Certification of the Deuterium-to-Hydrogen (D/H) ratio in a 1,1,3,3-tetramethylurea batch
Certified Reference Material
STA-003k

R. Zeleny, H. Emteborg, F. Ulberth

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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-003k

R. Zeleny, H. Emteborg, F. Ulberth

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Summary

This report describes the production of a tetramethylurea reference material (STA-003k), 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) spectroscopy 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 purified by removing most of the residual water and filled into amber glass bottles of 500 mL portions. Homogeneity was tested and no heterogeneity observed. Stability studies indicated no material degradation for 4 weeks at 60 °C and a very minor degradation over a period of 5 years at 20 °C which. Batch characterisation was accomplished in an inter-laboratory comparison using the SNIF-NMR technique exclusively.

The certified value was obtained as the unweighted mean of the laboratory means of the accepted sets of results. The expanded uncertainty associated (k = 2) includes contributions from (potential) heterogeneity, potential instability, characterisation, as well as the carried-over uncertainty from BCR-656 (96 % wine ethanol) that was used as the internal standard in the measurements.

The certified value and its associated uncertainty are:

	Amount-of-substance ratio	
	Certified value [1,2,4]	Uncertainty [3]
Deuterium-to-hydrogen (D/H) ratio	141.9×10^{-6}	0.9×10^{-6}

1) Traceable to V-SMOV (Vienna Standard Mean Ocean Water) by strictly adhering to the Community reference method (SNIF-NMR, site-specific natural isotope fractionation – nuclear magnetic resonance spectroscopy) as defined in Annex 8, Commission Regulation 2676/90. For V-SMOW, a value of 155.76 ppm was used (Tellus (1970) 22: 712-715).
2) This value was obtained by an inter-laboratory comparison (11 laboratories) employing SNIF-NMR at 46.1 (300), 61.4 (400), and 76.7 (500) MHz for ^2H (^1H). The value has been corrected for the material impurities (including water).
3) Expanded uncertainty with a coverage factor of k = 2, according to the Guide to the Expression of Uncertainty in Measurement, corresponding to a level of confidence of about 95 %.
4) The certified value has a measurement unit of one.

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Glossary

Å	Angström
ANOVA	Analysis of variance
b	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
δ	Chemical shift
df	Degrees of freedom
EU	European Union
FID	Flame ionization detection
GC	Gas chromatography
IHCP	Institute for Health and Consumer Protection
IRMM	Institute for Reference Materials and Measurements
IRMS	Isotope ratio mass spectrometry
ISO	International Organisation for Standardization
JRC	Joint Research Centre
k	Coverage factor
KFT	Karl Fischer titration
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
s	Standard deviation
s_{bb}	Between-bottle (in)homogeneity standard deviation
SNIF-NMR	Site-specific natural isotope fractionation – nuclear magnetic resonance
s_{wb}	Within-bottle standard deviation
t	t value
t_m^D	Alcoholic grade of BCR-656, expressed in mass %
TMU	Tetramethylurea
u_{bb}	*Standard uncertainty due to the between-bottle variation*
u^*_{bb}	Standard uncertainty due to the inhomogeneity that can be hidden by the method repeatability
$u_{BCR-656a}$	Uncertainty of D/H$_I$ value of BCR-656
$u_{BCR-656b}$	Uncertainty of the t_m^D value of BCR-656
u_{char}	Standard uncertainty from characterisation
U_{CRM}	Expanded uncertainty associated with the CRM
u_{lts1}	Standard uncertainty associated with long-term (in)stability
u_{lts2}	Additional uncertainty contribution to long-term (in)stability due to observed degradation
u_{lts}	Total uncertainty associated with long-term (in)stability
V-SMOW	Vienna Sea Mean Ocean Water

1 Introduction

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) outside 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®) [2]. To monitor wine authenticity, the measured values are compared with those from the respective authentic wines listed in the European Union (EU) Wine Databank [3], operated by the European Office for Wine, Alcohol and Spirit Drinks (BEVABS) at the Joint Research Centre (JRC) of the European Commission.

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 Institute for Reference Materials and Measurements (IRMM) – current batch "STA-003". This compound possesses appropriate physico-chemical properties, such as a low volatility, and excellent 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.
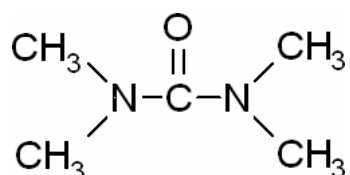
Figure 1. 1,1,3,3 – Tetramethylurea, CAS Number: 632-33-4

The first TMU reference material was certified measuring mixtures of TMU with V-SMOW (Vienna - Standard Mean Ocean Water) by SNIF-NMR [4]; 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, which is reflected in the high coverage factor (4.3) of the expanded uncertainty.

IRMM revised the certification strategy and produced a TMU master batch, to which all future secondary batches shall be linked. Isotope Ratio Mass Spectrometry was evaluated as an alternative methodology for value assignment, but was not found suitable due to a systematic bias between IRMS and NMR results [5]. Finally, SNIF-NMR was used, with BCR-656 (ethanol with 96 % m/m; [6]) serving as internal standard. Measurements were accomplished in a larger number of laboratories to reduce the coverage factor and thus the overall expanded uncertainty. Details about the master batch certification concept can be found in the certification report of IRMM-425 [7].

The STA-003k batch consists of the very same material as IRMM-425 (same TMU raw material, purified, and bottled – details see chapter 4). However, IRMM-425 was additionally aliquoted in amber glass ampoules and stored at -20 ºC until use [7], whereas STA-003k is stored in the dark at room temperature until sale.

It shall be noted that the certified value and its corresponding uncertainty are expressed as a ratio (141.9 ± 0.9 x 10^{-6}) with the measurement unit of one.

2 Time table of the project

Activity	Finalised by
Processing	April 2005
Homogeneity testing	January 2006
Short-term stability testing	January 2006
Long-term stability testing*	March 2007
Characterisation	April 2007

* data evaluation

3 Participants

Processing

Eurofins Scientific Analytics, Nantes, FR

Homogeneity and stability studies

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   (ISO/IEC 17025 accreditation; COFRAC Nº 1-0287)
Bundesinstitut für Risikobewertung, Berlin, DE
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European Commission, Joint Research Centre, Institute for Health
   and Consumer Protection, Ispra, IT
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   (ISO/IEC 17025 accreditation; IPAC L-0324)
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Landesuntersuchungsamt Rheinland-Pfalz, Speyer, DE
   (ISO/IEC 17025 accreditation; SAL-RLP-L 19.04.03)
Ministry of Health, State General Laboratory, Nicosia, CY
   (ISO/IEC 17025 accreditation; ESYD 290)
National Institute of Chemistry, Ljubljana, SI
*In alphabetical order

Project Management & Data evaluation

European Commission, Joint Research Centre, Institute for Reference
         Materials and Measurements, Geel, BE
   (ISO Guide 34 accreditation; BELAC 268-TEST)

4 Processing of the material

A batch of about 100 litres of TMU was acquired in China with a sufficiently high D/H amount-of-substance ratio (> 120 ppm). This material, which contained about 5 g/kg of water, was aliquoted in 4 L portions and further processed to remove most of the water from the product, as water hydrolyses TMU to trimethylurea and other degradation products. Effective removal of most of the water was accomplished by adding 500 grams of molecular sieve (type 3A, pore size 3 Å) to each TMU portion. After a contact time of 48 hours, the material was filtered to remove the molecular sieve, and pooled in a large vessel. After homogenisation, the TMU was aliquoted into 500 mL portions (unit size) in dark brown glass bottles, tightly closed and covered with a wax stopper. 180 bottles were produced in total.

