

An international comparison of mass fraction purity assignment of theophylline: The Comité Consultatif pour la Quantité de Matière (CCQM) Pilot Study CCQM-P20.e (Theophylline)

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Abstract

Under the auspices of the Organic Analysis Working Group (OAWG) of the Comité Consultatif pour la Quantité de Matière (CCQM) a laboratory comparison, CCQM-P20.e, was co-ordinated by the Bureau International de Poids et Mesures (BIPM) in 2006/2007. Nine national measurement institutes, two expert laboratories and the BIPM participated in the comparison. Participants were required to assign the mass fraction of theophylline present as the main component in two separate study samples (CCQM-P20.e.1 and CCQM-P20.e.2).

CCQM-P20.e.1 consisted of a high-purity theophylline material obtained from a commercial supplier. CCQM-P20.e.2 consisted of theophylline to which known amounts of the related structure compounds theobromine and caffeine were added in a homogenous, gravimetrically controlled fashion. For the CCQM-P20.e.2 sample it was possible to estimate gravimetric reference values both for the main component and for the two spiked impurities.

In addition to assigning the mass fraction content of theophylline for both materials, participants were requested but not obliged to provide mass fraction estimates for the minor components they identified in each sample.

The results reported by the study participants for the mass fraction content of theophylline in both materials showed good levels of agreement both with each other and with the gravimetric reference value assigned to the CCQM-P20.e.2 material. There was also satisfactory agreement overall, albeit at higher levels of uncertainty, in the quantification data reported for the minor components present in both samples. In the few cases where a significant deviation was observed from the consensus values reported by the comparison participants or gravimetric reference values where these were available, they appeared to arise from the use of non-optimal chromatographic separation conditions.

The results demonstrate the feasibility for laboratories to assign mass fraction content with associated absolute expanded uncertainties in the range 0.05-0.5 % for solid organic compounds of high purity (mass fraction of main component > 995 mg/g) and in the range 0.1-1 % for compounds of lower purity (mass fraction of main component > 980 mg/g).

Participants:

Institute	Acronym	Country
National Measurement Institute, Australia	NMIA	Australia
National Institute of Metrology	NIM	China
Federal Institute for Materials Research and Testing	BAM	Germany
National Metrology Institute of Japan	NMIJ	Japan
Centro Nacional de Metrologia	CENAM	Mexico
CSIR National Metrology Laboratory	CSIR	South Africa
National Institute of Metrology (Thailand)	NIMT	Thailand
Department of Medical Science, Ministry of Health, Thailand	DMSc	Thailand
LGC Ltd	LGC	United Kingdom
National Institute of Standards and Technology	NIST	USA
United States Pharmacopeia – Reference Standards Laboratory	USP	USA
Bureau International des Poids et Mesures	BIPM	

All the participating institutes submitted results for the study

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INTRODUCTION

The OAWG meeting at IRMM in September 2005 accepted the proposal by the BIPM to coordinate, in collaboration with LGC, the continuation of the CCQM-P20 study investigating the characterization of organic substances for chemical purity. For this round, designated CCQM-P20.e, two samples CCQM-P20.e.1 and CCQM-P20.e.2 were prepared. LGC prepared CCQM-P20.e.1, a relatively high purity sample of theophylline, and the BIPM prepared CCQM-P20.e.2, which consisted of high purity theophylline spiked with the structurally related compounds theobromine and caffeine. The BIPM subsequently characterized both materials and distributed them to the study participants. The mass fraction content of theophylline was greater than 975 mg/g for both the “pure” and “spiked” samples. The results reported by the study participants are the subject of this report.

The CCQM-P20 study was undertaken initially for National Metrology Institutes interested in the assessment of the purity of organic compounds and followed on from the earlier CCQM-P5 (previously known as CCQM-6) study on the same topic. Its purpose is to investigate current practice for the assignment of chemical purity to an organic compound intended for use as a primary standard or for the preparation of primary calibration solutions. The expected outcome of the study is to evaluate the scope, applicability, limitations and appropriateness of the various approaches and techniques used to assign purity property values to organic materials through a series of strategically planned exercises and, eventually, to produce a guidance document recommending appropriate experimental designs for the purity assessment of a given organic compound or class of compounds.

The study and its planned continuation into a key comparison will allow participating National Metrology Institutes to demonstrate capabilities for purity assessment where these are relevant to their existing or planned Calibration and Measurement Capability claims.

In previous rounds purity assignments were undertaken on tributyltin chloride (CCQM-P20.a),¹ xylene (CCQM-P20.b), atrazine (CCQM-P20.c)² and chlorpyrifos (CCQM-P20.d)³.

Background to Theophylline

Theophylline was selected as a suitable compound for the continuation of the CCQM-P20 series because it:

- was anticipated to permit study by a variety of analytical techniques
- could be provided in sufficient amounts to permit detailed investigation
- is stable and relatively non-toxic so transport, storage and safety were not anticipated to pose major difficulties
- is of specific interest within the framework of ongoing activities of the Joint Committee on Traceability in Laboratory Medicine (JCTLM)

The structure of theophylline (IUPAC name 3,7-Dihydro-1,3-dimethyl-1*H*-purine-2,6-dione; CAS Registry Number 58-55-9) and of a number of related structure compounds referred to in this report are shown in Figure 1. These compounds are all formally derived from methylation of the parent heterocyclic ring system xanthine. The numbering scheme for the xanthine ring system is also shown in Figure 1.

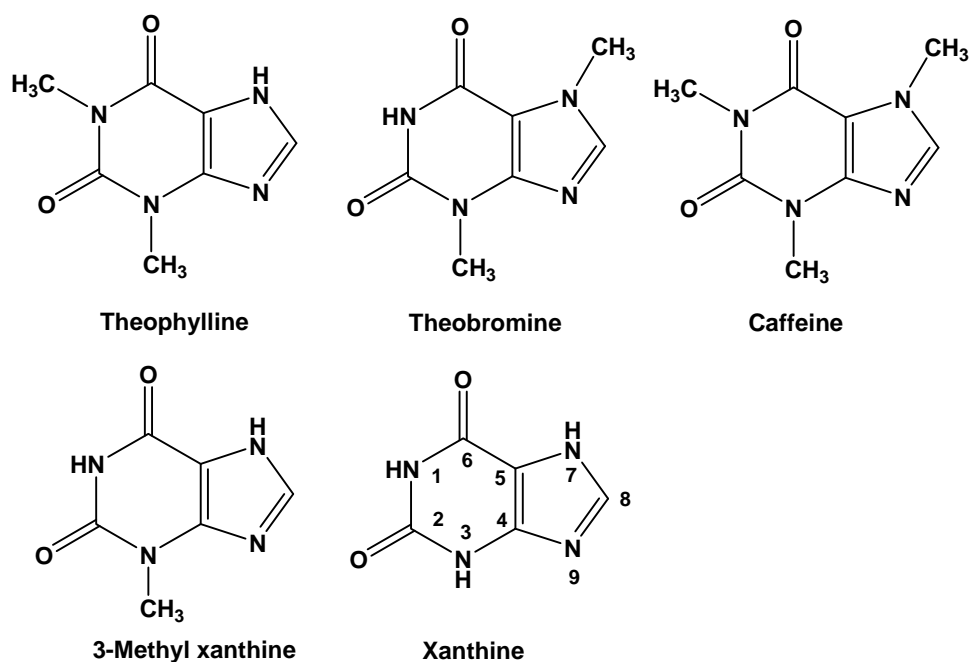


Figure 1 – Structures of theophylline and related compounds

Theophylline is a white crystalline powder with a reported melting point range of 270-274 °C. It has limited solubility in water, ethanol and chloroform, is readily soluble in dilute acid solutions but is only sparingly soluble in non-polar organic solvents. It is a widely used medication for treatment of a variety of respiratory diseases including asthma, despite having a relatively narrow therapeutic index. As a result of its widespread use and narrow efficacy range it is an important and widely used analyte for clinical testing and in medical laboratory proficiency test programmes. No pure substance certified reference material (CRM) is currently available for theophylline.

PRODUCTION AND DISTRIBUTION OF CANDIDATE MATERIALS

The candidate material for CCQM-P20.e.1 was prepared by the Reference Materials section of the Chemical Calibration facility of LGC Ltd. The source material was theophylline purchased from a commercial supplier which was used without further purification. The candidate material in the form of 200 sealed vials each containing 1 g of theophylline was supplied to the BIPM for characterization.

The candidate material for CCQM-P20.e.2 was prepared by the BIPM. The source materials were high purity theophylline, theobromine and caffeine obtained from a commercial supplier and used without further purification. In the context of the impurity profiles subsequently reported for both materials, it should be noted that the theophylline used to prepare both CCQM-P20.e.1 and CCQM-P20.e.2 was sourced from the same supplier and had the same product and batch number, although different individual units of the product were used in the production of the two study samples.

It required several attempts to develop a mixing protocol that afforded a final product with a suitable level of homogeneity for the incurred compounds. The successful protocol involved weighing each of the three source materials into a bulk container, and carrying out a preliminary mixing by simple rotation of the bulk material container. The bulk was divided into three approximately equal portions and each

portion was ground in a planetary ball mill using an agate sample jar and grinding balls. Finally the three milled portions were recombined and remixed by horizontal rotation prior to sub-division and sealing into 200 vials each containing 1 g of the bulk mixed material

Homogeneity study

The homogeneity of the theophylline content of both materials was estimated indirectly from assessment of the homogeneity of the minor components present in each study material.

1. CCQM-P20.e.1

Two UV-active minor components, 3-methyl xanthine and an unidentified UV-active component were inherently present in the CCQM-P20.e.1 candidate material. The homogeneity of each was determined using a LC-UV method and assessment by ANOVA. The uncertainty contributions due to between unit inhomogeneity ($u(bb)$) were not greater than 0.005 mg/g (2.7 % relative) for 3-methylxanthine and 0.005 mg/g (1.6 % relative) for the unidentified UV-active impurity when a 2.5 mg sample of CCQM-P20.e.1 was analysed. The study results are summarised in Table 1.

Table 1: Homogeneity study of UV-active impurities in CCQM-P20.e.1 (2.5 mg sample)

	3-Methylxanthine	Unidentified UV-active impurity
N	10	10
s_{wb}	5.7 %	3.5 %
s_{bb}	not calculable ($MS_{\text{between}} < MS_{\text{within}}$)	not calculable ($MS_{\text{between}} < MS_{\text{within}}$)
$u^*_{rel\ bb}$	2.7 %	1.6 %
$u_{rel\ bb}$	2.7 %	1.6 %
x_i (mg/g)	0.20	0.33
$u(bb_i)$ (mg/g)	0.005	0.005
F	0.73	0.85
F_{crit}	3.02	3.02

⁽¹⁾ Higher value of $u^*_{rel\ bb}$ or s_{bb} taken as $u_{rel\ bb}$, uncertainty estimate due to inhomogeneity ⁴

Based on the degree of homogeneity of the main impurities it was concluded that, provided a minimum sample intake per analysis of 2.5 mg was used, the homogeneity of theophylline in the CCQM-P20.e.1 candidate material was suitable for the intended purpose of the comparison.

2 CCQM-P20.e.2

The CCQM-P20.e.2 study material was spiked with theobromine and caffeine and needed to be prepared and homogenised using a rigorous process requiring the production of several trial batches before a candidate material was obtained which showed a suitable level of homogeneity for the incurred components. When individual analyses were undertaken on 25 mg samples the standard uncertainty contributions due to between unit inhomogeneity ($u(bb)$) of the spiked impurities were not greater than 0.44 mg/g (4.8 % relative) for theobromine and 0.34 mg/g (6.9 % relative) for caffeine. The $u(bb)$ for the spiked impurities was unacceptably large if a smaller sample size was tested.

It was concluded that, provided a minimum sample intake per analysis of 25 mg was used, the homogeneity of the CCQM-P20.e.2 candidate material was suitable both with respect to the main component, theophylline, and also the spiked impurities. The results for the homogeneity of the spiked impurities in CCQM-P20.e.2 are summarised in Table 2.

