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CERTIFICATION REPORT

The certification of the mass fraction of arsenobetaine in water: ERM®-AC626

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Abstract

This report describes the production of ERM-AC626, an aqueous solution material certified for the mass fraction of arsenobetaine (AB). The material was produced following ISO Guide 34:2009.
The starting material was 10 g of solid AB monohydrate. The purity of AB was assessed through measurements carried out by laboratories of demonstrated competence and within the scope of accreditation to ISO/IEC 17025:2005. An aqueous solution was prepared and ampouled into 2 mL amber glass ampoules under argon atmosphere.
Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006. Due to the inherent homogeneity of water solutions, determination of minimum sample intake (within-unit homogeneity) was not required. The sample intake used in the homogeneity study was adopted as the recommended minimum sample intake. The minimum sample intake is 50 µg.
The certified value was obtained from the gravimetric preparation of the solution, taking into account the purity of the starting material. The certified value was confirmed by independent analyses carried out by laboratories of demonstrated competence and adhering to ISO/IEC 17025.
Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties related to possible inhomogeneity and instability and to characterisation.
The material is intended to be used as a calibrant and quality control sample. The CRM is available in amber glass ampoules containing 1 mL of AB aqueous solution closed under argon atmosphere. The minimum amount of sample to be used is 50 µg.
The CRM was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

The certification of the mass fraction of arsenobetaine in water: ERM®-AC626

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Disclaimer

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

Summary

This report describes the production of ERM-AC626, an aqueous solution material certified for the mass fraction of arsenobetaine (AB). The material was produced following ISO Guide 34:2009 [1].

The starting material was 10 g of solid AB monohydrate. The purity of AB was assessed through measurements carried out by laboratories of demonstrated competence and within the scope of accreditation to ISO/IEC 17025:2005 [2]. An aqueous solution was prepared and ampouled into 2 mL amber glass ampoules under argon atmosphere.

Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [3]. Due to the inherent homogeneity of water solutions, determination of minimum sample intake (within-unit homogeneity) was not required. The sample intake used in the homogeneity study was adopted as the recommended minimum sample intake. The minimum sample intake is 50 µg.

The certified value was obtained from the gravimetric preparation of the solution, taking into account the purity of the starting material. The certified value was confirmed by independent analyses carried out by laboratories of demonstrated competence and adhering to ISO/IEC 17025.

Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible inhomogeneity and instability and to characterisation.

The material is intended to be used as a calibrant and quality control sample. The CRM is available in amber glass ampoules containing 1 mL of AB aqueous solution closed under argon atmosphere. The minimum amount of sample to be used is 50 µg.

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

The following value was assigned:

	Mass fraction	
	Certified value [1)] [mg/kg]	Uncertainty [2)] [mg/kg]
Arsenobetaine ($C_5H_{11}AsO_2$)	250.0	2.5

1) Calculated taking into account the thoroughly assessed purity of the starting material and the gravimetric preparation of the solution. The certified value and its uncertainty are traceable to the International System of Units (SI).

2) The certified uncertainty is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

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Glossary

AC	Arsenocholine
ANOVA	Analysis of variance
AB	Arsenobetaine
BCR®	One of the trademarks of CRMs owned by the European Commission; formerly Community Bureau of Reference
CRM	Certified reference material
CLSI	Clinical and Laboratory Standards Institute
DMA	Dimethylarsinic acid
ERM®	Trademark of European Reference Materials
ESI-MS	Electrospray ionisation - mass spectrometry
GC-FID	Gas chromatography-flame ionisation detection
GC-MS	Gas chromatography-mass spectrometry
GUM	Guide to the Expression of Uncertainty in Measurements
HPLC	High performance liquid chromatography
HS-GC-MS	Headspace-gas chromatography-mass spectrometry
IC	Ion chromatography
ICP-MS	Inductively coupled plasma-mass spectrometry
ICP-SFMS	ICP-sector field mass spectrometry
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
JRC	Joint Research Centre of the European Commission
k	Coverage factor
k_0NAA	k_0-neutron activation analysis
LOQ	Limit of quantification
M	Molar mass
$MS_{between}$	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
m/z	Mass-to-charge ratio
n	Number of replicates per unit
n.c.	Not calculated
OIML	International Organization of Legal Metrology
QC	Quality control
qNMR	Quantitative nuclear magnetic resonance
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material

RSD	Relative standard deviation
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added when appropriate
SI	International System of Units
s_{wb}	Within-unit standard deviation; an additional index "rel" is added when appropriate
t	Time
t_i	Time point for each replicate
t_{sl}	Proposed shelf life
t_{tt}	Chosen transport time
TMAO	Trimethylarsine oxide
u	Standard uncertainty
U	Expanded uncertainty
u^*_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_c	Combined standard uncertainty; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
$u_{impurities}$	Standard uncertainty of the impurity determination
u_Δ	Combined standard uncertainty of measurement result and certified value
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{meas}	Standard measurement uncertainty
U_{meas}	Expanded measurement uncertainty
u_{qNMR}	Standard uncertainty of the purity determination by qNMR
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
w	Mass fraction
$w_{impurities}$	purity of AB, determined using the "mass balance" approach
w_{qNMR}	purity of AB, determined by qNMR
$\bar{y}$	Mean of all results of homogeneity study

Δ_{meas}	Absolute difference between mean measured value and the certified value
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}

1 Introduction

1.1 Background

Arsenic is a metalloid which is released in the environment from natural and anthropogenic sources. The toxicity of the element strongly depends on its form. It is proven that the inorganic arsenic (As(III) and As(V)) are highly toxic, but the organoarsenic compounds are less toxic [5].

Arsenobetaine (AB), also known as 2-trimethylarsoniumylacetate, $C_5H_{11}AsO_2$, CAS no. 64436-13-1, is the major form of arsenic in marine fish and most other seafood. It has been shown by various toxicity tests that AB is a harmless compound [5]. The variety of arsenic compounds with different toxicity leads to the necessity of performing species determination rather than determination of total arsenic concentration in food [5].
To establish the level of arsenic in various foodstuffs, the European Commission recommended that EU member states conduct a monitoring program of inorganic As, total As and explicitly called also for the monitoring of other relevant As species [6].

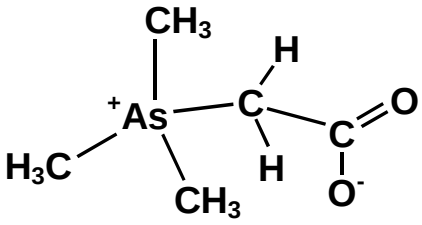

Figure 1: Structure of AB

The main analytical method used for separation of organoarsenic species is high performance liquid chromatography (HPLC) and AB is quantified by element specific detectors [5]. As HPLC is a relative method, which means that calibrants are needed to identify and quantify the compounds. This necessitates the use of calibrants with proven quality.ERM-AC626 is such a certified reference material intended to be used as a calibrant and quality control (QC) sample.

1.2 Choice of the material

ERM-AC626 is a certified reference material intended to replace BCR-626 (AB in solution). BCR-626 was widely used as a calibrant and quality control sample by laboratories dealing with food analysis.

ERM-AC626 consists of an aqueous solution prepared by dissolution of high purity AB in high purity water. The AB mass fraction was chosen to be approximately 250 mg/kg in contrast to BCR-626, which was four times higher – 1031 mg/kg. This choice was based on a survey among laboratories dealing with arsenic speciation and in particular AB determination.

1.3 Design of the CRM project

The purity of the base material was established by two independent approaches – determination of AB by quantitative nuclear magnetic resonance (qNMR) spectroscopy and determination of all known, expected or assumed impurities and their subtraction from unity. The certified value was obtained from the gravimetric preparation of the solution, taking into account the purity of the starting material. The certified value was confirmed by independent analyses performed by several laboratories using different methods.

The stability and homogeneity of the material were evaluated through dedicated studies.

2 Participants

2.1 Project management and evaluation

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

2.2 Syntesis of the starting material

Karl-Franzens Universität Graz, Institut für Chemie, Graz, AT

2.3 Processing

European Commission, Directorate General Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE

2.4 Homogeneity study

University of Aberdeen, Department of Chemistry, TESLA, Aberdeen, UK

2.5 Stability study

Karl-Franzens Universität Graz, Institut für Chemie, Graz, AT

2.6 Characterisation and Confirmation of the certified value

Bundesanstalt für Materialforschung und –prüfung (BAM), Berlin, DE
(Measurements performed under ISO/IEC 17025 accreditation; DAR DAP-PL-2614.14)

Karl-Franzens Universität Graz, Institut für Chemie, Graz, AT

Katholieke Universiteit Leuven (KUL), Faculteit Farmaceutische Wetenschappen, Leuven, BE

ProChem GmbH, Hildesheim, DE
(Measurements performed under ISO/IEC 17025 accreditation; DAkkS D-PL-14298-01-00)

Sigma-Aldrich Laborchemikalien GmbH, Seelze, DE
(Measurements performed under ISO/IEC 17025 accreditation; DGA DGA-PL-6670.09)

Solvias AG, Kaiseraugst, CH

Studiecentrum voor Kernenergie (SCK·CEN), Mol, BE
(Measurements performed under ISO/IEC 17025 accreditation; BELAC 015-TEST)

Universitat de Barcelona, Facultad de Química, Barcelona, ES

University of Aberdeen, Department of Chemistry, TESLA, Aberdeen, UK

3 Material processing and process control

3.1 Origin of the starting material

The starting material – AB monohydrate - was synthesised by the Institute of Chemistry, University of Graz, Graz, Austria. The four stage procedure included:

1. synthesis of $AsCl_3$

$$As_2O_3 + 3SOCl_2 \xrightarrow{60\ ^\circ C} 2AsCl_3 + 3SO_2$$

2. synthesis of $(CH_3)_3As$

$$CH_3I + Mg \xrightarrow{Dibutyl\ ether} CH_3MgI$$

$$3\,CH_3MgI + AsCl_3 \xrightarrow{Dibutyl\ ether,\ 0\ ^\circ C} (CH_3)_3As + 3\,MgICl$$

3. synthesis of AB bromide ($[(CH_3)_3AsCH_2COOH]^+Br^-$)

$$(CH_3)_3As + BrCH_2COOH \xrightarrow{Dibutyl\ ether\ /\ CH_3CN,\ 0\ ^\circ C} [(CH_3)_3AsCH_2COOH]^+ Br^-$$

4. conversion of AB bromide into AB monohydrate ($((CH_3)_3As^+CH_2COO^-\cdot H_2O$).