Material identity and purity was elaborated at Eurofins. The identity of TMU was investigated performing GC-MS measurements using an ion-trap mass spectrometer, and comparing the obtained spectrum with the NIST library spectrum of TMU. For all 3 independently sampled aliquots, identity could be confirmed.

Purity assessment was accomplished employing GC-FID, ^{1}H-NMR, and ^{13}C-NMR. GC-FID gave an average value of 99.89 ± 0.01 % (n = 3), calculated as TMU area to total peak area in the chromatogram. ^{1}H NMR (n = 3) and ^{13}C NMR (n = 3) agreed with literature data and did not show any detectable impurity. The final product exhibited a water content of 0.8 ± 0.1 g/kg (n = 4) as determined by Karl Fischer titration (KFT; shown in Table 1).

Taking into account these data and the NMR results, it was concluded that no major impurities remained undetected, and that combining GC-FID and KFT data represent a suitable approach to estimate the TMU purity and to calculate a realistic estimation of the uncertainty related to impurity. The sum of impurities detected by GC-FID and KFT was calculated, and the corresponding purity of TMU yielded 99.81 %. The uncertainty of the combined impurities detected by GC-FID and KFT was estimated by calculating the square root of the sum of the squares of the 2 contributions, and yielded 0.01 %.

Quality control after processing

After processing, four samples were submitted for water analysis by coulometric Karl Fischer titration. Table 1 shows the results, confirming the expected low water levels in the final TMU candidate reference material.

Table 1. Water content in STA-003k as measured by Karl Fischer titration

Bottle n.	Water content, [g/kg]
05018	0.8
05056	0.9
05124	0.8
05190	0.7

5 Analytical methodology used

All measurements were accomplished by employing site-specific nuclear isotope fractionation-nuclear magnetic resonance (SNIF-NMR) spectroscopy in accordance with Commission Regulation 2676/90 [1]. BCR-656 (96 vol% ethanol) was provided by IRMM and used as the internal standard. This material is certified for two site-specific ratios of deuterium to hydrogen in the ethanol molecule: D/H_I refers to the peak to the right of the TMU peak (methyl group, CH_2DCH_2OH), whereas D/H_{II} refers to the peak to the left of the TMU peak (methylene group, CH_3CHDOH), [1]. The hydroxy group signal (CH_3CH_2OD) was not evaluated (figure 3).

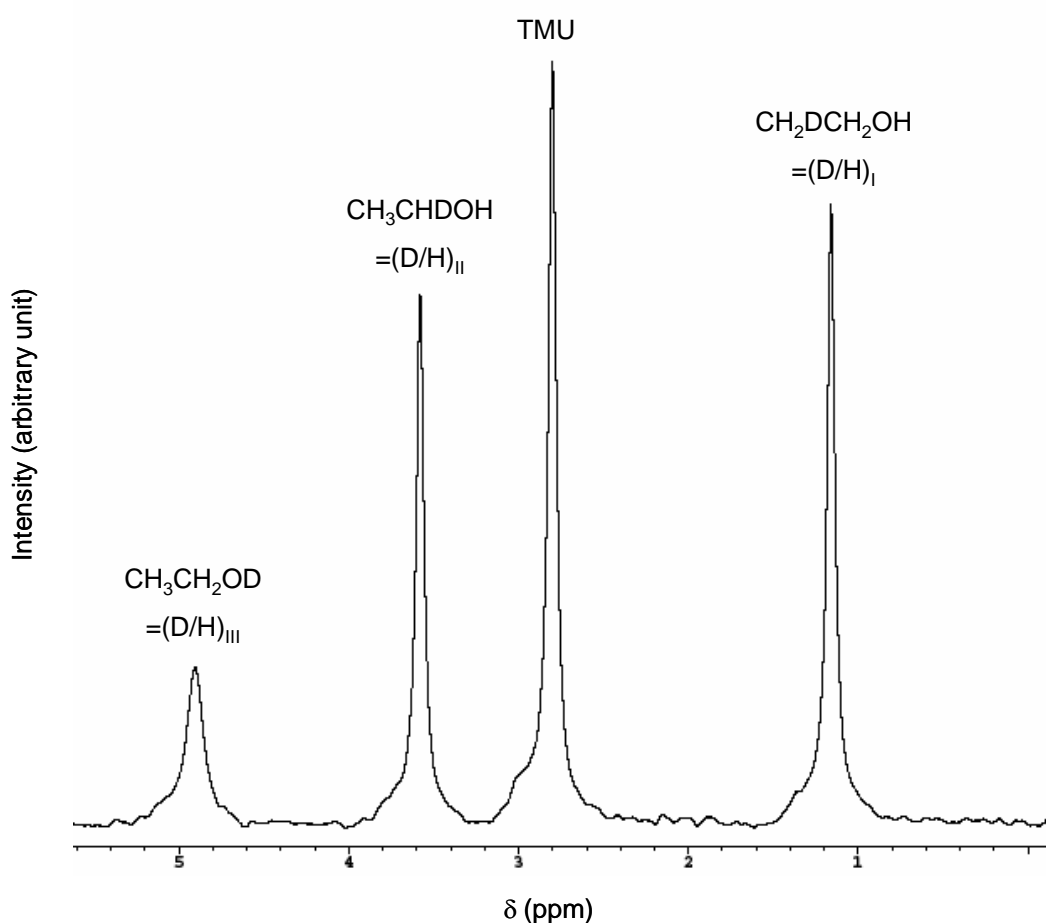**Figure 2**. Representative ^2H spectrum of ethanol/TMU mixture. Ethanol reveals three peaks, reflecting the position of the deuterium in the molecule

Measurements were acquired on 300, 400 or 500 MHz NMR appliances. 10 mm diameter NMR tubes were used.

For sample preparation, the TMU ampoules were opened and two NMR tubes were prepared immediately per ampoule (duplicate analysis). Sample preparation was done by weighing in

ca. 3.2 mL of reference alcohol BCR-656 (ca. 2.5 g) and ca. 1.3 mL of TMU (ca. 1.3 g); finally, some 100 - 200 μL (ca. 0.2 – 0.3 g) of the lock substance hexafluorobenzene 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 laboratory QC samples; most frequently, BCR-123 (3 different ethanols from beet, grape, and cane origin) was used [8]; some laboratories also used in-house ethanols distilled from wine with an assigned D/H value.

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 D).

The formulae depicted below and stipulated in [1] are used to calculate the ethanol D/H values using the certified TMU D/H value.

$$(D/H)_I = 1.5866 \times T_I \times \frac{m_{st}}{m_A} \times \frac{(D/H)_{St}}{t_m^D} \qquad\qquad (D/H)_{II} = 2.3799 \times T_{II} \times \frac{m_{st}}{m_A} \times \frac{(D/H)_{St}}{t_m^D}$$

with

T_I, height of signal I (CH_2DCH_2OH) divided by height of signal of TMU
T_{II}, height of signal II (CH_3CHDOH) divided by height of signal of TMU
m_{st}, mass of internal standard (TMU) to nearest 0.1 mg
m_A, mass of distilled wine ethanol (BCR-656) to nearest 0.1 mg
$(D/H)_{St}$, certified value of TMU
t_m^D, strength by mass of the wine ethanol in % (m/m), whereas

$$t_m^D = \frac{p - p'}{p} \times 100 \text{, whereas}$$

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

The factors of 1.5866 and 2.3799, respectively, derive from:

$$1.5866 = \frac{M_A}{M_{St}} \times \frac{P_{St}}{P_I} \qquad\qquad\qquad 2.3799 = \frac{M_A}{M_{St}} \times \frac{P_{St}}{P_{II}}$$

with
M_A, molecular mass of ethanol
M_{St}, molecular mass of internal standard (TMU)
P_{St}, number of hydrogen atoms in TMU molecule
P_I, number of hydrogen atoms at site I of the ethanol molecule
P_{II}, number of hydrogen atoms at site II of the ethanol molecule

In this exercise, the formula was inversed to calculate the TMU D/H value using either the certified D/H$_I$ or D/H$_{II}$ value of BCR-656.

