



CERTIFICATION REPORT

Certification of the Deuterium-to-Hydrogen (D/H) amount-of-substance ratio in a 1,1,3,3 – tetramethylurea batch

Certified Reference Material STA-003m

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Certified Reference Material STA-003m

R.Zeleny

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Summary

This report describes the production of a tetramethylurea (TMU) reference material (STA-003m), certified for its deuterium-to-hydrogen (D/H) amount-of-substance ratio. The material is to be used as an internal standard in site-specific natural isotope fractionation – nuclear magnetic resonance (SNIF-NMR) spectrometry measurements for determining the D/H amount-of-substance ratios of ethanol distilled from wines, an important measure in wine authenticity testing (Commission Regulation 2676/90, [1]).

Commercially obtained TMU with a sufficiently high D/H amount-of-substance ratio ($>120 \times 10^{-6}$) was first purified by removing most of the residual water and then filled into amber glass bottles of 500 mL. Between-unit homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [2]. Batch characterisation was accomplished by comparing STA-003m samples with samples of the TMU master batch (IRMM-425) under repeatability conditions and by adhering to the SNIF-NMR technique [1].

The certified value was calculated as the unweighted mean of 18 individual results obtained on 3 different NMR appliances. The uncertainty of the certified value was estimated in compliance with ISO Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM) [3]; it includes contributions from possible inhomogeneity, instability, and characterisation.

The certified value and its associated uncertainty are:

	Amount-of-substance ratio	
	Certified value ^{2,3)}	Uncertainty ^{2,4)}
Deuterium-to-hydrogen (D/H) ratio ¹⁾	153.7×10^{-6}	0.8×10^{-6}
1) The measurand is defined by strictly adhering to the Community reference method (SNIF-NMR, site-specific natural isotope fractionation – nuclear magnetic resonance spectrometry, as defined in Annex 8, Commission Regulation 2676/90). 2) The certified value and its uncertainty have the measurement unit one. 3) The value was obtained by comparing the mean value of 18 measurements of IRMM-425, tested together with 18 samples of STA-003m under repeatability conditions, with the certified value of IRMM-425. Measurements were performed on 3 different NMR appliances employing SNIF-NMR at 61.45/400 MHz, 61.43/400 MHz, and 61.42/400 MHz for ² H (¹ H) under repeatability conditions (values are indicating the transmitter frequency of the first and second channel, respectively). The value is traceable to the value of V-SMOW (Vienna Standard Mean Ocean Water) via using the master batch IRMM-425 and by employing SNIF-NMR as stipulated above. For V-SMOW, a value of 155.76 ppm was used (Hagemann, R., Nief, G., Roth, E. (1970), Tellus 22, 712). 4) Expanded uncertainty with a coverage factor of $k = 2$, 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. The number of degrees of freedom of the certified uncertainty, $\nu = 13$.		

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Glossary

Å	Angström
ANOVA	Analysis of variance
<i>b</i>	Slope of the linear regression
BCR	Community Bureau of Reference
BEVABS	European Office for Wine, Alcohol and Spirit Drinks
CRM	Certified reference material
D	Deuterium (² H)
D/H	Deuterium-to-hydrogen ratio
EC	European Commission
EU	European Union
GC	Gas chromatography
IRMM	Institute for Reference Materials and Measurements
IRMS	Isotope ratio mass spectrometry
ISO	International Organisation for Standardization
JRC	Joint Research Centre
<i>k</i>	Coverage factor
KFT	Karl Fischer titration
MB	Master batch
m/m	Mass/mass
MHz	Mega hertz
MS	Mass spectrometry
MS _{between}	Mean of squares between groups (ANOVA)
MS _{within}	Mean of squares within groups (ANOVA)
NIST	National Institute of Standards and Technology
ppm	Parts per million
QC	Quality control
RSD	Relative standard deviation
<i>s</i>	Standard deviation
<i>S</i> _{bb}	Between-bottle (in)homogeneity standard deviation
SB	Secondary batch
SNIF-NMR	Site-specific natural isotope fractionation – nuclear magnetic resonance
<i>S</i> _{wb}	Within-bottle standard deviation
<i>t</i>	<i>t</i> value
<i>t</i> _{<i>m</i>} ^{<i>D</i>}	Alcoholic grade of ethanol, expressed in mass % (strength by mass)
TMU	Tetramethylurea
<i>U</i> _{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
<i>U</i> [*] _{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
<i>U</i> _{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
<i>U</i> _{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
<i>U</i> _{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
<i>U</i> _{pur}	Standard uncertainty of material purity; an additional index "rel" is added as appropriate
<i>U</i> _{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
<i>U</i> _{lts}	Total uncertainty associated with long-term (in)stability
<i>v</i>	Degrees of freedom
V-SMOW	Vienna Standard Mean Ocean Water

1 Introduction

1.1 Background and choice of the material

The isotopic amount-of-substance ratio of deuterium to hydrogen (D/H) is regularly measured in ethanol distilled from wine to detect wine adulterations, such as mixing high with low quality wines, or the addition of sugar before fermentation (chaptalisation) above the allowed limits. The Community reference method to determine the wine ethanol D/H ratio, specified in Commission Regulation 2676/90 [1], is Site-specific Natural Isotope Fractionation measured by Nuclear Magnetic Resonance (SNIF-NMR[®]) [4]. To monitor wine authenticity, the measured values are compared with those from the respective authentic wines listed in the European Union (EU) Wine Databank [5], operated by the European Office for Wine, Alcohol and Spirit Drinks (BEVABS) at the Joint Research Centre (JRC) of the European Commission (EC).

The reference method specifies the internal standard in the SNIF-NMR measurements to be tetramethylurea (TMU, Figure 1) with a known D/H amount-of-substance ratio, available as a certified reference material (CRM) from the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (EC-JRC-IRMM). This compound possesses appropriate physico-chemical properties, such as a low volatility, and high miscibility with wine ethanol. Furthermore, it exhibits a convenient chemical shift in NMR analysis to avoid interference with ethanol peaks and it yields a signal width similar to that of the ethanol methyl signal.