The final product was 10 g of white powder, highly hygroscopic and photo labile.

3.2 Additional characterisation of the starting material

As AB is a highly hygroscopic compound, a sorption isotherm was determined and plotted using a moisture sorption analyser. The experiment was used to assess the hygroscopic behaviour of the base material and to get more information about its handling. The isotherm is presented in Figure 1.

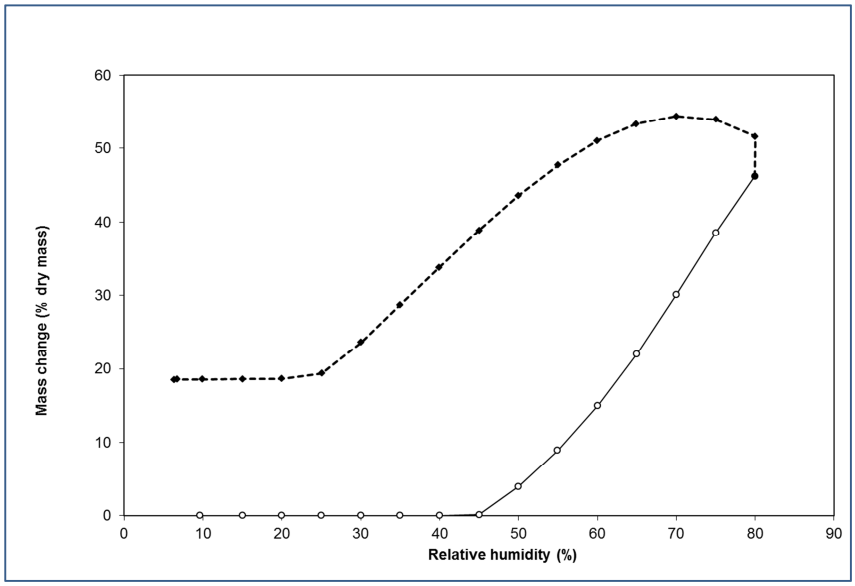

Figure 2: Sorption isotherm of the AB starting material open circles: sorption curve, full circles: desorption curve)

Based on the measurements shown above, the AB solution preparation was carried out in a glove box under argon atmosphere at low relative humidity – below 20 %. This stabilised environment caused negligible change in the AB mass during the substitution weighing leading to very low measurement uncertainty during this stage.

3.3 Processing

The AB solution (5 kg) was prepared gravimetrically by dissolution of 1.38755 g of the starting material into deionized water - 18.2 MΩ·cm at 25 °C (Millipore Q-POD® Element, Millipore, USA). The weighing procedure took place in a glove box under argon atmosphere, temperature 24.0 °C ± 0.5 °C, relative humidity of the argon atmosphere 14.5 % ± 0.5 %, atmospheric pressure 1012 hPa ± 1 hPa. To minimize the influence from static electricity increased by the low relative atmospheric humidity, the balance was automatically deionized and an aluminium weighing boat was used for the AB weighing. The AB starting material was weighed using a substitution weighing procedure in which a standard and an unknown weight are compared to determine the average difference between the two weights. In this way, calibration bias of the balance is eliminated since the balance is used just as a comparator (OIML international recommendation R111 [7]). When substitution weighing is performed, traceability to the International System of Units (SI) is directly realised by the mass standards, thus lowering the uncertainty of the measurement result. The linearity component in the balance uncertainty is negligible since the mass difference between mass standards and sample was sufficiently low.

Once the amount of AB starting material was weighed, it was transferred with the aluminium weighing boat into an amber glass bottle containing approximately 1 L deionized water. The bottle was filled with deionized water until the desired weight of 5 kg was reached. The solution was homogenised overnight using a magnetic stirrer.

A portion of 1 mL solution was filled into 2 mL amber glass ampoules. Special care was taken to prevent contamination of the solution during the ampoulation process. A magnetic stirrer was used to keep the solution homogenous. All ampoules were flushed with argon before and after the filling and flame sealed. Approximately 3000 ampoules were produced.

3.4 Process control

All the ampoules produced were checked for leaks. The unit number of each ampoule was given following the filling sequence. The filling sequence was checked for a potential trend during the homogeneity, short- and long-term stability studies. No significant trend was detected at a 99 % confidence level.

4 Homogeneity

A key requirement for any reference material aliquotted into units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, but it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 [1] requires RM producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample intake is respected, but determines the minimum size of an aliquot that is representative for the whole unit. Quantification of within-unit inhomogeneity is therefore necessary to determine the minimum sample intake.

A "Unit" is defined as an individual glass ampoule of ERM-AC626.

4.1 Between-unit homogeneity

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

The number of selected units corresponds to approximately the cubic root of the total number of the produced units. Fifteen units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch was divided into 15 groups (with a similar number of units) and one unit was selected randomly from each group. Six independent samples of 50 mg were taken from each selected unit, diluted 1000 times with deionised water (by weight), and analysed by cation-exchange HPLC-ICP-MS. The measurements were performed under repeatability conditions, i.e. during one analytical run, and in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence. The results are shown as graphs in Annex A.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trend in the filling sequence was visible at the 99 % confidence level. A significant (99 % confidence level) trend in the analytical sequence was visible, pointing at a signal drift in the analytical system. The correction of biases, even if they are statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value [8]. Correction of trends is therefore expected to improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. As the analytical sequence and the unit numbers were not correlated, the trend significant on at least a 95 % confidence level was corrected as shown below:

$$corrected\ \ result = measured\ \ \ result - b \cdot i \qquad \textbf{Equation 1}$$

b **=** slope of the linear regression

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

The trend-corrected dataset was tested again for trend in the filling sequence. No trend was visible at the 99 % confidence level.

The trend-corrected dataset was also checked for consistency using Grubbs outlier tests on a confidence level of 99 % on the individual results and the unit means. No outlying individual results and outlying unit means were detected.

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

Evaluation by ANOVA requires unit means which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. Distribution of the unit means was visually tested using histograms and normal probability plots. Although the distribution looks normal, too few data are available for the unit means to make a clear statement of the distribution. Therefore, it was visually checked whether all individual data follow a normal distribution using a histogram and normal probability plot. Both of them confirm the normality of the distribution. Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviation.

One has to bear in mind that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum inhomogeneity that could be hidden by method repeatability, was calculated as described by Linsinger *et al.* [9]. 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 ($s_{wb,rel}$), between–unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as:

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

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

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

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
$v_{MSwithin}$	degrees of freedom of MS_{within}

The results of the evaluation of the between-unit variation are summarised in

Table 11. The resulting values from the above equations were converted into relative uncertainties. In this case, the uncertainty contribution for homogeneity was determined by the method repeatability.

Table 1: Results of the homogeneity study

Analyte	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,rel}$ [%]
Arsenobetaine	1.76	n.c.	0.29	0.29

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

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore the between-unit standard deviation can be used as estimate of u_{bb}. As $MS_{between}<MS_{within}$, u^*_{bb} sets the limits of the study to detect inhomogeneity and it is adopted as an uncertainty contribution to account for potential inhomogeneity.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake The minimum sample intake is the minimum amount of sample that is representative of the whole unit and thus can be used in an analysis. Sample sizes equal to or above the minimum sample intake guarantee the certified value within its stated uncertainty.

The establishment of the minimum sample intake in this study was not specifically addressed due to the nature of the material itself (aqueous solution). The heterogeneity of solutions is known to be very small or even negligible. Nevertheless, this assumption was confirmed by the homogeneity study, where sample intakes of 50 µg were found to give acceptable repeatability, demonstrating that there is no intrinsic inhomogeneity or contamination at a sample intake of 50 µg. The minimum amount of sample to be used is therefore set at 50 µg.

5 Stability

Time, temperature and radiation were regarded as the most relevant influences on the stability of the materials. The influence of ultraviolet or visible radiation was minimised by the choice of amber glass ampoules for containment, which eliminates most of the incoming light. In addition, materials are stored and dispatched in the dark, thus eliminating the possibility of degradation by light. Therefore, only the influences of time and temperature were investigated.

Stability testing is necessary to establish conditions for storage (long-term stability) as well as conditions for dispatch to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C could be reached and stability under these conditions must be demonstrated if transport at ambient temperature will be applied.

The stability studies were carried out using an isochronous design [10]. In that approach, samples are stored for a certain time at different temperature conditions. Afterwards, the samples are moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples are analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

5.1 Short-term stability study

For the short-term stability study, samples of ERM-AC626 were stored at 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to 4 °C. Two units per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured by HPLC-ICP-MS. The measurements were performed under repeatability conditions during one analytical run and in a randomised sequence to be able to separate a potential analytical drift from a trend over storage time.

Regression analysis was performed to evaluate potential trends in the analytical sequence. No trend in the analytical sequence was visible at the 99 % confidence level. The obtained data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test. No outliers were detected.

Furthermore, the data were evaluated against storage time, and regression lines of mass fraction versus time were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to shipping conditions) and were found not significantly different from zero (at 99 % confidence level) at both 18 °C and 60 °C. The results of the measurements are shown in Annex B.

As no statistical outliers were detected, all results were used for the estimation of u_{sts}. No trend was statistically significant at a 99 % confidence level for any of the temperatures. The material can therefore be dispatched without further precautions under ambient conditions.

5.2 Long-term stability study

For the long-term stability study, samples were stored at 18 °C for 0, 4, 8 and 12 months. The reference temperature was set to 4 °C. Four ampoules per storage time were selected using a random stratified sampling scheme. From each ampoule, four samples were measured by HPLC-ICP-MS. The measurements were performed under repeatability conditions, in a random sequence to be able to separate any potential analytical drift from a trend over storage time.

Regression analysis was performed to evaluate potential trends in the analytical sequence. A significant trend in the analytical sequence was visible at the 99 % confidence level. The data were corrected using Equation 1.