Table 2: Homogeneity study of spiked impurities in CCQM-P20.e.2 (25 mg sample)

	Theobromine	Caffeine
N	10	10
s_{wb}	7.3 %	14.5 %
s_{bb}	4.8 %	not calculable ($MS_{\text{between}} < MS_{\text{within}}$)
$u^*_{rel\ bb}$	3.4 %	6.9 %
$u_{rel\ bb}^{(1)}$	4.8 %	6.9 %
x_i (mg/g)	9.3	4.9
$u(bb_i)$ (mg/g)	0.44	0.34
F	1.87	0.46
F_{crit}	3.02	3.02

⁽¹⁾ Higher value of $u^*_{rel\ bb}$ or s_{bb} taken as $u_{rel\ bb}$, uncertainty estimate due to inhomogeneity ⁴

The inherent minor impurities (3-methyl xanthine and an unidentified impurity) were present at similar levels and with a similar degree of homogeneity in CCQM-P20.e.2 as was found for the CCQM-P20.e.1 (see Table 1). They are not included in Table 2 because at the low absolute level at which they occur and higher degree of homogeneity they display relative to the spiked impurities they make a negligible contribution to the inhomogeneity of theophylline in the sample.

No trends in homogeneity due to filling order or analysis order were observed for either material.

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. Study sets were stored at the selected temperatures over eight weeks in total and units were transferred to reference temperature storage at two week intervals. Trend analysis of the HPLC data revealed no significant change in the relative composition of theophylline or of the minor UV-active components at any of the test temperatures.

The effect of temperature on water content as measured by Karl Fischer titration was also investigated. No significant changes were observed during storage at 4 °C. For the CCQM-P20.e.2 sample there was evidence of a minor reduction in water content on prolonged storage at ambient temperature and clear evidence of water loss on storage at 40 °C. It was concluded that the composition of the material was stable both for short-term transport and use at ambient temperature, provided the sample was not exposed to temperatures in excess of 40 °C, and for long term storage at 4 °C.

Gravimetric Reference Values for CCQM-P20.e.2

The equation used for the gravimetric assignment of the content of theophylline (TP), theobromine (TB) or caffeine (CA) in the CCQM-P20.e.2 comparison material at the recommended sampling size of 25 mg is:

$$W_{Grav\ XA} = H_{XA} * S_{XA} * \frac{m_{XA}}{m_{P20.e.2}} \quad (\text{Eqn. 1})$$

XA = Xanthine component (theophylline or spiked impurity) of CCQM-P20.e.2
 = Theophylline (TP) **or** Theobromine (TB) **or** Caffeine (CA)

$W_{Grav\ XA}$ = mass fraction of a given xanthine component in a 25 mg sample of CCQM-P20.e.2 calculated from gravimetric operations

H_{XA} = correction factor (assigned a value of 1.0 with an associated uncertainty) for the between-sample (intra- and inter-unit) homogeneity of the xanthine component in a 25 mg sample of CCQM-P20.e.2.

S_{XA} = correction factor (assigned a value of 1.0 with associated uncertainty) for the short-term stability of the xanthine component in CCQM-P20.e.2 under the recommended storage conditions.

$m_{P20.e.2}$ = mass of the production batch of CCQM-P20.e.2 candidate material
 = $z_{TP} + z_{TB} + z_{CA}$

where:

z_{TP} = mass of source material of theophylline used to prepare CCQM-P20.e.2

z_{TB} = mass of source material of theobromine used to prepare CCQM-P20.e.2

z_{CA} = mass of source material of caffeine used to prepare CCQM-P20.e.2

m_{XA} = mass of a given xanthine component in CCQM-P20.e.2

m_{TP} = mass of theophylline in CCQM-P20.e.2

$$= W_{TP,TP} * z_{TP} + W_{TP,TB} * z_{TB} + W_{TP,CA} * z_{CA}$$

m_{TB} = mass of theobromine in CCQM-P20.e.2

$$= W_{TB,TP} * z_{TP} + W_{TB,TB} * z_{TB} + W_{TB,CA} * z_{CA}$$

m_{CA} = mass of caffeine in CCQM-P20.e.2

$$= W_{CA,TP} * z_{TP} + W_{CA,TB} * z_{TB} + W_{CA,CA} * z_{CA}$$

where:

$W_{XA,TP}$ = mass fraction of xanthine component in source material of theophylline

$W_{XA,TB}$ = mass fraction of xanthine component in source material of theobromine

$W_{XA,CA}$ = mass fraction of xanthine component in source material of caffeine

Uncertainty Budget for Gravimetric Reference Values in CCQM-P20.e.2

The equation used to calculate the standard uncertainty of the mass fraction content of theophylline (TP), theobromine (TB) or caffeine (CA) in CCQM-P20.e.2 when the mass fraction is calculated using Eqn 1 is:

$$u_{Grav\,XA} = W_{Grav\,XA} * \sqrt{\left(\frac{u(H_{XA})}{H_{XA}}\right)^2 + \left(\frac{u(S_{XA})}{S_{XA}}\right)^2 + \left(\frac{u(m_{XA})}{m_{XA}}\right)^2 + \left(\frac{u(m_{P20.e.2})}{m_{P20.e.2}}\right)^2} \quad (\text{Eqn. 2})$$

The steps followed for the preparation of CCQM-P20.e.2 and their general contribution to the uncertainty budget for both theophylline and the spiked impurities are shown schematically in the following flow diagram (Figure 2).

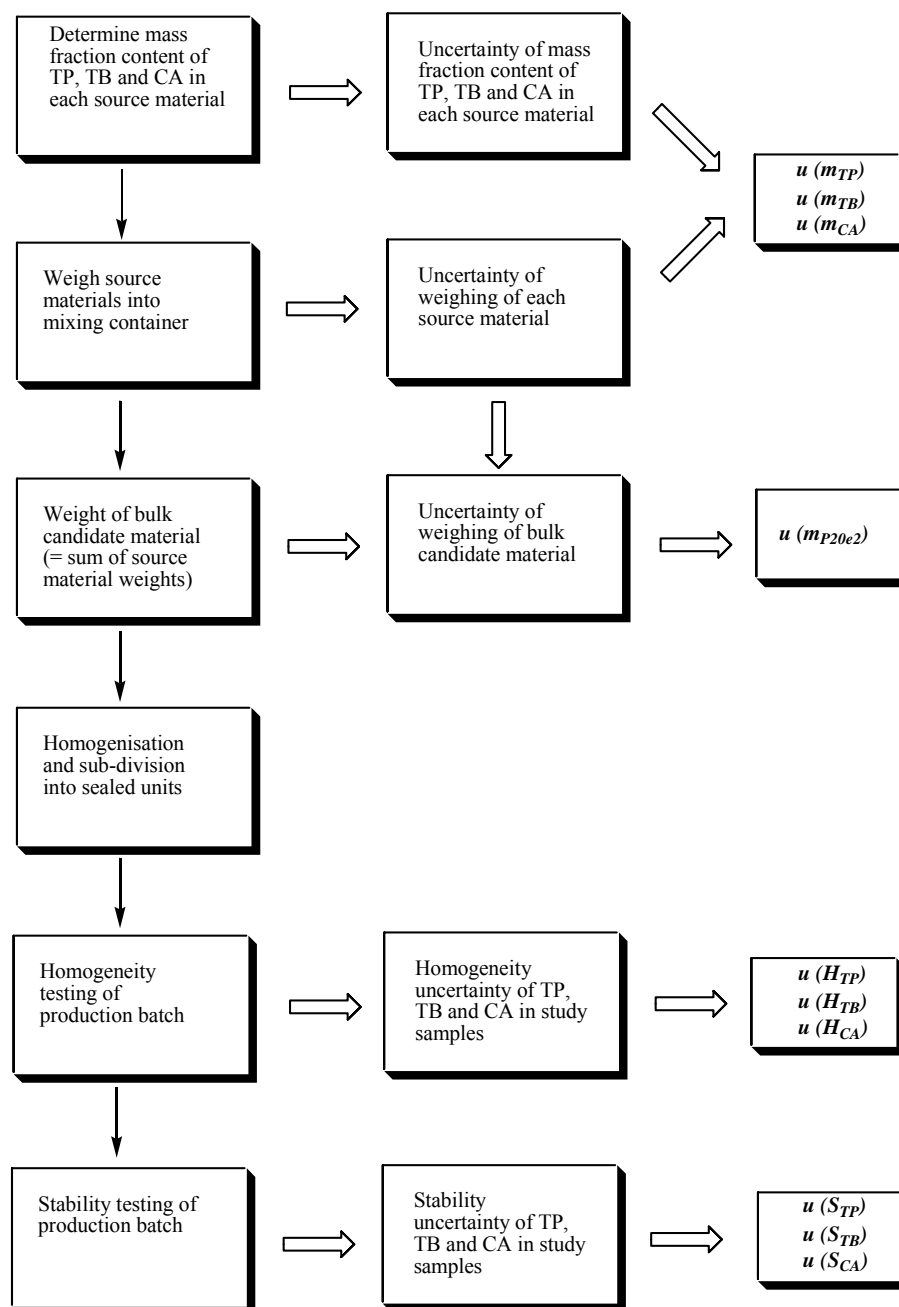


Figure 2 – CCQM-P20.e.2 material production process and MU contribution

- **Uncertainty of mass of xanthine components in CCQM-P20.e.2 ($u(m_{XA})$)**

The mass fraction of each xanthine component in each source material ($w_{XA, TP}$, $w_{XA, TB}$, $w_{XA, CA}$) was determined from the relative LC-UV peak area response of each compound in each material after correction for response factors and moisture content. The uncertainties in these estimates were calculated by combination of the repeatability of the LC-UV data with uncertainties for the response factor for each component and for the moisture content of each source material.

The uncertainty of the mass of each source material ($u(z_{TP})$, $u(z_{TB})$, $u(z_{CA})$) combined to produce the bulk candidate material for CCQM-P20.e.2 was calculated using the equation:

$$u(z_{XA}) = \sqrt{(s_{rep})^2 + \frac{2}{3}(s_{NL})^2 + \frac{1}{3}z_{XA}^2 \left[(s_{CAL})^2 + \frac{1}{3}(s_{TS} * d)^2 \right]} \quad (\text{Eqn. 3})$$

where:

s_{Rep} = balance repeatability, s_{NL} = non-linearity correction,

s_{CAL} = calibration mass tolerance and $s_{ts} * d$ = temperature sensitivity correction).

The manufacturer's recommended values for a Mettler AX504 balance were used.

The observed mass readings for each source material were buoyancy corrected but the uncertainty of the buoyancy correction was not taken into account in the uncertainty budget as it did not materially affect the result.

The overall uncertainty $u(m_{XA})$ was obtained by appropriate combination of the three mass fraction estimate uncertainties and the three individual weighing uncertainties.

- **Uncertainty of total mass of CCQM-P20.e.2 bulk material ($u(m_{P20e2})$)**

Three individual weighing operations were summed to give the mass of the combined CCQM-P20.e.2 material. The uncertainty of the mass of the candidate material ($u(m_{P20e2})$) was calculated by combination of the uncertainties of the individual weighing operations, which in turn were calculated using Eqn. 3.

$$u(m_{P20e2}) = \sqrt{(u(z_{TP}))^2 + (u(z_{TB}))^2 + (u(z_{CA}))^2}$$

- **Uncertainty of stability of xanthines in CCQM-P20.e.2 ($u(S_{XA})$)**

An isochronous stability study demonstrated that the uncertainty contribution to the gravimetric value arising from potential instability of the material was negligible and could be ignored. The assigned value for S_{XA} in eqn. 1 above was 1.0 in each case and the associated standard uncertainty in S_{XA} made no significant contribution to the overall uncertainty in the reference value.

- **Uncertainty of homogeneity of xanthines in CCQM-P20.e.2 ($u(H_{XA})$)**

The homogeneity factor for each xanthine component (H_{XA}) was assigned a value of 1.0 with an associated standard uncertainty ($u(H_{XA})$).

For theophylline in CCQM-P20.e.2 this uncertainty could not be derived directly from the homogeneity data, as ANOVA was unable to differentiate with sufficient precision between contributions due to the method repeatability and the true inhomogeneity of theophylline in the sample. Instead the uncertainty in the absolute level of the inhomogeneity of the main component was estimated by combination of the absolute standard uncertainty due to inhomogeneity of the main impurities at the recommended sampling level. Conversion of this absolute estimate into a value relative to the mass fraction estimate for theophylline in CCQM-P20.e.2 gave the estimate of the standard uncertainty in the homogeneity factor for theophylline.

