CCQM-K55.a (Estradiol)
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CCQM-K55.a Key Comparison on the Characterization of Organic Substances for Chemical Purity

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Introduction

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

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

Pilot Study Summary

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

The review of the results obtained in the course of the CCQM-P20 pilot study highlighted several of the challenges involved in implementing measurement procedures for purity assessment of organic compounds. The procedures must be robust, rigorous, reliable and efficient in order to provide results suitable to their intended application, particularly in a situation where a limited amount of material is available for characterization studies. The most common approach used was the "mass balance" or "summation of impurities" method for purity assessment, which aims to quantify on a mass fraction basis the orthogonal classes of impurity present in the material and by subtraction obtain a measure of the mass fraction of the main component. It is traditionally based on use of one or more high resolution hyphenated chromatographic methods, either LC- or GC-based, to resolve and quantify the related structure impurities present in the sample under investigation. This technique is complemented by additional methods to determine potential impurities, such as water, volatile organics or non-volatile compounds, that are invisible to the chromatographic technique used to establish the related substance impurity content.

The BIPM coordinated the final two rounds of the CCQM-P20 pilot study and developed a "molecular weight v. polarity" model to map the analytical space for the proposed key comparison. This provided objective criteria for the selection of the proposed measurands for the CCQM-K55 key comparison and for the drafting of the "How Far the Light Shines" statement

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

associated with each round. The OAWG meeting at Sèvres in April 2008 accepted the proposal of the BIPM to coordinate the key comparison as well as the specific measurand, 17β-estradiol, which was proposed for the first round (CCQM-K55.a). A parallel pilot study (CCQM-P117.a), was also undertaken. The NMIJ collaborated with BIPM in the production of the comparison material.

The comparison samples were distributed in early December 2008. The participant's individual results were returned to the comparison coordinator in March 2009 and the results were first discussed at the April 2009 meeting of the CCQM OAWG. Further studies were subsequently undertaken to resolve a disparity between the water content reported by participants in the original results. When this was resolved a KCRV was proposed and accepted at the November 2010 OAWG meeting.

Estradiol

Estradiol was selected as the measurand for CCQM-K55.a because it:

- provides a relevant analytical challenge indicative of the performance of a laboratory's measurement capability for the purity assignment of organic compounds of medium structural complexity and intermediate polarity (see "How Far The Light Shines" statement);
- is representative of steroids for which there are a number of existing Calibration and Measurement Capability claims in Appendix C of the BIPM Key Comparison Database;
- is an important analyte in its own right in clinical chemistry and was not available as a pure substance Certified Reference Material at the commencement of the study;
- is of specific interest within the framework of ongoing activities of the Joint Committee on Traceability in Laboratory Medicine (JCTLM);
- was safe and stable for transport and sufficient source material was available to permit production of a suitably sized batch of the comparison candidate material.

The structure and conventional ring numbering of 17β-estradiol are shown in Figure 1. The structures of related compounds referred to in this report are given in Annex 1.

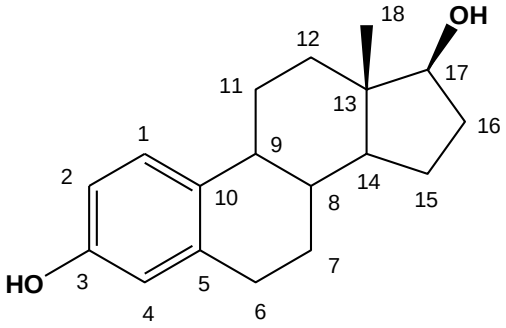


Figure 1: 17β-Estradiol

17β-Estradiol is a white crystalline powder with a reported melting range of 173-179 °C. It has limited solubility in water and alcoholic solvents and is sparingly soluble in non-polar organic solvents.[3] No pure substance CRM for 17β-estradiol was available at the time of the comparison, although reference substances were available from the U.S. and European pharmacopoeias.

KEY COMPARISON – MATERIALS AND CONDUCT OF STUDY

For the initial round of the key comparison, designated CCQM-K55.a (with parallel pilot study CCQM-P117.a), the NMIJ obtained a sample of 17β-estradiol sourced from a commercial supplier. 17β-Estradiol is normally supplied in a hemihydrate form but for the comparison the supplier subjected the bulk source material to extensive (but not exhaustive) drying in order to reduce the water content. The bulk material was subdivided into individual units each containing a minimum of 300 mg of the bulk material.

The individual units consisted of amber glass storage vials (5 ml capacity) which were fitted with a rubber insert, crimped with an aluminium cap and sealed in a laminated pouch. One hundred and fifty units of the material were shipped to the BIPM who investigated and characterised the minor components present in the material. The BIPM also investigated the homogeneity and stability of the material and shipped the material to the comparison participants.

The mass fraction of 17β-estradiol was initially assessed by the BIPM to be greater than 975 mg/g for the material, and its homogeneity and stability was determined to be suitable for the purposes of the comparison. A summary of the characterization results reported by the study participants are contained in this report.

Homogeneity studies

The homogeneity of components related in structure to 17β-estradiol in the material was assessed by high performance liquid chromatography with diode array UV-detection (LC-UV). The homogeneity of the water content of the material was assessed using Karl Fischer titration.

The uncertainty contributions due to the inhomogeneity of each related substance UV-active component ($u_{bb(rel)}$) were evaluated by ANOVA. This provided an estimate of the variation due to inhomogeneity of related substance impurities at a stated sampling size both between and within sample units. Acceptable uncertainty contributions due to inhomogeneity were observed for each of the resolved impurities present in the sample. The $u_{bb(rel)}$ for each impurity varied with the mass fraction of the specific impurity from 0.6 % for 4-methyl estradiol, present at approximately 5 mg/g in the comparison sample, to 4-6 % for impurities present at levels of approximately 0.5 mg/g. The absolute value of the contribution to the overall uncertainty from between unit inhomogeneity (u_{bb}) of the related substance impurities content of the material was conservatively calculated as 0.07 mg/g by quadratic combination of the individual inhomogeneity uncertainties for each impurity.

The contribution to the overall uncertainty from between unit inhomogeneity of the water content of the material ($u_{bb(Water)}$) was estimated at 0.28 mg/g from comparison of the within unit and between unit repeatability of the analysis of two (25-30) mg replicates from ten units of the comparison material. This was significantly larger than the combined $u_{bb(rel)}$ due to inhomogeneity of the related substance impurities content.

Both the uncertainty contribution due to inhomogeneity in related substance impurities and also in water content between units was taken into account when calculating a reference value for the material. The homogeneity of the study sample was assessed as appropriate for the comparison for evaluation of related structure components present at levels of 1 mg/g or higher when a sample size greater than 2.5 mg is used for analysis of the related substance content material and for analysis of the water content when sample sizes greater than 25 mg were used.

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 the relative composition of 17β-estradiol or of the minor UV-active components over this time for samples stored at any of the three temperature ranges.

The effect of temperature on water content was also investigated. No significant changes were observed after storage at 4 °C. There was evidence of a slow uptake of water after prolonged storage at 22 °C and significant uptake on storage at 40 °C. The effect of storage temperature on water content of the comparison material is displayed in Figure 2. Each data point is the average of three determinations and is plotted with the standard deviation of the three results as the error bar. It was concluded that for the purposes of the comparison the material was suitably stable for short-term transport at ambient temperature, provided it was not exposed to temperatures significantly in excess of 40 °C, and for longer term storage at 4 °C after opening.

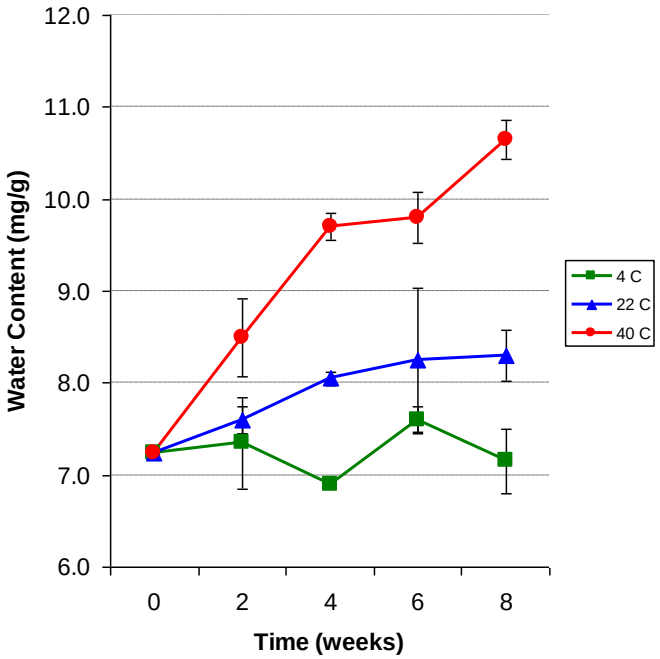


Figure 2: Effect of storage temperature on water content for CCQM-K55.a

To minimise the potential for changes in composition due to water absorption, participants were instructed to store the material at 4 °C and to perform all quantitative analyses within three weeks of the initial opening of the sample vial.

Sample distribution

Two units of the study sample, each containing a minimum of 300 mg of material, were distributed to each participant. Participants were asked to return a form acknowledging receipt of the samples and to advise the co-ordinator if any obvious damage had occurred to the vials 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. The monitor strips indicated that the vials supplied originally to INMETRO had been exposed to temperatures in excess of 50 °C during shipping. A replacement set of comparison samples was provided in this case. The second shipment was also exposed to an elevated temperature of 40 °C at some point in its shipment, however the time constraints of the study prevented the dispatch of another set of samples.

Quantities and Units

Participants were required to report the mass fraction of the major component, 17β-estradiol, in one of the supplied units of the comparison sample. The additional unit was provided for method development and trial studies. The reporting units for the mass fraction were mg/g.

Participants were encouraged to provide where possible mass fraction estimates for the minor components of the materials.

Results

1. Estradiol Content of CCQM-K55.a

The values for 17β-estradiol reported by the participants are summarised in Table 1.

Participant	**Estradiol Mass Fraction**			
	Value w (mg/g)	Standard Uncertainty $u(w)$ (mg/g)	Expanded Uncertainty $U_{95\%}$ (mg/g)	Relative Expanded Uncertainty (%)
BIPM	974.8	+ 0.81, -1.13	+ 1.6, -2.3	+ 0.18, - 0.24
NMIA	980.3	3.8	8.1 (k = 2.2)	0.83
NMISA	981.8	2.9	5.9	0.60
INMETRO	982.96	0.47	1.00 (k = 2.16)	0.10
NMIJ	983.6	1.0	2.0	0.20
NIST	983.8	N/R	+ 0.3, -2.9	+ 0.03, - 0.29
NRC-INMS	984.9	1.6	4.6 (k = 2.8)	0.47
NIM	988	2.5	5	0.51
GLHKSAR	989.1	0.36	0.7	0.07
BAM	990	2	4	0.40
CENAM	990.1	1.8	3.6	0.36
LGC	990.3	1.9	4.0 (k = 2.07)	0.40

Table 1: 17β-Estradiol content reported by participants for CCQM-K55.a
The coverage factor (k) for calculation of the $U_{95\%}$ was 2 unless indicated otherwise.

All participants in the key comparison used a hyphenated chromatographic technique (primarily LC-UV but in some cases GC-FID) to either identify and estimate the related substance impurity content of the material or to assign directly the estradiol content by comparison with a separate standard. This "mass balance" or "summation of impurities" approach, in which chromatography complemented by other techniques was used to estimate the total level of impurities, was the principal approach used to assign (by subtraction) the mass fraction of estradiol.

The NIST applied a novel variation of this approach by using a quantitative NMR (qNMR) approach to quantify the individual impurities (rather than the main component) and cross-checked their assignment using traditional chromatographic methods. In addition, in several cases the mass balance estimate was either compared to or combined with an independent value obtained by qNMR.

The basic measurement equations and approach to measurement uncertainty estimation used by each participant are outlined in Table 2.

