CCQM-K55.c (L-(+)-Valine)

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CCQM-K55.c Key Comparison on the Characterization of Organic Substances for Chemical Purity

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Introduction

The CCQM-K55 comparison was undertaken by the CCQM Organic Analysis Working Group (OAWG) for National Measurement Institutes (NMIs) and Designated Institutes (DIs) which provide measurement services in organic analysis under the CIPM Mutual Recognition Arrangement (MRA). The ability to perform suitable purity assessment on the materials that an NMI either makes available to external users as pure substance reference materials or that are used by an NMI as their primary calibrators for the assignment of the property values either of solution or matrix reference materials or for their reference measurement services is a core technical competency for the provision of measurement results in organic analysis that are traceable to the SI. The purity property value (generally reported for applications in organic analysis as the mass fraction[a] of the main component) assigned to the primary calibrator in a measurement hierarchy underpins the traceability chain for all results linked to that calibrator. All NMIs with ongoing programs in organic analysis were encouraged to participate in this series of comparisons.

The comparisons allow NMIs and DIs to demonstrate that their procedure for assignment of a purity property value and its associated uncertainty are fit for purpose for their intended application.

Summary of Previous Studies

The CCQM-P20 multi-round pilot study on purity determination was completed prior to the CCQM-K55 comparison. Studies were undertaken on the purity assessment of tributyl tin chloride (CCQM-P20.a), xylene (CCQM-P20.b), atrazine (CCQM-P20.c), chlorpyrifos (CCQM-P20.d), theophylline (CCQM-P20.e)[1] and digoxin (CCQM-P20.f)[2].

The "mass balance" or "summation of impurities" method for purity assessment, which aims to identify and quantify on a mass fraction basis all the orthogonal classes of impurity present in the material and by subtraction provides a measure of the mass fraction of the main component, was the most widely used approach by participants in the CCQM-P20 pilot studies. However the use of the quantitative nuclear magnetic resonance (qNMR) technique to obtain a direct measure of the content of the main component was increasingly being used.

The BIPM coordinated the final two rounds of the CCQM-P20 pilot study and developed a "molar mass v. polarity" model to map the analytical space for comparisons in this area. This model provided the criteria for the selection of the measurands for each of the four consecutive rounds – respectively CCQM-K55.a, CCQM-K55.b, CCQM-K55.c and CCQM-K55.d – that make up the initial CCQM-K55 key comparison. The relation based on this model between the proposed CCQM-K55 comparison materials and major areas of calibration and measurement capability (CMC) claims for the provision of primary calibrators and calibration solutions for organic analysis under the CIPM Mutual Recognition Arrangement is shown in Annex A.

The OAWG meeting at Sèvres in April 2008 accepted this overall strategy for the comparison as well as the specific measurand, 17β-estradiol, proposed for the first comparison round, CCQM-K55.a. A pilot study, CCQM-P117.a, was undertaken in parallel with the key comparison. The CCQM-K55.a comparison was completed in 2009 and the

[a] For the purposes of this comparison, the mass fraction of both the main component and associated impurities are expressed in units of mg/g. The upper limit value of 1000 mg/g corresponds to a "100 %" pure material.

Final Report was published in September 2012 in Appendix B of the BIPM Key Comparison Database.[3] A proposal for aldrin to be the measurand for the second comparison round, CCQM-K55.b, and accompanied by a parallel pilot study, CCQM-P117.b was approved at the April 2009 OAWG meeting at Sèvres. The CCQM-K55.b comparison was completed in 2012 and the Final Report was published in October 2012 in Appendix B of the BIPM Key Comparison Database.[4]

A proposal for L-(+)-valine to be the measurand for the third comparison round, CCQM-K55.c, and to be accompanied by a parallel pilot study, CCQM-P117.c, was approved at the April 2011 OAWG meeting at Sèvres. The comparison samples were distributed in May 2012. The individual results were communicated to the comparison coordinator in September 2012 and the results were first discussed at the November 2012 meeting of the OAWG in Hong Kong. Further investigations and data review were subsequently undertaken to resolve the apparent disparity between the results obtained by mass balance approaches and some of those obtained by qNMR, as well as separate reports by individual participants that the material contained significant amounts of D-(-)-valine enantiomer and of ammonium ion. The KCRV proposed in this report for valine in CCQM-K55.c is based on combination of separate KCRV estimates for contributing orthogonal impurity classes.

Valine

Valine was selected as the measurand for the second round of the comparison because it:

- provides an analytical challenge representative of a laboratory's capability for the purity assignment of organic compounds of low structural complexity and high polarity (see "How Far The Light Shines" statement);
- represents a sector for general CMC claims on the "analysis space" model (Annex A) which is distinct from the area already covered by the CCQM-K55.a and CCQM-K55.b measurands.
- is an amino acid relevant to a number of Calibration and Measurement Capability (CMC) claims currently in or in development for inclusion in either Appendix C of the BIPM Key Comparison Database (KCDB) or the Joint Committee on Traceability in Laboratory Medicine (JCTLM) Database of Higher Order Reference Materials;
- is an important measurand for the quantification of parent peptides and proteins via hydrolysis to their constituent amino acids;
- is safe and stable for transport in the amounts involved for the comparison and was available in sufficient amount to allow the preparation of a relatively large batch of the comparison sample.

The structure of L-(+)-valine (**1**) is shown in Figure 1 along with the conventional nomenclature (α-, β-, γ-) of the attached hydrogen atoms. The structure of amino acids reported as minor components of the comparison material are given in Annex B.

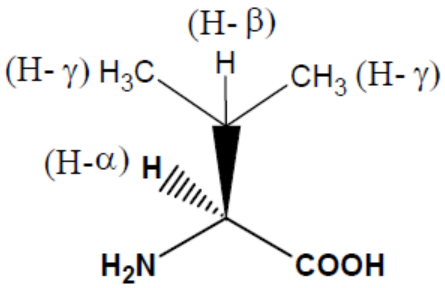

Figure 1 – Structure and hydrogen assignments of L-valine

L-Valine is a white crystalline powder with a reported thermal decomposition point at circa 296 °C. It has moderate solubility in water but is highly soluble in acidified or basified aqueous solution. It is moderately soluble in alcohols and polar organic solvents but generally insoluble in non-polar solvents. CMC claims for the measurement of L-valine, usually disseminated as a component of a standard solution CRM of stable amino acids, are listed in the BIPM KCDB Appendix C. Recently claims for both valine as a pure substance and as a component of a standard solution have been added to the JCTLM Database of Higher Order Reference Materials.

MATERIAL CHARACTERISATION AND CONDUCT OF STUDY

The comparison material for the CCQM-K55.c comparison and the parallel pilot study CCQM-P117.c was analytical grade L-valine purchased from a commercial supplier. The material was supplied as a white crystalline solid and was not subject to further purification. The analysis certificate provided with the material describes its purity as " ≥ 99.5% (NT)".

This material was subdivided into a batch of 175 individual units given the BIPM identifier OGP.015. Each unit of BIPM OGP.015 contained a minimum of 500 mg of L-valine in a 5 ml amber glass vial fitted with a rubber insert and crimped with an aluminium cap.

The impurity profile of the batch of sub-divided candidate material vials was determined at the BIPM, including assessment of the homogeneity and stability of the various components.

The mass fraction content of valine in the comparison material was assessed by the BIPM to be in excess of 990 mg/g and the homogeneity and stability of the valine and the associated impurity components were determined as being suitable for the purposes of the comparison.

A summary of the results for valine content and for characterization of the material's impurity profile reported by the comparison participants are contained in this report.

"How Far The Light Shines" Statement for CCQM-K55.c

The comparison is intended to demonstrate a laboratory's performance in determining the mass fraction of the main component in a relatively pure organic material. The measurement results should be indicative of the performance of a laboratory's measurement capability for the purity assignment of organic compounds of low structural complexity (molar mass range 100 g/mol-300 g/mol) and high polarity (pK_{OW} > -2) where K_{OW} is the octanol-water partition coefficient[5]. It is intended to be representative of compounds for which related structure impurities can be quantified directly by high performance liquid chromatography but not gas chromatography.

The expected overall outcome of the rounds making up the CCQM-K55 comparison is to evaluate through a series of strategically planned exercises the scope, applicability, limitations and appropriateness of the procedures used by an NMI to assign mass fraction property values to organic materials.

Characterisation study

The methods used to investigate, assign and confirm the quantitative composition of the CCQM-K55.c candidate material by the BIPM are summarised below.

Related structure impurity content was evaluated by:

a. LC-CAD
b. LC-MS/MS
c. GC-FID after derivatization[6] (related structure and enantiomeric purity)
d. ^{1}H and ^{13}C NMR

Water content was evaluated by:

a. coulometric Karl Fischer titration with heated oven transfer via dry nitrogen of water from the sample
b. thermogravimetric analysis (TGA) as a consistency check
c. microanalysis (% C,H content) as a consistency check

Residual solvent content was evaluated by:

a. GC-MS by direct injection
b. ^{1}H NMR
c. thermogravimetric analysis as a consistency check
d. microanalysis (% C,H content) as a consistency check

Non-volatile/ inorganics content :

a. ICP-MS for common elements (Na, K, Ca, Mg, Si, Fe, Al)
b. microanalysis (% C, H content) as a consistency check

Main component (Valine) content

a. qNMR

Homogeneity studies

i. Related structure components

The homogeneity of minor components related in structure to valine were assessed by sampling ten sub-units selected from across the candidate material batch with analysis by LC-MS/MS. The minimum sample size used to prepare each analysis sample was 2.5 mg.

ii. Water

The homogeneity of the material relative to water content was assessed by coulometric Karl Fischer titration using oven transfer and a minimum sample size of 50 mg per analysis on five sub-units representative of the candidate material batch

iii. Residual solvent

The homogeneity of the material relative to methanol content was assessed by direct injection GC-MS analysis using a minimum sample size of 5 mg per analysis on five sub-units representative of the candidate material batch.

iv. Inorganics content

Three units selected from across the production batch were analysed by ICP-MS and by elemental microanalysis for carbon and hydrogen. All gave metal content levels below the detection limits (25 ppm) for each element. Results for % C, H content were in accord with the molecular formula of valine

v. Valine

As a consistency check, the homogeneity of the valine content in the material was assessed using the ten sub-units selected for the related structure impurity study by the same LC-MS/MS methods developed for the related structure impurity characterisation. In addition a limited qNMR study was undertaken (two samples from two units of CCQM-K55.c), using maleic acid as the internal standard.

The uncertainty contributions due to the inhomogeneity of each related structure impurity component were evaluated by ANOVA. This provided an estimate of the variation due to inhomogeneity of each impurity at a stated sampling size both between and within sample units.

The uncertainty contributions due to the inhomogeneity of the major related structure components detected by LC-MS/MS ($u_{bb(rel)}$) were evaluated by ANOVA. This provided an estimate of the variation due to inhomogeneity of related structure impurities at the stated sampling size both between and within sample units. Acceptable uncertainty contributions due to inhomogeneity were observed for each of the resolved impurities present in the sample. Table 1 shows the estimated content, $u_{bb(rel)}$ and $u_{bb(abs)}$ for each of the major related structure impurities, and a combined value for the overall uncertainty contribution from between unit inhomogeneity (u_{bb}) of the related structure impurities content of the material. This was calculated as 0.038 mg/g by quadratic combination of the absolute inhomogeneity uncertainties for each impurity.

Impurity	Content (mg/g) from homogeneity study	$u_{bb(rel)}$ (%)	$u_{bb(abs)}$ (mg/g)
Alanine	2.77	0.90	0.025
Leucine	1.99	0.99	0.020
Isoleucine	1.92	1.11	0.021
Combined related structure impurities	6.68		0.038

Table 1: Homogeneity assessment for related structure impurities in CCQM-K55.c

For the homogeneity measurements, 5 vials taken at regular intervals from the filling sequence were analysed in duplicate (n = 2) in randomly stratified order for their water content using the Karl Fischer method described above. Sample portions of mass from 99.3 mg to 104.1 mg were weighed directly into the analysis vials and sealed. The result for each sample was not significantly different from those obtained by blank vials. It was not possible to make a direct evaluation of the homogeneity of the material as it was not distinguishable from the results obtained for blank vials under the same conditions.
The homogeneity testing of the water content of the CCQM-K55.c candidate material was consistent with the assigned value (0 mg/g) and showed no significant inhomogeneity beyond that attributable to the variability of the analytical process when a sample size of 100 mg was analysed.
For the assignment of a reference value no contribution due to the inhomogeneity of water content is considered either for the absolute value of water content or for its associated standard uncertainty.

Stability studies

An isochronous stability study was performed using a reference storage temperature of -20 °C and test temperatures of 4 °C, 22 °C and 40 °C. A set of units from the production batch were stored at each selected temperature over 8 weeks, with units transferred to reference temperature storage at 2-week intervals.
Trend analysis of the data obtained by LC-MS/MS analysis of the stability test samples under repeatability conditions indicated no significant change in the relative composition of valine or of the related structure components over this time at any of the test temperatures.
No significant changes in water content, which in any case were all below the level of quantification of our method, were observed after storage at 4 °C or 22 °C. There was some evidence of minor uptake of water but only after prolonged storage at 40 °C.

On the basis of these studies it was concluded that for the purposes of the comparison the material was suitably stable for short-term transport at ambient temperature, provided it was not exposed to temperatures significantly in excess of 40 °C, and for longer term storage at room temperature or below. To minimise the potential for changes in the material composition, participants were instructed to store the material at 4 °C.

Sample distribution

Two units of the study sample, each containing a minimum of 500 mg of material, were distributed to each participant. Participants were asked to return a form acknowledging receipt of the samples and to advise the comparison coordinator if any obvious damage had occurred to the vials during shipping. Recipients were asked to confirm that a monitoring strip inside the shipping container had not registered a temperature in excess of 37 °C during the transport process.

The monitor strips indicated that during two separate attempts the units supplied directly to NIM were exposed to temperatures in excess of 40 °C during shipping. A replacement set was finally delivered to NIM without exposure to elevated temperature by trans-shipment via Hong Kong.

Each of the twenty registered participants in the CCQM-K55.c comparison provided a result for their sample. In addition four of the participants in the key comparison, who assigned their value for the valine content in CCQM-K55.c using a mass balance approach, also obtained an estimate of valine content using a qNMR approach. The latter values were included in the results reported for the parallel pilot study CCQM-P117.c

Quantities and Units

Participants were required to report the mass fraction of L-(+)-valine, the major component of the comparison sample, in one of the two units supplied to them. The additional unit was provided for method development and trial studies.
In addition all participants who used a mass balance (summation of impurities) procedure to determine valine content were required to report the combined mass fraction assignment and associated uncertainty for some or all of the following sub-classes of impurity:

i. combined related structure organic substances

ii. water

iii. residual organic solvent / volatile organic compounds (VOCs)

iv. combined non-volatile organics & inorganics

Participants were encouraged but not required to identify and provide mass fraction estimates for the individual impurity components they reported in the comparison sample.

Mass Fraction of Valine in CCQM-K55.c

The values reported by participants for valine content in the comparison material are given in Table 2. The results are shown graphically, plotted against the Key Comparison Reference Value for valine, with their reported standard uncertainty in Figure 4 and with their expanded uncertainty for the 95% confidence range in Figure 5 on page 26 of this report.