The values and their corresponding uncertainties of BCR-656 [6] used in this study are listed in Table 2:

Table 2. Relevant certified values and uncertainties of BCR-656

	Certified value	Uncertainty*
D/H$_I$ in ppm	102.84	0.20
t_m^D in % (m/m)	94.61	0.05

* Expanded uncertainty, indicated as the half-width of the 95% confidence interval of the certified value

Preferentially, the D/H$_I$ value of BCR-656 is used for the calculation of the TMU value, due to the favourable stoichiometric factors - 3 H in the case of D/H$_I$ (CH$_2$D-CH$_2$-OH) signal, to 12 H in TMU - compared to 2 H in the case of D/H$_{II}$ (CH$_3$-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 [9], which also underpins the decision to use solely the certified value of D/H$_I$ of BCR-656 for calculating the D/H ratio of the TMU master batch and the uncertainty contributions.

Consequently, only the values calculated using the D/H$_I$ of BCR-656 were further on taken into account (homogeneity, stability, and characterisation measurements).

6 Homogeneity and minimum sample intake

6.1 Homogeneity

Four samples (about 2.2 % of the batch) 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 (500 MHz instrument). Measurements were performed under repeatability conditions.

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 heterogeneity. Data were scrutinised for outliers and normal distribution using normal probability plots. One outlier was detected in the filling sequence using the Grubbs test (95 % level of confidence), whereas no outlier was in the analytical sequence. The outlier was not excluded as data scrutiny revealed no technical reason to do so. Due to the limited amount of data, normal distribution of the data could be not be verified statistically; visual inspection however concluded suitability of data for regression analysis. The slopes of the regression lines were tested for their significance at 95 % and 99 % level of confidence: Table 3 summarizes the results of the homogeneity study; no significant trend was observed for neither filling order nor analytical sequence.

Table 3. Linear regression and statistical parameters associated to the homogeneity evaluation of STA-003k

Statistical parameters	Filling sequence	Analytical sequence
Slope (b)	-0.00265	0.00812
Standard error of slope (s_b)	0.0011	0.04674
Degrees of freedom (df)	2	6
$|b|/s_b$	2.41	0.17
$t_{(0.05;2)}$	4.30	-
$t_{(0.05;6)}$	-	2.14
Statistical significance (95% confidence interval)	No	No

The pure between-bottle variation s_{bb} was calculated using the following formula [10]:

$$s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}}$$

where $MS_{between}$ and MS_{within} are the mean of squares between and within groups, respectively (taken from ANOVA), and n is the number of replicates per sample

The value obtained was s_{bb} = 0.113 % using the certified D/H_l value of BCR-656. The calculated s_{bb} holds for the sample intake laid down in Commission Regulation 2676/90, that is about 2.5 g of ethanol and about 1.3 g of TMU, using a 10 mm diameter NMR tube (standard dimension). s_{bb} was taken as an estimate for u_{bb}, the standard uncertainty due to between-bottle (in)homogeneity.

Table 4. Summary of the homogeneity test

	Average	s_{bb}	s_{bb} %	s_{wb}	s_{wb} %
D/H ratio	141.842	0.161	0.113	0.238	0.168

The estimated value for s_{bb} is small, as can be expected for a liquid. 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 $MS_{between} < MS_{within}$, [7]). It can thus be concluded that STA-003k is a homogeneous material.

6.2 Minimum sample intake

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

7 Stability studies

7.1 Short-term stability

The data was taken from the study conducted in the frame of the master batch certification (IRMM-425).

A four weeks isochronous study [11] was performed to evaluate appropriate transport conditions. Samples were selected from the produced batch using a random stratified sample picking scheme. Measurements were performed under repeatability conditions.

Samples were stored at 18 °C and 60 °C and at a reference temperature of -20 °C. Three ampoules were stored at each temperature for 0, 1, 2, and 4 weeks. After the indicated storage periods, the samples were transferred to storage at -20 °C until analysis. Samples were analysed in duplicate in the order predefined at IRMM (randomised sample order) using SNIF-NMR as described above (400 MHz instrument). Care was taken that not more than 7 measurements were performed in between two replicate measurements of one given sample. This was a technical requirement as the two replicates from one sample (ampoule) are prepared at the same time, and due to the volatility of TMU evaporation might occur in the non-sealed NMR tubes (C. Fauhl, Berlin, personal communication). Measurements were performed under repeatability conditions.

Measurement results were evaluated for possible significant trends due to the storage conditions. One outlier was detected using the Grubbs test at 18 ºC (95 % level of confidence), whereas no outlier was detected at 60 ºC. The outlier was not excluded as data scrutiny revealed no technical reason to do so.

The measurement results and resulting graphs are presented in Annex B. The observed slopes were tested for significance using a t-test, with $t_{\alpha,\mathrm{df}}$ being the critical t-value (two-tailed) for a confidence level $\alpha = 0.05$ (95 % confidence interval). The slope was considered as statistically significant when $b/s_{\mathrm{b}} > t_{\alpha,\mathrm{df}}$. No significant slope at 95 % level of confidence was detected for both temperatures tested (Table 5).

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Table 5. Linear regression and statistical parameters associated to the short term stability evaluation of IRMM-425

Storage Temperature	18 ºC	60 ºC
Statistical parameters		
Slope (b)	-0.015	0.016
Standard error of slope (s_b)	0.038	0.048
Degrees of freedom *(df)*	22	22
$|b|/s_b$	0.39	0.33
$t_{(0.05;22)}$	2.07	2.07
Statistical significance (95% confidence interval)	No	No

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.

7.2 Long-term stability

In contrast to the short-term stability study, other data then those obtained for the master batch have been used to calculate the uncertainty contribution related to long-term storage. This was necessary, as the STA-003k batch is stored at room temperature, whereas the master batch is kept at -20 ºC. Stability data from a previous TMU batch produced at Eurofins was used to estimate the uncertainty contribution associated to long-term (in)stability. Measurements were performed over a period of 59 months, whereby on average every week one analysis was carried out. Reference ethanol (in-house samples) with an assigned D/H$_l$ ratio was used to calculate the D/H ratio of TMU. Sampling of TMU was done out of different bottles from a given TMU batch, and from each bottle sampling for about 3 – 15 measurements were performed, corresponding to a bottle usage period between 2 weeks and 4 months. Bottles were only closed, but not re-sealed between the sampling dates. This study set-up can therefore be considered a worst-case approach which renders a conservative estimation of the uncertainty contribution associated with long-term (in)stability.

Data evaluation was done in the same way as for the short-term stability study. No outliers were detected (single and double Grubbs test). Table 6 summarizes the results.

