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CERTIFICATION REPORT

The Certification of the Mass Fraction of Chloramphenicol in Pork Meat

Certified Reference Material ERM®-BB130

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The Certification of the Mass Fraction of Chloramphenicol in Pork Meat

Certified Reference Material ERM®-BB130

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Summary

This report describes the preparation of the pork meat matrix reference material ERM-BB130 and the certification of the content (mass fraction) of chloramphenicol.

The preparation and processing of the material, homogeneity and stability studies, and the characterisation are described hereafter and the results are discussed. Uncertainties were calculated in compliance with ISO Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM) [1] and include uncertainties due to possible heterogeneity, instability, and characterisation. The certified value is listed below:

Measurand in the reconstituted material	Certified value [1)] [µg/kg]	Uncertainty [2)] [µg/kg]	Number of accepted sets of results
Chloramphenicol	0.230	0.021	13

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

The assigned value and its uncertainty is based on a minimum sample intake of 5 g reconstituted material (corresponding to 1.25 g powder).

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1 Glossary

ANOVA ..... Analysis of variances
b ..... Slope of regression line
BCR ..... Community Bureau of Reference
C_{18} ..... Octadecyl silica
CAP ..... Chloramphenicol
CAS ..... Chemical Abstracts Services
CRM ..... Certified reference material
d_5- ..... Penta-deuterated compound
DAD ..... Diode-array detector
DSC ..... Differential scanning calorimetry
ECNI ..... Electron capture negative ionization
ERM ..... European Reference Material
ESI ..... Electrospray ionisation
GC ..... Gas chromatography
GC-MS ..... Gas chromatography-mass spectrometry
GUM ..... Guide to the Expression of Uncertainty in Measurement
HRMS ..... High resolution mass spectrometer
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
KFT ..... Karl Fischer titration
LC ..... Liquid chromatography
LC-MS/MS ..... Liquid chromatography-tandem mass spectrometry
LLE ..... Liquid liquid extraction
LRMS ..... Low resolution mass spectrometer
m/m ..... Mass-to-mass
MRL ..... Maximum residue limit
MRM ..... Multiple reaction monitoring
MRPL ..... Minimum required performance limit
MS ..... Mass spectrometry
$MS_{between}$ ..... Mean of squares between groups (ANOVA)
MS_{within} ..... Mean of squares within groups (ANOVA)
n ..... Number of replicates
ODS ..... Octadecyl silica
PBS ..... Phosphate buffered saline
PSA ..... Particle size analysis
QC ..... Quality control
qNMR ..... Quantitative nuclear magnetic resonance
RIVM ..... Rijksinstituut voor Volksgezondheid en Milieu
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
SIM ..... Selected Ion Monitoring
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

*t*BME	*tertiary* butyl methyl ether
TOF	Time-of-flight detector
TG-FTIR	Thermogravimetry coupled to fourier transform infrared spectrometry
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
$v_{MSwithin}$	Degrees of freedom of MS_{within}

2 Introduction

2.1 Background

2,2-dichloro-N-[(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitro-phenyl)ethyl]acetamide, termed chloramphenicol (CAP), also named as chloromycetin, is a broad-spectrum antibiotic and highly effective against many pathogenic Gram-positive and Gram-negative bacteria, rickettsiae and mycoplasmas. It exerts its action through inhibition of the protein biosynthesis [2]. CAP was used since the 1950s in veterinary practices. The administration to, and uptake of, CAP in humans, however, can cause serious haemotoxic effects such as agranulocytosis, aplastic anaemia, and leukemia [3-5]. Consequently, the use of CAP was banned for treatment of food-producing animals in the EU [6, 7] and several other countries, such as USA, Canada, Australia, and China. In 2003, Commission Decision 2003/181/EC [8] fixed a minimum required performance limit (MRPL) [9] of 0.3 µg/kg for residues of CAP in different matrices, including meat, eggs, milk, aquaculture products, honey, and urine.