Participant	Valine (mg/g)	u_c (mg/g)	Coverage factor	$U_{95\%}$ (mg/g)	Assignment method
UME	979.2	1.84	2	3.67	Combination of data from qNMR and mass balance
INMETRO	984.9	0.85	1.96	1.7	Mass balance
NMIA	985	2	2.03	4	qNMR
NRC	987	3.4	2	6.8	qNMR
NMISA	988.9	3.3	2	6.7	Mass balance
NIST	990.0	0.9	2	1.8	qNMR
CENAM	990.095	56.38	2	112.76	Combination of data from titration and mass balance
VNIIM	990.47	0.18	2	0.36	Mass balance
IRMM [a]	990.9	0.6	2	1.3	Combination of data from qNMR and mass balance
NIM [b]	990.9	1.14	2	2.28	Combination of data from qNMR and mass balance
BAM	991.22	0.16	2	0.31	Combination of data from qNMR and mass balance
KRISS	992	0.34	2.78	0.94	Mass balance
HSA [b]	992.1	1.6	2	3.2	Mass balance
NMIJ	992.6	0.51	2	1.1	Combination of data from qNMR, titration and mass balance
LGC [b]	992.7	2.3	2.09	4.8	Mass balance
GLHK	992.9	2.5	2	5.0	Mass balance
LNE [b]	992.95	0.85	2	1.7	Mass balance
SIRIM	993.0	1.5	2	3.0	qNMR
BIPM	993.2	+ 0.18, - 0.70	2	+ 0.36, - 1.40	Mass balance
NIMT	994.25	0.46	2	0.92	Mass balance

Table 2: Valine content (mg/g) of CCQM-K55.c reported by participant

a. qNMR data obtained by sub-contracting the service provision to BAM

b. An estimate of valine content using a qNMR approach only is reported in CCQM-P117.c

Impurity Profile and Key Comparison Reference Values (KCRVs) for Impurity Classes in CCQM-K55.c

All participants in CCQM-K55.c using a mass balance procedure to assign the valine content were required to give estimates for the mass fraction of the sub-classes of impurities they quantified in addition to their overall valine mass fraction estimate. At the November 2012 WG meeting it was agreed that, as for the previous comparisons, the comparison coordinator would propose an overall KCRV for the valine content of CCQM-K55.c based on the combination of individual KCRVs for the mass fraction of each of the orthogonal classes of impurity in the comparison sample.
This required the assignment of KCRVs for the mass fraction in CCQM-K55.c of:

- combined related structure impurities (w_{RS});
- water (w_{H2O});
- volatile organic solvent (w_{VOC});
- combined non-volatile organics and inorganics (w_{NV}).

i. KCRV for Related Structure impurity content (w_{RS})

The structures of the related structure impurities reported to be present in CCQM-K55.c by two or more participants are shown in Annex B. The major compounds identified by multiple participants as present at levels greater than 0.1 mg/g in CCQM-K55.c were: alanine (**2**), leucine (**3**), isoleucine (**4**), α-aminobutyric acid (**5**) and methionine (**6**). Information on the related structure impurity content was also provided for information purposes by some participants that used a qNMR assay to directly assign the valine content. Note that for the purposes of this comparison acetic acid was classified as a related structure impurity of valine. It could equally well be classified as a residual solvent/reagent impurity but as the majority of participants reported it as a related structure impurity for reporting purposes it is included under this classification in this report.

Due to the lack of a UV chromophore in the parent compound or the main impurities, LC-UV methods could not be used directly to determine the related structure impurity profile of the material. Use of a charged aerosol detector (CAD) or electrospray MS/MS techniques did allow for analysis of underivatised samples of the CCQM-K55.c material. An LC chromatogram for CCQM-K55.c, in this case with detection using a CAD (LC-CAD) is shown in Figure 2. The elution profile is representative of those obtained for underivatised samples of CCQM-K55.c under reverse phase LC conditions.
A number of participants used derivatisation strategies involving LC with fluorescence detection (FLD) or UV detection after derivatisation using orthophthaladehyde (OPA) or fluorescein, or GC-MS after silylation. The elution profiles obtained using these approaches were similar to those in Figure 2, although the derivatisation approaches included "noisier" baselines and some artefact peaks from the reagents. The advantage of the derivatisation approach using LC-FLD or LC-UV, which are well-established analytical methods for amino acid analysis, were greater sensitivity compared with LC-CAD or LC-MS/MS.
The majority of participants reported and identified alanine (**2**), leucine (**3**) and isoleucine (**4**) as the main related structure impurities in the comparison material. The presence of an additional impurity was reported by eleven participants, six identifying it as α-aminobutyric acid (**5**) while five participants reported it as an unidentified component. Relative retention

time studies by NIM using authentic standards of γ- and α-aminobutyric acid established that the impurity is α-aminobutyric acid (a-Ab in Figure 2).

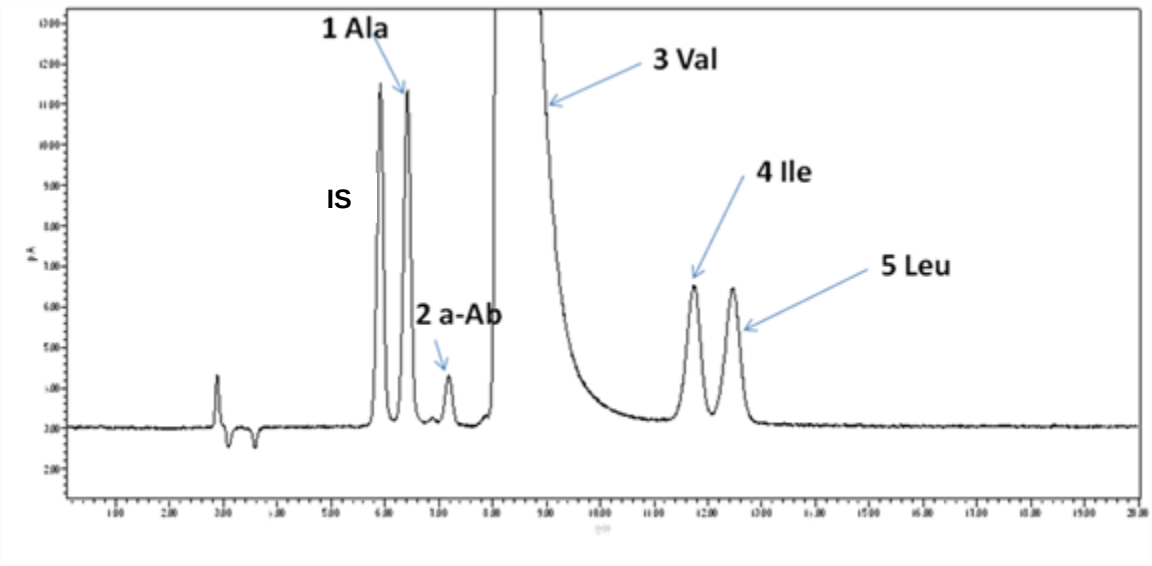

Figure 2 Representative LC-CAD chromatogram for CCQM-K55.c (reproduced from NIM report)

A summary of the combined quantification results reported for the main related structure impurities reported by more than one participant is provided in Table 3.

Compound	Participants reporting	Participants quantifying	Mean (mg/g)	Std. devn. (mg/g)
Alanine	18	17	2.54	0.34
Leucine	18	17	2.06	0.67
Isoleucine	18	17	1.79	0.32
Acetic acid	6	3	0.65	0.33
α-Aminobutyric acid	11[a]	6	0.35	0.06
Methionine	4	4	0.05	0.03

Table 3 – Estimates of individual related substance impurities in CCQM-K55.c

(a) Includes where reported as an unidentified component at a retention time corresponding to α-aminobutyric acid.

The reporting requirements required a value for combined related structure impurities for participants using the mass balance approach. The values for the combined related structure impurities and the associated standard uncertainty as well as the quantification assignments for individual impurities reported by all participants are summarised in Table 4.

Participant	TOTAL (u_c)	Ala	Leu	Ile	α-AB	Met	Acetic acid	Other	Method
NIMT	5.46 (0.14)	2.05	1.64	1.58				0.19	LC-FLD
LNE	6.58 (0.11)	2.45	2.13	2.00					GC-MS
BIPM	6.80 (0.11^{-}, 0.61^{+})	2.68	2.02	1.76			0.35		LC-MS/MS
GLHK	6.97 (2.07)	2.57	2.06	1.95				0.38	LC-FLD, LC-MS
IRMM	7.02 (0.54)	2.59	1.66	1.43	0.31		1.0		LC-IDMS, qNMR
LGC	7.12 (2)	2.45	2.13	2.00					GC-FID
HSA	7.16 (0.09)	2.59	1.96	1.92		0.045		0.63	LC-IDMS, LC-UV
NMIJ	7.58 (0.34)	2.676	2.184	1.866	0.455	0.1			LC-FLD
VNIIM	7.61 (0.17)	2.78	2.04	2.17	0.27			0.53	LC-MS, GC-MS
NMISA	7.67 (0.93)	1.76	1.93	1.79				2.19	LC-UV, GC-TOF
NIM	7.97 (0.52)	2.58	2.05	1.96	0.35	0.03		1	LC-CAD, LC-MS/MS, LC-UV
NIST [a]	8.00 (0.5)	2.6	2.2	2.1	0.37	0.04		0.65	LC-MS, qNMR
KRISS	8.02 (0.057)	3.3	1.84	1.73				1.19	LC-UV
BAM	9.13 (0.14)	2.83	1.92	2.08	0.33		0.6	1.37	GC-FID, qNMR
CENAM	9.68 (0.47)	2.258	4.512	1.048				1.863	LC-MS/MS, LC-FLD
INMETRO	13.81 (0.45)	*b*	*b*	*b*					GC-FID
UME	20.30 (0.133)								GC-FID, GC-MS
NMIA [a]		2.3	1.9	2.2					qNMR
NRC [a]		2.5	1.4	1.4					qNMR

Table 4 – Assignments of total and individual related structure impurities (mg/g) in CCQM-K55.c

a. Information value, not used in the assignment of valine content
b. Identified but not individually quantified

All the reported values that included the three major impurities (alanine, leucine and isoleucine) in their total value were included in the data used to assign w_{RS}. However there were some indications that participants using GC-FID after derivatisation under relatively forcing conditions (> 100 °C) may have introduced artifact peaks that increased their reported value. For this reason the median rather than the mean of the selected results was selected as the central tendency estimate to assign w_{RS} from this data set.

The associated standard uncertainty ($u_{wRS.}$) is the robust standard deviation of the median (MADe/√n, n = 16). This gives the following values for the KCRV for related structure impurity in CCQM-K55.c :

w_{RS} = 7.60 mg/g;
u_{wRS} = 0.24 mg/g

The results reported by participants for combined related structure impurity content with their associated standard uncertainties (k = 1) plotted against w_{RS} are shown in Annex C, Figure 7. The DoE table and plot for these results relative to the related structure impurity KCRV are given in Table 13 and Figure 11 respectively in Annex D.

Enantiomeric Purity of CCQM-K55.c

The measurement of the D-(-)-valine content of CCQM-K55.c was not a requirement of the comparison, however four participants including the coordinating laboratory reported on the enantiomeric purity of the material as part of their submission and one participant carried out a follow-up study after the original discussion of results.
Four different approaches were used and the results are summarized in Table 5.

Participant	Enantiomeric Assay Method	D-Valine estimate (mg/g)	Representative data in Annex E
BIPM	Derivatise with ethylchloro-formate then chiral GC-FID [7]	< LOD	Figure 15
INMETRO	Derivatise with Marfey's reagent[8] (L-FDAA) then LC-MS	18.6	Figure 16
NIM	LC-MS on chiral LC column	< LOD	Figure 17
NIST	NMR with chiral resolving agent	< LOD	Figure 18
HSA	Derivatise with Marfey's reagent (L-FDAA) then LC-DAD	< LOD	

Table 5 – Enantiomeric assay methods and results for valine in CCQM-K55.c

Examples of the results obtained by each participant for enantiomeric purity determinations for the CCQM-K55.c material are reproduced in Annex E.
All methods were able to demonstrate separation by either chromatographic retention time or NMR signal dispersion of the D- and L- enantiomeric forms of valine under the analysis conditions. Only the BIPM method reported detecting the L-Ala, L-Leu and L-Ile impurities also shown to be present in the material.
INMETRO, using the Marfey's reagent method, were the sole participant to report a significant level of D-valine in the sample. In a follow up study INMETRO reported that under their conditions the D-valine content in CCQM-K55.c was significantly higher than that observed for a high purity valine standard analysed under the same conditions. In a follow up study HSA also applied the Marfey method to the material and did not report any significant level of D-valine under their conditions.

As it was not a requirement for the comparison and enantiomeric purity has no effect on the total valine content it was decided at the April 2013 OAWG meeting to simply note the discrepancy between the INMETRO results and those reported by the other participants. For the purposes of finalizing the comparison undertaking further investigations to resolve the reason for the difference in reported results was not deemed to be warranted.

ii. KCRV for water content in CCQM-K55.c (w_{H2O})

The values for water content reported by the participants are summarised in Table 6.

