CCQM-K55.d (Folic acid) Final Report: April 2018

Project Name: **Key Comparison CCQM-K55.d**

Comparison: **Characterization of Organic Substances for Chemical Purity**

Date: 9/2015 to 2/2016

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I. INTRODUCTION

The CCQM-K55 series of key comparisons and parallel CCQM-P117 pilot studies are being 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 MRA.

The ability to undertake suitable value assessment on materials intended for use, either internally or externally, as pure substance reference materials is considered a core competency for the provision of SI-traceable measurement services in organic analysis. All NMIs with ongoing programs in this area were encouraged to participate in the comparison. It allows NMIs and DIs to provide objective evidence that the procedure(s) they use for purity assessment, and the property value with its associated uncertainty that is assigned through their procedure, are suitable for their intended purpose. For applications in organic analysis purity is reported as the mass fraction of the main component.[1] The application of the pure material can be for dissemination to external users as a pure substance or standard solution Certified Reference Material, or for internal use by the NMI as the primary calibrator of a reference measurement procedure.

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)[2] and digoxin (CCQM-P20.f).[3]

[1] 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

[2] An international comparison of mass fraction purity assignment of theophylline: CCQM Pilot Study CCQM-P20.e (Theophylline), *Metrologia*, **46** (2009) 1A, 08019

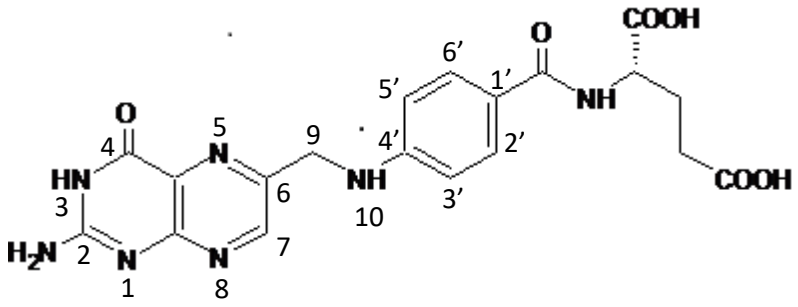
[3] An international comparison of mass fraction purity assignment of digoxin: CCQM Pilot Study CCQM-P20.f (Digoxin), *Metrologia* **48** (2011) 1A, 08013.

The BIPM has developed a "molar mass v. polarity" model to map the analytical space for key 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 CCQM-K55 series of key comparisons. 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 organic primary calibrators and calibration solutions 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 Final Report was published in September 2012 in Appendix B of the BIPM Key Comparison Database (BIPM KCDB).[4] 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 KCDB.[5] A proposal for L-valine to be the measurand for the third comparison round, CCQM-K55.c, and accompanied by a parallel pilot study, CCQM-P117.c was approved at the April 2011 OAWG meeting at Sèvres. The CCQM-K55.c comparison was completed in 2014 and the Final Report was published in May 2014 in Appendix B of the BIPM KCDB.[6]

A proposal for folic acid to be the measurand for the fourth comparison round, CCQM-K55.d, to be accompanied by a parallel pilot study, CCQM-P117.d, was approved at the April 2014 OAWG meeting at Sèvres. The comparison samples were distributed in September 2015. The individual results were communicated to the comparison coordinator in January 2016 and the results were first discussed at the April 2016 and October 2016 meetings of the OAWG in Sèvres.

The structure of folic acid (**1**) with the IUPAC- recommended ring numbering[7] is shown below.



(1)

Folic acid is a yellow-orange powder with a melting point of 250 °C. It is moderately soluble in water at neutral pH and more soluble in acidified or basified aqueous solution. It is effectively insoluble in non-polar organic solvents. Calibration and Measurement Capability (CMC) claims for

[4] Final report on key comparison CCQM-K55.a (Estradiol): An international comparison of mass fraction purity of estradiol, *Metrologia*, **49** (2012) 1A, 08009

[5] Final report on key comparison CCQM-K55.b (Aldrin): An international comparison of mass fraction purity of aldrin, *Metrologia*, **49** (2012) 1A, 08014

[6] Final report on key comparison CCQM-K55.c (L-Valine): An international comparison of mass fraction purity of valine, *Metrologia*, **51** (2014) 1A, 08010

[7] Nomenclature and Symbols for folic acid and related compounds *Pure Appl. Chem.* **59** (1987) 833

the measurement of folic acid as a component of serum, food and infant formula matrix materials are listed in Appendix C of the BIPM KCDB.

The names and structures of compounds related to folic acid identified by participants in the comparison as impurities present in the CCQM-K55.d material are shown in Annex B.

II. MATERIAL CHARACTERIZATION AND CONDUCT OF STUDY

The source material for the comparison was purchased from a commercial supplier. The material was described as being of pharmaceutical grade and was not subject to further treatment. In the analysis report provided with the material its composition is described as "corresponds to European Pharmacopoeia (Ph. Eur.) requirements".

This material was subdivided into a batch of 260 numbered units with the BIPM identifier code OGP.021. Each unit of BIPM OGP.021 used for the comparison contained in excess of 500 mg of folic acid in an amber glass vial with a rubber insert cap and crimped with an aluminium seal.

The impurity profile of the batch was determined at the BIPM, including assessment of the homogeneity and stability of the various components.
The mass fraction content of folic acid was assessed by the BIPM to be in excess of 900 mg/g and the homogeneity and stability of folic acid and the associated impurity components were determined as being suitable for the purposes of the comparison. Particular attention was paid to establishing the stability and homogeneity of the water content of the material.
A summary of the results reported for folic acid content and for characterization of the material's impurity profile by the CCQM-K55.d participants is provided in this report.

Folic acid was selected as the measurand for the fourth round of the CCQM-K55 comparison because it:

- provides a significant analytical challenge, representative of a laboratory's capability for the purity assignment of compounds of moderate complexity and high polarity;
- is a member of the water-soluble class of vitamins and is a representative analyte for a number of CMC claims for the estimation of folic acid derivatives present in food and infant formula matrices currently found in or in development for inclusion in Appendix C of the BIPM KCDB;
- is safe and stable for transport and readily available at suitable purity in an amount to allow the preparation of the required batch size for conduct of the comparison.

Seventeen NMIs or DIs as well as the BIPM registered to participate in the CCQM-K55.d comparison, and all submitted results.

Characterization study

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

Related structure impurity content was evaluated by:

a. LC-UV
b. LC-hrMS/MS
c. ^{1}H NMR

Water content was evaluated by:

a. coulometric Karl Fischer titration using heated oven transfer from the sample
b. thermogravimetric analysis (TGA)
c. microanalysis (% C,H content) as a consistency check
d. sorption balance (mass variation as a function of RH)

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. thermogravimetric analysis (TGA) under oxidative conditions
c. microanalysis (% C, H content) as a consistency check

Main component (folic acid) content direct assay:

a. qNMR

To address concerns regarding the stability of folic acid in solution during LC-UV analysis and the possibility of artefact peaks resulting from in situ decomposition, a repeatability study was undertaken whereby twenty injections from a solution containing the CCQM-K55.d comparison material were undertaken over a 24 h period. There was no observed change in the impurity profile observed in the resultant chromatograms over the period of repeated analysis.

Homogeneity studies

i. Related structure components
The homogeneity of the minor components related in structure to folic acid were assessed by sampling in triplicate from ten sub-units selected from the candidate material batch with analysis by LC-UV. The minimum sample size 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 10 mg per analysis on seven sub-units each sampled in duplicate from the candidate material batch

iii. Folic acid
The homogeneity of folic acid content in the material was assessed simultaneously from the data obtained with the ten sub-units selected for the related structure homogeneity study.

Stability studies

Isochronous stability studies of folic acid and related structure impurity content, and separately of the water content, were 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 candidate batch were stored at each selected temperature over eight weeks, during which two units were transferred to reference temperature storage at two-week intervals.

As for the homogeneity study, an LC-UV method was used to obtain data for analysis of the stability of the folic acid and related structure impurities and a method based on coulometric Karl Fischer titration using heated oven transfer was used to evaluate the water content stability of the samples.

Related Structure Impurity Content

a) Homogeneity

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-UV ($u_{bb(rel)}$) were evaluated by simple ANOVA. This provided an estimate of the variation due to inhomogeneity of each major related structure impurity 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. Quadratic combination of the absolute inhomogeneity uncertainties for each impurity gave the combined estimate as 0.117 mg/g. This value is dominated by the uncertainty in the homogeneity of the major impurity, PABA glutamate.

Impurity	Content (mg/g) from homogeneity study	$u_{bb(rel)}$ (%)	$u_{bb(abs)}$ (mg/g)
PABA glutamate	3.15	3.68	0.116
Pterinyl-6-carboxylate	0.30	1.39	0.004
6-Pterinyl folate	1.33	0.55	0.007
FA Isomer 1	1.66	0.85	0.014
Unknown impurity 1	0.26	1.41	0.004
Pteroic acid	0.52	0.24	0.001
FA Isomer 2	1.24	0.41	0.005
Unknown impurity 2	0.18	1.41	0.003
Unknown impurity 3	1.24	0.41	0.005
	Homogeneity uncertainty (in combined value)		0.117
	Relative homogeneity uncertainty (in combined value)		1.13 %

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

b) Stability

Trend analysis of the data obtained by LC-UV analysis of the stability test samples under repeatability conditions indicated a small but significant decrease in the composition of folic acid on prolonged storage at 40 °C (Figure 1) and an increase in PABA glutamate (Figure 2) under the same conditions. In absolute terms these corresponded to a decrease in the folic acid content from 98.9 % to 98.8% , and a corresponding increase in the PABA Glu content from 0.2% to 0.3% , of the total organic content by relative LC-UV response. Neither change, even in the worst case, would in practice have had a significant influence on the final assigned value for the comparison. None of the other related structure components exhibited a significant change even on prolonged storage at elevated temperature. The folic acid and PABA glutamate content were both unchanged after storage at 22 °C.

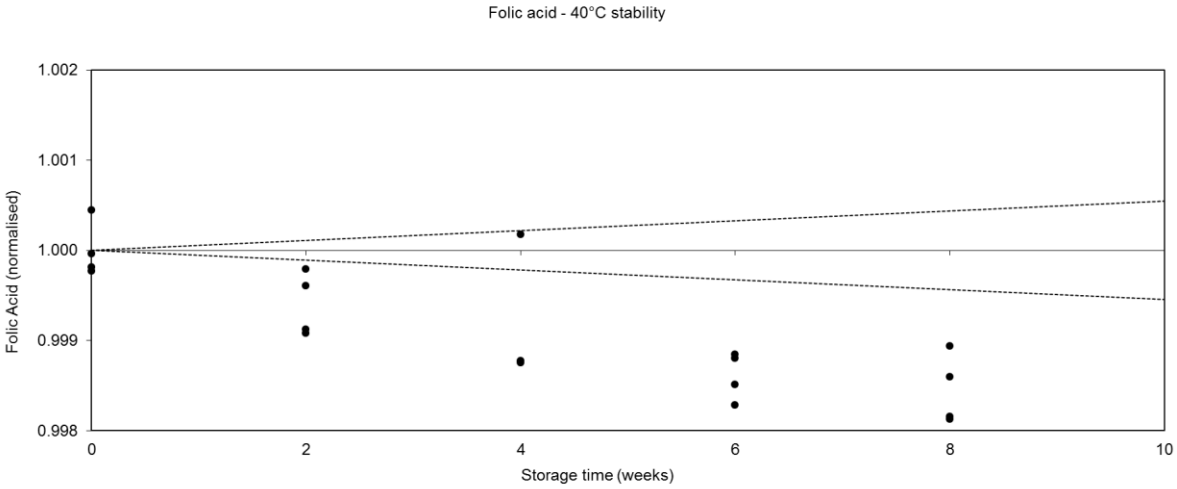


Figure 1: Stability profile of Folic acid on storage at 40 °C

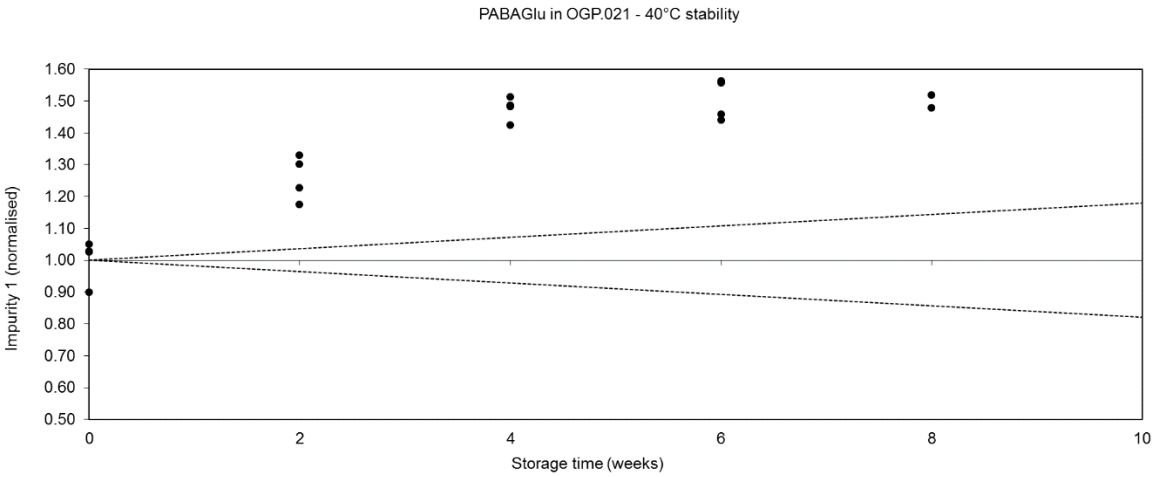


Figure 2: Stability profile of PABA Glu on storage at 40 °C

Water content

a) Homogeneity

For homogeneity measurements, ten vials taken at regular intervals from the filling sequence were analysed in triplicate (n = 3) in randomly stratified order for their water content using a Karl Fischer heated oven transfer method optimised for the analysis of folic acid. Sample portions of mass from 9.6 mg to 10.3 mg were weighed directly into the analysis vials and sealed. The directly observed results were corrected for an estimate for the blank vial water content.

