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

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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.f, was co-ordinated by the Bureau International des Poids et Mesures (BIPM) in 2007/2008. Nine national measurement institutes, four expert laboratories and the BIPM participated in the comparison. Participants were required to assign the mass fraction of digoxin present as the main component in the comparison sample (CCQM-P20.f) which consisted of digoxin material obtained from a commercial supplier stated to comply with USP requirements.

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

In contrast with the previous round of the CCQM-P20 series, in which the mass fraction content of theophylline in two comparison samples (CCQM-P20.e.1 and CCQM-P20.e.2) was determined, a wider range of results were reported for the mass fraction content of digoxin in the CCQM-P20.f comparison.

A minority of participants did not appear to use conditions capable of fully resolving and/or quantifying the major related structure impurities present in the comparison sample. Among those that did achieve suitable separations, there was further variation in their reported quantifications of the individual and total related substance content which reflected in part the limited availability of reference standards for these materials and the resulting assumptions that

had to be made regarding the structure and response factors relative to digoxin for each individual impurity. This was particularly relevant because of the span of molecular masses of the impurities present in the sample, which ranged from aglycones to glycones with tetrameric carbohydrate chains, relative to that of digoxin.

A significant additional factor also contributed to the observed variation of results. Unlike the CCQM-P20.e samples, in which the major impurities were solely related structure organic compounds, the CCQM-P20.f study material contained significant levels of residual organic solvents (ethanol, dichloromethane and to a lesser extent toluene). The majority of participants failed to detect and allow for the presence of this class of impurity, introducing a bias towards overestimation of digoxin content in most of the individual results.

However, the uncertainty budgets produced by several participants were sufficiently conservative such that their reported results were nevertheless consistent with the reference value for digoxin content assigned using a consensus mass balance approach.

The results of the comparison reinforces the conclusion from previous rounds of the CCQM-P20 study that care in developing and validating the suitability of the chromatographic separation method used to resolve the main component from the related structure impurities present is essential to obtaining reliable, comparable results when using the mass balance approach to estimate purity.

This specific comparison has demonstrated that, in addition to developing an appropriate chromatographic separation, it is also important to use complementary techniques capable of detecting all potential orthogonal classes of impurities if it is desired to demonstrate a general capability to assign purity with a small (< 0.2 % relative) standard uncertainty.

Participants:

Institute Name	Basis of Participation	Acronym	Country
National Measurement Institute, Australia	NMI	NMIA	Australia
National Research Council Canada - Institute for National Measurement Standards	NMI	INMS-MRC	Canada
National Institute of Metrology of China	NMI	NIM	China
National Metrology Institute of Japan	NMI	NMIJ	Japan
Centro Nacional de Metrologia	NMI	CENAM	Mexico
National Institute of Metrology (Thailand)	NMI	NIMT	Thailand
LGC Ltd	NMI	LGC	United Kingdom
National Institute of Standards and Technology	NMI	NIST	USA
DGKL Reference Institute for Bioanalysis, Germany	Expert Lab	DGKL	
Department of Medical Services, Ministry of Public Health, Thailand	Expert Lab	DMS	
Laboratorio de la Farmacopea de los Estados Unidos Mexicanos	Expert Lab	MP	
Unites States Pharmacopoeia – Reference Standards Laboratory	Expert Lab	USP	
Bureau International des Poids et Mesures		BIPM	

Co-ordinating laboratories

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Introduction

The OAWG meeting at Sèvres in April 2007 accepted the proposal by the BIPM to co-ordinate, in collaboration with the LGC, the continuation of the ongoing CCQM-P20 comparison investigating the characterization of organic substances for chemical purity. For this round, designated CCQM-P20.f, the LGC sub-divided a sample of purified digoxin, which was sourced from a commercial supplier and used as supplied, into individual units each containing 500 mg of the bulk material. The digoxin source material was stated by the supplier to "correspond with the requirements of current Ph. Eur.; JP; IP and USP".

The individual units consisted of amber glass storage vials (5 ml capacity) which were sealed with an inert rubber insert and crimped with an aluminium cap. Two hundred units were shipped to the BIPM who investigated and, where possible, identified the minor components present in the material. The BIPM characterized the homogeneity and stability of all identified minor components of the material and shipped the material to the study participants.

The mass fraction content of digoxin was assessed by the BIPM to be greater than 975 mg/g for the material, and the homogeneity and stability of the sample was confirmed to be suitable for the purposes of the comparison. The results reported by the study participants are the subject of this report.

The CCQM-P20 comparison 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. The overall purpose of the CCQM-P20 comparison is to investigate current practice for the assignment of mass fraction content 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 as well as to validate methods for use in planned CCQM Key Comparisons in this area.

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

Characterization of the comparison sample

Digoxin was selected for this round of the CCQM-P20 series because it:

- is an important analyte in clinical chemistry, was not available as a pure substance Certified Reference Material at the commencement of the study and is of specific interest within the framework of ongoing activities of the Joint Committee on Traceability in Laboratory Medicine (JCTLM)
- provided a significant analytical challenge and was representative of a structural class of organic compounds that had not been investigated in previous rounds
- could be provided in an amount that permitted sufficiently detailed investigation

The structure of digoxin is shown in Figure 1. The structures of a number of related compounds referred to in this report are shown in Annex 1. The compound provided without doubt the most significant analytical challenge thus far within the CCQM-P20 comparison series. Its size and structural complexity made it unsuitable for analysis by gas chromatography or differential scanning calorimetry. It was also the first CCQM-P20 analyte that contains centres of stereogenicity (chirality). As an indicator of one of the analytical challenges presented by digoxin, there are theoretically 2^{21} ($> 2 \times 10^6$!) stereoisomers of digoxin and an even larger number of regioisomeric forms. In addition, aglycones and related structure glycones comprised of the digoxin aglycone (digoxigenin) attached to carbohydrate chains of different length and structure, were also known to be potentially present as impurities.

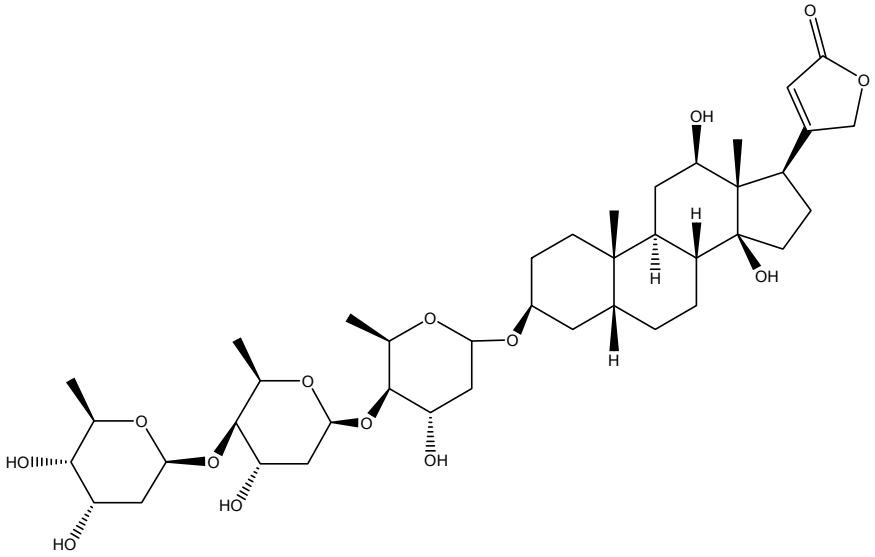


Figure 1 – Structure of digoxin

Digoxin is a white crystalline powder with a reported melting point of 249 °C. It has limited solubility in water and alcoholic solvents and is sparingly soluble in non-polar organic solvents. It is a widely used medication for the treatment of various heart conditions that cannot be controlled by other medications. Adverse, potentially fatal, drug reactions are possible because of its narrow therapeutic index. As a result of its widespread use, narrow efficacy range and the potential for fatal adverse reactions, it is an important analyte in clinical testing programmes and in medical laboratory proficiency testing schemes. No high-purity certified reference material (CRM) for digoxin was available at the start of the comparison, although reference substances are available from the U.S. and European pharmacopoeias.

Homogeneity studies

The homogeneity of the study material was assessed by use of two methods, both using high performance liquid chromatography and with either tandem mass spectrometry detection (LC-MS/MS) or diode array UV-detection (LC-UV). Both methods allowed for simultaneous determination of digoxin and a range of related structure steroid glycosides, but the LC-MS/MS method was more sensitive and displayed better repeatability compared to the LC-UV method. The uncertainty contributions due to the inhomogeneity of each analysed component was evaluated by an ANOVA approach. This approach provided an estimate of the variation due to inhomogeneity at a stated sampling size both between and with sample units. The inhomogeneity of the digoxin content of the material was estimated from combination of the individual assessments of the uncertainty contributions due to the between-bottle inhomogeneity of each of the minor components.

