The graph reports unit means \pm 95 % confidence interval of the means (same CI calculated from S_{wb} from ANOVA for all units)



Annex B: Results of the short-term stability measurements

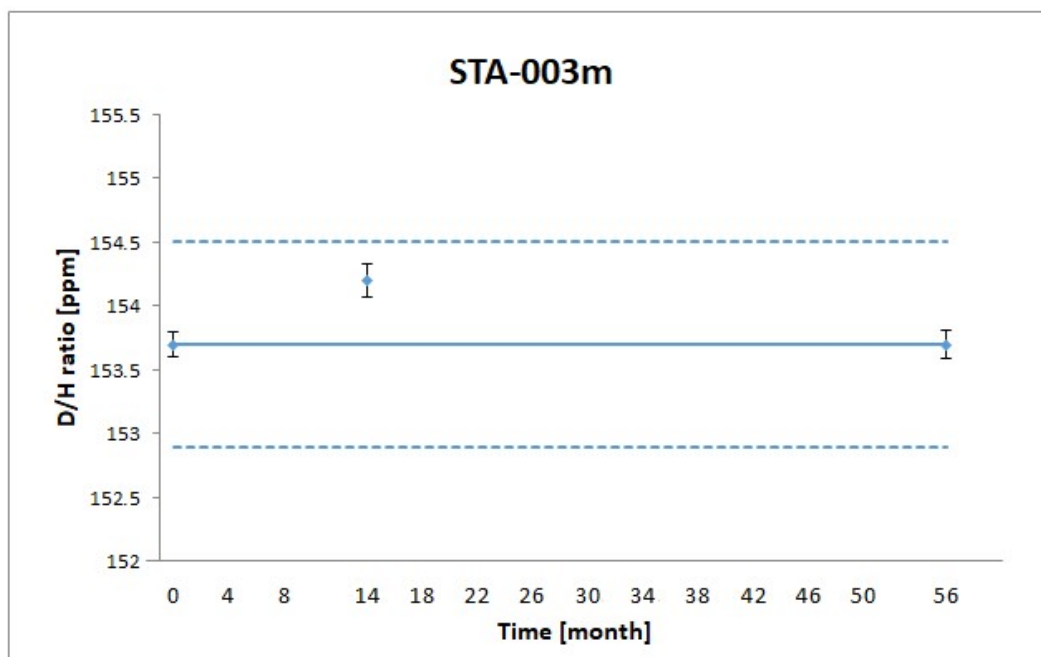
The graph reports unit means \pm 95 % confidence interval of the means (same CI calculated from s_{wb} from ANOVA for all units)



Annex C: Results of the long-term stability measurements

Post-certification stability monitoring results of STA-003m (18 °C). Time point 0 corresponds to the characterisation measurements, time points 14 months and 56 months correspond to the first and second stability monitoring measurements, respectively

The graph reports unit means \pm (standard error of the means)*2; certified value (solid line) \pm expanded uncertainty (dashed lines) of STA-003m