The trend-corrected dataset was screened for outliers using single and double Grubbs tests at a confidence level of 99 %. No outliers were detected.

Furthermore, the data were plotted against storage time and a linear regression line of mass fraction versus time was calculated. The slope of the regression line was tested for statistical significance (loss/increase due to storage conditions). The slope of the regression line was not significantly different from zero (at 99 % confidence level).

The results of the long term stability measurements are shown in Annex B.

As no outliers were observed and no trend over storage time was statistically significant at a 99 % confidence level, the material can be stored at 18 °C.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can rule out degradation of materials completely, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method repeatability, i.e. to estimate the uncertainty of stability. This means, even under ideal conditions, the outcome of a stability study can only be "degradation is 0 ± x % per time".

Uncertainties of stability during dispatch and storage were estimated as described in [11]. For this approach, the uncertainty of the linear regression line with a slope of zero is calculated. The uncertainty contributions u_{sts} and u_{lts} are calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

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

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

RSD relative standard deviation of all results of the stability study

t_i time elapsed at time point *i*

$\bar{t}$ mean of all t_i

t_{tt} chosen transport time (1 week at 60 ºC)

t_{sl} chosen shelf life (12 months at 18 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$ = 0.17 %

  the uncertainty of degradation during dispatch. This was estimated from the 60 °C studies. The uncertainty describes the possible change during a dispatch at 60 °C lasting for one week.

- $u_{lts,rel}$ = 0.34 %

  the stability during storage. This uncertainty contribution was estimated from the 18 °C study. The uncertainty contribution describes the possible degradation during 12 months storage at 18 °C.

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

No significant degradation during storage at 18 °C was found. Therefore, the material can be stored at 18 °C.

After the certification campaign, the material will be subjected to the JRC's regular stability monitoring programme to control its further stability.

6 Characterisation

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

The reference material characterisation was based on a primary method of measurement (gravimetric preparation) and the estimated purity of the starting material. The certified value was confirmed by independent analysis to exclude losses or contamination during the preparation and ampouling steps.

Laboratories are assigned a random code that does not correspond to the order of laboratories in Section 2. Datasets with the same number (L2a, L2b etc.) come from the same laboratory, but from different studies and/or were obtained by different methods.

6.1 Identity confirmation of the starting material

The identity of the synthesised AB starting material was confirmed by determination of its elemental composition and molecule structure. In addition, the hygroscopic behaviour of the material was examined in order to determine the best conditions for the AB solution preparation.

The measurements of the arsenic content were done by four different laboratories using several analytical methods. The results are summarized in Table 2. The results are given as reported by the laboratories. The measurement procedures used are presented in Annex C.

Table 2: Results (as reported by the laboratories) of the element content measurements of the starting material, [,] denominates ranges

Analyte	Theoretical value[e] ($AB{\cdot}H_2O$)	Mass fraction [g/kg]			
		L1	L2a	L3a	L4
C	306.3	306 ± 3[a] 305 ± 3[a]	[307.1, 307.8]		
H	66.8	67.0 ± 3[a] 66.3 ± 3[a]	[65.9, 66.7]		
O	244.8	242 ± 3[a] 241 ± 3[a]			
As	382.1		382 ± 4[b]	384 ± 9[c]	395 ± 18[d]

[a] standard measurement uncertainty
[b] standard deviation of 6 replicates (6 independent measurements)
[c] standard deviation of 3 replicates
[d] mean value of 3 replicates, expanded uncertainty, k=2
[e] based on the standard atomic weights of the elements by IUPAC [12]

The elemental composition of the starting material agrees with the chemical composition of AB monohydrate. The small difference between the measured and the theoretical values is explained by the presence of small amounts of impurities in the starting material.

The structural identification of the compound was carried out by 3 laboratories using three analytical techniques.

- High performance liquid chromatography-inductively coupled mass spectrometry (HPLC-ICP-MS): cation exchange and reversed phase HPLC-ICP-MS – the retention time of the starting material dissolved in deionised water matched with the retention time of AB

standard solution. A comparison between a chromatogram of the water solution of the starting material with a chromatogram of a standard solution of several organoarsenic compounds (AB, trimethylarsine oxide (TMAO), arsenocholine (AC), tetramethylarsonium ion) confirmed the base material as AB

- High performance liquid chromatography-electrospray mass spectrometry (HPLC-ESI-MS): HPLC-ESI-MS and HPLC-ESI-MS^2 – the peaks at m/z=179 in the ESI-MS mass spectra correspond to the protonated AB $(CH_3)_3As^+CH_2COOH$ (M=179). Due to the high concentration of AB in the solution, dimerisation at m/z 357 is also observed. Some in-source fragmentation, notably loss of the COO^-group (m/z 135), the loss of $-CH_2COOH$ (m/z 120) and loss of $-CH_2COOH + CH_3$ (m/z 105), occurred under the chosen ionisation conditions
- Nuclear magnetic resonance (NMR) spectroscopy: ^{1}H-NMR and ^{13}C-NMR – the 600 MHz ^{1}H-NMR signals of the base material were assigned to the structural formula of AB supported by a $^{13}C\{^1H\}$-NMR spectrum, two-dimensional H,H-correlation spectroscopy (2D H,H-COSY) and two-dimensional heteronuclear multiple bond correlation (H,C-HMBC) spectra. The signals in the ^{1}H-NMR spectra correspond to the AB molecule as well as to water and traces of methanol and acetone. The resonance signal at 1.805 ppm, corresponding to the methyl groups bound to As in the AB molecule, was used also for the quantitative determination of the AB mass fraction in the starting material.

A chromatogram or spectrum of the starting material analysis achieved by each technique is presented in Annex C. The analyses done confirmed that the starting material is AB.

6.2 Purity assessment of the starting material

The AB purity was assessed combining two independent approaches:

1) determination of AB by qNMR spectroscopy, and

2) "mass balance" approach: quantification of each impurity known, expected or assumed (to the best of knowledge) and its subsequent subtraction from unity.

For all measurements, the mass fraction of AB without crystal water was determined, although in solid state AB is usually monohydrate. Thus, the crystal water was considered as an impurity.

6.2.1 Determination of AB mass fraction by qNMR spectroscopy

The AB content was determined by qNMR spectroscopy using benzoic acid as an internal standard. The signal at 1.805 ppm in the ^{1}H-NMR spectrum was used for quantification. A mass fraction of 902.8 g/kg ± 1.2 g/kg (k=2) was reported. The result is based on 11 independent measurements. More details on the measurement parameters are presented in Annex C.

6.2.2 Determination of AB mass fraction based on the impurities

The impurities measured were:

1) related structure – trimethylarsin oxide (TMAO) and dimethylarsinic acid (DMA) (arsenic species as potential by-products);

2) residual solvents – methanol and acetone (re-crystallisation), dibutyl ether (solvent of the Grignard reaction) and n-butanol (impurity dibutyl ether);

3) water content - water (crystal water and moisture due to hygroscopicity);

4) non volatiles (inorganics) - arsenite/arsenate (inorganic arsenic species as potential by-products), magnesium and iodide (Grignard reaction), zinc, iron, copper, aluminium (typical

metals of labware), sodium (drying process), bromide (bromoacetic acid and residue of ion exchange chromatography).

The mass fraction of all impurities measured was calculated using the following rules:

- If the impurity mass fraction (w) was below the limit of quantification (LOQ), then w = LOQ/2 and u = w/√3 (assuming rectangular distribution) were used for the final calculations
- If two results for the same impurity were available and both of them were below the LOQ, then w = $LOQ_{lower\ value}$/2 and u = w/√3 were used
- If two results for the same impurity were available, then w = ($w_{low\ value}$ + $w_{high\ value}$)/2 and u = ($w_{high\ value}$ – $w_{low\ value}$)/(2*√3) were taken
- If the results for arsenic species were presented as mass fraction of As, then they were recalculated and taken as mass fraction of AsO_4^{3-} (for inorganic As), TMAO and DMA. The following molar masses were used: M(AsO_4^{3-}) = 138.918 g/mol; M(TMAO, C_3H_9AsO) = 136.023 g/mol; M(DMA, $C_2H_7AsO_2$) = 137.996 g/mol
- As the molar mass of the unknown arsenic compound is not known and the mass fraction is reported as mg As/kg, some additional calculations were done. It was assumed that the molar mass of the unknown arsenic compound is between M=74.922 g/mol (As) and M=178.058 g/mol (AB) and follows a rectangular distribution. Thus, the average M=126.490 g/mol was used for the recalculation of the mass fraction of the unknown compound. Furthermore, additional uncertainty contribution was added to the reported one taking into account a rectangular distribution between M(As) and M(AB).

The results are presented in Table 3 and all measurement methods in Annex C.

The presence of the main impurities was also confirmed by the qNMR spectra. In order to keep the independency of the two purity assessment approaches, no qNMR results were included into the "mass balance" approach.

Table 3: Results of impurity determination in the starting material. The column "Value used" corresponds to the value used for the calculation of the purity in the mass balance approach

Element/ Compound	Mass fraction [g/kg]							
	L2b	L5	L6	L3b	L7	L8[d]	Value used	
							value	u
TMAO	<1					<0.15	0.08	0.04
DMA	<1					<0.08	0.04	0.02
Unknown As compound				$(2.8\pm0.5)*10^{-3}$ [a]			0.005	0.002
Methanol		6.2±0.2[b]	3.7±1.8[b]				5.0	0.7
Acetone		1.43±0.06[b]	0.8±0.4[b]				1.1	0.2
Dibutylether		<0.01					0.005	0.003
n-butanol		<0.05					0.03	0.01
Water		91.0±4.0[b]			93.3±1.9[c]		92.2	0.7
Inorganic As	<1					<0.072[a]	0.07	0.04
Mg	<1			$<0.8*10^{-3}$			0.0004	0.0002
I	<1			$<0.8*10^{-3}$			0.0004	0.0002
Zn				$<2.6*10^{-3}$			0.001	0.001
Fe				$<0.5*10^{-3}$			0.0002	0.0001
Cu				$<94*10^{-6}$			0.00005	0.00003
Al				$<4*10^{-3}$			0.002	0.001
Na	<1						0.5	0.3
Br	<1			$<0.7*10^{-3}$			0.0004	0.0002

[a] results reported as As mass fraction but given as (hypothetical) AB mass fraction

[b] ±standard deviation of 3 replicates

[c] ±expanded uncertainty with k=2.78

[d] analysis of ERM-AC626 – AB in aqueous solution instead of the solid starting AB material

The sum of all mass fraction of all impurities detected is 99.0 g/kg, which corresponds to a purity of AB of 901.0 g/kg. The uncertainty of this purity is obtained by the square root of the squared uncertainties listed in the last column of Table 3. This is calculated as 1.1 g/kg. The purity from the mass balance approach is therefore 901.0 ± 1.1 g/kg, which agrees with the purity assessment of qNMR.

