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CERTIFICATION REPORT

The Certification of the Mass Fraction of Oxytetracycline in Partially Skimmed Milk

Certified Reference Materials
ERM®-BB492 and ERM®-BB493

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CERTIFICATION REPORT

The Certification of the Mass Fraction of Oxytetracycline in Partially Skimmed Milk

Certified Reference Materials
ERM®-BB492 and ERM®-BB493

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Disclaimer

Summary

This report describes the preparation of two milk powder matrix reference materials (ERM-BB492 and ERM-BB493) and the certification of their content (mass fraction) of oxytetracycline.

The preparation and processing of the material, homogeneity and stability studies, and the characterisation are described hereafter and the results are discussed. Uncertainties were estimated in compliance with ISO Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM) [1].

For ERM-BB492, the uncertainty contains contributions from possible heterogeneity, instability, characterisation, and purity of the common calibrant. The certified value is listed below:

Mass fraction in the reconstituted material	Certified value [1] [µg/kg]	Uncertainty [2] [µg/kg]	Number of accepted sets of results
Sum of oxytetracycline and 4-epi-oxytetracycline	101	11	10

1) The value represents the mass fraction based on the unweighted mean of means of accepted results.
2) Expanded uncertainty ($k = 2$) of the value defined in 1).

The certified value and its uncertainty is based on a minimum sample intake of 1.00 g reconstituted material.

For ERM-BB493, the following certified value has been assigned:

Mass fraction in the reconstituted material	Certified value [1] [µg/kg]
Sum of oxytetracycline and 4-epi-oxytetracycline	< 5

1) This value corresponds to the limit of quantification (LOQ) of the most sensitive method in the characterisation study. The certified value is below 5 µg/kg with a 95% level of confidence.

The indicative value is based on a minimum sample intake of 3.50 g reconstituted material.

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1 Glossary

ANOVA ....Analysis of variances
b......................................Slope of regression line
BCR ....Community Bureau of Reference
C_{18}...................................Octadecyl silica
CAS................................Chemical Abstracts Services
cKFT ....Coulometric Karl Fischer titration
CRM................................Certified reference material
DAD ....Diode-array detector
DSC ....Differential scanning calorimetry
EDQM ....European Directorate for the Quality of Medicines
EOTC..............................4-epi-oxytetracycline
ERM................................European Reference Material
ESI ....Electrospray ionisation
GUM................................Guide to the Expression of Uncertainty in Measurement
i.......................................Position of result in the analytical sequence (homogeneity study)
IRMM ....Institute for Reference Materials and Measurements
IUPAC ....International Union for Pure and Applied Chemistry
LC ....Liquid chromatography
LC-MS/MS.......................Liquid chromatography-tandem mass spectrometry
LLE..................................Liquid liquid extraction
MCAC ....Metal-chelate affinity chromatography
m/m ....Mass-to-mass
MRL ....Maximum residue limit
MRM ....Multiple reaction monitoring
MS ....Mass spectrometry
$MS_{\text{between-unit}}$....................Mean of squares between units from a 2-way ANOVA
$MS_{\text{within-sample (error)}}$ ....Mean of squares within a unit from a 2-way ANOVA
n.......................................Number of replicates
ODS ....Octadecyl silica
OTC ....Oxytetracycline
PET ....Polyethylene terephtalate
PSA.................................Particle size analysis
QC ....Quality control
qNMR..............................Quantitative nuclear magnetic resonance
RP...................................Reversed phase
RSD ....Relative standard deviation
RSD_{stab} ....Relative standard deviation of all results of the stability study
s......................................Standard deviation
s_{bb}....................................Between-bottle standard deviation
SI ....International Systems of Units
S/N..................................Signal-to-noise ratio
SPE.................................Solid phase extraction
s_{wb} ....Within-bottle standard deviation
$t_{\alpha,df}$....................................Critical t-value for a t-test, with a level of confidence of 1-α, and df degrees of freedom
TG-FTIR..........................Thermogravimetry coupled to Fourier-transform infrared spectrometry
TOF.................................Time-of-flight
u^{*}_{bb} ....Relative standard uncertainty due to the heterogeneity that can be hidden by the method repeatability
u_{bb} ....Relative standard uncertainty due to between-bottle heterogeneity

u_{cal} ....Relative uncertainty of common calibrant

u_{char} ....Relative uncertainty of the characterisation exercise

$u_{CRM, rel}$ ....Combined relative uncertainty of certified value

U_{CRM} ....Expanded uncertainty of certified value

$U_{CRM, rel}$ ....Expanded, relative uncertainty of certified value

u_{lts}.....................................Relative uncertainty of long-term stability

u_{meas} ....Uncertainty of measurement result

u_{sts} ....Relative uncertainty of short-term stability

u_{Δ} ....Combined uncertainty of certified value and measured value

U_{Δ}.....................................Expanded uncertainty of certified value and measured value

UPLC ....Ultra-performance liquid chromatography

x ....Pre-defined shelf life

x_i......................................Time point i in an isochronous stability study

Δ ....Difference between two measurement results

Δ_m....................................Difference between measured and certified value

vKFT ....Volumetric Karl Fischer titration

$v_{MSwithin}$ ....Degrees of freedom of MS_{within}

2 Introduction

2.1 Background

Oxytetracycline (OTC) is a broad-spectrum antibiotic highly effective against many bacteria. It is produced through fermentation by the soil bacterium *Streptomyces rimosus* [2]. OTC is approved for use in a variety of food-producing animals such as poultry, sheep, goat, swine, and fish, and widely used in the treatment of lactating and non-lactating dairy cattle for treatment of bacterial infections such as enteritis, pneumonia, shipping fever, diphtheria, and foot rot [3]. Residues of OTC can therefore be found in meat tissues, but also in inner organs (liver, kidney) and animal products such as eggs and milk, especially if the recommended dosage and/or the necessary withdrawal period are not respected.

In order to produce and safeguard reliable analytical results which are necessary to ensure effective consumer protection, a certified reference material (BCR-492) was made available by the Community Bureau of Reference. BCR-492 was certified in the 1990s with an OTC target concentration of 300 µg/L milk, based on the Official French screening method at that time [4]. Since that time, legal requirements in the European Union have changed, and for OTC (defined in European legislation as the sum of oxytetracycline and its 4-epimer), a provisional maximum residue limit (MRL) of 100 µg/kg in milk has been stipulated [5], and later on, this MRL has been confirmed and fixed at that concentration [6].

For both screening, but especially confirmation analysis, liquid chromatography - diode array detection (LC-DAD) as well as liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods are used, with a clear trend towards LC-MS/MS methods in recent years. An analytical challenge in the analysis of OTC and other tetracyclines is their isomerisation in solution. In slightly acidic medium, OTC is prone to a specific type of isomerisation known as epimerisation, which occurs at the C-4 atom (Figure 1) and results in the formation of 4-epi-oxytetracycline, EOTC [7]. The process is reversible and depends on the pH, the temperature, and the type of solution in which OTC is dissolved [7,8]. Moreover, epimerisation is catalysed by multi-valent cations, urea, citrate, and phosphate [9]. Also, the food matrix and its properties substantially influence the degree of epimerisation, with OTC epimerisation being reported in meat and inner organs [10], honey [11], and egg yolk [12], but not in milk [13]. Most currently used chromatographic methods do not distinguish between OTC and 4-epi-OTC (coelution of compounds); however, several papers have been published, demonstrating the feasibility of chromatographic separation of TCs and their 4-epimers [13 -16].

2.2 Choice of the material

Bovine milk was chosen as the material matrix in order to be consistent with BCR-492. In contrast to BCR-492 (skimmed milk), a partially skimmed milk (fat content 1.5 m/m %) was used as starting material. It was decided to process the milk to a powder by applying spray drying, a process which is used in the industry to produce milk powder. Moreover, experiments at IRMM revealed that the spray-dried powder could more easily be reconstituted than lyophilised milk powder (better and quicker dissolution).

The envisaged target concentration for candidate ERM-BB492 was 100 µg/kg. In addition, a blank milk powder (candidate ERM-BB493) was produced. Table 1 and Figure 1 define the analytes in ERM-BB492 and ERM-BB493.