Acceptable uncertainties due to within-unit and between-unit inhomogeneity were observed for the major component digoxin in the candidate study material when using the LC-UV method under repeatability conditions. Where minor components were identified and reference standards were available the more repeatable LC-MS/MS method was used to perform the homogeneity assessment. For unidentified impurities only the LC-UV method could be used for the homogeneity assessment. The study sample was assessed as being appropriate for use in CCQM-P20.f for the evaluation of inherent impurities present at mass fraction levels of 1 mg/g or higher when a sample size greater than 2.5 mg was used for analysis of the material. In four cases where authentic standards were available, an external calibration approach was used to provide the quantitative estimate of the amount of each identified minor component. External calibration should also have allowed for the detection of significant changes in homogeneity due to variations in the levels of other impurities such as water and residual organic solvent which are not directly determined by LC methods. The fact that no such significant change was observed with any of the materials assessed by external calibration indicated that there was also a suitable degree of homogeneity in these miscellaneous impurities within and between the sample batch units. Direct studies of the water content and residual organic solvent content of the material confirmed this assessment.

One component, identified as β-acetyl digoxin, displayed a higher apparent level of inhomogeneity than the other minor components. This was ascribed to *in situ* hydrolysis and/or rearrangement of the acetyl substituent under the sample preparation and analysis conditions rather than inherent inhomogeneity within the study sample. To minimize the influence of breakdown of the β-acetyl digoxin component participants were advised to analyse solutions of the study sample as soon as possible after their preparation and to discard solutions that were more than 24 h old.

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. Samples were stored at the selected temperatures over 8 weeks, with units transferred to reference temperature storage at 2-week intervals. Trend analysis of the data obtained by LC-UV analysis of the test samples indicated no significant

change in composition of digoxin or of the minor UV-active components over this time at any of the test temperatures.
The effect of temperature on water content (as measured by Karl Fischer assay) and volatile organic content (as measured by GC-MS analysis), which together made up the major non-UV active impurities found by the co-ordinating laboratory in the sample, was also investigated. No significant changes were observed after storage at 4 °C or 22 °C. There was evidence of a reduction in volatile organic content after prolonged storage at 40 °C. On the basis of these studies it was concluded that the material was suitably stable for short-term transport at ambient temperature, provided the sample was not exposed to temperatures in excess of 40 °C, and for longer term storage at 4 °C. Consistent with the results of the homogeneity study, there was apparent evidence of instability in the case of the minor component β-acetyl digoxin but this was ascribed to partial hydrolysis under the conditions used for LC-UV analysis, as observed previously for the homogeneity study, rather than instability of the material.

Sample distribution

One unit of the study sample, containing a minimum of 500 mg of material, was distributed to each participant. Participants were asked to sign and return a form acknowledging receipt of the samples and to advise the co-ordinator if any damage had occurred to the container or the vials containing the study samples during shipping. 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. No problems were reported with any of the comparison samples.

Quantities and Units

Participants were required to report the mass fraction of the major component, digoxin, in both materials. It was recognized that some measurement methods could provide purity estimates expressed as related quantities (e.g. amount of substance fraction) but study participants were asked to correct their results in this event.

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

Participants were encouraged to provide, where possible, 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 digoxin 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. Compliance with this recommendation was important for participants using a mass balance or summation of impurities **(100-x)** method to determine digoxin content, or those who wished to quantify the mass fractions of the minor components of each material.

Reported Mass Fraction Content of Digoxin in CCQM-P20.f

The estimates reported by the study participants for digoxin content of CCQM-P20.f are summarized in Table 1 and plotted in Figure 2.
USP reported results for the materials using both a USP Digoxin Assay and a USP Digoxin Related substances assay. The estimates reported by the study participants for digoxin content of CCQM-P20.f are summarized in Table 1 and plotted in Figure 2.

All but two participants used a formal mass balance approach based on high-performance liquid chromatographic separation of the digoxin from related structure impurities with supporting methods for other classes of impurity to obtain an estimate of the total impurity content, and by subtraction the mass fraction content of digoxin. The DGKL reported the digoxin content based solely on the total related structure impurity content as quantified by HPLC-UV and explicitly noted that this value would overestimate the digoxin content if a significant level of impurities not detectable by LC-UV were present.

The NRC-INMS was the only participant to rely solely on quantitative nuclear magnetic resonance (QNMR) to assign the digoxin content. However other participants used NMR as a contributing method to their overall characterization and two explicitly noted that due to the complex profile of related structure impurities in the sample it was not possible to identify a NMR signal that was unique to digoxin and therefore they could not obtain a reliable QNMR estimate of digoxin content by this approach. A contribution due to an undetected overlap of the digoxin signal selected for quantification with signal(s) due to one or more impurities of related structure may explain the relatively high value for digoxin content reported by NRC-INMS.

Participant	Digoxin content (mg/g)	Standard uncertainty (mg/g)	Coverage factor (95 % confidence)	Expanded Uncertainty at 95 % confidence (mg/g)
NMIA	979.0	6.0	2.1	12.0
BIPM	979.6	0.65	2.0	1.3
NIST	980.2	0.9	2.0	1.8
NMIJ	982.7	2.4	2.0	4.8
USP (Rel. Subst.)	983.6	0.42	2.0	0.84
USP (Direct Assay)	983.9	3.7	2.0	7.4
CENAM	984.7	4.3	2.0	8.6
LGC	984.8	2.0	2.0	4.0
NIM	985.0	3.0	2.0	6.0
NIMT	985.9	1.4	2.0	2.8
DMS	987.6	0.08	2.37	0.18
NRC-INMS	988.3	1.2	2.8	3.4
DGKL *	989.2	2.24	2.57	5.8
MP	990.3	0.27	2.0	0.5

Table 1 – Digoxin content estimates for CCQM-P20.f

*** DGKL submission reported LC-detectable impurities only**

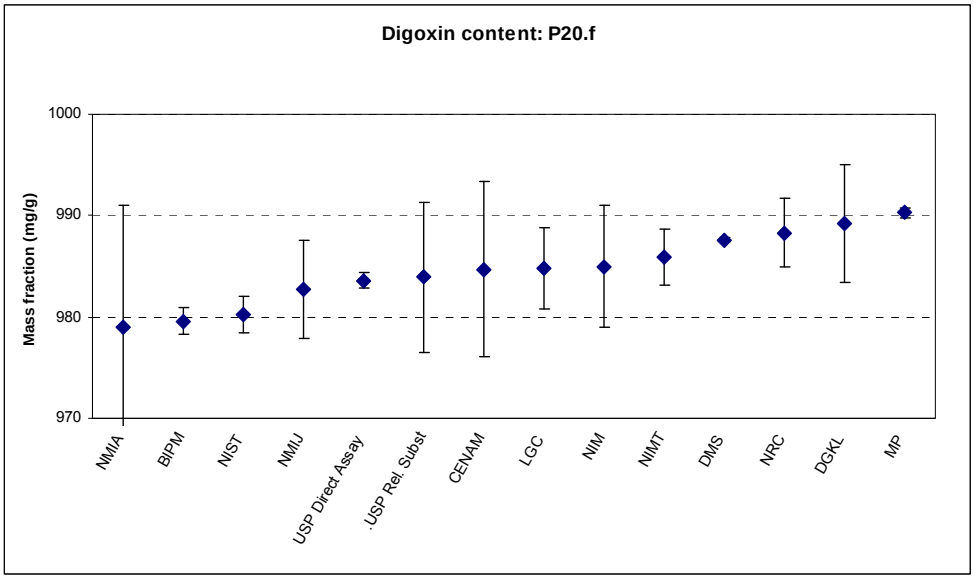


Figure 2 Mass fraction of Digoxin in CCQM-P20.f material (with expanded uncertainties)

Method Summaries and Measurement Equations

Lab ID	Measurement Equation	Method Summary
NMIA	Digoxin (mg/g) $= (100\ \%\text{-}I_{\text{"HPLC-all"}}) \times (100\%\text{-}I_{OT}) \times 10$ $I_{HPLC\text{-}all} = (R_{HPLC\text{-}raw} \times I_{HPLC\text{-}raw}) + I_{NR} + I_{ND}$ Where: $I_{HPLC\text{-}all}$ = total impurities by HPLC (percentage of normalized LC-UV response) assuming identical UV response factors $R_{HPLC\text{-}raw}$ = relative response factor of total impurities (assumed to be 1.0 with associated uncertainty) $I_{HPLC\text{-}raw}$ = total impurities (%) from LC-UV data I_{NR} = allowance for non-resolved impurities I_{ND} = allowance for impurities below detection limit I_{OT} = impurities not detectable by LC-UV	$I_{HPLC\text{-}raw}$ derived from normalized LC/UV peak area responses at 238 nm, solution conc. 2000 µg/ml. I_{OT} from water content by Karl Fischer titration and organic solvent impurities by ^{1}H NMR for thermogravimetric analysis (TGA) and elemental microanalysis were used to control the consistency of the result. ^{1}H NMR, elemental microanalysis and TGA were used to control the overall results.