6.2.3 Combination of qNMR and mass-balance approach

As the purity obtained by mass balance and by qNMR agree, the AB purity used for the certification of ERM-AC626 is equal to the average of the purities as determined by qNMR and the "mass balance" approach (Equation 7).

$$w_{purity} = \frac{w_{qNMR} + w_{mass\ balance}}{2}$$ **Equation 7**

w_{purity}	combined purity of the starting material
w_{qNMR}	purity as determined by qNMR
$w_{mass\ balance}$	purity as determined by the mass balance approach

The uncertainty was calculated according to Equation 8. Note that the denominator 4 in Equation 8 is the squared sensitivity coefficient of 1/2 , which comes from the division by 2 in Equation 7. Together with the uncertainty contribution of the two approaches for purity assessment, additional uncertainty is added. It accounts for the uncertainty arising from the difference between the purity results derived by the two approaches.

The results are reported in Table 4.

$$u_{c\_purity} = \sqrt{\frac{u_{qNMR}^2}{4} + \frac{u_{impurities}^2}{4}}$$ **Equation 8**

u_{qNMR}	uncertainty derived from the purity determination by qNMR
$u_{impurities}$	uncertainty derived from the impurity determination
w_{qNMR}	purity of AB, determined by qNMR
$w_{impurities}$	purity of AB, determined using the "mass balance" approach

Table 4: Arsenobetaine purity in the starting material

AB purity	Combined standard uncertainty
901.9 g/kg	0.63 g/kg

6.3 AB mass fraction in the solution

The AB mass fraction of the final solution was calculated from the masses of the AB (1.38755 ± 0.00030 g), its purity (901.9 ± 0.09 g/kg) and the mass of water used (5003.70 ± 0.14 g). The uncertainty of the AB mass fraction was estimated using the uncertainties of both weighings and the AB purity. The results are given in Table 5.

Table 5: : Mass fraction of AB in ERM-AC626

Mass fraction	u_{char}
250.03 mg/kg	0.18 mg/kg

6.4 Confirmation measurements

The mass fraction of AB in the calibration solution is certified on the basis of the gravimetric preparation and the estimated purity of the starting material. The certified value was confirmed by HPLC-ICP-MS, ICP-MS and k_0-neutron activation analysis (k_0NAA) to exclude losses or contamination during the preparation and ampouling steps.

Four laboratories were selected to perform confirmation analyses based on criteria that comprised both technical competence and quality management aspects. Each laboratory was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of AB/arsenic measurements by submitting results for intercomparison exercises or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2).

Each laboratory received three units of ERM-AC626 and was requested to provide two independent results per unit for the mass fraction of AB (three out of the four laboratories), arsenic (all four laboratories) and all other detected arsenic species (three out of the four laboratories). The units for confirmation analysis were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations (if necessary) and measurements had to be spread over three days (except k_0NAA) to ensure intermediate precision conditions.

Each participant received an aliquot (1 mL solution ampouled in a 2 mL amber glass ampoule) from unit number 174 of NMIJ CRM 7901-a (certified reference material produced by the National Metrology Institute of Japan, Japan) as a blinded quality control sample. The results for this sample were used to support the evaluation of the confirmation results.

Laboratories were also requested to give estimations of the expanded uncertainties of the mean value of the results of each unit. No approach for the estimation was prescribed, i.e. top-down and bottom-up were regarded as equally valid procedures.

The laboratories used HPLC-ICP-MS but with different set-up for the determination of AB and other arsenic species (three laboratories) and ICP-MS or k_0NAA for arsenic (four laboratories).

The results summarised in Table 6. All the measurement results of the quality control samples agreed with the certified value. The speciation analysis checked for the presence of trimethylarsine oxide, arsenocholine, dimethylarsinate, methylarsonate, arsenite As(III) and arsenate As(V). None of these species were detected by any of the laboratories. The mass fraction of AB, as well as the total As mass fraction agrees with the certified value. All the results therefore confirm the certified value.

Individual measurement results, description of the methods used and detailed graphical presentations of the data are included in Annex D.

Table 6: : Results of the confirmation analyses of ERM-AC626. Errors are expanded uncertainties (*k*=2). Results given as "<x" mean results smaller than the limit of quantification

Analyte	L2b	L3c	L4a (k0NAA)	L8
Total As [mg/kg]	103 ± 7	105.6 ± 3.7	109.7 ± 6.2	100.8 ± 6.7
AB [mg/kg]	241 ± 19	249.9 ± 9.0	Not tested	238.4 ± 16.4
DMA [mg/kg]	<0.9	<0.38	Not tested	< 0.012
TMAO [mg/kg]	<0.9	<0.39	Not tested	< 0.023
MA [mg/kg]	Not tested	Not tested	Not tested	< 0.014
AC [mg/kg]	Not tested	Not tested	Not tested	< 0.015
As (III) [mg/kg]	Not tested	sum <0.22	Not tested	< 0.008
As(V) [mg/kg]	Not tested		Not tested	< 0.020

7 Value Assignment

A certified value of the AB mass fraction was assigned. A certified value is a value that fulfils the highest standards of accuracy. A full uncertainty budget in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] was established.

The certified value is based on the masses of the starting AB material and the deionised water used in the gravimetrical preparation. The mass of the starting material was corrected for the AB purity determined beforehand by several laboratories using different analytical methods (Section 6).

The assigned uncertainty consists of uncertainties related to characterisation, u_{char} (Section 6), potential between-unit inhomogeneity, u_{bb} (Section 4) and potential degradation during transport (u_{sts}) and long-term storage, u_{lts} (Section 5). These different contributions were combined to estimate the expanded, relative uncertainty of the certified value ($U_{CRM,\,rel}$) with a coverage factor k as:

$$U_{CRM,rel} = k \cdot \sqrt{u^2_{char,rel} + u^2_{bb,rel} + u^2_{sts,rel} + u^2_{lts,rel}} \qquad \textbf{Equation 8}$$

- u_{char} was estimated as described in Section 6
- u_{bb} was estimated as described in Section 4
- u_{sts} was estimated as described in Section 5
- u_{lts} was estimated as described in Section 5

It is difficult to quantify the degrees of freedom of u_{char}. However, as seen in Table 7, u_{char} is a minor component of the total uncertainty budget. The degrees of freedom of u_{bb}, u_{sts} and u_{lts} sufficiently large to ensure that the degrees of freedom of the combined uncertainty is above 100, even if assuming that u_{char} has only 1 degree of freedom. Because of the sufficient numbers of the degrees of freedom of the different uncertainty contributions, a coverage factor k of 2 was applied to obtain the expanded uncertainties. The certified values and their uncertainties are summarised in Table 7.

Table 7: Certified value and its uncertainties for ERM-AC626

Analyte	Certified value [mg/kg]	$u_{char,\,rel}$ [%]	$u_{bb,\,rel}$ [%]	$u_{sts,\,rel}$ [%]	$u_{lts,\,rel}$ [%]	$u_{CRM,\,rel}$ [%]	U_{CRM}* [mg/kg]
AB mass fraction	250.0	0.07	0.29	0.17	0.34	0.48	2.5

* Expanded (k = 2) and rounded uncertainty

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

AB is a chemically clearly defined substance. Its identity was confirmed as described in Section 6.1. The characterisation stage included different approaches for purity assessment based on determinations performed by several laboratories using different methods for the sample preparation as well as for the final determination, demonstrating absence of measurement bias. The measurand is therefore structurally defined and independent of the measurement method.

Quantity value

The quantity value (AB mass fraction) has been derived from the gravimetric preparation of the CRM and the purity of AB. The purity has been assessed via two independent routes - qNMR determination of AB and mass balance approach (quantification of impurities and their subsequent subtraction from unity).

The traceability of the gravimetric preparation of the CRM is based on the use of calibrated balances and a thorough control of the substitution weighing procedure. The value is therefore traceable to the International System of Units (SI).

The qNMR determination of AB in the starting material was done by a validated method. The internal standard used was traceable to SI and all relevant input parameters were calibrated. Therefore, the value is traceable to the SI. All measurements of the impurities in the starting material used in the mass balance approach were done by validated methods and the relevant input parameters were calibrated. The realisation of the above-mentioned conditions demonstrates that the certified value is traceable to the International System of Units.

8.2 Commutability

Many measurement procedures include one or more steps, which are selecting specific (or specific) groups of analytes from the sample for the subsequent steps of the whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all the analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions expressing this concept. For instance, the CLSI Guideline EP30-A [13] recommends the use of the following definition for the term *commutability*:

> "The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and, thus, is a crucial characteristic in case of the application of different measurement methods. When commutability of a CRM is not established in such cases, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrant.

An AB solution intended to be used as a calibrant is usually prepared by dissolution of solid AB in deionised water. Therefore there is no reason to assume that ERM-AC626 would behave differently from other commercially available calibrants or calibrants prepared in-house by individual laboratories.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

9.2 Storage conditions

The materials shall be stored at 18 °C ± 5 °C in the dark.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened ampoules.

9.3 Preparation and use of the material

The unit shall be shaken by repeatedly turning it upside down for at least 30 s before opening to ensure material re-homogenisation.

9.4 Minimum sample intake

The minimum sample intake is 50 µg.

9.5 Use of the certified value

Use as a calibrant

The main purpose of this material is to be used as a calibrant for instrument calibration (e.g. external calibration, standard addition). The uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty.