The estimates for the uncertainty in the homogeneity of the main UV-active impurities in a 25 mg sample of CCQM-P20.e.2 are given in Table 2. An additional estimate for the absolute inhomogeneity of the sample moisture content, the only other impurity present at > 1 mg/g in CCQM-P20.e.2, is included in the overall calculation.

Combination gives the inhomogeneity uncertainty estimate for theophylline as:

$$\begin{aligned}
 u(bb_{TP}) &= \sqrt{(u(bb_{TB}))^2 + (u(bb_{CA}))^2 + (u(bb_{H_2O}))^2} \\
 &= \sqrt{(0.44)^2 + (0.34)^2 + (0.22)^2} \\
 &= 0.61 \text{ mg/g}
 \end{aligned}$$

The standard uncertainty in the homogeneity factor ($u(H_{TP})$) was calculated from:

$$\begin{aligned}
 u(H_{TP}) &= u_{rel}(bb_{TP}) \\
 &= \frac{u(bb_{TP})}{w_{GravTP}} \\
 &= 0.0006
 \end{aligned}$$

For theobromine and caffeine the $u_{rel}(bb)$ estimated from the homogeneity test data (Table 2) was used directly as the estimate for the standard uncertainty in the assigned value of 1.0 for the homogeneity factor for each spiked impurity.

Summary

The gravimetric reference values and the associated uncertainty budgets calculated from Equation 1 and Equation 2 for theophylline, theobromine and caffeine in CCQM-P20.e.2 are summarised below.

1. Theophylline

Uncertainty budget for reference value of theophylline in CCQM-P20.e.2

Uncertainty component	x_i	$u(x_i)$	Percent contribution
Inhomogeneity factor	1.0	0.0006	67
Stability factor	1.0	negligible	negligible
Mass of theophylline in candidate material (g)	199.07	0.086	33
Mass of candidate material (g)	202.50	0.001	negligible
Theophylline content (mg/g)	983.1	0.73	
Expanded uncertainty U (C.I.95%, k = 2) (mg/g)		1.5	

2. Theobromine

Uncertainty budget for reference value of theobromine in CCQM-P20.e.2

Uncertainty component	x_i	$u(x_i)$	Percent contribution
Inhomogeneity factor	1.0	0.048	99
Stability factor	1.0	negligible	negligible
Mass of theobromine in candidate material (g)	1.88	0.006	1
Mass of candidate material (g)	202.50	0.001	negligible
Theobromine content (mg/g)	9.28	0.45	
Expanded uncertainty U (C.I.95%, k = 2) (mg/g)		0.89	

3. Caffeine

Uncertainty budget for reference value of caffeine in CCQM-P20.e.2

Uncertainty component	x_i	$u(x_i)$	Percent contribution
Inhomogeneity factor	1.0	0.069	96
Stability factor	1.0	negligible	negligible
Mass of caffeine in candidate material (g)	1.00	0.014	4
Mass of candidate material (g)	202.50	0.001	negligible
Caffeine content (mg/g)	4.94	0.35	
Expanded uncertainty U (C.I.95%, k = 2) (mg/g)		0.69	

Sample distribution

One unit of each of the study samples, each containing a minimum of 1 g of material, were distributed to each participant. Participants were asked to sign and return a form acknowledging receipt of the samples and to advise the coordinator if any damage had occurred to the container or the vials containing the study samples. Recipients were also 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.

Quantities and Units

Participants were required to report the mass fraction of the major component, theophylline, in both materials. It was recognized that individual measurement methods could provide purity estimates expressed as related quantities (e.g. amount of substance fraction), but in this case participants were required to correct these results into a mass fraction estimate.

The units for reporting the mass fraction content of theophylline were mg/g.

Participants were also encouraged to provide mass fraction estimates for the minor components of the materials. The ability to identify and quantify minor components is regarded as an important competency for the high-level characterization of organic materials.

Recommended Minimum Sample Size

Investigations at BIPM of the homogeneity of theophylline (CCQM-P20.e.1 and CCQM-P20.e.2) and the spiked impurities (CCQM-P20.e.2) (see discussion above) identified a minimum sampling size for each material which reduced to an acceptable level the effect of within bottle and between bottle inhomogeneity. The recommended minimum sample size was 2.5 mg per analysis replicate for material CCQM-P20.e.1 and 25 mg for material CCQM-P20.e.2. Compliance with this recommendation was important for participants using a summation of impurities (100 - x) method to determine theophylline content, or who wished to quantify the mass fractions of the minor components of each material.

Instructions for Use

Each participant received one vial of each of the study samples. The materials were provided in amber glass crimp sealed vials fitted with an inert stopper.

Participants were asked to apply the methods and procedures they would use in their laboratory to obtain an estimate of the mass fraction content of the material and (optionally) the mass fraction content of the minor components.

RESULTS

Mass Fraction of Theophylline in CCQM-P20.e.1

BAM reported two results, one by a summation of impurities approach based on LC-UV analysis and the other by quantitative ¹H NMR (QNMR). The BAM NMR group identified a significant calculation error affecting both their QNMR estimates subsequent to their initial submission of results. Only their corrected value is given.

USP reported results for the materials using both the USP Theophylline Assay (“direct assay” method) and the USP Theophylline Related substances assay (“related substances” method).

The estimates reported by study participants for the theophylline mass fraction content of CCQM-P20.e.1 with some summary statistics are reported in Table 3 and plotted in Figure 3. The statistics were calculated using the revised BAM QNMR result and excluding both the CSIR result which was biased due to overestimation of the moisture content and the USP “direct assay” result. In addition to the raw mean and median the weighted mean of the reported values was calculated along with its 95% confidence range⁵. The weighted mean values are shown in red in Figure 3.

Where participants reported expanded uncertainty ranges extending above 1000 mg/g they have been truncated at the physical constraint in Figure 3.

Table 3 – Theophylline content estimates for CCQM-P20.e.1

Institute	Theophylline content (mg/g)	Expanded Uncertainty at 95 % confidence (mg/g)	Relative Expanded Uncertainty at 95 % confidence (%)
NIM	995	5.0	0.50
CSIR	997.3 ^a	0.6	0.06
BAM QNMR	998.3 ^b	2.6	0.26
CENAM	998.3	8.6	0.86
USP (Related Subst.)	998.64	1.47	0.15
NMIA	998.7	4.8	0.48
NIST	998.9	0.6 / 0.9 ^c	0.06 / 0.09 ^d
BAM (LC-UV)	999	1.0 / 2.0 ^c	0.10 / 0.20 ^d
NMIJ	999	1	0.10
BIPM	999.1	0.4	0.04
NIMT	999.2	2.5	0.25
DMSC	999.36	16.3	1.63
LGC	999.5	2.7	0.27
USP (Direct Assay)	999.8 ^a	7.6	0.76
Mean	998.6	0.75	
Weighted Mean ⁵	999.0	0.31	
Median	999.0		

- a. Data not included in calculation of summary statistics
- b. Corrected for a calculation error identified subsequent to original submission
- c. Asymmetric expanded uncertainty with upper / lower ranges reported
- d. Relative expanded uncertainty of upper / lower ranges reported

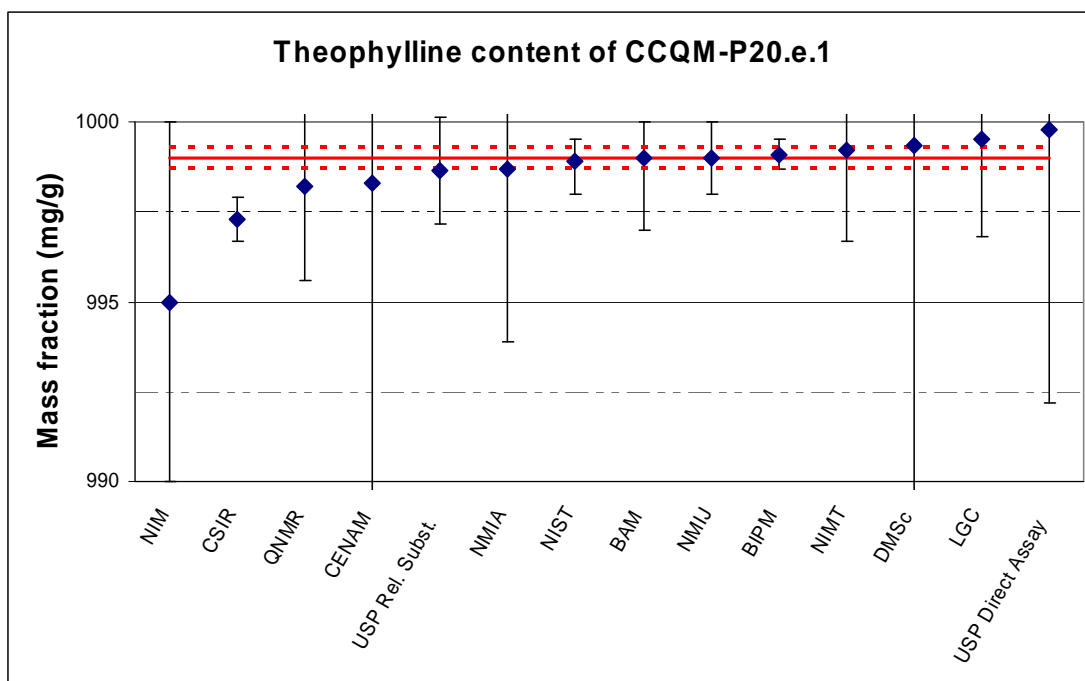


Figure 3 Mass fraction of Theophylline in CCQM-P20.e.1 material (Weighted mean with 95% confidence range in red)

Mass Fraction of Theophylline in CCQM-P20.e.2

The estimates reported by the study participants for the theophylline content of CCQM-P20.e.2 are shown in Table 4 and plotted in Figure 4. The weighted mean of the reported values was calculated along with the 95% confidence range.⁵

The results can be compared to a gravimetric reference value calculated, as described above, from the amounts of each source material (theophylline, theobromine and caffeine) used in its preparation. The gravimetric reference value for theophylline with its associated expanded uncertainty is shown in red in Figure 4 and the difference from the gravimetric reference value for each result ($D_i = w_i - w_{\text{grav TP}}$) is plotted in Figure 5. The expanded uncertainty U_i at the 95% confidence level of the difference from the reference value for each result was calculated as

$$U(D_i) = 2 * \sqrt{u(w_i)^2 + u(w_{\text{grav}})^2}$$

Table 4 – Theophylline estimates for CCQM-P20.e.2

Institute	Theophylline content (mg/g)	Expanded Uncertainty (<i>U</i>) at 95% confidence level (mg/g)	Relative Expanded Uncertainty at 95% confidence level (%)
CSIR	980.9	4.9	0.50
BAM QNMR	983.4 ^a	3.0	0.31
NIST	983.5	7.4 / 4.2 ^b	0.75 / 0.43 ^c
USP (Rel. Subst.)	983.5	1.38	0.14
NMIA	983.8	5.7	0.58
LGC	983.8	2.3	0.23
BIPM	983.8	1.1	0.11
CENAM	983.9	8	0.81
USP (Direct Assay)	984.1 ^d	7.8	0.79
NIM	985	5.0	0.51
DMS	985.5	26	2.64
NIMT	986.5	2.6	0.26
BAM (LC-UV)	987	6	0.61
NMIJ	990	4	0.40
Mean	984.7	1.3	
Weighted mean ⁵	984.1	0.76	
Median	983.8		
Reference Value (Gravimetric)	983.1	1.5	

- a. Corrected for a calculation error found subsequent to original submission**
- b. Asymmetric expanded uncertainty with upper / lower ranges shown**
- c. Relative expanded uncertainty of upper / lower ranges respectively**
- d. Result not included in calculation of summary statistics**