Lab ID	Measurement Equation	Method Summary
BIPM	$w_{TP} = \frac{m_D}{m_{P20.f}} = \frac{m_D}{m_D + \sum m_i + \sum m_j}$ $= \frac{1}{1 + \left(\sum \frac{A_i}{R_i} \cdot \frac{1}{A_D} \right) + \left(\sum \frac{m_j}{m_D} \right)}$	The LC-UV peak area responses at 215 nm were used with a correction applied to take account of UV response factors relative to digoxin. Where minor components were identified by comparison with authentic standards and could be quantified by LC-MS, the LC-MS value was used in preference to the less sensitive and less selective LC-UV data. A correction for water content by Karl Fischer titration and residual solvent by GC-MS was also applied to the LC-UV data. ^{1}H NMR, elemental microanalysis and TGA were used to confirm no significant impurity sources had been overlooked.
NIST	$\frac{\text{mg digoxin}}{\text{g sample}} = \frac{\left(1000 - \sum \text{mg}_{\text{ICabsolute}}\right)}{1 + \sum \left(\frac{\text{Area}_{\text{IC}}}{\text{Area}_{\text{digoxin}}} \right) \left(\frac{\text{RF}_{\text{IC}}}{\text{RF}_{\text{digoxin}}} \right) \left(\frac{\text{MM}_{\text{IC}}}{\text{MM}_{\text{digoxin}}} \right)}$	Mass fraction estimates: related structure components by LC-UV (UV area response at 220 nm), inorganic components by X-ray fluorescence spectrometry, water by ^{1}H NMR and volatile organics by headspace GC-MS. Confirmatory methods included LC-MS, LC-ELSD, Karl Fischer titration and loss on drying at 105 °C.

Where:

w_D = mass fraction (g/g) of digoxin in P20.f sample

m_D = mass (g) of digoxin in a P20.f test sample

$m_{P20.f}$ = mass (g) of a P20.f test sample

m_i = mass (g) of individual LC-UV detectable minor components in a P20.f test sample

m_j = mass (in g) of components in the test sample not quantified by LC-UV. For P20.f these included related impurities quantified by LC-MS, water content by Karl Fischer titration and organic solvents quantified by GC-MS.

A_i = Normalized UV peak area of minor component *i*

A_D = Normalized UV peak area of digoxin

R_i = UV response factor (on a mass basis) of component *i* relative to digoxin

NOTE: Mass fraction (mg/g) of digoxin in P20.f sample $= w_D \times 1000$

$\text{mg}_{\text{IC absolute}}$ = mass in mg of impurity components is the mass in mg of known impurity components determined relative to mass of sample (here, the inorganic and identified glycoside impurities), Area_{IC} and $\text{Area}_{\text{digoxin}}$ are the peak areas of the impurity and digoxin, RF_{IC} and $\text{RF}_{\text{digoxin}}$ are the molar response factors for the impurity and digoxin, and MM_{IC} and $\text{MM}_{\text{digoxin}}$ are the molar masses for the impurity and digoxin. For $^{1\text{H}}$NMR assignments, the RF_{IC}s are proportional to the number of equivalent hydrogens. For LC/UV_{220} assignments, the RF_{IC}s are assumed to follow the distribution of the RFs determined for glycoside standards. The molar masses of the unidentified glycoside impurities were assumed to follow a distribution defined by the molar mass of digoxin and the available evidence for the minimum number of sugar moieties present.

Lab ID	Measurement Equation	Method Summary
NMIJ	$x_p = 1 - \Sigma x_i(\text{HPLC}) - x(\text{KF}) - x(\text{ND})$	Mass fraction from LC-UV peak area response at 220 nm and LC-MS data. Water content from Karl Fischer titration with cross check by TGA
USP	(1) Direct Assay Value for P20.f test material : Response factor for P20.f sample / Response factor for USP Digoxin RS where: Response factor = UV area response / concentration (2) Related Substances Method: Individual impurity level (mg/g) = $\frac{C_{\text{ref sol}}}{C_{\text{test sol}}} * \frac{r_{\text{imp}}}{r_{\text{ref sol}}} * 1000$ Where: $C_{\text{ref sol}}$ = concentration of digoxin reference solution* $C_{\text{test sol}}$ = concentration of P20.f test solution r_{imp} = peak area of the impurity in P20.f test solution $r_{\text{ref sol}}$ = peak area of digoxin in reference solution * for digoxigenin quantification was carried out against a digoxigenin reference solution rather than digoxin Digoxin content calculated by subtraction including the volatiles (in mg/g) found by loss on drying analysis.	Direct Assay: LC-UV method using the ratio of response factor for digoxin in CCQM-P20.f sample (UV response area at 218 nm/ concentration) to the response factor for USP digoxin reference standard at a similar concentration. Corrected for volatiles content as determined by loss on drying at 105 °C. Related Substances: UV peak area response relative to a digoxin reference standard solution equivalent to the 0.1 % at 218 nm (or digoxigenin reference solution for determination of digoxigenin content) corrected for volatiles by loss on drying at 105 °C.
CENAM	$\text{Purity}\% = 100 - \text{moisture}\% - \%\,\text{Chrom imp}$ $\%\,\text{Chrom imp} = \left(\frac{A_{total} - A_D}{A_{total}}\right) * 100$ Moisture % = Moisture content (g/100g) Chrom imp = impurities obtained by HPLC A_{total} = Total chromatographic area A_D = chromatographic area for digoxin	Impurities identified by LC-UV at 220 nm. Digoxin estimate was obtained by subtraction.
LGC	$w_{dig} = P_{tot} = \frac{1}{1 + \frac{\sum(area_{UVimp} \times R_{imp})}{area_{dig}}} \times \left[1 - \left(\frac{m_{water}}{m_{P20f}} + \frac{m_{IR}}{m_{P20f}}\right)\right]$ w_{dig} = mass faction (g/g) of digoxin in P20.f sample R_{imp} = UV response factor of minor components detectable by UV relative to digoxin $area_{UV\ imp}$ = peak area of components detectable by UV $area_{dig}$ = peak area of digoxin $area_{tot}$ = total area after correction for response factors m_{water} = water in sample by Karl Fischer titration m_{IR} = inorganic residue in sample by ICP-OES	Combination of mass fraction estimates obtained by LC-UV (normalized UV peak area responses at 220 nm), after subtraction of the water content by Karl Fischer titration and inorganic content by ICP-OES. LC-MS confirmed the identity of the main component and was used as a cross check for the LC-UV data.

Lab ID	Measurement Equation	Method Summary
NIM	$X_{(digoxin)}=X_i-X_{water}$ $X_i = \frac{fiAi}{\sum(f_i A_i)} \times 100\%$	Mean of two methods based on LC-UV with detection at 219 nm and 207 nm, after correction for water content.
NIMT	$W_{digoxin} = \frac{A_{digoxin} \times 1000}{A_{total}} - M_{water}$ $A_{digoxin}$ = normalized area response for digoxin A_{total} = total area M_{water} = mass fraction of water by Karl Fisher titr	From normalized LC-UV peak area response at 217 nm., after correction for water content.
DMSc	Content (%) = $\frac{\text{Area of each component}}{\text{Total area}} \times 100$ Content (mg/g) = Content (%) × 1000	From normalized LC-UV peak area response at 218 nm
NRC	$P_{dig} = \frac{I_{dig}}{I_{cal}} \cdot \frac{\rho_{cal}}{\rho_{dig}} \cdot \frac{MW_{dig}}{MW_{cal}} \cdot \frac{m_{cal}}{m_{dig}} \cdot P_{cal}$ P_{dig} = purity of digoxin I_{dig} = integrated signal area digoxin ρ_{dig} = number of protons integrated for digoxin I_{cal} = integrated signal area calibrator ρ_{cal} = number of protons integrated for calibrator MW_{dig} = molar mass digoxin MW_{cal} = molar mass calibrator m_{dig} = weight of digoxin m_{cal} = weight of calibrator P_{cal} = purity of calibrator	Benzoic acid (NIST SRM 350b) used as internal calibrator. Peak areas for a 1H signal at 5.91 ppm (for digoxin) and a 2H signal at 8.02 ppm (for benzoic acid) were used, without correction for satellites, to determine I_{dig}/I_{cal}.
DGKL	C [mg/g] = Area (Dig) * rf (Dig) / [Area (Dig) * rf(Dig) + Area (X) *rf(X) + Area (Y) * rf(Y)] ***Dig = Digoxin*** ***X*** = unidentified impurity 1 ***Y*** = unidentified impurity 2 ***Z*** = unidentified impurity 3	From normalized LC-UV peak area response in range 200 nm to 240 nm. The potential for the presence of additional impurities not detectable by LC-UV was noted but could not be investigated by the laboratory.
MP		From normalized LC-UV peak area response in range 200 nm to 400 nm with corrections for total volatiles and ashing residue

