Influence of the approach to calibration on accuracy and traceability of certified values in certified reference materials

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Examples:
Chloramphenicol in pork (ERM-BB130)

Oxytetracycline in milk (ERM-BB492)

Outline

Certification exercises at IRMM

Calibration approaches – common calibrant; in-house calibrant
Pros and cons

The two examples (background information, materials, methods)
ERM-BB130 (chloramphenicol in pork)
ERM-BB492 (oxytetracycline in milk)

Techniques used for calibrant purity and identity assessment

Results
Interlaboratory comparisons – differences

Conclusions, take-home messages

Certification approach at IRMM:

Typically by intercomparison among expert laboratories

Qualification of laboratories:

a.) documented evidence of competency

validated method (fit-for-purpose for this exercise) - compulsory

method under scope of accreditation (ISO17025) - asset

satisfactory results in recent intercomparisons (e.g. PT studies) with same or

similar analyte/matrix combination

b.) dedicated study (preliminary intercomparison); blinded RMs as samples

Key:

Suitable study design

Clear instructions to participants

Joint
Research
Centre

2 calibration approaches compared:

"common calibrant"

Commercially available substance, extensively characterised for its purity and identity at IRMM and elsewhere (fulfils RM requirements); shipped to ILC participants

"in-house calibrant"

Commercially available substance (different sources), defined in SOP of laboratory method; usual approach: purity taken as indicated by provider

Each laboratory:

2 calibration curves made for each measurement
1.) one with the common calibrant
2.) one with the in-house calibrant
2 results submitted

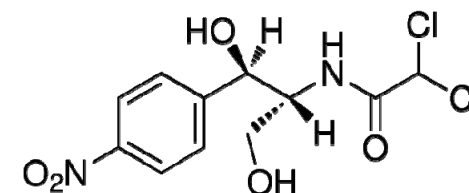## 2 calibration approaches compared – some pros and cons

Common calibrant

+ Proper estimation of uncertainty related to purity

+ Transparent and unequivocal situation as concerns metrological traceability

- Unavailability of calibrant – new batch with different purity

In-house calibrant

+ More rugged data if (trustworthy) calibrants from different sources are used

+ Avoid potential systematic bias

- Very often, "standards", "reference standards", "reference materials" (…) do not fulfil RM requirements!

- Purity value not clear ("only" HPLC/GC area % ?; water included ?; what is the ± value ?, etc.)

Chloramphenicol (CAP) in pork (ERM-BB130)

Broad-spectrum antibiotic and highly effective against many pathogenic Gram-positive and Gram-negative bacteria, rickettsiae and mycoplasmas

Banned in the EU since 1994 as the uptake of CAP in humans can cause serious haemotoxic effects such as agranulocytosis, aplastic anaemia, and leukemia

MRPL (minimum required performance limit) in the EU since 2003: 0.3 μg/kg in various food matrices (Commission Decision 2003/181/EC)

Processing: incurred pork meat, lyophilised, cryogenically milled, mixed with blank pork powder to desired target concentration around 0.3 μg/kg

Methods

Sample preparation part
Differences in:
- Sample intake (1 – 5 g reconstituted material)
- Type of extraction solution (e.g. ethyl acetate; McIlvaine buffer)
- Clean-up (e.g. none; defatting with isooctane; reverse-phase SPE)
- Type of internal standards (e.g. d_5-CAP; $^{37}Cl_2$-CAP)

Calibration: neat standards, matrix-matched

Analytical part
LC-ESI-MS/MS (neg. ESI, tripe quad; MRM)
Differences in : type of C_{18} column; solvents, HPLC and MS used, HPLC settings (flow rate, injection amount); compound-dependent parameters (dwell time) as well as in source/gas-related MS settings (temperature; curtain gas)