2.3 Definition of analyte and chemical structure

Table 1. Definition of the oxytetracycline analytes comprised in ERM-BB492 and ERM-BB493

| Trivial name and abbreviation	IUPAC name	CAS number	Chemical formula	Molecular mass (g/mol)
Oxytetracycline (OTC)	(2E,4S,4aR,5S,5aR,6S,12aS)-2-[amino(hydroxy)methylidene]-4-(dimethylamino)-5,6,10,11,12a-pentahydroxy-6-methyl-4,4a,5,5a-tetrahydrotetracene-1,3,12-trione	79-57-2	$C_{22}H_{24}N_2O_9$	460.43
4-epioxytetracycline (4-epi-OTC)	(2E,4R,6S,12aS)-2-[amino(hydroxy)methylidene]-4-(dimethylamino)-5,6,10,11,12a-pentahydroxy-6-methyl-4,4a,5,5a-tetrahydrotetracene-1,3,12-trione	14206-58-7	$C_{22}H_{24}N_2O_9$	460.43
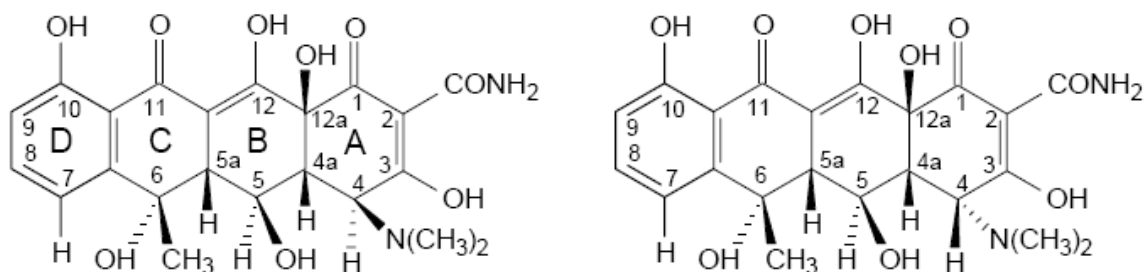
Fig. 1: Chemical structures of oxytetracycline (left) and 4-epi-oxytetracycline (right)

3 Participants

Project management and evaluation:
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(Work performed under ISO Guide 34 accreditation; BELAC-268-Test)

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(Measurements performed under ISO/IEC 17025 accreditation, DAP-PL-2614.14)

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(Measurements in conformity with GLP according to Directive 2004/9/EC)

4 Processing of the material

The processing of partially skimmed bovine milk into a spray-dried powder was carried out at the Staatliche Milchwirtschaftliche Lehr- und Versuchsanstalt, Wangen im Allgäu, Germany. First, whole milk and skimmed milk were mixed to obtain milk with 1.5 m/m % fat content. The milk was thermally preconcentrated in an evaporator by a factor of 2.8; this step was necessary to ensure that the subsequent spray-drying yields a powder of suitable properties (particle size, residual water content, and reconstitutability).

The blank material was spray-dried.

For the spiked material, a spike solution was prepared by dissolving pure crystalline OTC dihydrate (Sigma Aldrich, Bornem, Belgium) in 20 (v/v) % methanol. 2 L of preconcentrated milk were then spiked with the OTC solution, and after extensive stirring, the spiked milk was added stepwise into 54 L of preconcentrated milk to yield the final product with a target OTC content of about 100 µg/kg. The milk was again stirred extensively, and then subjected to spray-drying. All spiking and dilution steps were performed on calibrated balances.

The process yield after spray-drying was 12 kg of both blank milk powder and OTC-containing milk powder. These were packed under inert gas and shipped to IRMM. Both milk powders were extensively mixed in a 3-dimensional mixer (Dynamix CM-200 WAB, Basel, Switzerland) for 1 hour to ensure homogenisation. Due to the hygroscopic behaviour of the material, filling was performed in a glove box under nitrogen atmosphere. A vibrating feeder (Fritsch, Idar-Oberstein, Germany) was used to fill 5.5 g portions of powder in 30 mL amber glass vials which were then closed with rubber stoppers. The vials were then sealed with metal caps, using a Bausch & Ströbel capping machine (Ilshofen, Germany). After labelling, sample sets were produced by putting together one vial of ERM-BB492 (spiked material) and one vial of ERM-BB493 (blank material) and sealing those in a metalized PET pouch for additional protection from light, humidity, and entrainment of air.

In total, 1300 bottles each of ERM-BB492 and 493 were processed; they were stored after production at -70 ºC.

5 Material characterisation measurements

5.1 Water content
The water content in the final materials was measured by volumetric Karl Fischer titration (vKFT) [17]. Ten bottles of the batch were chosen using a random stratified sample picking scheme and analysed in duplicate. The determined mean water content and its standard deviation was 2.50 ± 0.08 g/100 g for ERM-BB492 and 2.69 ± 0.11 g/100 g for ERM-BB493.

5.2 Particle size measurements
Particle size analysis (PSA) was performed using laser diffraction spectrometry. Five bottles were chosen from the final material using a random stratified sample-picking scheme and analysed over a range of 0.5 to 1750 µm using a Helos laser light scattering instrument (Sympatec GmbH System-Partikel-Technik, Clausthal-Zellerfeld, DE). The determined top particle size for the milk powder was 360 µm. About 50 % of all particles were smaller than 30 µm and approximately 10 % of all particles were smaller than 9 µm (same values for both materials).

6 Homogeneity study

For the homogeneity study, 15 samples of ERM-BB492 were chosen using a random stratified sample selection scheme and analysed in triplicate for their OTC content (sum of OTC and 4-epi-OTC). Measurements were performed with an in-house validated LC-ESI-MS/MS method compliant with Commission Decision 2002/657/EC. Matrix-matched calibration was performed by spiking neat standard solutions to blank material (lyophilized pork powder provided by IRMM). No internal standard was used. One g of milk powder was reconstituted with 8.15 g water, and 1 g of the resulting milk was used as the sample intake for analysis.

As not all samples could be analysed in one analytical series, they were split over different series and run over different days in such a way that, for each bottle, one replicate was analysed on each analysis day (n=3). Within a series (day), samples were analysed in a random order. Moreover, two consecutive injections were made of each sample replicate, and the average was taken as the result for the replicate.

Data were checked for single and double outliers by applying the Grubbs test at a confidence level of 95% and 99%; no outlier was detected. Regression analysis was performed to detect possible trends regarding the filling sequence or analytical sequence. In the filling sequence, the slope was indistinguishable from zero at both 95 and 99% level, whereas in the analytical sequence a significant slope (test value 2.43, critical value 2.02) was observed at the 95% level for the analytical sequence (no significant slope at 99% level).

A t-test (data not shown) revealed a significant difference of day means, which made it necessary to analyse the data by 2-way ANOVA, thereby differentiating between sample-to-sample, day-to-day, and random effects.

In conclusion, the distribution of OTC in the material can be regarded as sufficiently homogeneous. Furthermore it was checked whether the data followed a normal or unimodal distribution using normal probability plots and histograms, respectively. Individual data and sample averages showed a unimodal distribution. Finally, the uncertainty contribution from possible heterogeneity was estimated by a two-way analysis of variance (ANOVA) [18]:

Method repeatability (s_{wb}) expressed as a relative standard deviation is given as follows:

$$s_{wb} = \frac{\sqrt{MS_{within-sample(error)}}}{\overline{y}}$$

$MS_{within-sample\ (error)}$: mean square within a bottle from a 2-way ANOVA
$\overline{y}$: average of all results of the homogeneity study

Between-unit variability (s_{bb}) expressed as a relative standard deviation is given by the following equation:

$$s_{bb} = \frac{\sqrt{\dfrac{MS_{between-unit} - MS_{within-sample(error)}}{n}}}{\overline{y}}$$

$MS_{between-unit}$: mean square among units from a 2-way ANOVA
n: number of replicates for a sample

The heterogeneity that can be hidden by method repeatability [19] is defined as follows:

$$u_{bb}^{*} = \frac{s_{wb}}{\sqrt{n}} \sqrt[4]{\frac{2}{\nu_{MSwithin-sample(error)}}}$$

$\nu_{MSwithin-sample\ (error)}$: degrees of freedom of $MS_{within-sample\ (error)}$

The larger value of s_{bb} or u^*_{bb} was used as uncertainty contribution for homogeneity, u_{bb} (see Table 2 for a summary of results, values were converted into relative uncertainties).

Table 2. Homogeneity study results for ERM-BB492

RSD [%]	5.761
$MS_{within\ (error)}$	15.17435
$MS_{between\ sample}$	57.10739
$MS_{between\ day}$	522.33550
s_{wb} [%]	3.125
s_{bb} [%]	2.999
u^*_{bb} [%]	0.933
u_{bb} [%]	**2.999**

6.1 Minimum sample intake

For ERM-BB492, 1 g was the sample intake used in the homogeneity and stability studies. Therefore, the minimum sample intake is 1 g of reconstituted material, proving that the samples are homogeneous at least at this level. The difference between the amount of reconstituted material and the minimum sample intake shall be noted: the laboratory performing the analyses reconstituted 1 g of powder with 8.15 g of water, and from the 9.15 g of reconstituted milk, an aliquot of 1 g was taken for analysis.