Table 6. Evaluation of long-term stability study, 60 months

Storage Temperature	20 ºC
Statistical parameters	
Slope (b)	-0.007
Std error slope (s_b)	0.001
df	205
$|b|/s_b$	6.94
$t_{(0.05;34)}$	1.74
Statistical significance (95 % confidence interval)	Yes

A statistically significant instability was detected, therefore the corresponding uncertainty associated to long-term (in)stability, u_{lts}, was calculated as follows.

The standard uncertainty associated with (in)stability, u_{lts1}, was estimated for a shelf-life of 5 years using the following formula [12]:

$$u_{lts1} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot x$$

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

As the observed data revealed a statistically significant degradation, a second uncertainty term ("additional uncertainty contribution to long-term (in)stability due to observed degradation") had to be introduced [13]. It was calculated using the following formula, whereby a rectangular distribution was assumed:

$$u_{lts2} = \frac{b \cdot x}{Intercept} \cdot \frac{1}{\sqrt{3}}$$

where

b is the slope of the regression line using all data

$Intercept$ is the intercept of the regression line using all data

The total uncertainty associated with long-term (in)stability was calculated using the following formula:

$$u_{lts} = \sqrt{u_{lts1}^2 + u_{lts2}^2}$$

The relationship between u_{lts} and shelf-life is illustrated Annex C.

In conclusion, the TMU batch was found to exhibit a minor degradation (about 0.25 % of the certified value after 5 years at room temperature). However, 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 ºC in the dark.

8 Characterisation

Batch characterisation was accomplished in an inter-laboratory comparison. The data from IRMM-425 was used [7], because the D/H ratio for the master batch and secondary batch are the same. This is justified, as the material is the same except the additional processing step for the master batch. Stability data revealed very similar u_{lts} values for the master batch and a previous TMU processed and handled exactly the same way as the secondary STA-003k batch, suggesting very limited to neglible degradation of the materials under the applied storage conditions. Finally, the mean value of the homogeneity study performed on STA-003k ($141.57 \pm 0.28 \times 10^{-6}$, n = 8, value after correction of purity) is in excellent agreement with the value submitted by Eurofins for IRMM-425 ($141.78 \pm 0.27 \times 10^{-6}$, n = 6, same NMR appliance, value after correction of purity) and the certified value of IRMM-425 ($141.9 \pm 0.7 \times 10^{-6}$).

Laboratories needed to have proven experience in the isotopic analysis of wine ethanol samples by SNIF-NMR and were selected according to the quality criteria required by IRMM. In several cases, laboratories were accredited to ISO 17025; otherwise, the required measurement performance was demonstrated by successful participation in PT schemes, respective quality control charts, and scientific publications in the field.

8.1 Discussion of results

Laboratories had to comply strictly with 2676/90 [1] and thus used the methodology outlined above. Small method variations were mainly due to different analytical appliances, software versions used, the exact amounts of TMU, BCR-656 and lock substance, and differences in calculations as outlined below (correction of water content or not, etc.). Methodical details such as NMR spectrometer type, software and QC measures are listed in table 1 (Annex E). Each of the 11 laboratories received three units of IRMM-425 randomly selected from the batch. Laboratories were asked to analyse the samples in duplicate under intermediate precision conditions, i.e. 2 days à 3 measurements, whereby replicates of a given sample had to be analysed on different days (day 1: sample 1A, 2A, 3A; day 2: samples 1B, 2B, 3B). Appropriate performance of instrument and method had to be indicated by providing data on QC samples before and after the series. Laboratories were asked to provide all raw data and calculation of the D/H values, a representative spectrum and methodical details.

Data were scrutinized at IRMM and eventually re-calculated, whereby the following considerations applied:
- The value for one sample (replicate), being the mean of 10 acquired spectra, can be calculated in two ways: either the average of the peak heights (areas) for each signal is

taken, and the respective values are then used in the formula to calculate the D/H value (approach 1), or the obtained ethanol and TMU peak heights (areas) in each spectra are taken and used in the formula, and then the average of the 10 calculated values is taken as the result for one sample (approach 2); the differences were found to be very minor in all cases (< 0.02 x 10^{-6}). Approach 2 was used to re-calculate data (when necessary) at IRMM.

- Minor rounding differences (< 0. 02 x 10^{-6}) were sometimes observed between the values calculated and submitted by the laboratory, and re-calculations of the provided raw data performed at IRMM.

Laboratory-specific remarks:

Lab 3: The result of one spectrum in each of two of the samples was indicated to be an outlier (in-house laboratory statistical analysis). However, as no technical reason was found to exclude them, those values were retained and used for the calculation of the certified value.

Lab 5: Due to a lack of sufficient amount of BCR-656, only 1.7 mL of ethanol could be used for preparation of the last sample (usual amount is ca. 3.2 mL per sample); since this individual result did not deviate from the other five which were obtained strictly following the procedure laid down in [1], it was retained in the laboratory data set.

Lab 6: In one sample, considerably worse repeatability was obtained among peak heights within the acquired 10 spectra. No technical reason could be attributed to this finding, and the overall mean of this sample complied well with mean values of the other five samples. The value was thus retained. Furthermore, this laboratory was the only one where a considerable difference for the TMU value was obtained when using either the D/H_I value of BCR-656 or its D/H_{II} value. No reasonable explanation could be given for this finding. However, as explained earlier, only the values calculated using the D/H_I value of BCR-656 are taken into account for establishing the certified value.

Lab 7: One of the ampoules provided was also checked for the water content, which was found to be < 1.1 g/kg. Due to a lack of sufficient amount of BCR-656, only 1.9 mL of ethanol could be used for preparation of the last sample (usual amount is ca. 3.2 mL per sample); since this individual result did not deviate from the other five which were obtained strictly following the procedure laid down in [1], it was retained in the laboratory data set.

Lab 8: One of the ampoules provided was also checked for the water content, which was found to be < 1.1 g/kg. Due to a lack of sufficient amount of BCR-656, a laboratory in-house

ethanol (established D/H$_I$ value of 103.00 ppm) was used instead for the preparation of the last sample; the result was retained in the laboratory data set.

Lab 9: One of the ampoules provided was also checked for the water content, which was found to be < 1.1 g/kg.

Lab 10: The D/H value for TMU was calculated by correcting for residual water (1.6 g/kg as determined by Karl Fischer titration at the laboratory); furthermore, rounded molecular masses for TMU and ethanol of 116 and 46 g/mol were used, respectively. Therefore, the TMU D/H values were recalculated at IRMM, using the coefficients in 2676/90 and leaving out the correction for the residual water.

Lab 11: The laboratory re-examined the alcoholic grade of the 2 bottles of BCR-656 provided and reported values of 94.38 and 94.45 % (m/m), respectively, which were then used in the calculation of the TMU D/H value; therefore, the TMU D/H values were re-calculated at IRMM. The laboratory repeated the analysis of the 3rd ampoule due to instrumental problems in the first series for this sample. Analysis was done on 2 different days.

Finally, all data sets were subjected to visual and statistical analysis. These analyses included Dixon test, Nalimov t-test and Grubbs test to detect possible outliers, Cochran and Bartlett test to verify homogeneity of variances, and skewness and kurtosis test to check for normal distribution of laboratory means.