<table>
<tr><th>Participant</th><th>Measurement Equation</th><th>Estimation of Measurement Uncertainty</th></tr>
<tr><td>BAM</td><td>$$w = \frac{A_{estradiol}}{\sum A} \cdot (1 - w_{water})$$
w: mass fraction of estradiol in the sample
$A_{estradiol}$: peak area of estradiol by HPLC-DAD
$\sum A$: sum of peak areas by HPLC-DAD
w_{water}: mass fraction of water, by KF titration</td><td>Source — Contribution to u_c (mg/g)
Repeatability 0.05
Non-linearity 0.14
Response factors 1.61
Water correction 0.02
TOTAL 1.62</td></tr>
<tr><td>BIPM</td><td>$$w_E = \frac{m_E}{m_{K55.a}} = \frac{m_E}{m_E + \sum_1^i m_i + \sum_1^j m_j}$$
$$= \frac{1}{1 + \left(\sum_1^i \frac{A_i}{L_i R_i} \cdot \frac{1}{A_E} \right) + \left(\sum_1^j \frac{m_j}{m_E} \right)}$$
w_E = mass fraction (g/g) of estradiol in K55.a
m_E = mass (g) of 17β-estradiol in CCQM-K55.a
$m_{K55.a}$ = mass (g) of a CCQM-K55.a test sample
m_i = mass (g) of a LC-UV detectable minor component i in CCQM-K55.a sample
m_j = mass (in g) of component j in the test sample not detected by LC-UV (water for K55.a).
A_i = Normalised UV area of minor component i
A_E = Normalised UV area of 17β-estradiol
L_i = Linearity of response (on a mass basis) of component i relative to 17β-estradiol
R_i = UV response factor (on mass basis) of component i relative to 17β-estradiol</td><td>
<table>
<tr><th>Uncertainty component</th><th>x_i (mg/g)</th><th>$u_c^+(x_i)$</th><th>% of u_c^+</th><th>$u_c^-(x_i)$</th><th>% of u_c^-</th></tr>
<tr><td>Water</td><td>7.48</td><td>0.44</td><td>30</td><td>0.44</td><td>15</td></tr>
<tr><td>Estriol</td><td>1.39</td><td>0.004</td><td>< 1</td><td>0.004</td><td>< 1</td></tr>
<tr><td>9,11-Dehydro estradiol</td><td>0.43</td><td>0.075</td><td>1</td><td>0.075</td><td>< 1</td></tr>
<tr><td>4-Methyl estradiol</td><td>4.81</td><td>0.016</td><td>< 1</td><td>0.016</td><td>< 1</td></tr>
<tr><td>Estrone</td><td>1.21</td><td>0.173</td><td>5</td><td>0.173</td><td>2</td></tr>
<tr><td>Unidentified UV-active impurity 1</td><td>0.21</td><td>0.082</td><td>1</td><td>0.735</td><td>43</td></tr>
<tr><td>Unidentified UV-active impurity 2</td><td>0.52</td><td>0.069</td><td>< 1</td><td>0.069</td><td>< 1</td></tr>
<tr><td>Unidentified UV-active impurity 3</td><td>0.44</td><td>0.069</td><td>< 1</td><td>0.069</td><td>< 1</td></tr>
<tr><td>Unidentified UV-active impurity 4</td><td>0.74</td><td>0.097</td><td>1</td><td>0.097</td><td>< 1</td></tr>
<tr><td>Unidentified UV-active impurity 5</td><td>2.48</td><td>0.337</td><td>17</td><td>0.337</td><td>9</td></tr>
<tr><td>Unidentified UV-active impurity 6</td><td>2.08</td><td>0.282</td><td>12</td><td>0.282</td><td>6</td></tr>
<tr><td>Unidentified UV-active impurity 7</td><td>3.38</td><td>0.45</td><td>32</td><td>0.45</td><td>16</td></tr>
<tr><td>Volatile solvent and non volatiles < LOD</td><td>0</td><td>0</td><td>0</td><td>0.29</td><td>7</td></tr>
<tr><td colspan="2">17b-Estradiol content</td><td>974.8</td><td colspan="2">0.81</td><td>1.13</td></tr>
<tr><td colspan="4">Expanded uncertainty $U+$ (C.I.95%, k = 2)</td><td colspan="2">1.6</td></tr>
<tr><td colspan="4">Expanded uncertainty $U-$ (C.I.95%, k = 2)</td><td colspan="2">2.3</td></tr>
</table>
</td></tr>
</table>

Table 2 : Measurement equation and MU overview for CCQM-K55.a reported by participant

Participant	Measurement Equation	Estimation of Measurement Uncertainty
CENAM	Estradiol (mg/g) = 1000-Estrone (mg/g)-Water (mg/g) - Major impurity by LC-UV (mg/g)- other impurities (mg/g)	Major components of the overall uncertainty budget: 0,36 % U represents the 100 % Repeatability using LC/DAD 2,4 % Repeatability using GC-FID and CG-MS 5,2 % Uncertainty of quantification of Estrone 0,1% Uncertainty of quantification by area normalization of major impurity 21,3% Uncertainty of the quantified minor impurities by area normalization and estimation of the unknown impurities 19,8% Co elution of the main peak and one impurity 51,3 % Uncertainty of the water content in the sample 0,2%
GLHKSAR	$X_{PC} = 1 - \Sigma X_{IC}$ where X_{PC} – mass fraction of the principle component ; X_{IC} – mass fraction of impurities components	U(XPC) = U(∑XIC) Major components of U(XIC) include guess estimation for unknown impurities (77% total), precision, recovery and etc make up the remainder.
INMETRO	Mass fraction equation (expressed as mass fractions in mg/g): 17β-estradiol = 1000 - estrone - 17α-estradiol - methylated analog - unidentified impurities - water Organic impurities by HPLC: impurity (mg/g) = (impurity area in a concentrated sample × 1000) / ((17β- estradiol area in a diluted sample × dilution factor) + (sum of impurities areas in the concentrated sample))	Component / *u* (mg/g) / relative contribution (%)* 17α-estradiol 0.00039 0.00004 estrone 0.039 0.00397 methylated analog 0.062 0.00631 unidentified impurities by HPLC 0.19 0.01933 water 0.42 0.04273 **Total** 0.47 0.04781 * Standard uncertainty (u) expressed in terms of 17β-estradiol mass fraction
LGC	$P_{org} = 100\% - \sum \frac{\text{Area imp} \times 100}{\text{Area total} \times \text{RF}}$ $P_{total} = \left[1 - \left[\frac{\%\text{water}}{100} + \frac{\%\text{IR}}{100} + \frac{\%\text{res solvent}}{100}\right]\right] \times P_{org}$ $MF(\text{mg/g}) = P_{total} \times 10$ $RF = \frac{\text{Reponse slope impurity (conc/peak area)}}{\text{Response slope } 17\beta\text{ - estradiol (conc/peak area)}}$	Component / *u* (mg/g) 6β-hydroxyestradiol (HPLC) 0.039 9,11-didehydroestradiol (HPLC) 0.006 17-epi-estradiol (HPLC) 0.023 Estrone (HPLC) 0.026 4-methyl estradiol (HPLC) 0.140 UV-active organic unknown impurities (4) 0.918 FID-active unknown 0.353 Co-elution, not detected (HPLC) 1.0 Co-elution, not detected (GC) 1.0 Water 0.81 Inorganic residues 0.008 Residual solvent (methanol) 0.1 **Total** 1.9

Table 2 (continued from previous page): Measurement equation and MU overview for CCQM-K55.a reported by participant

Participant	Measurement Equation	Estimation of Measurement Uncertainty
NIM	$X_i = \frac{fiAi}{\sum(f_i A_i)} \times 100\%$ $X1$- 17β-estradiol content(%) , fi- response factor of ingredient (i=1~6); Ai- peak area of ingredient **The final result: *X(estradiol)=Xi-Xwater***	Two main contributors identified: (1) The uncertainty of method repeatability (Type A) = 0.12 % (rel) (2) The uncertainty of LC-UV response of impurities $u_{B\text{-}i}$ (Type B) = 0.22% (rel) $u_{STD} = \sqrt{u_A^{\,2} + u_B^{\,2}} = 0.25\%$
NIST	$\frac{\text{mg 17-β Estradiol}}{\text{g sample}} = \frac{(1000 - \sum \text{mg}_{\text{ICabsolute}})}{1 + \sum\left(\frac{\text{Area}_{\text{IC}}}{\text{Area}_{\text{estradiol}}}\right)\left(\frac{\text{RF}_{\text{IC}}}{\text{RF}_{\text{estradiol}}}\right)\left(\frac{\text{MM}_{\text{IC}}}{\text{MM}_{\text{estradiol}}}\right)}$ $\text{Mg}_{\text{ICabsolute}}$ is the mass in mg of known impurity components determined relative to mass of sample, Area_{IC} and $\text{Area}_{\text{estradiol}}$ are the peak areas of the impurities and estradiol, RF_{IC} and $\text{RF}_{\text{estradiol}}$ are the molar response factors, and MM_{IC} and $\text{MM}_{\text{estradiol}}$ are the molar masses. For qNMR area assignments, the RF_{ICs} are proportional to the number of equivalent hydrogens. For LC/UV_{225}, the RF_{ICs} are based of measurement of chromatographically related compounds.	$U_{95}(\text{mg}_{\text{water}})$ = skewed distribution, ranging from low 6.5 ± 0.3 mg/g (volumetric Karl Fischer) to 9.1 mg/g (1H-qNMR area). $u(\text{mg}_{\text{other identified ICs}})$ = 0.25 mg $U_{95}(\text{mg}_{\text{unassigned signals}})$ = skewed distribution, ranging from low of 0.5 mg/g to 2.0 mg/g.
NMIA	$\text{I}_{\text{GC-all}} = \text{I}_{\text{GC-raw}} + \text{I}_{\text{NR}} + \text{I}_{\text{ND}}$ $\text{I}_{\text{GC-raw}} = \text{I}_{\text{GC-I.D.}} + \text{I}_{\text{GC-Non I.D.}}$ $\text{Purity} = (100\% - \text{I}_{\text{GC-all}}) \times (100\% - \text{I}_{\text{OT}})$ $\text{I}_{\text{CC-all}}$ = total impurities by GC-FID (percentage of normalized response) allowing for non-resolved and non-detected components assuming identical response factors $\text{I}_{\text{GC-raw}}$ = total impurities (%) from GC-FID data $\text{I}_{\text{GC-ID}}$ = total identified impurities $\text{I}_{\text{GC-nonID}}$ = total non-identified impurities I_{NR} = allowance for impurities not resolved from estradiol I_{ND} = allowance for impurities below detection limit I_{OT} = mass fraction of impurities not detectable by GC-FID	$u_{\text{Purity}} = \text{Purity} \times \sqrt{\left(\frac{u_{\text{GC-all}}}{\text{I}_{\text{GC-all}}}\right)^2 + \left(\frac{u_{\text{OT}}}{\text{I}_{\text{OT}}}\right)^2}$ Major components of the uncertainty budget: The standard deviation of the raw GC-FID data = 0.023% The standard uncertainty of GC-FID non-identified = 0.29% The standard uncertainty of the non resolved component(s) = 0.06% The standard uncertainty of the non detected component(s) = 0.02% The standard uncertainty of the I_{OT} = 0.24% The uncertainty associated with I_{OT} is a combination of the standard uncertainty of the Karl Fischer results (0.18%) and the standard uncertainty of volatile content (0.12%) and non volatile residue (0.12%), both below the limit of detection of 0.2% respectively.