For ERM-BB493 (blank material), no homogeneity and short-term stability study was performed. The minimum sample intake is 3.5 g , which was the sample intake of laboratory 12 in the characterisation study, operating the method with the lowest LOD (see page 22).

7 Stability studies

7.1 Short-term stability study

A four weeks isochronous study [20] was performed to evaluate stability of ERM-BB492 during transport. Twenty samples were selected from the produced batch using a random stratified sample picking scheme.

Samples were stored at +4 °C, +18 °C, +60 °C and at a reference temperature of -70 °C. Two bottles were stored at each temperature for 0, 1, 2, and 4 weeks. After the indicated storage periods, the samples were transferred to storage at -70 °C until analysis. Samples were dispatched to the testing laboratory on dry ice. In total, 20 samples (3 temperatures x 3 storage times x 2 units/time point = 18 samples, and 2 samples stored at reference termperature throughout the isochronous study) were analysed in triplicate, so 60 measurements were performed in total. Measurements were carried out under intermediate precision conditions, whereby on each of the four measurement days, five samples were analysed in triplicate (15 analyses per day). Samples were analysed in a randomised order. The same LC-MS/MS method as for the homogeneity study was used, except that new calibration solutions were prepared and used.

Data (Annex B) were first checked for single and double outliers by applying the Grubbs test at confidence levels of 95 % and 99 %, respectively. No outliers were detected. Data points were plotted against time and the regression lines were calculated (see Table 3 for a summary). The observed slopes were tested for significance using a t-test, with $t_{\alpha,df}$ being the critical t-value (two-tailed) for a confidence level $\alpha = 0.05$ (95 % confidence interval). The slope was considered as statistically significant when $b/s_b > t_{\alpha,df}$.

Whereas the slope was found to be indistinguishable from zero for storage temperatures of 4 °C and 18 °C, a significant slope was found when the samples were stored at 60 °C (95 % confidence level, t value 2.61, t_{crit} 2.07, no significant slope at 99 % confidence level). It was concluded that the uncertainty of the short-term stability (u_{sts}) can be assumed to be negligible if sample shipment is carried out at temperatures below 18 °C.

Table 3. Short-term stability results for ERM-BB492

Statistical parameters	4 °C	18 °C	60 °C
Slope (b) [%/week]	-1.17	0.01	- 1.83
$|b|/s_b$	1.758	0.012	2.350
Statistical significance (95% conf. interval) [1]	no	no	yes
u_{sts} [%/week]	0.693	0.607	1.314

[1] $t_{0.05;22} = 2.074$

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7.2 Long-term stability study

A twelve months isochronous study [20] was performed to evaluate the stability of ERM-BB492 and ERM-BB493 during storage, taking into account existing knowledge about stability of BCR-492. For ERM-BB493 (blank material), the concept of stability testing was to spike each sample with 100 µg/kg of OTC at the beginning of the sample preparation, and to verify potential alteration of the matrix during the storage time at different temperatures by analysing the samples.

For ERM-BB492 and ERM-BB493, samples were picked from the produced batch using a random stratified sample picking scheme. Samples were stored at +4 °C and -20 °C, and at a reference temperature of -70 °C. Three bottles were stored at each temperature for 0, 4, 8, and 12 months, respectively. After the indicated periods, samples were transferred to -70 °C until analysis. Samples were dispatched on dry ice and kept at -70 ºC in the laboratory until analysis. In total, 21 samples (2 temperatures x 3 storage times x 3 units/time point = 18 samples, and 3 samples stored at reference temperature throughout the isochronous study) were analysed in triplicate (for ERM-BB492) or in duplicate (for ERM-BB493). Moreover, two consecutive injections were made of each replicate, and the average was taken as the result for the replicate. In total, 126 measurements were performed for ERM-BB492, and 84 measurements were performed for ERM-BB493. Samples were analysed under intermediate precision conditions (3 measurement days for ERM-BB492, 2 measurements days for ERM-BB493). For this, one replicate of each sample was analysed on each analysis day.

For both materials, the same LC-MS/MS method as for the short-term stability study was applied, except that a different type of reverse-phase LC column (Symmetry$^{®}$ C$_{18}$, Waters, instead of Aqua™, Phenomenex) was used and the aqueous mobile phase was slightly adapted.

For both ERM-BB492 and ERM-BB493, significantly different day means were obtained (verified by a t-test, data not shown). Consequently, values were normalised to the respective day means.

Data (Annex C) were checked for single and double outliers by applying the Grubbs test at confidence levels of 95 % and 99 %, respectively. For ERM-BB492, one outlier was detected (95 % and 99 % confidence level); in-depth data inspection showed a considerably smaller peak area compared to other samples, but a possible extraction and/or pipetting problem during sample preparation could not be confirmed by the laboratory, therefore the data was retained. No outlier was detected in the series of ERM-BB493.

Data points were plotted against time and the regression lines were calculated to check for significant trends (degradation, enrichment) due to storage conditions. The observed slopes were tested for significance using a t-test, as described in section 7.1.

Finally, the uncertainty of stability u_{lts} [21] was calculated for a pre-defined shelf life of 2 years as:

$$u_{lts} = \frac{RSD_{stab}}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot x$$

with RSD_{stab} being the relative standard deviation of all 36 individual results of the relevant stability study, x_i being the time point for each replicate, $\bar{x}$ being the average of all time points and x being the pre-defined shelf life. Results are summarized in Table 4.

Table 4. Long-term stability results for ERM-BB492 and ERM-BB493

	ERM-BB492		ERM-BB493	
Statistical parameter	-20 ºC	4 ºC	-20 ºC	4 ºC
Slope (b) [%/year]	-1.11	3.69	-0.07	-0.23
$|b|/s_b$	0.56	2.69	0.04	0.11
Statistical significance (95% conf. interval) [1]	no	yes	no	no
u_{lts} [%/2 years]	3.907	5.056	3.979	3.893

[1] $t_{0.05;34}$= 2.032 for ERM-BB492, $t_{0.05;22}$= 2.074 for ERM-BB493

For ERM-BB492, a statistically significant positive trend was obtained at 4 ºC (95 % confidence level, no trend at 99 % level). However, this trend can be regarded technically irrelevant, as (i) the content of OTC in the material cannot increase, (ii) the results for the blank material ERM-BB493 show the same variance in overall results and no degradation of the matrix, and (iii) because of a random agglomeration of data points around 1.03 (normalised values) at the time point 12 months, which makes the trend statistically significant (Annex C, figure C2).

For ERM-BB493, no significant slopes at the 95% level of confidence were detected, demonstrating stability of the material under these conditions. For both materials, -20 ºC was chosen as the storage temperature for the batch.

8 Characterisation

8.1 Calibrants used

8.1.1   Oxytetracycline (OTC)

A common calibrant (OTC hydrochloride, pure substance) was purchased from Sigma-Aldrich (Bornem, BE), and distributed by IRMM. Prior to calibrant dispatch to the laboratories, the purity was assessed by a set of methods, which included liquid chromatography with diode array detection (LC-DAD), coulometric Karl Fischer titration (cKFT), differential scanning calorimetry (DSC), total ash content, and residual solvent determination using thermogravimetry coupled to Fourier-transformed infrared spectrometry (TG-FTIR). Additionally, both material identity and purity were assessed by quantitative nuclear magnetic resonance (qNMR) spectrometry. Finally, LC coupled to time-of-flight (TOF) mass spectrometry was applied to monitor the presence of compounds other than OTC (EOTC) in the material preparation.

Differential scanning calorimetry (DSC) was not applicable due to decomposition of the compound during sample heating.

LC-TOF analysis revealed 3 detectable impurities (peaks), which accounted for about 3.1 area % of the total peak area including OTC. The largest impurity peak (2.3 area %) was identified as 2-acetyl-2-decarboxamidooxytetracycline (ADOTC) and the second largest peak (0.5 area %) as tetracycline (TC); this was accomplished by comparing the exact masses with those in the literature. This result corroborates findings concerning major impurities of manufactured OTC being ADOTC and TC [23]. The identity of the third peak (0.3 area %) could not be elucidated.

Total ash determination according to the European Pharmacopeia 6.0 method yielded the result "below limit of detection of the method" (< 0.05 m/m %).

HPLC-DAD purity (mean ± SD, n=3), expressed as a ratio of OTC peak area to sum of areas from all peaks in chromatogram was 98.7 ± 0.1 % and 99.1 ± 0.1 %, respectively. Results were obtained by two methods that differed by type of LC column (reversed-phase; styrene-divenylbenzene copolymer R), mobile phase (phosphate pH 6.5; phosphate pH 7.5 containing 2-methyl-2-propanol, tetrabutylammonium hydrogen sulphate, and EDTA), gradient, and detection wavelength (240 and 220 nm).

cKFT measurements indicated a water content of 4.96 ± 0.37 g/100g (mean ± SD, n=5).