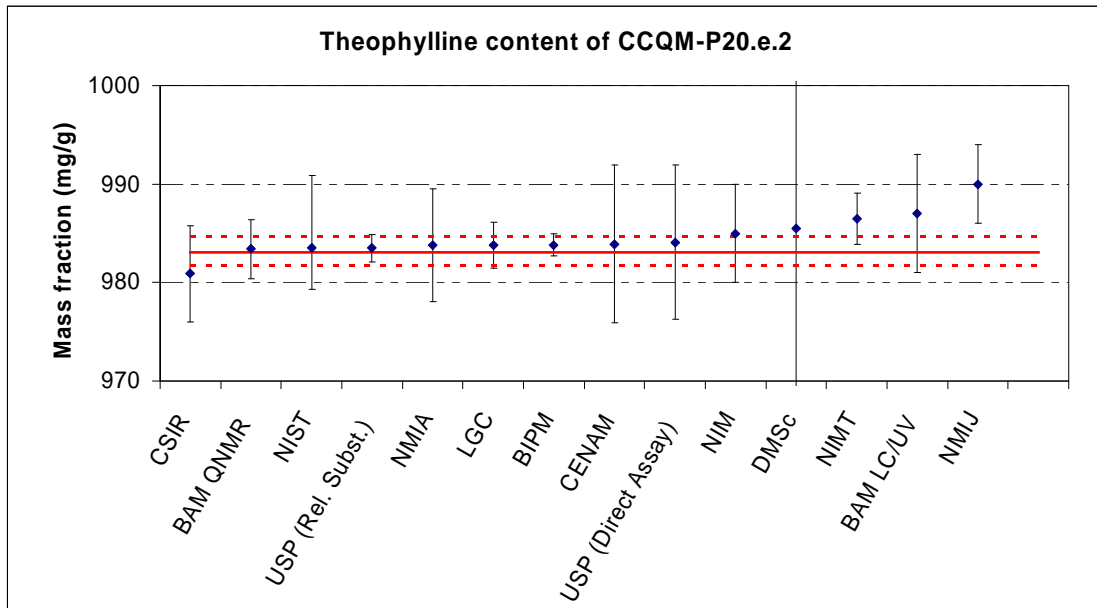


Figure 4 Reported mass fraction of theophylline in CCQM-P20.e.2 material (Gravimetric reference value and 95% expanded uncertainty plotted in red)

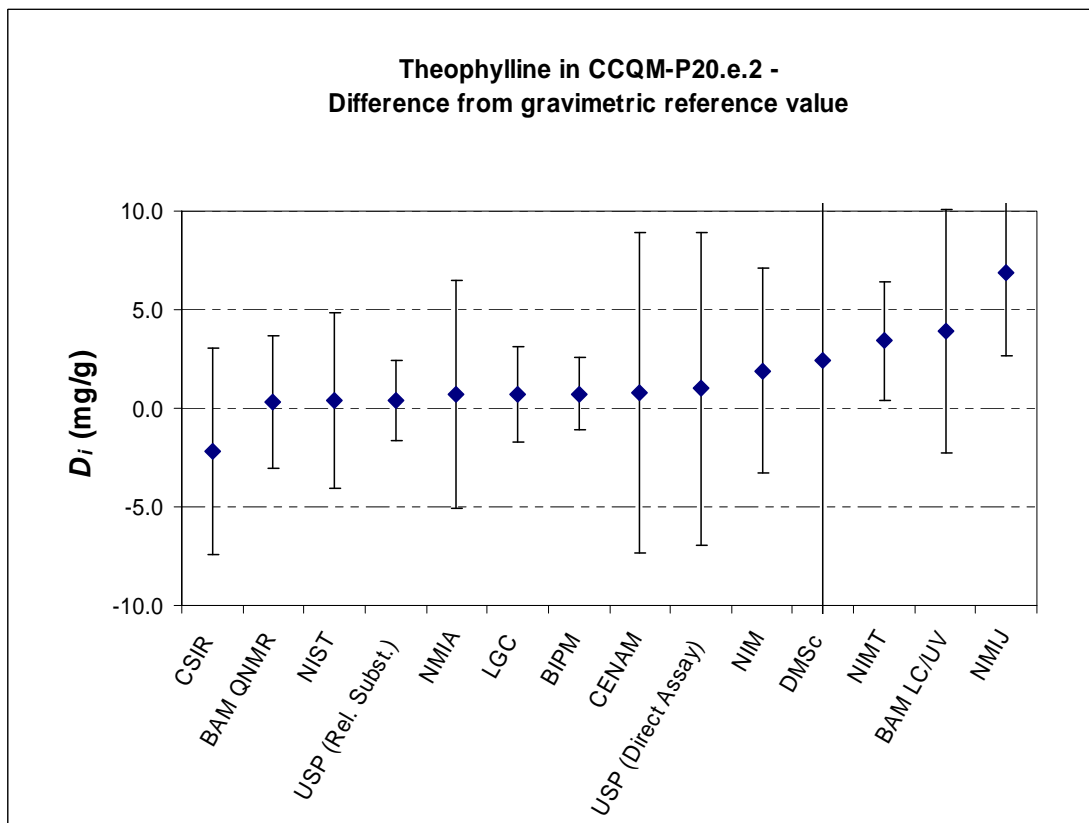


Figure 5 Difference of CCQM-P20.e.2 theophylline estimates from gravimetric reference value ($w_{\text{grav}} = 983.1 \pm 1.5$ mg/g)

Method Summaries and Measurement Equations

Lab ID	Measurement Equation	Method Summary
BAM (LC-UV)	$w_{theoph*} = w_{theoph} \cdot (1 - w_{water})$ <p> w_{theoph}^*: mass fraction in the sample w_{theoph}: mass fraction by normalised LC-UV w_{water}: mass fraction of water by Karl Fischer titration </p>	<p>From LC-UV peak area response at 245 nm Water content by Karl Fischer titration. Theobromine and caffeine content of CCQM-P20.e.2 checked by external calibration LC-UV</p>
BAM (QNMR)	$k_X = \frac{I_X}{I_{Std}} \cdot \frac{N_{Std}}{N_X} \cdot \frac{M_X}{M_{Std}} \cdot \frac{m_{Std}}{m} \cdot f_{Std}$ <p> k_X = mass fraction of the analyte I_X and I_{Std} = signal areas of analyte and standard N_X and N_{Std} = number of nuclei for the respective signal M_X, M_{Std} = molar masses of analyte and standard m and m_{Std} = weights of sample and standard f_{Std} = purity factor of the standard </p>	<p>From relative peak area integral for CH₃-signal at δ 3.39 ppm in theophylline to the integral (corrected for ¹³C satellite signals) at δ 7.44 pm (2H) and δ 7.56 pm (1H) for a high purity benzoic acid standard. Analyte and standard were weighed accurately and co-dissolved in DMSO.</p>
CSIR	<p>Theophylline content = (1000 – Loss on drying – caffeine- theobromine) mg/g</p>	<p>From normalized LC-UV peak area response at 274 nm, corrected for water content as determined by loss on drying at 105 °C.</p>
NIST	$MF_{theophylline,water=0} = \frac{\bar{X}_{LC} + \bar{X}_{GC} + \bar{X}_{NMR}}{3} - MF_{water}$ <p> X_{LC} = (anhydrous) mass fraction estimate by LC-UV X_{GC} = (anhydrous) mass fraction estimate by GC-FID X_{NMR} = (anhydrous) mass fraction by NMR </p>	<p>Mass fraction estimates (ignoring water) by LC-UV (normalized UV area response at 280 nm), GC-FID (normalized peak areas) and NMR (summation of mole fractions to mass fraction). Average of each method, after subtraction of water by qNMR, gave theophylline estimate.</p>
USP	<p>(1) Direct Assay Value for P20.e test material : Response factor for P20.e sample / Response factor for USP Theophylline RS where: Response factor = UV area response / concentration (2) Related Substances Method:</p> $\text{Individual impurity level (mg/g)} = \frac{C_{ref\ sol}}{C_{test\ sol}} \cdot \frac{r_{imp}}{r_{ref\ sol}} \cdot 1000$ <p>Where:</p> <p> $C_{ref\ sol}$ = concentration of reference solution $C_{test\ sol}$ = concentration of P20.e test solution r_{imp} = peak area of the impurity in P20.e test solution $r_{ref\ sol}$ = peak area of theophylline in reference solution </p> <p>Theophylline content calculated by subtraction including the volatiles (in mg/g) found by loss on drying analysis.</p>	<p>(1) USP Direct Assay: LC-UV method using the ratio of response factor for theophylline in CCQM-P20.e samples (UV response area at 280 nm/concentration) to the response factor for USP theophylline reference standard at a similar concentration. Corrected for water content as determined by loss on drying at 105 °C.</p> <p>(2) USP Related Substances: Normalized UV peak area response at 272 nm relative to a theophylline reference standard solution equivalent to the 0.1% limit, corrected for volatiles by loss on drying at 105 °C.</p>

Method Summaries (Ctd)

NMIA Theophylline (mg/g) = (100%-I^{HPLC-all}) x (100%-I^{OT}) x 10
 $I_{\text{HPLC-all}} = I_{\text{HPLC-raw}} + I_{\text{NR}} + I_{\text{ND}}$

Where:

$I_{\text{HPLC-all}}$ = total impurities by HPLC (percentage of normalized LC-UV response) allowing for non-resolved and non-detected components and assuming identical UV response factors

$I_{\text{HPLC-raw}}$ = total impurities (%) from LC-UV data

I_{NR} = allowance for non-resolved impurities

I_{ND} = allowance for impurities below detection limit

I_{OT} = mass fraction of impurities not detectable by LC-UV

LGC For CCQM-P20.e.1 a combination (average ?) of purity as determined by LC-UV and by DSC with a cross check by GC-FID. An independent correction for moisture content determined by Karl Fischer titration was used.

For CCQM-P20.e.2 the levels of theobromine and caffeine were determined by external calibration LC-UV and the LC-UV relative areas were corrected for the response factors for these two compounds. LC-UV value was cross-checked by DSC and GC-FID. Moisture content determined by Karl Fischer titration.

BIPM

$$w_{\text{TP}} = \frac{m_{\text{TP}}}{m_{\text{P20.e}}} = \frac{m_{\text{TP}}}{m_{\text{TP}} + \sum m_i + \sum m_{\text{other}}}$$
$$= \frac{1}{1 + \left(\sum \frac{A_i}{R_i} \cdot \frac{1}{A_{\text{TP}}} \right) + \left(\sum \frac{m_{\text{other}}}{m_{\text{TP}}} \right)}$$

Where:

w_{TP} = mass fraction (g/g) of theophylline in a P20.e sample

m_{TP} = mass (g) of theophylline in a P20.e test sample

$m_{\text{P20.e}}$ = mass (g) of a P20.e test sample

m_i = mass (g) of a resolved, LC-UV detectable minor component in a CCQM-P20.e test sample

m_{other} = mass (in g) of components in a CCQM-P20.e test sample not detectable by LC-UV

A_n = Normalised peak area of UV-active component i

A_{TP} = Normalised peak area of theophylline

R_i = UV response factor (mass basis) of UV-active component i relative to theophylline

NOTE: Mass fraction (mg/g) of theophylline in P20.e sample
 $= w_{\text{TP}} \times 1000$

From normalized LC-UV peak area responses at 271 nm, with correction for water content determined by Karl Fischer titration. NMR used to check for organic solvent impurities and to confirm the theobromine and caffeine estimate by LC-UV of CCQM-P20.e.2.

Thermogravimetric analysis (TGA) and elemental microanalysis were used to check for non-volatiles and to confirm the overall consistency of the result.

Combination of mass fraction estimates obtained by LC-UV (normalized UV peak area responses at 273 nm) and DSC, after subtraction of the water content by Karl Fischer titration. GC-FID and GC-MS confirmed the identity of the main component and used as cross check.

From the LC-UV peak area responses at 273 nm with a mass-based correction applied to take account of UV response factors relative to theophylline. LC-MS was used to identify minor components and to check by external calibration the 3-methyl xanthine, theobromine and caffeine estimates obtained by LC-UV for both materials. A correction for water content by Karl Fischer titration was applied. GC-MS was used to check for volatile organics and TGA to cross check the volatiles estimate.