GC-MS (ECNI, SIM)
Differences in : type of capillary GC column (coating); GC and MS settings (e.g. injection amount and type; carrier gas and flow rate; temperature gradient)
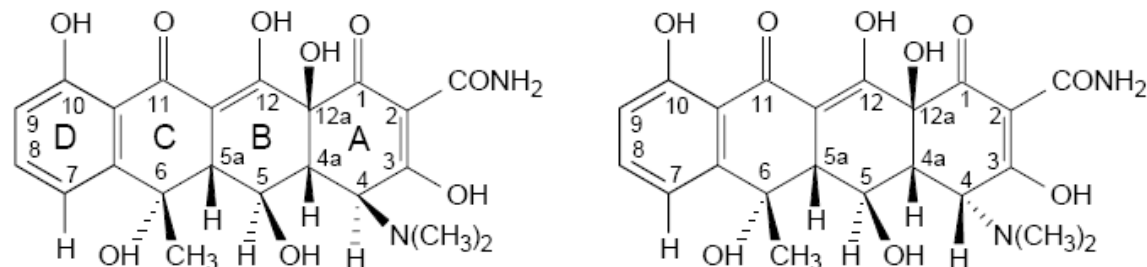
Oxytetracycline in milk (ERM-BB492)

Oxytetracycline (OTC): broad-band antibiotic

Approved for use in food-producing animals, e.g. in dairy cattle: treatment of bacterial infections such as enteritis, pneumonia, and diphtheria

Maximum residue limit (MRL): 100 μg OTC/kg milk [Commission Regulation (EC) No. 508/1999]   NOTE: OTC is defined as the sum of OTC and its 4-epimer

Epimerisation: isomerisation at asymmetric C-atom in slightly acidic solution

Chemical structures of oxytetracycline (left) and 4-epi-oxytetracycline (right)

Processing of material: skimmed milk, spiked with OTC, spray-dried

Methods

Sample preparation part
Differences in:
• Sample intake (0.5 – 6 g reconstituted material)
• Type of extraction solution (e.g. trichloroacetic acid; succinate buffer)
• Clean-up (e.g. ultrafiltration; mixed-mode or reverse-phase SPE)
• Type of internal standards (e.g. none; demeclocycline)

Calibration (all: matrix-matched)

Analytical part
LC-ESI-MS/MS (pos. ESI, tripe quad; MRM)
Differences in : type of C_{18} column; solvents, HPLC and MS used, HPLC settings (flow rate, injection amount); compound-dependent parameters (dwell time) as well as in source/gas-related MS settings (temperature; curtain gas)

LC-UV
Differences in : type of LC column (reverse-phase coating); injection amount, detection wavelength

epi-OTC found in tissues such as kidney or liver, but not in milk (literature)

most routine methods (laboratories accredited to ISO17025 measuring OTC in food matrices) do not separate the two epimers by chromatography (which is easy to achieve), calibration only done with OTC

Techniques applied for purity and identity assessment

qNMR (BAM, Spectral Service)

HPLC-UV/DAD: different columns, solvent systems, detection wavelengths

      (IRMM, KU Leuven)

Coulometric Karl-Fischer titration (IRMM)

Total ash: Pharmacopeia 6.0 (SOLVIAS)

TG-FTIR (SOLVIAS)

DSC (IRMM)

LC-QTOF-MS (BVL)

Calibrant CAP

Specification: >99.5 % (CoA indicated 99.8 area% HPLC)

Verification measurements:
1.) cKFT: 0.09 ± 0.02 m/m %
2.) HPLC 200 nm, 99.73 ± 0.02%; 273 nm, 99.83 ± 0.01 %;
3.) ash: <0.05 % (below LOD of method)
4.) TG-FTIR: < 0.05 % (below LOD of method)
5.) DSC: no impurity detected
6.) qNMR: 99.6 ± 0.03 m/m % (k=2)

Identity check: LC-QTOF-MS, no other compounds detected

Purity assumed: 99.5%
Uncertainty: 0.5 * (100-Purity)/SQRT(3), 0.145 m/m %

Calibrant OTC

Specification: 98.1 area% HPLC, 264 nm (CoA)

Verification measurements:
1.) cKFT: 4.96 ± 0.37 m/m %
2.) HPLC 220 nm, 98.7 ± 0.1%; 240 nm, 99.1 ± 0.1%;
3.) ash: <0.05% (below LOD of method)
4.) TG-FTIR: 5.32 ± 0.13 m/m % (sum of water and residual solvents)
5.) DSC: not applicable (decomposition of compound)
6.) qNMR: 94.34 ± 0.061 m/m %