The results obtained demonstrated the level of homogeneity of the water content of the material both within and between the individual units of the comparison material. The relative uncertainty contribution due to potential inhomogeneity of the water content (u_{bb}) was not greater than the repeatability estimate obtained from validation of the Karl Fischer method for water content estimation of folic acid materials. This corresponded to a relative uncertainty of 0.65 % (0.52 mg/g absolute) for the analysis of the set of samples used for the homogeneity test.

b) Stability

For stability study, a reference storage temperature of -20 °C and test temperatures of 4 °C, 22 °C and 40 °C were used. A set of units from the production batch were stored at each selected temperature over eight weeks and two units were transferred to reference temperature storage at two-week intervals.

Trend analysis of the data obtained by Karl Fischer titration using heated oven transfer of the set of stability test samples indicated no significant change in the water content of folic acid even on prolonged storage at 40 °C (Figure 3)

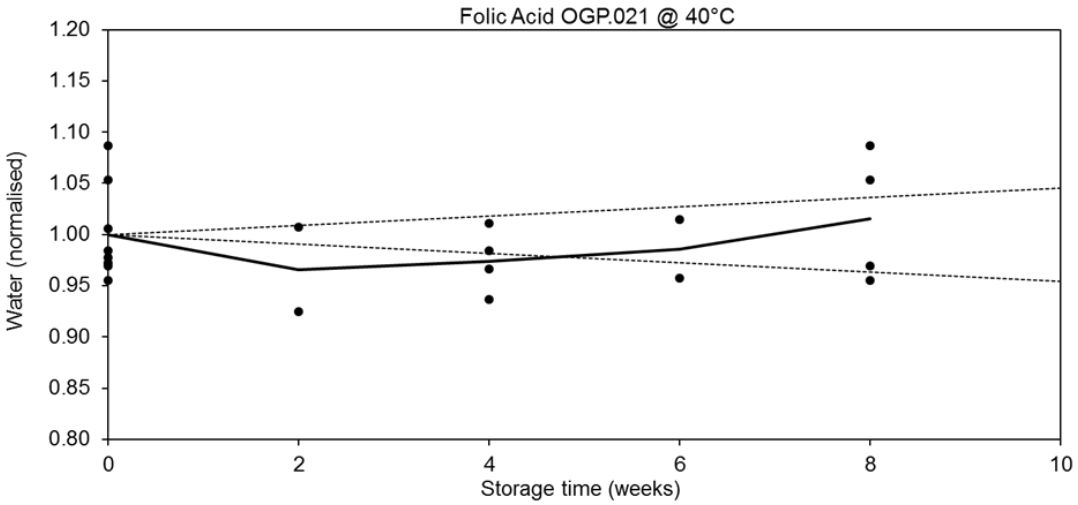


Figure 3: Stability profile of water in folic acid on storage at 40 °C

It was concluded that for the purposes of the comparison that the material was stable for short-term transport, provided it was not exposed to temperatures in excess of 40 °C, and for longer term storage at room temperature or below. To minimise potential changes in the material composition, participants were instructed to store the material, on receipt and after opening, at 4 °C.

Sorption Balance

The influence of relative humidity (RH) and temperature on the observed mass of samples of folic acid (OGP.021), the candidate material for CCQM-K55.d, were investigated using a sorption balance analysis. The plot in Figure 4 shows the percentage change in sample mass (red) of a 2 mg sample of CCQM-K55.d material as a function of RH (blue) at constant temperature. A plot of mass change of a similar size sample as a function of temperature at constant RH showed no significant change in the range 22 °C to 28 °C.

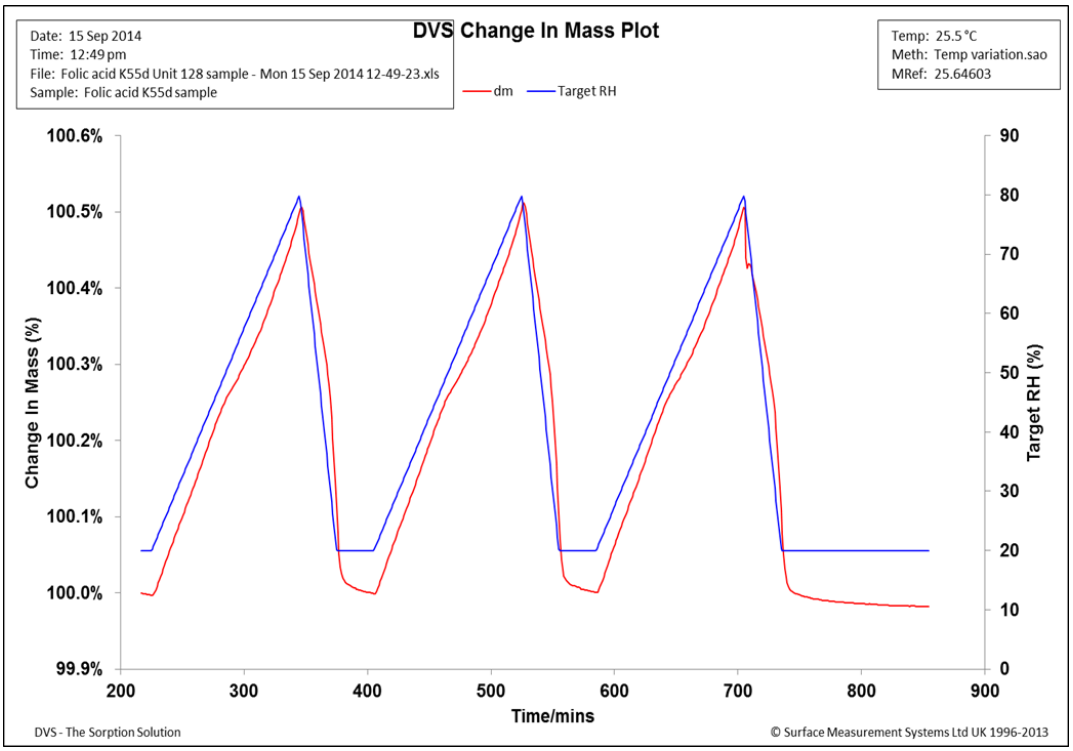


Figure 4: Change in sample mass (red) as a function of RH (blue) for folic acid

The results indicated:

i. no effect of variation of temperature on water content of folic acid in range 22 °C to 28 °C;

ii. rapid, reversible adsorption/desorption of water due to change in RH at a rate equivalent to 1 mg/g mass (0.1%) change per 10 % change in the sample RH environment.

It was concluded that for the purposes of the comparison the material was stable for short-term transport, but that the potential influence of the laboratory environment at the time of measurement needed to be controlled. It was also appropriate to recognise the potential for non-equivalence in water content measurements between laboratories solely due to differences in the local relative humidity at the time of measurement.

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, and to indicate whether a monitoring strip inside the shipping container had registered a temperature in excess of 37 °C during the transport process.

Apart from an extended delay encountered in obtaining local Customs clearance for the delivery of the samples to three participants no particular difficulties shipping were encountered and there was no indication of any material being exposed to temperatures above 37 °C during transport, even in the cases when there was a delay for Customs clearance.

Each of the registered participants in the CCQM-K55.d comparison provided a result for their sample. In addition four of the participants in the key comparison, who assigned their value for the folic acid content in CCQM-K55.d using a mass balance approach, also obtained an independent result using a qNMR approach. One participant (BIPM) provided a mass balance result using thermogravimetric analysis (TGA) results rather than KFT measurements for the estimation of water content. These five results, which were additional to the CCQM-K55.d results, were reported within the parallel pilot study CCQM-P117.d

Quantities and Units

Participants were required to report the mass fraction of folic acid in one of the supplied units. Participants who used a mass balance procedure were required to report the combined mass fraction assignment and associated uncertainty for the contributing sub-classes of impurity; total related structure organic impurities, water, residual solvent and total non-volatiles/inorganics content. Assessment of the enantiomeric purity of the material was not required.
The mass fraction content of folic acid and impurities in CCQM-K55.d are reported in units of mg/g. Participants using a mass balance approach were encouraged but not required to also provide mass fraction estimates for the main related structure impurity components they identified in the comparison sample.

III. REPORTED MASS FRACTION CONTENT OF FOLIC ACID IN CCQM-K55.d

The results reported for the folic acid content of CCQM-K55.d are listed in Table 2 and a summary plot is shown in Figure 5. Unless otherwise stated, throughout this summary results are plotted with their associated standard uncertainty ($\pm u_c$, $k = 1$).

The results are shown in Figure 6 with their expanded uncertainties, reported as a 95% confidence range.

The approaches used to assign purity for the CCQM-K55.d results were mass balance, qNMR or the combination of data obtained using both these approaches.

Participant	Folic acid (mg/g)	u (mg/g)	k	U (mg/g)	General Approach
NRC	858.3	6.3	2	12.6	qNMR
NMIJ	898.6	7.7	2	15.4	Mass balance & qNMR
INMETRO	900.3	4.2	2	8.4	Mass balance & qNMR
BAM	900.95	0.36	2	0.72	qNMR
SIRIM	902.0	2.0	2	4.0	qNMR
LGC	902.9	1.6	2	3.2	Mass balance & qNMR
NMIA	903	4	2	8	Mass balance & qNMR
NIM	905.21	5.27	2	10.54	Mass balance (K55.d) & qNMR (P117.d)
NIST	905.9	1.60	-	-3.1 / +3.5	Mass balance & qNMR
NMISA	906.8	2.6	2	5.10	Mass balance (K55.d) & qNMR (P117.d)
GLHK	909.6	1.99	2	3.98	Mass balance
HSA	910.8	2.4	2	4.8	Mass balance
NIMT	911	5.18	2	11	Mass balance
BIPM	911.1	2.15	2	4.3	Mass balance (K55.d) & qNMR (P117.d)
UME	911.365	3.62	2	7.24	Mass balance (K55.d) & qNMR (P117.d)
VNIIM	912.07	1.56	2	3.12	Mass balance
KRISS	912.9	0.27	2.45	0.66	Mass balance
CENAM	935.4	5.17	2	10.33	Mass balance

Table 2: Folic acid mass fraction estimates for CCQM-K55.d

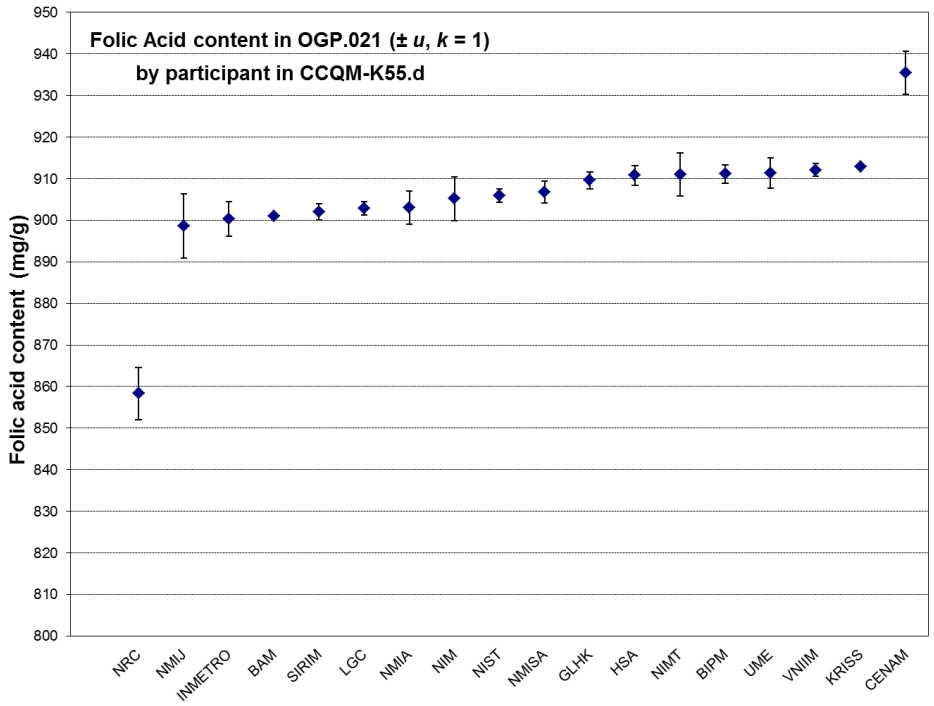


Figure 5 Folic acid content by participants in CCQM-K55.d (with associated standard uncertainties, $k = 1$)

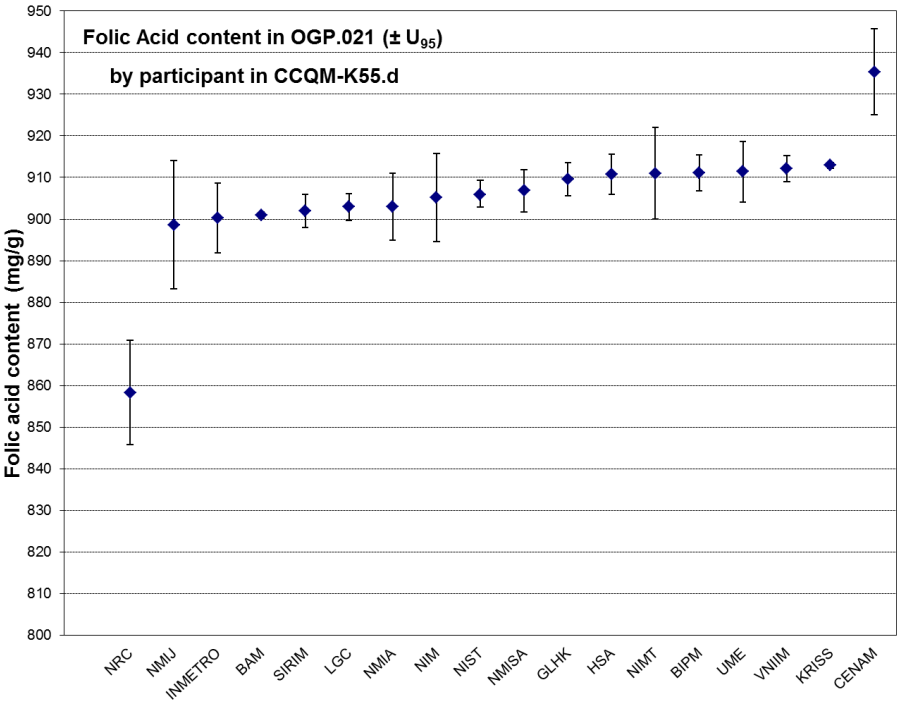


Figure 6 Folic acid content by participants in CCQM-K55.d (with expanded uncertainties, 95% confidence range)

IV. IMPURITY PROFILE AND KEY COMPARISON REFERENCE VALUES (KCRVs) FOR IMPURITY CLASSES IN CCQM-K55.d

All participants in CCQM-K55.d using a mass balance procedure were required to give estimates for the mass fraction of the sub-classes of impurities they quantified and combined to obtain their overall folic acid mass fraction estimate. After the initial discussion of results at the April 2016 OAWG meeting it was agreed that, for purposes of discussion of the KCRV the comparison coordinator would estimate the folic acid content of CCQM-K55.d based on the combination of consensus values for the mass fraction of each of the orthogonal classes of impurity in the comparison sample.