Method Summaries (Ctd)

CENAM	<p>Theophylline = (1000 – caffeine content – theobromine content) mg/g</p>	<p>Impurities identified by LC-UV at 270 nm quantified by external calibration. Theophylline estimate was obtained by subtraction.</p>
NIM	<p>(1) External standard LC-UV method</p> $P_s = \frac{A_s}{\frac{1}{2} \left(\frac{A_L}{P_{th} C_L} + \frac{A_H}{P_{th} C_H} \right) C_s}$ <p>P_s: mass fraction of sample (g/g); P_{th}: mass fraction of standard theophylline from NRCCRM ; A_s: peak area of sample solution; A_L, A_H: peak areas of standard solution of low and high concentration respectively; C_s: concentration ($\mu\text{g/g}$) of sample solution; C_L, C_H: low and high concentrations ($\mu\text{g/g}$) of standard solution.</p> <p>(2) Internal standard LC-UV method</p> $P_s = \frac{RA_s}{\frac{1}{2} \left(\frac{P_{cf} RA_L}{P_{th} RC_L} + \frac{P_{cf} RA_H}{P_{th} RC_H} \right) RC_s}$ <p>RA_s, RA_L, RA_H: peak area of sample solution to caffeine standard solution, low theophylline standard solution to caffeine standard solution and high theophylline standard solution to caffeine standard solution, respectively; RC_s, RC_L, RC_H: concentrations ($\mu\text{g/g}$) ratio of sample solution to caffeine standard solution, low theophylline standard solution to caffeine standard solution and high theophylline standard solution to caffeine standard solution, respectively; P_{th}: mass fraction of std. theophylline from NRCCRM; P_{cf}: mass fraction of std. caffeine from NRCCRM</p>	<p>Mean of two methods based on LC-UV with detection at 275 nm and 254 nm. One method used external calibration against a theophylline reference material and the other internal calibration against a caffeine reference material.</p>
DMS	<p>Content (%) = $\frac{\text{Area of each component}}{\text{Total area}} \times 100$</p> <p>Content (mg/g) = Content (%) x 1000</p>	<p>From normalized LC-UV peak area response at 272 nm</p>
NIMT	<p>Content (%) = $\frac{\text{Area of theophylline}}{\text{Total area}} \times 100$</p> <p>Content (mg/g) = Content (%) x 1000</p>	<p>From normalized LC-UV peak area response at 270 nm. The value obtained was cross checked by DSC and impurities were identified by GC-MS</p>
NMIJ	$x_p = \frac{x_p(\text{DSC}) + x_p(\text{HPLC})}{2}$	<p>Mean of mass fraction estimates obtained by stepwise scan DSC and from normalized LC-UV peak area response at 274 nm. For the DSC data the mole fraction estimate obtained directly by DSC was corrected into a mass fraction estimate based on the relative molecular weights of theophylline and the identified impurities.</p>

Measurement Uncertainty Budgets

Lab ID

Measurement Uncertainty Budgets

BAM Contributing uncertainty sources are

(LC-UV)

- repeatability
- non-linearity
- response factor (i.e. detection wavelength) influence
- trueness/recovery

Contribution	sample e.1	sample e.2
repeatability	0.000024	0.000058
non-linearity	0.000002	0.000348
wavelength influence	0.000072	0.001226
inhomogeneity	-	0.001989
trueness/recovery	0.000992	0.000992
total	0.000995	0.002563

BAM
(QNMR)

$$\frac{u(k_x)}{k_x} = \sqrt{\left(\frac{u(I_x/I_{Std})}{I_x/I_{Std}}\right)^2 + \left(\frac{u(M_x)}{M_x}\right)^2 + \left(\frac{u(M_{Std})}{M_{Std}}\right)^2 + \left(\frac{u(m)}{m}\right)^2 + \left(\frac{u(m_{Std})}{m_{Std}}\right)^2 + \left(\frac{u(f_{Std})}{f_{Std}}\right)^2}$$

Uncertainty budget for P20.e.1

Uncertainty component

	x_i	$u(x_i)$	$\frac{u(x_i)}{x_i}$
Integration (initial values)	992.9	0.49	0.05 %
Integration (corrected values)	998.3	0.50	0.05 %
Molar mass analyte in g mol ⁻¹	180.167	0.006	0.003 %
Molar mass standard in g mol ⁻¹	122.121	0.006	0.005 %
mass sample for aliquot I in mg	44.90	0.03	0.07 %
mass standard for aliquot I in mg	30.95	0.03	0.10 %
Purity factor standard	0.999958	0.000012	0,0012 %
Combined uncertainty		1.3	0.13%
Expanded uncertainty U (C.I.95%)		2.6	

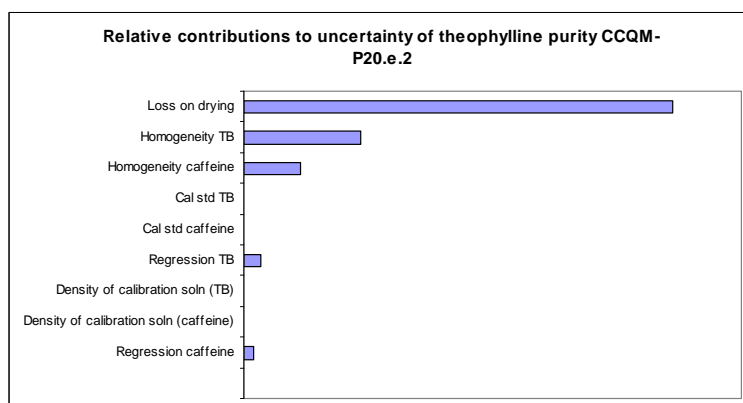
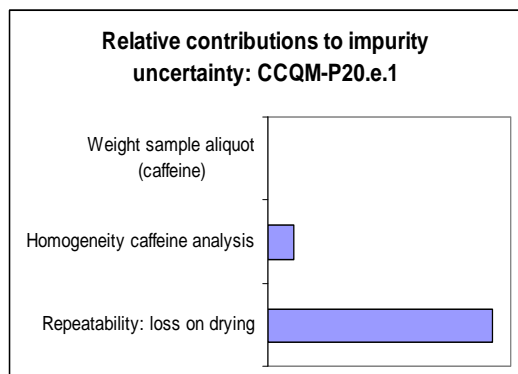
Uncertainty budget for P20.e.2

Uncertainty component

	x_i	$u(x_i)$	$\frac{u(x_i)}{x_i}$
Integration (initial values)	978.1	1.01	0.10 %
Integration (corrected values)	983.4	1.02	0.10 %
Molar mass analyte in g mol ⁻¹	180.167	0.006	0.003 %
Molar mass standard in g mol ⁻¹	122.121	0.006	0.005 %
mass sample for aliquot I in mg	45.30	0.03	0.07 %
mass standard for aliquot I in mg	32.25	0.03	0.09 %
Purity factor standard	0.999958	0.000012	0,0012 %
Combined uncertainty		1.5	0.15%
Expanded uncertainty U (C.I.95%)		3.0	

Measurement Uncertainty Budgets (Ctd)

CSIR



NIST

$$U_{95}^{-}(MF_{\text{theophylline}}) = MF_{\text{theophylline, water}=0} - 2 \times u_c$$

$$U_{95}^{+}(MF_{\text{theophylline}}) = \text{minimum}(999.5, MF_{\text{theophylline, water}=0} + 2 \times u_c + Bias_{\text{water}})$$

Relative contributions of the components of the overall uncertainty budget for:
CCQM-P20.e.1

Sample heterogeneity at 2.5 mg: $u_{\text{heterogeneity}} = 0.44$ mg/g

Agreement among methods: $u_{\text{method}} = 0.14$ mg/g

Bias from inexact knowledge of water: $bias_{\text{water}} = 0.43$ mg/g

CCQM-P20.e.2

Sample heterogeneity at 25 mg: $u_{\text{heterogeneity}} = 1.6$ mg/g

Agreement among methods: $u_{\text{method}} = 1.4$ mg/g

Bias from inexact knowledge of water: $bias_{\text{water}} = 3.2$ mg/g

USP

Relative contributions of the components of the overall uncertainty budget for:

CCQM-P20.e.1 by direct assay method

Type A uncertainty from standard deviation of individual injection data = 2.2 mg/kg

Type B uncertainty for gravimetric and volumetric operations, including loss on drying data = 3.1 mg/kg

Combined standard uncertainty = 3.8 mg/kg, coverage factor = 2

CCQM-P20.e.1 by the related substances assay method

Type A uncertainty from standard deviation of individual data = 0.236 mg/kg

Type B uncertainty for gravimetric and volumetric operations, including loss on drying data. Uncertainty in the reference peak height included. = 0.694 mg/kg

Combined standard uncertainty = 0.734 mg/kg, coverage factor = 2

Measurement Uncertainty Budgets (Ctd)

USP

CCQM-P20.e.2 by direct assay method

Type A uncertainty from standard deviation of individual injection data = 2.6 mg/kg

Type B uncertainty for gravimetric and volumetric operations, including loss on drying data = 2.9 mg/kg

Combined standard uncertainty = 3.9 mg/kg, coverage factor = 2

CCQM-P20.e.2 by the related substances assay method

Type A uncertainty from standard deviation of individual data = 0.187 mg/kg

Type B uncertainty for gravimetric and volumetric operations, including loss on drying data. Uncertainty in the reference peak height included. = 0.666 mg/kg

Combined standard uncertainty = 0.691 mg/kg, coverage factor = 2

NMIA

$$u_{\text{HPLC-all}} = [(u_{\text{HPLC-raw}})^2 + (u_{\text{NR}})^2 + (u_{\text{ND}})^2]^{1/2}$$

The major components of the uncertainty budget are:

- Standard deviation of the raw HPLC results
- The HPLC correction factor (assigned as 1) but with associated uncertainty
- U_{ND} = Standard uncertainty of non detected impurities
- U_{NR} = Standard uncertainty of non resolved impurities
- U_{IOT} = Standard uncertainty of “other” impurities (non volatiles, solvent and water)

LGC

Contributing uncertainty sources are

- Standard uncertainties of the analytical techniques (from historical method performance data or

$$u_{\text{Meth}} = \frac{\sigma}{\sqrt{n}} \text{ where } \sigma = \text{s.d of the analysis and } n = \text{number. of independent analyses}$$

- Standard uncertainties for the determination of the individual impurities by HPLC
- Standard uncertainty for the determination of moisture
- Between method standard uncertainty $u_{\text{BM}} = \frac{\sigma}{\sqrt{2}}$ (σ = standard deviation of the method means)

BIPM

Uncertainty budget for CCQM-P20.e.1

Uncertainty component i	x_i	$u(x_i)$	Percent contribution
Normalised response of 3-Me xanthine	0.222	0.030	12
Mass conversion response factor for 3-methyl xanthine relative to theophylline	1.09	0.023	negligible
Normalised response of unknown impurity	0.658	0.031	1
Mass conversion response factor for unknown impurity relative to theophylline	2.0	0.981	50
LC-MS estimate of caffeine content	0.2	0.115	25
Water content estimate	0.2	0.04	12
Theophylline content	999.1	0.2	
Expanded uncertainty U (C.I.95%, k = 2)		0.4	

Measurement Uncertainty Budgets (Ctd)

BIPM

Uncertainty budget for CCQM-P20.e.2

Uncertainty component i	x_i	$u(x_i)$	Percent contribution
Normalised relative response of 3-Me xanthine	0.215	0.009	negligible
Response factor for 3-methyl xanthine relative to theophylline	1.09	0.023	negligible
Normalised relative UV response of theobromine	9.253	0.405	50
Response factor for theobromine relative to theophylline	1.04	0.23	13
Normalised relative UV response of unknown impurity	0.662	0.026	negligible
Response factor for unknown impurity relative to theophylline	2.0	0.981	9
Normalised relative UV response of caffeine	4.33	0.110	4
Response factor for caffeine relative to theophylline	0.95	0.023	4
Water content estimate	0.223	0.025	21
Theophylline content	983.8	0.55	
Expanded uncertainty U (C.I.95%, k = 2)		1.1	

CENAM

Contributing uncertainty sources are

- Repeatability and reproducibility of caffeine and theobromine estimates
- Uncertainty due to calibration curves for caffeine and theobromine
- Repeatability of theophylline concentration

NIM

Uncertainty of external standard method

Relative standard uncertainties	source	value	Comment
	P_{th}	0.15%	Uncertainty by reference material of theophylline
	A_s	0.11%	RSD of determination of HPLC
	A_L	0.08%	RSD of determination of HPLC
	A_H	0.08%	RSD of determination of HPLC
	C_s	0.06%	Weighing of sample and its dilution
	C_L	0.06%	Weighing of reference material and its dilution to low standard
	C_H	0.06%	Weighing of reference material and its dilution to high standard
Relative standard uncertainty	P_s	0.25%	$\sqrt{u_r(P_{std})^2 + u_r(A_L)^2 + u_r(A_H)^2 + u_r(A_s)^2 + u_r(C_L)^2 + u_r(C_H)^2 + u_r(C_s)^2}$
Standard uncertainty	u_C	0.25%	0.25%
Expand uncertainty	U	0.5%	$k=2$