Comparing an analytical result with the certified value

The reference material can be also used to assess the trueness of the value of own in-house prepared calibration solutions. In this case, the measured value of the CRM is compared with the certified value using the following procedure.

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, www.erm-crm.org [14].

For assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is described here in brief:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %
- If $\Delta_{meas} \leq U_{\Delta}$ no significant difference between the measurement result and the certified value, at a confidence level of about 95 % exists.

Use in quality control charts

The materials can be used for quality control charts. Different CRM-units will give the same result as inhomogeneity was included in the uncertainties of the certified values.

10 Acknowledgments

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Annexes

Annex A: Homogeneity study

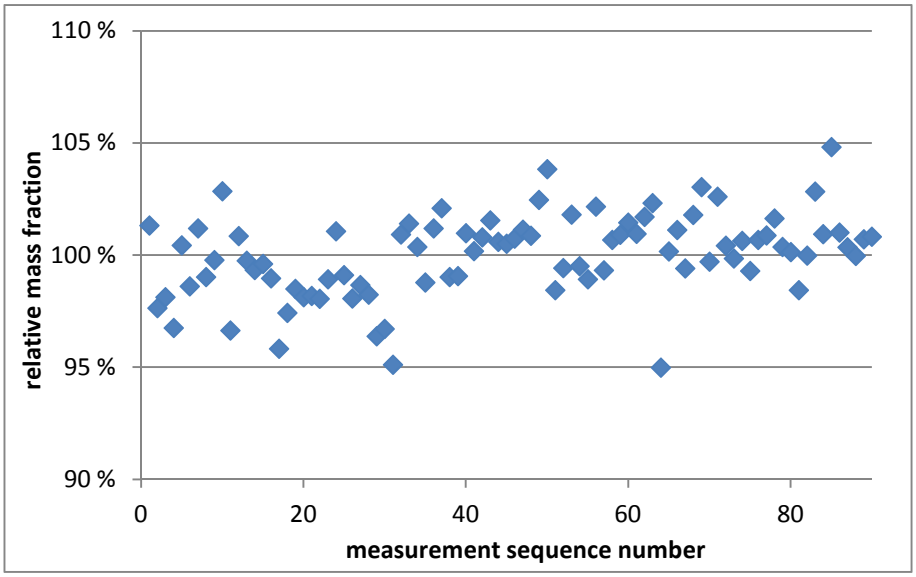

Figure A.1: Mass fractions of individual measurement replicates relative to the mean against sequence number before correction for analytical trend

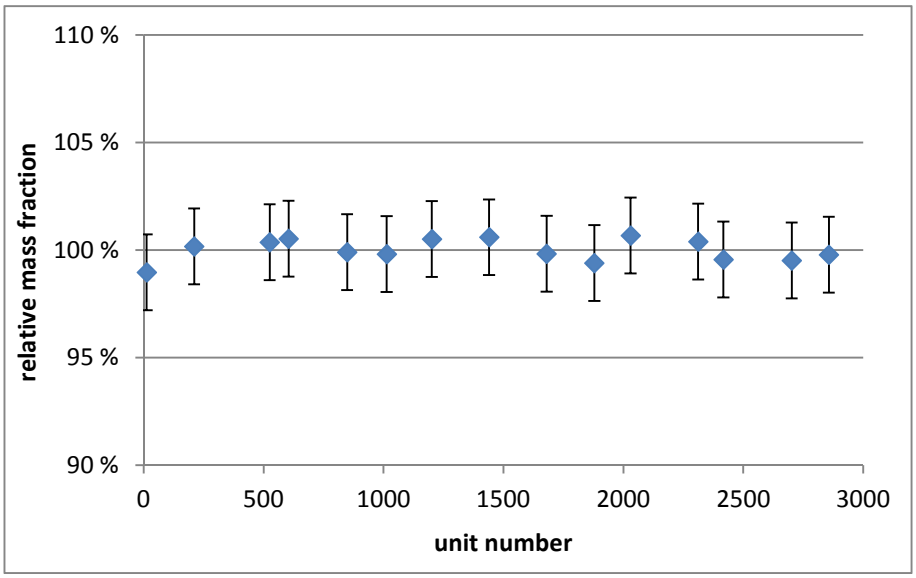

Figure A.2: Mass fractions of the mean values relative to the grand mean against unit number after correction for analytical trend. Vertical bars represent the 95 % confidence interval of the means

Annex B: Stability studies

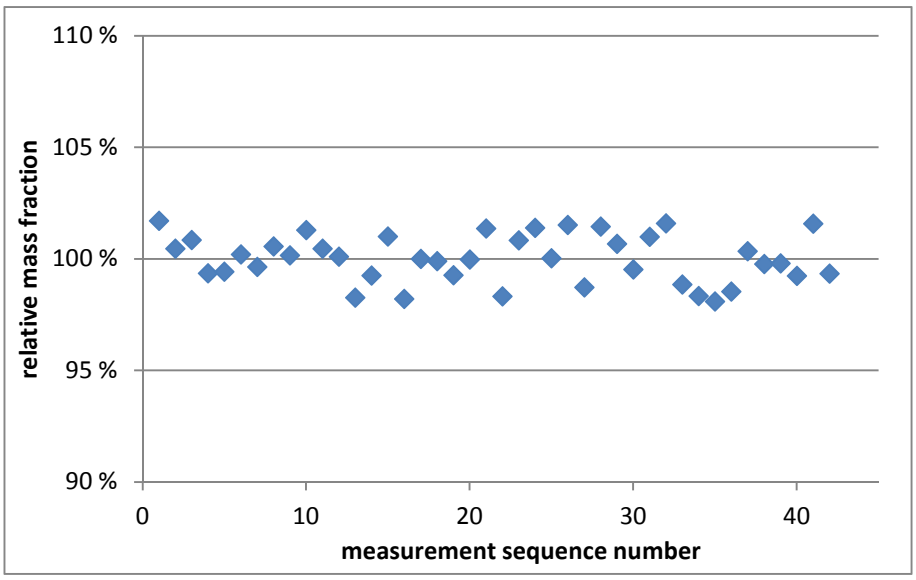

Figure B.1: Mass fractions of individual measurement replicates of AB relative to the mean against sequence number (short-term stability study)

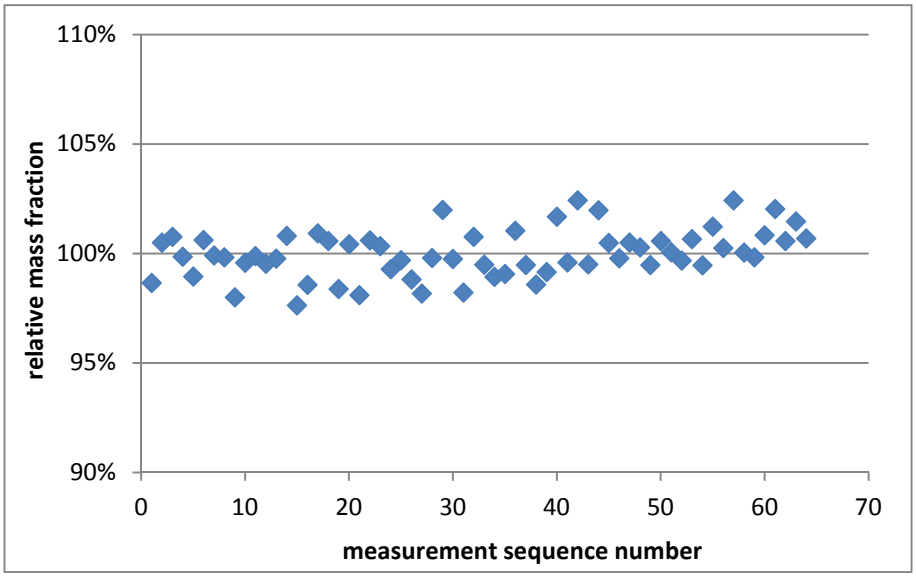

Figure B.2: Mass fractions of individual measurement replicates of AB relative to the mean against sequence number (long-term stability study)

The graphs below show the mass fractions of the mean values (per time and temperature) relative to the grand mean and their 95 % confidence intervals of the six replicates (2 units, 3 replicates per unit). Confidence intervals are based on the pooled repeatability standard deviation as obtained by ANOVA.