This required the assignment of reference values for the mass fraction in CCQM-K55.d of:

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

a. Total related structure impurity content (w_{RS}) and KCRV

The main impurities related to folic acid identified by at least three participants in the comparison material were, in order of chromatographic elution, *p*-aminobenzoic acid glutamate (PABA Glu), pterin-6-carboxylic acid, pterin-6-aldehyde, 6-pterinyl folic acid and pteroic acid. The chemical structures of each of these compounds are shown in Annex B.
In addition the presence of two folic acid isomers of unassigned structure and a longer retention time impurity were also reported by a number of participants. Various additional trace level impurities were observed by most participants.
The estimates for total related structure impurity content by each participant are listed in Table 3.
All participants used LC-UV methods to analyse the materials and although the chromatographic separations reported by participants shared common features, there was variation in the retention time, resolution and number of the related structure impurities observed and reported between each participant. Some representative chromatograms provided by participants are reproduced in Annex C to illustrate these similarities and differences. The chromatogram reported by the NMIJ was notable for showing significantly fewer impurities than those supplied by other participants.
A compilation of the mass content estimates reported for the individually identified impurities is given in Table 4. The components independently identified by two or more participants are listed in Table 4 in approximate order of their chromatographic retention time. PABA-Glu and pteroic acid are also referred to respectively as “Impurity A” and “Impurity D” in the Ph. Eur. monograph for folic acid.

Some suggestions to account for the variations in the mass fractions of related structure impurities as reported by the participants that were proposed during discussions were:

1. non-equivalent determination of the relative LC-UV response due to failing to control for:
    a. water content of folic acid
    b. UV-response on a mass content basis of related impurities relative to folic acid
    c. pH dependency of the UV response for individual components
2. saturation in some cases of the detector response of folic acid relative to the impurity components at the sample concentration used for the analysis
3. instability of folic acid in solution, particularly in solution at pH above 7, leading to the formation of artefact peaks misidentified as true impurities

NMIJ suggested the explanation for the range of results was due to artefacts being formed by breakdown of folic acid during sample preparation and analysis. While other laboratories (NRC, BIPM, UME, NIM) confirmed that folic acid was not stable for an extended period in solution, the coordinating laboratory reported that they found no evidence of a significant change in the impurity profile when a single sample of folic acid taken up in solution was analyzed by HPLC immediately after preparation. Repeated injection of aliquots of this sample with analysis by LC-UV over a 24 h period resulted in no significant alteration over time of the observed impurity profile. In addition NMIA isolated a purified fraction containing exclusively folic acid by HPLC and saw no evidence of its decomposition to reform the initially observed impurity profile when a sample of the purified fraction was reinjected for the same HPLC analysis.

One caveat regarding the stability of folic acid in solution was the observation by two participants that pterin-6-aldehyde (4) rather than pterin-6-carboxylic acid (3) was the primary form of impurity containing a pterin ring present in folic acid. They reported that observed pterin carboxylic acid was an artefact arising from *in situ* oxidation in solution of initially present pterin aldehyde. Some participants reported the presence of both impurities. No firm conclusion was reached on this issue, and in itself it was not sufficient to account for the observed differences in the reported values for total related impurity content.

After review of the results CENAM requested that their value for total related structure impurities be withdrawn from consideration for assignment of a reference value for this impurity category.

Although the reported results were in relatively good agreement given the complexity and lability of folic acid and the range and number of related structure impurities present, there was nevertheless a range of reported results for both the total and individual impurity content and variable observed impurity profiles in addition to different assumptions made in processing the results by each participant. In discussions it was emphasised that not all participants may be appropriately correcting for differences in response factor when using relative area response as a measure for relative related structure impurity content and that care should be exercised when converting these values into absolute mass fraction estimates.

In principle further studies could have been undertaken to resolve these issues, but it was agreed at the October 2016 OAWG meeting that given the lack of consensus of the source of the inconsistency the time and resources required for a meaningful further investigation were not justified. For the purposes of assigning a reference value it was also agreed by the OAWG that due to uncertainty regarding the equivalency of the components being measured by each participant it was not justified to use a standard deviation of the mean approach (or its robust statistical equivalent) to assign the uncertainty for a reference value.

Instead the mean of the data set is used as the assignment for the KCRV for total related structure impurity content and the standard deviation of the data as an appropriate if conservative estimate for the uncertainty of this KCRV.

Figure 7 is a plot of the sixteen individual results, the mean of the results excluding CENAM, with the standard deviation as the uncertainty estimate (red lines).

The mean and the standard deviation of the data set were selected as the estimator for the KCRV for total related structure impurity present in CCQM-K55.d:

w_{RS} = 12.6 mg/g;
u_{wRS} = 3.14 mg/g

The results reported by participants for combined related structure impurity content with their associated standard uncertainties (k = 1) plotted against the KCRV are shown in Figure 7. The DoE table and plot for these results are given in Table 14 and Figure 16 in Annex D.

Participant	Total Related Structure impurity (mg/g)	u_c (mg/g)	Coverage factor	$U_{95\%}$ (mg/g)
CENAM	0.433	0.021	2	0.042
NMIJ	7.24	0.11	2	0.23
HSA	7.71	0.314	2	0.627
BIPM	10.37	0.89	2	1.78
NMISA	10.5	1.4	2	2.8
UME	11.31	0.244	2	0.488
KRISS	11.6	0.20	1.97	0.40
GLHK	11.87	1.26	2	2.52
VNIIM	12.2	1.51	2	3.02
LGC	13.8	2	2	4
NIM	14.91	4.01	2	8.02
NIST	15.2	2.1	n.a.	-3.8 / +4.4
NMIA	15.8	1	2	2
NIMT	16.69	0.17	2	0.34
INMETRO	17.3	4	2	7.9

Table 3: Assignments of total related structure impurity content reported by participants in CCQM-K55.d using mass balance methods

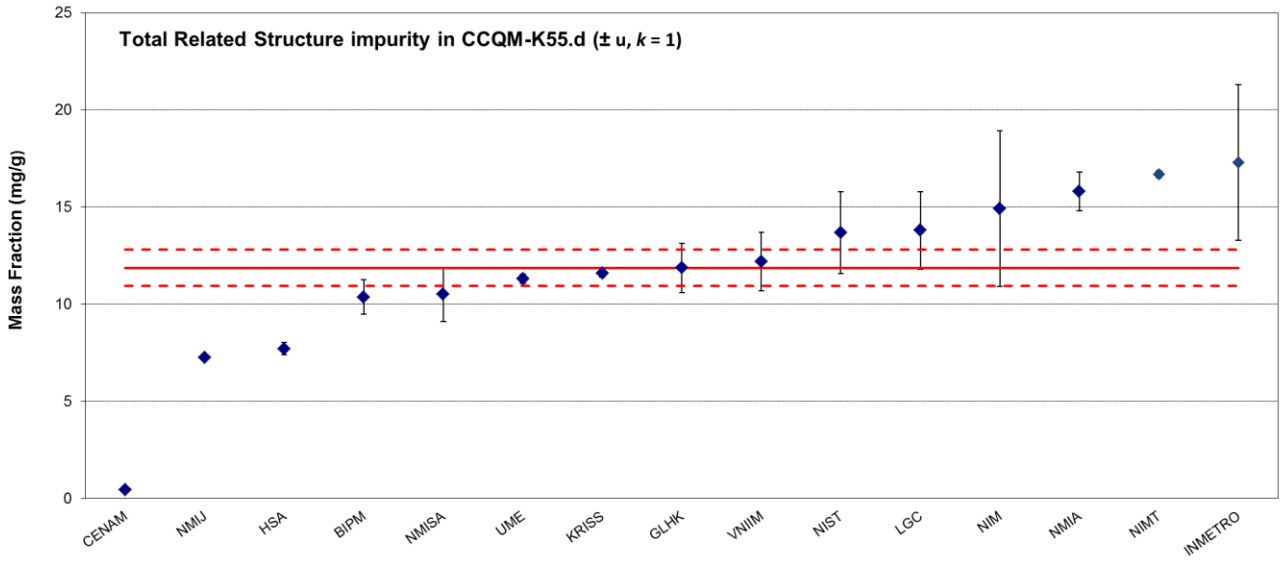


Figure 7 Total related structure impurity estimates in CCQM-K55.d (± u, k = 1)
The red line is the KCRV (12.61 mg/g) calculated excluding the CENAM result and the bracketing dashed lines the KCRV standard uncertainty (k = 1, KCRV ± 3.14 mg/g).

Compound	Participants	Mean (mg/g)	Std. dev (mg/g)
PABA-Glu (Impurity A, **2**)	13	3.08	0.55
Pterin-6-carboxylic acid (**3**)	5	0.26	0.31
Pterin-6-aldehyde (**4**)	2	2.75	-
Folic acid Isomer 1	6	1.55	0.27
6-Pterinyl folate (**5**)	6	1.62	0.36
Folic acid Isomer 2	5	1.90	0.80
Pteroic acid (Impurity D, **6**)	10	0.53	0.11
Long RT unknown	6	1.93	0.83

Table 4 – Assigned related structure impurities reported in CCQM-K55.d

b. Water content (w_{H2O}) and KCRV

The values for water content in CCQM-K55.d reported by the participants are listed in Table 5.

All reported water content used coulometric Karl Fischer titration, either by direct addition of the sample into the titration cell or using heated oven transfer. An overview of the individual methods used to determine and confirm water content estimates is given in Table 6.

To address concerns of the influence due to the hygroscopicity of folic acid on water content determinations in separate laboratory environments, particularly the influence of variations in ambient relative humidity, participants were requested to report the temperature and relative humidity at which they undertook both gravimetric operations and performed KFT measurements. A plot of the reported laboratory relative humidity conditions against the reported value for water content under these conditions is given in Figure 8.

Participant	Water (mg/g)	u_c (mg/g)	Coverage factor	$U_{95\%}$ (mg/g)
CENAM	63.97	5.17	2	10.33
NIMT	72	5.15	2	11
UME	75.225	1.597	2	3.194
VNIIM	75.61	0.39	2	0.78
KRISS	76.06	0.21	3.18	0.67
NIST	77	0.18	2	0.35
BAM	77.5	0.2	2.57	0.6
NRC	77.5	1.2	2	2.4
GLHK	78.4	1.16	2	2.32
INMETRO	78.5	0.56	2	1.1
NIM	78.7	3.37	2	6.73
NMIA	79	2	2	4
NMISA	79	2.1	2	4.1
BIPM	79.3	1.9	2	3.8
LGC	79.52	1.61	2	3.22
NMIJ	79.81	1.35	2	2.70
HSA	81.08	1.94	2	3.88
SIRIM	81.31	2.373	2	4.746

Table 5 – Estimates of water content reported by participant for CCQM-K55.d

Participant	Water content (± U_{95}, mg/g)	Method summary	Laboratory Environment
CENAM	63.97 ± 10.33	KFT; 10 – 12 mg	T = 21.06 °C, RH = 39.6 %
NIMT	72 ± 11	KFT; direct addition, 5 x 15 mg	T = 19 °C, RH = 49 %
UME	75.225 ± 3.194	KFT; oven transfer @ 160 °C, 3 x 15 mg	T = 19 °C, RH = 33 %
		TGA (25 °C – 850 °C, 10 mg)	
VNIIM	75.61 ± 0.78	KFT; oven transfer @ 160 °C, 6 x 50 mg	T = 23 °C, RH = 33 %
KRISS	76.06 ± 0.67	KFT; oven transfer @ 170 °C, 4 x 40 mg	T = 22.8 °C, RH = 32.5
NIST	77 ± 0.35	KFT; direct addition, 1 x 25, 1 x 26 mg	T = 21.8 °C, RH = 25%-26%
		TGA (22 °C – 200 °C, 5 x 7 -10 mg)	
BAM	77.5 ± 0.6	KFT; oven transfer @ 150 °C, 6 x 50 mg	T = 23±1 °C, RH = 40 ± 5%
NRC	77.5 ± 2.4	KFT; oven tfr @ 140 °C, 3 x 10 - 15 mg	
		TGA (10 °C – 120 °C, 3 x 5 -7 mg)	
GLHK	78.4 ± 2.32	KFT; oven transfer at 160 °C	T = 26 °C, RH = 44%
INMETRO	78.5 ± 1.1	KFT; direct addition, 4 x 20 mg	T = 20.7 °C, RH = 43.8 %
NIM	78.7 ± 6.73	KFT; direct addition, 11 x 9 - 14 mg	T = 20±2 °C, RH = 10-20 %
NMIA	79 ± 4	KFT; direct addition, 2 x 13 mg	T = 21.9 °C, RH = 50 %
		TGA (25-120 °C,15 min to 160 °C 15 min)	
		% CHN consistent with KFT	
NMISA	79 ± 4.1	KFT; direct and oven transfer @ 150 °C	T = 22.2 °C, RH = 67 %
		TGA (25 °C - 950 °C)	
BIPM	79.3 ± 3.8	KFT; oven transfer @ 170 °C, 10 x 10 mg	T = 22 °C, RH = 45% - 48 %
		% CHN (6 x 5 mg) consistent with KFT	
		TGA (22 °C – 150 °C, 9 x 5 mg)	
LGC	79.52 ± 3.22	KFT; oven transfer @ 150 °C ; 6 x 12 mg	T = 22 °C, RH = 47 %
		TGA (22 °C – 150 °C, 5 x 3.5 mg)	
NMIJ	79.81 ± 2.70	KFT; oven transfer @ 150 °C, 5 x 20 mg	T = 25 °C, RH = 41.5-42.6%
HSA	81.08 ± 3.88	KFT; oven transfer @ 170 °C ; 3 x 20 mg	T = 22 °C, RH = 49-50 %
SIRIM	81.31 ± 4.746	KFT; direct addition, 5 x 14 mg	T = 22 °C, RH = 56-60 %

Table 6 – Reported values and method outlines for water content assignment for CCQM-K55.d

After review of the participant results CENAM requested that their value for water content be withdrawn from consideration for assignment of a reference value, due to concerns with the quality of the reagent used to calibrate their KF apparatus.