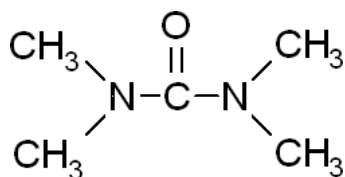


Figure 1. 1,1,3,3 – Tetramethylurea, CAS number: 632-33-4

1.2 Design of the project

The first TMU reference material was certified by measuring mixtures of TMU with V-SMOW (Vienna - Standard Mean Ocean Water [6]) by SNIF-NMR [7]. All subsequent TMU batches, however, were certified using reference ethanols, which themselves were calibrated against the penultimate TMU batch. This procedure has some drawbacks, such as the dependence of the certified value on a former material, resulting in an increasing uncertainty and an elongated traceability chain from batch to batch. In addition, value assignment was accomplished using

only three dedicated NMR spectrometers operated independently in one laboratory, which is reflected in the high coverage factor (4.3) of the expanded uncertainty of STA-003.

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 [8]. Consequently, SNIF-NMR has to be used for characterisation measurements.

IRMM revised the certification strategy and produced a TMU master batch (MB), IRMM-425, to which all future secondary batches (SB) are to be linked. The certified value of the MB was obtained by means of an international laboratory intercomparison [9]. 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 repeatability conditions, with the certified value of the MB. Details are described in section 7).

It shall be noted that the certified value and its corresponding uncertainty are expressed as a ratio with the measurement unit of one. Commonly however, SNIF-NMR results are expressed in parts per million (ppm).

STA-003m will replace the currently available secondary batch STA-003k [10] once exhausted. The material represents the "sample of standard TMU" to be used as stipulated in paragraph 3.3.1 of Annex 8, Commission Regulation 2676/90 [1].

2 Participants

Processing

Eurofins Scientific Analytics, Nantes, FR

Purity assessment of the candidate material

Eurofins Scientific Analytics, Nantes, FR
(ISO/IEC 17025 accreditation; COFRAC N° 1-0287)

Homogeneity and stability studies

Eurofins Scientific Analytics, Nantes, FR
(ISO/IEC 17025 accreditation; COFRAC N° 1-0287)

Characterisation

Eurofins Scientific Analytics, Nantes, FR
(ISO/IEC 17025 accreditation; COFRAC N° 1-0287)

Project management & data evaluation

European Commission, Joint Research Centre, Institute for Reference
Materials and Measurements, Geel, BE

3 Material processing and process control

Material processing (from acquisition of raw material to bottling and storage of the candidate CRM) was performed at Eurofins Scientific Analytics. Briefly, a batch of about 150 L of TMU was acquired from VWR Ltd. (Lutterworth, UK) with a sufficiently high D/H amount-of-substance ratio (> 120 ppm). This material, which contained about 3 g/kg of water, was dried overnight over 3 Å molecular sieve, filtered to remove the molecular sieve, and aliquoted into 500 mL portions (unit size) in dark brown glass bottles, tightly closed with a Teflon cap and additionally sealed with a wax stopper. 300 bottles were produced in total.

Material identity and purity was elaborated at Eurofins. The identity of TMU was investigated by performing gas chromatography - mass spectrometry (GC-MS) measurements using an ion-trap mass spectrometer, and comparing the obtained spectrum with the NIST library spectrum of TMU. From each of the 14 bottles of STA-003m (units selected for homogeneity study and characterisation measurements, see below), one measurement was performed, and identity could be confirmed as follows: 7 points over the elution time of the TMU peak were taken and compared with the reference spectrum. The software considered the following parameters for calculating a probability factor for compound identity: the presence the same mass signals as those found in the reference spectrum, the relative abundances of the individual mass signals had to be comparable to that of reference spectrum, and finally the elemental composition of the compound had to match that of the reference. TMU was the only compound found in the data base matching with the experimentally derived data (probability value of 93 % indicated by the software).

Purity assessment was accomplished employing GC-MS, $^1\text{H-NMR}$, and $^{13}\text{C-NMR}$. Again, from each of the 14 bottles of STA-003m (see above), one measurement was performed with each technique. All GC-MS results indicated that no other peak besides TMU could be detected; moreover, the mass spectra matched with the reference spectrum. Likewise, all acquired $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra agreed with literature data and did not show any detectable impurity. As the applied GC-MS method (scan range 40 - 300 Da) was not capable of detecting water, coulometric Karl Fischer titration (KFT) was performed. The final product exhibited a water content of 0.45 ± 0.11 g/kg (mean \pm standard deviation; $n = 14$).

Taking into account these data, it was concluded that no major impurities remained undetected. Considering the detected impurity (residual water), the purity of candidate reference material thus amounts to 99.95 %. The uncertainty of material purity (u_{pur}), estimated by taking the standard deviation of the 14 KFT measurements, yielded 0.011 %.

4 Analytical methodology used

All measurements including those for homogeneity and stability were accomplished by employing site-specific natural isotope fractionation-nuclear magnetic resonance (SNIF-NMR) spectrometry in accordance with Commission Regulation 2676/90 [1]. In-house ethanols with assigned values for D/H_I and ethanolic strength by mass were used in the measurements. Figure 2 depicts a representative spectrum of an ethanol/TMU mixture.