Identity check: LC-QTOF-MS, 3 organic impurities identified

Purity assumed: 94.34 %
Uncertainty: 0.5 * (highest – lowest qNMR result)/SQRT(3), u_{cal} = 0.569 m/m %

Characterization intercomparisons

ERM-BB130, CAP in pork

   16 laboratories

   each lab: 2 days, 4 analyses/day: 8 independent analyses

   all measurements under ISO17025 accreditation

ERM-BB492, OTC in milk
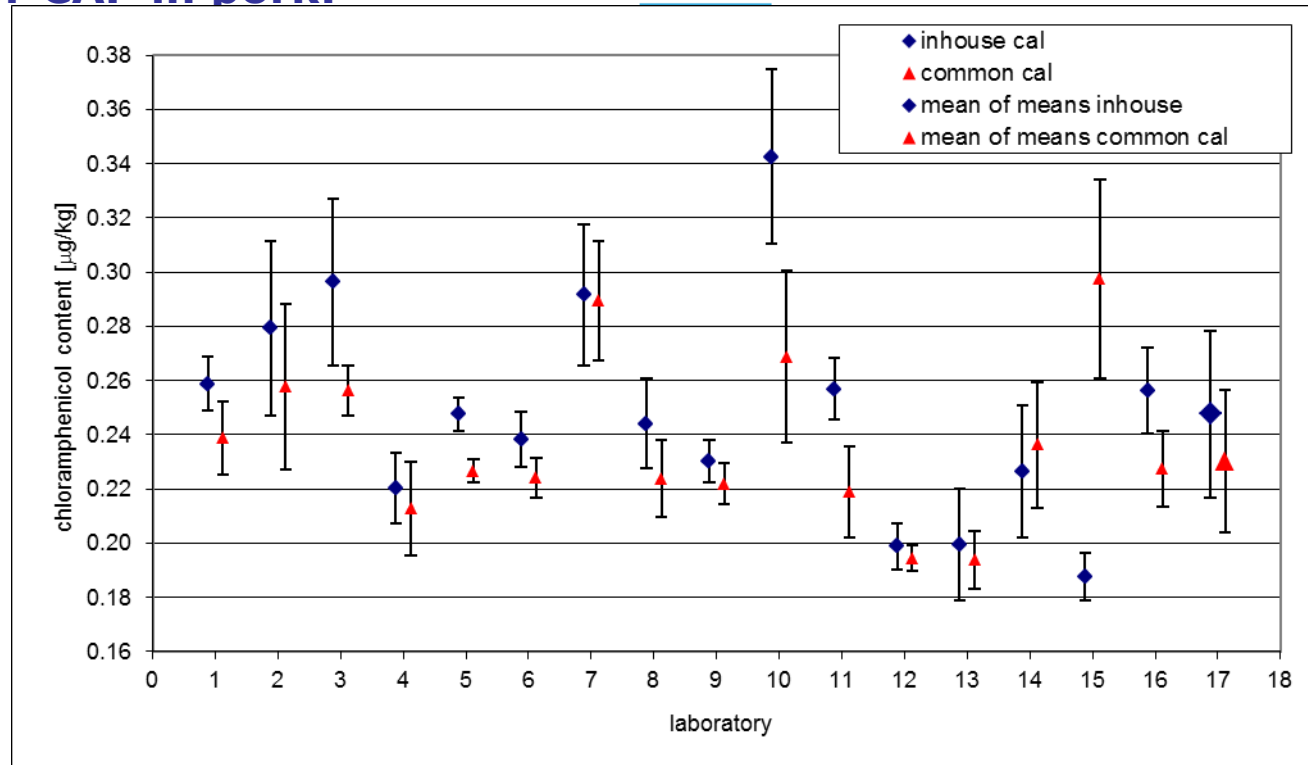
   12 laboratories