In order to produce and safeguard reliable analytical results which are necessary to ensure effective consumer protection, a certified reference material (BCR-445) was made available by the Community Bureau of Reference; that material was certified in the early 1990s using the maximum residue limit (MRL) of 10 µg/kg existing at that time [10]. Due to the current legal requirement to have suitable methods available which can reliably detect, identify, and quantify CAP at 0.3 µg/kg [8], a new reference material was required, supporting validation (trueness determination) as well as performance verification of modern analytical methods. Liquid chromatography-tandem mass spectrometry (LC-MS/MS), and gas chromatography-mass spectrometry (GC-MS), are now the commonly used methods, as they are most suitable to comply with Commission Decision 2002/657/EC concerning requirements for confirmatory methods [9]. A clear trend towards LC-MS/MS methods has been observed in the past years.

2.2 Choice of the material

Pork was chosen as the material matrix in order to be consistent to the existing BCR material. An incurred material which closely resembles a typical sample analysed in the laboratory in terms of comparable analyte extractability was considered necessary. An incurred pig muscle material was blended with a blank pig muscle material to obtain a reference material with the envisaged target concentration around the MRPL. Table 1 and Figure 1 define the analyte in ERM-BB130.

2.3 Definition of analyte and chemical structure

Table 1. Definition of the chloramphenicol analyte comprised in ERM-BB130

Trivial name and abbreviation	IUPAC name	CAS number	Chemical formula	Molecular mass (g/mol)
Chloramphenicol (CAP)	2,2-dichloro-*N*-[(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitro-phenyl)ethyl]acetamide	56-75-7	$C_{11}H_{12}Cl_2N_2O_5$	323.13

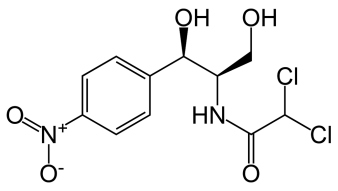

Fig. 1: Chemical structure of chloramphenicol

3 Participants

Project management and evaluation:

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, Geel, BE
(Work performed under ISO Guide 34 accreditation; BELAC-268-Test)

Raw material provision:

Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, Berlin, DE

Processing:

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, Geel, BE
(Processing performed under ISO Guide 34 accreditation; BELAC-268-Test)

Homogeneity and stability measurements:

C.E.R. Groupe, Laboratoire d'Hormonologie, Marloie, BE
(Measurements performed under ISO/IEC 17025 accreditation; BELAC 073-TEST)

Characterisation analysis:

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(Measurements performed under ISO/IEC 17025 accreditation; COFRAC 1-0247)

Agri-Food and Biosciences Institute, Veterinary Sciences Division, Belfast, UK
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(Measurements performed under ISO/IEC 17025 accreditation; COFRAC 1-1706)

Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), Berlin, DE
(Measurements performed under ISO/IEC 17025 accreditation; AKS-PL-12005)

Central Laboratory of Veterinary Control and Ecology (CLVCE), Sofia, BG
(Measurements performed under ISO/IEC 17025 accreditation; BAS 51)

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Norges Veterinærhøgskole, Oslo, NO
(Measurements performed under ISO/IEC 17025 accreditation; NORSK AKKREDITERING TEST137)

Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Wien, AT
(Measurements performed under ISO/IEC 17025 accreditation; Bundesministerium für Wirtschaft und Arbeit, Id 189)

RIKILT – Institute of Food Safety, Wageningen, NL
(Measurements performed under ISO/IEC 17025 accreditation; RvA L014)

Ústav pro státní kontrolu veterinárních biopreparátů a léčiv (ÚSKVBL), Brno, CZ
(Measurements performed under ISO/IEC 17025 accreditation; Czech Accreditation Institute, 621/2007)

4 Processing of the material

Incurred raw material (ca. 6 kg) and blank material (ca. 50 kg) were delivered in the frozen state from the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), Berlin (DE).

A similar procedure to that described for ERM-BB124 (nitroimidazoles in pork) [11] was applied in order to optimise processing steps and their order, and to minimize possible material disruption during processing.