Annex D: Results of the repeated use studies

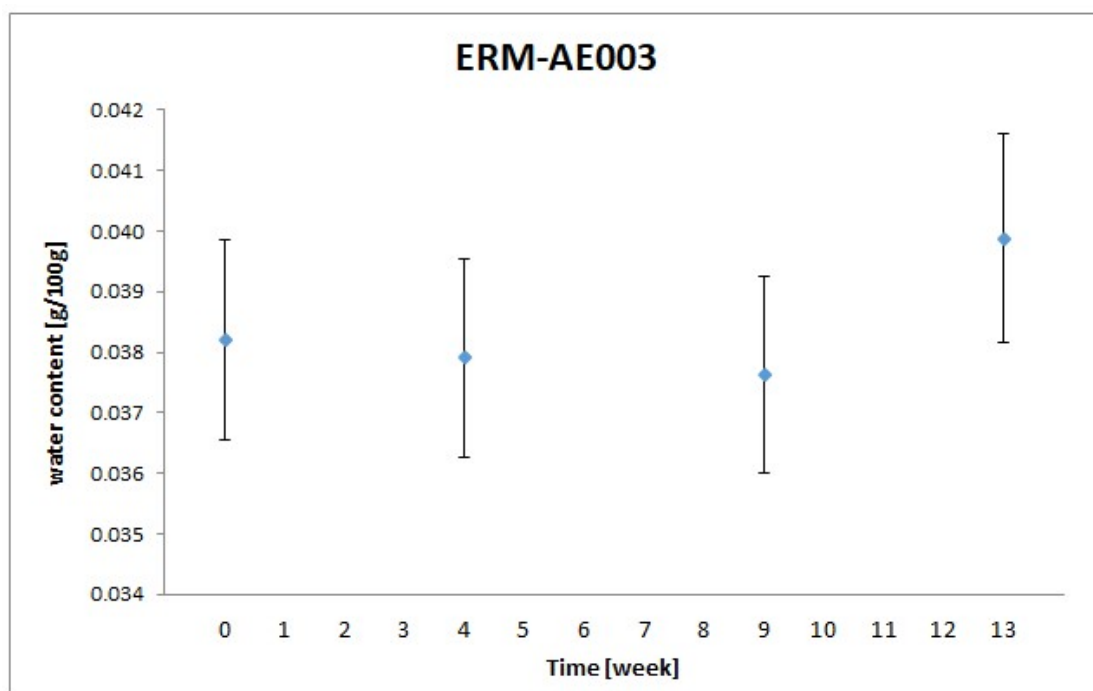


Figure D1. Water content by C-KFT. The graph reports the means per time point \pm expanded uncertainty ($k = 2$)

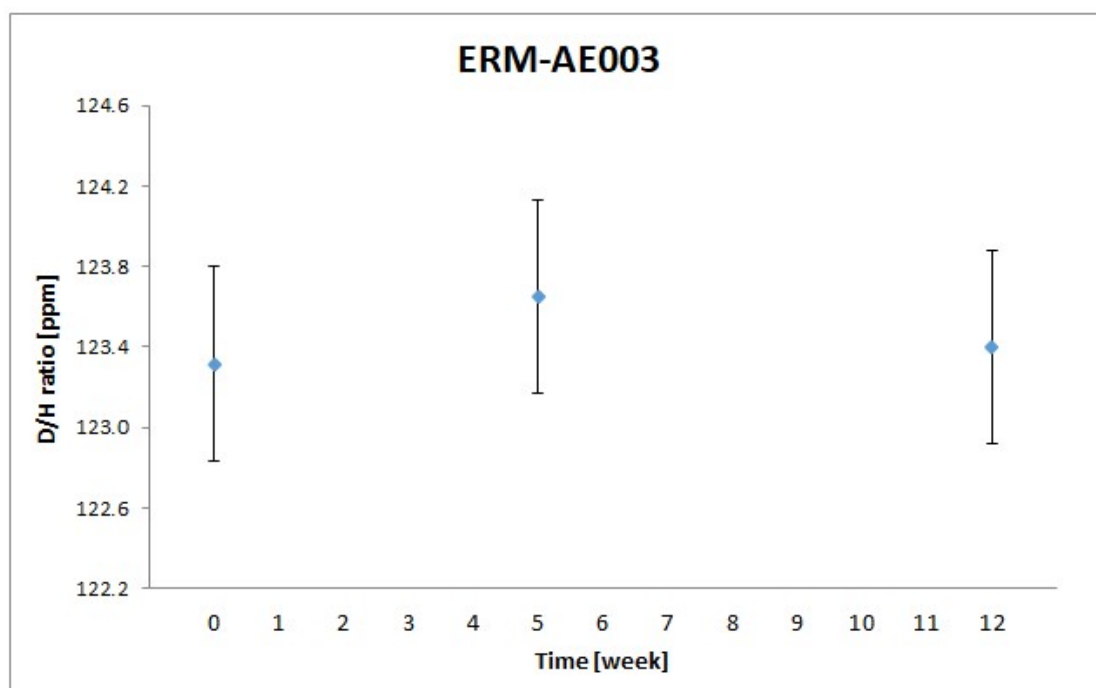


Figure D2. D/H amount-of-substance ratio by SNIF-NMR. The graph reports the means per time point \pm expanded uncertainty ($k = 2$)

Annex E: Summary of methods used in the characterisation study

Table E1. Method information as reported by the laboratories

Laboratory code	NMR spectrometer parameters	Signal property used for calculation	NMR appliance; software	QA/QC measures; in-house ethanol characteristics
L1	constant probe temperature: 300 K acquisition time: 6.68 s relaxation delay: 0.1 s spectral width: 19.947 ppm SF01 = 61.44 MHz SF02 = 400.23 MHz number of scans: 150	Height	Bruker Avance III 400 MHz ultrashield; Topspin 3.2 Eurospec	BCR-123, Ethanol M; D/H _i = 101.61; ethanolic strength by mass = 92.69 %
L2	constant probe temperature: 303 K acquisition time: 6.7 s relaxation delay: 0.1 s spectral width: 19.895 ppm SF01 = 61.42 MHz SF02 = 400.13 MHz number of scans: 150	Height	Bruker Avance III 400 MHz; TopSpin Software - NMR Data Analysis	BCR-123, Ethanol L and M; D/H _i = 99.58; ethanolic strength by mass = 94 %
L3	constant probe temperature: 300 K acquisition time: 6.68 s relaxation delay: 0.1 s spectral width: 19.949 ppm SF01 = 61.43 MHz SF02 = 400.18 MHz number of scans: 200	Height	Bruker Ascend 400 MHz; Topspin 3.2 Eurospec	BCR-123 Ethanol L, H and M; D/H _i = 101.28; ethanolic strength by mass = 91.74 %