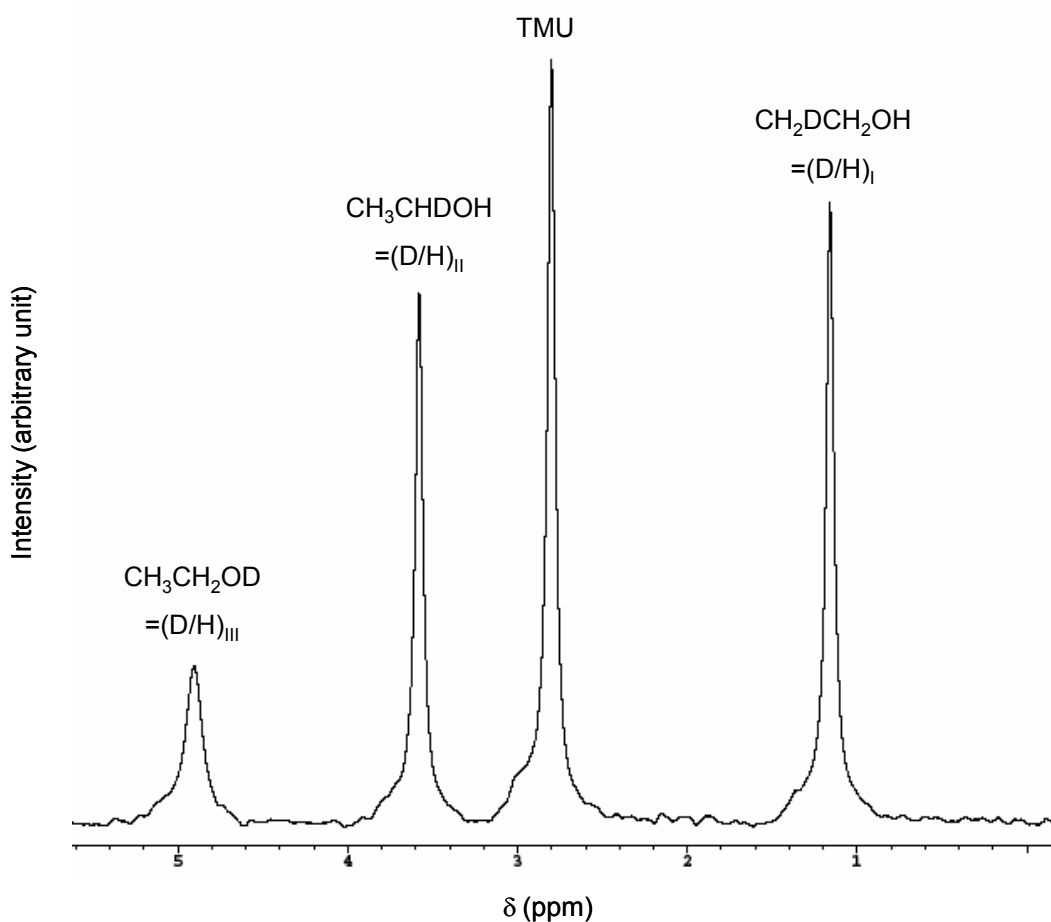


Figure 2. Representative 2H spectrum of ethanol/TMU mixture. Ethanol reveals three peaks, reflecting the position of the deuterium in the molecule [1,11].

Measurements were acquired on 400 MHz NMR appliances. 10 mm diameter NMR tubes were used. Sample preparation was done by weighing in 3.2 mL of the in-house ethanol and 1.3 mL of TMU; finally, 150 μ L of field-frequency stabilization substance (mixture of 10 vol hexafluorobenzene and 1 vol trifluoroacetic acid) was added. All exact weights and sample numbers were recorded and reported. In addition, date and time of measurements were documented. SNIF-NMR instrument performance was verified by analysing BCR-123 (3 different ethanols from beet, grape, and cane origin [12]) and in-house ethanols distilled from wine with assigned D/H_I and ethanolic strength by mass values were used. One analysis

consisted of 10 recorded spectra. Finally, 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 Annex C).

The formula depicted below and stipulated in [1,11] is used to calculate the ethanol D/H_I value using the certified TMU D/H value.

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

with

$(D/H)_{IA}$, 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. CH₂DCH₂OH) divided by height of signal of TMU

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), whereas

$$t_m^D = \frac{p - p'}{p} \cdot 100 \quad \text{Equation 2}$$

with

p , mass of wine ethanol

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

In this exercise, the formula was inversed to calculate the TMU D/H value using the assigned values of D/H_I and t_m^D of the "QC 02424616" ethanol, an in-house ethanol with assigned values for D/H_I value (102.2 ppm) and ethanolic strength by mass (92.98 %):

$$(D/H)_{TMU} = \frac{P_I}{P_{TMU}} \cdot \frac{M_{TMU}}{M_A} \cdot \frac{1}{T_I} \cdot \frac{m_A}{m_{TMU}} \cdot t_m^D \cdot (D/H)_{IA} \quad \text{Equation 3}$$

The D/H_I value of the ethanol is used for the calculation of the TMU 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 [1] tends to slightly overestimate the D/H_{II} ratio due to field distortion effects [13]. This underpins the decision to use solely the assigned value of D/H_I of the ethanol for calculating the D/H ratio of the TMU batch.

5 Homogeneity

5.1 Between-unit homogeneity

Ten samples were selected from the produced batch using a random stratified sample picking scheme. Samples were analysed in duplicate to allow detection of possible trends in the analytical sequence or the filling order, using SNIF-NMR as described above. Measurements were performed under repeatability conditions.

Data were scrutinised for outliers and checked for normal/unimodal distribution using normal probability plots and histograms. No outlier was detected in the filling sequence and the analytical sequence using the Grubbs test (95 % level of confidence). Data appeared to be normally distributed and were therefore suitable for regression analysis. Results were evaluated using linear regression analysis to detect possible trends regarding the filling sequence or analytical sequence and to estimate the uncertainty contribution of potential inhomogeneity. The slopes of the regression lines were tested for their significance at 95 % level of confidence: Table 1 summarizes the results of the homogeneity study. No significant trend was observed for neither filling order nor analytical sequence.

Table 1. Linear regression and statistical parameters associated to the homogeneity evaluation of STA-003m

Statistical parameters	Filling sequence	Analytical sequence
Slope (b)	-0.00068	-0.00202
Standard error of slope (s_b)	0.00078	0.01027
Degrees of freedom (ν)	8	18
$ b /s_b$	0.87	0.20
$t_{(0.05;8)}$	2.31	-
$t_{(0.05;18)}$	-	2.10
Statistical significance (95% confidence interval)	No	No

The between-unit standard deviation (s_{bb}) and the maximum inhomogeneity that could be hidden by method repeatability (u_{bb}^*) were calculated as estimation of the uncertainty due to inhomogeneity as described by Linsinger *et al.* [14]. u_{bb}^* is comparable to the limit of detection of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup. Method repeatability ($s_{wb,rel}$), $s_{bb,rel}$ and $u_{bb,rel}^*$ were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \quad \text{Equation 4}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \quad \text{Equation 5}$$

$$u_{bb,rel}^* = \frac{\sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{v_{MS_{within}}}}}{\bar{y}} \quad \text{Equation 6}$$

MS_{within} mean square within a unit from an ANOVA
 $MS_{between}$ mean squares between-unit from an ANOVA
 \bar{y} mean of all results of the homogeneity study
 n mean number of replicates per unit
 $v_{MS_{within}}$ degrees of freedom of MS_{within}

Table 2: Results of the homogeneity study for STA-003m

	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u_{bb,rel}^*$ [%]	$u_{bb,rel}$ [%]
D/H ratio	0.155	0.066	0.073	0.073

The estimated value for u_{bb} is small, as can be expected for a liquid (Table 2). This uncertainty contribution is in the same order of magnitude as the one calculated for the master batch (u_{bb}^* 0.06 %; s_{bb} could not be calculated as $MS_{between} < MS_{within}$, [9]). 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 (u_{bb}).

5.2 Minimum sample intake

This parameter was not assessed, as the method predefines the sample intake (Commission Regulation 2676/90, [1]): for 10 mm diameter NMR tubes, 1.3 g have to be used.