Participant	Water (mg/g)	u_c (mg/g)	Coverage factor	$U_{95\%}$ (mg/g)	Method
BIPM *	0	+ 0.14 /- 0	2	+ 0.28 / -0	Oven transfer KFT, 2 x 100 mg @ 180 °C
KRISS *	0	+ 0.28 /- 0	4.3	+1.2 / -0	Oven transfer KFT, 3 x 20 mg @ 120 °C
BAM	0.06	0.02	2	0.03	Oven transfer KFT, 2 x 100 mg @ 120 °C
NMIJ	0.062	0.020	2	0.040	Oven transfer KFT, 2 x 55 mg @ 160 °C
LNE	0.069	0.019	2	0.038	Oven transfer KFT, 3 x 100 mg @ 200 °C
GLHK	0.12	0.03	2	0.05	Oven transfer KFT, @ 160 °C
LGC	0.15	0.06	4.303	0.27	Oven transfer KFT, 3 x 30 mg @ 95 °C
NIST	0.16	0.04	2	0.08	Direct addn. KFT, 2 x 50 mg
INMETRO *	0.2	+ 0 /- 0.058	2	+ 0 / - 0.11	Oven transfer KFT, 1 x 250 mg @ 170 °C
CENAM	0.222	0.0016	2	0.0032	Oven transfer KFT, 3 x 100 mg @ 150 °C
NIM	0.27	0.025	2	0.05	Direct addn. KFT, TGA
NIMT	0.28	0.20	2	0.40	Direct addn. KFT, 4 x 60 mg
UME	0.35	0.0192	2	0.039	Oven transfer KFT, 3 x 50 mg @ 150 °C
NMISA *	0.64	0.11	2	0.21	Direct addn. KFT, 3 x 50 mg, heated cell
HSA *	0.72	0.25	2	0.50	Oven transfer KFT, @ 280 °C
VNIIM *	1.84	0.04	2	0.08	Oven transfer KFT, 3 x 50 mg @ 220 °C
IRMM *	2.47	0.37	2	0.74	Vaporisation KFT , 3 x 35 mg @ 105 °C
NRC *	7.65	0.57	2	1.14	Direct addn. KFT, heated solution in DMF

Table 6: Assignments of water content for CCQM-K55.c

* Result not included in dataset for estimation of w_{H2O}

All participants used coulometric Karl Fischer titration as either their sole or a major method to determine water content. Both direct addition of the comparison material into the titration cell as a solid or evaporative transfer through oven heating to deliver the water content into the titration cell were used.
Results obtained using heated oven transfer from a solid sample of CCQM-K55.c at temperatures above 220 °C or where a solution of CCQM-K55.c was heated to maintain solubility of the valine prior to KFT analysis indicated a larger amount (> 0.6 mg/g) of water was present than the results of procedures where material was not heated above 200 °C. The discrepancy in these results was accounted for as due to formation *in situ* of water by a condensation reaction between the amine and carboxylic acid functional groups present in the compound. This reaction appeared to occur at a detectable rate in the solid at temperatures above 220 °C and more rapidly when valine was heated in solution. TGA thermograms for valine confirm that at temperatures above 200 °C it commences to decompose and the mass of the material never subsequently reaches a plateau level.
For KCRV calculations all values for water obtained using heating above 220 °C or in solution were excluded. The IRMM result was anomalous, being larger than other non-solution KFT results but obtained at a relatively low temperature. The difference in absolute value and relatively high variability compared with other results was ascribed to the relatively small sample size used and it was decided to also exclude this value.
After exclusion of values in which a bias due to water formation under the analysis conditions may have occurred, and three results where water content was reported as below the method quantification level, the median of the resulting data set was selected as the KCRV for water content (w_{H2O}) in CCQM-K55.c. The standard uncertainty of the KCRV (u_{H2O}) was the robust estimate of the standard deviation of the median (MADe/√n, n = 10).

w_{H2O} = 0.155 mg/g;
u_{H2O} = 0.042 mg/g

Data from other techniques (qNMR, elemental analysis, TGA) provided cross checks for this assignment and were consistent with the KCRV. The results reported by participants with their associated standard uncertainties (k = 1) plotted against the w_{H2O} are shown in Annex C, Figure 8. The DoE table and plot of individual results relative to the water content KCRV are given in Table 14 and Figure 12 respectively in Annex D.

iii. KCRV for VOCs in CCQM-K55.c (w_{VOC})

Fourteen participants provided estimates for the volatile organics content of CCQM-K55.c. GC-MS approaches with detection from either the heated headspace or by direct injection in solution, or headspace GC-FID analysis were predominantly used to test for the presence of traces levels of solvent. No significant residual solvent was identified in the material by any participant using these techniques.
NIST reported a low level of ether and a trace level of t-butyl ethyl ether identified through an extended NMR experiment that was not attempted by any other participant.
NMIJ reported the presence of a trace level of 2-methylpropanal identified by GC-MS and retention time. The results for residual solvent content reported by the participants are listed in Table 7.

Participant	Residual Solvent (mg/g)	u_c (mg/g)	Coverage factor	$U_{95\%}$ (mg/g)	Method
BIPM	0	+ 0.1 / - 0	2	+ 0.2 / - 0	hsGC-FID, GC-MS, TGA
CENAM	0	-	-	-	GC-FID
KRISS	0	+ 0.02 / - 0	1.96	0.04	GC-FID
HSA	0	+ 0.58 / - 0	-	+ 1.16 / - 0	GC-MS, TGA
IRMM	0	+ 0.16 / - 0	2	+ 0.32 / - 0	qNMR
LGC	0	+ 1.1 / - 0	2	2.2	TGA-MS, hsGC-MS
GLHK	0	+ 1 / - 0	2	2	hsGC-MS
LNE	0	+ 0.82 / - 0	2	1.6	hsGC-MS
NMISA	0	+ 0.75 / - 0	2	1.50	hsGC-TOFMS
NMIJ	0.0017	0.0007	2	0.002	hsGC-MS
NIMT	0.01	+ 0.30 / - 0	2	0.60	hsGC-MS
NIM	0.021	0.011	2	0.022	hsGC-MS, GC-FID, qNMR
VNIIM	0.02	+ 0.1 / - 0	-	-	hsGC-TOFMS
BAM	0.1	+ 0 / - 0.1	-	-	hsGC-MS, FID
NIST	0.16	0.03	2	0.06	qNMR, SPME-GC/MS

Table 7 – Assignments of residual solvent content in CCQM-K55.c

There is no evidence of a significant level of residual solvent in the material and if present it was below the detection limits of the methods reported by the majority of NMIs. However it was not possible to exclude the NIST result. As the majority of results are below their detection limit simple statistical techniques cannot be applied and a type B estimate is required. After discussion within the OAWG at the April 2013 meeting it was proposed that the best compromise was that the KCRV for residual solvent (w_{VOC}) be assigned as 0.0 mg/g with an associated asymmetric uncertainty calculated assuming an equal probability up to an upper limit of 0.2 mg/g. This gives u_{VOC+} of 0.12 mg/g (= 0.2/√3) and u_{VOC-} of 0.0 mg/g.

$$w_{VOC} = 0.0 \text{ mg/g}$$
$$u_{VOC+} = 0.12 \text{ mg/g}$$
$$u_{VOC-} = 0.0 \text{ mg/g}$$

The results reported by participants with their associated standard uncertainties (k = 1) plotted against the w_{VOC} are shown in Annex C, Figure 9. The DoE table and plot of individual results relative to the residual solvent KCRV are given in Table 15 and Figure 13 respectively in Annex D.

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iv. KCRV for non-volatile organics & inorganics content in CCQM-K55.c (w_{NV})

The values reported for combined non-volatile organics and inorganics content by the comparison participants are listed in Table 8. Various methods including ICP-MS, ICP-OES, XRF spectrometry, TGA and combinations thereof were used and participants generally reported negligible levels of this impurity class, often below the quantification limits of their methods.
The presence of trace levels of cations in the material (Fe^{2+}, Ca^{2+}, Mg^{2+}, NH_4^+) was noted by some participants but the combined levels of these impurities was below 0.2 mg/g.
It was decided by the participants that the information available from the ensemble of reported results was best interpreted as consistent with a rectangular distribution of possible values up to a maximum of 0.5 mg/g. The KCRV for non-volatiles content (w_{NV}) in this case was the mid point of the range and the associated standard uncertainty of the KCRV is the half range divided by √3.

w_{NV} = 0.25 mg/g;

u_{NV} .= 0.144 mg/g

Data from other techniques (qNMR, elemental analysis, TGA) provided cross checks for this assignment and were consistent with the proposed value. The results reported by participants with their associated standard uncertainties (k = 1) plotted against the w_{NV} are shown in Annex C, Figure 10. The DoE table and plot of results relative to the non-volatiles content KCRV are given in Table 16 and Figure 14 respectively in Annex D.

Participant	Non-vols (mg/g)	u_c (mg/g)	Coverage factor	$U_{95\%}$ (mg/g)	Methods used
BIPM	0	+ 0.28/- 0	2	+0.56/-0.0	ICP-MS, TGA, EA
GLHK	0	+ 1/- 0	2	+2/-0	ICP-MS
HSA	0	+ 1.44/- 0	2	+2.88/-0	ICP-OES, TGA
KRISS	0	+ 0.19/- 0	2	+0.38/-0	TGA
LGC	0	+ 0.28/- 0	2	+0.8/-0	TGA-MS, ICP-MS, EA
NMIA	0	+ 1.15/- 0	2	+2.3/-0	TGA
NMIJ	0	+ 0.18/- 0	2	+0.36/-0	TGA
BAM	0	+ 0.28/- 0	2	+0.56/-0	ICP-OES
CENAM	0.00253	0.00007	2	0.00014	ICP-MS
VNIIM	0.083	0.02	2	0.04	ICP-MS
IRMM	0.12	0.12	2	0.24	ICP-OES, ICP-MS
NIM	0.19	0.09	2	0.18	ICP-MS, IC
NIST	0.37	0.12	2	0.24	TGA, XRF, IC
LNE	0.4	0.1	2	0.2	ICP-MS
NIMT	0.5	0.25	2	0.50	TGA
INMETRO	1.3	0.72	2	1.44	TGA
UME	2.05	0.0009	2	0.0018	ICP-MS, TGA
NMISA	2.8	1.16	2	2.32	TGA

Table 8: Assignments of non-volatiles/inorganics content in CCQM-K55.c

Direct Assay Methods for Assignment of Valine in CCQM-K55.c

i. Quantitative NMR (qNMR)

qNMR was the predominant assay method used for obtaining a direct estimate of the valine content of the comparison material. Four participants (NMIA, NRC, NIST and SIRIM) used qNMR as their sole method for assigning the valine content while a further five (BAM, UME, IRMM, NIM and NMIJ) used it as a contributing method, combined with data obtained by one or more additional methods, to provide their final value.

In addition four participants who used a mass balance approach or mass balance combined with qNMR data to assign their value for the key comparison submitted a separate value based solely on qNMR for inclusion in the parallel pilot study CCQM-P117.c. Two key comparison participants reported their qNMR data for information purposes without using it for their value assignment.

Table 9 provides key information on the results that contributed (in part or full) to the assignment of the participant's value for valine content in CCQM-K55.c. The value reported by IRMM was obtained by sub-contracting qNMR service provision to BAM on an aliquot from the IRMM comparison sample.

Participant	Valine (mg/g) by qNMR	Solvent	qNMR Internal Standard	Use of qNMR result
UME	981.05 (u = 1.82)	D_2O	Benzoic acid	Contributes to value for K55.c
NMIA	985 (u = 2.03)	D_2O	Dimethyl sulfone	Sole value for K55.c
NRC	987.0 (u = 3.4)	D_2O CD_3OD	KHP (internal) Benzoic acid (external)	Sole value for K55.c
NIST	990.0 (u = 0.9)	D_2O	KHP	Sole value for K55.c
NIM	990.27 (u = 1.81)	D2O	Creatinine / KHP	Contributes to value for K55.c
IRMM	991.3 (u = 0.54)	CD_3OD/D_2O	Benzoic acid	Contributes to value for K55.c
BAM	991.72 (u = 0.27)	CD_3OD/D_2O	Benzoic acid	Contributes to value for K55.c
SIRIM	993.0 (u = 1.5)	CD_3OD/D_2O	Benzoic acid	Sole value for K55.c
NMIJ	993.78 (u = 1.82)	D_2O/OD-	KHP	Contributes to value for K55.c

Table 9 – qNMR conditions and estimates for valine used in CCQM-K55.c (KHP = Potassium hydrogen phthalate)

Table 10 summarises results qNMR reported for CCQM-P117.c or supplementary data not used for value assignment but provided by participants in CCQM-K55.c.

Participant	Valine (mg/g) by qNMR	Solvent	qNMR Internal Standard	Use of qNMR result
LNE	983.0 (u = 1.3)	D_2O	Benzoic acid	Value for P117.c
HSA	987.7 (u = 4.8)	D_2O	KHP / maleic acid	Value for P117.c
NIM	990.27 (u = 1.81)	D_2O	Creatinine / KHP	Value for P117.c
EXHM	992.0 (u = 2.2)	D_2O	Maleic acid	Value for P117.c
LGC	993.1 (u = 2.8)	D_2O/D+	Benzoic acid	Value for P117.c
INMETRO	987.2 (u = 3.8)	D_2O	Maleic acid	Information only
BIPM	994 (u = 2.7)	D_2O/OD-, D+	KHM	Information only

Table 10 – qNMR estimates for valine reported in CCQM-P117.c or for information only (KHP = Potassium hydrogen phthalate, KHM = Potassium hydrogen maleate)

The combined qNMR data obtained for valine are plotted in Figure 3.

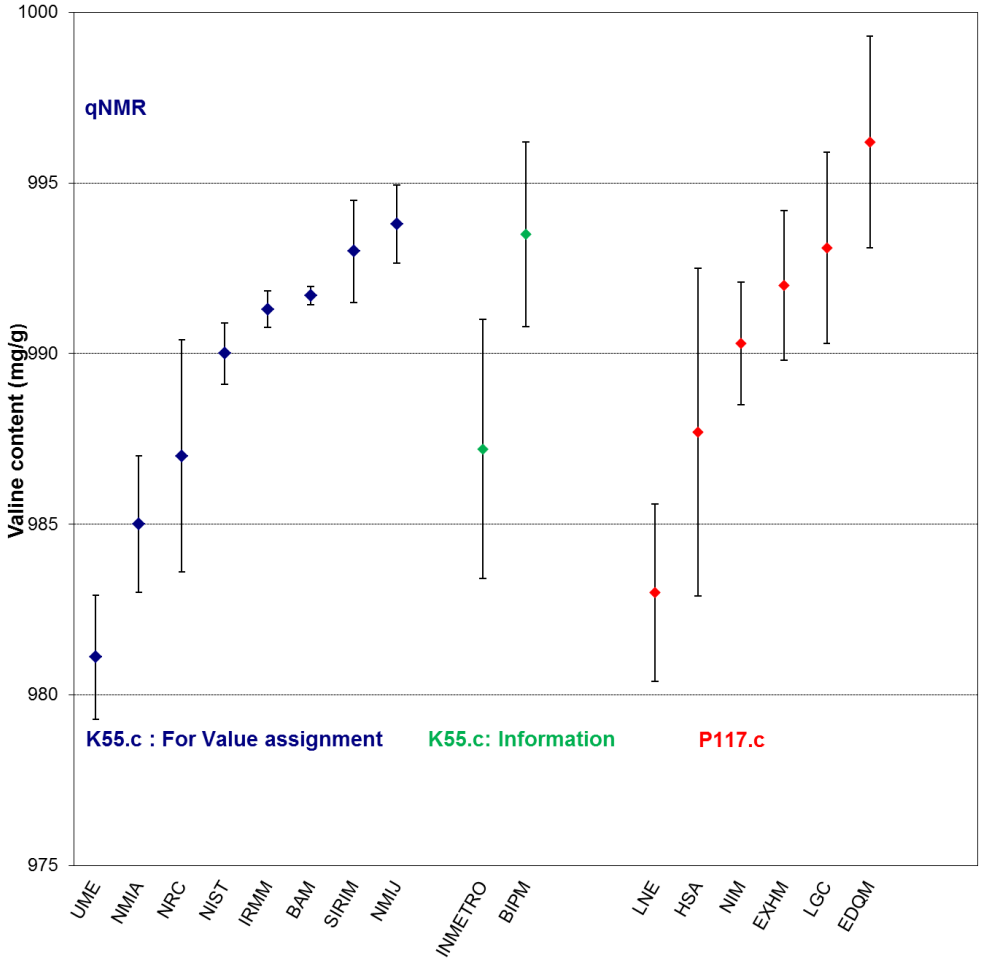


Figure 3 qNMR values reported for Valine content with u_c (where reported) or standard deviation, k = 1
◆ = reported in CCQM-K55.c result (alone or combined)
◆ = not used for CCQM-K55.c result, information only
◆ = reported in CCQM-P117.c

The assignment of content used integration of the signal due to the valine β-H, which was assumed to be distinct from interference due to signals from the main impurities, though it is possible that an acetic acid impurity overlapped with this signal under some conditions. The signal from the α-H could also be used providing a correction was applied for the clearly resolved signals due to associated impurities. Representative ^{1}H and ^{13}C NMR spectra for CCQM-K55.c, with an expansion of the α- and β-H region of the ^{1}H spectrum, are reproduced in Annex F, Figures 19 to 22.