The measurement model used for assignment of the KCRV for water content and its associated uncertainty was

$$w_{H2O} = H * \Delta RH * \overline{w}$$

where:

$\bar{w}$ = mean of participant data (excluding CENAM) for water content
= 78.0 mg/g
$u(w_{H2O})$ = standard deviation of the mean
= 0.56 mg/g
$u_{rel}(w_{H2O})$ = relative standard uncertainty of the mean water content
= (0.56/78.0)*100 %
= 0.72 %

H = homogeneity factor (1.0)
$u_{rel}(H)$ = 0.66 % [from coordinating lab characterization studies, see page (8)]

ΔRH = factor (1.0) for differential sorption of water by folic acid as function of laboratory RH.

The range of laboratory RH reported by participants was 15% - 65%. The relative change in the total sample mass due to water sorption over this range estimated from the characterization studies (see page (9)) would be 0.5%, corresponding to an absolute change in water content of 5 mg.

$u_{rel}(\Delta RH)$ = relative uncertainty from a rectangular distribution of variation of water content in folic acid due to sorption/water content

$u_{rel}(\Delta RH)$ = $(5/\sqrt{12}) / \bar{w}$
= (1.44/78.0) *100 %
= 1.851 %

w_{H2O} = 78.0 mg/g

u_{H2O} = $w_{H2O} * \sqrt{((0.661\%)^2 + (1.851\%)^2 + (0.718\%)^2)}$
= 1.63 mg/g

The results reported by participants for water content with their associated standard uncertainties (k =1) plotted against the KCRV are shown in Figure 9. The DoE table for water content estimates and a DoE plot relative to the KCRV are given in Annex D in Table 15 and Figure 17 respectively.

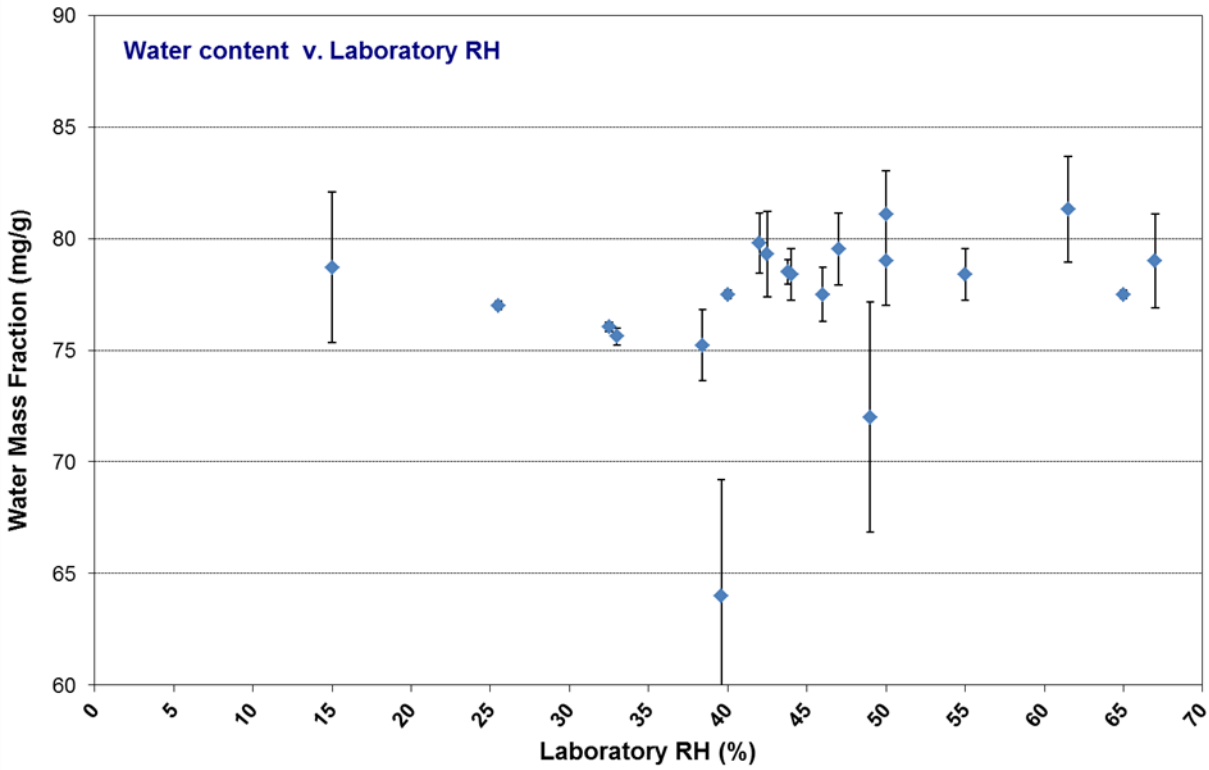


Figure 8 Water content (± *u*, *k* =1) in CCQM-K55.d obtained by KFT as a function of laboratory RH

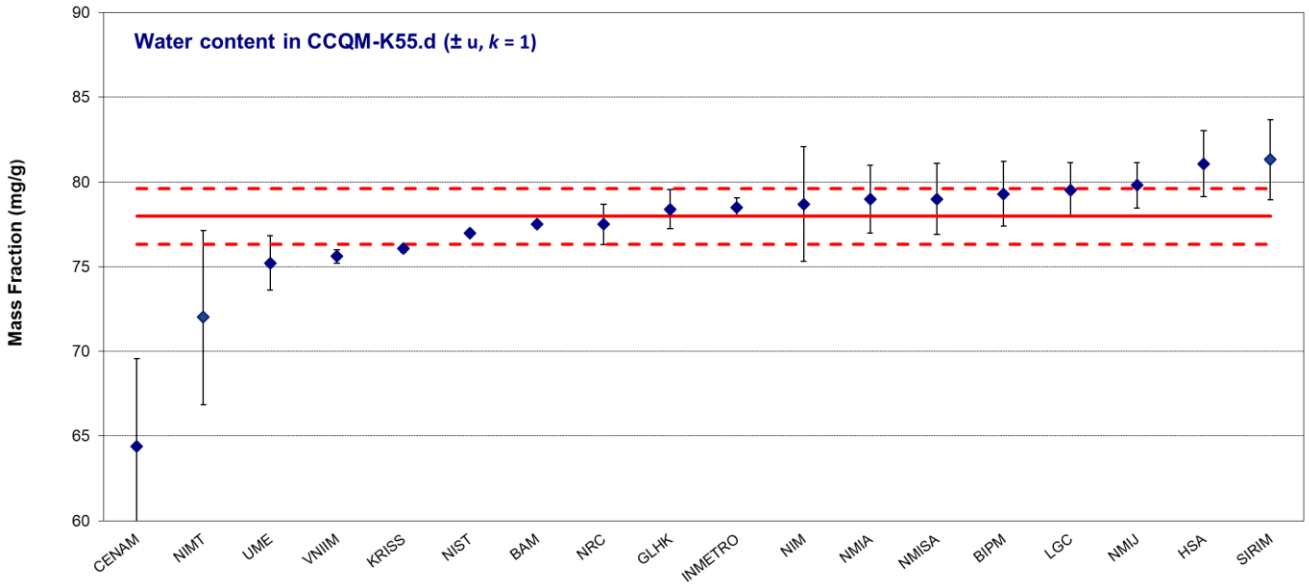


Figure 9 Water content estimates by participants in CCQM-K55.d (± *u*, k = 1)
The KCRV for water content (w_{H2O}) is 78.0 mg/g. The dashed lines correspond to the KCRV standard uncertainty (*k* = 1, KCRV (w_{H2O}) ± 1.63 mg/g).

c. Volatile organic compound content (w_{VOC}) content and KCRV

Seventeen participants provided estimates for the volatile organics content of CCQM-K55.d. Nine participants did not report the presence of residual solvent at levels above their method detection limits. Eight reported the presence of a small level of acetone. Two reported the presence of acetic acid, though at significantly different levels.

As the majority of results were below their individual method detection limit, simple statistical techniques cannot be applied to the combined results. The KCRV for volatile organics content (w_{VOC}) was assigned as the median of the eight results that reported the quantification of acetone and other solvents with the robust uncertainty of the standard deviation of the median (1.25*MADe/√8) as the uncertainty estimate. This gives the KCRV for w_{VOC} of 0.374 mg/g and u_{VOC} of 0.083 mg/g

The results reported by participants with their associated standard uncertainties (k = 1) are listed in Table 7 and are plotted against the KCRV in Figure 10. The DoE table and plot relative to the residual solvent impurity KCRV are given in Annex D in Table 16 and Figure 17 respectively.

Participant	Solvent (mg/g)	u_c (mg/g)	Coverage factor	$U_{95\%}$ (mg/g)
BAM	0	0.01		
BIPM	0	- 0, + 0.15	2	- 0, + 0.3
CENAM	0			
KRISS	0	0.083	2.12	0.18
NIMT	0			
NIST	< 0.01			
NIM	< 0.01			
GLHK	< 0.1			
UME	< 0.5			
VNIIM	0.044	0.0048	2	0.0096
NMIJ	0.05	0.03	1.65	0.05
NRC	0.29	0.03	2	0.06
LGC	0.373	0.274	2	0.549
HSA	0.374	0.153	2	0.305
INMETRO	0.467	0.012	2	0.025
NMISA	0.5	0.12	2	0.25
NMIA	0.9	0.36	2	0.72

Table 7 – Estimates of volatile organics content reported by participant for CCQM-K55.d

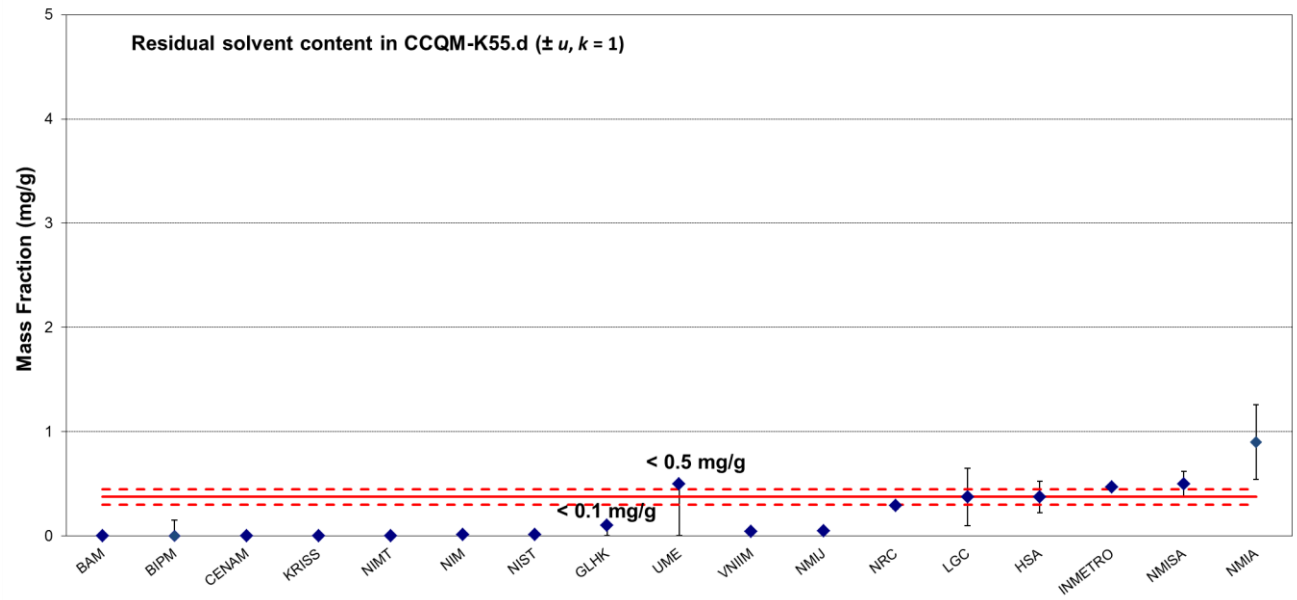


Figure 10 Volatile organics estimates plotted with their standard uncertainties, k = 1. The KCRV for volatile organics content (w_{VOC}, red line) is 0.374 mg/g. The dashed lines correspond to the KCRV standard uncertainty (k = 1, KCRV (w_{VOC}) ± 0.083 mg/g).

d. Non-volatiles/inorganics (w_{NV}) content and KCRV

The majority of participants reported low but detectable levels of non-volatile material in the sample. Na^+ species were identified as the main component of this impurity class. The combined value was small in all cases with only two participants reporting a total in excess of 0.5 mg/g.

The values assigned for combined non-volatile organic and inorganics content are listed in Table 8 and plotted against the KCRV in Figure 11.