Outlier testing revealed one outlier (laboratory 11) with the Nalimov t-test, whereas the other two tests did not indicate any outliers. After data inspection (laboratory data including the QC samples), it was decided to retain this value for the calculation of the overall mean. Both Cochran and Barlett test indicate the homogeneity of variances, and skewness and kurtosis test revealed normal distribution of laboratory mean values. In conclusion, no data was rejected, and u_{char} was calculated as

$$u_{char} = \frac{s}{\sqrt{n}}$$

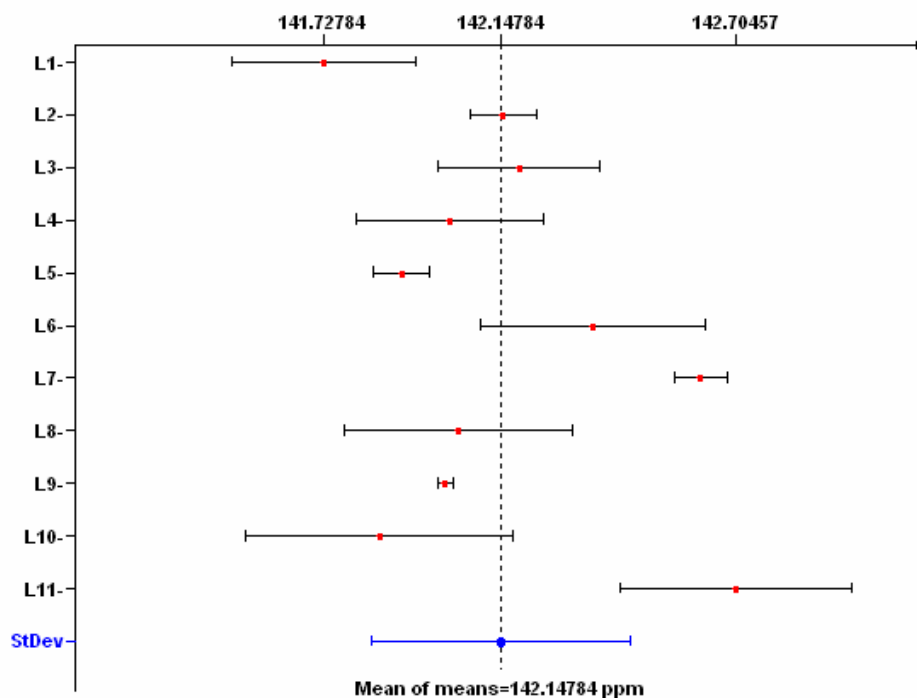s being the standard deviation of the laboratory means, and n the number of accepted data sets (laboratories).

Figure 3. Mean of the laboratory means and its standard deviation, and means from all accepted data sets ± standard deviations (n = 6) from the characterisation of STA-003k

Table 7. Mean of the means of the accepted data sets of results, standard uncertainty from characterisation (u_{char}) and relative uncertainty from characterisation (u_{char}%)

Property	Mean of means [1]	Accepted data sets (p)	u_{char} [1]	u_{char} [%] [2]
D/H ratio	142.147 x 10^{-6}	11	0.092 x 10^{-6}	0.065

[1] Mean value is a ratio; commonly it is expressed in parts per million (ppm)

[2] Relative u_{char} (in percent of the certified value)

The overall mean value finally was corrected for the purity of the material (99.81 % m/m, page 8), thus yielding a value of 141.877 x 10^{-6} (141.877 ppm).

8.2 Certified value and uncertainty budget

Uncertainties of the certified value of an individual unit (U_{CRM}) were calculated using the formula depicted below. In addition to the usually included contributors for homogeneity, long-term stability, and characterisation, also the uncertainties carried over from BCR-656 (D/H$_I$ value, alcoholic strength value) were taken into account. The calculated uncertainty

due to purity, however, was not included due to its negligible contribution to the total uncertainty.

$$U_{CRM} = k \cdot \sqrt{u_{bb}^2 + u_{lts}^2 + u_{char}^2 + u_{BCR-656_a}^2 + u_{BCR-656_b}^2}$$

where U_{CRM} is the expanded uncertainty of the certified value

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

u_{bb} is the uncertainty associated with between bottle (in)homogeneity

u_{lts} is the total uncertainty associated with long-term (in)stability (page 17)

u_{char} is the uncertainty associated with the characterisation of the material

$u_{BCR656a}$ is the uncertainty of the D/H_I value of BCR-656

$u_{BCR-656b}$ is the uncertainty of the t_m^D value of BCR-656

Table 8. Uncertainty budget for STA-003k

u_{bb} [%][1]	0.113
u_{lts} [%][1]	0.245
u_{char} [%][1]	0.065
$u_{BCR-656\ a}$ [%][1]	0.089
$u_{BCR-656\ b}$ [%][1]	0.024
u_{pur} [%][1]	negligible
u_{CRM} [%][1]	0.293

[1] The relative uncertainties are listed (percent of the certified value)

[2] Values are ratios; commonly they are expressed in parts per million (ppm)

Table 9. Certified value and expanded uncertainty of the D/H ratio of STA-003k

Certified value [1]	141.9 x 10^{-6}
U_{CRM} (k = 2) [1]	0.9 x 10^{-6}

[1] Values are ratios; commonly they are expressed in parts per million (ppm)

9 Metrological Traceability

The certified value is linked to BCR-656, which itself is linked to the penultimate TMU batch. This TMU was certified using reference ethanols, which themselves were calibrated against a former TMU batch. The first TMU reference material [4] was certified measuring mixtures of TMU with V-SMOW (Vienna Standard Mean Ocean Water).

The certified value is thus traceable to V-SMOW by 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 (Tellus (1970) 22: 712-715).

10 Instructions for use

10.1 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.

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 by including the following ratio: (100 – 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 Safety precautions

The following health and safety clauses apply:

S 23 Do not inhale gas/fumes/vapour/spray

S 28 After contact with skin, wash immediately with plenty of water

S 37 Wear suitable gloves

S 45 In case of accident or if you feel unwell, seek medical advice immediately

11 Acknowledgments

The authors would like to thank M. Lees (Eurofins) and C. Guillou (JRC/IHCP/BEVABS) for fruitful discussions on a suitable strategy for the certification of the batch, and T. Linsinger and G. Roebben (IRMM) for reviewing certification report and certificate.

12 Annexes

Annex A: Results of the homogeneity study
Annex B: Results of the short-term stability study
Annex C: Results of the long-term stability study
Annex D: Characterisation – method-related information
Annex E: Results of the Characterisation study

13 References

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Annex A - Homogeneity data

Results of the homogeneity study of STA-003k. Data was calculated according to formula on page 11 [1], taking the $(D/H)_I$ value from BCR-656 into account.