6 Stability

6.1 Short-term stability

No dedicated short-term stability study was conducted, as the material (purified TMU) is very similar to IRMM-425 (TMU, purified by same means to comparable purity and final water content), for which a short-term stability study has been performed with the following conclusion: no detectable degradation was observed, even at storage conditions of 60 °C for 4 weeks. Therefore, no special precautions are required during transport; the material can be dispatched under ambient temperature.

u_{sts} , the uncertainty of short-term stability, was estimated to be 0.034 % (1 week, 60 °C). Details about the short-term stability study of IRMM-425 can be found elsewhere [9].

6.2 Long-term stability

In contrast to the short-term stability study, other data than those obtained for the master batch have been used to evaluate the uncertainty contribution related to long-term storage. This was necessary, as the STA-003m batch is stored at room temperature, whereas the master batch is kept at -20 °C. Stability data from the currently available TMU batch STA-003k [10] was used to estimate the uncertainty contribution associated to long-term (in)stability. Measurements were performed over a period of 38 months, whereby on average every week 4-5 analyses were carried out. Reference Ethanol (in-house samples) with assigned D/H₁ ratios were used to calculate the D/H ratio of TMU. Sampling of TMU was done out of different bottles from the STA-003k batch, and from each bottle sampling for several dozens of measurements was performed, corresponding to a bottle usage period of several months. Bottles were only closed, but not re-sealed between the sampling dates. The study set-up can therefore be considered a conservative approach for the estimation of the uncertainty contribution associated with long-term (in)stability.

As different ethanol solutions with different D/H₁ ratios were used over the testing period, it was necessary to normalize the obtained values in order to combine the results: for each sub-study (dedicated ethanol), the results were divided by the respective sub-study average result. The results of the long-term stability study are illustrated in figure B1 of Annex B. The y axis representing the normalised D/H values is substantially enlarged. For different sub-studies, different degrees of rounding were applied, which explains the apparent different resolution of data points in the figure.

No outliers were detected (single Grubbs test at 95 % confidence level). Table 6 summarizes the results. The measurement results were then evaluated for a possible trend due to the storage condition. Linear regression analysis was performed, and the observed slope was tested for significance using a t -test, with $t_{\alpha,v}$ being the critical t -value (two-tailed) for a confidence level of 95 %. The slope was considered statistically significant when $b/s_b > t_{\alpha,v}$. No significant slope at 95 % confidence level was detected for the storage temperature of 20 °C (Table 3).

Table 3. Evaluation of the long-term stability study

Storage temperature	20 °C
Study duration	38 months
Slope (b)	0.00000722418
Std error slope (s_b)	0.00000634883
Degrees of freedom (ν)	709
$ b /s_b$	1.14
$t_{(0.05;709)}$	1.96
Statistical significance (95 % confidence level)	No

The standard uncertainty of the long-term stability, u_{lts} , was estimated for a shelf-life of 5 years using the following formula [15]:

$$u_{lts} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot x \quad \text{Equation 7}$$

where

x_i are the time points for each replicate

x is the shelf life (in months)

RSD is the relative standard deviation of all data in this series

In conclusion, the TMU batch was found to potentially exhibit a very minor degradation (0.038 % of the certified value after 5 years at room temperature). Taking into account the study set-up (considerably worse conditions than those applying to the batch units which are stored unopened till dispatch to the customers), it can be concluded that the material is stable when stored at 20 °C in the dark.

7 Characterisation

7.1 Study set-up

Batch characterisation was accomplished by comparing the mean value of 18 samples of the SB, tested together with 18 MB samples under repeatability conditions, with the certified value of the MB. The official method described in section 4 was thoroughly followed. Moreover, these measurements were performed in one laboratory on 3 different NMR appliances (PG400, DPX400, AVIII, all from Bruker) to obtain a larger set of results allowing a more robust estimate of the true value (6 samples each of SB and MB measured on each NMR appliance). One measurement consisted of sample preparation and 10 recorded spectra. The average of the 10 single results was reported as measurement result. Different TMU units were used on the 3 NMR appliances. All measurements were performed using an in-house ethanol ("QC 02424616") with assigned values for D/H₁ value (102.2 ppm) and ethanolic strength by mass (92.98 %). The influence of the employed ethanol is cancelled out, as the initially determined D/H ratio of STA-003m is compared with that of the master batch IRMM-425 (D/H ratio determined using the same ethanol).

The rationale for the experimental set-up described below was to keep the consumption of units for the various studies as low as possible and thus maximise the number of units available for distribution. For measurements on the DPX400 and the AVIII instruments, 2 units each of IRMM-425 (MB) and candidate CRM STA-003m (SB) were measured in triplicate, whereas on the PG400 instrument 3 units each of MB and SB, respectively, were measured in duplicate. This allowed the combination of homogeneity and characterisation measurements in one analytical series on the PG400 appliance (duplicate analysis planned for homogeneity study, 10 x 2 measurements on SB, see above; 2 x 3 MB measurements). The measurement results on the 3 units of the homogeneity study which were considered for the characterisation were determined by random assignment in the course of the planning phase of the project. The entire work was carried out in a laboratory with the SNIF-NMR method accredited to ISO 17025. Table D1 in Annex D lists the individual results obtained by measuring the SB and MB samples, respectively. Table 4 summarizes those results.

Table 4: Results of characterisation of batch STA-003m by comparison with MB IRMM-425

	Number of analyses (<i>n</i>)	Mean value ¹⁾ (meas _{SB} or meas _{MB})	Standard deviation ¹⁾ (<i>s</i> _{SB} or <i>s</i> _{MB})	Relative standard deviation (%)
STA-003m (SB)	18	153.458	0.266	0.173
IRMM-425 (MB)	18	141.722	0.196	0.138

¹⁾ Values are ratios with the measurement unit one; commonly they are expressed in parts per million (ppm)

To calculate the certified value for STA-003m, one needs the certified value of the MB (IRMM-425). Table 5 shows the main data, taken from the Certificate of Analysis of IRMM-425:

Table 5: Data from certification of the MB, IRMM-425

	D/H ratio, certified value ¹⁾	Standard uncertainty ¹⁾	Relative standard uncertainty (%)
IRMM-425	141.9 x 10 ⁻⁶	0.7 x 10 ⁻⁶	0.247

¹⁾ Values are ratios with the measurement unit one; commonly they are expressed in parts per million (ppm)

The values obtained for STA-003m were then compared to those obtained for the MB as follows:

$$x_{\text{mod STA-003m}} = \frac{CV_{\text{IRMM-425}}}{x_{\text{IRMM-425}}} \cdot x_{\text{prel STA-003m}} \quad \text{Equation 8}$$

$x_{\text{mod STA-003m}}$ being the modified individual result for STA-003m on a given appliance after correction (listed in Table D2 of Annex D), $CV_{\text{IRMM-425}}$ being the certified value of the MB, $\bar{x}_{\text{IRMM-425}}$ being the mean value of 6 MB measurements on a given appliance, and $x_{\text{prel STA-003m}}$ being the preliminary individual result for STA-003m on a given appliance.