As illustrated by Figure 3, a relatively wide range of values for valine content were reported using qNMR, particularly in comparison with the values obtained by mass balance approaches. In a follow up from the initial discussion of results in November 2012 a questionnaire on the parameters used to obtain and process qNMR data was distributed to participants in CCQM-K55.c to try and shed further light on the source of the variation. A copy of the questionnaire is reproduced in Annex G, Fig 23.

The responses were reviewed by John Warren (LGC) and the comparison coordinator Steven Westwood (BIPM) and were discussed at the OAWG April 2013 meeting.

Summary of qNMR parameter review

a. Integration Ranges

Two participants integrated the valine signal within the confines of its ^{13}C satellites and reported low values. All other participants used integration ranges sufficiently wide to ensure no significant impact on their determined purity value was expected. Where benzoic acid was used as the internal standard integration of the benzoic acid aromatic protons was one source of variation with participants either integrating the *ortho* doublet or the entire aromatic envelope. It should be noted that on any instrument of less than 600 Mhz, the ^{13}C satellites of the benzoic acid signals are not sufficiently resolved to allow clean integration of the *ortho* signal and its ^{13}C satellites alone. The relation of integration range to reported purity is shown in Annex G, Fig. 24

b. Choice of NMR internal Standard

No correlation was seen between the choice of reference material used and purity value of valine determined. The relation of standard to observed purity is summarised in Annex G, Fig. 25.

c. Relaxation delays and T_1 values

A range of relaxation delays between 30 s and 120 s was used, corresponding to a variety of T_1 for the internal standard chosen (0.7 s to 10.4 s). Reported relaxation delays were at least $8T_1$ for all but one case where the ratio was 5.2. No influence of relaxation time on reported purity values was evident or anticipated based on the relaxation delays selected.

d. Baseline correction

With integration ranges employed of over 1 ppm in some cases and the potential for interference due to broad exchangeable signals made this a challenging material for qNMR, particularly in comparison with the case for aldrin in CCQM-K55.b.

Participants who used manual baseline correction on individual spectral regions gave a consistent set of purity values on the higher end of the reported results that were in agreement with the KCRV assigned using the consensus mass balance approach. The results reported relying on autocorrection by the NMR processing software were more widely spread. The observation that manual baseline correction and integration generally provides a more reliable qNMR value is consistent with findings from previous CCQM Pilot studies on the qNMR technique[9] and literature recommendation.[10,11] The variation in reported purity with baseline correction mode are plotted in Annex G, Fig. 26.

Overall there did not seem to be a sufficiently good understanding of the observed variability in the qNMR results to justify use of this data in the assignment of the KCRV for valine content in this case, despite the widespread use of the technique for this comparison. It was noted that qNMR values for valine content provided by participants using manual baseline correction in their data processing procedure were both consistent with each other and, within their reported uncertainties, with the KCRV for valine. Where automated baseline correction was used the range of reported values was larger and in some cases no longer agreed with the KCRV.
In summary, the qNMR results for valine content of CCQM-K55.c show:

- no correlation between valine content and
  - nature of internal standard (IS)
  - solvent
  - concentration of analyte and standard
  - pulse delay and T_1 parameters
  - use of "in-house" versus "external" service provision;
- integration ranges appeared suitable except in two cases where the result may have been biased low due to selection of an insufficiently wide range;
- participants using manual baseline correction and integration obtained higher values within a consistent set of qNMR values for valine content and these values were also equivalent within their reported uncertainties with the KCRV.

The main recommendation from the review of the combined data is for participants using this method to validate their baseline correction approach taking into account that manual baseline correction and peak integration currently appears to be the most reliable approach.

ii. Titration methods

Two participants reported purity assignments for valine based on titration. Their values for the valine content of the CCQM-K55.c material were:

Participant	Valine (mg/g)	Method
NMIJ	991.7 (u = 0.94)	Non-aqueous titration with perchloric acid of amine content as a solution in acetic acid (3 x 30 mg samples)
CENAM	996.1 (u = 22.8)	Non-aqueous titration with perchloric acid of amine content as a solution in acetic acid (1 x 100 mg sample)

The value reported by NMIJ included a correction of the raw titration value to allow for the contribution due to amino acid impurities identified in other studies as present in CCQM-K55.c

iii. Differential Scanning Calorimetry

Two participants (NIST and NMISA) reported investigating the use of DSC to determine the content of valine in the comparison material. In both cases they found that the thermal transition properties of valine were not suitable for purity assessment using this technique.

Key Comparison Reference Value (KCRV) for Valine in CCQM-K55.c

It was agreed by the participants during the initial discussion of results at the October 2012 OAWG meeting for the comparison coordinator should follow the precedent of the approach used in the CCQM-K55.a and CCQM-K55.b comparisons and propose individual KCRVs for the mass fraction of each of the orthogonal classes of impurity present in the comparison material and use these values to assign an overall KCRV for valine content.

Assignment of KCRV for Valine in CCQM-K55.c

The measurement equation (Eqn. 1) to assign the KCRV of valine (in mg/g) is:

$$w_{Val} = 1000 - [w_{RS} + w_{H2O} + w_{VOC} + w_{NV} + H_{RS}] \quad \textit{(Eqn. 1)}$$

w_{Val} = KCRV for mass fraction of valine in CCQM-K55.c

w_{RS} = KCRV for mass fraction of valine-related impurities in CCQM-K55.c

w_{H2O} = KCRV for mass fraction of water in CCQM-K55.c

w_{VOC} = KCRV for mass fraction of residual solvent/volatile organics in CCQM-K55.c

w_{NV} = KCRV for mass fraction of non-volatile organics/inorganics in CCQM-K55.c

H_{RS} = Correction for between unit inhomogeneity of related structure impurities in the CCQM-K55.c material. Assigned value of 0 mg/g with associated uncertainty ($u_{H\,RS}$)

Units for reporting mass fraction (w) are mg/g throughout.

The standard uncertainty associated with the mass fraction estimate is calculated from equation (2):

$$u_{w_{Val}} = \sqrt{(u_{w_{RS}})^2 + (u_{w_{H2O}})^2 + (u_{w_{VOC}})^2 + (u_{w_{NV}})^2 + (u_{H_{RSt}})^2} \quad \textit{(Eqn. 2)}$$

The KCRVs for the contributing impurity classes used for calculation of a mass balance KCRV for valine in the CCQM-K55.c comparison and their combined value are summarised in Table 11.

Input factor *w*	**KCRV (mg/g)**	***n***	**u_c (+) (mg/g)**	**u_c (-) (mg/g)**
Related structure organics	7.60	16	0.24	0.24
Water	0.155	10	0.042	0.042
Residual solvent	0.0	15	0.12	0.0
Non-volatiles/ inorganics	0.25	15	0.144	0.144
Homogeneity - related structure impurities	0.0	large	0.038	0.038
Combined value	8.01		0.29	0.31

Table 11: Input values and final result for combined impurities and associated standard uncertainty in CCQM-K55.c.

When substituted into the equations (1) the KCRV (w_{Val}) for valine content becomes:

$$w_{Val} = 1000 - [w_{RS} + w_{H2O} + w_{VOC.} + w_{NV.} + H_{RS}] \text{ mg/g}$$

$$= 1000 - [7.6 + 0.155 + 0 + 0.25 + 0]) \text{ mg/g}$$

$$= 992.0 \text{ mg/g}$$

As a result of the asymmetry in the uncertainty assignment for residual solvent content, the u_{Val} calculated using equation (2) is also asymmetric.

$$u_{wVal}(-) = \sqrt{(u_{w_{RS}})^2 + (u_{w_{H2O}})^2 + (u_{w_{VOC}})^2 + (u_{w_{NV.}})^2 + (u_{H_{RSt}})^2}$$

$$= \sqrt{(0.24)^2 + (0.042)^2 + (0.12)^2 + (0.144)^2 + (0.038)^2}$$

$$= 0.31 \text{ mg/g}$$

$$u_{wVal}(+) = \sqrt{(u_{w_{RS}})^2 + (u_{w_{Water}})^2 + (u_{w_{VOC}})^2 + (u_{w_{NV.}})^2 + (u_{H_{RSt}})^2}$$

$$= \sqrt{(0.24)^2 + (0.042)^2 + (0.0)^2 + (0.144)^2 + (0.038)^2}$$

$$= 0.29 \text{ mg/g}$$

Note that in Table 11 the assigned uncertainties for the KCRV of each impurity class are designated as (+) or (-) as a function of their influence on the uncertainty of the assigned value for that impurity. However when these uncertainties are combined in the uncertainty budget for the KCRV of valine, their influence on the final value for valine is reversed. For this reason the signs of the uncertainty values for the individual and combined impurities in CCQM-K55.c are the opposite of those for the assigned value for valine in CCQM-K55.c

Figures 4 shows the participant results with their reported standard uncertainties plotted against w_{Val} (solid red line) and $w_{Val} \pm u_{wVal}$ (dotted red lines).

Figures 5 shows the participant results with their reported expanded uncertainties ($U_{95\%}$) plotted against w_{Val} (solid red line) and $w_{Val} \pm U_{wVal}$ (dotted red lines).

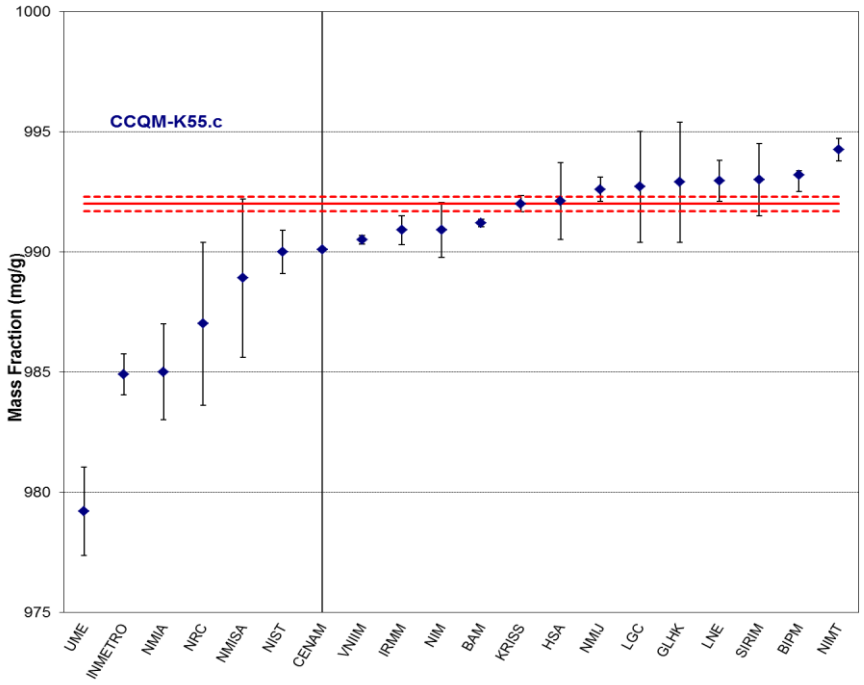


Figure 4: Mass fraction estimates by participant for valine in CCQM-K55.c with their reported uncertainty (*u*). KCRV for valine (solid red line) is 992.0 mg/g . Dashed red lines show $w_{Val} \pm u_{Wval}$ (k = 1)

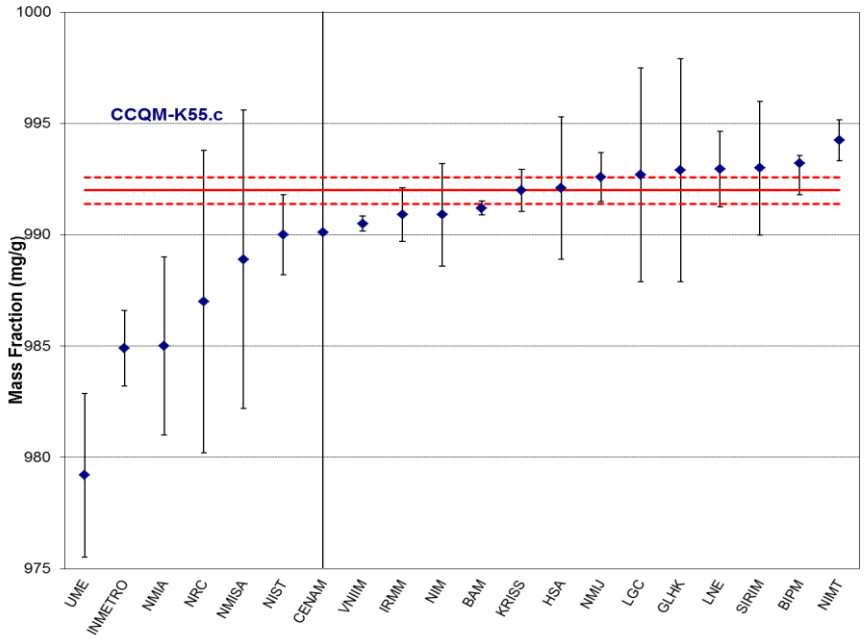


Figure 5: Mass fraction by participant for valine in CCQM-K55.c with their reported expanded uncertainty ($U_{95\%}$). KCRV for valine (solid red line) is 992.0 mg/g . The dashed red lines show $w_{Val} \pm U_{Wval}$.

Degree of equivalence plot with KCRV for Valine in CCQM-K55.c

The degree of equivalence of a result with the KCRV (D_i) is given by: $D_i = w_i - w_{Val}$

The expanded uncertainty U_i at the 95% coverage level associated with D_i was calculated:

$$U_{95\%}(D_i) = 2 * \sqrt{u(w_i)^2 + u(w_{Val})^2}$$

Table 12 records the degree of equivalence (D_i) of each result with the valine KCRV.