Table 2 (continued from previous page): Measurement equation and MU overview for CCQM-K55.a reported by participant

<table>
<tr><th>Participant</th><th>Measurement Equation</th><th>Estimation of Measurement Uncertainty</th></tr>
<tr><td>NMIJ</td><td>$$w_{estradiol} = \frac{w_{estradiol,sum} + w_{estradiol,NMR}}{2}$$
$w_{estradiol}$ = Mass fraction of 17β-estradiol
$w_{estradiol,sum}$ = Mass fraction of 17β-estradiol by summation of impurities
$w_{estradiol,NMR}$ = Mass fraction of 17β-estradiol by qNMR</td><td>$$u^2(w_{estradiol}) = \left(\frac{|w_{estradiol,sum} - w_{estradiol,NMR}|}{2}\right)^2 + \left(\frac{1}{2}\right)^2 \cdot u^2(w_{estradiol,sum}) + \left(\frac{1}{2}\right)^2 \cdot u^2(w_{estradiol,NMR})$$
$$= \left(\frac{|983.9 - 983.2|}{2}\right)^2 + \left(\frac{1}{2}\right)^2 \cdot (1.0)^2 + \left(\frac{1}{2}\right)^2 \cdot (1.6)^2$$
$u(w_{estradiol}) = 1.0$ mg g^{-1}
Uncertainty component — Value (x_i) — Standard uncertainty $u(x_i)$ — Relative Contribution
Difference between $w_{estradiol,sum}$ and $w_{estradiol,NMR}$ — 983.6 — 0.35 — 0.12
$w_{estradiol,sum}$ — 983.9 — 0.5 — 0.25
$w_{estradiol,NMR}$ — 983.2 — 0.8 — 0.63</td></tr>
<tr><td>NMISA</td><td>$w_{β\text{-}E2} = 1000 - w_{H2O} - w_{E1} - w_{\Sigma impHPLC220nm}$
$w_{β\text{-}E2}$ = Mass fraction of 17β-Estradiol in K55a (mg/g)
w_{H2O} = Mass fraction of water in K55a (mg/g) determined by Karl Fischer coulometric titration
w_{E1} = Mass fraction of Estrone (mg/g) determined by HPLC using external calibration at 220 nm
$w_{\Sigma impHPLC220nm}$ = Mass fraction of organic impurities by HPLC peak area % at 220 nm (mg/g) of anhydrous K55a</td><td>Uncertainty budget for impurities (18.15 mg/g)
<table>
<tr><th></th><th>u</th><th>u^2</th><th></th><th></th></tr>
<tr><td>u_{rel}(E1)</td><td>0.03556</td><td>0.001264</td><td></td><td></td></tr>
<tr><td>u_{rel}(imp)</td><td>0.01786</td><td>0.000319</td><td></td><td></td></tr>
<tr><td>u_{rel}(H$_2$O)</td><td>0.07118</td><td>0.005067</td><td></td><td></td></tr>
<tr><td></td><td></td><td>1.48</td><td>u(impurities)</td><td></td></tr>
<tr><td></td><td></td><td>2.96</td><td>U(impurities) k=2</td><td></td></tr>
</table>
<table>
<tr><td>Total impurities</td><td>18.1554</td><td>+/-</td><td>2.961</td><td>mg/g</td><td>(k=2)</td></tr>
<tr><td>Impurity range:</td><td>15.194</td><td>-</td><td>21.116</td><td>mg/ g</td><td></td></tr>
<tr><td>β-Estradiol range:</td><td>978.884</td><td>-</td><td>984.806</td><td>mg/ g</td><td></td></tr>
<tr><td>β-Estradiol measurement result</td><td>981.84</td><td>+/-</td><td>5.92</td><td>mg/ g</td><td>(k=2)</td></tr>
<tr><td></td><td>981.8</td><td>+/-</td><td>5.9</td><td>mg/ g</td><td></td></tr>
</table></td></tr>
<tr><td>NRC-INMS</td><td>$$w_{estradiol} = \left(\frac{I_{an}}{I_{cal}} \cdot \frac{\rho_{cal}}{\rho_{an}} \cdot \frac{M_{an}}{M_{cal}} \cdot \frac{m_{cal}}{m_{an}} \cdot \frac{V_{an}}{V_{cal}} \cdot P_{cal}\right) - w_{sri}$$
P = purity
I = integrated signal area
ρ = number of protons integrated
M = molar mass (g/mol)
m = weighed mass (g)
n = amount of substance (mol)
V = volume by mass (g) - for external standards only
$w_{estradiol}$ = mass fraction of estradiol (mg/g)
w_{sri} = mass fraction of structurally related impurities not resolved by NMR (mg/g)
w_{nmr} = mass fraction by qNMR (mg/g)</td><td>$$u_{cnmr} = P_{an}\sqrt{\left(\frac{u(I_{an}/I_{cal})}{I_{an}/I_{cal}}\right)^2 + \left(\frac{u(M_{an})}{M_{an}}\right)^2 + \left(\frac{u(M_{cal})}{M_{cal}}\right)^2 + \left(\frac{u(m_{an})}{m_{an}}\right)^2 + \left(\frac{u(m_{cal})}{m_{cal}}\right)^2 + \left(\frac{u(V_{cal})}{V_{cal}}\right)^2 + \left(\frac{u(V_{an})}{V_{an}}\right)^2 + \left(\frac{u(P_{cal})}{P_{cal}}\right)^2}$$
overall uncertainty: $u_c = \sqrt{u(w_{nmr})^2 + u(w_{sri})^2}$
<table>
<tr><th>Component (units)</th><th>x_i</th><th>$u(x_i)$</th><th>$u(x_i)/x_i$ (%)</th></tr>
<tr><td>w_{nmr} (mg g^{-1}) n=5</td><td>986.9</td><td>1.6</td><td>0.1621</td></tr>
<tr><td>$MW_{estradiol}$ (g mol^{-1})</td><td>272.382</td><td>0.003</td><td>0.0011</td></tr>
<tr><td>MW_{cal} (g mol^{-1})</td><td>122.121</td><td>0.002</td><td>0.0016</td></tr>
<tr><td>$\rho_{estradiol}$</td><td>1 or 2</td><td>0</td><td>0</td></tr>
<tr><td>ρ_{cal}</td><td>2 or 5</td><td>0</td><td>0</td></tr>
<tr><td>$m_{estradiol}$ (mg)[a]</td><td>16.898</td><td>0. 003</td><td>0.0178</td></tr>
<tr><td>m_{cal} (mg)[a]</td><td>8.811</td><td>0. 003</td><td>0.0340</td></tr>
<tr><td>m sol. $_{estradiol}$ (g)</td><td>3.55128</td><td>0.00004</td><td>0.0011</td></tr>
<tr><td>m sol. $_{cal}$ (g)</td><td>3.58596</td><td>0.00004</td><td>0.0011</td></tr>
<tr><td>P_{cal}</td><td>0.999978</td><td>0.000022</td><td>0.0022</td></tr>
<tr><td>w_{sri} (mg/g) n=5</td><td>2.0</td><td>0.2</td><td>10.0</td></tr>
<tr><td>$w_{estradiol}$ (mg/g)</td><td></td><td>984.9</td><td></td></tr>
<tr><td>Combined uncertainty</td><td></td><td>1.6</td><td></td></tr>
<tr><td>Expanded uncertainty $U_{c\ 95\%}$ (k = 2.8)</td><td></td><td>4.6</td><td></td></tr>
</table>
[a] minimum weights of calibrator and estradiol in series of replicates</td></tr>
</table>

Table 2 (continued from previous page): Measurement equation and MU overview for CCQM-K55.a reported by participant

Impurity Profile of CCQM-K55.a

All the CCQM-K55.a participants provided some information on the minor components (impurity content) present in the study sample. The data reported is summarised by participant in Table 3, by each related structure component reported by two or more participants in Table 4 and by individual estimates for the water content of the comparison sample in Table 5.
Related structure compounds identified by more than one participant included 4-methyl estradiol (2), estrone (3), 17α-estradiol (4), 9,11-didehydroestadiol (5), 17β-dihydroequilenin (6) and 1-methyl-17β-estradiol (7). The structures of each of these compounds are given in Annex A.
Four participants (NIST, NMIJ, GLHKSAR and LGC) noted that analysis of the material by LC-UV was potentially accompanied by artefact formation under neutral conditions. GLHKSAR and NMIJ demonstrated that this artefact formation was suppressed when the eluting solvent was acidified. Chromatographic data provided by GLHKSAR clearly demonstrating the formation of artefacts and their suppression under acidic conditions are shown in Figures 3a and 3b below.
NIST provided literature precedent for the formation of dimer and trimer artefacts during LC analysis of estradiol-like compounds.[4] The artefacts arise from oxidative coupling involving the phenol ring A sub-structure.

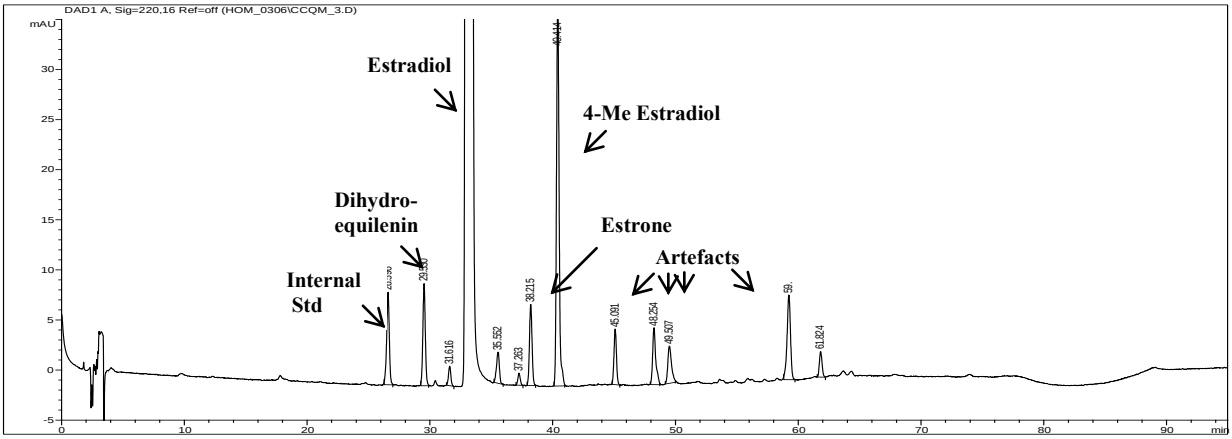


Figure 3a: Artefact formation visible in LC-UV chromatogram of CCQM-K55at 220 nm when eluting with neutral aqueous solvent

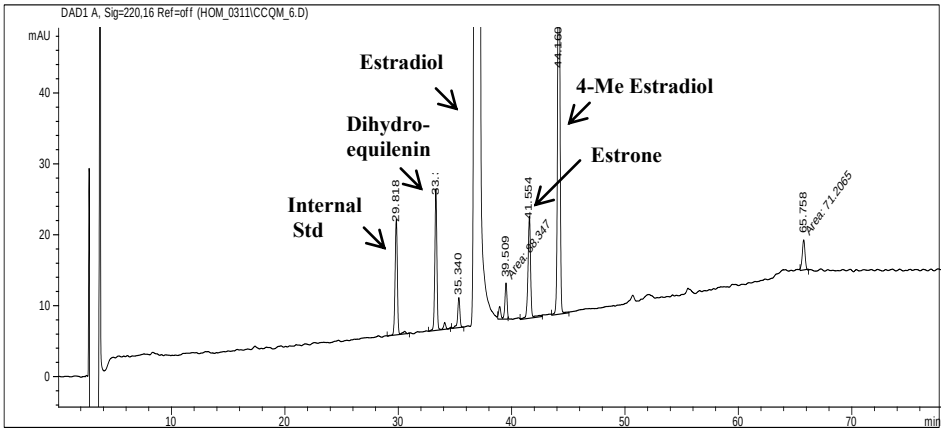


Figure 3b: Artefact formation suppressed in LC-UV chromatogram of CCQM-K55.a at 220 nm when eluting with acidified (0.05% TFA) solvent

Participant	Impurities reported in CCQM-K55.a	Mass Fraction (mg/g)		
		w	*u(w)*	$U_{95\%}$
BAM	Major impurity (Me estradiol ?)	4.9	0.2	0.4
	Combined minor organics	3.0	0.92	1.9
	Estrone	1.22	0.02	0.04
	Water	0.79	0.03	0.06
BIPM	Combined real and artefact minor "organics" [(a)]	7.94	0.63	1.26
	Water	7.48	0.44	0.88
	4-Methyl estradiol	4.81	0.016	0.03
	Combined real minor organics [(b)]	1.91	0.37	0.74
	Estriol	1.39	0.004	0.01
	Estrone	1.21	0.17	0.34
	9,11-Didehydroestradiol	0.43	0.075	0.15
	Organic solvent	< LOD	+ 0.29, - 0.0	+ 0.6, 0
	Inorganic residues	< LOD	+ 0.29, - 0.0	+ 0.6, 0
CENAM	Major impurity (Me estradiol ?)	5.06	0.84	1.68
	Combined minor organics	2.24	0.81	1.62
	Estrone	1.37	0.049	0.098
	Water	0.57	0.007	0.014
GLHKSAR	4-Methyl estradiol	5.98	0.12	0.24
	Combined minor organics	1.78	0.32	0.64
	Water	1.42	0.12	0.24
	Estrone	1.2	0.027	0.054
	β-Equilenol	0.29	0.007	0.014
	17α-Estradiol	0.11	0.013	0.026
	6-Dehydroestradiol	0.10	0.007	0.014
	Inorganic impurities	0.04	0.008	0.016
INMETRO	Water	10.30	0.42	0.95 (k = 2.07)
	Major impurity (Me estradiol ?)	4.30	0.062	0.13 (k = 2.09)
	Combined minor organics	1.30	0.19	0.39 (k = 2.07)
	Estrone	1.06	0.039	0.08 (k = 2.07)
	17α-Estradiol	0.077	0.0004	0.0008 (k = 2.26)
LGC	4-Methyl estradiol	3.9	0.14	0.35 (k = 2.45)
	Combined minor organics	2.0	0.918	2.25 (k = 2.45)
	Water	1.3	0.81	1.63
	6-Hydroxyestradiol	1.2	0.039	0.10 (k = 2.45)
	Estrone	0.8	0.026	0.07 (k = 2.45)
	Methanol	0.2	0.1	0.2
	9,11-Didehydroestradiol	0.1	0.006	0.02 (k = 2.45)
	17α-Estradiol	0.1	0.023	0.06 (k = 2.45)
	Combined inorganics	0.03	0.008	0.016
NIM	Water	1.2	0.2	0.4
	Estrone	1.12	0.10	0.20
NMIA	Water	10.7	1.8	3.6
	Major impurity (Me estradiol)	4.9	0.08	0.19 (k = 2.32)
	Estrone	1.7	0.12	0.28 (k = 2.32)
	Unknown impurity	0.4	0.01	0.033 (k = 2.26)
NMISA	Combined organic impurities	11.62	0.21	0.42
	Water	6.75	0.48	0.96
NRC-INMS	Water	6.0	0.3	N/R
	4-Methyl estradiol	5.12	0.20	0.56 (k = 2.8)
	Estrone	1.22	0.04	N/R
	17α-Estradiol	0.10	0.01	N/R