7.2 Evaluation of results

The obtained results were subjected to visual and statistical analysis. These analyses included Dixon test, Nalimov t-test and Grubbs test to detect possible mean outliers, Cochran and Bartlett test to verify homogeneity of variances, and skewness and kurtosis test to check for normal distribution of instrument means. Outlier testing revealed no outlier, neither for instrument means nor for variances. As ANOVA followed by Snedecor *F*-test indicated that differences among and within instruments are not significant, all 18 individual values were taken into account for calculating the certified value. The uncertainty associated with the characterisation of the SB, u_{char} , was calculated as a combined uncertainty with contributions from the master batch uncertainty, as well as uncertainties derived from MB and SB measurements as follows:

$$u_{\text{char}} = \sqrt{\left(\frac{u_{\text{MB}}}{CV_{\text{MB}}}\right)^2 + \left(\frac{\frac{S_{\text{finSTA-003m}}}{\sqrt{n}}}{x_{\text{finSTA-003m}}}\right)^2} \quad \text{Equation 9}$$

with u_{MB} being the standard uncertainty of the MB, CV_{MB} the certified value of the MB, and $S_{\text{finSTA-003m}}$ and $\bar{x}_{\text{finSTA-003m}}$ being the standard deviation and mean value of all n (18) measurements of the SB after correction with the master batch, respectively. The effective number for degrees of freedom (ν_{eff}) for u_{char} is obtained using the Welch-Satterthwaite equation [2]:

$$v_{eff} = \frac{u_c^4}{\sum_{i=1}^N \frac{u_i^4}{v_i}}$$

Equation 10

Table 6: Uncertainty budget for u_{char}

	Source of uncertainty	Measured value ¹⁾	Standard uncertainty ¹⁾	Probability distribution	Relative uncertainty (%)	Degrees of freedom (v)
CV _{MB}	Certification of MB	141.9	0.311 ²⁾	Normal	0.219 ²⁾	10
meas _{SB}	Comparison of SB and MB under repeatability conditions	153.651	0.050	Normal	0.033	17
				u_{char} (%)	0.222	10 ³⁾
				u_{char}	0.341	

¹⁾ Values are ratios with the measurement unit one; commonly they are expressed in parts per million (ppm)

²⁾ The unrounded u from IRMM-425 was taken

³⁾ Welch-Satterthwaite equation, v_{eff}

8 Value assignment

The certified value of the SB (cv_{SB}) was calculated as arithmetic mean of the 18 individual results obtained after comparison to the MB (Table D2, Annex D). It amounts to 153.651×10^{-6} . The expanded uncertainty of the certified value (U_{CRM}) was calculated using the formula depicted below:

$$U_{CRM} = k \cdot \sqrt{u_{pur}^2 + u_{bb}^2 + u_{sts}^2 + u_{lts}^2 + u_{char}^2}$$

Equation 11

Where k is the coverage factor; a factor of 2 is applied to give approximately 95 % confidence

u_{pur} was estimated as described in section 3

u_{bb} was estimated as described in section 5.1

u_{sts} was estimated as described in section 6.1

u_{lts} was estimated as described in section 6.2

u_{char} was estimated as described in section 7.1

It was decided to include all five individual uncertainties in the uncertainty budget for calculation of the expanded uncertainty, U . u_{pur} , u_{sts} , and u_{lts} can be considered negligible (smaller than one third of the largest uncertainty contribution, u_{char}), since the finally calculated value for U (0.8×10^{-6}) doesn't change irrespective of including only u_{char} and u_{bb} or all standard uncertainties. Nevertheless, the combined standard uncertainty is thus calculated by combining contributions

from purity, homogeneity, short-term stability, long-term stability, and characterisation. The budget is summarized in table 7.

Table 7: Uncertainty budget for STA-003m

	Source of uncertainty	Relative value (%)	Absolute value ¹⁾	Degrees of freedom
u_{pur}	Purity of the material	0.011	0.017	13
u_{bb}	Homogeneity of SB	0.073	0.112	19
u_{sts} ²⁾	Short-term stability of SB	0.034	0.052	22
u_{lts} ³⁾	Long-term stability of SB	0.038	0.058	709
u_{char}	Characterisation of SB	0.222	0.341	10
u_{CRM}	Combined standard uncertainty	0.237	0.364	13
U_{CRM}	Expanded uncertainty, $k=2$	0.474	0.728	

¹⁾ Values are ratios with the measurement unit one; commonly they are expressed in parts per million (ppm)

²⁾ taken from the short-term stability study of IRMM-425

³⁾ taken from the long-term stability study of STA-003k

The relevant numbers of degrees of freedom calculated using the Welch-Satterthwaite equation [2] is sufficiently large to justify the use of a coverage factor $k = 2$ to expand the confidence level to about 95 %.

Table 8. Certified value and expanded uncertainty of the D/H ratio of STA-003m

	Certified value ¹⁾	Expanded uncertainty ($k=2$) ¹⁾
D/H ratio in STA-003m	153.7×10^{-6}	0.8×10^{-6}

¹⁾ Values are ratios with the measurement unit one; commonly they are expressed in parts per million (ppm)

9 Metrological Traceability

The certified value is traceable to the value of V-SMOW via using the master batch IRMM-425 and by testing samples of STA-003m and IRMM-425 under repeatability conditions, thereby strictly adhering to the Community reference method (SNIF-NMR) as defined in Commission Regulation 2676/90 [1]. For V-SMOW, a value of 155.76 ppm was used [6].

10 Instructions for use

10.1 Safety information

The following health and safety clauses apply:

H302	Harmful if swallowed
H361d	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 protection equipment as required
P301+P312	IF SWALLOWED: call a POISON CENTER or doctor/physician if you feel unwell
P308+P318	If exposed or concerned: get medical advice/attention
P330	Rinse mouth

10.2 Storage

Upon receipt, the material should be stored at ambient temperature in the dark. It is recommended to aliquot the content of the bottle in smaller flasks (amber glass) of about 50-60 mL. Flasks shall be tightly closed and kept in a desiccator. After prolonged storage, TMU might turn slightly yellow. This colour change does not significantly alter the isotopic content. Due to the hygroscopicity of TMU, the water content shall be checked periodically (at the beginning and at the end of a smaller flask). If the water content exceeds the value of 3 g/kg, a correction needs to be applied in the numerator of the formula in 5.1 of Annex 8 of Commission Regulation 2676/90 [1] by including the following ratio: $(100 - \text{measured water content in g/100 g})/99.74$. However, the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened samples.