   each lab: 3 days, 3 analyses/day: 9 independent analyses

   10 laboratories operated ISO17025 accredited methods

   1 laboratory in the process of have its method ISO17025 accredited

   1 laboratory operated a GLP-compliant method
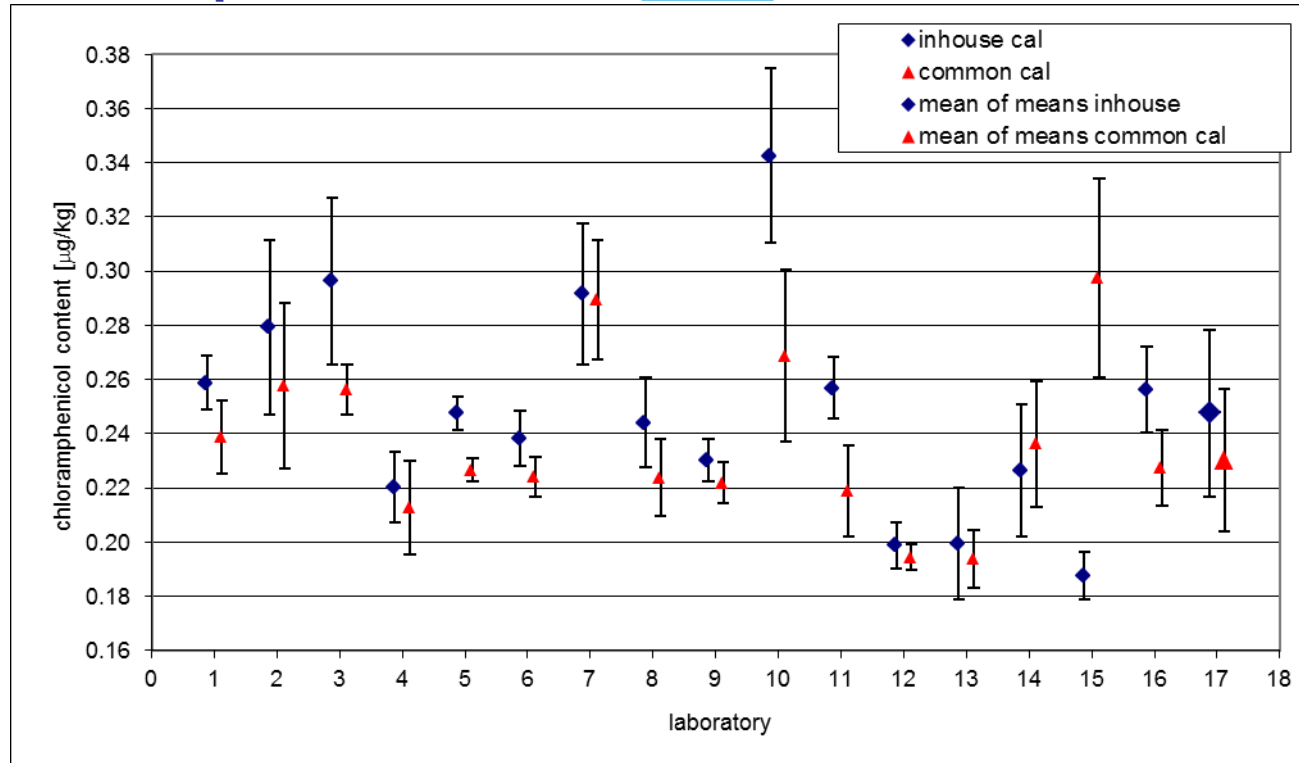
Result for CAP in pork:

Overestimation of purity of in-house calibrants in one case (lab 15)

data set lab 15 excluded, as technically invalid (non-linear calibration line)