Participant	D_i (mg/g)	U_D (mg/g)
UME	-12.80	3.73
INMETRO	-7.10	1.80
NMIA	-7.00	4.04
NRC	-5.00	6.82
NMISA	-3.10	6.63
NIST	-2.00	1.89
CENAM	-1.90	112.76
VNIIM	-1.50	0.68
IRMM	-1.10	1.33
NIM	-1.10	2.35
BAM	-0.80	0.66
KRISS	0.00	0.89
HSA	0.10	3.25
NMIJ	0.60	1.17
LGC	0.70	4.64
GLHK	0.90	5.03
LNE	0.95	1.80
SIRIM	1.00	3.06
BIPM	1.20	+ 0.70, -1.52
NIMT	2.25	1.09

Table 12: Degrees of equivalence (D_i) and U_D for valine results

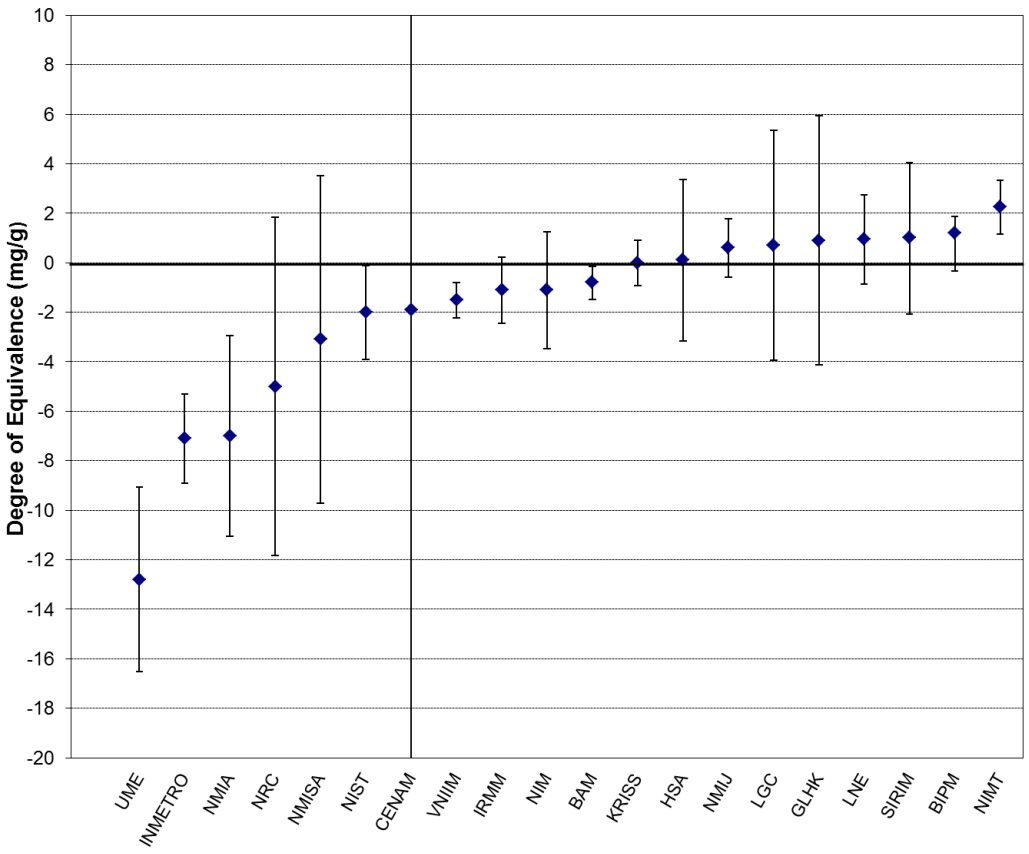


Figure 6: **Degree of equivalence with the valine KCRV for each participant. Points are plotted with the expanded uncertainty in the degree of equivalence corresponding to an approximately 95% coverage range.**

Degree of equivalence plots for Mass Balance KCRVs in CCQM-K55.c

The motivation for assigning KCRVs for the impurity classes in CCQM-K55.c was to assess the fitness of the individual mass balance methods and to confirm that an overall value for the main component in agreement with the KCRV for valine did not arise through cancellation of errors in the contributing impurity assignments.

The combined DoE plots by participant for each impurity class quantified are shown in Appendix B. To aid in assessment and comparison, the DoE of the final result for valine is plotted at the right (green data point). Where a participant used a mass balance approach but provided no information on a particular class of impurities a "pseudo" DoE is shown in this case as a red data point. This provides information on the validity of the participant's implicit assumption that the particular impurity component does not make a significant contribution to the overall purity. The derived DoE plots also allow for a visualization of specific problem areas for this comparison, regardless of whether overall agreement with the KCRV for valine was achieved.

CONCLUSIONS AND HOW FAR THE LIGHT SHINES

Valine was selected to be a representative high polarity, low complexity organic compounds capable of direct analysis by HPLC but not GC methods. It was anticipated to provide an analytical measurement challenge representative for the value-assignment of compounds of broadly similar structural characteristics.
There was good agreement between the majority of participants in both the identification and the quantification of the related structure impurity content of the sample, confirming the conclusion of previous rounds of CCQM-K55 that measurement of this general class of impurities is performed satisfactorily by most NMIs. In the case of amino acids in general and valine in particular, LC-based and qNMR methods appeared to be more consistent and sensitive and less variable than GC methods requiring a preliminary derivatization step.
There was good agreement on the quantification of the (relatively low) water, residual solvent and non-volatile contents of the material, though some results for water content appeared to have been influenced by the formation of water as a byproduct of internal condensation reactions under harsher analysis conditions.
As discussed in the report, the main area of disparity in the overall results arose from variability in the reported results obtained by qNMR. After review of the qNMR parameters used by the various participants it appears that the principal source of variability was the baseline correction protocol implemented, with those reporting using manual correction and integration obtaining results in agreement with the KCRV while more variable results were obtained if autocorrection by the analysis software was relied on.
In summary, the major conclusions from the comparison were:

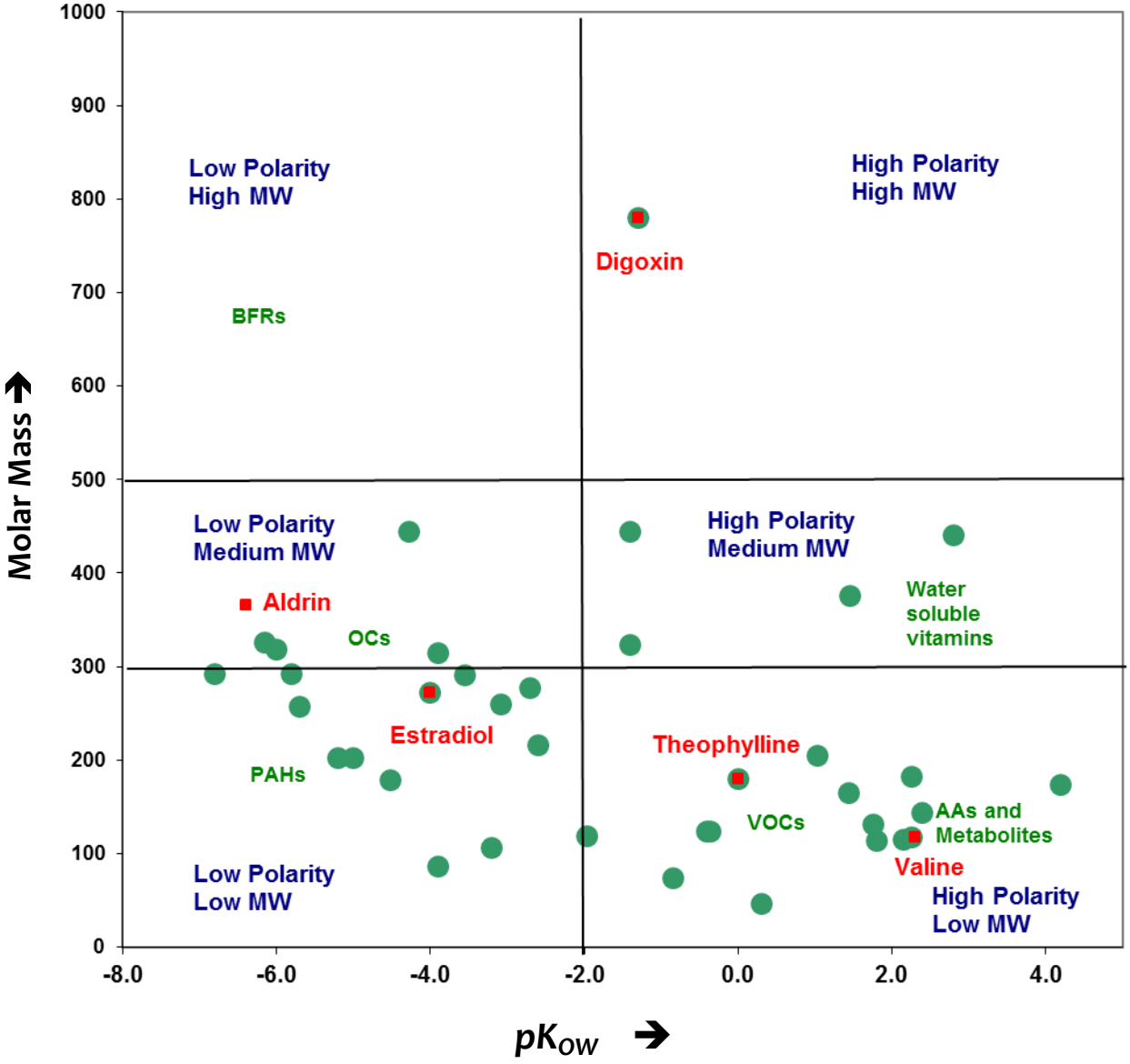
- generally good agreement in the mass balance method results for valine content and in the mass fraction assignments for each class of impurity in CCQM-K55.c;
- in cases where a participant's mass balance result for valine was not in agreement with the KCRV the likely source of the deviation could be identified;
- the implementation of qNMR for assignment of the purity of valine provided more variable results in the assigned value with larger associated uncertainty compared with results obtained using mass balance approaches;
- the selection of appropriate qNMR parameters and an understanding of their potential influence on the final result is critical for reliable implementation of the method, particularly when either or both of the peaks to be quantified are complex multiplet signals;
- manual baseline correction and integration of all quantified peaks is the recommended approach for qNMR quantifications.

The comparison shows that in the case of amino acids (mass fraction > 990 mg/g), purity assignment can be achieved with a relative expanded uncertainty below 0.5 % using a mass balance approach, an appropriately implemented qNMR approach or a combination of results from both methods.

"How Far The Light Shines" Statement for CCQM-K55.c

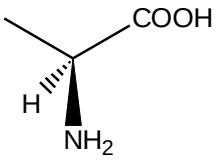
The comparison was intended to demonstrate a laboratory's performance in determining the mass fraction of the main component in a high purity organic material. Successful participation should be indicative of the performance of a laboratory's measurement capability for the mass fraction purity assignment of organic compounds of low structural complexity (molar mass range 100-300) and high polarity ($pK_{OW} > -2$) and for which related structure impurities can be quantified by high performance liquid chromatography either directly or after preliminary derivatisation with fluorescence detection.

Annex A: Analysis Space Model for Organic Primary Calibrators

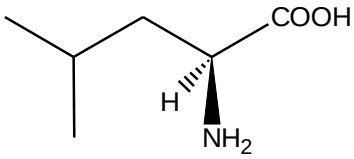


- ■ CCQM-P20 & CCQM-K55 measurands
- ● CMC claims for pure substance calibrators or calibration solutions

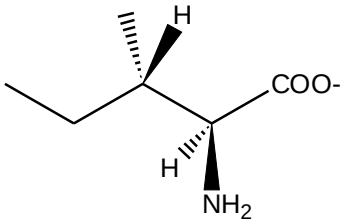
Annex B: Amino acid impurities reported in CCQM-K55.c



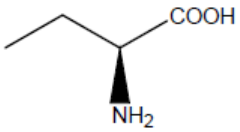
L-Alanine **(2)**



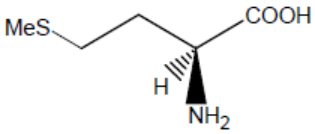
L-Leucine **(3)**



L-Isoleucine **(4)**



α-Aminobutyric acid **(5)**



L-Methionine **(6)**

Annex C: Participant Results Relative to Impurity Category KCRVs

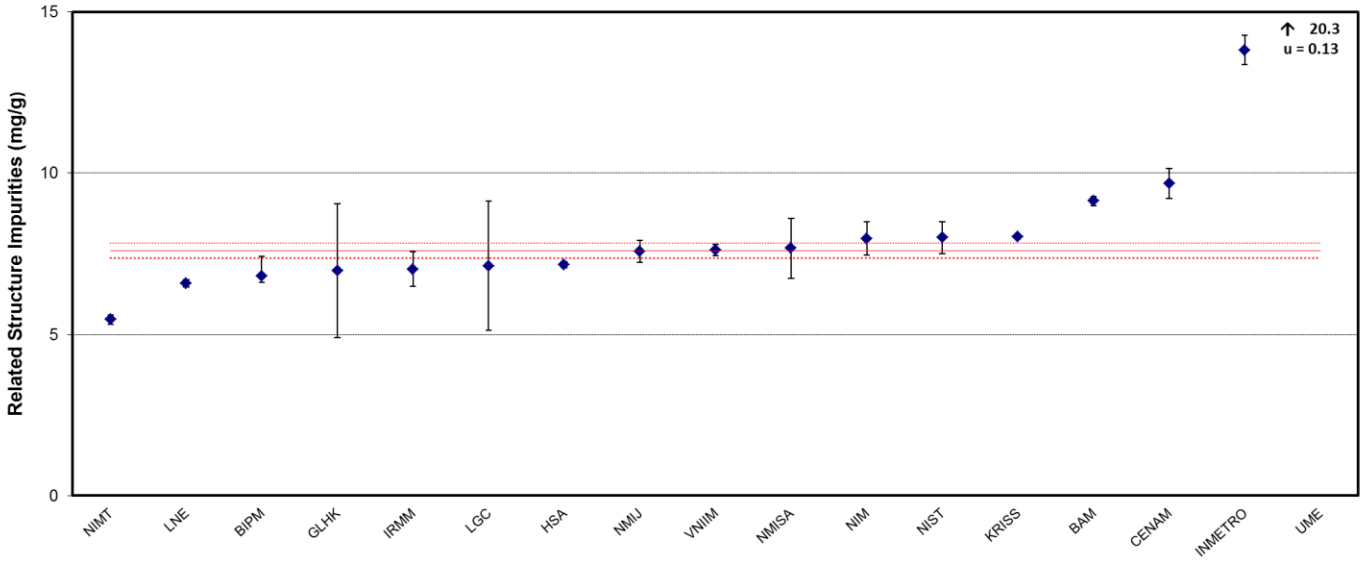


Figure 7 Total related structure impurity in CCQM-K55.c with standard uncertainties (± u_c , k = 1). The KCRV for related structure impurity (w_{RS}, solid red line) is 7.60 mg/g. The dashed red lines show $w_{RS} \pm u_{wRS}$ (k = 1) where $u_{wRS.}$ = 0.24 mg/g

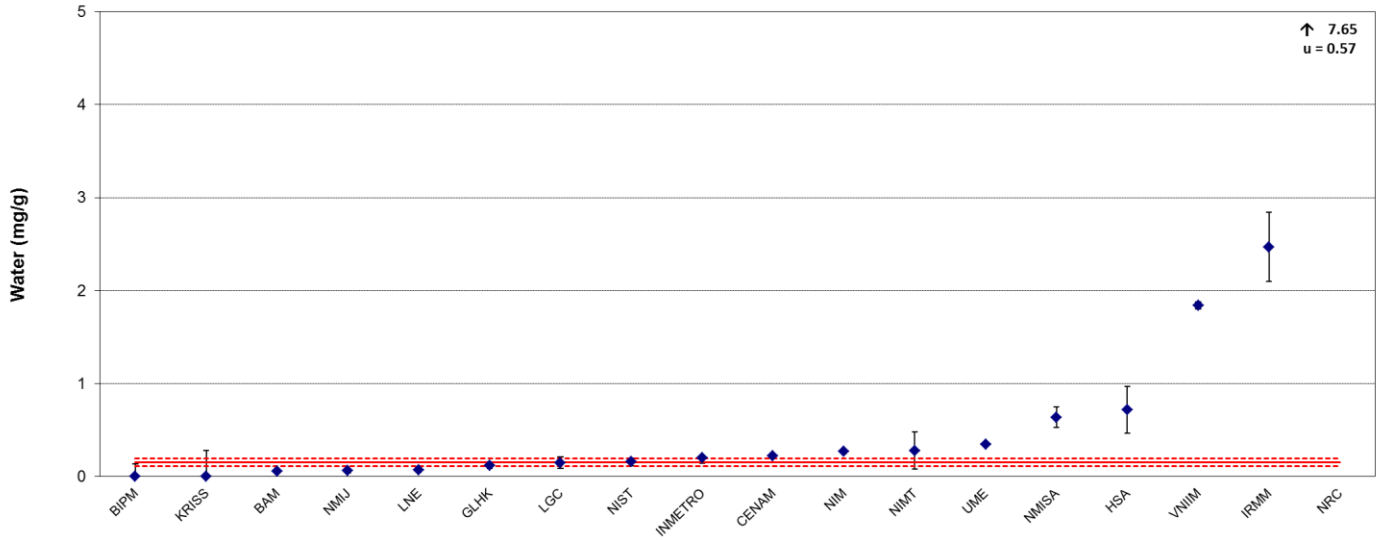


Figure 8 Estimates for water in CCQM-K55.c plotted with their uncertainties (k = 1). The KCRV for water content (w_{H2O}, solid red line) is 0.155 mg/g. Dashed red lines show $w_{H2O} \pm u_{wH2O}$ (k = 1) where u_{wH2O} = 0.042 mg/g.