Thermogravimetry coupled to Fourier-transform infrared spectrometry (TG-FTIR) analysis determined residual solvents, reported as sum of water and methanol, with 5.32 ± 0.13 g/100g (mean ± SD, n=3).

qNMR has been used in pharmaceutical sciences for several applications, including purity determination of pharmaceutical and chemical compounds [22]. Two laboratories performed six independent qNMR measurements each; both laboratories used 600 MHz instruments, but methods differed by the exact instrumental settings, the internal standard used for quantification, and the sample amount used per analysis. Table 5 lists the results obtained (g/g %). All qNMR results were taken into account to calculate the purity of the common calibrant. It amounts to 94.34 ± 0.61 g/g % (mean ± SD, n=12). This value could be nicely confirmed, taking into account the results obtained by the different purity methods, which yielded a total impurity of about 6 g/g % (sum of LC-DAD, and TG-TFIR which includes water).

Table 5. qNMR results (in g/g %) for the OTC
calibrant used in the characterisation exercise

Sample	Laboratory A	Laboratory B
1	94.25	94.12
2	94.24	95.18
3	93.79	94.88
4	94.03	94.77
5	93.59	95.09
6	93.32	94.87
Average	93.87	94.82
SD	0.37	0.37
RSD%	0.40	0.39

As a conservative estimation of the corresponding relative uncertainty of the common calibrant u_{cal}, the half-width of a rectangular distribution between the highest and lowest value of all NMR results was taken:

$$u_{cal} = \frac{|\text{highest result - lowest result}|}{2 \cdot \sqrt{3}}$$

u_{cal} amounts to 0.537 g/g %, and u_{cal} [%] to 0.569.

8.1.2   4-epi-oxytetracycline (EOTC)

qNMR indicated a purity of 82.9 ± 2.2 g/g % (mean ± SD, n=6). This value was corroborated by the results obtained by other purity measurements (see details about methods above): 1.9 ± 0.1 area % organic impurities (HPLC-DAD), 17.87 ± 0.33 g water/100g (cKFT), 16.59 ± 0.39 g/g % water (TG-FTIR), total ash <LOD of method, which combined indicates about 81 % purity of the commercial EOTC.

8.2 Design of the study

Exclusively LC-UV and LC-MS/MS methods were applied for the characterisation of the reference material, as these are by far the predominantly used techniques nowadays.

Twelve laboratories were selected for analysing ERM-BB492 based on the following criteria: validated methods were an indispensable requirement for participation; accreditation of the laboratory for this method was considered an asset. Ten laboratories operated methods executed under the scope of their accreditation, one laboratory was not yet accredited for these measurements, and one laboratory operated a method in compliance with GLP according to Directive 2004/9/EC [24]. The laboratories had to prove their measurement capabilities and had to demonstrate previous experience in OTC analysis in milk (e.g. results from recent proficiency testing rounds).

For ERM-BB493 (blank material), the seven laboratories operating methods with an LOD < 15 µg/kg for the sum of OTC and EOTC (method validation data commuciated by the laboratories, see table 11) were participating in the intercomparison.

For the characterisation study, each laboratory was provided with the following samples:
- 3 vials of ERM-BB492 (and 3 vials of ERM-BB493, when applicable)
- 1 bottle (ca. 60 g) of blank milk powder
- 1 vial each of pure OTC and EOTC neat substance (ca. 250 mg and 100 mg, respectively)

Laboratories were required to apply their validated LC-UV or LC-MS/MS methods and were required to use the provided neat substance for calibration purposes. Preparation of calibration curves was done according to the laboratories' method working instructions (neat standard solution calibration or matrix-matched calibration). Three independent sub-samples of each vial had to be prepared and analysed, amounting to nine analysis of ERM-BB492. Measurements had to be performed on three different days (three analyses/day), with independent calibrations on each day.

Reconstitution of the samples was prescribed by IRMM and was performed as follows: to 1.00 g powder, 8.15 g of distilled water was added. Higher or lower amounts of milk powder could be used if required by the laboratory's working instruction, whereby the 1:8.15 m/m ratio of powder to water had to be maintained. The sample was homogenised by adding a magnetic stirring bar to each powder/water mixture, and stirred for 10 - 15 min at room temperature.

The blank milk powder provided was used for the preparation of quality control (QC) samples (blank matrix sample, sample spiked at low µg/kg level), and for the preparation of matrix-matched calibration when applicable. - The same study set-up held for ERM-BB493, except that two independent sub-samples of each vial had to be prepared and analysed, which amounted to six analyses (two per day) in total.

8.3 Results and technical evaluation

Only the methods applied in laboratories 2, 6, and 7 chromatographically separated OTC from EOTC, in all other methods co-elution of the two epimers was obtained. The results in laboratories 2, 6 and 7 showed that ERM-BB492 did not contain detectable amounts of EOTC. This supports previously published data (EOTC below LOD of methods in milk, [13]), and indicates that an *in vivo*-formation of EOTC from OTC is not taking place in the material. Moreover, apparently OTC does not epimerize to EOTC during sample preparation in detectable amounts with the procedures applied.

The individual methods employed by the laboratories are summarised in Tables 6 - 9 (sample preparation and calibration; overview LC-MS/MS and LC-UV methods; transitions and ions used for quantification). Only laboratories 2, 6, and 7 operated methods which separate OTC from EOTC; consequently, calibration was performed using both neat OTC and neat EOTC. All other methods did not separate OTC and EOTC, and calibration was performed with neat OTC exclusively.

It can be seen that the laboratory methods varied substantially in terms of employed extraction solution and clean-up procedure. Also, sample intakes varied more than one order of magnitude. All laboratories applied matrix-matched calibration. Only half of the laboratories used an internal standard, the others reported that according to their experience the use of an internal standard does not enhance the quality of the obtained data.

LC-MS/MS methods differed in the type of reversed-phase column used (dimension, particle size), eluents, and specific compound-dependent MS parameters (dwell time, collision energy) as well as in source/gas-related MS-settings (temperature at ionisation point, ion spray voltage, curtain gas, etc.). All laboratories operated their ionisation source in the positive ESI mode, and the mass spectrometer was used as a triple quadruple spectrometer in the multiple reaction monitoring (MRM) mode. The majority of labs used transition 461>426 for quantification, and laboratories 2 and 4 used two transitions (Table 9).

Table 6. Methods in the characterisation study – sample preparation and calibration

Lab code	Sample intake [g][1]	Extraction solution	Clean-up	Calibration[2]	Internal standard
1	5	Succinate buffer	MCAC, SPE (lipophilic-hydrophilic copolymer)	Matrix-matched (powder)[3]	-
2	5	Trichloroacetic acid	SPE (lipophilic-hydrophilic copolymer)	Matrix-matched (powder)[4]	4-epidemeclocycline HCl, EPE0440000, lot 1, LGC Promochem (origin EDQM)
3	3	Water, EDTA, acetonitrile	-	Matrix-matched (powder)[3]	-
4	6	Carrez I and II[5], McIlvaine buffer[6]	SPE (reverse-phase)	Matrix-matched (powder)[3]	Demeclocycline HCl, D6140, lot 066K1213, Sigma Aldrich
5	5	Trichloroacetic acid, Mc Ilvaine buffer[6]	SPE (lipophilic-hydrophilic copolymer)	Matrix-matched (powder)[3]	Demeclocycline HCl, 46161, lot 6129X, Riedel-de-Haën
6	5	Succinate buffer, trichloroacetic acid	SPE (lipophilic-hydrophilic copolymer)	Matrix-matched (powder)[4]	Demeclocycline HCl, D6140, lot 1438182, Sigma Aldrich
7	1	Trichloroacetic acid, Mc Ilvaine buffer[6]	SPE (lipophilic-hydrophilic copolymer)	Matrix-matched (powder)[4]	-
8	2	Triochloroacetic acid	-	Matrix-matched (powder)[3]	Demeclocycline HCl, C12128000, lot 70920, Dr. Ehrenstorfer
9	2	Water, EDTA, McIlvaine buffer[6], Trichloroacetic acid	SPE (reverse-phase)	Matrix-matched (powder)[3]	Demeclocycline . HCl, D6140, lot 1438182, Sigma Aldrich
10	5	EDTA, hydrochloric acid	SPE (cyclohexyl phase)	Matrix-matched (powder)[3]	-
11	0.5	EDTA, McIlvaine buffer[6]	Ultrafiltration (30 kD)	Matrix-matched (powder)[3]	-
12	3.5	Succinate buffer, EDTA	Delipidation with heptane; SPE (reverse-phase)	Matrix-matched (powder)[3]	-

[1] reconstituted material
[2] powder: blank provided by IRMM
[3] laboratory used exclusively OTC for calibration
[4] laboratory used OTC and 4-epi-OTC for calibration
[5] Carrez I, zinc acetate solution; Carrez II, potassium hexacyanoferrate solution
[6] citric acid/disodium hydrogen phosphate buffer

LC-UV methods differed in the type of reversed-phase column, eluents, and detection wavelength.