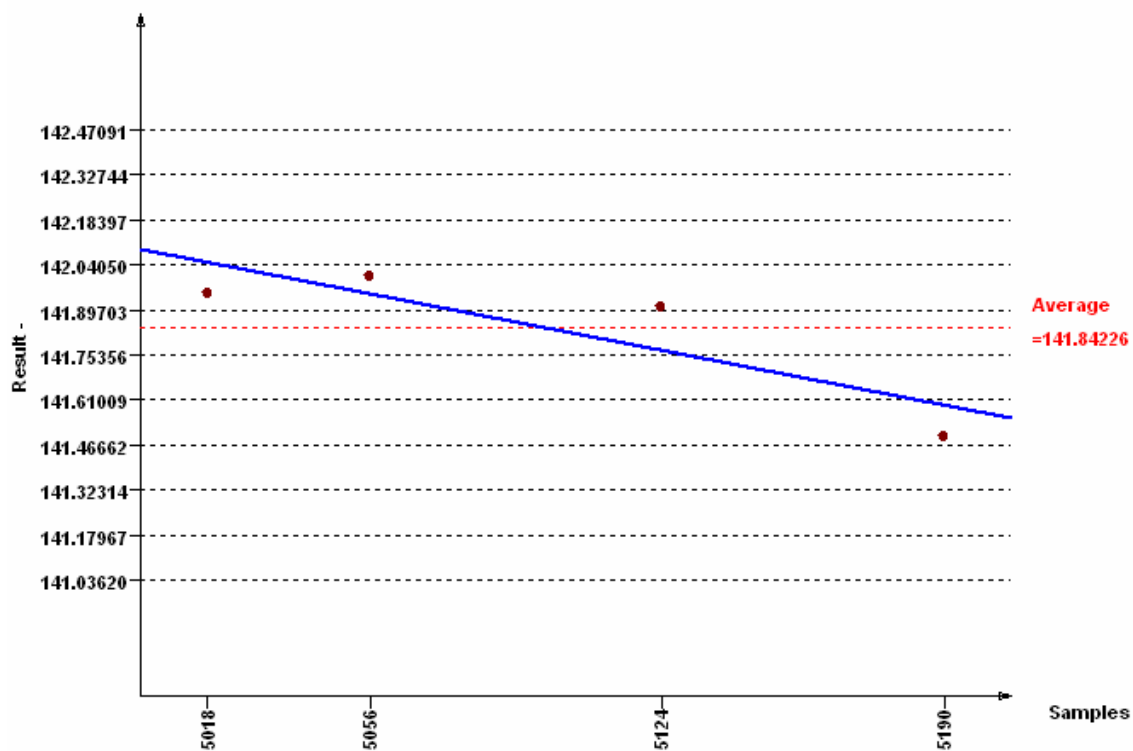| Ampoule number	Replicate 1 (ppm)	Replicate 2 (ppm)	Average (ppm)
Series 1, $(D/H)_I$ signal taken			
05018	141.91	142.00	141.94
05056	142.00	142.02	141.80
05124	141.58	142.24	141.89
05190	141.43	141.57	141.74

Figure A1.Homogeneity of STA-003k. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of duplicate measurements.
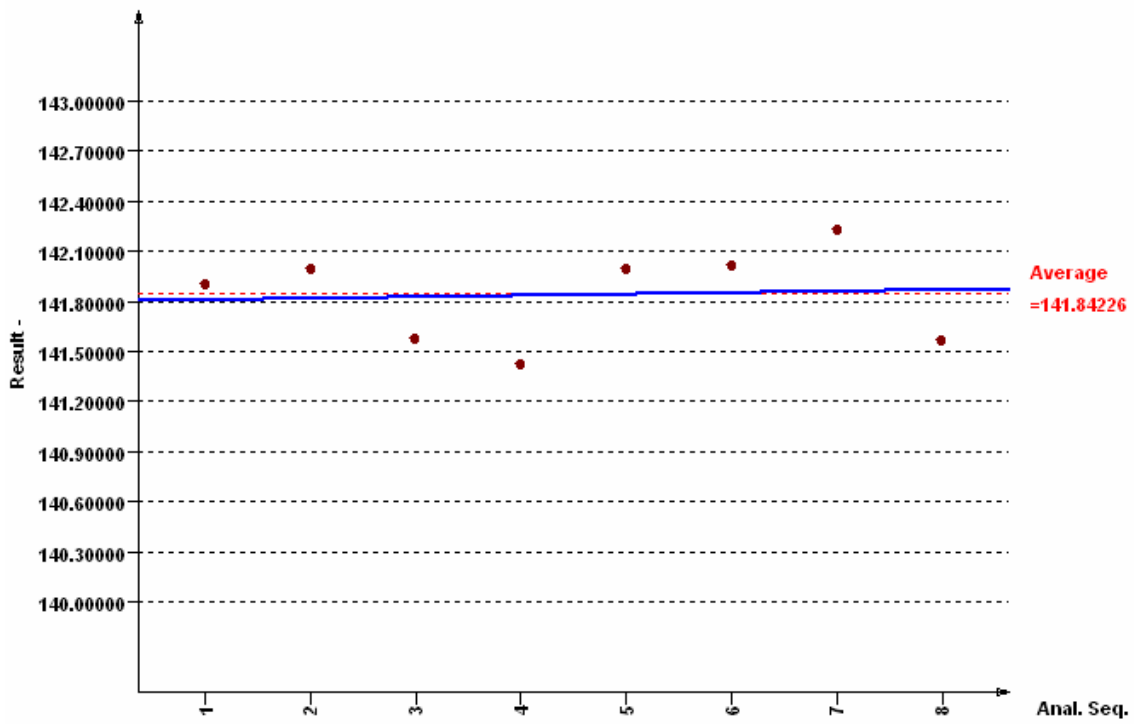
Figure A2. Homogeneity of STA-003k. The x axis depicts the order of measurements (analytical sequence).

Annex B – Short-term stability

Results of the short-term stability study of STA-003k. Data was calculated according to formula on page 11 [1], taking the $(D/H)_I$ value from BCR-656 into account.