10.3 Minimum sample intake

The sample intake is 1.3 g of TMU when using 10 mm diameter NMR tubes (Commission Regulation 2676/90 [1]).

10.4 Intended use

The material shall be used as internal standard in SNIF-NMR analysis for the determination of the D/H ratios of wine ethanol according to Commission Regulation 2676/90, Annex 8 [1].

11 Acknowledgments

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Annex A - Homogeneity data

Table A1. Results of the homogeneity study of STA-003m. Data was calculated according to formula on page 11 [1], taking the $(D/H)_i$ value from ethanol "QC 02424616" into account.

Ampoule number	Replicate 1 (ppm)	Replicate 2 (ppm)
12	153.95	153.77
51	153.56	153.34
74	153.92	153.74
116	153.29	153.88
140	153.34	153.57
165	153.71	153.60
186	153.09	153.29
222	153.23	153.96
242	153.63	153.80
272	153.52	153.61

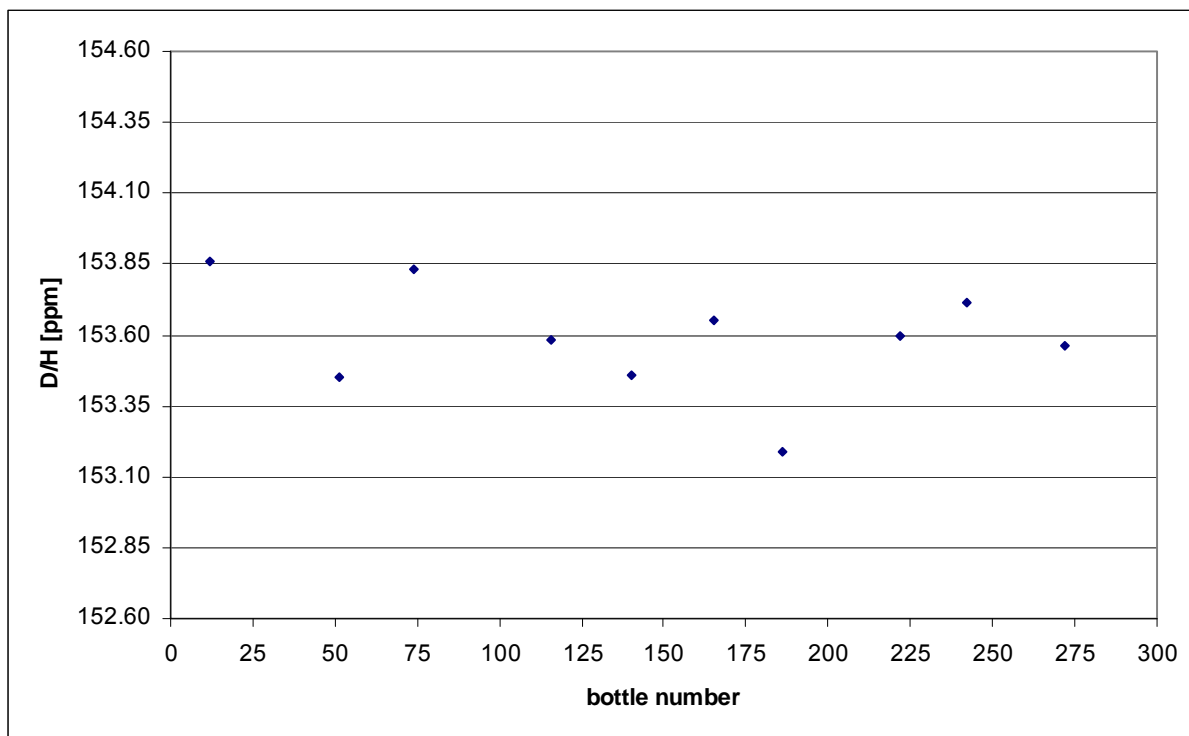


Figure A1. Homogeneity of STA-003m. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of duplicate measurements.

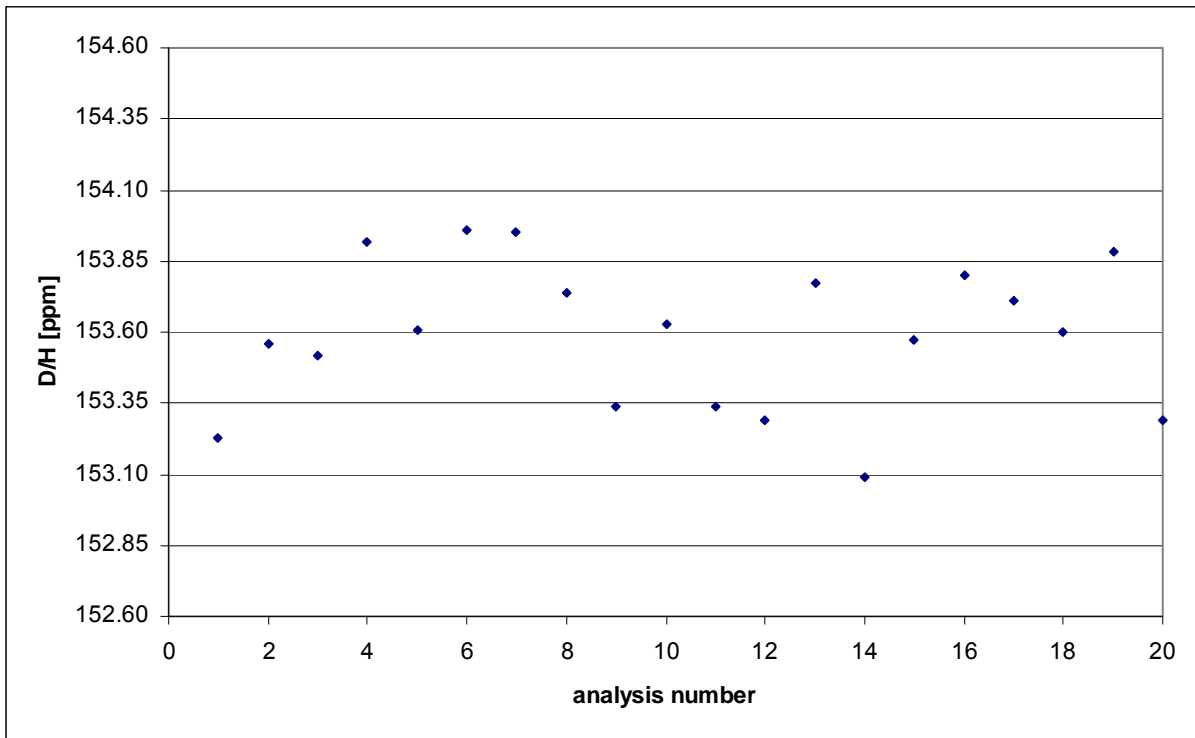


Figure A2. Homogeneity of STA-003m. The x axis depicts the order of measurements (analytical sequence).