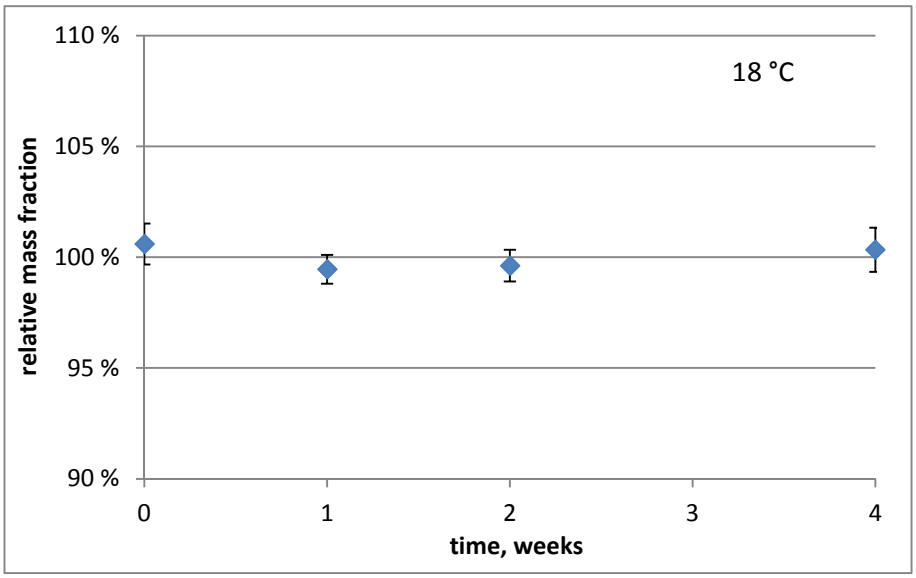

Figure B.3: Short-term stability study of AB at 18 °C

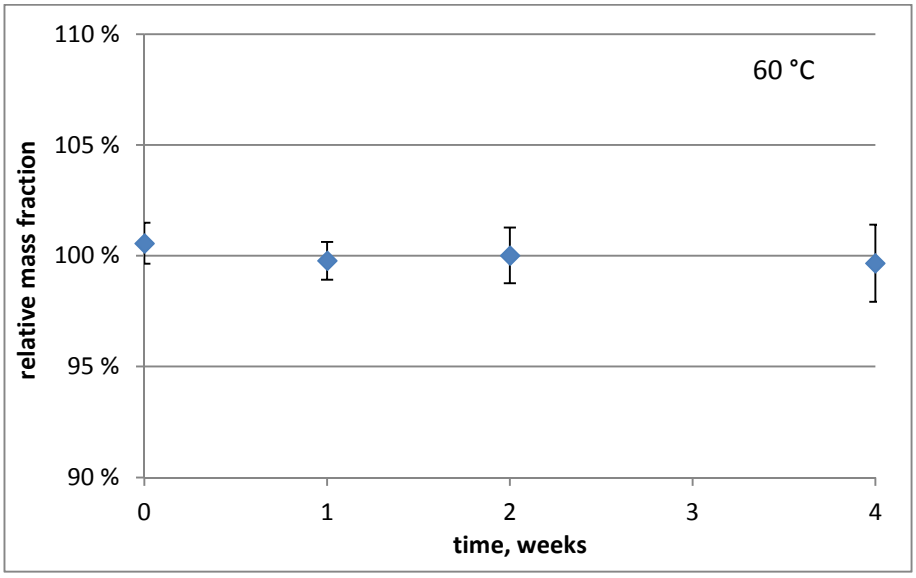

Figure B.4: Short-term stability study of AB at 60 °C

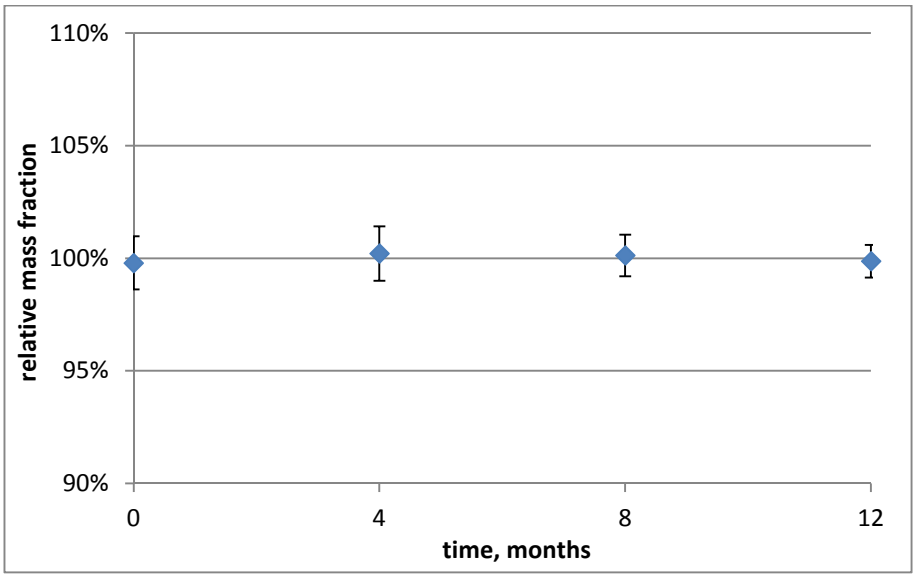

Figure B.5: Long-term stability study of AB at 18 °C

Annex C: Characterisation of the starting material

Table C.1: Measurement parameters of the qNMR used for the characterisation of the starting material

Parameter	Reagent	
Internal standard	Benzoic acid NIST SRM 350b	
Solvent	$DMSO_{d6}$	
Parameter	Value	Unit
Spectral width	5387.9	Hz
Acquisition time	6.08	s
Number of scans	40	
Excitation pulse	2.53	µs
Pulse angle	30	○

Table C.2: Analytical methods used for the characterisation of the starting material

Lab code	Analyte	Sample preparation method	Measurement technique
L1	C, H		Elemental analyzer
	O		Oxygen analyzer
L2a	C,H		Elemental analysis system
	As total	Ultraclave microwave digestion with nitric acid	ICP-MS (collision cell, He as collision gas)
L3a	As	Acid digestion	ICP-SFMS at high resolution mode
L4	As		k_0NAA
L2b	As inorganic, Trimethylarsine oxide, Dimethylarsinic acid		HPLC-ICP-MS
	Na, Mg, I, Br	Ultraclave microwave digestion	ICP-MS (collision cell, He as collision gas)
	Br		IC with conductivity detection
L5	Methanol, Ethanol, Aceton, Dietylether, n-butanol, Dibutylether		HS-GC-MS
	Water	Dissolved in methanol	Volumetric Karl Fischer titration
L6	Acetone, Methanol,		GC-FID, GC-MS

	Ethanol		
L3b	Mg, Al, Fe, Cu, Zn	Acid digestion	ICP-SFMS at medium resolution mode
	I	Alkaline digestion	ICP-SFMS at low resolution mode
	Br	Alkaline digestion	ICP-SFMS at high resolution mode
L7	Water		Volumetric Karl Fischer titration
L8	*Inorganic As, Trimethylarsine oxide, Dimethylarsinic acid		HPLC-ICP-MS

*Method used for the confirmation of the certified value of ERM-AC626

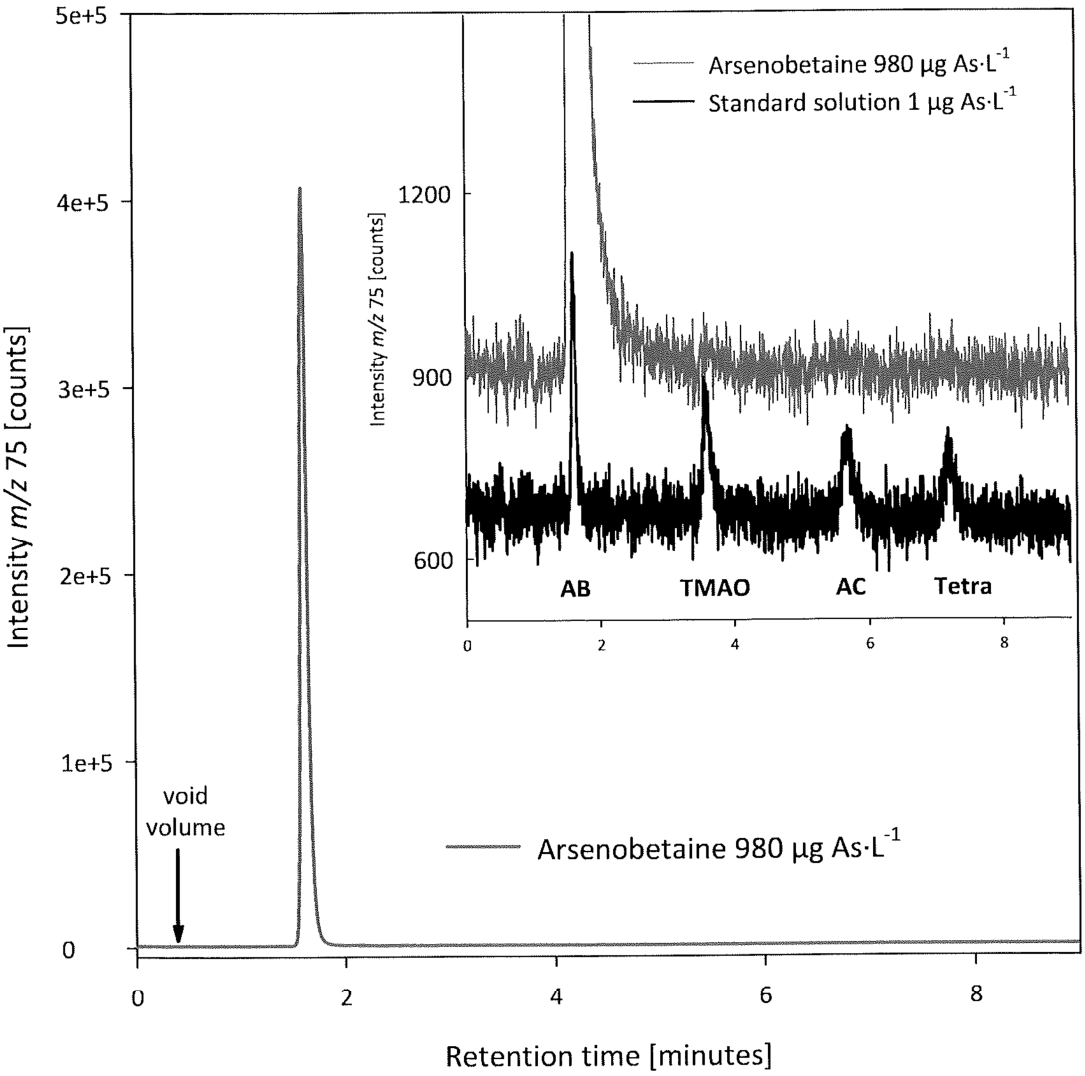

Figure C.1: Chromatogram of the AB starting material. Analysis by cation-exchange HPLC-ICP-MS. For better clarity the chromatograms of AB (980 µg As/L) and mix standard (1 µg As/L of cationic species AB (AB), trimethylarsine oxide (TMAO), arsenocholine (AC) and tetramethylarsonium ion (Tetra)) are overlaid with an offset of 300 counts