Time (weeks)	D/H_{TMU} (in ppm)	
	18°C	60°C
0	141.71	141.71
0	141.48	141.48
0	141.59	141.59
0	141.55	141.55
0	140.92	140.92
0	141.45	141.45
1	141.58	140.97
1	141.69	141.46
1	141.49	141.53
1	141.13	141.56
1	141.51	141.88
1	141.42	140.84
2	141.00	141.41
2	141.35	141.61
2	140.61	141.07
2	141.59	141.07
2	141.26	140.91
2	141.52	140.86
4	141.64	141.65
4	141.13	141.07
4	141.46	142.10
4	141.44	141.69
4	141.27	141.37
4	141.61	141.24
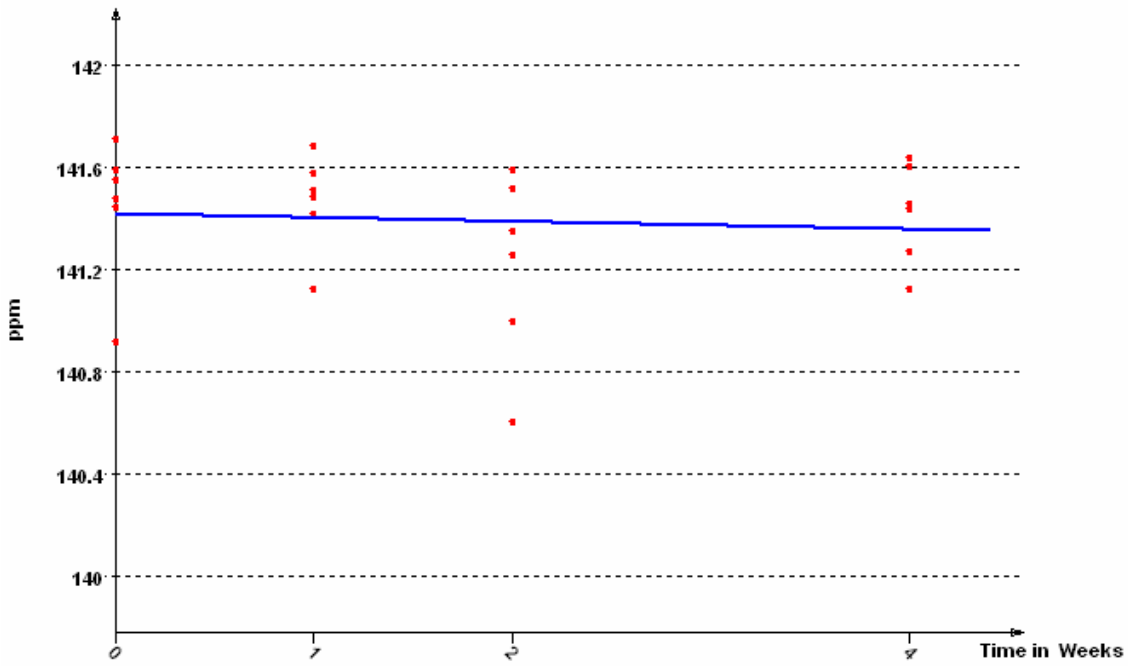
Figure B1. Short-term stability of STA-003k at 18 ⁰C (data taken from IRMM-425, see page 15).
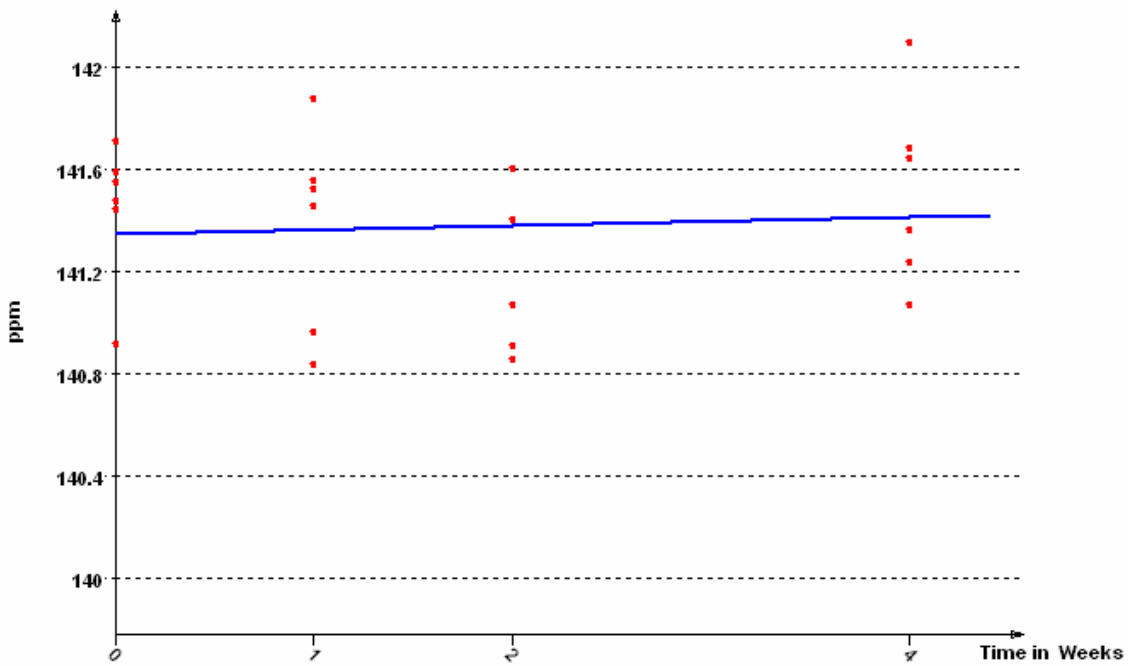

Figure B2. Short-term stability of STA-003k at 60 ⁰C (data taken from IRMM-425, see page 15).

Annex C – Long-term stability

Results of the long-term stability study of STA-003k at 20 ºC. Data listed have been acquired at Eurofins between 1997 and 2002.

Time (months)	D/H$_{TMU}$ (in ppm)	Time (months)	D/H$_{TMU}$ (in ppm)
0.00	102.81	15.43	102.60
0.40	102.94	16.03	102.62
0.50	103.29	16.70	102.88
1.13	103.34	16.87	102.98
1.53	102.99	16.97	102.60
1.53	102.72	17.10	102.65
1.70	103.26	17.17	103.06
1.93	102.72	17.73	102.78
2.63	102.79	18.00	102.96
2.63	102.75	18.20	102.90
3.07	102.98	18.63	102.78
3.10	102.34	18.70	102.82
3.57	102.68	18.93	102.55
3.67	103.03	19.20	102.72
3.87	102.99	19.20	102.74
4.10	103.05	19.30	103.25
4.40	103.35	19.63	103.31
4.43	102.27	19.87	102.90
4.50	102.80	20.00	102.95
5.17	102.99	20.03	103.29
5.27	102.68	20.23	102.74
5.30	103.10	20.43	102.81
5.57	102.81	21.73	102.33
5.70	102.76	22.07	102.74
6.53	102.76	22.23	102.63
6.73	103.21	22.40	102.29
6.93	102.18	22.43	103.08
7.07	102.97	22.77	102.87
7.20	102.76	23.80	102.67
7.50	102.87	23.83	103.26
7.60	102.93	23.87	102.63
7.67	102.58	23.97	102.91
8.10	102.63	24.00	102.75
8.30	103.06	24.20	102.82
8.33	103.09	25.03	102.65
8.43	102.93	25.30	102.86
8.67	102.91	26.27	102.53
9.27	102.50	26.33	102.60
9.83	102.67	26.57	102.80
10.30	103.10	26.80	102.70
11.00	103.10	27.07	102.73
11.33	103.10	28.50	102.25
11.87	102.70	29.07	102.76
11.97	102.71	29.07	102.37
12.13	103.13	29.30	102.47
12.17	102.93	29.47	102.93
12.90	102.36	29.70	102.11
13.27	102.98	30.17	102.42
13.37	102.66	30.37	102.19
14.43	102.97	30.73	102.29
14.60	102.78	30.97	102.63
14.73	102.68	31.27	102.29
14.73	103.40	31.87	102.49