Measurement Uncertainty Budgets

Lab ID **Measurement Uncertainty Budgets**

NMIA

$$u_{Purity} = Purity \times \sqrt{\left(\frac{u_{HPLC\text{-}all}}{I_{HPLC\text{-}all}}\right)^2 + \left(\frac{u_{OT}}{I_{OT}}\right)^2}$$

Where

$$u_{HPLC\text{-}all} = \sqrt{\left(P_{HPLC} \times \sqrt{\left(\frac{u_R}{R_{HPLC\text{-}raw}}\right)^2 + \left(\frac{u_{HPLC\text{-}raw}}{P_{HPLC\text{-}raw}}\right)^2}\right)^2 + (u_{NR})^2 + (u_{ND})^2}$$

The major components of the uncertainty budget are:

- Standard deviation of the raw HPLC results (for 10 samples run in duplicate)
- U_{ND} = Standard uncertainty of non-detected impurities
- U_{NR} = Standard uncertainty of non-resolved impurities
- U_R = The uncertainty associated with the HPLC correction factor (R, assigned as 1)
- U_{OT} = Standard uncertainty of "other" impurities (non volatiles, solvent and water)

BIPM

Uncertainty budget for CCQM-P20.f

Uncertainty component	$x_{i\ (mg/g)}$	$u(x_i)$
Water	1.10	0.18
Ethanol	2.50	0.15
Dichloromethane	1.00	0.10
Toluene	0.10	0.02
Gitoxin	0.63	0.02
Digitoxin	0.63	0.01
Unidentified UV-active impurity 1	2.37	0.28
Unidentified UV-active impurity 2	3.63	0.42
Unidentified UV-active impurity 3	1.81	0.22
Unidentified UV-active impurity 4	1.92	0.23
Digoxigenin tetradigitoxide	3.16	0.05
β-Acetyl digoxin	0.53	0.03
Combined minor UV-active impurities (including digoxigenin)	1.00	0.03
Digoxin content	979.60	0.65
Expanded uncertainty U (C.I.95 %, k = 2)		1.30

Lab ID **Measurement Uncertainty Budgets**

NIST

u (mg $_{\text{IC absolute}}$) = 0.2 mg
u (mg impurities/gm digoxin, ^{1H}NMR) = 0.3 mg
u (glycoside impurity area / digoxin area, LC/UV$_{220}$) = 0.1 mg
u (glycoside impurity RF, LC/UV$_{220}$) = 0.4 mg
u (glycoside impurity molar mass, LC/UV$_{220}$) = 0.6 mg

The above uncertainty components were evaluated via Monte Carlo propagation of uncertainty estimates for individual impurity components. The individual component uncertainties were determined by repeated experiment and/or expert opinion for identified impurities. They were extrapolated from experimental evidence and expert opinion for the unidentified chromatographic peaks.

NMIJ

Impurity	x / (mg/g)	$u(x)$ / (mg/g)
HPLC	16.92	2.16
KF	0.41	0.049
Not detected component	0	1.0
Total	17.33	2.38

USP

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

CENAM

$$U_p = \sqrt{(uc_{inj})^2 + (uc_{sample})^2 (uc_{IT})^2 + (uc_{ss})^2 + (uc_{moisture})^2 + (uc_{\text{int}})^2}$$

uc $_{inj}$ =uncertainty by injection
uc $_{sample}$ =uncertainty by sample preparation
uc $_{IT}$ =Total impurities
uc $_{ss}$ =uncertainty by difference between sub samples
uc $_{moisture}$ =uncertainty by moisture determination
uc $_{int}$ =uncertainty by integration

Lab ID **Measurement Uncertainty Budgets**

LGC

$$u_{w_{dig}} = \sqrt{\sum (u_{UV\ imp})^2 + (u_{org\ non-UV\ imp})^2 + (u_{IR})^2 + (u_{water})^2}$$

with v_{eff} calculated from the Welch-Satterthwaite equation

NIM

Uncertainty includes two parts: A kind and B kind uncertainty

uncertainty	A	Assess with RSD of nine measurement	0.21 %
	B	Assess different response of impurity	0.22 %

Contributing uncertainty elements include:

(1) Uncertainty of individual impurities assessed with the formula.

$$u_{B-i} = \frac{A_{i\max\lambda} - A_{i\lambda}}{\Sigma A_{i\lambda}}$$

$u_{B\text{-}i}$: uncertainty of every impurity

$A_{i\ max\ \lambda}$:peak area of impurity of i at max absorbed wavelength of impurity i (mAu·s)

$A_{i\ \lambda}$: peak area of impurity of i at max absorbed wavelength of digoxin (mAu·s)

Overall uncertainty in digoxin impurities (u_1) :

$$u_1 = \sqrt{\sum u_{B-i}^{\ 2}}$$

(2) Uncertainty of instrument linearity

(3) Uncertainty of solution preparation

NIMT

Source of uncertainty	Typical values	Standard uncertainty	Degree of freedom	Type of uncertainty
Normalized response of digoxin (mg/g)	987.7	0.7819	17	A
Water content estimate (mg/g)	1.8	0.78	3	A
Resolution of LC	1.0	0.866	Large	B

u_c = 1.4 mg/g

Lab ID **Measurement Uncertainty Budgets**

DMS

$$\text{Standard Uncertainty} = \sqrt{\left(\frac{SD^2}{n}\right)}$$

Expanded Uncertainty = 2.365 x Standard Uncertainty

SD = Standard deviation of content of Digoxin

n = Number of sample

2.365 = t-value (df = 7, 95% confidence)

NRC

$$u_c(P_{dig}) = P_{dig}\sqrt{\left(\frac{u(I_{dig}/I_{cal})}{I_{dig}/I_{cal}}\right)^2 + \left(\frac{u(MW_{dig})}{MW_{dig}}\right)^2 + \left(\frac{u(MW_{cal})}{MW_{cal}}\right)^2 + \left(\frac{u(m_{dig})}{m_{dig}}\right)^2 + \left(\frac{u(m_{cal})}{m_{cal}}\right)^2 + \left(\frac{u(P_{cal})}{P_{cal}}\right)^2}$$

Component (units)	x_i	$u(x_i)$	$u(x_i)/x_i$ (%)
$\boldsymbol{P}_{dig}$ (mg g^{-1})	988.3	1.2[a]	0.12
$\boldsymbol{MW}_{dig}$ (g mol^{-1})	780.938	0.003	0.0004
$\boldsymbol{MW}_{cal}$ (g mol^{-1})	122.121	0.001	0.0008
$\boldsymbol{\rho}_{dig}$	1	0	0
$\boldsymbol{\rho}_{cal}$	2	0	0
$\boldsymbol{m}_{dig}$ (mg)[b]	16.6441	0.0003	0.0018
$\boldsymbol{m}_{cal}$ (mg)[b]	6.9050	0.0003	0.0043
$\boldsymbol{P}_{cal}$	0.999978	0.000022	0.0022
Combined uncertainty	1.2		
Expanded uncertainty $Uc_{95\,\%}$ (k = 2.8)[c]	3.4		

[a] standard deviation of the mean of five independent determinations
[b] minimum weights of calibrator and digoxin in series of replicates
[c] $k = t_{(0.05, 4)}$

DGKL

Major components of uncertainty:

1) Dispersion of peak area measurements

→ calculated from 5 repetitive measurements

2) Response factors for the recording in a range from 200 nm to 240 nm

→ Since the identity of the minor components is not known, the response factors have been set to =1.

MP

Not reported

Impurity Profile of CCQM-P20.f

All submissions except the NRC QNMR report provided information on the minor components (impurity content) of the study material. As was to be expected given the biological origin and complex structure of digoxin, which is prepared commercially by purification of plant extract obtained from the *digitalis* species, numerous significant (> 0.1 % by relative LC-UV response) related structure impurities were present in the study sample. A representative chromatogram of the digoxin comparison sample (reproduced from the USP submission) with structural assignments of the minor components identified by the USP is shown in Figure 3.