(a) Contribution to BIPM result subsequently shown to arise from artefacts, not true impurities
(b) Contribution to BIPM result from true minor impurities in CCQM-K55.a

Table 3 : Impurity content for CCQM-K55.a reported by participant (ctd over page)

Participant	Impurities in CCQM-K55.a	Mass Fraction (mg/g)		
		w	*u(w)*	$U_{95\%}$
NIST	Water	6.7	N/A	- 0.6, + 2.4
	4-Methyl estradiol	4.9	0.2	0.4
	Estrone	1.10	0.02	0.05
	17β-Dihyroequilenin	0.30	0.02	0.03
	1-Methylestradiol	0.30	0.02	0.04
	9-Dehydroestradiol	0.16	0.01	0.03
	?-Hydroxyestradiol	0.16	0.06	0.11
	Si as SiO_2	0.14	0.04	0.08
	17α-Estradiol	0.13	0.03	0.05
	Ethanol	0.09	0.02	0.03
NMIJ	Water	7.07	0.53	1.06
	4-Methyl estradiol	5.41	0.32	0.64
	Estrone	1.16	0.024	0.05
	1-Methylestradiol	0.32	0.014	0.03
	17β-Dihydroequilenin	0.28	0.006	0.02
	17α-Estradiol	0.12	0.012	0.03
	6-Dehydroestradiol	0.08	0.002	0.01
	Estradiol 3-methyl ether	0.04	0.01	0.02

Table 3 (ctd) : Impurity content for CCQM-K55.a reported by participant

Component	Participant	Mass Fraction in CCQM-K55.a (mg/g)		
		w	*u(w)*	$U_{95\%}$
4-Methylestradiol	LGC	3.9	0.14	0.35 (*k* = 2.45)
	INMETRO	4.3 [a]	0.062	0.13 (*k* = 2.09)
	BIPM	4.81	0.016	0.032
	BAM	4.9 [a]	0.2	0.4
	NMIA	4.9 [a]	0.08	0.19 (*k* = 2.32)
	NIST	4.9	0.2	0.4
	CENAM	5.06 [a]	0.84	1.68
	NRC-INMS	5.12	0.20	0.56 (*k* = 2.8)
	NMIJ	5.41	0.32	0.64
	GLHKSAR	5.98	0.12	0.24
Estrone	LGC	0.8	0.026	0.07 (*k* = 2.45)
	INMETRO	1.06	0.039	0.08 (*k* = 2.07)
	NIST	1.10	0.02	0.05
	NIM	1.11	0.1	0.2
	NMIJ	1.16	0.024	0.05
	GLHKSAR	1.2	0.027	0.054
	BIPM	1.21	0.17	0.34
	BAM	1.22	0.02	0.04
	NRC-INMS	1.22	0.04	N/R
	CENAM	1.37	0.049	0.098
	NMIA	1.7	0.12	0.28 (*k* = 2.32)
17α-Estradiol	INMETRO	0.08	0.0004	0.0008
	NRC-INMS	0.10	0.01	N/R
	LGC	0.10	0.023	0.06 (*k* = 2.45)
	GLHKSAR	0.11	0.013	0.026
	NMIJ	0.12	0.012	0.03
	NIST	0.13	0.03	0.05
9,11-Didehydroestradiol	LGC	0.1	0.006	0.02 (*k* = 2.45)
	NIST	0.16	0.01	0.03
17β-Dihydroequilenin	NMIJ	0.28	0.006	0.02
	NIST	0.30	0.02	0.03
1-Methylestradiol	NIST	0.30	0.02	0.04
	NMIJ	0.32	0.014	0.03

Table 4: Estimates for specific impurities in CCQM-K55.a by participant

a. Identified as "methylated estradiol" only

4-Methyl estradiol was the principal related structure impurity identified in the sample. It was resolved and quantified and its identity was partially or fully reported by all participants in CCQM-K55.a. Estrone was also identified and quantified in the sample by all participants. As can be seen from Table 4, overall there was excellent agreement between participants for the quantification of the major individual related structure components. The overall estimates for total amounts of related structure impurities reported by the laboratories were also in good agreement, as is discussed later in the context of the assignment of a consensus value for this class of impurities for use in calculating a KCRV.

The other significant minor component in the comparison sample was water. The source for the study material was a commercial sample of estradiol hemihydrate that had been extensively (but not exhaustively) dried by the supplier. The level of uncertainty due to inhomogeneity in water content between units ($u_{bb(Water)}$) was estimated by BIPM at 0.28 mg/g. The original results reported for water content are given in Table 5 and include a summary of the information provided by participants on the method(s) they used to obtain their result.

Participant	**Method summary**	**Mass Fraction water (mg/g)**		
		w	*u(w)*	$U_{95\%}$
CENAM	Coulometric KF titration; direct addition as soln. in EtOH	0.57	0.007	0.014
BAM	Coulometric KF titration with oven transfer at 105 °C ; 2 x 90 mg	0.79	0.03	0.06
NIM	Coulometric KF titration with oven transfer at 150 °C, 7 x 20^{+} mg	1.2	0.2	0.4
LGC	Coulometric KF titration with oven transfer at 150 °C ; 2 x 75 mg	1.3	0.81	1.6
GLHKSAR	Coulometric KF titration with oven transfer at 130 °C and GC-TCD	1.42	0.12	0.24
NRC-INMS	Coulometric KF titration; 2 x 20 mg by addition as soln. in DMF	6.0	0.3	N/R
NIST	Estimated by qNMR, checked by Volumetric KF titration	6.7	N/A	-0.6, +2.4
NMISA	Coulometric KF titration; 6 x 20 mg by direct addition	6.75	0.48	0.96
NMIJ	Coulometric KF titration with oven transfer at 185 °C ; 4 x 10 mg	7.07	0.53	1.06
BIPM	Coulometric KF titration; 5 x 30 mg by direct addition ; consistent with %C,H analysis	7.48	0.44	0.88
INMETRO	Coulometric KF titration; 10 x 10 mg by direct addition	10.3	0.42	0.95 (k = 2.07)
NMIA	Coulometric KF titration; 6 x 10-15 mg by direct addition ; consistent with %C,H analysis	10.7	1.8	3.6

Table 5: Results for water content of CCQM-K55.a (N/A = not applicable N/R = not reported)

The range in values reported for water content by the participants was greater than could be explained on the basis of between bottle inhomogeneity. It constitutes the major source of variation in the purity values reported by participants for 17β-estradiol.

Water content was determined by most participants using a variation of Karl Fischer (KF) titration. Direct addition of the comparison sample as a solid, addition as a solution in anhydrous solvent or heated sample oven transfer to release water (as water vapour) from the solid sample for transfer by dry gas were all used to introduce the water content of the material into the titration cell. Other methods used to independently measure water content or check the consistency of an estimate obtained by KF titration included GC-TCD, thermogravimetric analysis (TGA), qNMR and elemental microanalysis.

Participants who used Karl Fischer techniques with heated oven transfer at temperatures below 170 °C all reported low values (< 1.5 mg/g) for the total water content. By contrast when direct addition or heated transfer with oven temperatures greater than 170 °C was used, only values in excess of 6 mg/g were reported. The temperature dependence of the KF result on oven temperature was reported by several participants and it was demonstrated that water release was not complete until the melting point of estradiol (176 °C) had been exceeded. This is illustrated in a representative thermogravimetric analysis (TGA) over 80 °C to 200 °C shown in Annex B below. A related study by TGA-MS undertaken by LGC subsequent to the discussion of the initial results confirmed this result and is also reproduced in Annex B. MS analysis of the volatile material confirmed that only water (m/z = 18) was released, with no evidence for the oxidative formation of CO_2 (m/z = 44) under these conditions.

The thermogravimetric data indicates two distinct stages of water release from the sample – an initial release (of adsorbed water?) complete by 120 °C and subsequent release of the residual water (of crystallization) when the solid structure of the material is broken down at temperatures above the melting point.

The relatively high values originally reported by NMIA and INMETRO (> 10 mg/g) may have arisen from water adsorption by the sample either from exposure to relatively elevated temperatures during transport or due to prolonged storage after initial opening of the sample vial. When these laboratories repeated the analysis using samples sent at a time of cooler local ambient temperatures and following the recommendation to perform all water quantifications within three weeks of opening the vial they obtained markedly lower values for water content that were in good agreement with the proposed KCRV estimate.

The results obtained by the non-KF methods for independently estimating or checking the water content result were consistent with values for water in CCQM-K55.a in the range between 6 mg/g and 8 mg/g with one exception. The initial GC-TCD result reported by GLHKSAR as supporting evidence gave a value for water content of 2.5 mg/g. However a subsequent repeat analysis by GC-TCD on a new sample provided for follow-on studies gave a water content of 8.2 mg/g, in reasonable agreement with the KF result of 7.1 mg/g obtained using a higher transfer oven temperature.

In addition to the related structure impurities and water, the levels of volatile organic solvents and non-volatile residues were also investigated or at least controlled for by most participants and generally found to be either present at very low levels or below the limit of detection of methods such as TGA and elemental analysis.

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

The initial discussion of the results lead to the conclusion that assignment of a KCRV for the CCQM-K55.a based solely on the reported results for overall estradiol content was not justified, given the evidence for bias in the results for water content and in some cases for combined organic impurities due to artefact formation during LC-UV analysis. After initial discussion at the April 2009 CCQM OAWG meeting follow-on studies to investigate and resolve these issues were undertaken by a number of participants. Subsequent discussion of this data continued at the OAWG meetings in November 2009 and April 2010. The study coordinator was finally asked to follow the precedent of the approach used in the CCQM-P20.f comparison and to propose an overall KCRV for the estradiol content of CCQM-K55.a based on the combination of individual KCRVs for the mass fraction of each of the orthogonal classes of impurity in the CCQM-K55.a comparison sample.

This required the assignment of separate KCRVs for:

- total structurally related impurities;
- water;
- volatile organic solvent;
- non-volatiles/inorganics.

It was noted that the establishment of KCRVs for each impurity category was not included in the original comparison proposal and although information on the mass fraction assignments of individual impurities was requested participants were not asked to provide an estimate for total related structure impurities.

However it was recognized during discussion of results that it is possible for a mass balance approach to give an overall value in apparent agreement with the KCRV for the main component that arises solely due to mutually cancelling errors in the assignment of the individual types of impurity. That is to say that, where a mass balance procedure is used to assign purity, agreement with the main component KCRV does not provide in isolation sufficient information on the fitness of the methods used to make the assignment. Given that the mass balance approach was the dominant one used by NMIs to value assign estradiol in CCQM-K55.a, it was decided that the performance of this approach by an NMI could only be properly assessed if KCRVs were established for each major impurity class.

Each key comparison participant was requested to review their original data and to provide to the study coordinator, where they considered it justified, an estimate of the mass fraction of each class of impurity. Participants could only use their original data but were allowed to undertake further studies, in particular to identify contributions due to artefact impurities, which could be removed from consideration when estimating the total structurally related compounds. They could also review and assess the validity of their original method for water content estimation.

A form for submission of impurity estimates for calculation of KCRVs for each impurity class was circulated to participants in September 2009 by the comparison coordinator.

Assignment of KCRVs for Individual Impurity Classes in CCQM-K55.a

1. KCRV for Total Related Structure impurities

The data submitted by participants for estimates for this category of impurity is shown in Table 6. INMETRO, NIM and NMISA did not provide a value and BIPM provided a revised value from their original data corrected for identifiable artifact peaks.