| Time (months)	D/H$_{TMU}$ (in ppm)	Time (months)	D/H$_{TMU}$ (in ppm)
31.97	102.18	46.37	102.44
32.03	102.27	46.63	103.22
32.30	102.36	46.87	102.66
32.53	102.71	47.20	102.48
32.73	102.58	47.40	102.57
32.83	102.55	47.67	102.64
32.87	102.20	48.00	102.52
33.63	102.50	48.23	103.08
34.47	102.22	48.53	102.78
35.67	102.82	48.70	102.24
37.53	102.68	48.97	102.93
37.80	103.08	49.20	102.69
39.13	102.75	49.40	102.53
39.80	102.81	49.53	102.71
39.80	102.61	50.30	102.50
39.80	102.88	50.33	102.89
39.87	103.11	50.63	102.44
39.97	102.67	51.53	102.38
40.17	102.68	51.93	102.71
40.37	102.60	52.20	102.71
40.57	102.26	52.43	102.87
40.63	102.50	52.80	103.03
40.83	102.94	53.00	102.49
40.97	102.44	53.37	102.96
41.03	102.81	53.60	102.84
41.27	102.97	53.87	102.52
41.37	102.17	54.27	102.78
41.43	102.25	54.40	102.59
41.43	102.53	54.60	102.29
41.60	102.83	54.90	102.78
41.73	102.62	55.20	102.63
41.97	103.03	55.23	102.60
42.17	102.87	55.73	102.24
42.27	102.52	55.73	102.26
42.50	102.86	55.93	102.64
42.70	102.95	56.07	102.18
42.93	102.67	56.23	102.60
43.13	102.32	56.53	102.37
43.43	102.31	56.77	102.48
43.83	102.70	57.10	102.44
44.13	103.00	57.30	102.43
44.40	102.79	57.60	102.43
44.60	102.38	57.67	102.14
44.70	102.81	57.87	102.29
44.77	102.69	58.23	102.02
45.07	102.31	58.47	102.66
45.30	102.63	58.70	102.17
45.67	102.62	58.83	102.27
45.77	102.79	59.03	102.24
45.93	102.84	59.07	102.29
46.13	102.63		
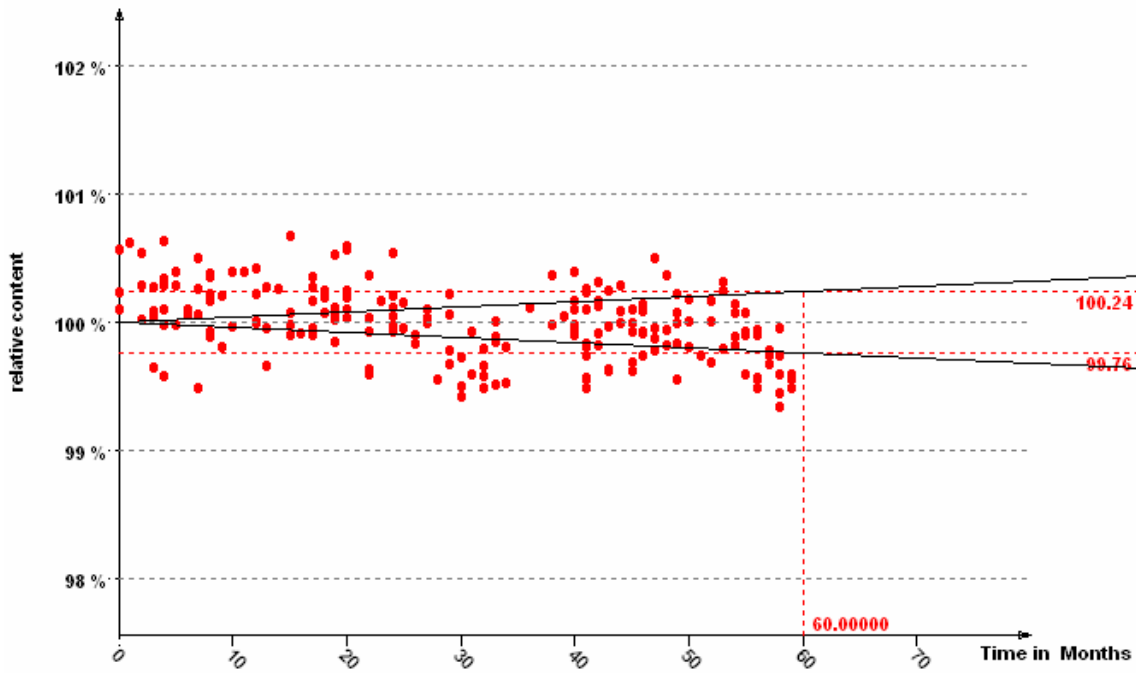
Figure C1. Long-term stability of STA-003k at 20 ºC. u_{lts} is estimated for a shelf life of 5 years using the measurement data on pages 31-32. The depicted uncertainty includes both the standard uncertainty associated with (in)stability and the additional uncertainty contribution due to the observed degradation (see formulas on page 17).

Annex D – Characterisation – method-related information

Laboratory code	Signal property used for calculation	NMR appliance and software	QC measures (instrument verification, QC sample(s) used)
1	Height	Bruker Avance 400, Topspin 1.3	BCR-656, samples of latest TMU batch included in sequence
2	Height	Bruker Avance 400, Topspin 1.3	BCR-123, in-house alcohol
3	Height	Bruker Avance 400, X-Win NMR	BCR-123, TMU calibrated in-house
4	Height	Bruker Avance 400, Icon NMR	BCR-123, in-house wine ethanol sample (95 vol%)
5	Height	Varian Unity Inova 300, VNMRJ rev. 1.1D	BCR-123
6	Area	Bruker ARX 500, X-Win NMR 2.6	BCR-123
7	Height	Bruker AM 500, Aspect X32	BCR-123
8	Height	Bruker AMX 500, X-Win NMR 2.6	BCR-123
9	Height	Bruker DPX 400, Topspin 1.3.6	BCR-123
10	Height	Bruker Avance 400, X-Win NMR 3.1	BCR-123
11	Height	Bruker DXR 500, X-Win NMR 2.3	BCR-123

Annex E – Characterisation data

Characterisation data of STA-003k. Data (ratio of deuterium to hydrogen) is expressed in ppm and has been acquired from TMU/BCR-656 mixtures strictly adhering to 2676/90 [1]. Values were calculated using the D/H_l value of BCR-656 and the formula depicted on page 12. All 6 individual measurements (2 days, 3 samples in duplicate) per laboratory are presented. Data were eventually re-calculated at IRMM performing a harmonised way of calculation (see page 22).

Laboratory code	Day 1			Day 2		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
1	141.42	141.26	141.91	141.93	141.83	142.02
2	142.48	142.06	142.28	141.82	142.10	142.19
3	142.36	142.15	142.36	142.40	141.84	142.24
4	141.72	142.14	142.26	142.00	141.74	142.30
5	142.08	142.30	142.19	141.57	141.59	141.72
6	142.34	142.39	141.86	142.27	142.93	142.41
7	142.61	142.52	142.63	142.53	142.86	142.56
8	142.31	142.14	141.57	142.23	142.11	141.93
9	142.09	141.92	141.97	141.97	142.12	142.03
10	141.85	141.82	142.41	141.47	141.56	142.04
11	143.34	143.58	143.12	142.22	142.29	141.69

Abstract

This report describes the production of a tetramethylurea reference material (STA-003k), 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) spectroscopy 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 (> 120×10^{-6}) was purified by removing most of the residual water and filled into amber glass bottles of 500 mL portions. Homogeneity was tested and no heterogeneity observed. Stability studies indicated no material degradation for 4 weeks at 60 $^{\circ}$C and a very minor degradation over a period of 5 years at 20 $^{\circ}$C which. Batch characterisation was accomplished in an inter-laboratory comparison using the SNIF-NMR technique exclusively.

The certified value was obtained as the unweighted mean of the laboratory means of the accepted sets of results. The expanded uncertainty associated (k = 2) includes contributions from (potential) heterogeneity, potential instability, characterisation, as well as the carried-over uncertainty from BCR-656 (96 % wine ethanol) that was used as the internal standard in the measurements.

The certified value and its associated uncertainty are:

	Amount-of-substance ratio	
	Certified value [1,2,4]	Uncertainty [3]
Deuterium-to-hydrogen (D/H) ratio	141.9×10^{-6}	0.9×10^{-6}

1) Traceable to V-SMOV (Vienna Standard Mean Ocean Water) by strictly adhering to the Community reference method (SNIF-NMR, site-specific natural isotope fractionation – nuclear magnetic resonance spectroscopy) as defined in Annex 8, Commission Regulation 2676/90. For V-SMOW, a value of 155.76 ppm was used (Tellus (1970) 22: 712-715).
2) This value was obtained by an inter-laboratory comparison (11 laboratories) employing SNIF-NMR at 46.1 (300), 61.4 (400), and 76.7 (500) MHz for ^{2}H (^{1}H). The value has been corrected for the material impurities (including water).
3) Expanded uncertainty with a coverage factor of k = 2, according to the Guide to the Expression of Uncertainty in Measurement, corresponding to a level of confidence of about 95 %.
4) The certified value has a measurement unit of one.

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