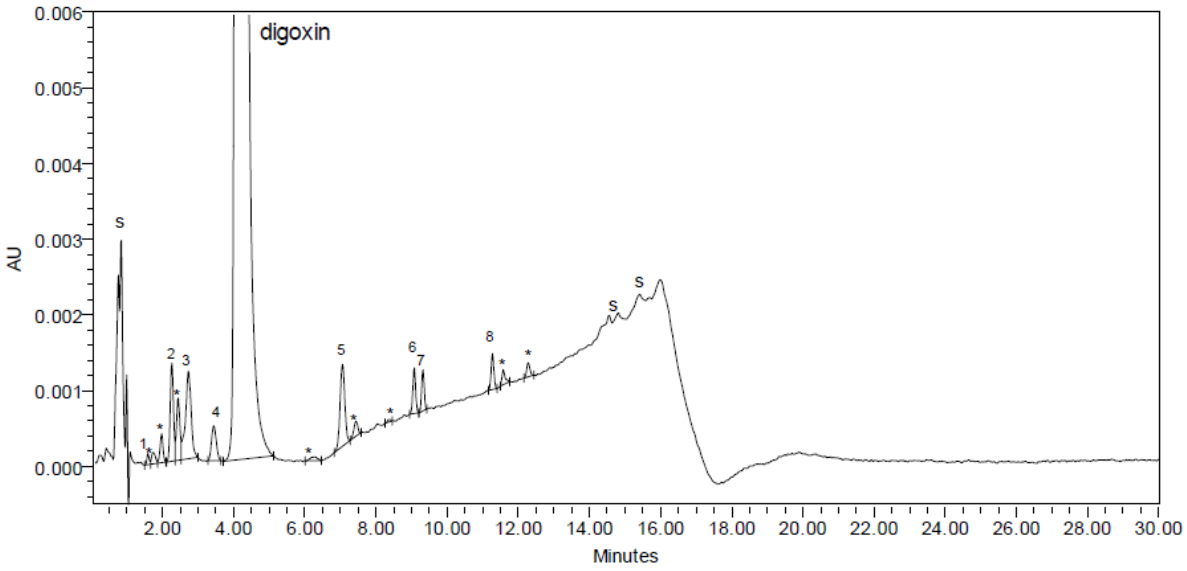


Figure 3 Representative LC-UV chromatogram for CCQM-P20.f

Compounds identified in the comparison material by more than one participant included digitoxin, gitoxin, digoxigenin, β-acetyl digoxin and digoxigenin tetradigitoxide.

Participants who identified some of the minor components reported in addition the presence of several unidentified UV-active materials. These impurities were all assumed to be steroid glycosides of related structure, either on the basis of the similarity of their UV spectra to that of digoxin or on LC-MS evidence or both. NIM made tentative structural assignments of a number of these components based on MS data and USP identified more of the minor components than any other participant through comparison with authentic standards.

Several participants (NMIA, NIST, BIPM, NIM, NMIJ, LGC) used analysis by LC-MS as well as LC-UV to provide further qualitative and quantitative information on the impurity content.

The total UV-active impurities, with structural assignments where available, by participant are given in Table 2. Several participants provided a listing, by elution time relative to the main component, of the main resolved impurities. A comparative summary of these impurities with identification and quantification where available, is given in Table 3.

Participant	Mass fraction estimate (mg/g) – total UV impurities	u (mg/g)	No. of impurities (UV and/or MS active)	Detection method(s)
NMIA	17.9	0.7	9	LC-UV and LC-MS
BIPM	15.7	0.6	10	LC-UV and LC-MS
NIST	13.2	0.7	n/r	LC-UV and LC-MS
NIMJ	16.9	2.2	33	LC-UV and LC-MS
USP	14.1	0.2	16	LC-UV
CENAM	15	4	n/r	LC-UV
LGC	14.6	1.4	13	LC-UV and LC-MS
NIM	14.4	3	11	LC-UV and LC-MS
NIMT	12.3	0.8	n/r	LC-UV
DMS	12.3	0.08	10	LC-UV
DGKL	10.8	2.2	3	LC-UV

Table 2 – Estimates of UV-active impurity content for CCQM-P20.f

Component (from Fig. 3)	Participant	Identified as:	Mass fraction estimate (mg/g)	Method
1	USP	Digoxigenin	0.08	**LC-UV @ 218 nm Comparison with RM**
	LGC	Digoxigenin	0.16	**LC-UV @ 220 nm Comparison with RM and LC-MS**
	NIST	Digoxigenin	0.13	**LC-UV @ 220 nm Comparison with RM and LC-MS**
	NMIJ	Digoxigenin	0.12	**LC-UV @ 220 nm Comparison with RM and LC-MS**
	NIM	Digoxigenin	N/R	LC-UV @ 220 nm. ID suggested by LC-MS
2	USP	Diginatin	2.12	**LC-UV @ 218 nm Comparison with RM**
	BIPM	Unknown 1	2.37	**LC-UV @ 220 nm**
	LGC	Impurity 3	1.59	**LC-UV @ 220 nm**
	NMIA	Digitoxin	2.7	**LC-UV @ 238 nm. Identity by LC-MS**
3	USP	Digoxigenin bisdigitoxoside	2.46	**LC-UV @ 218 nm Comparison with RM**
	NIM	Digoxigenin bisdigitoxoside	N/R	LC-UV @ 220 nm. Identity by LC-MS
	BIPM	Unknown 2	3.63	**LC-UV @ 220 nm**
	LGC	Impurity 4	1.99	**LC-UV @ 220 nm**
	NMIA	Impurity 2	1.9	**LC-UV @ 238 nm**
4	USP	Neodigoxin	1.11	**LC-UV @ 218 nm Comparison to RM**
	BIPM	Unknown 4	1.92	**LC-UV @ 220 nm**
	LGC	Impurity 7	1.91	**LC-UV @ 220 nm**
	NIM	Neodigoxin	N/R	**LC-UV @ 220 nm. Identity by LC-MS**
	NMIA	Gitoxin	1.3	**LC-UV @ 238 nm. Identity by LC-MS**

Table 3 – Estimates of UV-active impurity content for CCQM-P20.f (continued over page)

Table 3 (Continued) – Estimates of UV-active impurity content for CCQM-P20.f

Component	Participant	Identified as:	Mass fraction estimate (mg/g)	Method
5	USP	**Digoxigenin tetrakisdigitoxoside**	2.59	**LC-UV @ 218 nm Comparison to RM**
	BIPM	**Digoxigenin tetrakisdigitoxoside**	3.16	**LC-MS / LC-UV @ 220 nm Comparison to RM**
	NMIJ	**Digoxigenin tetrakisdigitoxoside**	4.8	**LC-MS / LC-UV@ 220 nm Comparison to RM**
	NIM	**Digoxigenin tetrakisdigitoxoside**	N/R	**LC-UV @ 220 nm. Identity by LC-MS**
	LGC	**Digoxigenin tetrakisdigitoxoside**	2.94	**LC-UV @ 220 nm. Identity by LC-MS**
	NMIA	Unknown 6	3.3	**LC-UV @ 220 nm**
6	USP	β-Acetyl digoxin	0.84	**LC-UV @ 218 nm Comparison to RM**
	BIPM	β-Acetyl digoxin	0.53	**LC-MS / LC-UV @ 220 nm Comparison to RM**
	LGC	β-Acetyl digoxin	0.49	**LC-UV @ 220 nm. Identity by LC-MS**
	NIM	α-Acetyl digoxin	N/R	
7	USP	Gitoxin	0.70	**LC-UV @ 218 nm Comparison to RM**
	NIST	Gitoxin	0.65	**LC-UV @ 220 nm MS Comparison with RM**
	BIPM	Gitoxin	0.63	**LC-MS / LC-UV @ 220 nm Comparison to RM**
8	USP	Digitoxin	0.67	**LC-UV @ 218 nm Comparison to RM**
	NIST	Digitoxin	0.67	**LC-UV @ 220 nm MS Comparison with RM**
	BIPM	Digitoxin	0.63	**LC-MS / LC-UV @ 220 nm Comparison to RM**
	LGC	Digitoxin	1.42	**LC-UV @ 220 nm Comparison to RM**
	NMIJ	Digitoxin	0.67	**LC-MS / LC-UV@ 220 nm Comparison to RM**
	NIM	Digitoxin	N/R	**LC-UV @ 220 nm. Identity by LC-MS**
	NMIA	Diginatin	0.7	**LC-UV @ 238 nm. Identity by LC-MS**

Other impurity components identified in CCQM-P20.f included water (quantified directly by Karl Fischer titration or as a component of total volatiles by loss-on-drying measurements) and residual organic solvents (identified directly by GC-MS or NMR or as a component of total volatiles by loss-on-drying measurements). The results reported by each participant are summarized in Table 4. NIST reported that loss-on-drying methods only measured water content of the material and did not in this case provide information on the residual solvent content. They observed that their headspace GC estimates for residual solvent did not alter after extensive preliminary drying of the material at 105 °C. This conclusion is supported by the observation that thermogravimetric analysis, carried out separately by NMIA, BIPM and NMIJ, also failed to detect significant volatile content. Estimates for total inorganic content of the material were obtained using X-ray fluorescence spectrometry (NIST), ICP-OES (LGC) or by residue after ignition (MP).