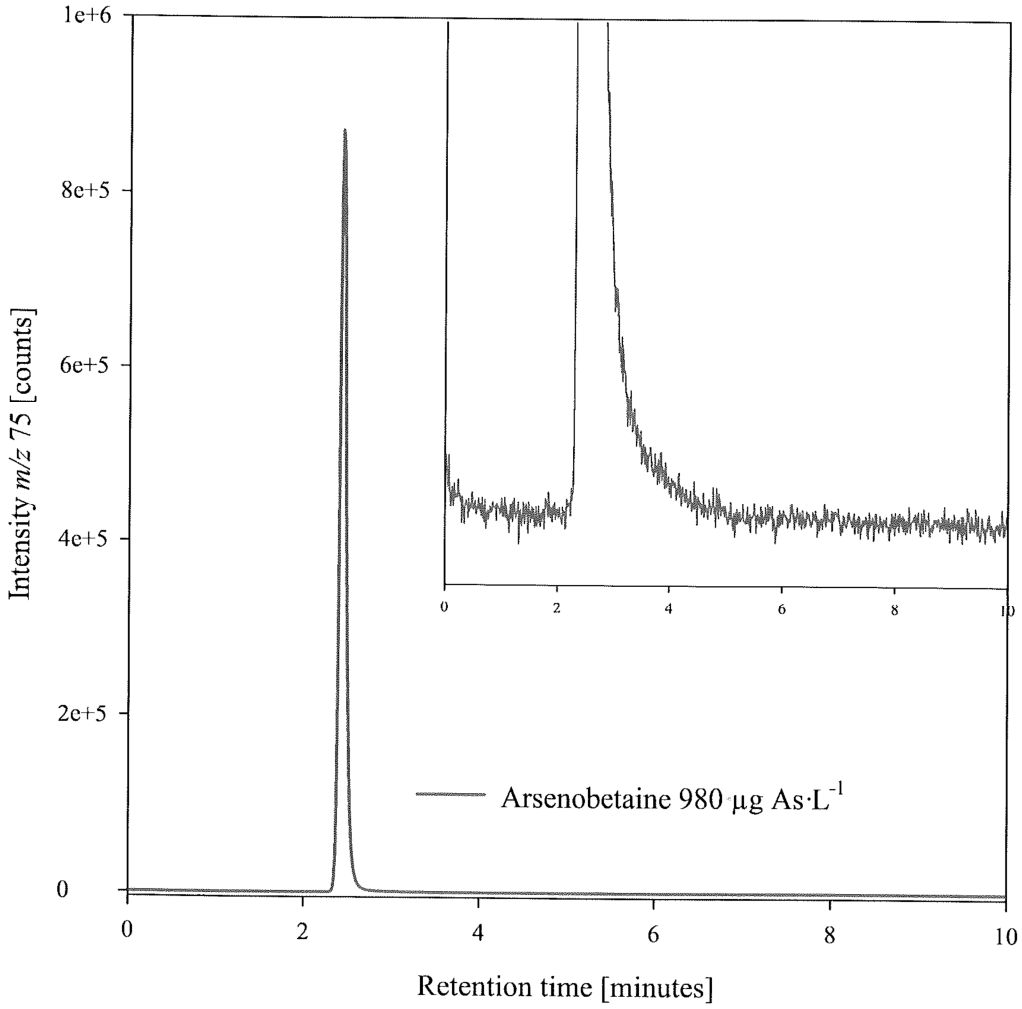

Figure C.2: Chromatogram of the AB starting material. Analysis by reversed phase HPLC-ICP-MS