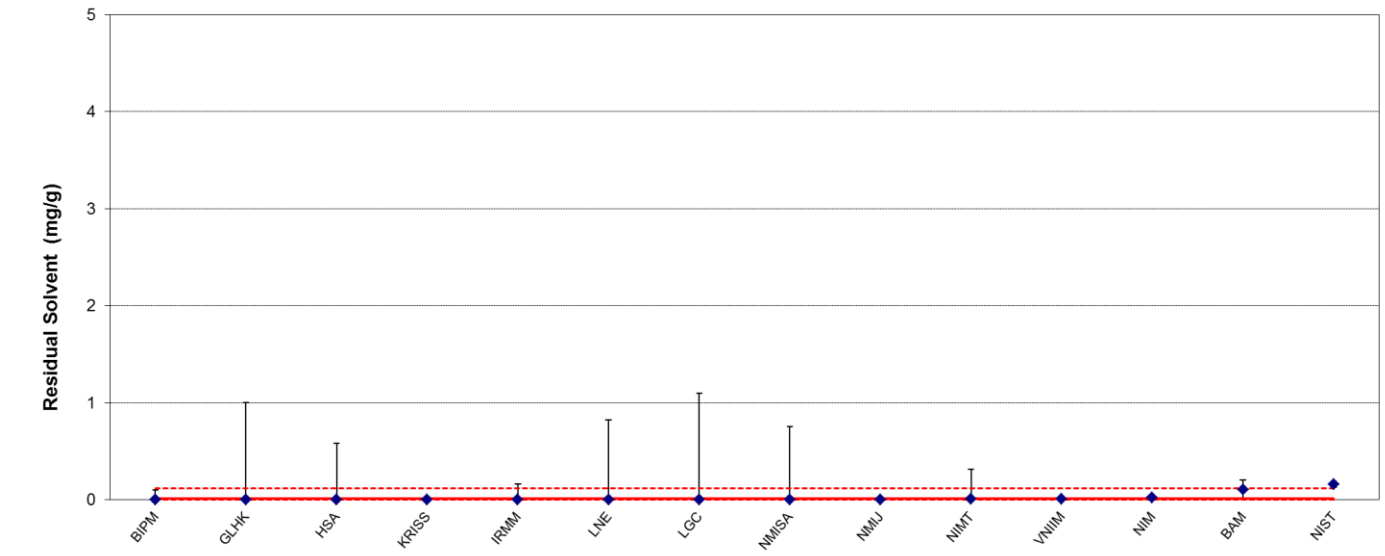


Figure 9 Estimates for residual solvent in CCQM-K55.c plotted with their uncertainties (k = 1). The KCRV for residual solvent (w_{VOC}, solid red line) is 0 mg/g. Dashed red line shows the $w_{VOC} + u_{VOC+}$ (k = 1) where u_{VOC+} = 0.12 mg/g.

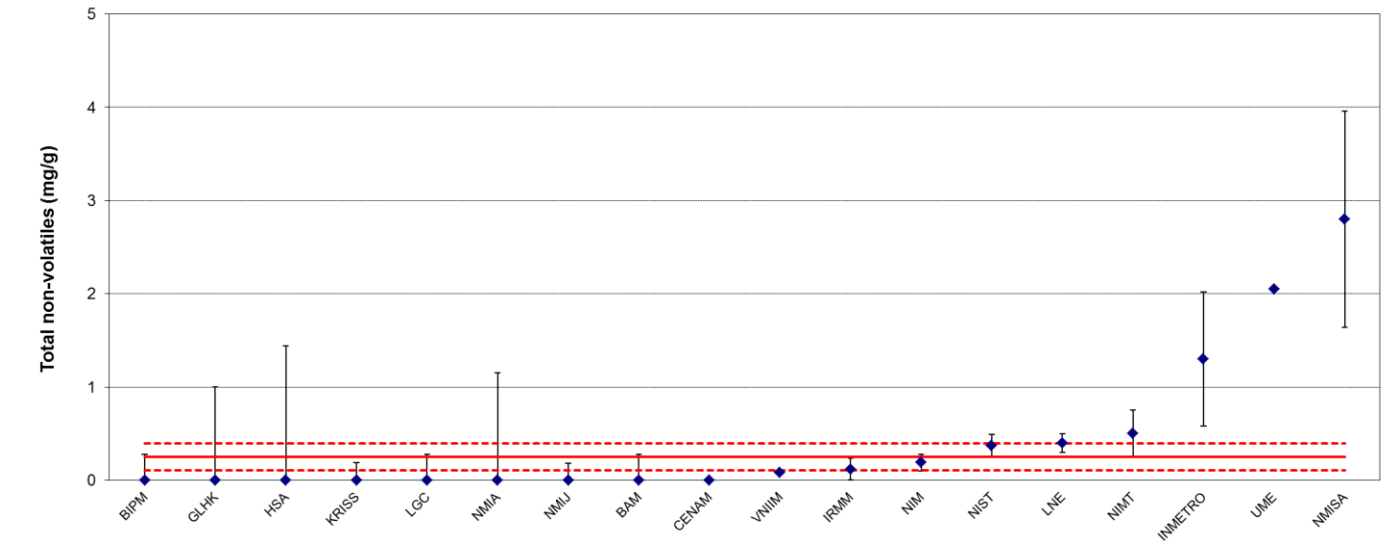


Figure 10 Estimates for non-volatiles/inorganics in CCQM-K55.c with their uncertainties (k = 1). The KCRV for non-volatiles in CCQM-K55.c (w_{NV} = 0.25 mg/g, solid red line) Dashed red lines show the $w_{NV} \pm u_{NV}$ (k = 1) where u_{NV} = 0.144 mg/g.

Annex D: DoE Tables and Plots for Impurity Category KCRVs

Degree of equivalence (D_i) of results for related structure impurities.

Participant	D_i (mg/g)	U_{D+} (mg/g)	U_{D-} (mg/g)
NIMT	-2.14	0.55	0.55
LNE	-1.02	0.52	0.52
BIPM	-0.80	1.31	0.53
GLHK	-0.63	4.17	4.17
IRMM	-0.58	1.18	1.18
LGC	-0.48	4.03	4.03
HSA	-0.44	0.51	0.51
NMIJ	-0.02	0.83	0.83
VNIIM	0.01	0.59	0.59
NMISA	0.07	1.92	1.92
NIM	0.37	1.14	1.14
NIST	0.40	1.11	1.11
KRISS	0.43	0.49	0.49
BAM	1.54	0.55	0.55
CENAM	2.09	1.05	1.05
INMETRO	6.22	1.02	1.02
UME	12.71	0.54	0.54

Table 13: Degrees of equivalence (D_i) and U_D for total related substance impurities

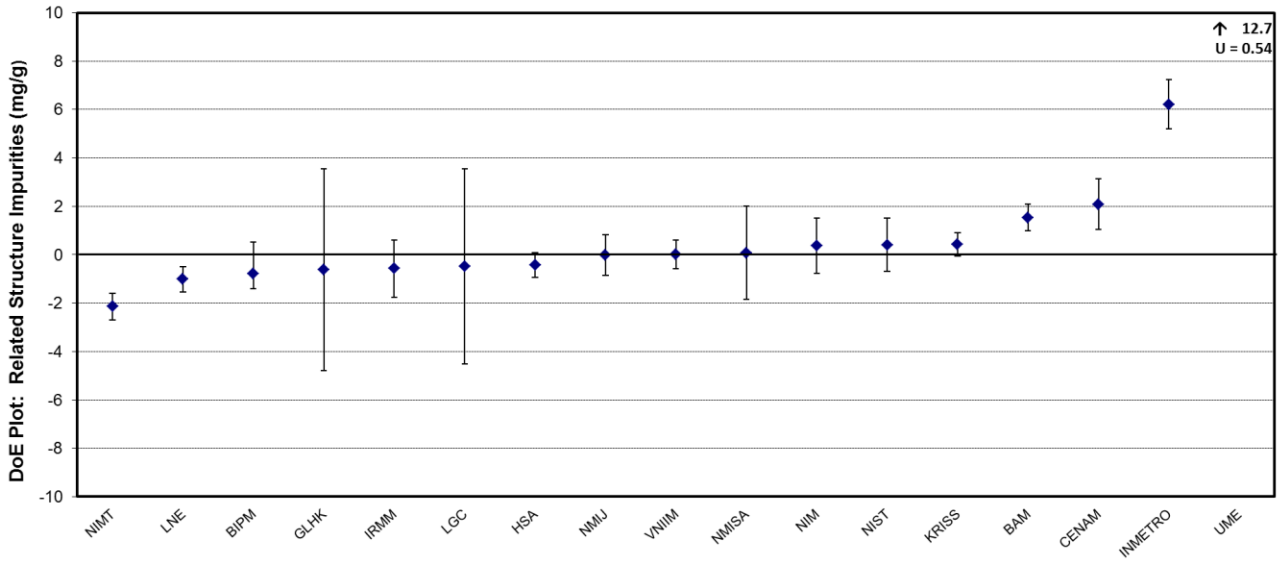


Figure 11 DoE Plot for total related structure impurities in CCQM-K55.c

Degree of equivalence (D_i) of results for water in CCQM-K55.c.

Participant	D_i (mg/g)	U_{D+} (mg/g)	U_{D-} (mg/g)
BIPM	-0.16	0.292	0.084
KRISS	-0.16	0.566	0.084
BAM	-0.10	0.093	0.093
NMIJ	-0.10	0.093	0.093
LNE	-0.09	0.092	0.092
GLHK	-0.04	0.103	0.103
LGC	-0.01	0.146	0.146
NIST	0.01	0.116	0.116
INMETRO	0.05	0.084	0.143
CENAM	0.07	0.084	0.084
NIM	0.12	0.098	0.098
NIMT	0.13	0.409	0.409
UME	0.20	0.092	0.092
NMISA	0.49	0.235	0.235
HSA	0.57	0.507	0.507
VNIIM	1.69	0.116	0.116
IRMM	2.32	0.745	0.745
NRC	7.50	1.143	1.143

Table 14: Degrees of equivalence (D_i) and U_D for water content

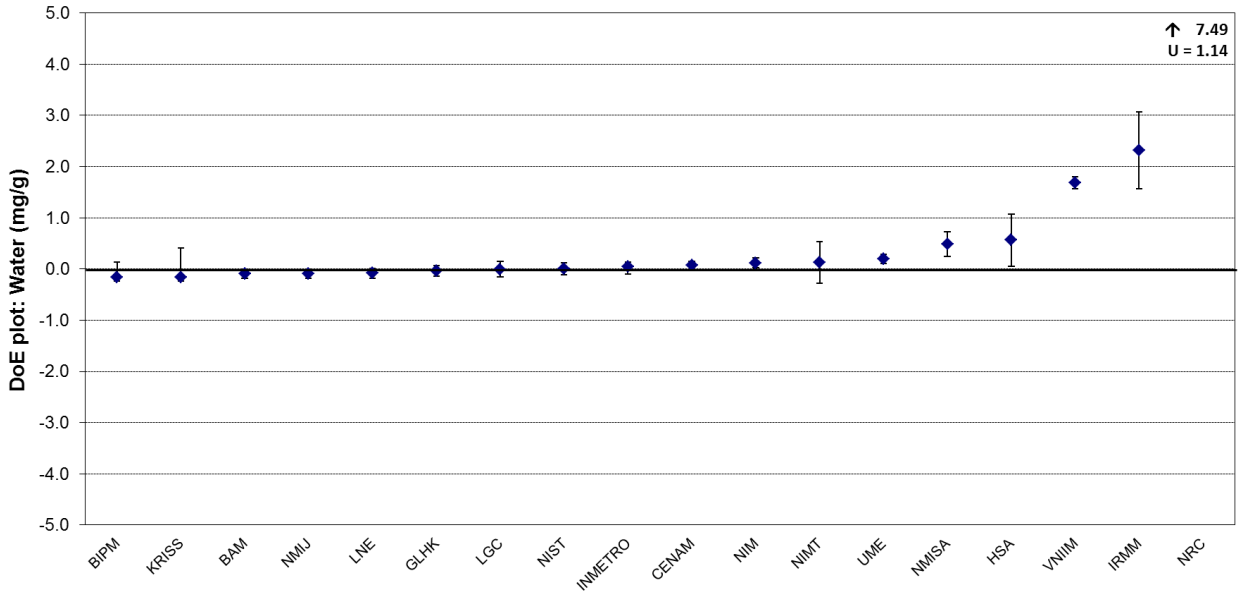


Figure 12 DoE Plot for water in CCQM-K55.c

Degree of equivalence (D_i) of results for residual solvent in CCQM-K55.c.