Participant	Value for KCRV calculation (mg/g)	
	w	***u(w)***
NRC-INMS	7.1	0.3
LGC	8.1	1.8
GLHKSAR	8.24	0.12
CENAM	8.67	1.8
NMIJ	8.93	0.65
BIPM	8.96*	0.43
NMIA	9.1	0.41
BAM	9.1	2.0
NIST	9.6	- 0.3, + 1.0
INMETRO	Not reported	
NMISA	Not reported	
NIM	Not reported	

$w_{Rel\ Subst.}$ = Mean = 8.65 mg/g ;
$u_{wRel\ Subst.}$ = Standard error of mean = 0.16 mg/g

Table 6: Estimates for total related impurity in CCQM-K55.a used for calculation of KCRV
*** original data after removal of contributions due to identified artefact impurities**

The mean of the submitted results was selected as the estimate of the KCRV for related structure impurity content ($w_{Rel\ Subst.}$). The associated standard uncertainty of the KCRV ($u_{wRel\ Subst.}$) is the standard deviation of the mean of the data set. The individual results with their associated standard uncertainties (k = 1) plotted against the KCRV are shown in Figure 4.

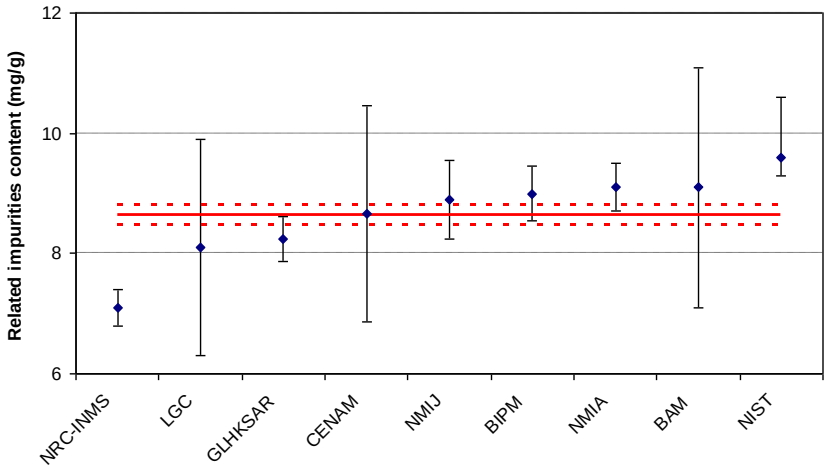


Figure 4 **Estimates of related structure impurities used to calculate the KCRV plotted with their reported standard uncertainties (± u_c , k = 1). The KCRV for related substance impurity ($w_{Rel.\ Subst.}$) content (solid red line) is 8.65 mg/g. The standard uncertainty of the KCRV is 0.16 mg/g. Dashed red lines show the KCRV ± u_c (k = 1).**

2. KCRV for water content

After review and follow-on studies, seven participants reported their original comparison data estimates for the water content of CCQM-K55.a, as given in Table 5, for use in assigning a KCRV for water content. Five participants (BAM, LGC, GLHKSAR, INMETRO and NMIA) decided after consideration that their original method did not provide an accurate value and withdrew their results.

BAM, LGC and GLHKSAR reported that their original method provided values for water in CCQM-K55.a that were too low. By contrast, INMETRO and NMIA concluded that their initially reported values for water in CCQM-K55.a were too high. For information purposes only, each laboratory reported revised values for the water content of CCQM-K55.a. For each of these participants their original results, some information on their revised method and the values obtained using this method are tabulated in Table 7. These revised values, while in good agreement with the final KCRV for water, could not be and were not used to assign the KCRV. They are provided below for information only.

Participant	Original water content (mg/g - ref Table 5)	Revised Method	Revised water content (mg/g – for info. only)
BAM	0.79 ± 0.06	Coulometric KF titration with oven transfer at 200 °C ; 2 x 100 mg	6.6 ± 0.5
GLHKSAR	1.42± 0.12	Coulometric KF titration with oven transfer at 180 °C ; 2 aliquots	7.07 ± 0.12
GLHKSAR	1.42± 0.12	GC-TCD ; 2 x aliquots	8.17
LGC	1.3 ± 1.6	Coulometric KF titration with oven transfer at 185 °C ; 3 x aliquots	6.35 ± 1.3
NMIA	10.7 ± 1.8	Direct addition, 4 x aliquots	7.57 ± 1.3
INMETRO	10.3 ± 0.95	Direct addition, 3 x 20 mg	7.7 ± 1.3

Table 7: Participants reporting revised values for water in CCQM-K55.a after follow-up studies

The results submitted by participants from their original data for use in calculation of the water KCRV is listed in Table 8. After review of the submissions and the methods used to obtain the data and after further discussion at subsequent OAWG meetings, the study coordinator proposed to exclude values below 1.5 mg/g from the calculation of the KCRV on the grounds that there was significant evidence (see discussion on the determination of water content) that the methods used to obtain those values did not completely release water from the sample.

Participant	Value for KCRV calculation (mg/g)	
	w	***u(w)***
*CENAM**	*0.57*	*0.007*
*NIM**	*1.2*	*0.2*
NRC-INMS	6.0	0.3
NIST	6.7	- 0.3, + 1.2
NMISA	6.75	0.48
NMIJ	7.07	0.53
BIPM	7.48	0.44

Table 8: Estimates for water content in CCQM-K55.a used for calculation of KCRV

** Results not used for calculation of the KCRV for water*

The median of the five results for water in the range 6-7.5 mg/g was selected as the KCRV for water content (w_{H2O}). The associated standard uncertainty of the KCRV (u_{wH2O}) was assigned as the robust standard deviation of the median (MADe/√n) of the data set

$$w_{H2O} = \textbf{Median} = \textbf{6.75 mg/g ;}$$

$$u_{wH2O} = \frac{MADe}{\sqrt{n}} = \textbf{0.21 mg/g}$$

The individual results for water content plotted against the KCRV are shown in Figure 5.

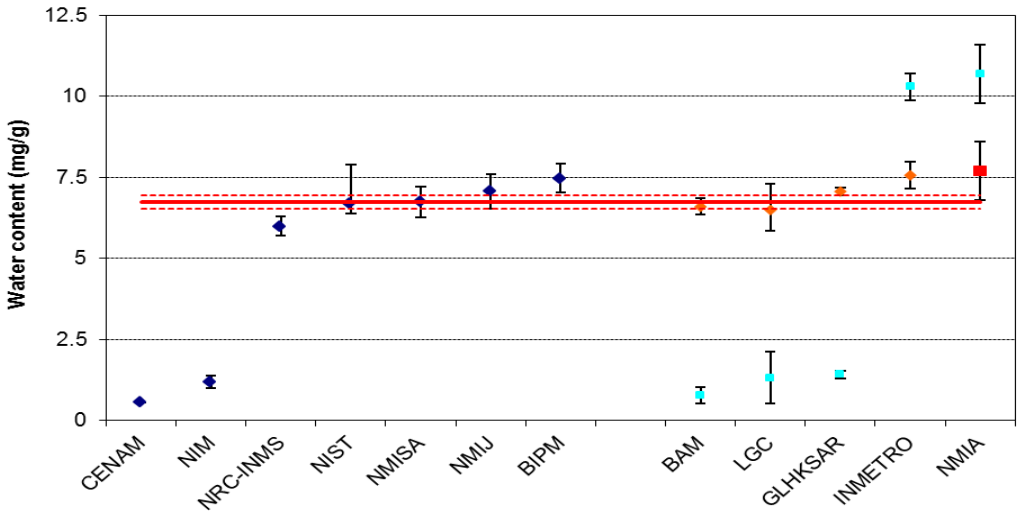


Figure 5 Mass fraction of water in CCQM-K55.a plotted with reported standard uncertainties (*k* = 1). The KCRV for water content of CCQM-K55.a (solid red line) is 6.75 mg/g. The calculated combined standard uncertainty of the KCRV (dashed lines, *k* = 1) is 0.21 mg/g. Dark blue: data submitted by participants for use for KCRV calculations. Light blue: original results withdrawn by participants from KCRV calculation. Orange: Information values for water content of CCQM-K55.a obtained by follow-up studies.

3. KCRV for volatile organic compound content

Seven participants provided estimates for the volatile organics content of CCQM-K55.a, as given in Table 8, for use in assigning a KCRV for this class of impurity. Methods used to investigate volatile solvent content included NMR, headspace or direct injection GC-MS and TGA. The participants that did detect solvent reported low levels (0.2, 0.09 and 0.055 mg/g respectively).

Participant	Value for KCRV calculation (mg/g)	
	w	***u(w)***
BAM	< LOD	-
BIPM	0.0	+ 0.29
NMIA	0.0	+ 1.2
GLHKSAR	< 0.01	
NMIJ	0.055	0.007
NIST	0.09	0.02
LGC	0.2	0.1

Table 8: Estimates for VOC content in CCQM-K55.a for calculation of KCRV

For calculation purposes the KCRV estimate for this class of impurity was assigned as a rectangular distribution in the range 0.0 - 0.2 mg/g. This gave the KCRV as the mid-point of the range and the associated uncertainty the standard approximation of the half-range divided by the square root of three.

$w_{NonVol.}$ = 0.1 mg/g

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

4. KCRV for non-volatile content

Six participants provided estimates for the non-volatile content of CCQM-K55.a, as given in Table 9, for use in assigning a KCRV for this class of impurity.

Participant	Value for KCRV calculation (mg/g)	
	w	***u(w)***
NIST	0.42	0.06
NMIA	0.0	+ 1.2
LGC	0.03	0.008
GLHKSAR	0.04	0.008
NMIJ	0.0	+ 0.46
BIPM	0.0	+ 0.29

Table 9: Estimates for non-volatile content in CCQM-K55.a for calculation of KCRV

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 (< 0.05 % on a relative mass fraction basis) of this general class of impurity. Participants using more sensitive methodologies (XRF spectrometry, ICP-OES) were able to detect and provide quantitative estimates for the presence of some inorganic components.

Given the lack of evidence from other techniques for the presence of total non volatile components at a combined level in excess of 0.4 mg/g, the mass fraction estimate for contributions due to this class of impurity was assigned as a rectangular distribution in the range (0.0-0.4) mg/g. This gave the following KCRV as the mid-point of the range and the associated uncertainty the standard approximation of the half-range divided by the square root of three.

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

$u_{w\,Non\,Vol.}$ = 0.12 mg/g

Homogeneity

In addition to KCRVs for the mass fraction of each impurity class, in order to calculate an overall KCRV for estradiol uncertainty, contributions due to inhomogeneity of the impurity content of the material need to be included. As described earlier (see above under “Homogeneity Studies”, p. 5) the uncertainty contribution due to the inhomogeneity of water content was estimated at 0.28 mg/g and the separate contribution due to inhomogeneity of the total related impurities content was estimated at 0.07 mg/g. Uncertainty due to inhomogeneity of the other impurity classes made no significant contribution and is not included.

Assignment of KCRV for Estradiol in CCQM-K55.a

The measurement equation (Eqn. 1) to assign the KCRV of estradiol in CCQM-K55.a (in mg/g) is:

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

$w_{Estradiol}$ = KCRV for mass fraction of estradiol in CCQM-K55.a

$w_{Rel.Subst.}$ = KCRV for mass fraction of estradiol-related impurities in CCQM-K55.a

w_{Water} = KCRV for mass fraction of water in CCQM-K55.a

$w_{Org.Solv.}$ = KCRV for mass fraction of volatile organic solvents in CCQM-K55.a

w_{NonVol} = KCRV for mass fraction of non-volatiles/inorganics in CCQM-K55.a

H_{Water} = Correction for between unit inhomogeneity of water in the CCQM-K55.a material. Assigned value of 0 with associated uncertainty ($u_{H\ water}$)

$H_{Rel.Subst.}$ = Correction for between unit inhomogeneity of estradiol-related impurities in the CCQM-K55.a material. Assigned value of 0 with associated uncertainty ($u_{H\ Rel\ Subst.}$)

Note: Units for reporting mass fraction (w) are mg/g throughout.
The standard uncertainty associated with the mass fraction was calculated from equation (2):

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

The KCRVs for the impurity classes used for calculation of a mass balance KCRV for estradiol in the CCQM-K55.a comparison are summarised in Table 10.