The individual results as obtained are listed in Annex D.

Table 7. LC-MS/MS methods in the characterisation study – separation and quantification

Lab code	LC column	Solvent system	HPLC system	Mass spectrometer[1,2]
2	Alltima™ C_{18}, 150 x 2.1 mm, 3 µm (Alltech)	Formic acid/ (methanol/acetonitrile)	Alliance 2695 (Waters)	Quattro LCZ (Micromass)
3	GENESIS® C_{18}, 100 x 2.1 mm, 4 µm (Grace)	(Formic acid/oxalic acid)/acetonitrile	1200 (Agilent)	6410 (Agilent)
4	Gemini® C_{18}, 150 x 4.6 mm, 3 µm (Phenomenex)	Formic acid/ acetonitrile	Alliance 2695 (Waters)	Quattro Micro API (Waters)
6	Acquity™ UPLC BEH-C_{18}, 50 x 2.1 mm, 1.7 µm (Waters)	Formic acid/ acetonitrile	Acquity™ UPLC (Waters)	Quattro Premier XE (Micromass)
7	Symmetry® C_{18}, 150 x 3.9 mm, 5 µm (Waters)	Formic acid/ acetonitrile	1100 (Agilent)	API 3000 (Applied Biosystems)
8	Gemini®-NX C_{18}, 150 x 2 mm, 3 µm (Phenomenex)	Formic acid/ methanol	Alliance 2695 (Waters)	Quattro Premier (Micromass)
9	Symmetry® C_{18}, 100 x 2.1 mm, 3.5 µm (Waters)	Pentafluoropropionic acid/acetonitrile	1100 (Agilent)	API 4000 (Applied Biosystems)
10	Kinetex™ C_{18}, 50 x 2 mm, 2.6 µm (Phenomenex)	Formic acid/ acetonitrile	1200 (Agilent)	API 4000 (Applied Biosystems)
12	Symmetry® C_{18}, 150 x 2.1 mm, 5 µm (Waters)	Trifluoroacetic acid/acetonitrile[1]	Alliance 2695 (Waters)	Quattro Ultima Pt (Waters)

[1] isocratic elution

Table 8. LC-UV methods in the characterisation study – separation and quantification

Lab code	LC column	Solvent system	HPLC system	Detector, detection wavelength
1	Zorbax SB C_{18}, 150 x 4.6 mm, 3.5 µm (Agilent)	Oxalic acid/ acetonitrile[1]	Module: 305 pump, 231 XL autosampler, 402 dilutor (Gilson)	UV-975 UV/VIS (Jasco), 350 nm
5	Superspher® 60 RP-8 endcapped, 250 x 4 mm, 5 µm (Merck)	Citric acid/ acetonitrile	Module: LC-10 AT VP pumps, SILL 10 AT VP injector, CTO 10 AT VP column oven, FCV 11 ALL mixer, SCL 10 A controller (Shimadzu)	SPD-M10A (Shimadzu), 355 nm
11	Purospher RP C_{18} endcapped, 125 x 4 mm, 5 µm (Merck)	Oxalic acid/ acetonitrile[1]	Module: P 4000 pump, AS300 autosampler, Chromquest data station (Thermo)	UV 6000 LP (Thermo), 354 nm

[1] isocratic elution

Table 9. LC-MS/MS methods in the characterization study - MRM transitions[1] used for quantification.

Lab. code	OTC
2	461>444 461>426
3	461>426
4	461>426 461>443
6	461>426
7	461>426
8	461>426
9	461>426
10	461>426
12	461>443

[1] Values represent the parent (molecular ion) and the daughter ion, respectively

ERM-BB492

Laboratories 1 and 10 (no internal standard used) corrected their results for recovery obtained in the QC samples within the analytical series (blank milk powder provided by IRMM, spiked with common calibrant), which was about 82 and 60 %, respectively. Laboratories 3, 7 and 11 (no internal standard used) did not correct their results, as the QC samples indicated recoveries close to 100 %. All other laboratories used internal standards, and did not correct their results for recovery.

All results were subjected to technical evaluation. The obtained data sets from laboratories 2 and 8 were rejected as the data did not meet the specified quality requirements of IRMM (cases of non-linear calibration lines, reported possible error in the preparation of the calibrant stock solution, some recovery values of QC samples either substantially changing from day to day or largely exceeding 100 %). For laboratory 12, the data of the first day had to be rejected, as the laboratory reported problems with obtaining a linear calibration line due to saturation problems with the ion source of the mass spectrometer (problem solved on days 2 and 3, linear calibration lines).

In total, 87 results from 10 laboratories were accepted after technical scrutiny and subjected to statistical data assessment. The accepted sets of results were submitted to the following statistical tests:
  - Scheffe's multiple t-test to check if the means of two labs are significantly different
  - Dixon's test to detect outlying lab means
  - Grubb's test to detect single and double outliers
  - Cochran test to check for outlying lab variances
  - Bartlett test to check for homogeneity of lab variances
  - Skewness and Kurtosis test to assess the normality of the lab means distribution.

Datasets were first subjected to the Cochran test to identify outlying laboratory variances. No outlying variances were detected. The results of the statistical tests of the finally considered data for ERM-BB492 are summarized in Table 10. It shall be noted that this mean value had to be multiplied with 0.9434, the relative purity of the common calibrant. Also, the data in Annex D refers to the results as obtained, where the laboratories when reporting their data were requested to assume 100% purity for the common calibrant. The correction was only made to the mean of means and its absolute combined uncertainty and expanded uncertainty.

u_{char} was estimated as the standard error of the mean of laboratory means:

$$u_{char} = \frac{s}{\sqrt{n}}$$

with s being the standard deviation of the mean of means, and n the number of accepted set of data

Table 10. Summary of statistical evaluation for ERM-BB492

Parameter	
Number of data sets	10
Number of replicate measurements[1]	87
Mean of means [µg/kg]	107.495
Mean of means [µg/kg] after correction with common calibrant purity (0.9434)	101.411
Relative standard deviation of mean of means [%]	5.165
Relative standard error of mean of means [%], u_{char}	1.633
All data sets compatible two by two? (Scheffe's test)	No
Outlying means? (Dixon test)	No
Outlying means? (Grubbs test)	No
Outlying lab variances? (Cochran test)	No
Lab variances homogeneous? (Bartlett test)	No
Distribution of means normal? (Skewness & kurtosis, normal probability plot)	Yes

α = 0.05 unless stated otherwise

[1] only 6 measurements for laboratory 12 (mass spectrometer ion source problems on day 1)

ERM-BB493
Only laboratories with a LOD < 15 µg/kg were participating in analysing ERM-BB493; these were laboratories 1, 4, 6, 7, 8, 9, and 12. The results for OTC were reported as "not detected" by all laboratories. The result of laboratory 8 was not taken into account, as a consequence of the insufficient data quality of the ERM-BB492 measurements (see above).

Table 11 lists the LOD values of the methods; the values were established in the laboratories during validation of the methods, and were calculated as indicated in the table. For laboratories 6 and 7, values represent the LOD of OTC, whereas for all other labs, values are the LOD of the sum of OTC and EOTC.

Table 11. LOD values for OTC (sum of OTC and EOTC)
in the methods of the characterization study

Lab.	LOD [µg/kg]	Basis of value
1	1.9	S/N = 3 (matrix blank)
4	5	Spiked sample
6	0.12	Extrapolation to S/N = 3 from sample spiked at 10 µg/kg
7	10	S/N = 3 (matrix blank)
9	5	S/N = 3 (matrix blank)
12	1.4	S/N = 3 (matrix blank)

In addition to the information provided, chromatograms of blank milk and lowest level calibration curve samples (data of characterization exercise) were inspected in detail during results evaluation at IRMM to verify that the communicated LODs are meaningful.

Laboratory 6 reported a LOD which is considerably lower than that of the other five laboratories. In-depth data evaluation revealed that this LOD (estimated by extrapolation from a spiked sample at an approximately 100-fold higher concentration) is questionable; therefore this value was not taken into account for further calculations. The validity of the other five values could be confirmed. Therefore, the lowest of the remaining five values (1.4 µg/kg) was taken, and multiplied by 3.3 (conservative estimation, [25]) to obtain the corresponding LOQ, yielding 4.6 µg/kg.