Measurement Uncertainty Budgets (Ctd)

NIM

Uncertainty of internal standard method

Relative standard uncertainties	source	value	Comment
Relative standard uncertainties	P_{th}	0.15%	Uncertainty by reference material of theophylline
	P_{cf}	0.10%	Uncertainty by reference material of caffeine
	RA_s	0.07%	RSD of determination of HPLC
	RA_L	0.06%	RSD of determination of HPLC
	RA_H	0.06%	RSD of determination of HPLC
	RC_s	0.08%	Weighing of sample and its dilution
	RC_L	0.08%	Weighing of reference material and internal standard, and their dilution to low standard
	RC_H	0.08%	Weighing of reference material and internal standard, and their dilution to high standard
Relative standard uncertainty	P_s	0.25%	$\sqrt{u_r(P_{std})^2 + u_r(RP_{std})^2 + u_r(RA_L)^2 + u_r(RA_H)^2 + u_r(RA_s)^2 + u_r(RC_L)^2 + u_r(RC_H)^2 + u_r(RC_s)^2}$
Standard uncertainty	u_C	0.25%	0.25%
Expand uncertainty	U	0.5%	$k=2$

DMSc

Major components of the uncertainty budget

- Peak area of each component
- Peak area of total components

Summary of relative contributions of the major components of the overall uncertainty budget

Measurand = content of Theophylline (mg/g)

$$\mu_{\text{content}} = U_{\text{content}} * \sqrt{\left(\frac{u_i}{U_i}\right)^2 + \left(\frac{u_T}{U_T}\right)^2}$$

$$U_{\text{content}} = \text{Content (mg/g)}$$

μ_i = standard uncertainty of Theophylline: repeatability of Theophylline
(Standard deviation of peak area of Theophylline)

U_i = Average peak area of Theophylline

μ_T = standard uncertainty of total peak area: standard deviation of total peak area

U_T = Average total peak area

Measurement Uncertainty Budgets (Ctd)

NIMT

The major components are tabulated below.

Source of uncertainty	Typical values	Standard uncertainty	Degree of freedom	Type of uncertainty
Minor 1 (%)	0.0265	0.0020	9	A
Minor 2 (%)	0.0568	0.0027	9	A
Resolution of LC	0.1000	0.0866	Large	B
Contamination of HPLC column	0.1000	0.0866	Large	B

$$u_c = 1.2 \text{ mg/g}$$

$$U = 2.5 \text{ mg/g, } k = 2$$

NMIJ

The major components are tabulated below.

Source of uncertainty	$u(x_i)$	c_i	$c_i u(x_i)$
purity (DSC)	0.7	0.5	0.35
purity (HPLC)	0.2	0.5	0.1
difference between techniques	0.2	1	0.2

Relative contributions of the major components are tabulated below.

source	$c_i u(x_i)$	relative contribution
purity (DSC)	0.35	0.71
purity (HPLC)	0.1	0.06
difference between DSC and HPLC	0.2	0.23

Impurity Analysis of CCQM-P20.e.1 and CCQM-P20.e.2

All participants except the BAM (NMR) submission reported the impurity content of both materials. All identified caffeine and theobromine as the two incurred impurities in CCQM-P20.e.2. Two additional significant impurities (relative area response > 0.1 % of the summation of area of UV responses) were observed by LC-UV in both materials by the majority of participants. One impurity had a short LC-retention time and was independently identified as 3-methyl xanthine by NIM, NIST and BIPM by comparison with an authentic standard. An impurity was reported at the same relative retention time without structural identification by BAM, NMIJ, LGC, NMIA, DMSC, USP and NIMT .

The other UV-active impurity had a relative LC retention time intermediate between that of theophylline and caffeine and could be resolved from the two when appropriate isocratic elution conditions were used. It was reported by BAM, NIM, NIST, USP, BIPM, LGC, NMIA, DMSC and NIMT but was not definitively identified by any participant. During the production of the study materials the coordinating laboratory was able to undertake testing and comparison with authentic standards of a range of structurally-related xanthine compounds as well as known theophylline breakdown and hydrolysis products to try and identify this impurity but without success. The conclusion of the coordinating laboratory was that it is not structurally related to theophylline and is possibly a plasticizer or related non-polar

UV active compound that was introduced into the commercial source material for theophylline during its production.

There were some differences in the impurity profiles reported for both materials. While the results obtained by participants using isocratic elution were very consistent, they were different from those reported when participants used gradient elution. These anomalies were probably due to co-elution of the longer retention time impurities when gradient elution was used. Some participants who had used gradient methods for the original study subsequently repeated the analysis using an isocratic method and obtained results in agreement with those obtained by the other participants who used this approach throughout.

Water content was estimated for each material either by Karl Fischer titration (BAM, BIPM, LGC, NMIA) by quantitative NMR (NIST) or by loss on drying (USP, CSIR). There was an acceptable level of agreement between the Karl Fischer titration and NMR estimates. The loss on drying methods are inherently less accurate and overestimated the water content, particularly for CCQM-P20.e.1 which only contained a very low level of water.

Impurity profile of CCQM-P20.e.1

An example of a typical LC-UV chromatographic profile obtained for the P20.e.1 material by participants using isocratic elution methods is shown in Figure 6.⁶ Two significant peaks were generally observed in addition to the theophylline main component. The nature and amount of the minor components of CCQM-P20.e.1 reported by each study participant are given in Annexe 1.

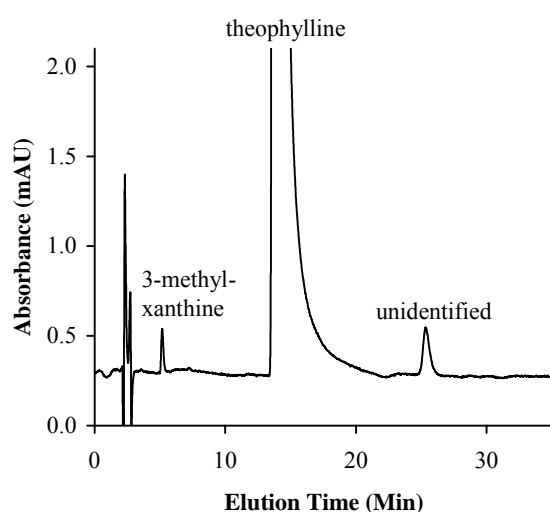


Figure 6 LC-UV chromatogram for CCQM-P20.e.1 (isocratic elution)⁵

Impurity profile of CCQM-P20.e.2

An example of a typical LC-UV chromatographic profile obtained for CCQM-P20.e.2 using an isocratic elution method is shown in Figure 7.⁶ The two spiked compounds (theobromine and caffeine) are clearly resolved from theophylline as are two UV-active impurities innately present in the theophylline source material. The four minor component peaks, correspond (in retention time order) to 3-methylxanthine, theobromine, an unidentified UV-active compound and caffeine.

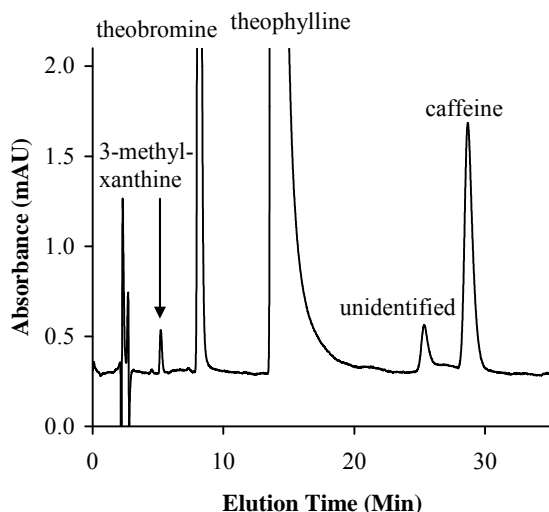


Figure 7 LC-UV chromatogram for CCQM-P20.e.2 (isocratic elution)⁶

Quantification of spiked impurities in CCQM-P20.e.2

A secondary aim of the study was to test the ability of participating laboratories to identify and quantify the minor components present in a material. As the amount of theobromine and caffeine added into the theophylline source material was known and the material displayed a sufficient degree of homogeneity when analysed at the recommended sample size, it was possible to calculate a gravimetric reference value and associated uncertainty for the two spiked compounds. This allowed for direct comparison with the values reported by study participants. The estimates reported for the theobromine and caffeine content of CCQM-P20.e.2 are given in Table 5 and Table 6 respectively. The difference from the gravimetric reference value of each result ($D_i = w_i - w_{grav}$) are shown in Figure 8 for theobromine and Figure 9 for caffeine. The expanded uncertainty U_i at the 95% confidence level of the difference from the reference value was calculated for each result as $U(D_i) = 2 * \sqrt{u(w_i)^2 + u(w_{grav})^2}$.

Table 5 – Theobromine mass fraction assignments for CCQM-P20.e.2

Institute	Theobromine content (mg/g)	Expanded Uncertainty (U_i) at 95% confidence level (mg/g)	Relative Expanded Uncertainty at 95% confidence level (%)
NMIJ	6.4	2.0	31
BAM	6.8	0.6	9
NIMT	7.6	0.4	5
CENAM	8.06	0.96	12
USP (Rel. Subst.)	8.45	0.31	4
NMIA	8.7	2.0	23
LGC	8.80	0.12	1
BIPM	8.88	0.87	10
NIM	8.9	0.5	6
DMSC	9.24	0.26	3
NIST	9.6	1.3	14
CSIR	9.89	0.60	6
Mean	8.4	0.67	
Weighted mean ⁵	8.7	0.10	
Median	8.75		
Reference Value (Gravimetric)	9.28	0.89	

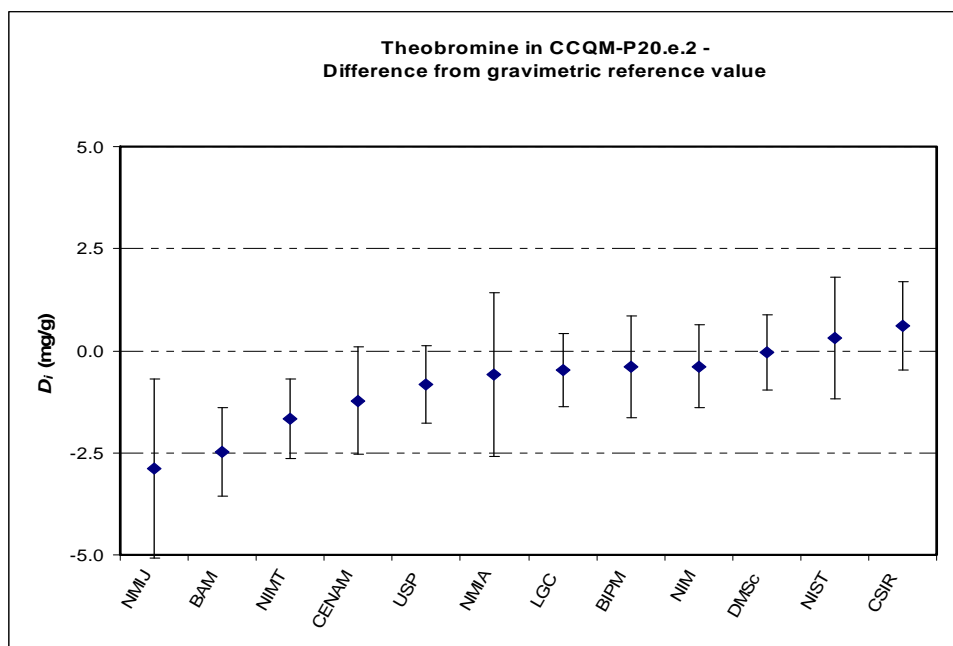


Figure 8 Difference of CCQM-P20.e.2 theobromine estimates from gravimetric reference value (9.28 ± 0.9 mg/g)