Component	Institute	Mass fraction content (mg/g)	Method	Details
Water	NMIA	1.9 (u = 0.5)	Coulometric KF	4 × 20 mg, 2 × 50 mg samples – direct addn.
	BIPM	1.1 (u = 0.2)	Coulometric KF	2 × 200 mg sample – oven transfer at 180 °C
	NIST	1.39 (u = 0.05)	^{1}H NMR	With cross check by loss on drying, Karl Fischer titration and TGA
	NMIJ	0.41 (u = 0.05)	Coulometric KF	110 mg, 170 mg sample – direct addition
	LGC	0.50 (u = 0.05)	Coulometric KF	1 × 320 mg sample – oven transfer at 110 °C
	NIM	1.3 (u = 0.4)	Coulometric KF	7 × 15 mg samples – direct addition
	NIMT	1.8 (u = 0.8)	Coulometric KF	N/A
Ethanol	BIPM	2.5 (u = 0.15)	GC-MS	Direct injection on thick-film carbowax column; confirmation by ^{1}H NMR
	NIST	2.9 (u = 0.3)	^{1}H NMR	Confirmation by headspace GC-MS
Dichloromethane	NMIA	1.1	^{1}H NMR	
	BIPM	1.0 (u = 0.1)	GC-MS	Direct injection on thick-film carbowax column; confirmation by ^{1}H NMR
	NIST	1.1 (u = 0.2)	^{1}H NMR	Confirmation by headspace GC-MS
Toluene	BIPM	0.1 (u = 0.1)	GC-MS	Direct injection on thick-film carbowax column; confirmation by ^{1}H NMR
	NIST	0.1 (u = 0.2)	^{1}H NMR	Confirmation by headspace GC-MS
Pyridine	NIST	0.02	GC-MS	
Acetone	NIST	0.01	GC-MS	

Table 4 – Estimates of water and volatile organic impurities (continued over page)

Component	Institute	Mass fraction content (mg/g)	Method
Total volatiles	NMIA	< LOD	TGA
	USP	2.26	Loss on drying for 1 h @ 105 °C under vacuum
	MP	0.84 (u = 0.00001)	Loss on drying for 1 h @ 105 °C under vacuum
Total inorganics	NMIA	< LOD	TGA
	MP	0.13	Residue on ignition @ 800 °C
	LGC	0.12 (u = 0.02)	ICP-OES
Si	NIST	0.18 (u = 0.06)	XRF spectrometry
Fe	NIST	0.12 (u = 0.04)	XRF spectrometry
Al, Ni, Cr	NIST	< 0.05	XRF spectrometry

Table 4 (continued) – Estimates of water and volatile organic impurities

Reference Value for Digoxin in CCQM-P20.f

An assignment of a reference value for the digoxin content of CCQM-P20.f based on the study results as a whole did not appear to be justified. There is evidence of a bias in some of the submissions due to incomplete resolution of related substance impurities from the main component peak, and in most of the results due to insufficient allowance for the significant levels of organic solvent residues present in the sample.
Several participants independently confirmed in follow-up studies undertaken after circulation of the study results that ethanol and dichloromethane were present in the sample at the levels reported by NIST and BIPM. NIST demonstrated that these components were not measured by simple loss on drying methods or other techniques for estimating total volatile content, but required use of a more selective test such as GC-MS or NMR. When these latter methods were used, good agreement was found between the estimates obtained for residual ethanol, dichloromethane and toluene in the study sample.
Following the initial discussion of the results at the April 2008 meeting of the CCQM OAWG, the study co-ordinator was asked to propose a mass fraction content with associated uncertainty for the digoxin content of the CCQM-P20.f study sample based on summation of a “consensus estimate” for each of the independent classes of impurities identified in the comparison material derived from the ensemble of results submitted by the comparison participants.
The calculation of the proposed value was discussed at the November 2008 OAWG meeting and approval was given to assign a reference value for the comparison based on this approach.
Four orthogonal classes of impurity were detected in the CCQM-P20.f study sample:

- structurally related compounds
- water
- volatile organic solvent
- non-volatiles/inorganics

The measurement equation (1) used to assign the mass fraction content of digoxin (in mg/g) is:

$$w_{Digoxin} = H_{Digoxin} * (1000 - [w_{Rel.Subst} + w_{Water} + w_{Org.Solv.} + w_{NonVol.}]) \quad \textit{(Eqn. 1)}$$

where:

$w_{Digoxin}$ = mass fraction of digoxin in CCQM-P20.f

$H_{Digoxin}$ = homogeneity correction factor (for between sample inhomogeneity of digoxin at the recommended minimum sample size of CCQM-P20.f). Assigned value = 1 with associated uncertainty

$w_{Rel.Subst.}$ = mass fraction of digoxin-related minor components in CCQM-P20.f in mg/g

w_{Water} = mass fraction of water in CCQM-P20.f in mg/g

$w_{Org.Solv.}$ = mass fraction of volatile organic solvents in CCQM-P20.f

w_{NonVol} = mass fraction of non-volatiles/inorganics in CCQM-P20.f

Note: Units for mass fraction content (w) of each contributor are mg/g throughout.

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

$$u_{w_{Digoxin}} = \sqrt{(u_{w_{Rel\ Subst}})^2 + (u_{w_{Water}})^2 + (u_{w_{OrgSolv.}})^2 + (u_{w_{NonVol.}})^2 + (u_{H_{Digoxin}})^2} \quad \textit{(Eqn. 2)}$$

Consensus Estimates of Individual Impurity Classes

1. Related Structure components

There was reasonable agreement between most participants as to the identity and amount of UV-active impurities, despite the complex nature of the related substance impurities present in the study sample and the difficulties they posed for LC-UV analysis. The estimates for total UV-active impurities by participant are given in Table 2 and are plotted in Figure 4 below.

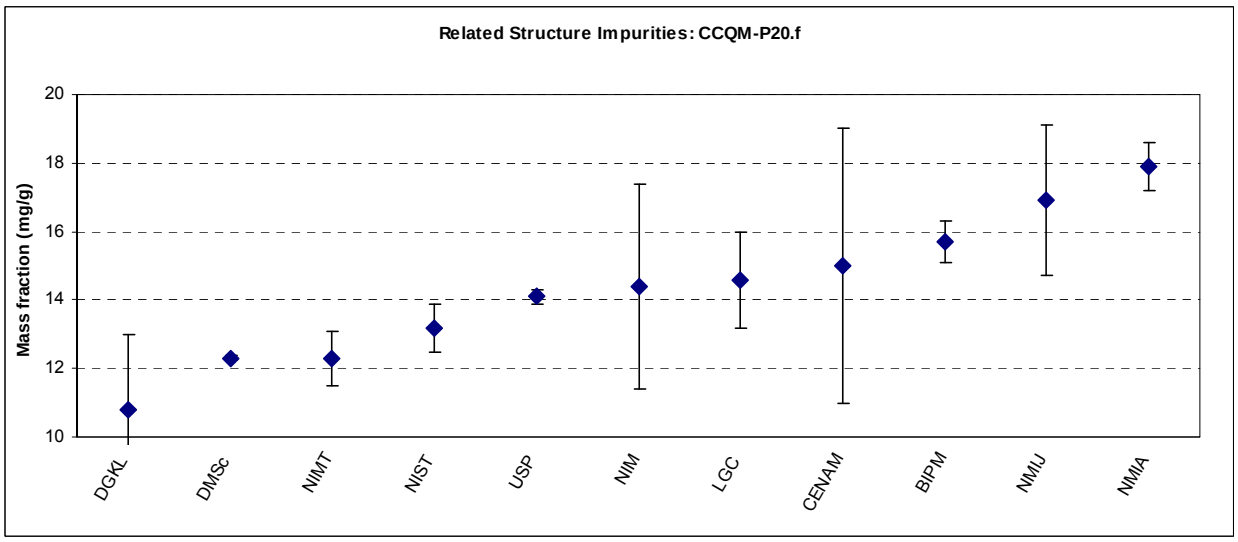


Figure 4 Mass fraction of total related structure impurities in CCQM-P20.f material (with expanded uncertainties corresponding to 95 % coverage interval)

In the absence of evidence of obvious outliers or significant difference between the mean and median, the participant mean was used as the estimate of the related structure impurity content and the standard deviation of the mean for the eleven submissions as the standard uncertainty associated with this value.

Mean = $w_{Rel.Subst.}$

= 14.3 mg/g

SD = 2.09 mg/g

SD_{mean} = SD/√ 11

= $u_{w\,Rel.Subst.}$

= 0.63 mg/g

2. Water content

Only the results obtained by participants using methods that provide a direct measurement of water content - Karl Fischer titration or NMR - were used to assign an overall estimate for the water content of CCQM-P20.f. The mass fraction estimates for water content reported by each participant using these techniques are summarized in Table 4 and plotted in Figure 5.