Input factor *w*	**KCRV (mg/g)**	***n***	***u(w)* (mg/g)**
Related structure organics	8.65	9	0.16
Water	6.75	5	0.21
Volatile organics	0.1	7	0.06
Non-volatiles/inorganics	0.2	6	0.12
Homogeneity - water	0	large	0.28
Homogeneity - related structure impurities	0	large	0.07

Table 10: KCRV values for impurities used for calculation of estradiol KCRV and associated combined standard uncertainty in CCQM-K55.a

When substituted into the equations (1) and (2) described previously, the overall KCRV for the estadiol content becomes:

$$w_{Estradiol} = 1000 - [w_{Rel.Subst} + w_{Water} + w_{Org.Solv.} + w_{NonVol.} + H_{water} + H_{relsubst}] \text{ mg/g}$$
$$= 1000 - [8.65 + 6.75 + 0.1 + 0.2]) \text{ mg/g}$$
$$= 984.3 \text{ mg/g}$$

$$u_{w_{Estradiol}} = \sqrt{(u_{w_{Rel\ Subst}})^2 + (u_{w_{Water}})^2 + (u_{w_{OrgSolv.}})^2 + (u_{w_{NonVol.}})^2 + (u_{H_{Water}})^2 + (u_{H_{\mathrm{Re\ lSubst}}})^2}$$
$$= \sqrt{(0.16)^2 + (0.212)^2 + (0.06)^2 + (0.12)^2 + (0.28)^2 + (0.07)^2} \text{ mg/g}$$
$$= 0.41 \text{ mg/g}$$

This is a conservative estimate for the standard uncertainty that is likely to be double counting to some extent the contribution due to the inhomogeneity of the water and related impurity content. Figure 6 shows the participant results with their reported standard uncertainties plotted against the proposed KCRV (solid red line) and its associated standard uncertainty (k = 1). Figure 7 shows the same results with their expanded uncertainty and the KCRV with the corresponding expanded uncertainty for an approximately 95% coverage range (dashed red lines).

Degree of equivalence plots of participant results for CCQM-K55.a with the Estradiol KCRV

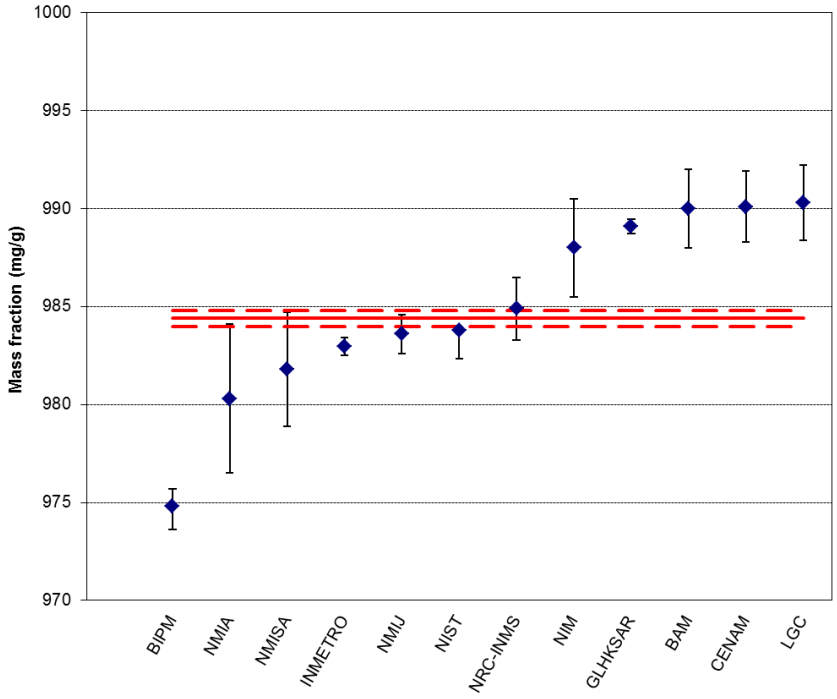


Figure 6: Mass fraction estimates by participants for estradiol in CCQM-K55.a with their reported combined standard uncertainty (u). Key Comparison Reference Value for CCQM-K55.a (solid red line) is 984.3 mg/g . The calculated combined standard uncertainty of the KCRV is 0.41 mg/g. Dashed red lines show KCRV ± u_c (k = 1)

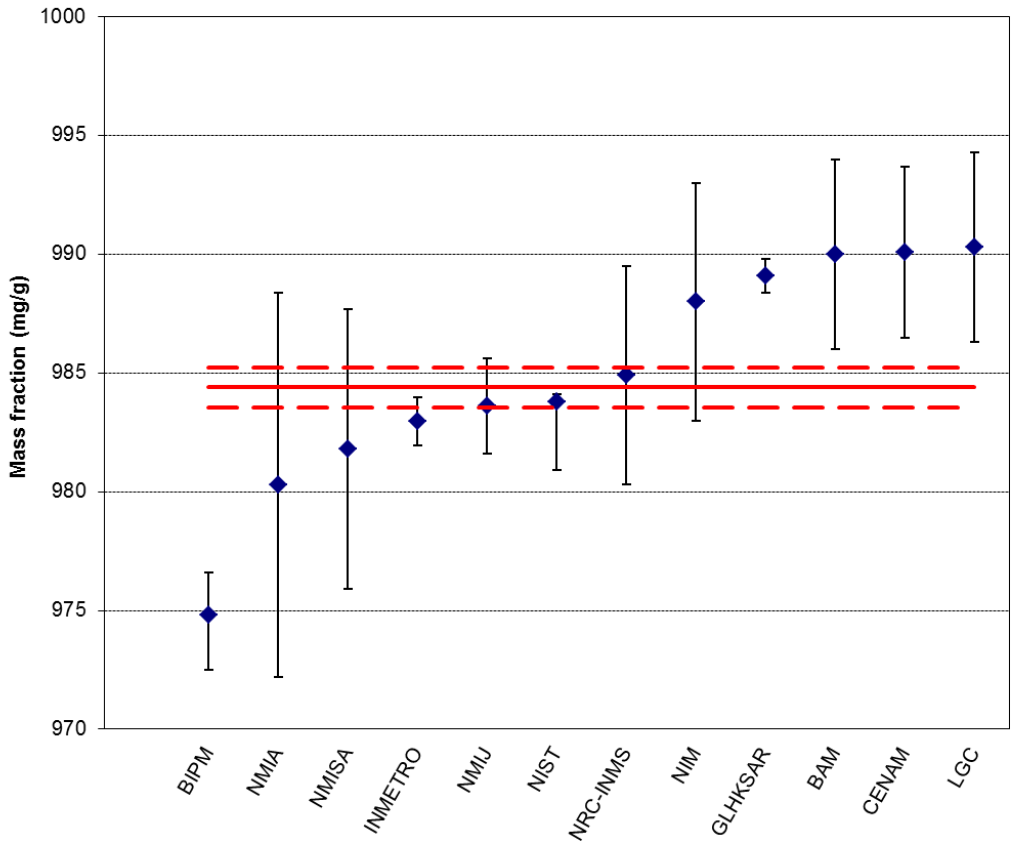


Figure 7: Mass fraction estimates by participants for estradiol in CCQM-K55.a with reported expanded uncertainty corresponding ($U_{95\%}$). Key Comparison Reference Value for CCQM-K55.a (solid red line) = 984.3 mg/g . The expanded uncertainty for 95% coverage range of the KCRV (dashed red lines) is 0.82 mg/g.

The degree of equivalence of a participant's result with the KCRV (D_i) is given by:

$D_i = w_i - w_{Estradiol}$

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

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

Table 11 records the degree of equivalence (D_i) of each key comparison participant's result with the proposed KCRV. These results are also shown graphically in Figure 8.

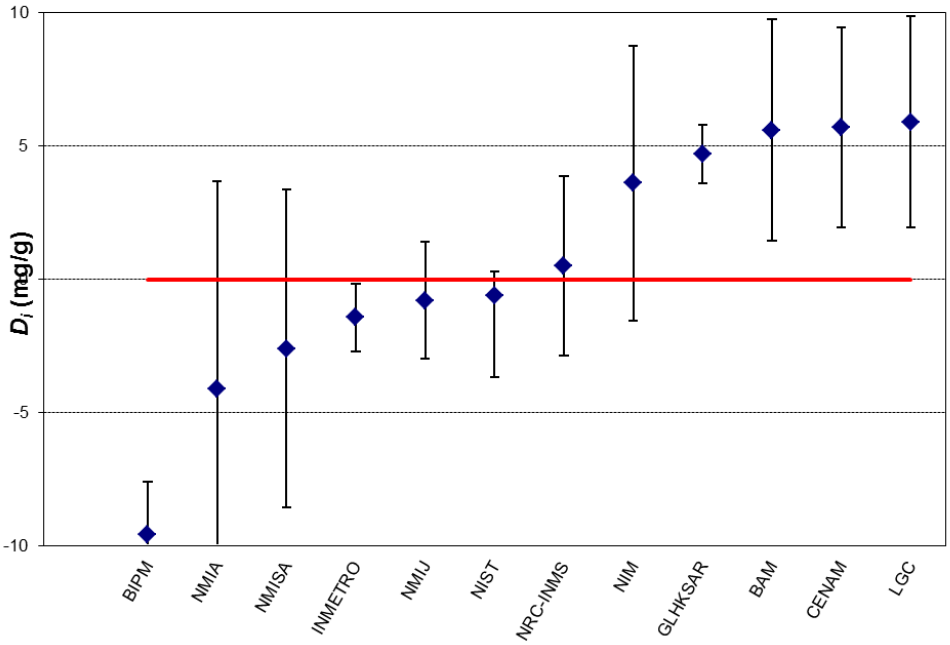


Figure 8: **Degree of equivalence with the estradiol KCRV for each participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to an approximately 95% coverage range.**

Participant	D_i (mg/g)	U_D (mg/g)
BIPM	-9.5	+ 2.00, -2.50
NMIA	-4.0	7.65
NMISA	-2.5	5.86
INMETRO	-1.3	1.27
NMIJ	-0.7	2.18
NIST	-0.5	+ 0.97, -3.04
NRC-INMS	0.6	3.31
NIM	3.7	5.07
GLHK	4.8	1.12
BAM	5.7	4.09
CENAM	5.8	3.70
LGC	6.0	3.90

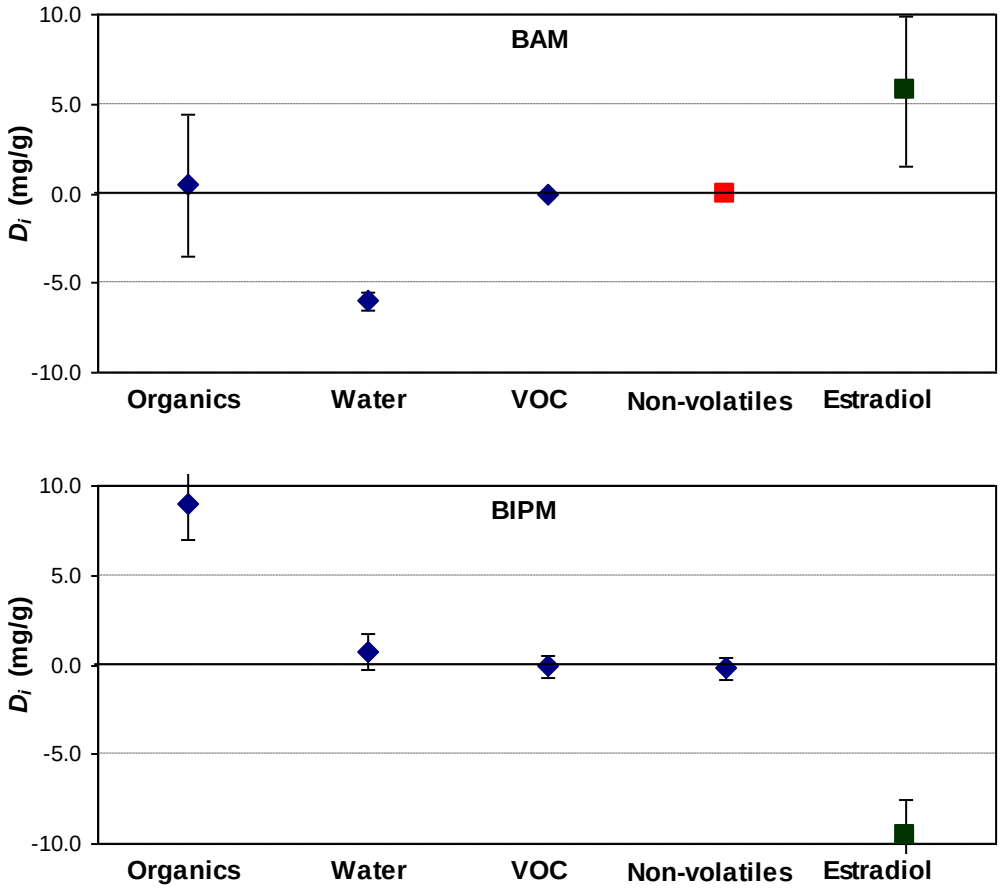
Table 11: **Degrees of equivalence D_i and expanded uncertainties U_D at approximately 95% coverage range in mg/g for estradiol in CCQM-K55.a**

Degree of equivalence plots for impurity KCRVs in CCQM-K55.a

The motivation for assigning KCRVs for the contributing impurity classes in CCQM-K55.a was to assess the fitness of mass balance methods, to confirm that an overall value for the main component in agreement with the KCRV for estradiol did not occur through cancellation of errors in contributing impurity assignments and to allow identification of problem areas when overall agreement with the KCRV for estradiol was not achieved.