Participant	D_i (mg/g)	U_{D+} (mg/g)	U_{D-} (mg/g)
BIPM	0.00	0.31	0.0
GLHK	0.00	2.01	0.0
HSA	0.00	1.18	0.0
KRISS	0.00	0.24	0.0
IRMM	0.00	0.40	0.0
LNE	0.00	1.66	0.0
LGC	0.00	2.21	0.0
NMISA	0.00	1.52	0.0
NMIJ	0.00	0.23	0.0
NIMT	0.01	0.64	0.0
VNIIM	0.02	0.31	0.0
NIM	0.02	0.23	0.02
BAM	0.10	0.24	0.20
NIST	0.16	0.24	0.06

Table 15: Degrees of equivalence (D_i) and U_D for residual solvent content

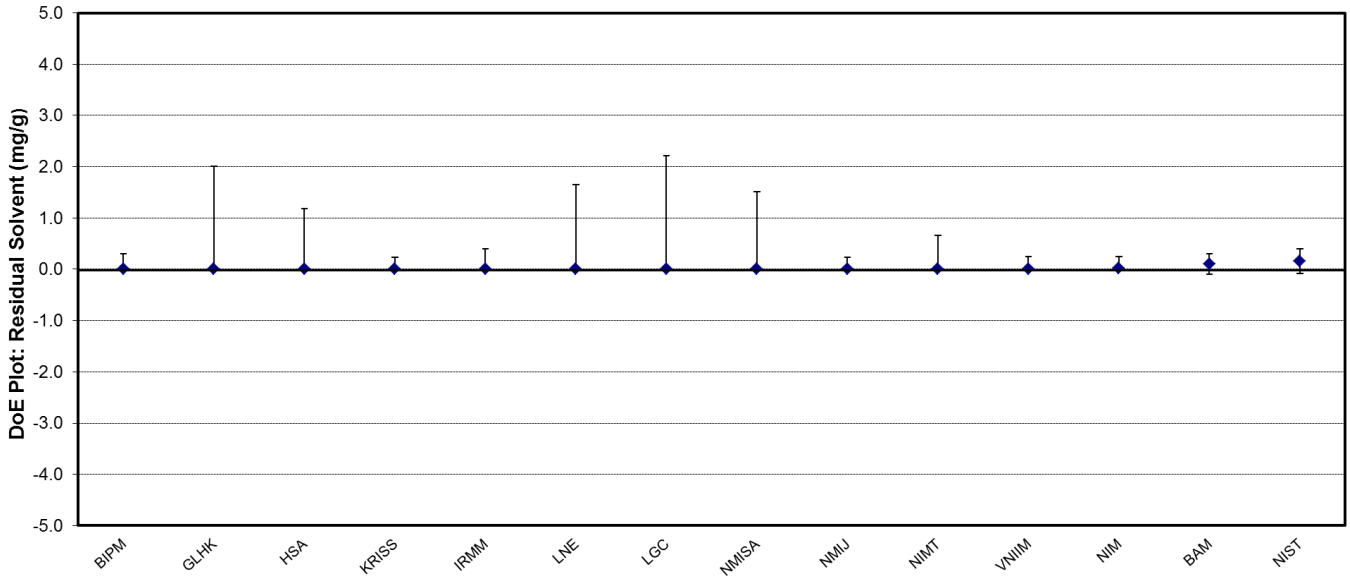


Figure 13 DoE Plot for residual solvent in CCQM-K55.c

Degree of equivalence (D_i) of results for non-volatiles & inorganics in CCQM-K55.c.

Participant	D_i (mg/g)	U_{D+} (mg/g)	U_{D-} (mg/g)
BIPM	-0.25	0.63	0.29
GLHK	-0.25	2.02	0.29
HSA	-0.25	2.89	0.29
KRISS	-0.25	0.48	0.29
LGC	-0.25	0.63	0.29
NMIA	-0.25	2.32	0.29
NMIJ	-0.25	0.46	0.29
BAM	-0.25	0.63	0.29
CENAM	-0.25	0.29	0.29
VNIIM	-0.17	0.29	0.29
IRMM	-0.13	0.37	0.37
NIM	-0.06	0.34	0.34
NIST	0.12	0.37	0.37
LNE	0.15	0.35	0.35
NIMT	0.25	0.58	0.58
INMETRO	1.05	1.47	1.47
UME	1.80	0.29	0.29
NMISA	2.55	2.34	2.34

Table 16: Degrees of equivalence (D_i) and U_D for non-volatiles content

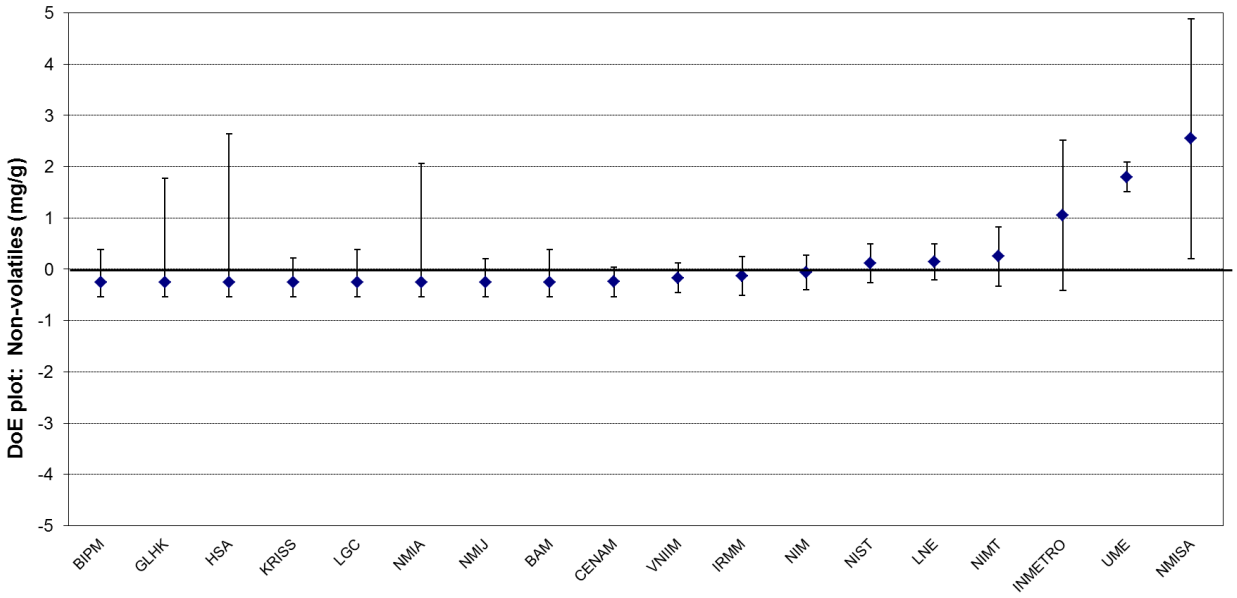


Figure 14 DoE Plot for combined non-volatiles in CCQM-K55.c

Annex E – Enantiomeric purity analyses of CCQM-K55.c

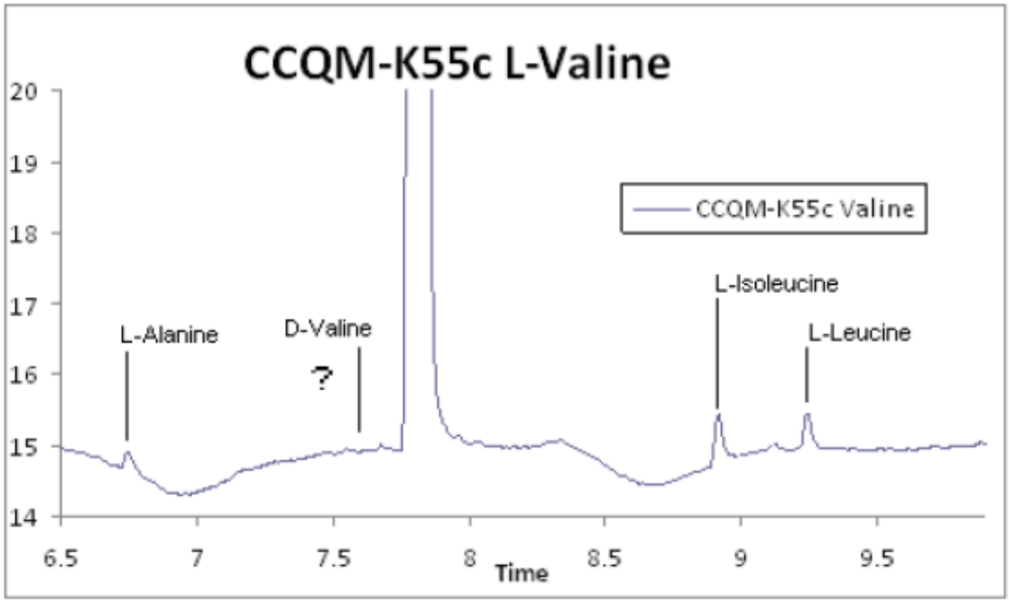


Figure 15: Chirasil GC-FID chromatogram of ECF-derivatised CCQM-K55.c
Retention time of D-Valine under same conditions (7.6 min) indicated for comparison

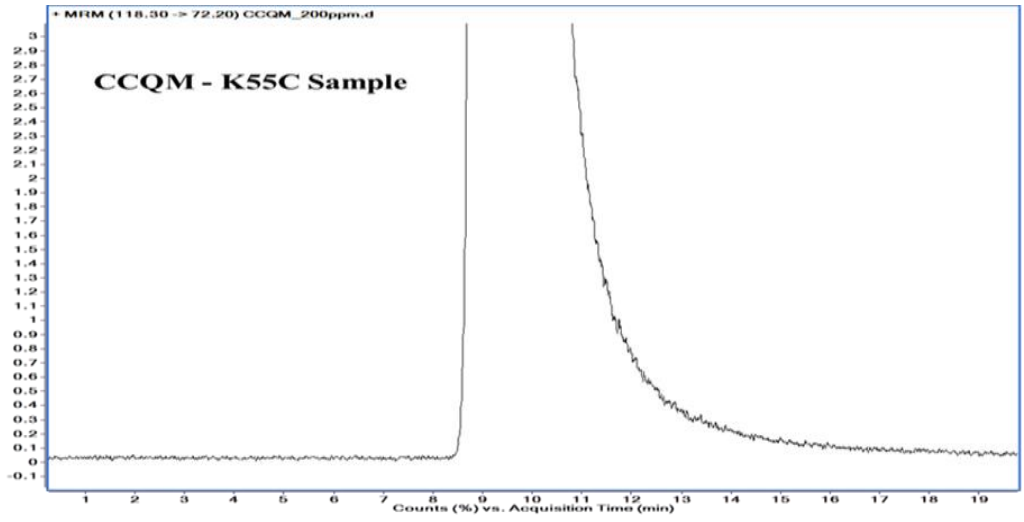


Figure 16 LC-MS chromatogram of CCQM-K55.c on chiral LC column
Retention time of D-Valine under same conditions is 14.8 minutes

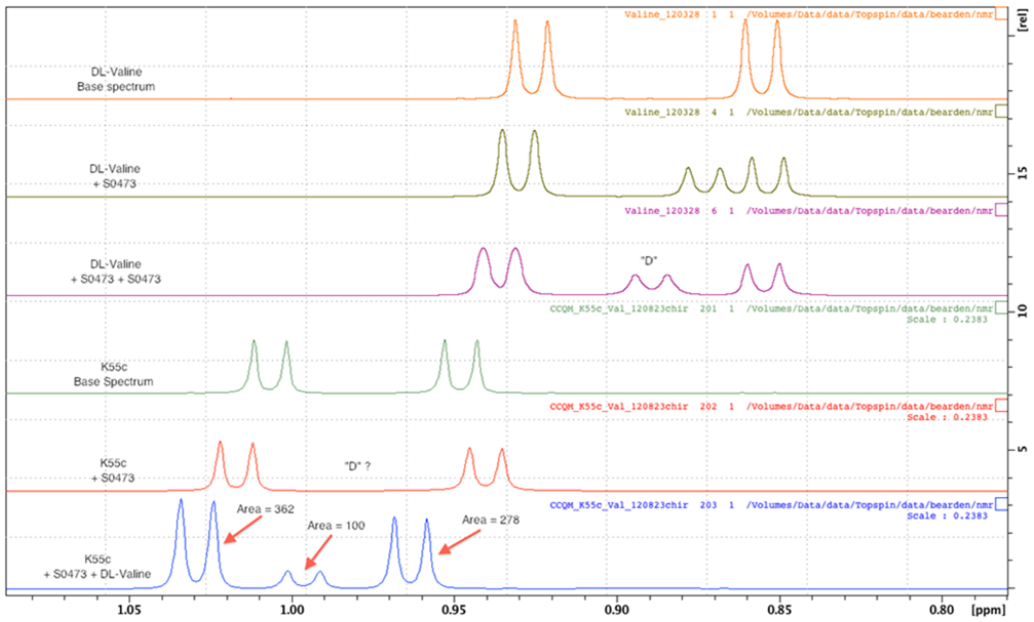


Figure 17 Effect of chiral complexing agents on the NMR spectra of DL-Valine and CCQM-K55c.

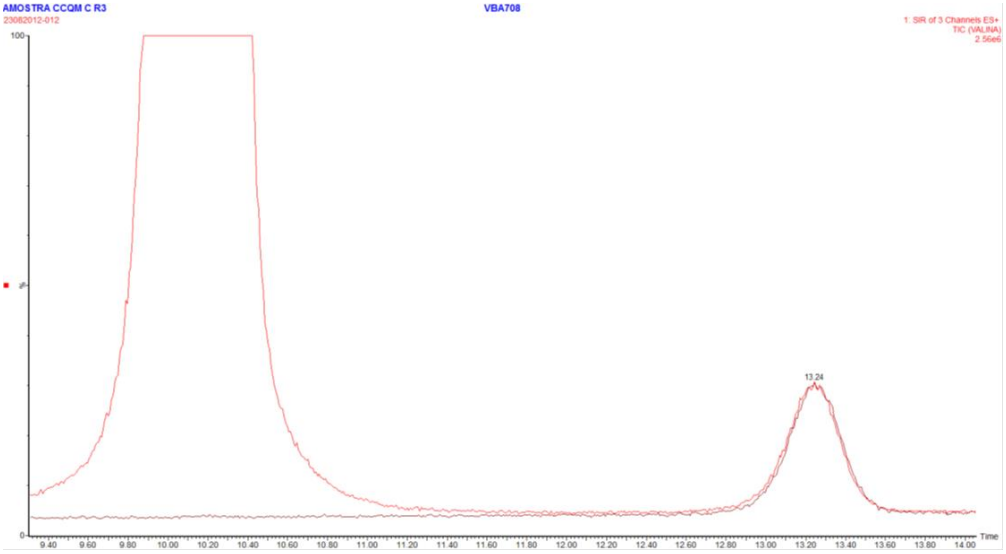


Figure 18 LC-MS chromatogram of CCQM-K55.c (red) and D-Valine (brown) after derivatisation with Marfey's reagent as reported by INMETRO.