9 Certified values and uncertainties

The certified value for ERM-BB492 is calculated as the mean of means of the accepted data sets. The standard error of the mean of means was used as an estimation of the uncertainty contribution of the characterisation exercise. The standard error is calculated as the standard deviation divided by the square root of the number of accepted data sets.

The combined uncertainty of the certified value includes contributions from the between-bottle heterogeneity, long-term storage, the characterisation study, and from the common calibrant (purity). The relative combined uncertainty is calculated as the square root of the sum of squares of the relative uncertainties of the individual contributions, according to:

$$u_{CRM} = \sqrt{u_{bb}^2 + u_{lts}^2 + u_{char}^2 + u_{cal}^2}$$

Table 12 summarizes the individual uncertainty contributions and the resulting expanded uncertainty, and indicates the certified value and its uncertainty after rounding.

Table 12. Certified value and uncertainty for ERM-BB492

Parameter	
u_{bb} [%]	2.999
u_{lts} [%][1]	3.907
u_{char} [%]	1.633
u_{cal} [%]	0.569
$u_{CRM, rel}$ [%]	5.220
$U_{CRM,rel}$ (k=2) [%]	10.440
Certified value [µg/kg]	**101**
U_{CRM} (k=2) [µg/kg]	**11**

[1] shelf life 24 months

For ERM-BB493, the certified value (mass fraction) for the sum of OTC and EOTC has been assigned as "< 5 µg/kg with a 95% level of confidence". This value corresponds to the LOQ of the most sensitive method in the characterisation study.

10 Metrological traceability

The measurement results for assigning a mass fraction value for the sum of OTC and EOTC to the material were obtained by employing methods with different sample preparation procedures (from extraction with different organic solvent and/or aqueous buffers, without any clean-up, up to extensive sample preparation metal-chelate affinity chromatography and solid-phase extraction). Therefore, independence of the results from the sample preparation part can be concluded.

LC-UV and LC-MS/MS methodologies were used for analyte separation and quantification, thereby defining the measurand. For the LC-MS/MS methods, the liquid chromatography parts of the methods mainly differed in type of eluents used, the type of reversed phase columns applied (particle size, column dimension), and LC system differences (HPLC systems, flow rate, column temperature, injected sample amount). The mass spectrometry parts exclusively used positive electro-spray ionisation and utilised the instruments in the triple quadrupole configuration by applying tandem mass spectrometry in the multiple reaction monitoring mode. Most of the methods used the same transition (parent ion, daughter ion) for quantification. Nevertheless, MS methods differed in some compound-dependent parameters (dwell times, collision energies) as well as in source/gas-related MS-settings (temperature at ionisation point, ion spray voltage, curtain gas, etc.). The applied LC-UV methods differed in the type of column, mobile phases, injection amount, and detection wavelength. Therefore, independence of the results can be concluded as long as LC-UV and LC-MS/MS methodologies are applied.

The common calibrant (crystalline substance) was provided by IRMM. The purity was assessed by qNMR (two data sets) and verified using a set of purity assessment methods including LC-DAD (two methods), cKFT, total ash determination according to European Pharmacopoeia 6.0, TG-FTIR, and LC-TOF. The certified value is traceable to the International System of Units (SI) via the common calibrant used.

11 Instructions for use

11.1 Safety precautions

The usual laboratory safety precautions apply.

11.2 Reconstitution of the material

- Allow the bottle to warm up to ambient temperature; shake vigorously for at least 30 s before opening.
- Weigh accurately an aliquot of 1.00 ± 0.01 g. The weighing should be performed immediately after opening of the vial to minimise water uptake by the lyophilised powder.
- Add an accurately weighed amount of 8.15 ± 0.01 g of distilled water to the powder.
- The sample has to be homogenised by adding a magnetic stirring bar to each powder/water mixture, and stirring for 10 - 15 min at room temperature.
- In case the working instruction of the laboratory's method foresees a higher/lower sample intake than 9.15 g of reconstituted material, the 1:8.15 (m/m) ratio of powder to distilled water has to be maintained. It shall be noted that most methods have smaller sample intakes per analysis, and that the sample intake usually is an aliquot of the reconstituted milk.

11.3 Intended use

This material is intended to be used for method performance control and validation purposes (trueness determination). For assessing the method performance, the measured values of the CRMs are compared with the certified values following a procedure described in ERM Application Note 1 [26]. The procedure is described here in brief:

- Calculate the absolute difference between mean measured value and the certified value (Δ_m).
- Combine measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_\Delta = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_Δ) from the combined uncertainty (u_Δ) using a coverage factor of two ($k = 2$), corresponding to a confidence interval of approximately 95 %
- If $\Delta_m \leq U_\Delta$ then there is no significant difference between the measurement result and the certified value, at a confidence level of about 95 %.

11.4 Storage conditions

The materials should be stored at a temperature of -20 ± 5 °C. However, the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of open samples.

12 Acknowledgements

13 References

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Annex A. Homogeneity data

Table A1. Results of the homogeneity study. Values are the sum of OTC and EOTC in µg/kg

Bottle number	1st replicate	2nd replicate	3rd replicate
39	120.7	125.4	137.2
149	124.2	127.7	129.7
205	121.5	126.2	137.2
325	124.0	121.8	133.8
361	125.3	118.7	128.8
444	119.9	112.8	125.5
553	126.2	123.8	134.4
623	123.4	123.0	141.6
766	121.2	126.9	142.5
841	115.7	108.8	119.9
918	121.5	119.5	123.5
981	116.8	119.1	123.5
1079	122.3	118.3	136.3
1128	120.1	116.7	128.0
1235	129.7	117.5	129.1
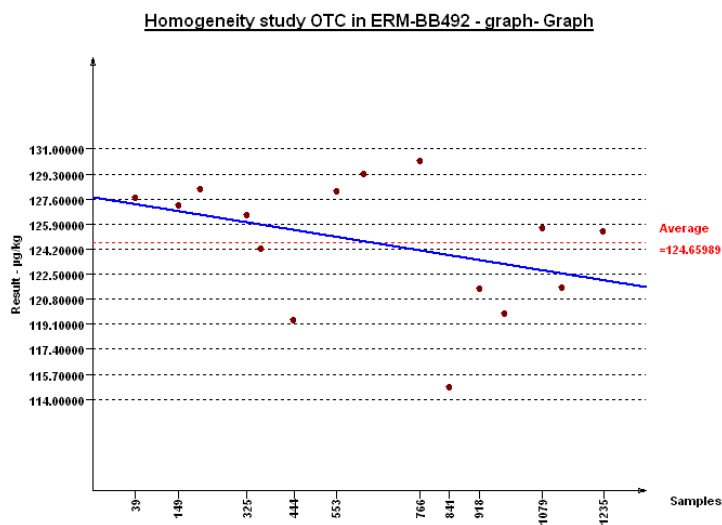
Figure A1. Homogeneity of OTC in ERM-BB492. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of triplicate measurements.

Annex B. Short-term stability data

Table B1. Results of the short-term stability study. Values are the sum of OTC and EOTC in µg/kg