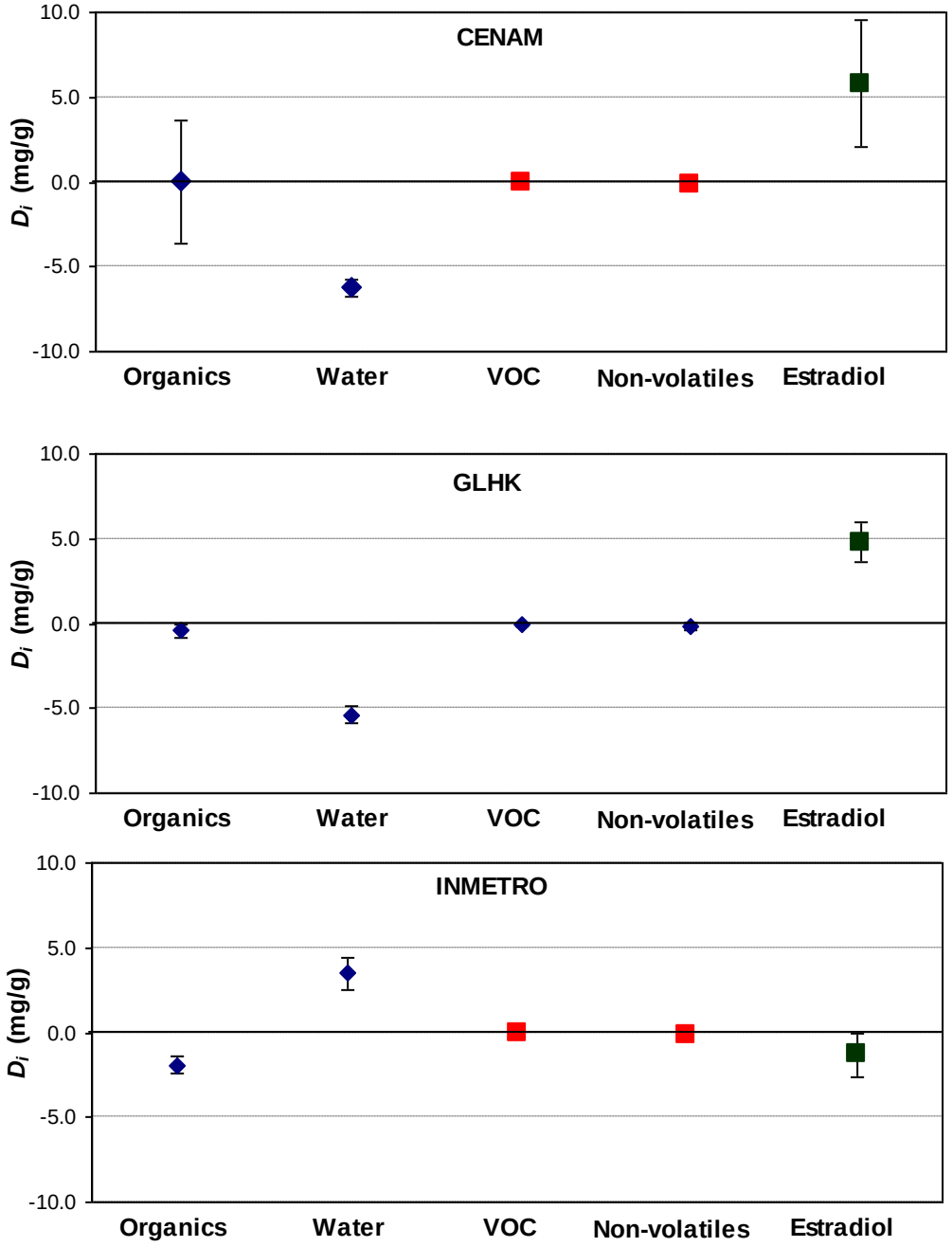
The combined DoE plots by participant for each impurity class quantified are shown below. To aid in assessment and comparison, the DoE of the result for the main component (cf Figure 8) is also plotted (green data point). Where a participant provided no information on a particular class of impurities (in this case VOCs and/or non-volatile content) the data point is shown as a red square, and a nominal D_i is plotted on the implicit assumption that the impurity makes no contribution to the overall purity assignment .

Mass Balance KCRV DoEs by Participant:



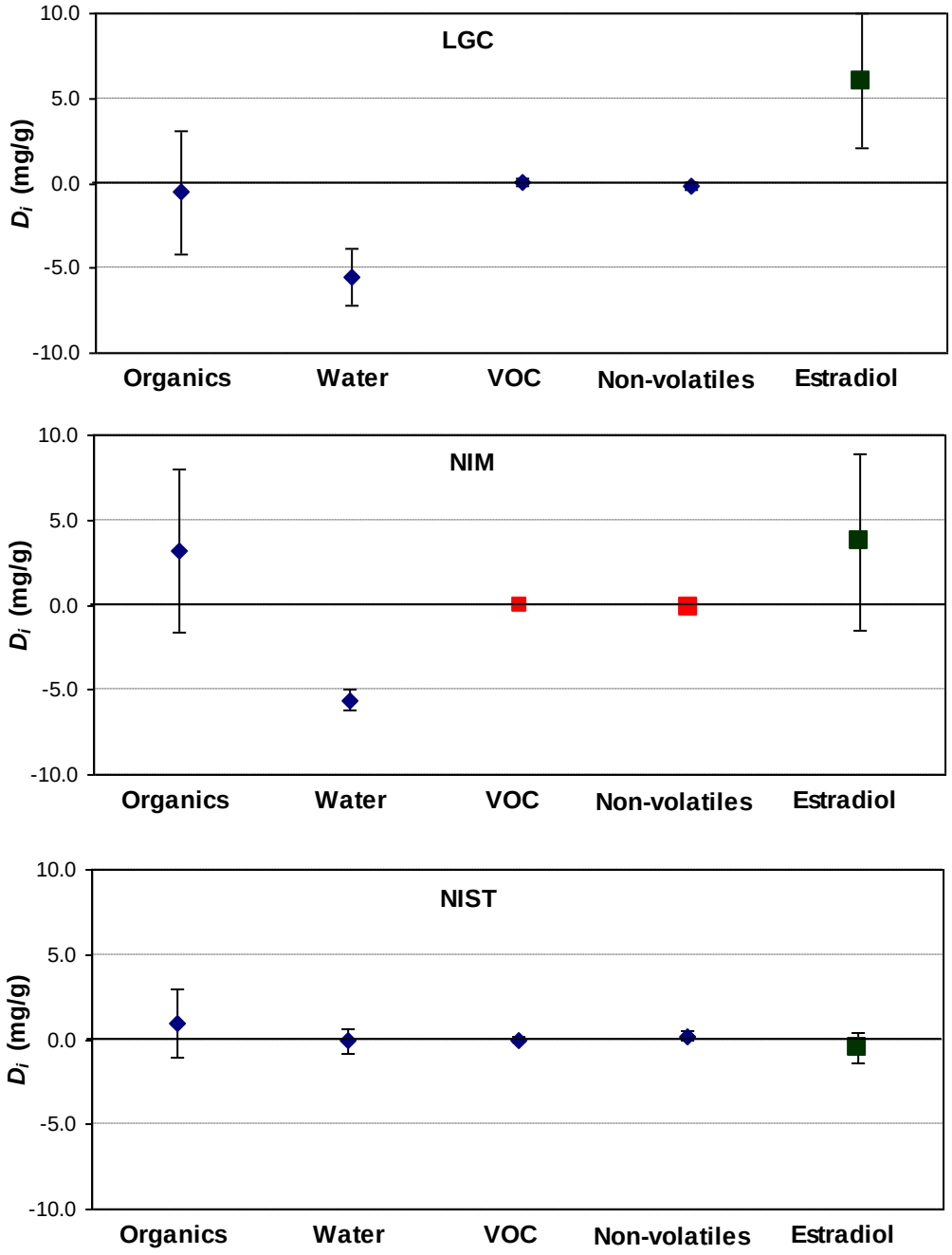
Key: ♦ = DoE for reported impurity; ■ = Nominal "DoE" when no value reported; ■ = DoE for estradiol in CCQM-K55.a

Mass Balance KCRV DoEs by Participant (Ctd):



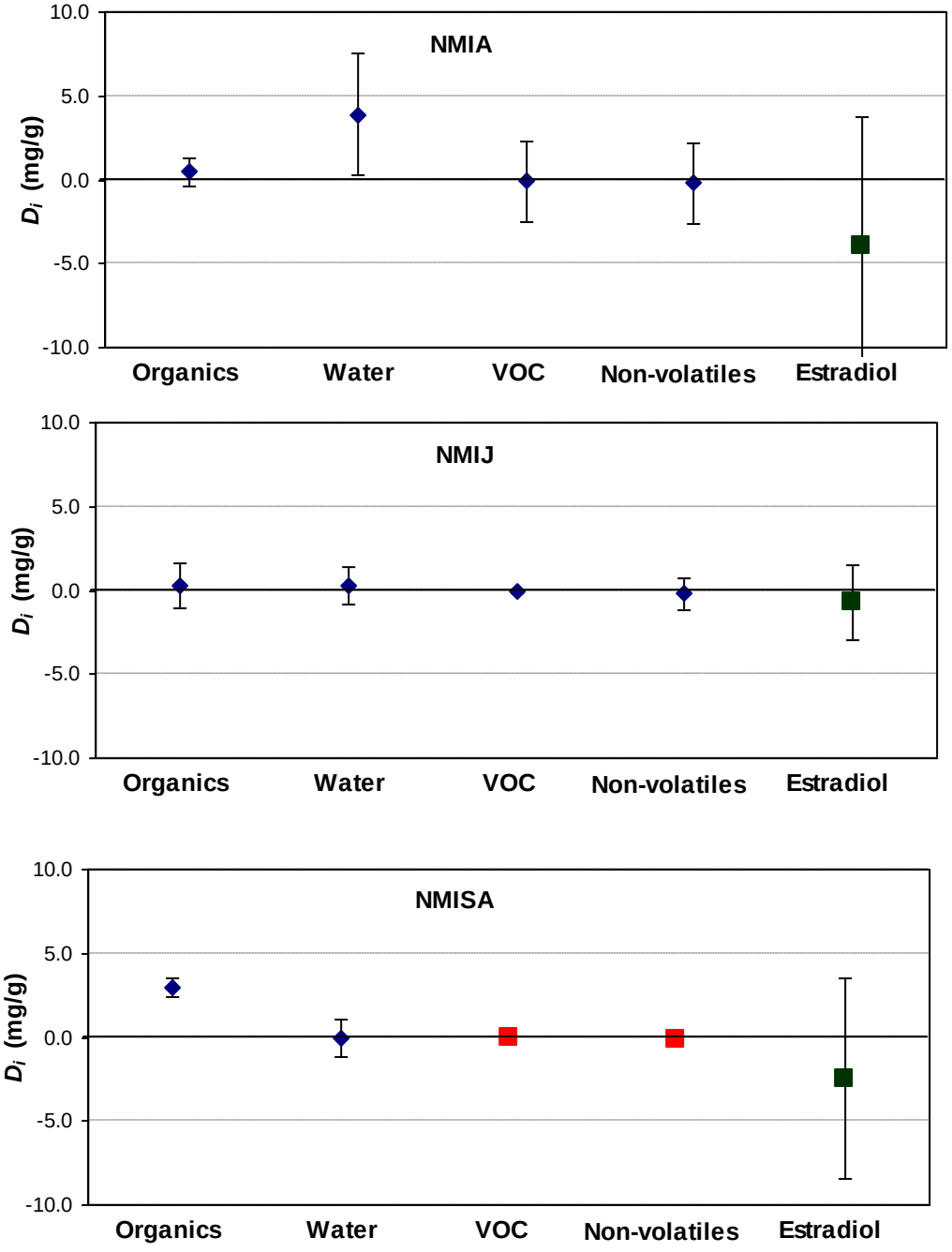
Key: ♦ = DoE for reported impurity; ■ = Nominal "DoE" when no value reported; ■ = DoE for estradiol in CCQM-K55.a

Mass Balance KCRV DoEs by Participant (ctd):



Key: ♦ = DoE for reported impurity; ■ = Nominal "DoE" when no value reported; ■ = DoE for estradiol in CCQM-K55.a

Mass Balance KCRV DoEs by Participant (ctd):



Key: ♦ = DoE for reported impurity; ■ = Nominal "DoE" when no value reported; ■ = DoE for estradiol in CCQM-K55.a

CONCLUSIONS AND HOW FAR THE LIGHT SHINES

Estradiol was selected to be representative of low polarity, moderately complex organic compound capable of analysis by GC or LC methods. It was anticipated to provide an analytical measurement challenge representative for the value-assignment of compounds of broadly similar structural characteristics.