Annex B – Long-term stability

Results of the long-term stability study of STA-003k at 20 °C. Data listed have been acquired at Eurofins between June 2008 and July 2011.

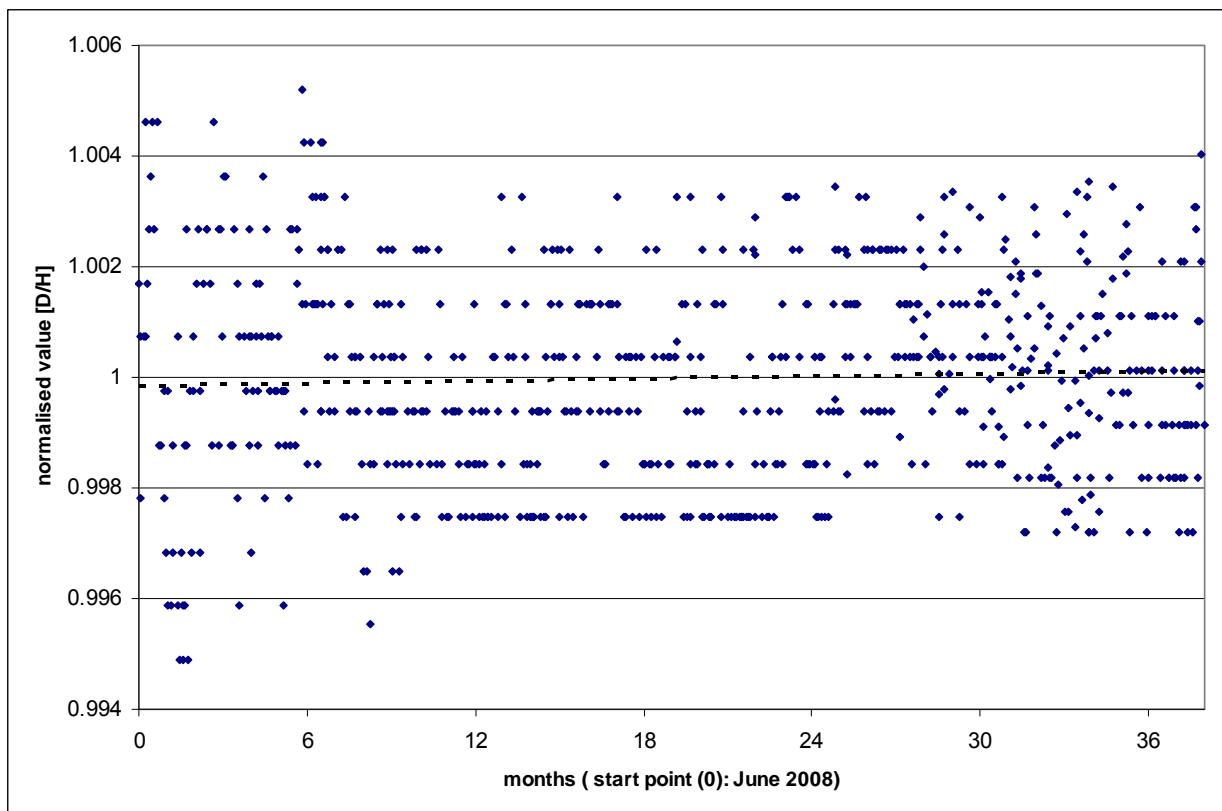


Figure B1. Long-term stability of STA-003k at 20 °C. u_{ITS} was estimated for a shelf life of 5 years using measurement data over a period of 38 months (711 individual measurements done with different ethanols and from different bottles of STA-003k). For comparability reasons and for combining all results, data had to be normalised: for each sub-study (dedicated ethanol), the results were divided by the respective sub-study average result.

Annex C – Characterisation – method-related information

Table C1. The laboratory (3 appliances used) strictly adhered to Community reference method (Comm. Reg. 2676/90, Annex 8, [1]). Method details are listed below.

Data set	Short description of method parameters	Signal property used for calculation	NMR appliance and software	QC measures (instrument verification, QC sample(s) used)
1	Constant probe temperature: 29 °C; acquisition time 6.68 s, relaxation delay 0.1 s; spectral width 19.94 ppm, SFO1 = 61.45 MHz, SFO2 = 400.23 MHz, number of scans: 150	Height	Bruker PG400; in-house software Eurospec	BCR-123, in-house alcohol. Evaporation of ethanol from NMR tube controlled my weighing of tube before and after measurement
2	Constant probe temperature: 29 °C; acquisition time 6.81 s, relaxation delay 0.1 s; spectral width 39.13 ppm, SFO1 = 61.42 MHz, SFO2 = 400.13 MHz, number of scans: 200	Height	Bruker DPX400; in-house software Eurospec	BCR-123, in-house alcohol. Evaporation of ethanol from NMR tube controlled my weighing of tube before and after measurement
3	Constant probe temperature: 29 °C; acquisition time 6.81 s, relaxation delay 0.1 s; spectral width 39.12 ppm, SFO1 = 61.43 MHz, SFO2 = 400.23 MHz, number of scans: 152	Height	Bruker AVIII; in-house software Eurospec	BCR-123, in-house alcohol. Evaporation of ethanol from NMR tube controlled my weighing of tube before and after measurement

Annex D – Characterisation data

Table D1. Characterisation data of STA-003m. Data (ratio of deuterium to hydrogen) is expressed in ppm and has been acquired from TMU/ethanol mixtures strictly adhering to 2676/90 [1]. Values were calculated using the D/H_i value of the in-house ethanol used at Eurofins during the time of the measurements ("QC 02424616") and the formula depicted in section 4. All 6 individual measurement results per instrument for both STA-003m and IRMM-425 are presented.