Table 6 – Caffeine mass fraction assignments for CCQM-P20.e.2

Institute	Caffeine content (mg/g)	Expanded Uncertainty (U_i) at 95% confidence level (mg/g)	Relative Expanded Uncertainty (%)
BAM	3.7	0.6	16
NIST	3.9	0.9	23
USP	3.95	0.25	6
CSIR	4.12	0.18	4
NMIA	4.20	0.9	21
DMSC	4.26	0.51	12
NMIJ	4.30	1.0	23
BIPM	4.55	0.32	7
NIM	4.7	0.6	13
NIMT	4.7	0.4	9
LGC	4.80	0.6	2
CENAM *	8.02	0.95	12
Mean	4.3	0.22	
Weighted mean ⁵	4.6	0.07	
Median	4.3		
Reference Value (Gravimetric)	4.94	0.69	

* Result not included in summary statistics

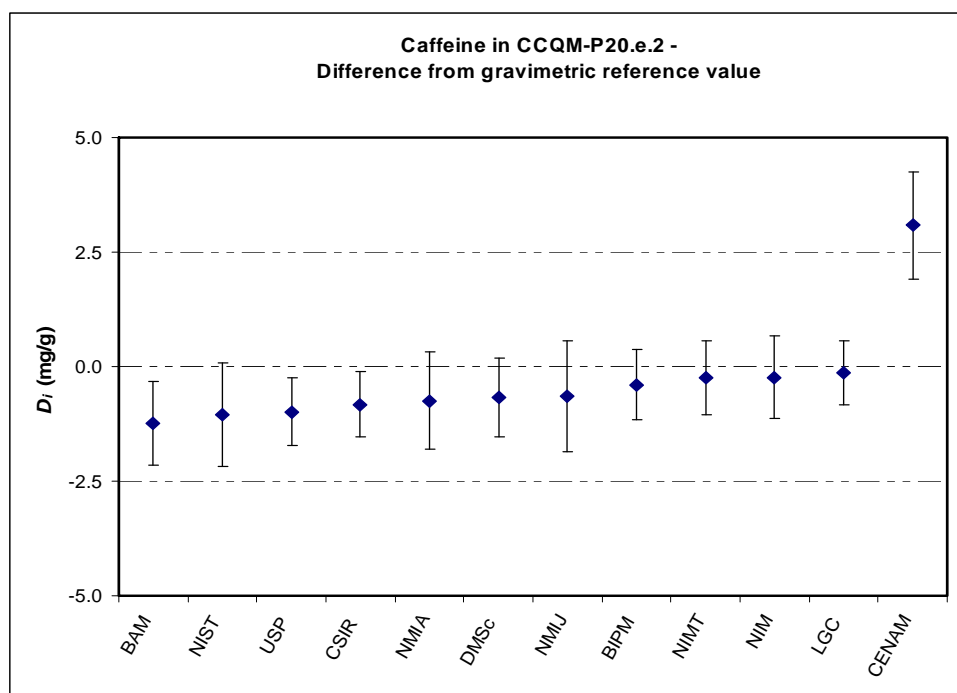


Figure 9 Difference of CCQM-P20.e.2 caffeine estimates from gravimetric reference value (4.94 ± 0.7 mg/g)

While there appears to be a negative bias in the reported results for caffeine relative to the gravimetric value, this is due in part to the assumption of equivalent UV-response factors (on a unit mass basis) by some participants. When the response factor was taken into account or external calibration against a caffeine standard was used the reported results generally agreed more closely with the gravimetric value.

Quantification of minor impurities in CCQM-P20.e.1 and CCQM-P20.e.2

The minor impurity components reported for both materials are listed in Annexe 2. These impurities were innately present in the two theophylline source materials used to prepare each study sample and there was very good agreement in the levels of these compounds found by the participants.

Ten participants reported the presence of low levels of a short LC-retention time impurity which eluted before theobromine and three laboratories (NIST, NIM and BIPM) independently identified it as 3-methyl xanthine.

A second UV-active impurity with an LC-retention time intermediate between that of theophylline and caffeine (using isocratic elution conditions) was reported by nine participants however none was able to make a definitive assignment of the structure of the compound. The co-ordinating laboratory compared its LC-MS properties with a wide range of related structure xanthines and products known to derive from breakdown of theophylline however none corresponded by LC retention time or MS to the unidentified impurity. It is interesting to note that while the reported levels of the two minor organic impurities (3-methyl xanthine and the unidentified UV-active compound) were essentially the same in the both CCQM-P20.e.1 and CCQM-P20.e.2, there appeared to be a clear difference in the water content of the two materials.

There is an apparent discrepancy between the small level of caffeine reported to be present in CCQM-P20.e.1 by USP and BIPM and the significantly higher levels reported by NMIJ, CSIR and CENAM. However the higher reported levels were all obtained when gradient elution LC-UV analysis was used and can be explained from the inability to resolve caffeine from the unidentified UV-active impurity under these conditions.

SUMMARY

The reported estimates for the mass fraction content of theophylline in both materials displayed a good level of agreement both with each other and, in the case of the CCQM-P20.e.2 sample, with a gravimetric reference value. The abilities of the participant laboratories to assign mass fraction content to high purity materials of this general class of compound appear to be justified. There were sufficient cross checks by other methods (LC-MS, NMR, GC-FID, DSC) to be confident that no significant bias resulted from the general reliance on HPLC-UV for the actual quantification.

For the higher purity CCQM-P20.e.1 material, relative expanded uncertainties in the range 0.05 – 0.5 % were reported in the mass fraction content of the main component. For the artificially spiked CCQM-P20.e.2 sample the range of expanded uncertainties reported in the mass fraction content was wider (0.1 – 1 %) as was to be expected given the larger number and higher level of minor components it contained. These results were achieved despite the application of a variety of measurement equations and budgets for the estimation of measurement uncertainty. There was in addition excellent agreement for the CCQM-P20.e.2 sample between the participant's reported results and the theophylline gravimetric reference value.

The agreement between the levels reported for the spiked components and their respective gravimetric values for CCQM-P20.e.2 was satisfactory overall. The relative expanded uncertainties in the assigned values were wider (1-30 %) than those for the main component. It is worth noting that the results of all laboratories that reported relative expanded uncertainties of less than 5 % in the mass fraction content assigned to the spiked impurities were validated in terms of their level of agreement with the gravimetric values.

Overall there was good agreement in the identification and quantification of the impurity content of the study samples, both for the incurred impurities in CCQM-P20.e.2 as well as the minor components found innately in both materials. The impurity profiles and identifications reported by participants using LC-UV with isocratic elution were in excellent agreement with each other. Some differences were found when compared with the results from participants using LC-UV gradient elution methods. However these can be ascribed to the co-elution of longer retention time materials under gradient conditions.

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5. W. Hasselbarth, W. Bremser, R. Pradel; Uncertainty-based evaluation of certification study data (*Fresenius J. Anal. Chem.* 1998, **360**, 317-321)
6. Reproduced with permission from the NIST CCQM-P20.e report

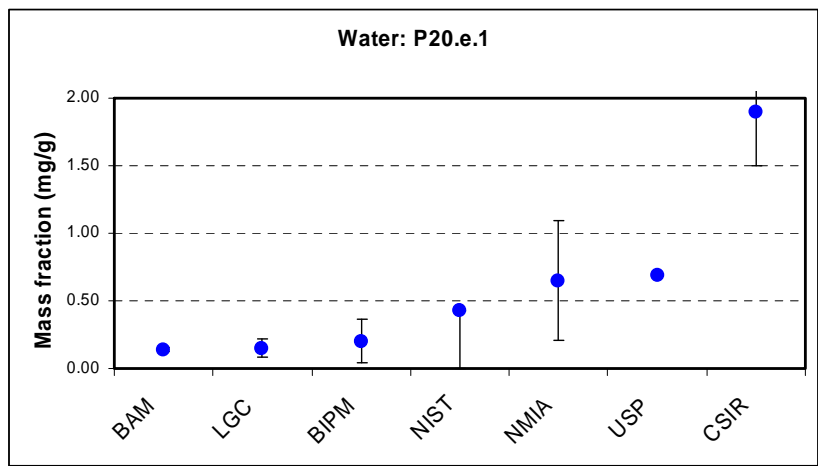
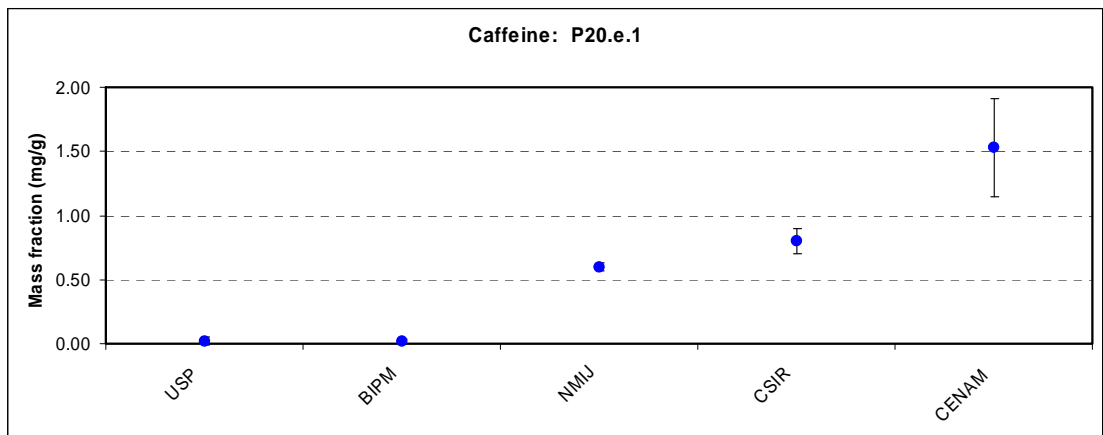
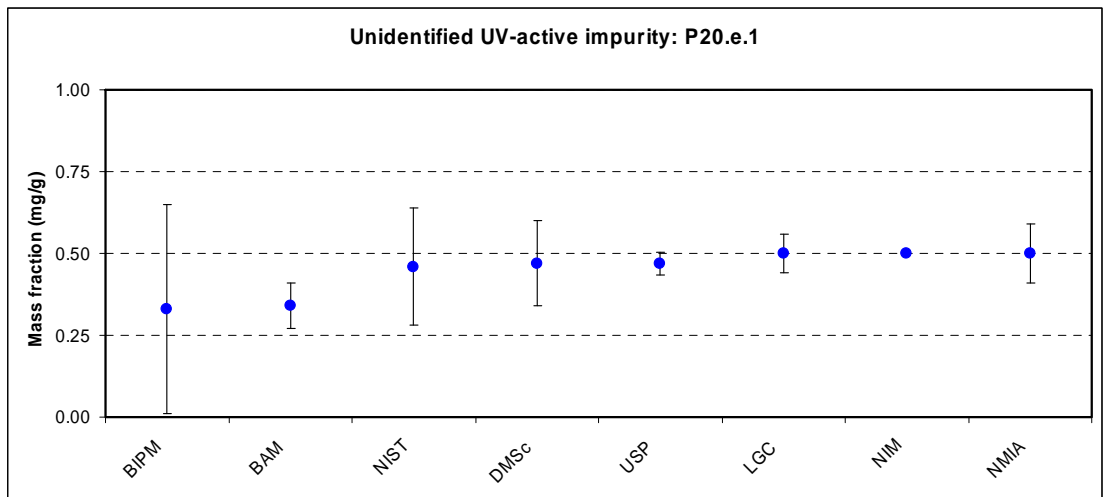
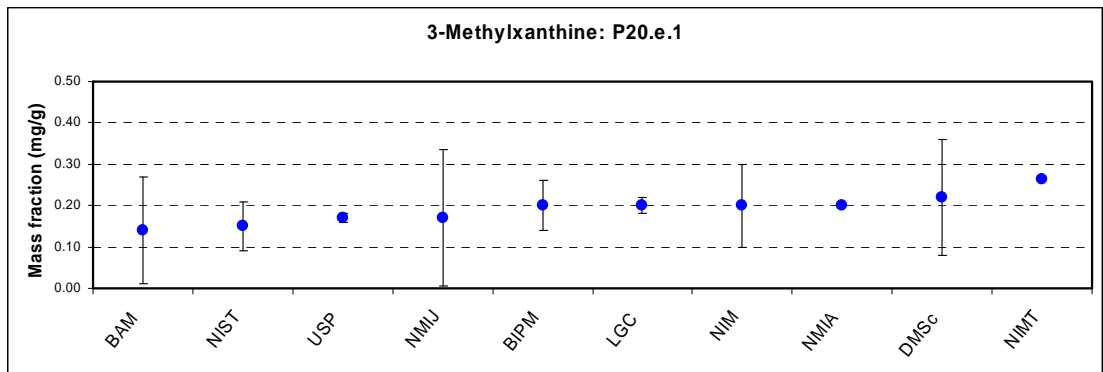
Annexe 1 – Reported minor components for CCQM-P20.e.1