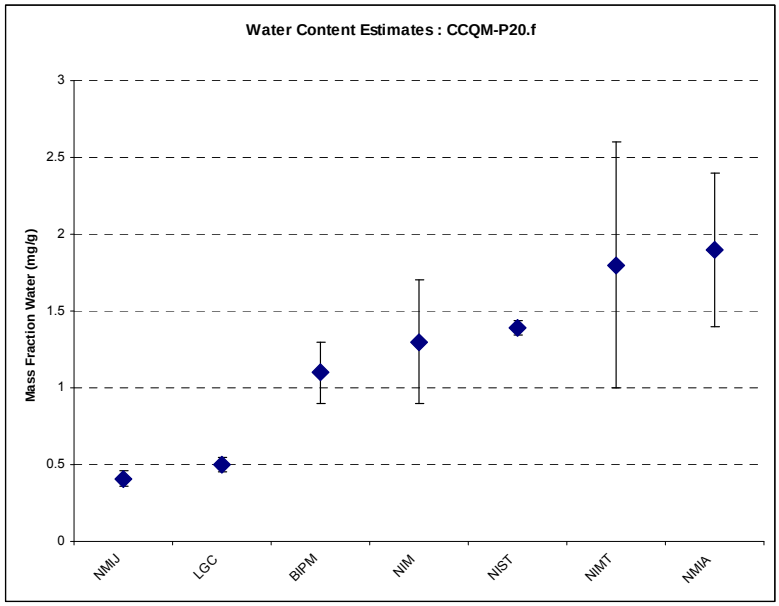


Figure 5 **Mass fraction of water in CCQM-P20.f material (with expanded uncertainties corresponding to 95 % coverage interval)**

The median value (1.3 mg/g) was selected as the water content best estimate w_{Water}.

Given the relatively wide variation in results and the limited amount of data used to assign each value, most participants relied on only one or two data points to make their assignment. The robust standard deviation (MADe) (rather than the robust standard deviation of the mean, MADe/√n) was selected as a more appropriate estimate of the associated standard uncertainty.

Median = w_{Water}

= 1.3 mg/g

MADe = Median Absolute Deviation from Median (MAD) * 1.483

= $u_{wWater.}$

= 0.74 mg/g

3. Volatile solvent content

Two participants identified the presence of ethanol, chloroform and toluene residues in the material at significant levels. The presence of these impurities was confirmed in characterization information provided by the source material supplier. A third laboratory identified chloroform only. The mass fraction estimates for solvent residues reported by each participant are also summarized in Table 4.

For calculation purposes the mean value of the reported results and the more conservative uncertainty estimate for each contributor were used to obtain the estimates of $w_{Org\ Solv.}$ and its associated standard uncertainty.

$w_{Org.Solv.}$ = 2.7 + 1.1 + 0.1 mg/g

= 3.9 mg/g

$$u_{w_{OrgSolv}} = \sqrt{(u_{w_{Ethanol}})^2 + (u_{w_{Dichloromethane}})^2 + (u_{w_{Toluene}})^2}$$

$$= \sqrt{0.3^2 + 0.2^2 + 0.02^2}$$

= 0.36 mg/g

4. Non-volatiles/inorganic residues

Participants investigated a variety of methods (TGA, ash residue, elemental microanalysis) for obtaining a global estimate of non-volatile content of the study sample but none detected significant levels (< 0.3 % on a relative mass fraction basis) of this general class of impurity. Two participants used more sensitive methodologies (XRF spectrometry, ICP-OES) that were able to detect and provide quantitative estimations for some individual inorganic components. The mass fraction estimates for inorganic materials present at greater than 0.1 mg/g are reported as the final entries in Table 4.

Given the lack of evidence from other techniques for the presence of total non volatile components at greater than 0.3 mg/g, the mass fraction estimate for contributions due to this class of impurity was assigned as a rectangular distribution in the range 0.1 mg/g to 0.3 mg/g. This gives the following assigned values for calculation purposes:

$w_{NonVol.}$ = 0.2 mg/g

$u_{w\,Non\,Vol.}$ = 0.06 mg/g

5. Homogeneity factor

The uncertainty contribution due to inhomogeneity of the total impurity content of the material was estimated at equivalent to 0.16 mg/g, based on the results of single factor ANOVA analyses of each of the UV-active impurities present in the material, obtained from homogeneity studies by LC-UV of the study material undertaken during the material characterization.

Digoxin content reference value assignment

The values assigned from the combined results to the four classes of impurities are:

Impurity class	Content in CCQM-P20.f (mg/g)	*u* (mg/g)
Related structure organics	14.3	0.63
Water	1.3	0.74
Volatile organics	3.9	0.36
Non-volatiles/inorganics	0.2	0.06

When substituted into the equations (1) and (2) described previously, the calculated values for the reference value for the comparison are:

$$w_{Digoxin} = H_{Digoxin} * (1000 - [w_{Rel.Subst} + w_{Water} + w_{Org.Solv.} + w_{NonVol.}]) \text{ mg/g}$$
$$= 1.0 * (1000 - [14.3 + 1.3 + 3.9 + 0.2]) \text{ mg/g}$$
$$= 980.2 \text{ mg/g}$$

$$u_{w_{Digoxin}} = \sqrt{(u_{w_{Rel\ Subst}})^2 + (u_{w_{Water}})^2 + (u_{w_{OrgSolv.}})^2 + (u_{w_{NonVol.}})^2 + (u_{H_{Digoxin}})^2}$$
$$= \sqrt{(0.63)^2 + (0.74)^2 + (0.36)^2 + (0.06)^2 + (0.16)^2} \text{ mg/g}$$
$$= 1.0 \text{ mg/g}$$

Figure 6 shows the individual participant results plotted against the proposed reference value (solid red line) and its associated expanded uncertainty corresponding to a 95 % coverage range (dashed red lines). The expanded uncertainty was calculated from the combined standard uncertainty estimate of 1.0 mg/g using a coverage factor (k) of 2.

Figure 7 shows the difference of the participant results from the reference value (D_i) where:

$D_i = w_i - w_{Digoxin}$

The expanded uncertainty U_i at the 95 % coverage level of the difference from the reference value was calculated for each result as:

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

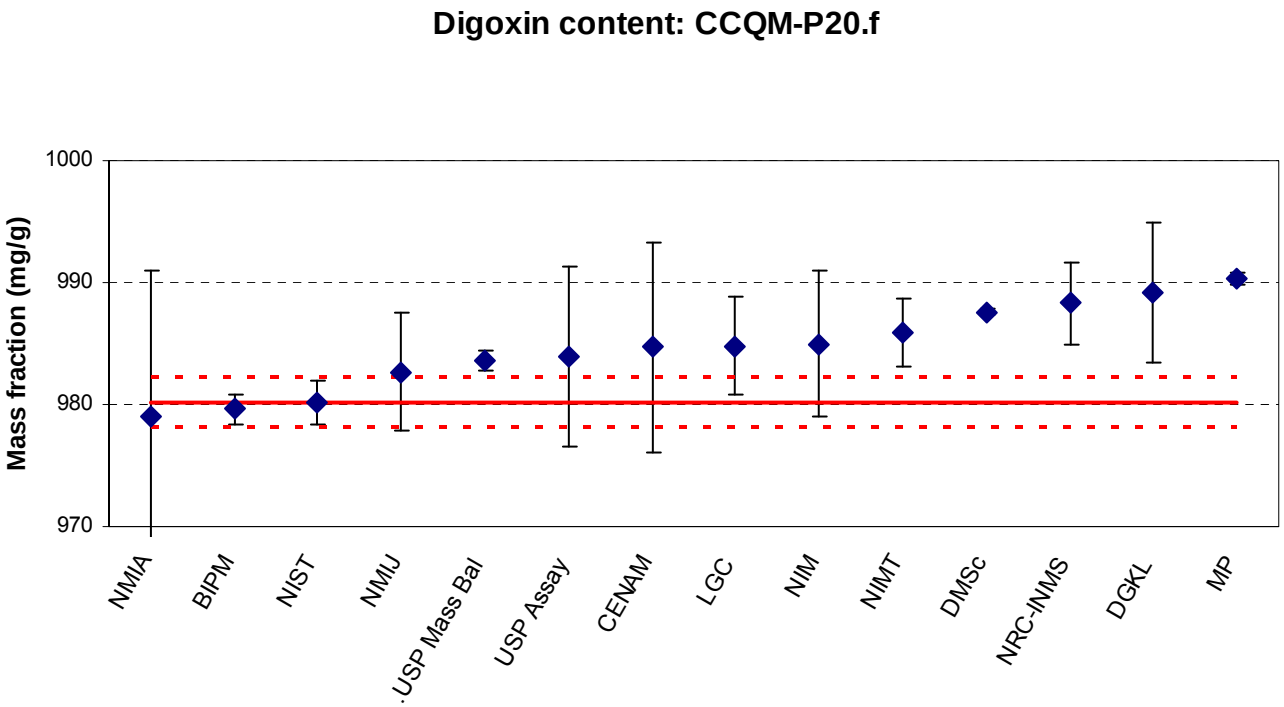


Figure 6: Mass fraction estimates reported by participants for digoxin in CCQM-P20.f with associated expanded uncertainty corresponding to 95 % coverage.
Mass balance reference value for CCQM-P20.f (solid red line) = 980.2 ± 2.0 mg/g
The expanded uncertainty of the reference value (dashed red lines) corresponds to a 95 % coverage range using a coverage factor (k) of 2.