Annex F – NMR spectra of CCQM-K55.c

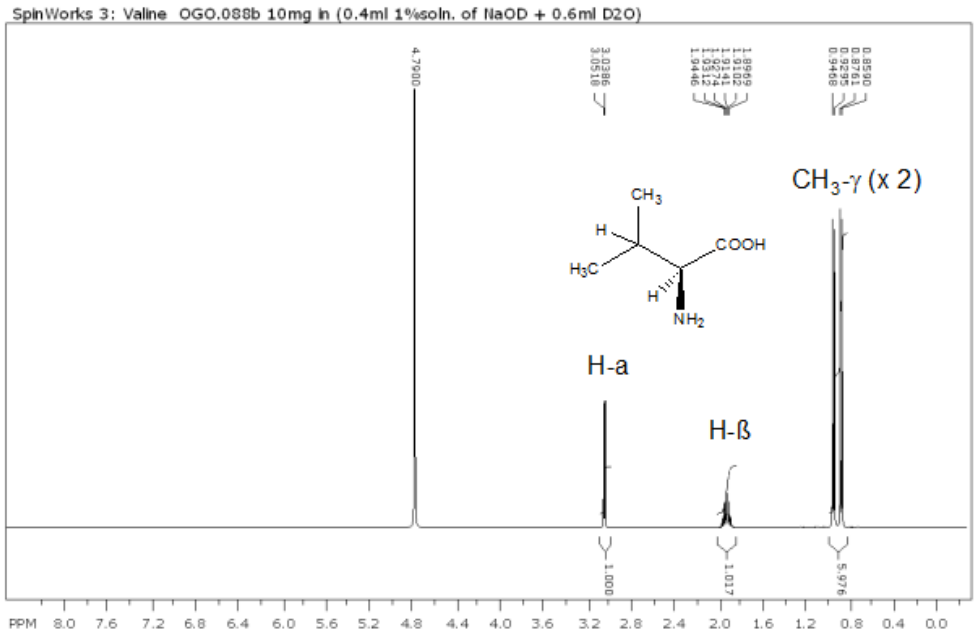


Fig. 19: ^{1}H NMR spectrum of CCQM-K55.c in D_2O (full scale)

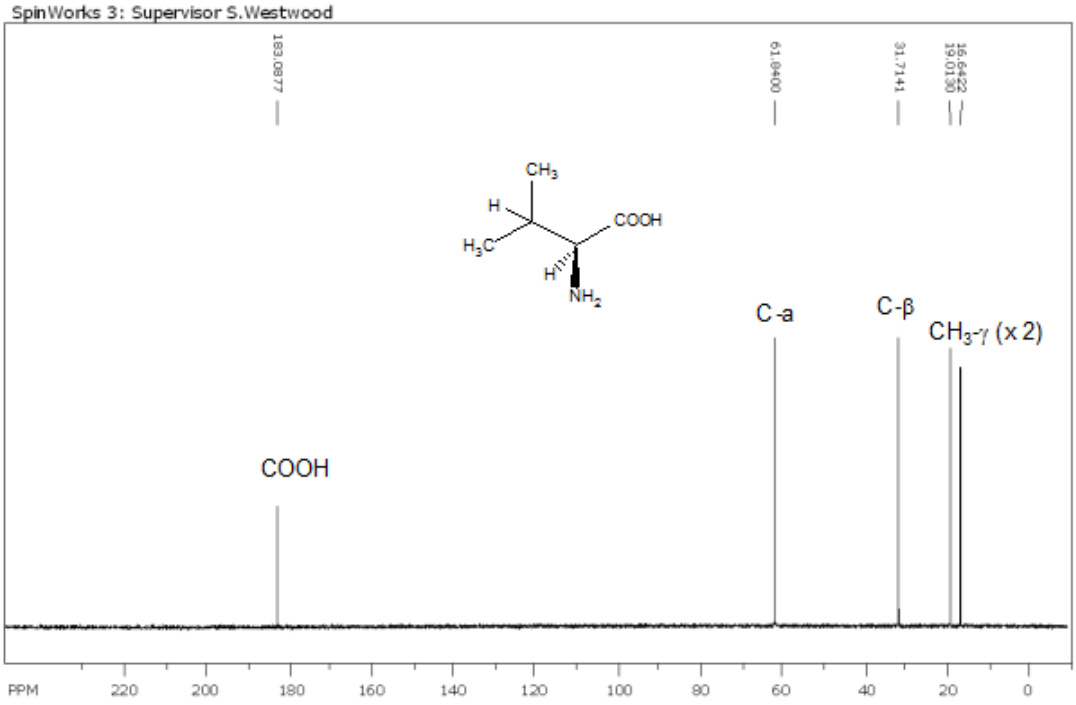


Fig. 20: ^{13}C NMR spectrum of CCQM-K55.c in D_2O (full scale)

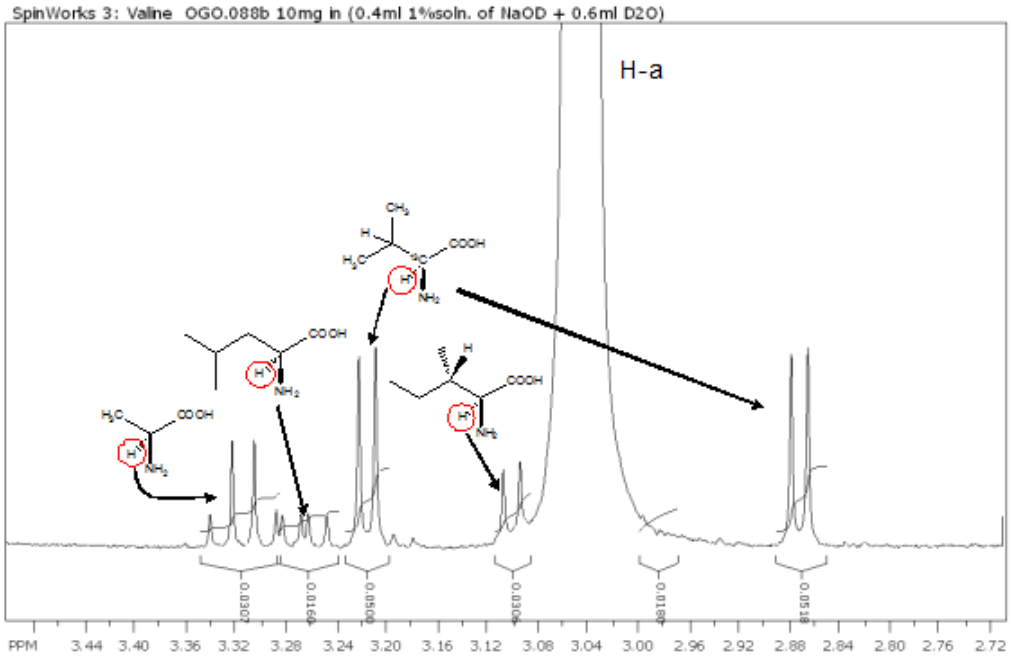


Fig. 21: ^{1}H NMR spectrum - expansion of α-H region

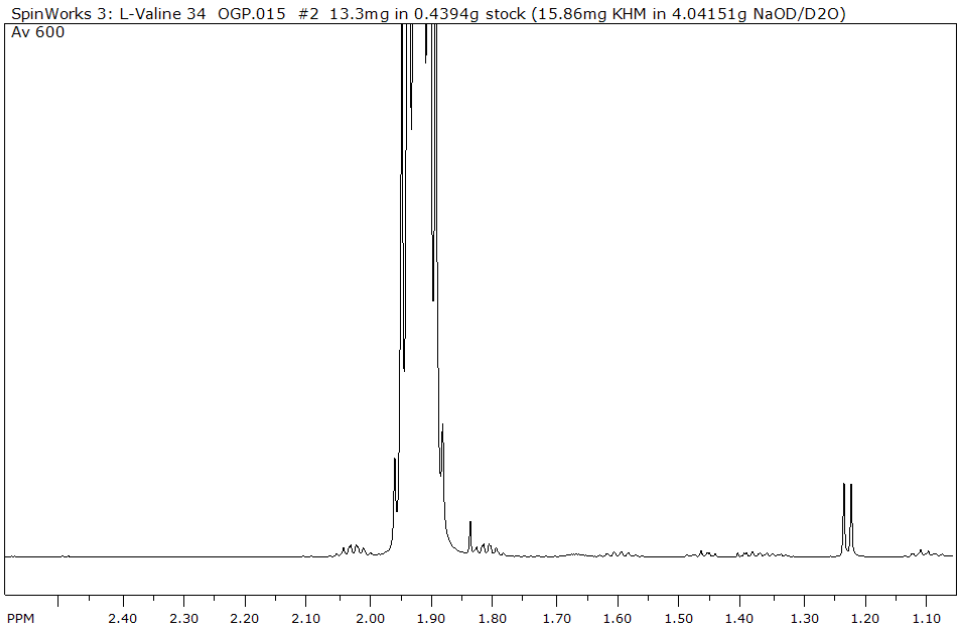


Fig. 22: ^{1}H NMR spectrum - expansion of β-H region

Annex G – Influence of qNMR Parameters on Valine Assignment in CCQM-K55.c

	Valine	**Internal Standard**
Signal used (ppm)		
T1(s)		
Integration range (Hz)		
Line width (full width half height, Hz)		
Weight of sample (mg)		

Receiver delay (s)						
^{13}C decoupling	Yes		No			
Integration type	Standard		Standard with Slope /bias adjustment		Deconvolution	
Baseline correction	none		polynomial		spline	
Baseline correction	Automatic		Manual (whole spectrum)		Manual (individual regions)	

Fig. 23: Questionnaire on qNMR parameters

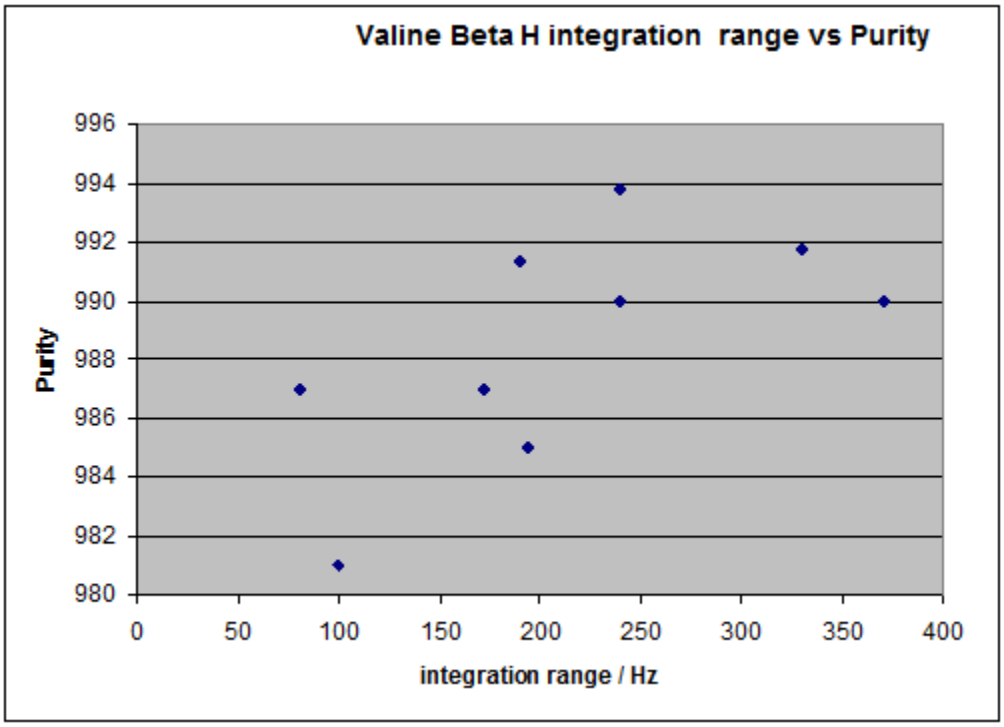


Fig. 24: Integration range of β-H signal v. reported purity by qNMR

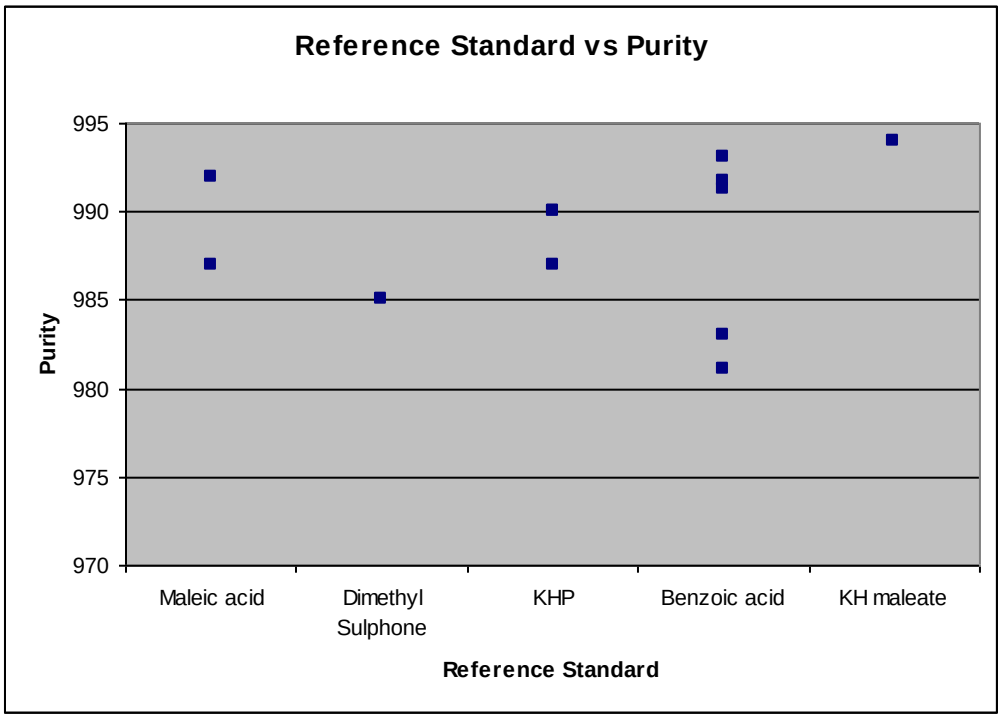


Fig. 25: Reference standard v. reported purity by qNMR

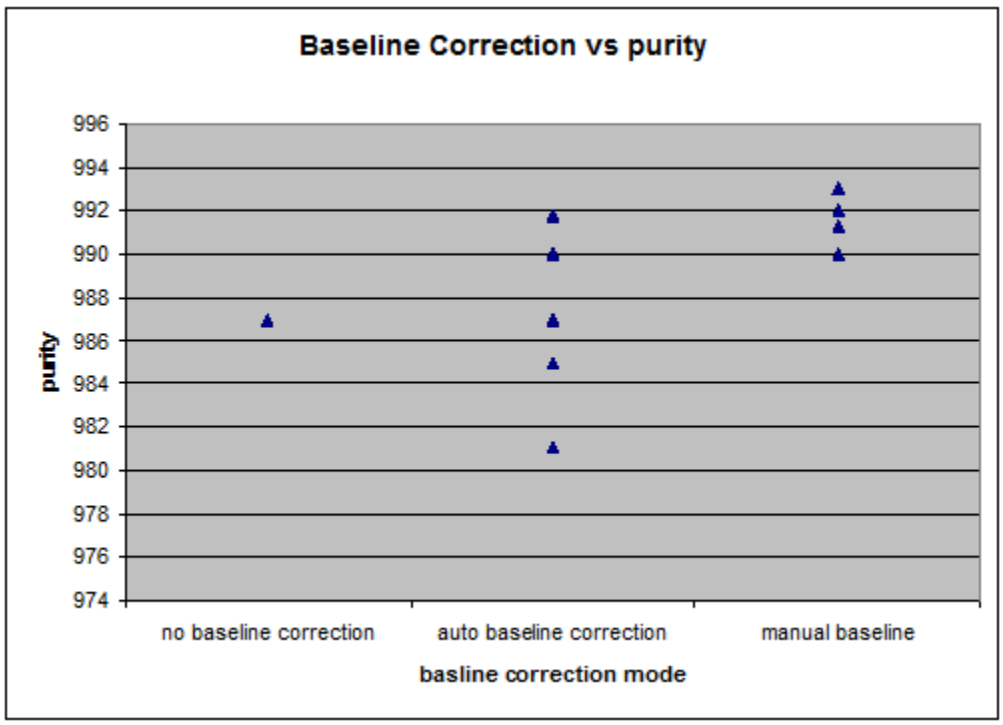


Fig. 26: Baseline correction mode v. reported purity by qNMR

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