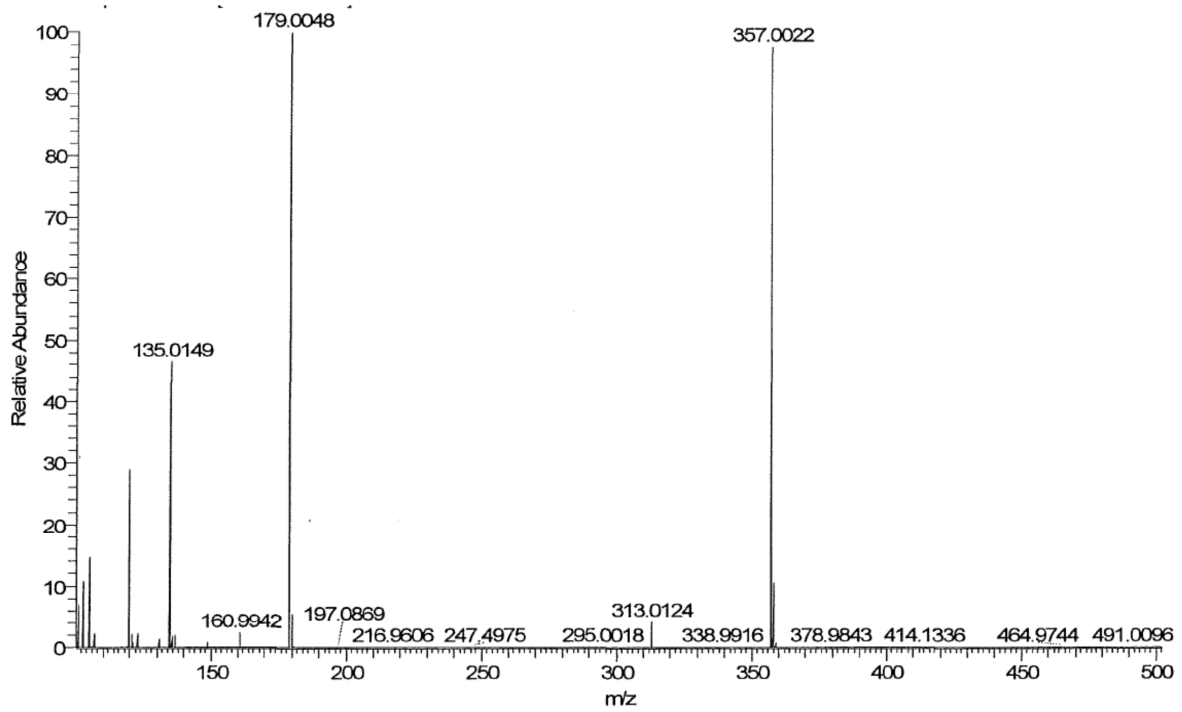

Figure C.3: HPLC-ESI-MS spectrum of the AB starting material

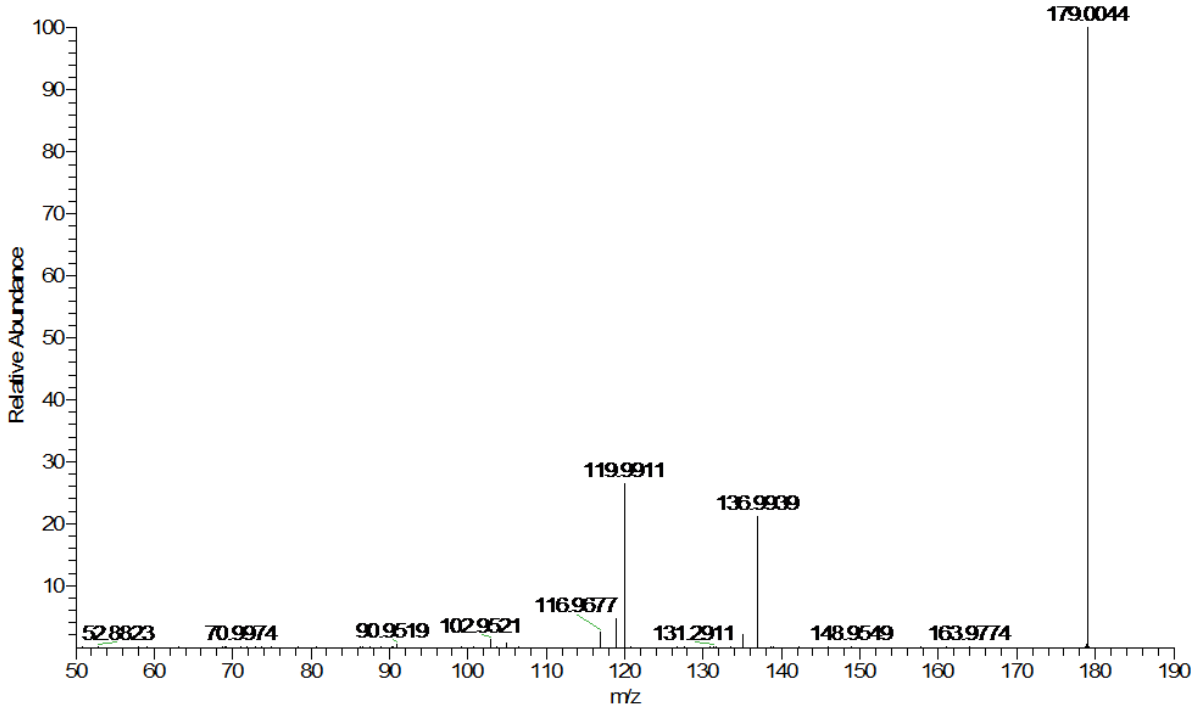

Figure C.4: HPLC-ESI-MS^2 spectrum of the AB starting material

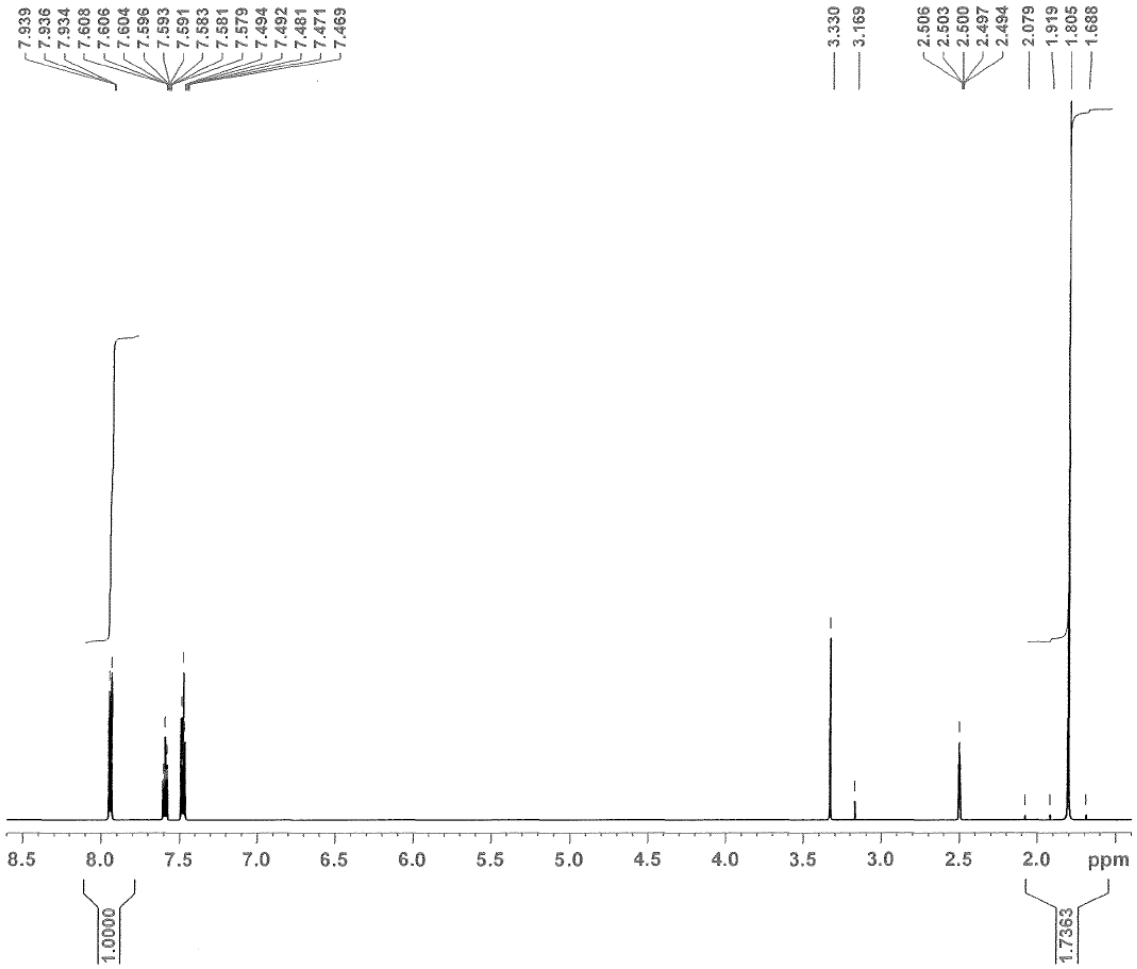

Figure C.5: ^{1}H-NMR spectrum of AB + benzoic acid

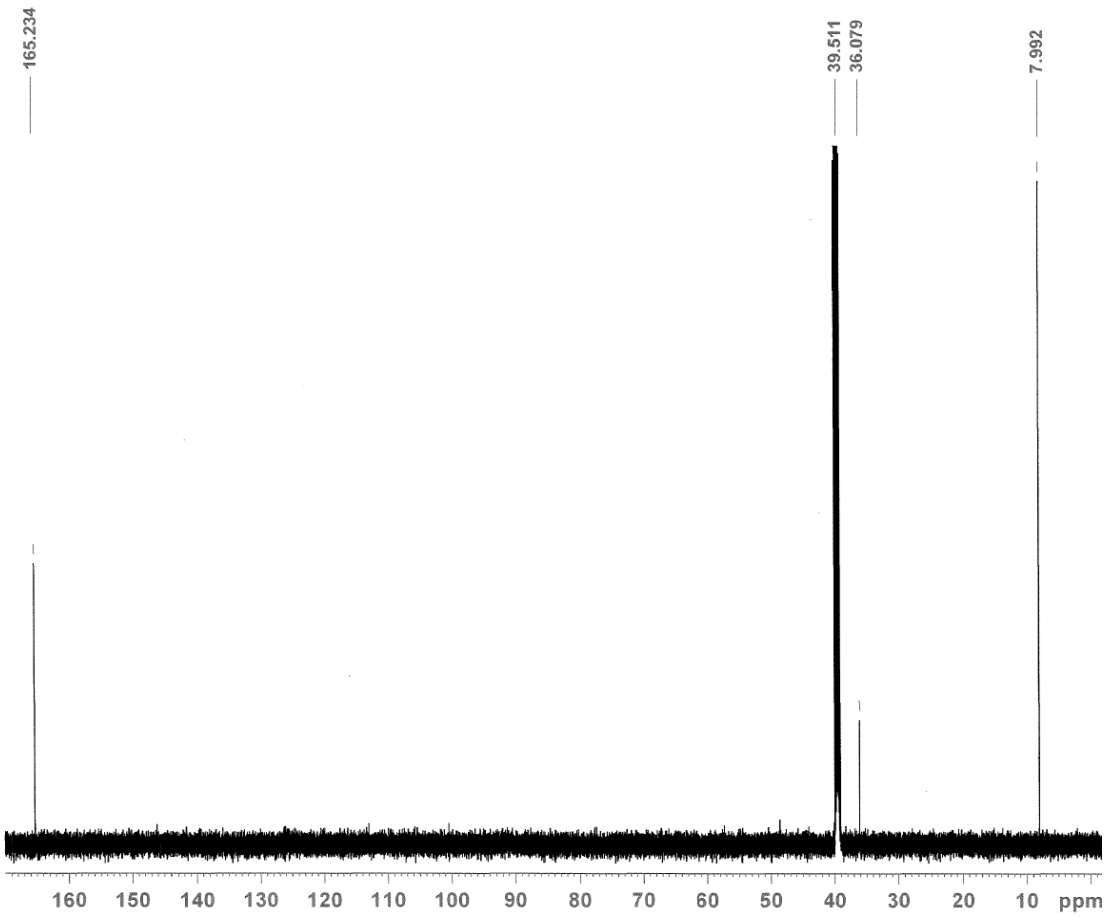

Figure C.6: $^{13}C\{^{1}H\}$ NMR spectrum of AB

Annex D: Confirmation analysis of the AB certified value

Table D.1: Results of the confirmation measurements of the certified value and the analytical methods used

Laboratory code	Analyte	Mean mass fraction [mg/kg]	Expanded uncertainty, k=2 [mg/kg]	Method
L2b	Arsenobetaine	241	19	HPLC-ICP-MS
L3c	Arsenobetaine	248.9	9.04	HPLC-ICP-MS
L8	Arsenobetaine	238.4	16.4	HPLC-ICP-MS
L2b	Arsenic	103	7	ICP-MS
L3c	Arsenic	105.6	3.74	ICP-MS
L4	Arsenic	109.7	6	k_0NAA
L8	Arsenic	100.8	6.7	ICP-MS

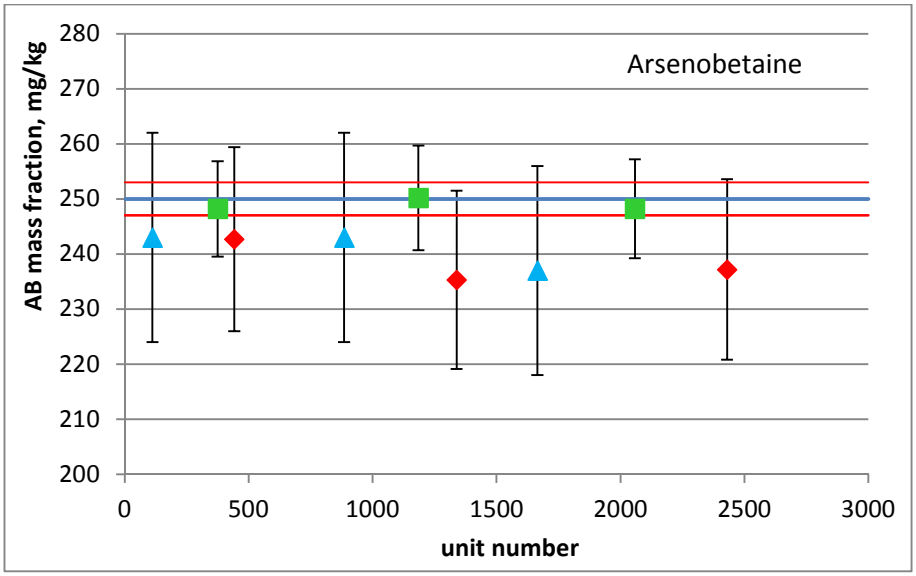

Figure D.1: Results of the confirmation analysis of the AB certified value. The blue line represents the certified value and the red lines outline the confidence interval with a level of confidence of about 95 %. The different colours of the value points correspond to: blue – L2b, green – L3c, red – L8

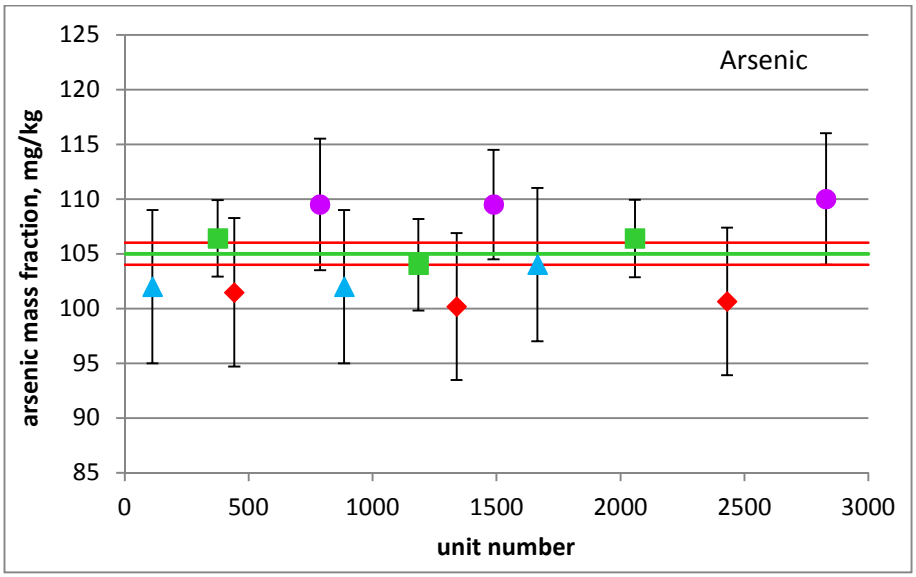

Figure D.2: Results of the arsenic determination used only for information because the As mass fraction is not certified. The green line represents the certified value of AB expressed as arsenic mass fraction (mg As/kg) and the red lines outline the confidence interval with a level of confidence of about 95 %. The different colours of the value points correspond to: blue – L2b, green – L3c, red – L8, purple – L4

European Commission

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