The KCRV for total non-volatiles (w_{NV}) was assigned as the median of the twelve participants that reported a value for non-volatile content. Given the relatively large range of values and the different methods used to estimate content, the robust standard deviation of the data set (1.25*MADe) , rather than robust standard deviation of the median, was the (conservative) associated KCRV uncertainty estimate. This gives w_{NV} of 0.305 mg/g and the associated u_{NV} of 0.203 mg/g

Participant	NV (mg/g)	u_c (mg/g)	k	$U_{95\%}$ (mg/g)
HSA	0	1.44	2	2.89
NIMT	0	0.52	2	1.04
UME	< 1.0			
NMIA	< 2.0			
VNIIM	0.076	0.0035	2	0.007
GLHK	0.17	1	2	2
CENAM	0.18	0.022	2	0.044
INMETRO	0.211	0.017	2	0.034
NIST	0.27	0.05	2	0.10
NRC	0.28	- 0.28, + 0.31	1, 2	- 0.28, + 0.63
KRISS	0.33	0.11	3.18	0.34
NMIJ	0.35	0.43	1.65	0.71
BIPM	0.4	0.2	2	0.4
LGC	0.429	0.107	2	0.214
NIM	1.18	0.59	2	1.19
NMISA	3.2	0.59	2.78	1.6

Table 8 – Estimates of non-volatiles content for CCQM-K55.d and CCQM-P117.d

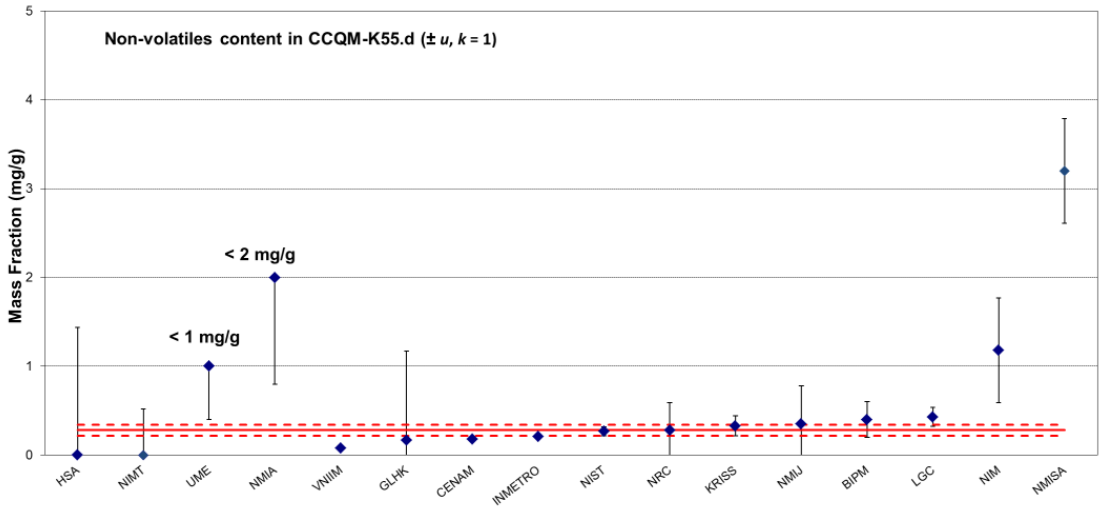


Figure 11 Estimates reported for non-volatile content (with standard uncertainties, k = 1). The KCRV for non-volatile content (w_{NV}, red line) is 0.305 mg/g. The dashed lines correspond to the KCRV standard uncertainty (k = 1, KCRV (w_{NV}) ± 0.203 mg/g).

V. qNMR RESULTS FOR FOLIC ACID CONTENT

The majority of participants in the comparison used a qNMR study in their assessment of the material. qNMR assignments were used as:

- the sole method to assign the reported folic acid mass fraction content (BAM, NRC, SIRIM)
- a result combined with a mass balance result to assign the value for overall folic acid content in the CCQM-K55.d material reported by the participant in Table 1 (INMETRO, NMIJ, LGC, NIST, NMIA)
- supporting data when a mass balance assignment was reported as the comparison result (NIM, NMISA, BIPM, UME, KRISS).

Four NMIs which reported folic acid content obtained by a mass balance method as their result for CCQM-K55.d obtained an independent estimate by qNMR which they submitted as their result within the parallel CCQM-P117.d study.

The signal for the hydrogen at position 7 of the pterin ring was the basis for quantification by all participants. A summary of the results reported, solvent and quantification standards used by each participant providing a qNMR value are shown in Table 9. As mentioned above, these results were in some cases combined with a mass balance result to give an overall assignment for folic acid content. For this reason the qNMR results in Table 9 do not necessarily correspond to the result reported previously by the same participant for CCQM-K55.d in Table 1.

The low solubility of folic acid presented challenges for analyte dissolution and sample preparation for qNMR. As seen in Table 9, half the participants reporting qNMR results made use of the higher solubility of folic acid in basified aqueous solution whilst the others prepared sample solutions in deuterated DMSO, generally with the addition of some D_2O as co-solvent.

Participant	^{1}H NMR Field strength (MHz)	Solvent	Internal Standard	Folic acid (u_c) (mg/g)
CCQM-K55.d				
NRC	600	D_2O/NaOD	Maleic acid	858.3 (6.3)
INMETRO	500	D_2O/NH_4OH	Dimethyl sulfone	897 (3.45)
NMIJ	600	D_2O/NaOD	DSS-d_6	898.6 (5.2)
BAM	600	d_3-ACN/d_6-DMSO	1,3,5-TMB	900.95 (0.36)
NMIA	500	D_2O/NaOD	KH maleate & Gly	901 (4.0)
LGC	600	D_2O/Na_2CO_3	Maleic acid	901.1 (1.5)
SIRIM	600	d_6-DMSO	TCNB	902 (2)
NIST	600	D_2O/KD_2PO_4/K_2HPO_4	Dimethyl maleate & dimethyl sulfone	905.8 (1.5)
CCQM-P117.d				
NIM	800	d_6-DMSO/D_2O	K-Acesulfame	896.37 (3.8)
NMISA	600	d_6-DMSO	Maleic acid	907.5 (4.0)
UME	600	d_6-DMSO/D_2O	Maleic acid & 1,3,5-TMB	909.78 (1.28)
BIPM	400	d_6-DMSO/D_2O	Maleic acid	912.9 (2.2)
Information value				
EDQM[8]	400	D_2O/Na_2CO_3	Dimethyl sulfone & DSS-d_6	903.6 (0.95)
KRISS	700	d_6-DMSO	Dimethyl sulfone	915.2 (7.3)

Table 9 – qNMR values and conditions for folic acid in CCQM-K55.d

[8] EDQM = European Directorate for the Quality of Medicines. Result reported in parallel pilot study CCQM-P117.d

For information purposes, the combined reported values for folic acid content by qNMR (green data points) and by mass balance approaches (purple data points) from participants in both the key comparison and parallel pilot study are plotted side by side in Figure 12.

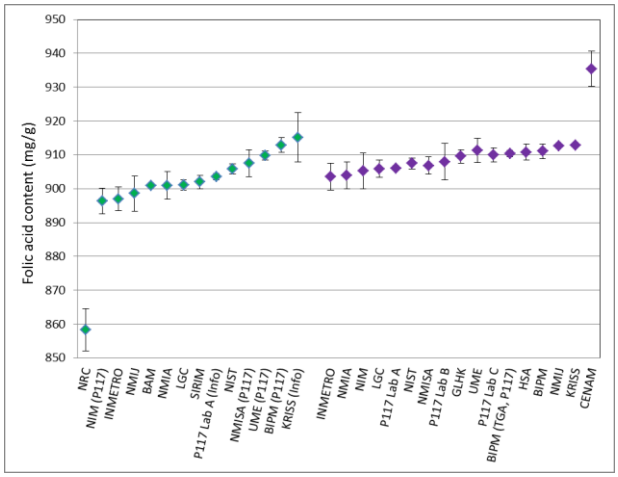


Figure 12 qNMR values (green) and mass balance values (purple) for folic acid content of the comparison material (with standard uncertainties, $k = 1$)

Solutions of folic acid, particularly in aqueous solution at elevated pH, were shown by a number of participants to undergo decomposition over time, particularly at elevated pH. The NRC result for CCQM-K55.d, which was based solely on a qNMR result, was withdrawn from consideration for the KCRV calculation when follow up studies confirmed that the folic acid in the NMR sample solutions had undergone significant decomposition between sample preparation and the acquisition of the qNMR data. NMIJ, BIPM and NIM also demonstrated that over time folic acid undergoes decomposition in solution at these concentrations.
For the purposes of the final KCRV assignment it was assumed that, apart from the one withdrawn result, bias due to decomposition was either not significant or was adequately controlled for and did not introduce a significant bias to the reported qNMR results.

VI. KEY COMPARISON REFERENCE VALUE FOR FOLIC ACID

The comparison coordinator followed the precedent from the previous comparisons and proposed for initial discussion, based on the comparison results, individual KCRVs for the mass fraction of each of the orthogonal classes of impurity present in the comparison material and used these values to propose an overall KCRV for folic acid content. This proposal was the starting point for a discussion on an approach to the KCRV at the October 2016 OAWG meeting. After review of the data NRC and CENAM agreed that their results should not be included within the overall KCRV calculation.

Some general conclusions were reached after the OAWG discussions in April and October 2016 and some follow up studies undertaken by the comparison participants. Although the overall consistency of the results was encouraging given the complexity of the measurand and the variety of measurement challenges associated with it, it was agreed that there remained a degree of non-equivalence in the results at their reported uncertainties, and in particular between results based on a mass balance approach and those based on qNMR. This difference could be ascribed in part to inhomogeneity in the material, in particular to the water content, but this did not account for all the observed difference. No generally accepted rationalization to account for the additional difference

was proposed and it was decided that it was not justified to devote further resources of time and effort, resulting in further delay in preparing the Final Report, to attempt to resolve it.
Instead it was proposed that an appropriate estimate for the folic acid content of the comparison material should use data obtained by qNMR, mass balance or combinations of both approaches and not be based solely on the "consensus" mass balance approach used for the KCRV assignment in previous CCQM-K55 comparisons.

The discrepancy between the mass balance and qNMR result assignments was recognized as the main contributor to the spread of the reported results. For results based on the mass balance approach a further contribution arose from the range of related structure impurity profiles obtained and the different assumptions made in processing the results by each participant. It was decided that as a result of this lack of confidence that all participants were effectively measuring the same entity, it was not justified to use a standard deviation of the mean approach (or its robust statistical equivalent) to calculate the estimate of the uncertainty of the assigned reference value.

Instead a conservative proposal was made to use the simple mean of the data set, after exclusion of the two results removed from consideration at the request of the individual NMI, as the KCRV assignment for the folic acid content in the CCQM-K55.d material and to use the standard deviation of the data set as an appropriate if again cautious estimate for the uncertainty of the KCRV.

This gives the KCRV for folic acid content in the CCQM-K55.d material as:

w_{FA} = 906.5 mg/g

The estimate of the uncertainty of the KCRV assignment is:

$u(w_{FA})$ = 4.8 mg/g

Figure 13 plots the KCRV against participant results, all with their associated standard uncertainties

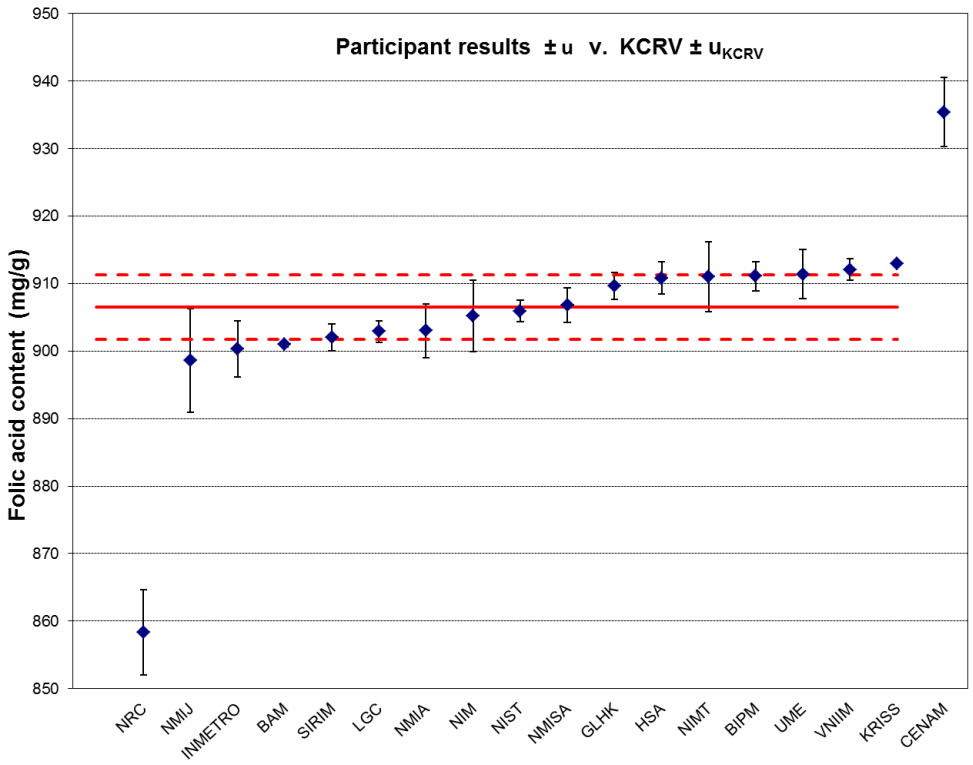


Figure 13: Mass fraction reported of folic acid in CCQM-K55.d with reported uncertainty (± *u*). KCRV for folic acid is the red line. KCRV ± u_{KCRV} (*k* = 1) correspond to the dashed lines

The degree of equivalence (D_i), expanded uncertainty of D_i (U_D) and the relative expanded uncertainty of D_i (U_{rel}) of each participant's reported value with the KCRV for folic acid in the CCQM-K55.d material is shown in Table 10 and plotted in Figure 14.