Annex F: Results of the characterisation measurements

N.B.1: Values in tables are ratios with the measurement unit mol/mol, commonly expressed in parts per million (ppm); the rounding is as reported by the laboratories

N.B.2: the certified value is calculated as the arithmetic mean of all 18 measurements

Table F1. D/H ratio values of ERM-AE003 and IRMM-425 as reported by the laboratories

Laboratory code	CRM	replicate 1 [ppm]	replicate 2 [ppm]	replicate 3 [ppm]	replicate 4 [ppm]	replicate 5 [ppm]	replicate 6 [ppm]	mean [ppm]	expanded uncertainty [ppm]
L1	ERM-AE003	123.43	123.02	123.27	123.34	122.74	123.04	123.14	0.2
	IRMM-425	142.17	141.71	142.16	141.82	141.79	142.22	141.98	0.2
L2	ERM-AE003	123.38	123.19	122.86	123.21	123.26	123.01	123.15	0.8
	IRMM-425	142.26	142.24	142.12	142.22	141.87	141.95	142.11	0.8
L3	ERM-AE003	123.03	122.71	123.14	123.22	123.20	123.36	123.02	0.48
	IRMM-425	141.61	141.92	141.92	142.24	141.92	142.07	141.95	0.55

Table F2. D/H ratio values of ERM-AE003 after calculation upon comparison with IRMM-425 certified value

Laboratory code	replicate 1 [ppm]	replicate 2 [ppm]	replicate 3 [ppm]	replicate 4 [ppm]	replicate 5 [ppm]	replicate 6 [ppm]	mean [ppm]	expanded uncertainty [ppm]
L1	123.36	122.95	123.20	123.27	122.67	122.97	123.07	0.2
L2	123.20	123.01	122.68	123.03	123.08	122.83	122.97	0.8
L3	122.99	122.67	123.10	123.18	123.16	123.32	123.07	0.48

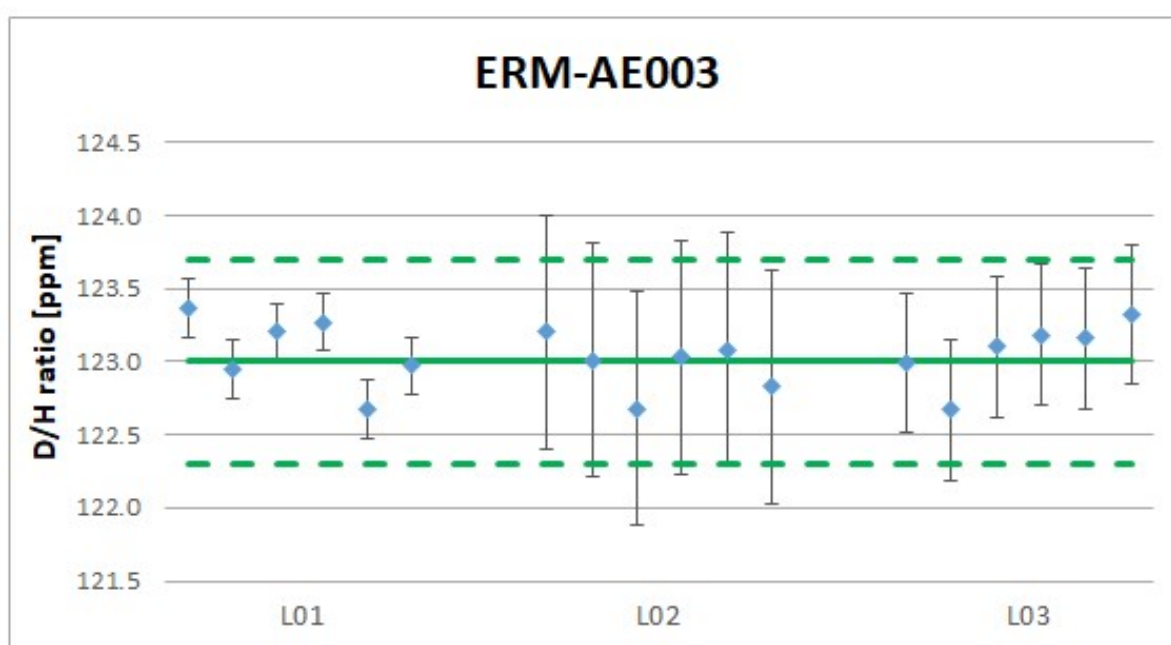


Figure F1. Certified value (solid line) \pm expanded uncertainty (dashed lines) for the D/H amount-of-substance ratio in ERM-AE003, $123.0 \times 10^{-6} \pm 0.7 \times 10^{-6}$; error bars of the individual laboratory means correspond to the expanded uncertainty

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