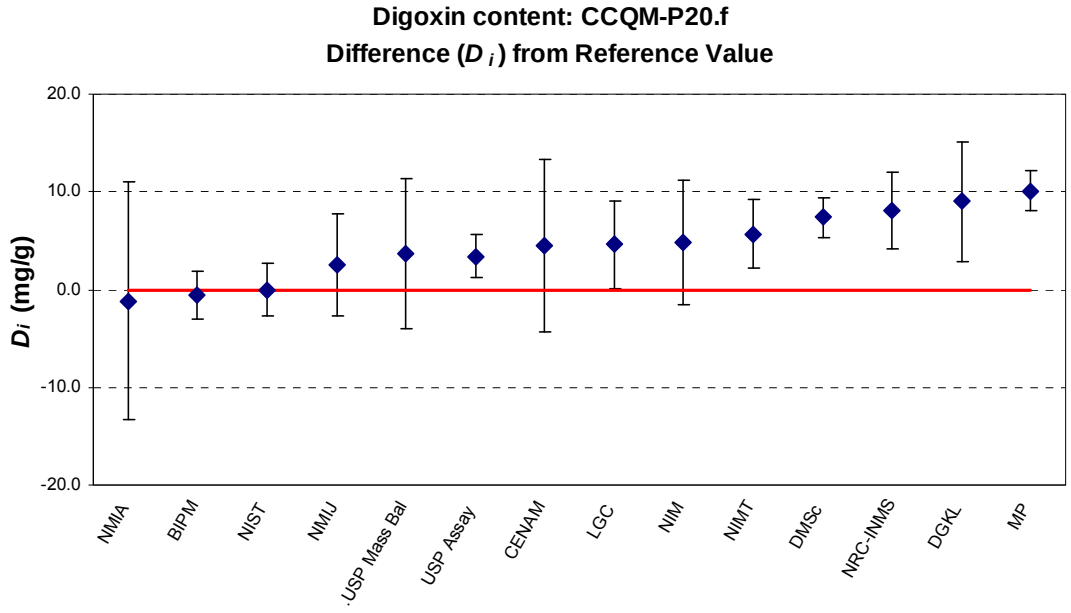


Figure 7: Difference of reported digoxin value from the reference value for each participant. Each point is plotted with the associated expanded uncertainty in the difference corresponding to a 95 % coverage range.

SUMMARY

In contrast with the previous round of the CCQM-P20 series, in which the mass fraction content of theophylline in two comparison samples (CCQM-P20.e.1 and CCQM-P20.e.2) was determined, a wider range of results were reported for the mass fraction content of digoxin in the CCQM-P20.f comparison.

In part, this disparity was expected given the significantly greater analytical challenge posed by digoxin which is structurally more complex, contained a wider variety of impurities and exhibited a lower sensitivity for detection by either UV or MS techniques than the CCQM-P20.e measurands. This was reflected in a bimodal distribution of the estimates for related structure impurity content.

A minority of participants did not appear to use conditions capable of fully resolving and/or quantifying the major related structure impurities present in the comparison sample. Among those that did achieve suitable separations, there was further variation in their reported quantifications of the individual and total related substance content which reflected in part the limited availability of reference standards for these materials and the resulting assumptions that had to be made regarding the structure and response factors relative to digoxin for each individual impurity. This was particularly relevant because of the span of molecular masses of the impurities present in the sample, which ranged from aglycones to glycones with tetrameric carbohydrate chains, relative to that of digoxin.

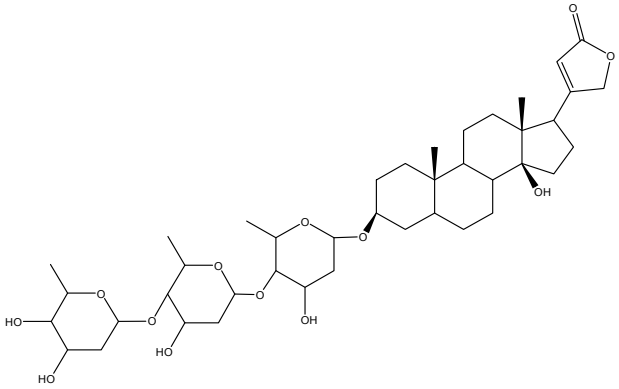
A significant additional factor also contributed to the observed variation of results. Unlike the CCQM-P20.e samples, in which the major impurities were solely related structure organic compounds, the CCQM-P20.f study material contained significant levels of residual organic solvents (ethanol, dichloromethane and to a lesser extent toluene). The majority of participants failed to detect and allow for the presence of this class of impurity, introducing a bias towards overestimation of digoxin content in most of the individual results.

However, the uncertainty budgets produced by several participants were sufficiently conservative such that their reported results were nevertheless consistent with the reference value for digoxin content assigned using a consensus mass balance approach.

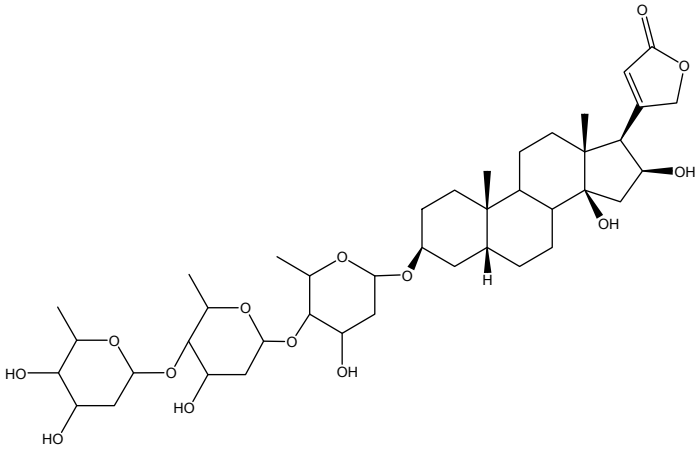
The results of the comparison reinforces the conclusion from previous rounds of the CCQM-P20 study that care in developing and validating the suitability of the chromatographic separation method used to resolve the main component from the related structure impurities present is essential to obtaining reliable, comparable results when using the mass balance approach to estimate purity.

This specific comparison has demonstrated that, in addition to developing an appropriate chromatographic separation, it is also important to use complementary techniques capable of detecting all potential orthogonal classes of impurities if it is desired to demonstrate a general capability to assign purity with a small (< 0.2 % relative) standard uncertainty.

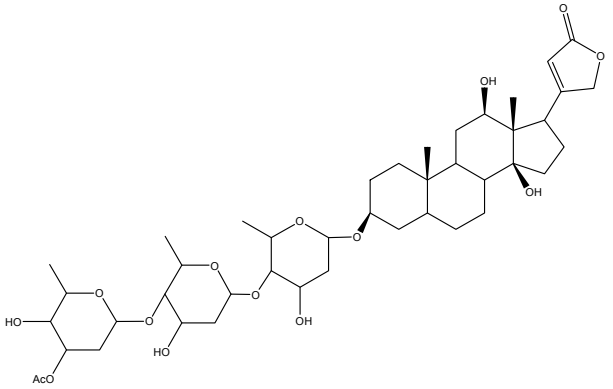
Annex A – Related structure compounds present in CCQM-P20.f



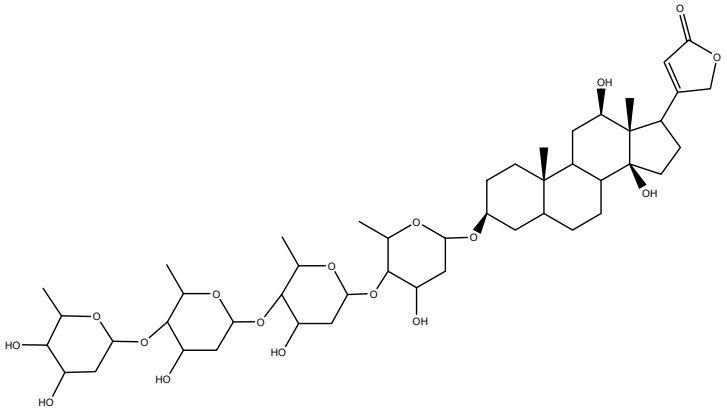
Digitoxin



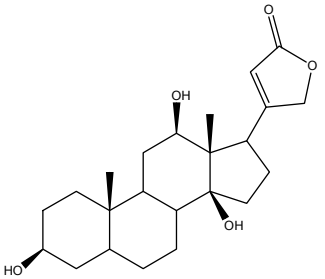
Gitoxin



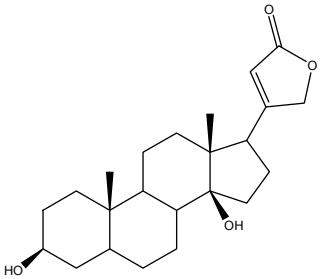
β-Acetyl digoxin



Digoxigenin tetradigoxoside



Digoxigenin



Digitoxigenin