Participant	D_i (mg/g)	U_D (mg/g)	U_{rel} (%)
NRC	-48.23	15.8	1.75%
NMIJ	-7.93	18.1	2.00%
INMETRO	-6.23	12.7	1.41%
BAM	-5.58	9.6	1.06%
SIRIM	-4.53	10.4	1.15%
LGC	-3.63	10.1	1.11%
NMIA	-3.53	12.5	1.38%
NIM	-1.33	14.2	1.57%
NIST	-0.63	10.1	1.11%
NMISA	0.27	10.9	1.20%
GLHK	3.07	10.4	1.14%
HSA	4.27	10.7	1.18%
NIMT	4.47	14.1	1.56%
BIPM	4.57	10.5	1.16%
UME	4.83	12.0	1.32%
VNIIM	5.54	10.1	1.11%
KRISS	6.37	9.6	1.06%
CENAM	28.87	14.1	1.56%

Table 10: Degrees of equivalence (D_i), U_D and U_{rel} for folic acid in CCQM-K55.d

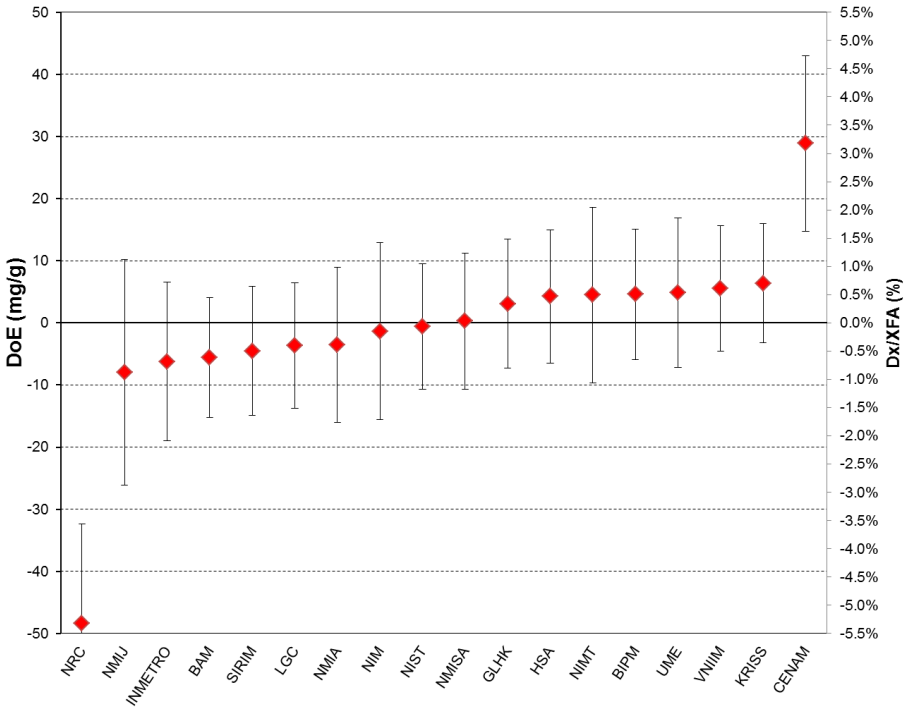


Figure 14: DoE plot for the folic acid KCRV using the participant results. Data are plotted with the expanded uncertainty in the DoE, corresponding to a 95% coverage range.

For information and comparison purposes, values for folic acid content could also be calculated using a "mass balance" approach from the consensus values from the combined participant results assigned for the individual impurity classes and also using a "qNMR" results-only approach.
As described previously, the consensus assigned values for each impurity type are shown in Table 11 as well as the estimate of the combined impurity content in the CCQM-K55.d material.
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Impurity type	**KCRV (mg/g)**	***u* (mg/g)**
Related structure organics	12.6	3.14
Water	78.0	1.64
Residual solvent	0.374	0.083
Non-volatiles/ inorganics	0.305	0.203
Combined value	91.3	3.54

Table 11: KCRV values and final result for combined impurities in CCQM-K55.d.

This leads to a "consensus mass balance" estimate ($w_{FA(MB)}$) for the content of folic acid of:

$w_{FA(MB)} = 1000 - [w_{RS} + w_{H2O} + w_{VOC.} + w_{NV.}]$ mg/g

= 1000 – [91.3] mg/g
= 908.7 mg/g

$u(w_{FA(MB)})$ = 3.6 mg/g

A combined qNMR assignment for folic acid content was obtained separately based on seven individual qNMR values that either directly or in contribution with a mass balance value were used to obtain a final result for CCQM-K55.d. As these seven results were not consistent within their individual stated uncertainties, their mid-range, taking into account the measurement uncertainty of the minimum and maximum reported qNMR value, was used as the estimate for a "combined" qNMR value. The uncertainty associated with this value was assigned as a rectangular distribution based on the half-range. This gave a "combined"qNMR estimate ($w_{FA(NMR)}$) for folic acid of 899.5 mg/g with $u(w_{FA(NMR)})$ of 5.4 mg/g.

The three assignments for folic acid mass fraction content – the KCRV calculated as the mean of all results, the "consensus" mass balance and the "combined" qNMR value - are listed in Table 12 and are plotted with their associated uncertainties (k = 1) in Figure 15.

Folic acid assignment	**FA content (mg/g)**	***u* (mg/g)**
KCRV	906.5	4.8
Combined Mass balance	908.7	3.6
Combined qNMR	899.5	5.4

Table 12: Assignments of folic acid content in CCQM-K55.d.

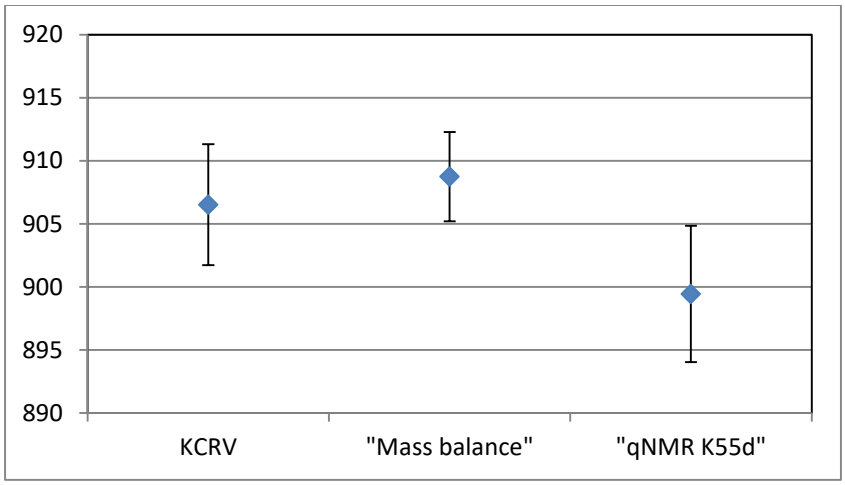


Figure 15: Comparison of KCRV, mass balance only and qNMR only assignments of folic acid content in CCQM-K55.d. Results are reported ± *u* (*k* = 1).

The combined "mass balance" and "qNMR" assignments agree with the KCRV within the standard uncertainty of each, and with each other within their expanded uncertainties. The difference between the mass balance and qNMR based values, whilst not statistically significant, is relatively large in absolute terms. As discussed above it could be ascribed to the inclusion within the qNMR result of impurity content not quantified by the mass balance methods, to a bias to lower values in the qNMR result due to instability of folic acid in (basic) aqueous solution over the time of the qNMR sample preparation and analysis, or to a combination of both factors.

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

The motivation for assigning KCRVs for the impurity classes in CCQM-K55.d is 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 folic acid does not arise through cancellation of errors in the contributing impurity assignments. The combined DoE plots for each impurity class quantified are shown in Annex D to this report.

CONCLUSIONS AND HOW FAR THE LIGHT SHINES

Folic acid was selected to be representative of polar (pK_{OW} > -2), medium molar mass (300 g/mol – 500 g/mol) organic compounds that are capable of analysis by LC but not by direct GC methods. It was anticipated to provide an analytical measurement challenge representative for the value-assignment of compounds of broadly similar structural characteristics.

Given the significant analytical challenge posed by the compound due to its relative instability, sensitivity to light and instability in solution, complexity of structure and high water content, the overall agreement and consistency of the majority of the participant results was encouraging. It indicates that lessons learned in the course of the CCQM-K55 series are being reflected in greater appreciation of the factors influencing the uncertainty of purity assignments.

There was a degree of consistency between participants in the identification of the related structure impurity content of the sample, although one issue that did arise was the relatively large spread of the quantification results reported for total related structure impurity content despite all participants using methods based on LC-UV detection. No single reason for this variability was identified. A contributing factor suggested was inconsistency or unsuitability in the response factors assigned relative to folic acid for related structure impurities. In particular the assumption of identical response factors was shown to be highly questionable given the different molecular weight and UV absorption properties of most of the identified impurities relative to folic acid. In addition *in situ* formation of artefact impurities depending on the sample preparation, chromatographic conditions, influence of pH on the UV absorption profile of specific impurities, sensitivity of chromatographic resolution from the main component to the elution system used and the existence of a degree of inhomogeneity in the material also potentially contributed to the variability of results. It was emphasised in discussions that when using a mass balance approach based around LC-UV methods for detection and quantification of related structure impurities there is a need to put sufficient effort into investigating the effects of different molecular weights and different UV absorption properties of impurity components in order to establish with appropriate confidence the metrological traceability of a result assigned using this approach.
The overall agreement of the water quantification results was encouraging given the significant amount of water present, the relative hygroscopicity of folic acid and the potential for the generation of water from breakdown of the structure as a decomposition artefact if forcing conditions were used. There was adequate agreement on the quantification of the (low) levels of residual solvent and non-volatile content in the material.
As discussed in more detail in the report, the main area of disparity in the overall results arose from the larger variability and generally lower assigned value of the reported results obtained using qNMR based assignment compared with those based on mass balance.

Some observations and conclusions from the comparison and participant performance were:

- suitable agreement between participants was achieved for the measure of water content and of residual solvent and non-volatile residue (inorganics);
- 12 of the 17 participants detected and quantified occluded acetone, while one participant also reported the presence of a significant level of acetic acid;

- mass fraction assignments reported for total related structure impurity content ranged from 7.24-17.3 mg/g. Although the range was not inconsistent with performance in previous comparisons, there continue to be issues with the approaches used for the assignment of this class of impurity that should continue to be addressed in future comparisons;
- consistent implementation of qNMR assignment of folic acid, giving results lower than but generally equivalent within their stated uncertainties with values obtained by mass balance;
- the observed difference between the combined mass balance and combined qNMR results may indicate either the presence of a small level of impurity in the comparison material not quantified using the mass balance approach, or a bias to lower qNMR values due to degradation of folic acid in the solvent system under the conditions used for qNMR analysis, or a combination of both factors;
- caution is needed to justify reporting purity assignments for analytically challenging compounds such as folic acid with a small associated standard uncertainty (< 0.5%).

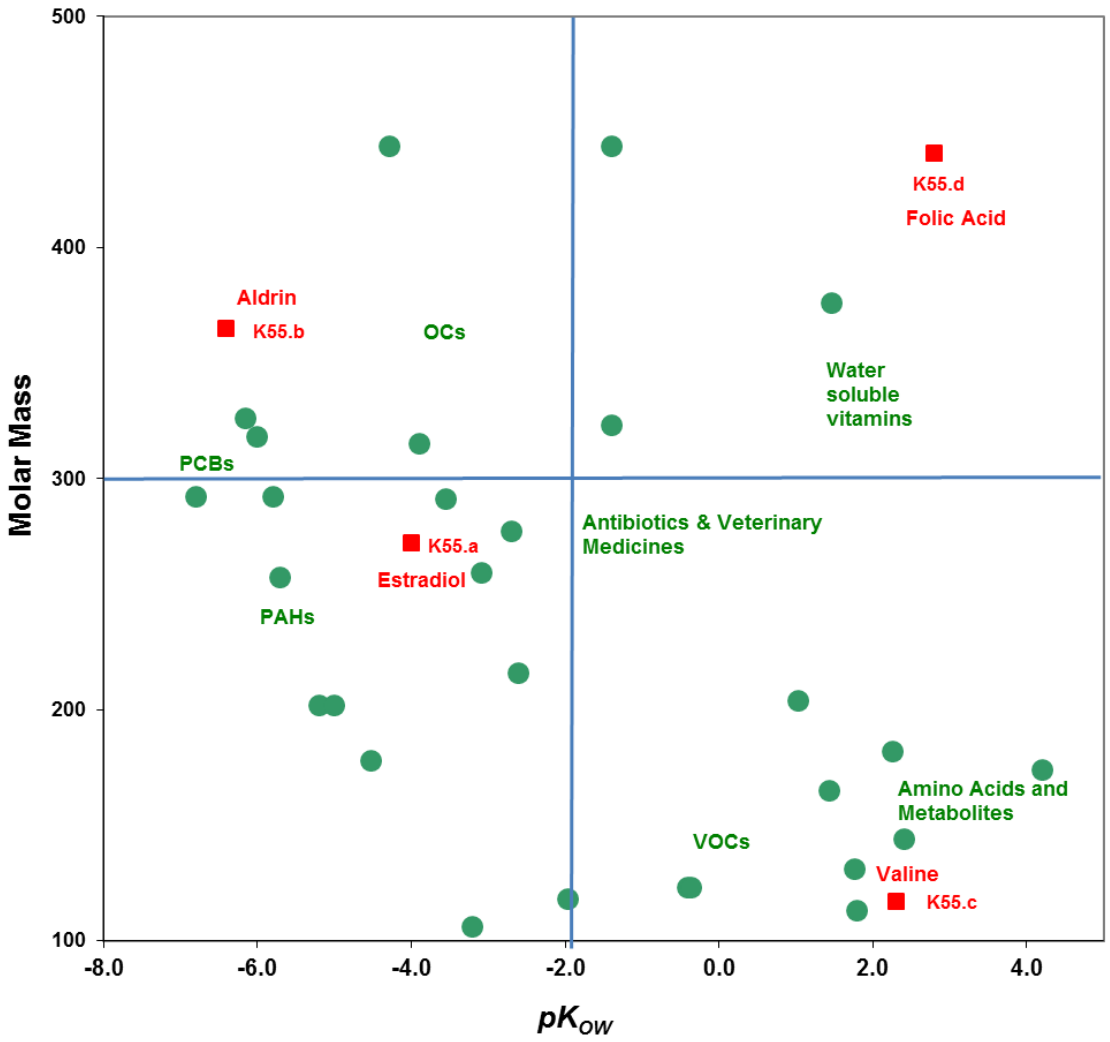
The comparison demonstrates that for folic acid when present as the main component at a mass fraction of around 900 mg/g, purity assignment can be achieved within a relative standard uncertainty in the assigned value of at least 0.5 % using a mass balance, a qNMR approach or a combination of results obtained from both methods.