Time (weeks)	4 ºC	18 ºC	60 ºC
0	119.5	119.5	119.5
0	119.3	119.3	119.3
0	112.4	112.4	112.4
0	111.2	111.2	111.2
0	124.1	124.1	124.1
0	124.6	124.6	124.6
1	111.9	114.8	122.0
1	120.5	117.3	119.1
1	113.0	113.6	109.7
1	130.4	115.3	128.3
1	125.4	115.6	128.7
1	129.4	123.5	119.4
2	118.5	120.0	118.8
2	113.5	115.2	122.5
2	118.3	124.4	126.8
2	116.8	110.1	117.5
2	117.8	117.9	116.0
2	118.1	109.5	123.9
4	110.2	107.7	107.3
4	116.5	114.1	110.8
4	106.0	118.7	103.8
4	116.6	124.5	115.8
4	116.9	121.2	103.5
4	120.2	123.0	123.3
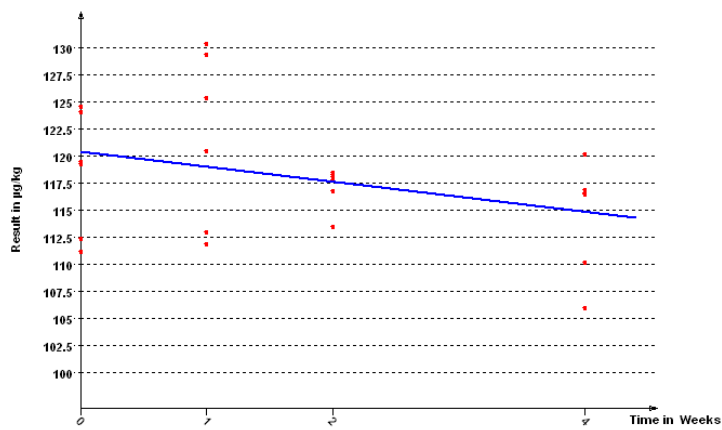
Figure B1. Short-term stability for OTC at 4 ºC.
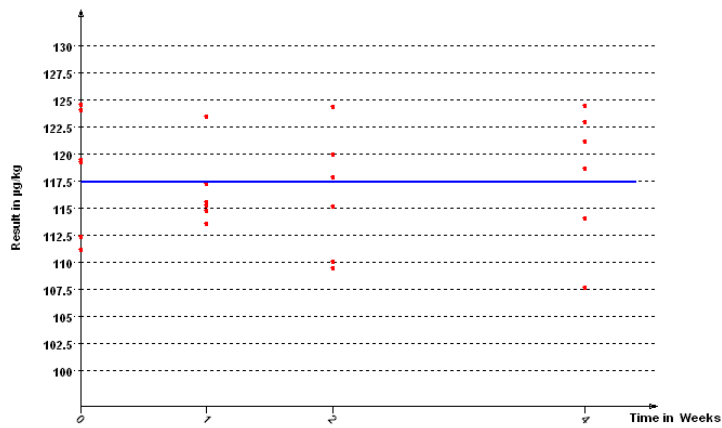
Figure B2. Short-term stability for OTC at 18 ºC.
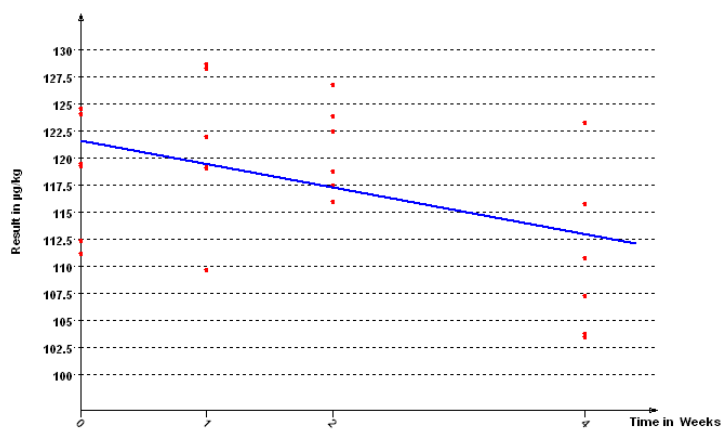
Figure B3. Short-term stability for OTC at 60 ºC.

Annex C. Long-term stability data

Table C1. Results of the long-term stability study. Values are the sum of OTC and EOTC in µg/kg

ERM-BB492			ERM-BB493		
Time (months)	4 ºC	-20 ºC	Time (months)	4 ºC	-20 ºC
0	111.90	111.90	0	100.50	100.50
0	99.20	99.20	0	110.80	110.80
0	109.50	109.50	0	98.45	98.45
0	104.35	104.35	0	97.90	97.90
0	95.80	95.80	0	95.90	95.90
0	100.10	100.10	0	95.25	95.25
0	101.25	101.25	4	103.00	99.35
0	96.10	96.10	4	101.80	100.05
0	101.20	101.20	4	96.05	97.55
4	102.00	103.05	4	107.85	106.60
4	99.45	96.65	4	98.25	96.95
4	102.60	106.50	4	100.05	98.10
4	113.10	113.00	8	97.65	99.00
4	99.20	102.00	8	99.70	100.40
4	103.40	104.00	8	95.75	101.05
4	106.70	111.55	8	106.40	108.90
4	101.15	99.45	8	98.25	92.60
4	100.70	105.05	8	97.90	103.95
8	112.70	106.90	12	99.45	95.90
8	102.80	102.25	12	102.50	104.60
8	108.10	105.90	12	101.20	94.40
8	113.55	90.55	12	103.40	103.40
8	101.40	95.55	12	95.00	97.55
8	104.25	103.15	12	99.50	100.40
8	106.00	115.35			
8	96.90	96.65			
8	106.20	99.80			
12	112.10	107.25			
12	102.55	95.50			
12	106.75	98.70			
12	102.90	108.00			
12	102.00	102.85			
12	107.85	95.95			
12	110.35	109.90			
12	97.75	97.50			
12	107.60	100.65			
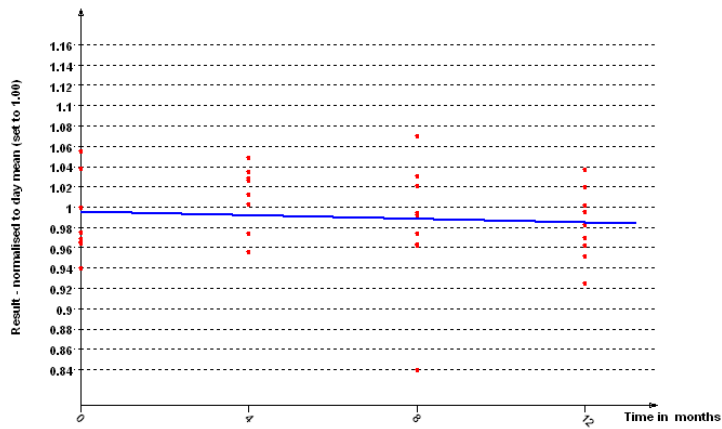
Figure C1. Long-term stability for OTC in ERM-BB492 at -20 ºC.
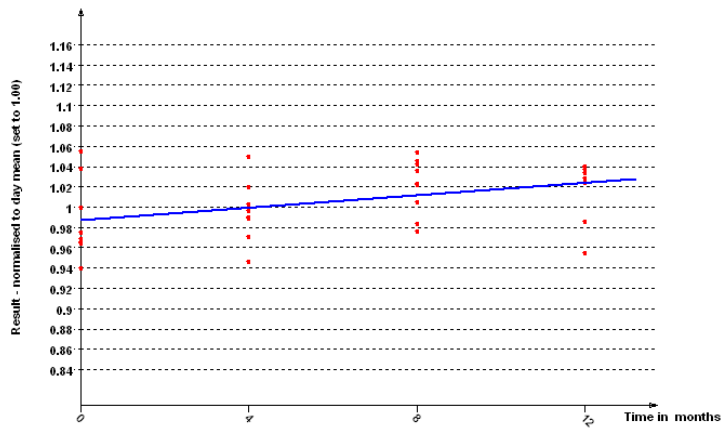
Figure C2. Long-term stability for OTC in ERM-BB492 at 4 ºC.

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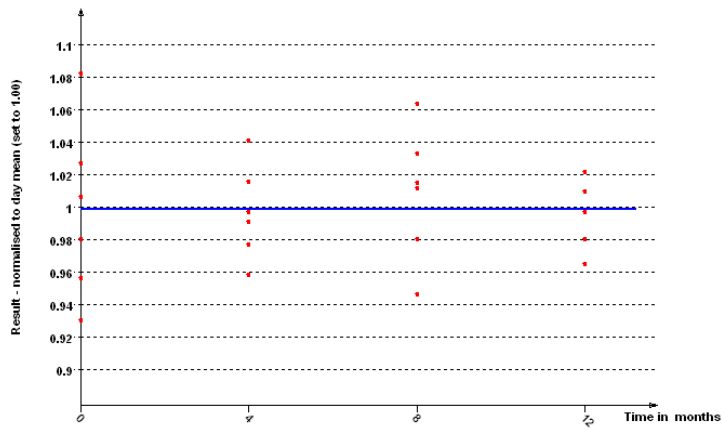
Figure C3. Long-term stability for OTC in ERM-BB493 at -20 ºC.