Component	Institute	Mass fraction content $\pm U_j$ at 95% conf. level (mg/g)	Method
3-Methylxanthine	BAM	0.14 \pm 0.13	Relative area by LC-UV at 245 nm. Identity not established.
	NIST	0.15 \pm 0.06	Relative area by LC-UV at 280 nm. Identity established by MS, NMR and comparisons with standard.
	USP	0.17 \pm 0.01	Relative area by LC-UV at 272 nm.
	NMIJ	0.17 \pm 0.17	Relative area by LC-UV at 274 nm. Identity not established.
	BIPM	0.20 \pm 0.06	Relative area by LC-UV at 273 nm, corrected for UV response factor and confirmed by LC-MS. Identity by MS and comparison with standard .
	LGC	0.20 \pm 0.02	Relative area by LC-UV at 273 nm. Identity not established.
	NIM	0.2 \pm 0.1	Relative area by LC-UV at 275 and 254 nm. Identity established by MS.
	NMIA	0.2	Relative area by LC-UV at 271 nm. Identity not established.
	DMSC	0.22 \pm 0.14	Relative area by LC-UV at 272 nm. Identity not established.
	NIMT	0.27 \pm 0.04	Relative area by LC-UV at 270 nm. Identity not established
UV-active impurity	BIPM	0.33 \pm 0.32	Relative area by LC-UV at 273 nm. Identity unknown but not consistent with a xanthine-related structure
	BAM	0.34 \pm 0.07	Relative area by LC-UV at 245 nm. Identity not established.
	NIST	0.46 \pm 0.18	Relative area by LC-UV at 280 nm. Identity possibly trimethylxanthine.
	USP	0.47 \pm 0.04	Relative area by LC-UV at 272 nm. Identity not established.
	DMSC	0.47 \pm 0.13	Relative area by LC-UV at 272 nm. Identity not established.
	LGC	0.50 \pm 0.06	Relative area by LC-UV at 273 nm. Identity possibly trimethylxanthine
	NMIA	0.50 \pm 0.09	Relative area by LC-UV at 271 nm. Identity not established.
	NIMT	0.57 \pm 0.06	Relative area by LC-UV at 270 nm. Identity not established.
	NIM	Observed, not quantified	Observed by LC-UV at 275 and 254 nm. Identity not established.

Annexe 1 – Reported minor components for CCQM-P20.e.1 (ctd)

Component	Institute	Mass fraction content $\pm U$, at 95% conf. level (mg/g)	Method
Caffeine	USP	0.02 \pm 0.03	Relative area by LC-UV at 272 nm. Identity established by comparison with standard
	BIPM	0.02 \pm 0.02	Quantitative LC-MS. Identity established by comparison with standard. Present slightly below detection limit for LC-UV.
	NMIJ	0.60 \pm 0.03	Relative area by LC-UV at 274 nm. Identity established by comparison with a standard.
	CSIR	0.8 \pm 0.1	Relative area by LC-UV at 274 nm. Identity established by comparison with reference standard
	CENAM	1.5 \pm 0.4	Relative area by LC-UV at 270 nm. Identity established by comparison with standard
Water	BAM	0.14 \pm 0.02	Coulometric Karl Fischer titration 3 x 150 mg samples
	LGC	0.15 \pm 0.07	Coulometric Karl Fischer titration 3 x 150 mg samples
	BIPM	0.20 \pm 0.16	Coulometric Karl Fischer titration 2 x 200 mg samples
	NIST	\leq 0.43	Quantitative NMR
	NMIA	0.65 \pm 0.44	Coulometric Karl Fischer titration 6 x 30 mg samples
	USP	0.69	Loss on drying at 105 °C
	CSIR	1.9 \pm 0.4	Loss on drying at 105 °C

Other potential non-related components (inorganic salts, volatile organics) were tested for by one or more participants without being detected at significant levels.



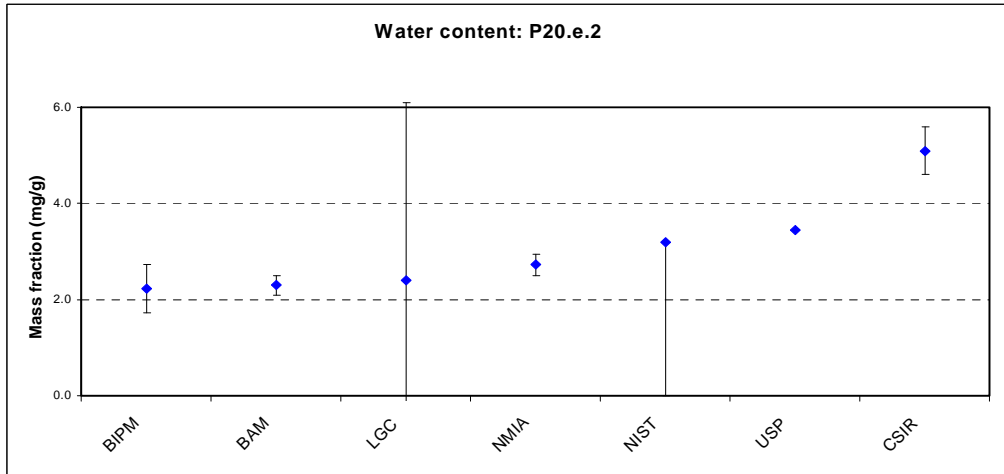
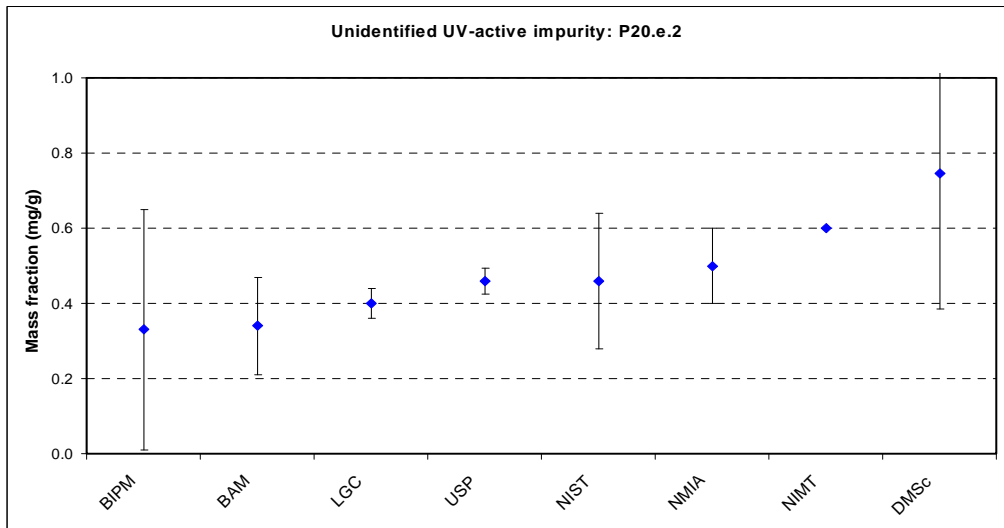
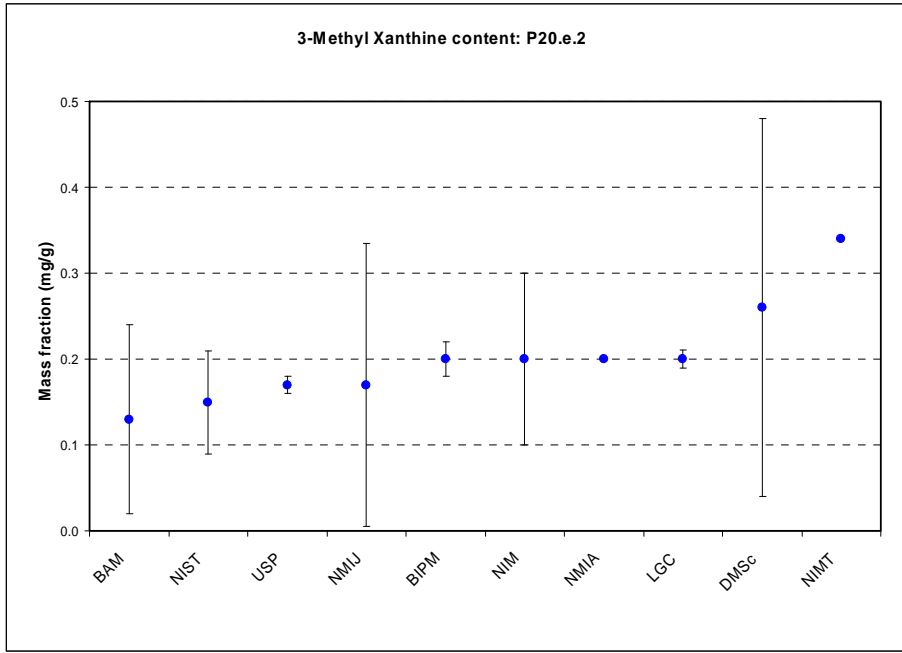
Mass fraction estimates reported for minor components in CCQM-P20.e.1

Annexe 2 – Reported minor components for CCQM-P20.e.2

Component	Institute	Mass fraction content $\pm U_i$ at 95% conf. level (mg/g)	Method
3-Methylxanthine	BAM	0.13 - 0.13 + 0.22	Relative area by LC-UV at 245 nm. Identity not established.
	NIST	0.15 \pm 0.06	Relative area by LC-UV at 280 nm. Identity established by MS, NMR and comparisons with standard.
	USP	0.17 \pm 0.01	Relative area by LC-UV at 272 nm.
	NMIJ	0.17 \pm 0.17	Relative area by LC-UV at 274 nm. Identity not established.
	BIPM	0.19 \pm 0.02	Relative area by LC-UV at 273 nm, corrected for UV response factor and confirmed by LC-MS. Identity by MS and comparison with standard
	NIM	0.2 \pm 0.1	Relative area by LC-UV at 275 and 254 nm. Identity established by MS.
	NMIA	0.2	Relative area by LC-UV at 271 nm. Identity not established.
	LGC	0.20 \pm 0.01	Relative area by LC-UV at 273 nm. Identity not established.
	DMSC	0.26 \pm 0.22	Relative area by LC-UV at 272 nm. Identity not established.
	NIMT	0.34 \pm 0.06	Relative area by LC-UV at 270 nm. Identity not established
UV-active impurity	BIPM	0.33 \pm 0.32	Relative area by LC-UV at 273 nm. Identity unknown but not consistent with a xanthine-related structure
	BAM	0.34 \pm 0.13	Relative area by LC-UV at 245 nm.
	LGC	0.40 \pm 0.04	Relative area by LC-UV at 273 nm. Possibly a trimethylxanthine
	USP	0.46 \pm 0.04	Relative area by LC-UV at 272 nm.
	NIST	0.46 \pm 0.18	Relative area by LC-UV at 280 nm. Possibly a trimethylxanthine.
	NMIA	0.5 \pm 0.1	Relative area by LC-UV at 271 nm.
	NIMT	0.60 \pm 0.04	Relative area by LC-UV at 270 nm.
	DMSC	0.75 \pm 0.36	Relative area by LC-UV at 272 nm.

Annexe 2 – Reported minor components for CCQM-P20.e.2 (ctd)

Component	Institute	Mass fraction content $\pm U_i$ at 95% conf. level (mg/g)	Method
Water	BAM	2.3 ± 0.2	Coulometric Karl Fischer titration – 5 x 150 mg samples
	LGC	2.4 ± 3.7	Coulometric Karl Fischer titration – 3 x 150 mg samples
	BIPM	2.23 ± 0.5	Coulometric Karl Fischer titration – 2 x 200 mg samples
	NIST	≤ 3.2	Quantitative NMR
	NMIA	2.72 ± 0.22	Coulometric Karl Fischer titration – 6 x 30 mg samples
	USP	3.45	Loss on drying at 105 °C
	CSIR	5.1 ± 0.5	Loss on drying at 105 °C



Mass fraction estimates reported for minor components in CCQM-P20.e.2