2 data sets (labs 10 and 14) not considered, as the measurand was different (free CAP versus total CAP) – only those labs performed a β-glucuronidase digestion during sample preparation
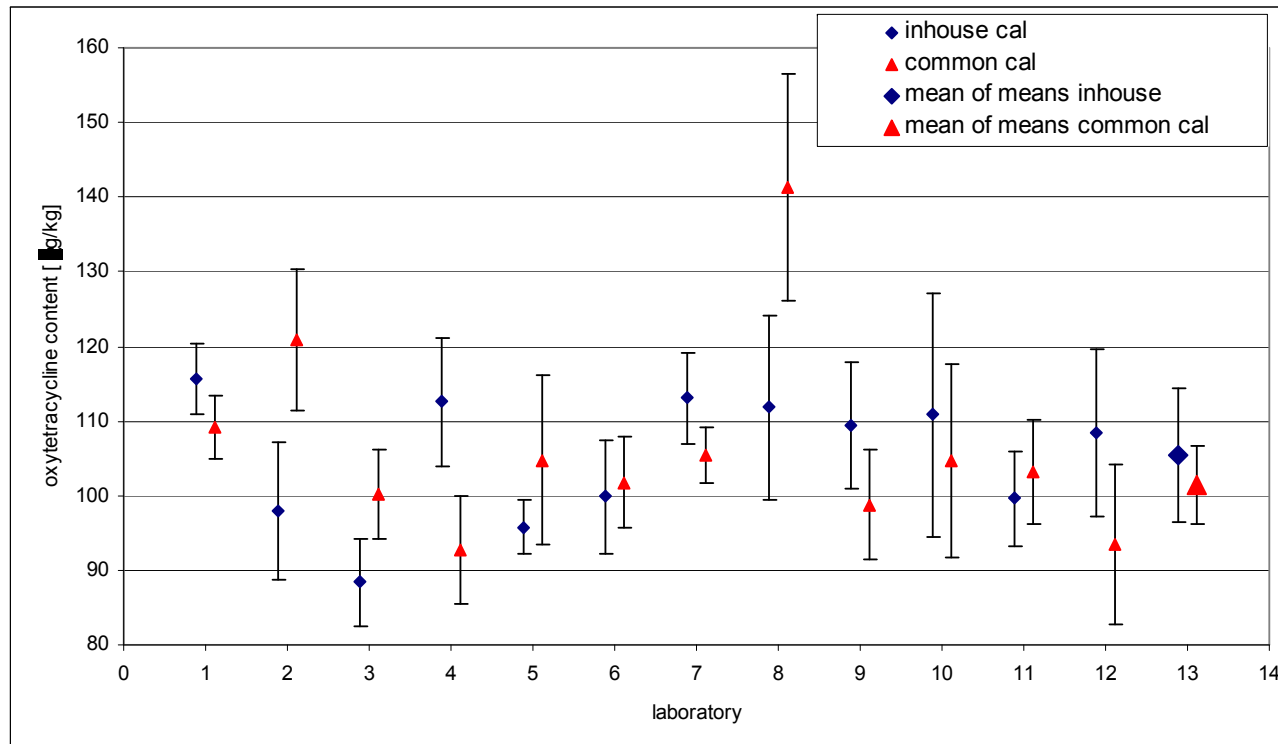
Result for CAP in pork:

Consequences on certified value and its uncertainty are significant!

u_{char}[%] drops from 3.46 to 3.16 if the common calibrant is used

Certified value and uncertainty change from 0.248 ± 0.024 µg/kg to 0.230 ± 0.021 µg/kg
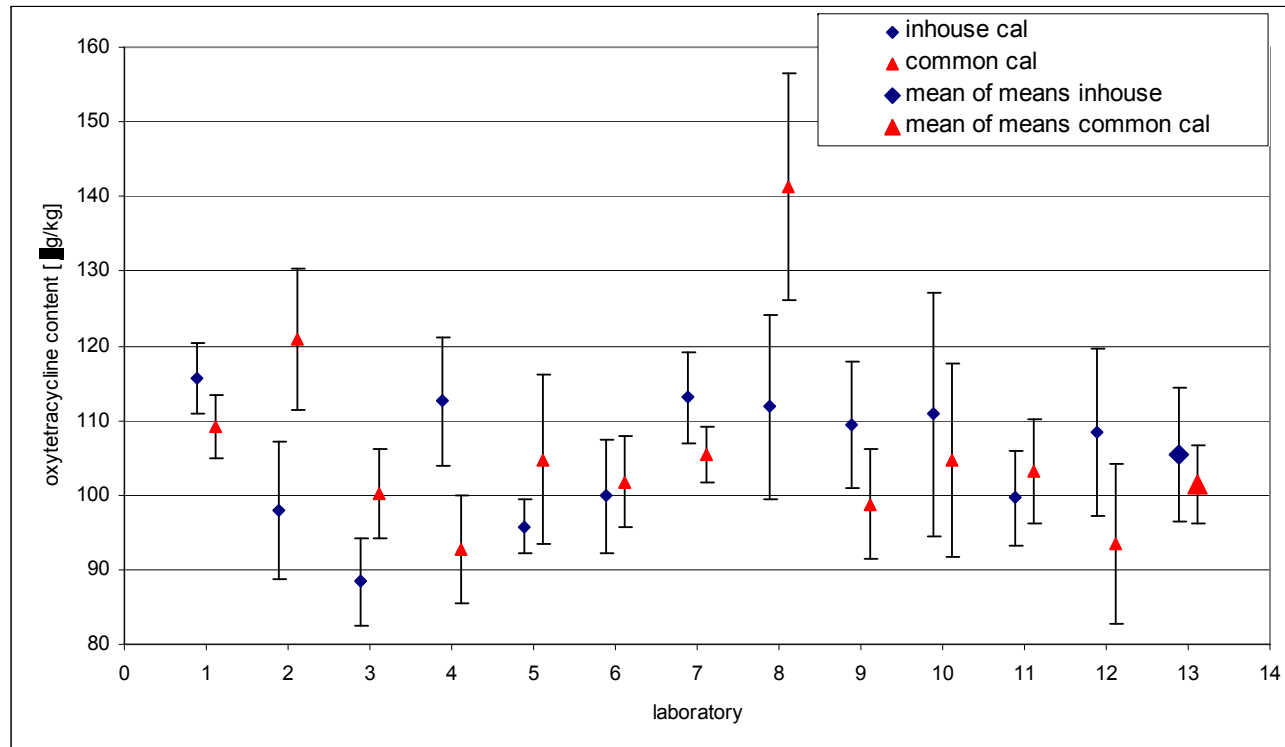
Result for OTC in milk:

Overestimation of purity of in-house calibrants in some cases (lab 2, 8)

Data sets labs 2 and 8 are technically invalid (not meeting requirements set by IRMM: non-linear calibration curve, inconsistent recovery values for QC sample)

Result for OTC in milk:

Consequences on certified value and its uncertainty are significant!

u_{char}[%] drops from 2.68 to 1.63 if the common calibrant is used

Certified value and uncertainty change from 105 ± 12 µg/kg to 101 ± 11 µg/kg

Some more considerations for one or the other approach

Certification measurements versus "routine measurements"

What typically is good enough for routine measurements, might not be

sufficient for certification purposes

Calibration /calibrant uncertainty in context with expanded U of material, is it

relevant?

Metrological traceability for property values reference materials from IRMM:

1. identity of the measurand, either:

        structurally defined (e.g. total Cd)

        operationally defined (e.g. total dietary fibre according to AOAC 2009.01)

2. quantity value of measurand, either:

        to SI

        to artefact

Koeber, R., Linsinger, T.P.J., Emons, H. (2010) Accred. Qual. Assur. 15, 255-262

ERM policy statement for metrological traceability

(http://www.erm-crm.org/)

Related to a combination of 5 attributes

1. body/matrix identification (e.g. partially skimmed milk)
2. property/component identification (e.g. OTC as defined by xxx)
3. quantity/kind of quantity: (e.g. mass fraction)
4. number and its uncertainty: (e.g. 101 $\pm$ 11)
5. measurement unit (e.g. μ g/kg)

Traceability statement on certificate (OTC)

PARTIALLY SKIMMED MILK		
	Mass fraction	
	Certified value [2)] [μg/kg]	**Uncertainty [3)] [μg/kg]**
Oxytetracycline (sum of oxytetracycline and 4-epi-oxytetracycline) [1)]	101	11
1) Oxytetracycline as measured by liquid chromatography - tandem mass spectrometry and liquid chromatography - diode array detection. Different sample preparation procedures (extraction and clean-up) were applied. 2) The value is applicable to the material when reconstituted according to the specified procedure (page 3). The certified value is the unweighted mean of 10 accepted set of results, each set being obtained in a different laboratory, using the calibration substance provided. The value is traceable to the International System of Units (SI).		
3) Expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of approximately 95 % estimated in accordance with ISO Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM), ISO, 2008.		

Value traceable to the SI via the use of the common calibrant

Conclusions and take-home messages

Different requirements for routine measurements and measurements used to certify reference materials

Degree of difference among the two calibration approaches is case-depending (analyte, matrix, performances of methods)

The two examples: some hints to overestimation of purity of in-house calibrants

Type of calibration influences both accuracy and traceability

Common calibrant approach favourable if calibrant purity is not well established

Hope to welcome you soon at IRMM!