The majority of participants used a mass balance approach for value assignment. The NIST were the first laboratory to use qNMR to quantify the impurities present in the sample, rather than the main component itself as is normal practice when using of qNMR methods, within the context of a mass balance approach.

Given the predominance of the mass balance approach, it was decided to assign the KCRV for estradiol by combination of KCRVs for each orthogonal impurity class, following the general approach that had already been used to assign a reference value for CCQM-P20.f. This allows participants to demonstrate the efficacy (or otherwise) of their implementation of the mass balance approach. In particular it allows participants to demonstrate that their assigned value for the main component agrees with the KCRV through use of internally consistent contributing methods rather than that the agreement was achieved by mutual cancellation of biased contributing results.

The KCRV and associated uncertainty for the material indicate that a relative expanded uncertainty for the purity assignment of 0.1 % is a reasonable estimate of the best achievable result for a material of this complexity atthis level of purity. The relative expanded uncertainties reported by laboratories having results consistent with the KCRV ranged from 0.1 % to 0.8 %.

Inspection of the results that were biased from the KCRV showed that the major analytical challenge posed by the material, which is not normally encountered with low polarity organic compounds, was the measurement of its water content. The results having a positive bias relative to the KCRV result can be explained as resulting from underestimation of the water content of the material. This is shown clearly by inspection of the individual participant degree of equivalence plots of the assigned values by impurity class and by estradiol content. Convincing evidence was provided that the material retained a significant amount of water that was only released once the crystalline structure of the sample was broken down. This could only be achieved thermally if the material was heated above its melting point.

There was good agreement in most cases between participants in the identification and the quantification of the related structure impurity content of the sample. The exception was the BIPM who, although they detected and quantified the “real” impurities in agreement with the results obtained by other participants, overestimated the total related structure impurity content through a failure to identify a contribution from artefacts formed *in situ* under their LC analysis conditions.

The results of the comparison reinforce one of the main conclusions from the CCQM-P20 study - the importance of using complementary, independent techniques capable of confirming estimates for all orthogonal classes of impurities if it is desired to demonstrate a general capability to assign purity through a mass balance approach with a small expanded uncertainty ($U_{95\%} < 0.2$ % relative) and suitable degree of trueness. Reliance on one measurement technique to quantify a particular class or group of impurities without control by an independent method is accompanied

by the risk of introducing a significant bias, as was demonstrated by the results for water content determinations in this comparison.

The comparison also demonstrated the utility of high-field ^{1}H NMR for both quantitative and qualitative analysis of high purity compounds. It is noted that all the participants who used qNMR as a major or contributing technique and included it as part of, combined it or confirmed it with a conventional "mass balance" data estimate obtained results agreeing with the KCRV.

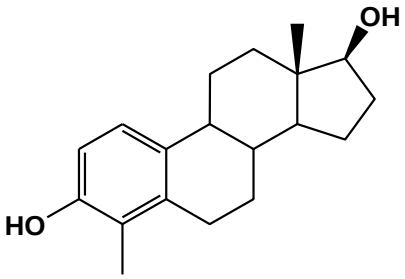
"How Far The Light Shines" Statement for CCQM-K55.a

The comparison was intended to demonstrate a laboratory's performance in determining the mass fraction of the main component in a high purity organic material. The measurement results were intended to be indicative of the performance of a laboratory's measurement capability for the purity assignment of organic compounds of medium structural complexity [molecular weight range (300-500) Da] and low polarity (pK_{OW} < -2) for which related structure impurities can be quantified by capillary gas phase chromatography (GC) or by high performance liquid chromatography (LC).

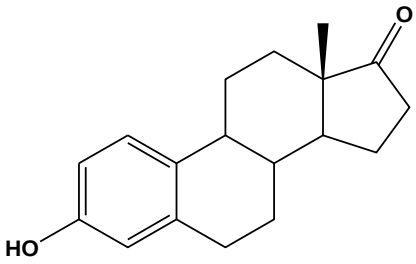
ACKNOWLEDGEMENT

The assistance of colleagues from the Organic Analytical Chemistry Division of the National Metrology Institute of Japan in the preparation of the CCQM-K55.a comparison material is gratefully acknowledged.

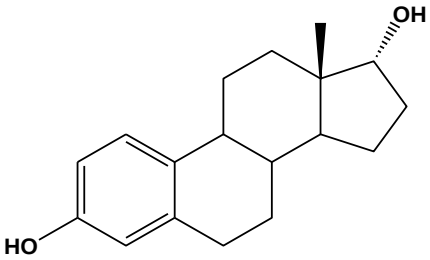
Annex A – Structure of compounds reported as impurities in CCQM-K55.a



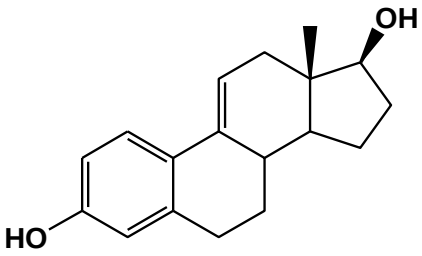
4-Methylestradiol **(2)**



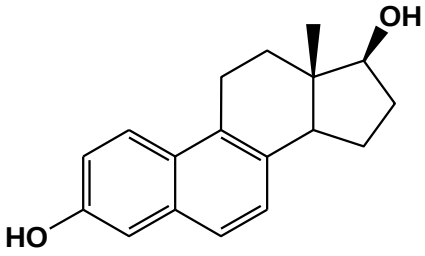
Estrone **(3)**



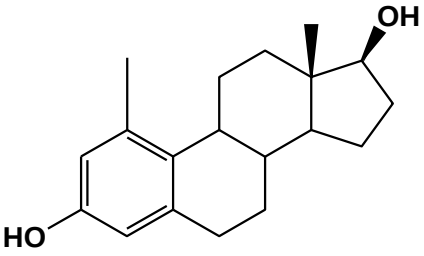
17α-Estradiol **(4)**



9,11-Dehydroestradiol **(5)**

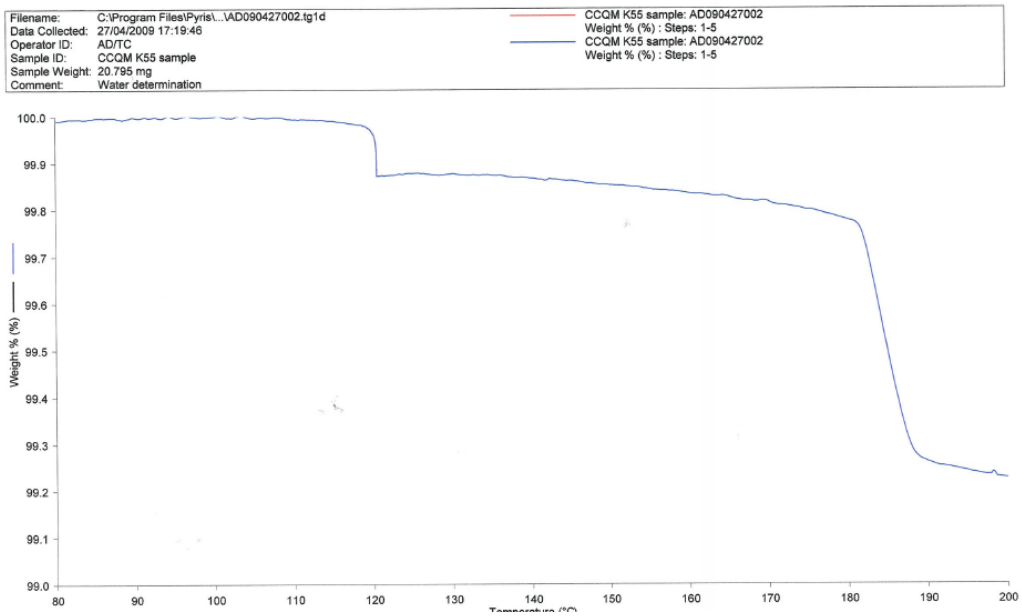


17β-Dihydroequilenin **(6)**

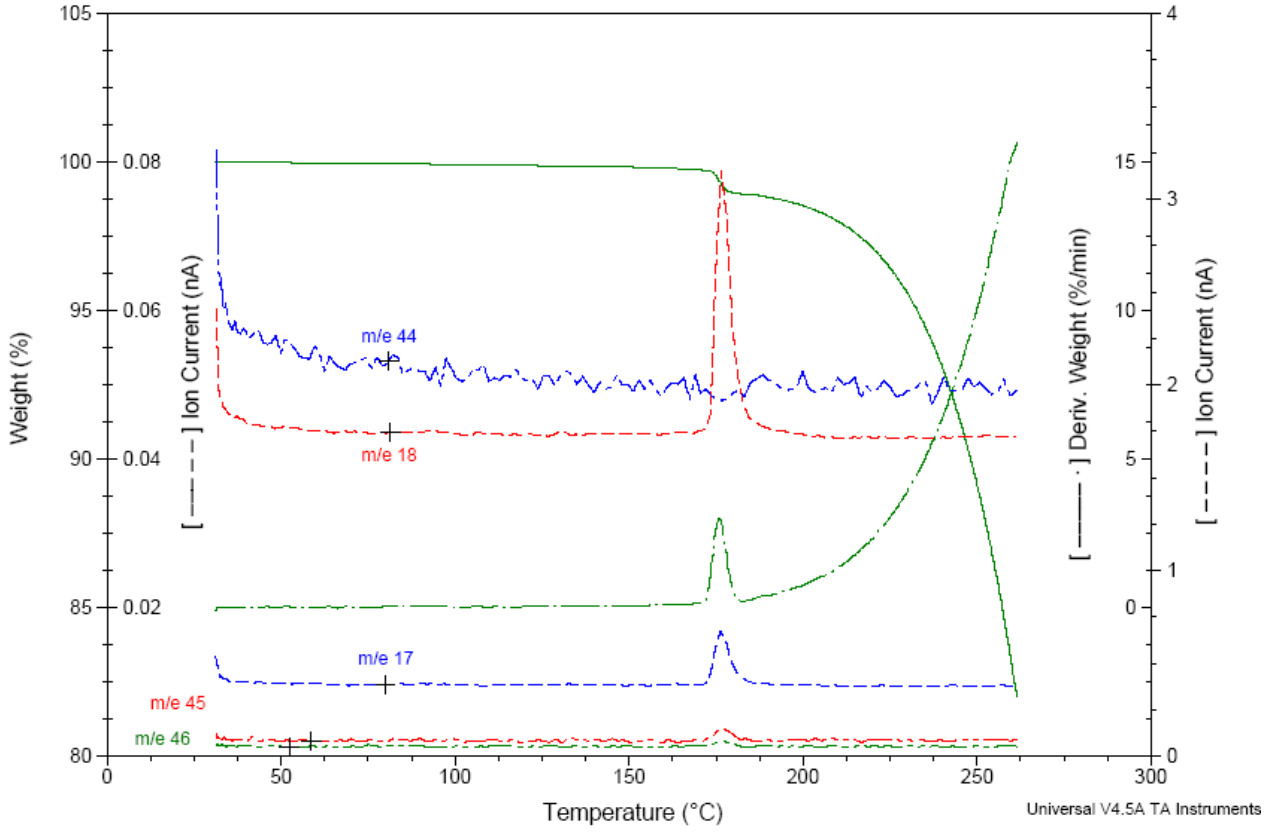


1-Methylestradiol **(7)**

Annex B – Thermogravimetric behaviour of CCQM-K55.a



TGA data for CCQM-K55.a in range 80 °- 200 °C showing an initial mass loss (ca 1.5 mg/g, 0.15 % relative) at 120 °C and subsequent mass loss (ca 6 mg/g) above 170 °C



TGA-MS of CCQM-K55.a in range 30 °C – 250 °C showing mass loss (solid green line), mass change derivative (alternating green lines) at 170 °C and ion current (dashed lines) for selected *m/z* from liberated volatile material

References

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