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

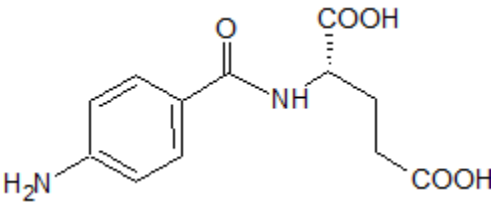
The comparison demonstrates a laboratory's performance in determining the mass fraction of the main component in a high purity organic material. Successful participation is indicative of a laboratory's measurement capability for the mass fraction purity assignment of polar organic compounds ($pK_{OW} > -2$) of medium structural complexity (molar mass range 300 g/mol - 500 g/mol) for which related structure impurities can be quantified by high performance liquid chromatography.

Annex A: Analysis Space Model for Organic Primary Calibrators

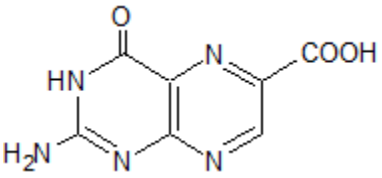
- CCQM-K55 measurands
- CMC claims for pure substance calibrators or calibration solutions



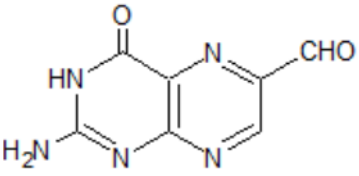
Annex B – Related structure impurities reported in CCQM-K55.d



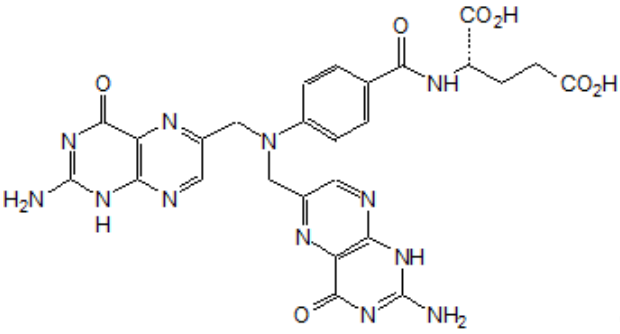
PABA-Glu (**2**, “Impurity A”)



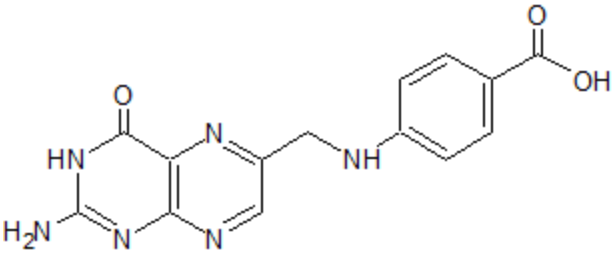
Pterin-6-carboxylic acid (**3**, P-6-CA)



Pterin-6-aldehyde (**4**, P-6-A)

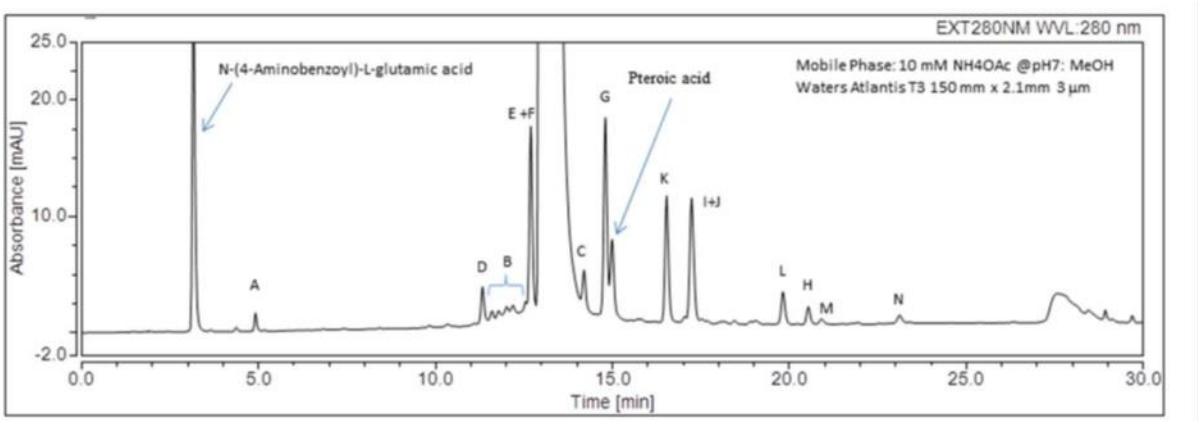


6-Pteryl-folic acid (**5**)

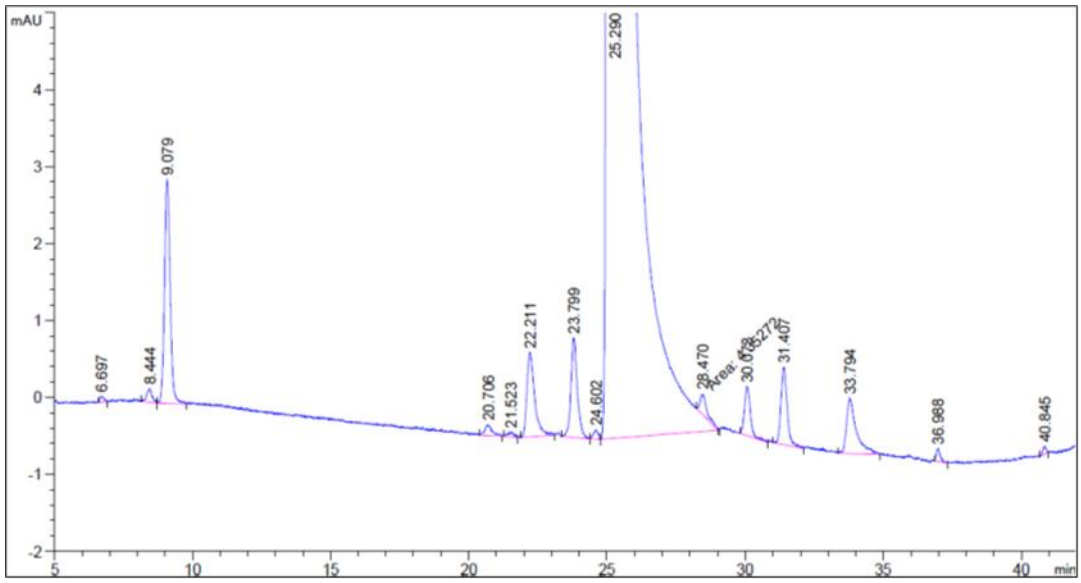


Pteroic acid (**6**, PTA, “Impurity D”)

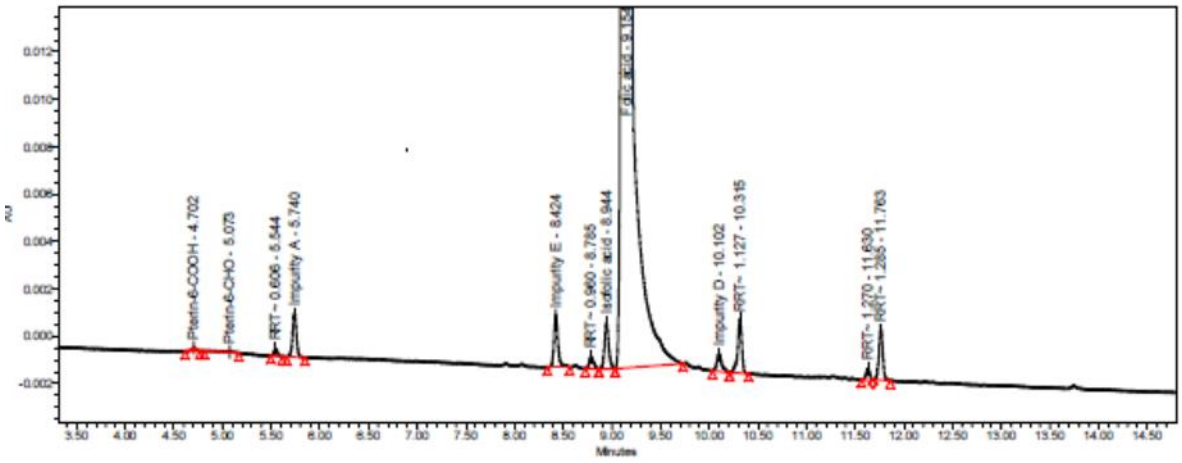
Annex C – Chromatographic profiles for CCQM-K55.d



GLHK (LC-UV @ 280 nm)

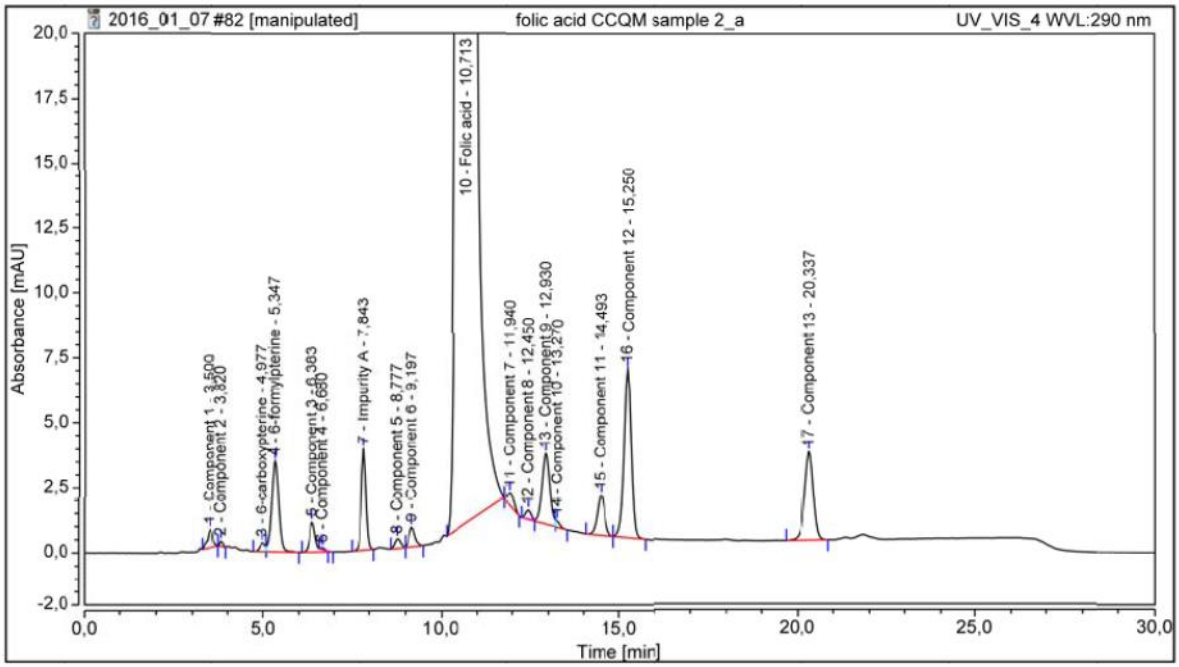


HSA (LC-UV @ 284 nm)

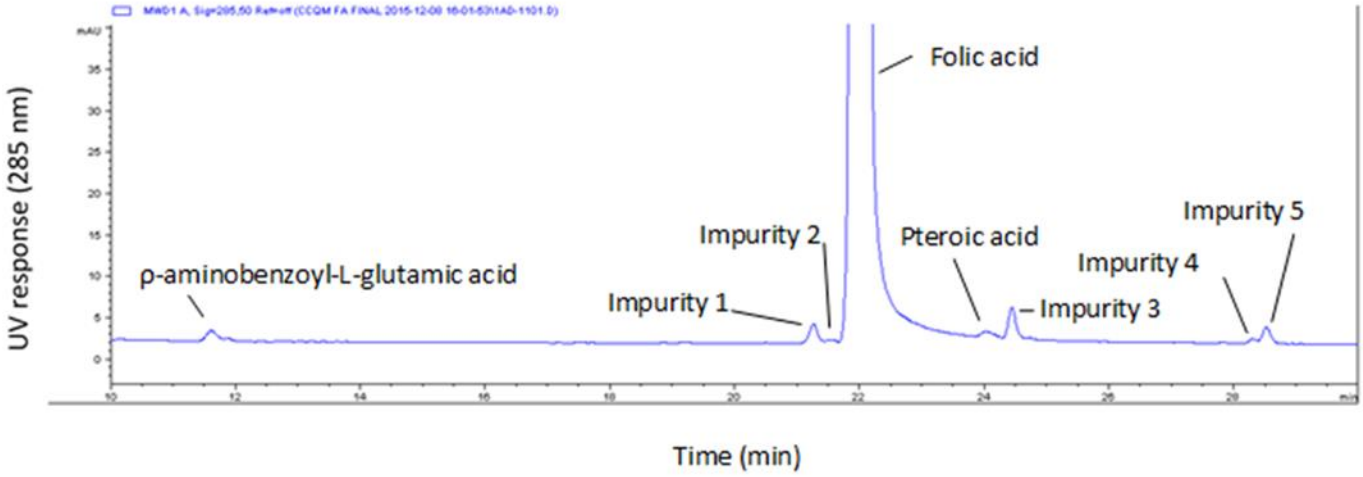


LGC (LC-UV @ 284 nm)

(b) HPLC-PDA of the sample at 290 nm



Example LC-UV chromatogram of CCQM 55.d folic acid



Annex D: DoE Tables and Plots for Impurity Category KCRVs

Degree of equivalence (D_i) for related structure impurity assignment.