Figure C4. Long-term stability for OTC in ERM-BB493 at 4 ºC.
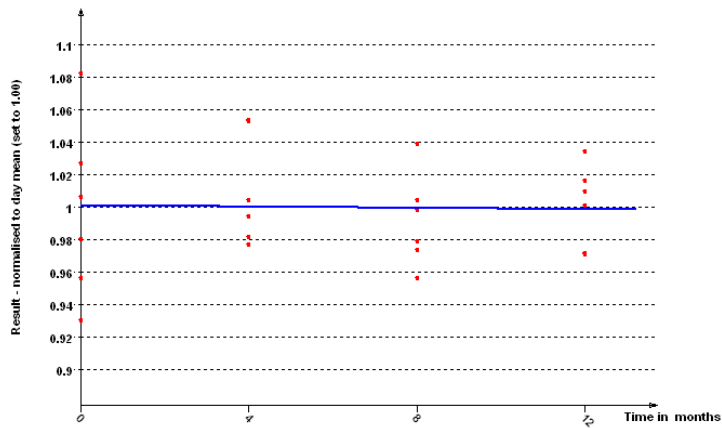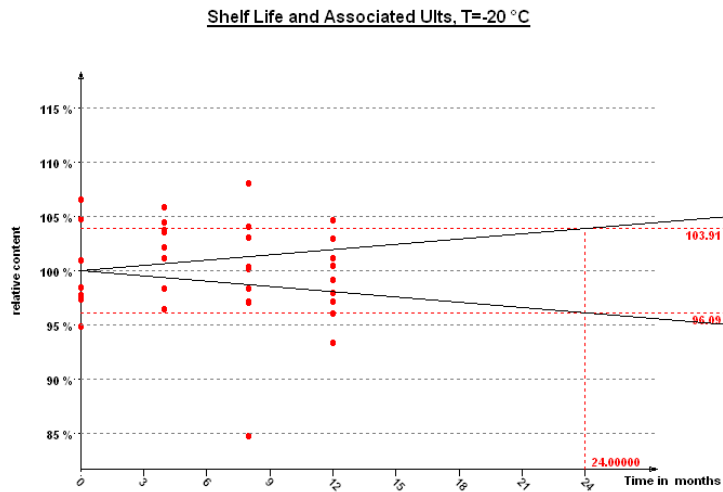
Figure C5. Long-term stability for OTC in ERM-BB492 at -20 ºC with associated u_{lts} for storage period of 24 months
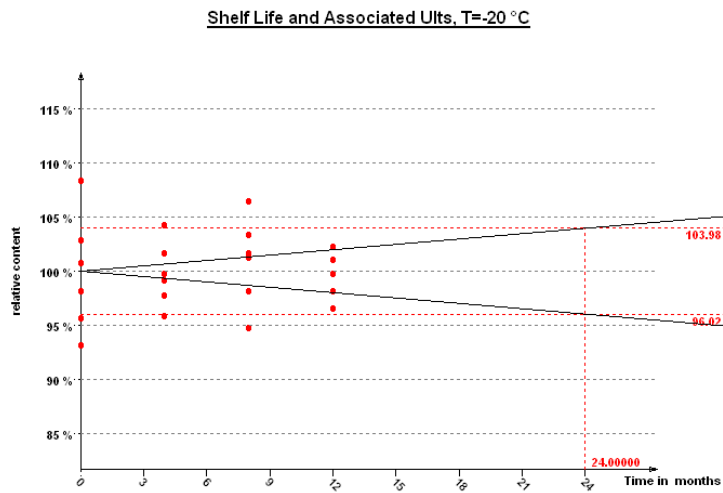

Figure C6. Long-term stability for OTC in ERM-BB493 at -20 ºC with associated u_{lts} for storage period of 24 months

Annex D. Characterisation data

Table D1. Results of the characterisation measurements for oxytetracycline. Values (sum of OTC and EOTC in µg/kg) as obtained, not corrected for common calibrant purity. The graph shows laboratory mean values and the mean of means. Error bars are standard deviations. Results with a low standard deviation may well have a large measurement uncertainty.

Lab code	Day1/1	Day1/2	Day1/3	Day2/1	Day 2/2	Day 2/3	Day 3/1	Day 3/2	Day3/3
1	118.02	112.67	118.09	118.25	122.15	119.49	108.68	111.90	111.87
2[1]	129.91	125.25	133.29	145.04	130.58	129.16	114.18	133.11	112.47
3	113.71	114.22	111.68	108.61	104.84	104.13	101.48	101.09	96.87
4	102.40	101.60	91.76	97.78	107.81	109.42	90.48	95.53	87.55
5	132.87	118.60	123.98	98.22	99.70	98.78	108.13	109.89	109.15
6	119.70	109.40	109.00	98.50	103.90	112.00	100.20	111.20	106.90
7	116.36	110.37	111.52	115.18	106.21	111.28	107.13	109.94	117.43
8[1]	132.80	143.37	153.48	119.00	149.79	157.86	153.86	169.41	167.98
9	113.05	115.83	101.93	106.56	92.66	101.00	112.12	96.37	102.85
10	110.28	95.74	92.51	94.50	122.16	112.03	126.53	118.82	125.85
11	103.71	99.20	102.29	107.14	116.84	104.02	116.26	117.69	116.93
12	*129.45*	*139.33*	*143.82*	111.02	106.43	104.80	87.66	102.13	82.68

[1]data sets rejected for technical reason (see page 21)
[2] Laboratory 12, day 1, data (in italic): not taken into account for calculating laboratory mean due to technical reason (see page 21)
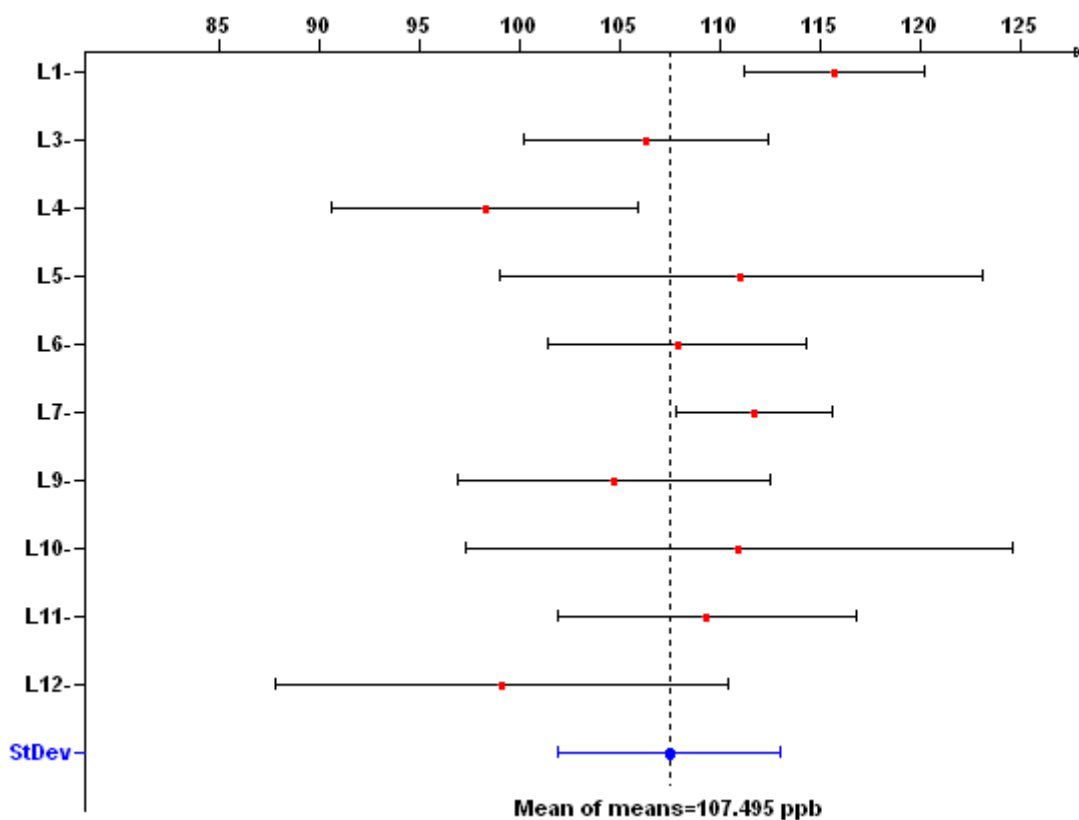

Figure D1. Laboratory means, mean of means and their standard deviations for sum of OTC and EOTC as obtained (not corrected for common calibrant purity)

Abstract

This report describes the preparation of two milk powder matrix reference materials (ERM-BB492 and ERM-BB493) and the certification of their content (mass fraction) of oxytetracycline.

The preparation and processing of the material, homogeneity and stability studies, and the characterisation are described hereafter and the results are discussed. Uncertainties were estimated in compliance with ISO Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM) [1].

For ERM-BB492, the uncertainty contains contributions from possible heterogeneity, instability, characterisation, and purity of the common calibrant. The certified value is listed below:

Mass fraction in the reconstituted material	Certified value [1] [µg/kg]	Uncertainty [2] [µg/kg]	Number of accepted sets of results
Sum of oxytetracycline and 4-epi-oxytetracycline	101	11	10

1)   The value represents the mass fraction based on the unweighted mean of means of accepted results.
2)   Expanded uncertainty ($k = 2$) of the value defined in 1).

The certified value and its uncertainty is based on a minimum sample intake of 1.00 g reconstituted material.

For ERM-BB493, the following certified value has been assigned:

Mass fraction in the reconstituted material	Certified value [1] [µg/kg]
Sum of oxytetracycline and 4-epi-oxytetracycline	< 5

1)   This value corresponds to the limit of quantification (LOQ) of the most sensitive method in the characterisation study. The certified value is below 5 µg/kg with a 95% level of confidence.

The indicative value is based on a minimum sample intake of 3.50 g reconstituted material.

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