Instrument 1	"PG400"					
	Sample 1 1 st replicate	Sample 1 2 nd replicate	Sample 2 1 st replicate	Sample 2 2 nd replicate	Sample 3 1 st replicate	Sample 3 2 nd replicate
STA-003m	153.56	153.34	153.29	153.88	153.63	153.80
IRMM-425	141.89	142.09	142.06	141.57	141.58	141.75

Instrument 2	"DPX400"					
	Sample 4 1 st replicate	Sample 4 2 nd replicate	Sample 4 3 rd replicate	Sample 5 1 st replicate	Sample 5 2 nd replicate	Sample 5 3 rd replicate
STA-003m	153.54	153.13	153.16	153.39	153.01	153.16
IRMM-425	141.59	141.54	141.75	141.58	141.53	141.42

Instrument 3	"AVIII"					
	Sample 6 1 st replicate	Sample 6 2 nd replicate	Sample 6 3 rd replicate	Sample 7 1 st replicate	Sample 7 2 nd replicate	Sample 7 3 rd replicate
STA-003m	153.50	153.84	153.28	153.74	153.68	153.32
IRMM-425	141.77	141.90	141.51	141.69	141.91	141.87

Table D2. Individual values obtained for STA-003m after comparison with master batch samples and correction with certified value of MB, as described in equation 8 of section 7.1.

Instrument 1	"PG400"					
	Sample 1 1 st replicate	Sample 1 2 nd replicate	Sample 2 1 st replicate	Sample 2 2 nd replicate	Sample 3 1 st replicate	Sample 3 2 nd replicate
STA-003m	153.64	153.42	153.37	153.96	153.71	153.88

Instrument 2	"DPX400"					
	Sample 4 1 st replicate	Sample 4 2 nd replicate	Sample 4 3 rd replicate	Sample 5 2 nd replicate	Sample 5 1 st replicate	Sample 5 3 rd replicate
STA-003m	153.90	153.49	153.52	153.75	153.37	153.52

Instrument 3	"AVIII"					
	Sample 6 1 st replicate	Sample 6 2 nd replicate	Sample 6 3 rd replicate	Sample 7 2 nd replicate	Sample 7 1 st replicate	Sample 7 3 rd replicate
STA-003m	153.64	153.98	153.42	153.88	153.82	153.46

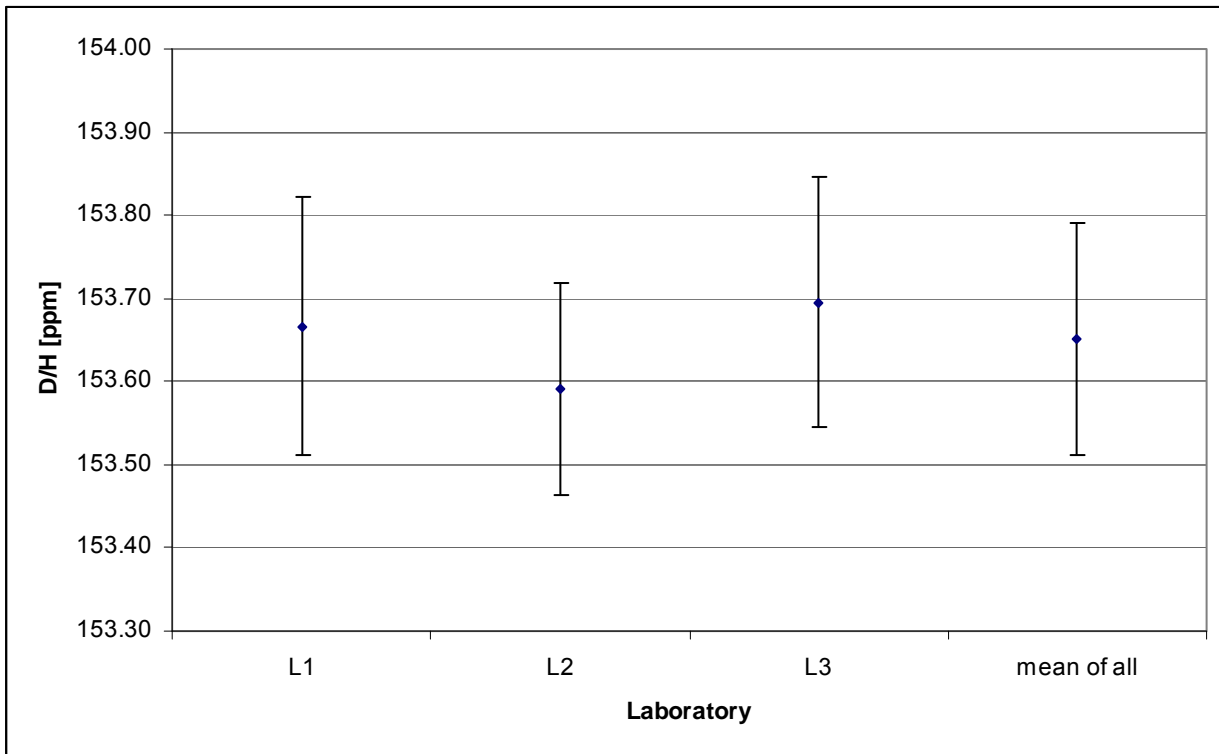


Figure D1. Means from accepted data sets \pm standard deviations ($n = 6$), and mean of all 18 values and standard deviation from the characterisation of STA-003m. It shall be noted that for estimation of u_{char} , the 18 individual result were taken into account.

European Commission

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Abstract

This report describes the production of a tetramethylurea (TMU) reference material (STA-003m), certified for its deuterium-to-hydrogen (D/H) amount-of-substance ratio. The material is to be used as an internal standard in site-specific natural isotope fractionation – nuclear magnetic resonance (SNIF-NMR) spectrometry measurements for determining the D/H amount-of-substance ratios of ethanol distilled from wines, an important measure in wine authenticity testing (Commission Regulation 2676/90. Commercially obtained TMU with a sufficiently high D/H amount-of-substance ratio ($>120 \times 10^{-6}$) was first purified by removing most of the residual water and then filled into amber glass bottles of 500 mL. Between-unit homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006. Batch characterisation was accomplished by comparing STA-003m samples with samples of the TMU master batch (IRMM-425) under repeatability conditions and by adhering to the SNIF-NMR technique. The certified value was calculated as the unweighted mean of 18 individual results obtained on 3 different NMR appliances. The uncertainty of the certified value was estimated in compliance with ISO Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM); it includes contributions from possible inhomogeneity, instability, and characterisation.

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