Participant	D_i (mg/g)	U_D (mg/g)	U_{rel} (%)
CENAM	-12.17	6.28	-96.6%
NMIJ	-5.37	6.28	-42.6%
HSA	-4.90	6.31	-38.8%
BIPM	-2.24	6.52	-17.7%
NMISA	-2.11	6.87	-16.7%
UME	-1.30	6.29	-10.3%
KRISS	-1.01	6.29	-8.0%
GLHK	-0.74	6.76	-5.8%
VNIIM	-0.41	6.96	-3.2%
LGC	1.19	7.44	9.5%
NIM	2.30	10.18	18.3%
NIST	2.59	7.55	20.6%
NMIA	3.19	6.59	25.3%
NIMT	4.08	6.28	32.4%
INMETRO	4.69	10.17	37.2%

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

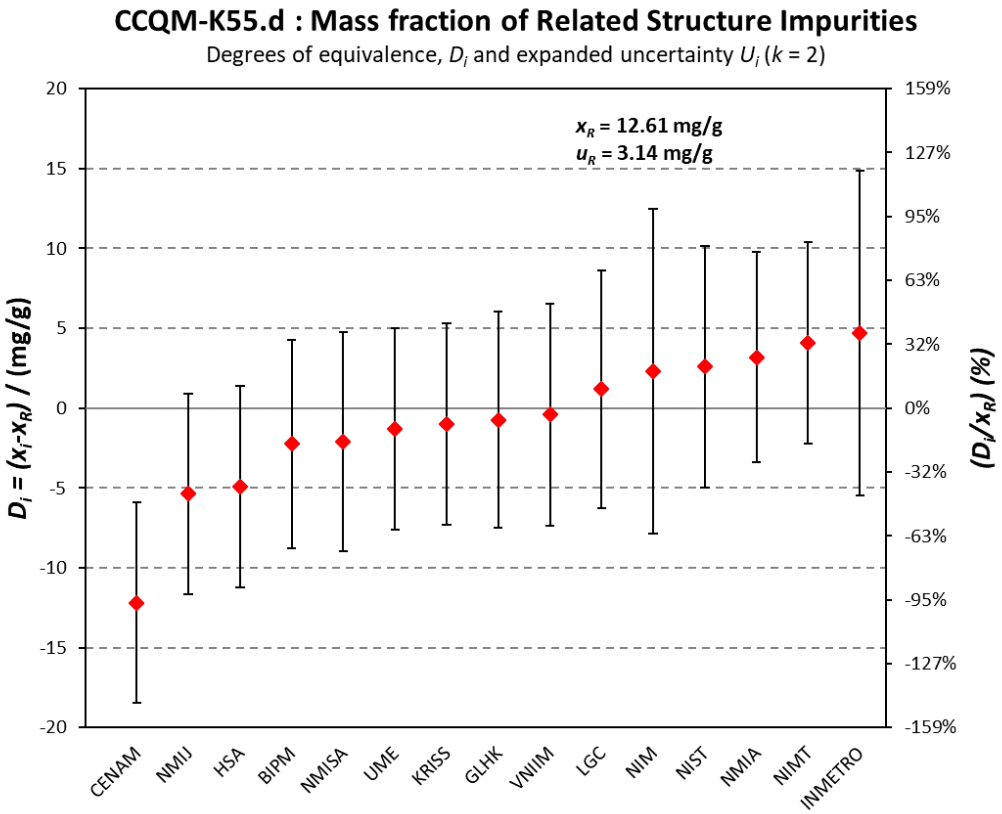


Figure 16: DoE Plot for total related structure impurities in CCQM-K55.d

Degree of equivalence (D_i) for water content.

Participant	D_i (mg/g)	U_D (mg/g)	U_{rel} (%)
CENAM	-13.57	10.84	-17.4%
NIMT	-5.97	10.80	-7.7%
UME	-2.75	4.57	-3.5%
VNIIM	-2.36	3.36	-3.0%
KRISS	-1.91	3.29	-2.5%
NIST	-0.97	3.28	-1.2%
BAM	-0.47	3.29	-0.6%
NRC	-0.47	4.05	-0.6%
GLHK	0.43	4.00	0.5%
INMETRO	0.53	3.45	0.7%
NIM	0.73	7.49	0.9%
NMIA	1.03	5.16	1.3%
NMISA	1.03	5.32	1.3%
BIPM	1.33	5.01	1.7%
LGC	1.55	4.58	2.0%
NMIJ	1.84	4.24	2.4%
HSA	3.11	5.07	4.0%
SIRIM	3.34	5.75	4.3%

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

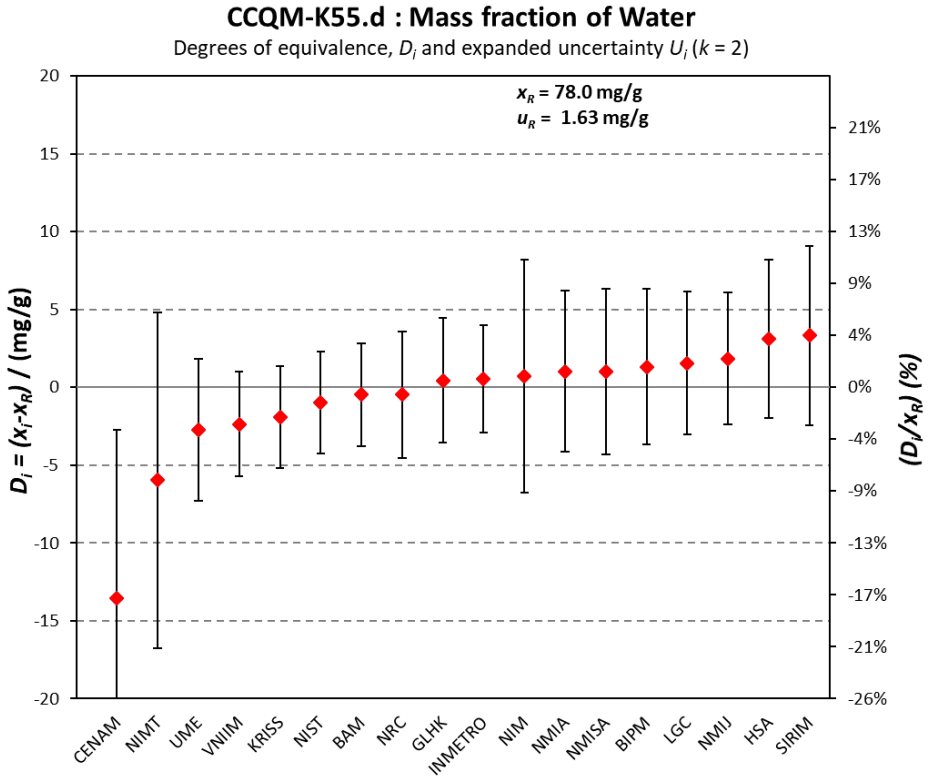


Figure 17: DoE Plot for water content in CCQM-K55.d

Degree of equivalence (D_i) for volatile organics content.

Participant	D_i (mg/g)	U_D (mg/g)
BAM	-0.37	0.17
BIPM	-0.37	0.34
CENAM	-0.37	0.17
KRISS	-0.37	0.23
NIMT	-0.37	0.17
NIM	-0.36	0.17
NIST	-0.36	0.17
GLHK	-0.27	0.17
UME	0.13	0.17
VNIIM	-0.33	0.17
NMIJ	-0.32	0.18
NRC	-0.08	0.18
LGC	0.00	0.57
HSA	0.00	0.35
INMETRO	0.09	0.17
NMISA	0.13	0.29
NMIA	0.53	0.74

Table 15: Degrees of equivalence (D_i) and U_D for volatile organics content

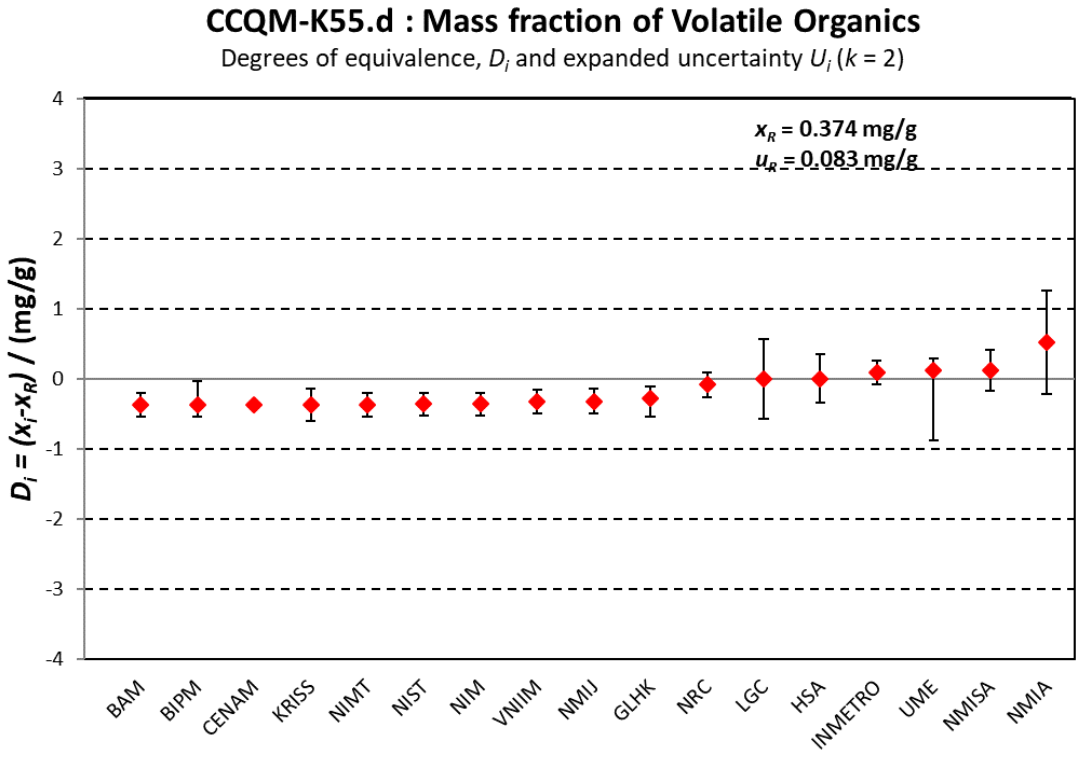


Figure 18: DoE Plot for residual solvent content

Degree of equivalence (D_i) for total non-volatiles content.

Participant	D_i (mg/g)	U_D (mg/g)
HSA	-0.31	2.91
NIMT	-0.31	1.12
UME	0.70	0.41
NMIA	1.70	0.41
VNIIM	-0.23	0.41
GLHK	-0.14	2.04
CENAM	-0.13	0.41
INMETRO	-0.09	0.41
NIST	-0.04	0.42
NRC	-0.03	0.74
KRISS	0.03	0.46
NMIJ	0.04	0.95
BIPM	0.10	0.57
LGC	0.12	0.46
NIM	0.88	1.25
NMISA	2.90	1.25

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

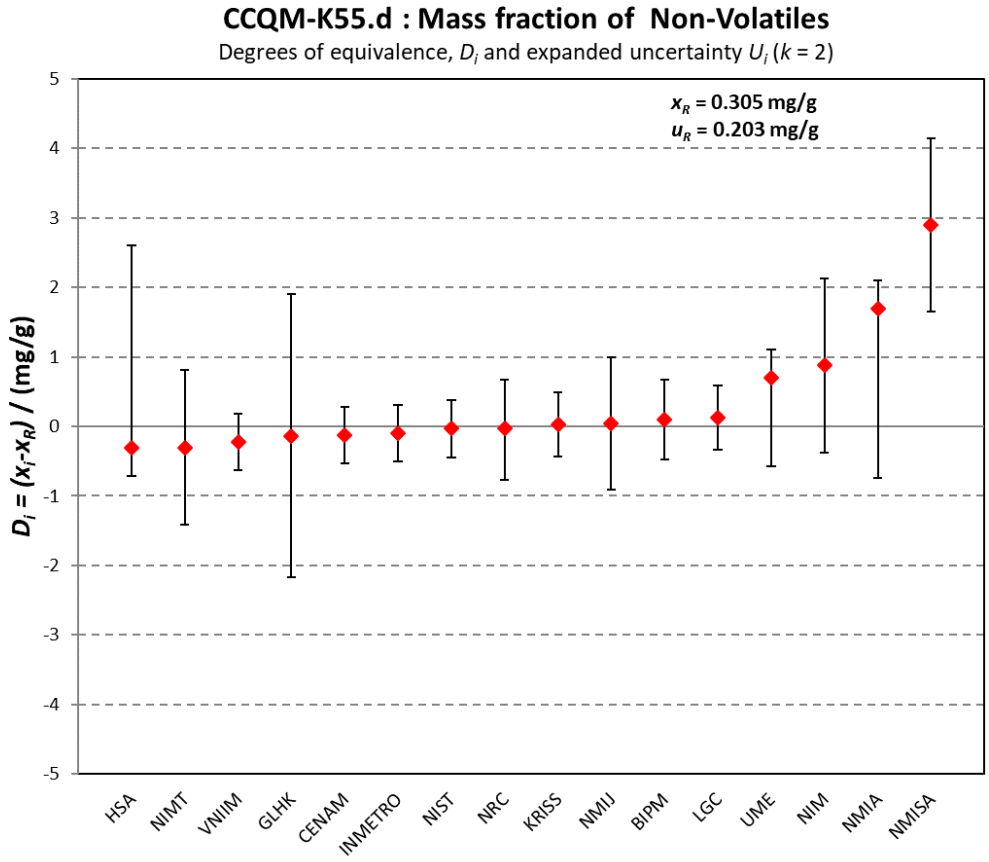


Figure 19: DoE Plot for non-volatile solvent content in CCQM-K55.d