The delivered muscle tissue portions were thawed overnight in a refrigerator, manually cut into small cubes, and lyophilised in an Epsilon 2-85D freeze dryer (Martin Christ, Osterode, DE). The yield (mass ratio of freeze-dried matter to meat tissue) was determined gravimetrically on calibrated balances and calculated to be 24.8 m/m %. The blank freeze-dried matter was immersed in liquid nitrogen overnight, and milled to a powder in a Palla VM-KT vibrating cryogenic mill (KHD Humboldt Wedag, Köln, DE). The powder was sieved through a 710 µm stainless steel sieve (Model 17300, Russel Finex Industrial sieve, London, UK). Coarse particles retained in the sieve were collected and cryogenically milled and sieved again. Thereafter, the incurred freeze-dried matter was handled in the same manner. The resulting incurred powder was then tested at BVL for its CAP content using a fully validated GC-MS method. In a three-step dilution process, the incurred powder was blended with the blank powder in a Dyna-MIX CM200 mixer (WAB, Basel, CH) to achieve the envisaged analyte target concentration around 0.3 µg/kg. The blend was vacuum-dried to approximately 2.5 m/m % and again homogenised in the Dyna-MIX CM200 mixer. Finally, 7.5 g portions of powder were filled into amber glass bottles (100 mL) using an automatic filling machine (All Fill, Sandy, UK). Bottles were closed in the freeze-dryer under inert gas atmosphere after manual insertion of lyo-inserts. Capping and labelling was performed using a Bausch & Ströbel device (Ilshofen, DE). In total, 1285 bottles of ERM-BB130 were processed; they were stored after production at -70 ºC.

5 Material characterisation measurements

5.1 Water content

The water content in the final material was measured by volumetric Karl Fischer titration [12]. Five vials 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 2.05 ± 0.07 g/100 g.

5.2 Particle size measurements

Particles size analysis (PSA) was performed using laser diffraction spectrometry on five bottles 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 lyophilised pork muscle material was 700 µm. About 50 % of all particles were smaller than 80 µm and approximately 3 % of all particles were smaller than 5 µm. Despite careful processing, some long fibres were found in the final material (micrograph analysis) which might impair PSA measurements.

6 Homogeneity study

For the homogeneity study, 10 samples of ERM-BB130 were chosen using a random stratified sample picking scheme and analysed in quadruplicate for their chloramphenicol content. Samples were dispatched to the testing laboratory on dry ice. Measurements were performed with an in-house validated LC-MS/MS method compliant with Commission Decision 2002/657/EC; matrix-matched calibration was performed using blank pork powder provided by IRMM. Deuterated internal standards were spiked to the samples in the beginning of the extraction procedure.

Samples were measured in a random order (predefined at IRMM and communicated to the laboratory) to allow distinction between an analytical trend and a trend in the filling sequence. Measurements were performed under repeatability conditions.

Data were checked for single and double outliers by applying the Grubbs test at a confidence level of 95% and 99%. No outlier in the filling sequence was detected, but one outlier was found in the analytical sequence (95% level) which was scrutinised and retained as no technical reason was found to eliminate it. Regression analysis was performed to detect possible trends regarding the filling sequence or analytical sequence.

The observed slope (*b*) was 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) and *df* degrees of freedom. The slope was considered as statistically significant when $|b|/s_b > t_{\alpha,df}$, with s_b being the standard error of the slope. No significant slope was obtained for the filling sequence, but a significant slope (95% confidence level) was detected in the analytical sequence (*t*-value 2.25, $t_{0.05,38}$ 2.02; no significant slope at 99% level). However, as the analytical sequence was randomised and is not correlated with the filling sequence, trends in the analytical sequence were corrected in order to improve the sensitivity of the study for potential between-unit heterogeneity:

$$corrected\ result = measured\ result - b \cdot i$$

b slope of the linear regression
i position of the result in the analytical sequence

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 one-way analysis of variance (ANOVA) [13]:

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

$$s_{wb} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$

MS_{within}: mean square within a bottle from an ANOVA
$\bar{y}$: 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:

$$s_{bb} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\overline{y}}$$

$MS_{between}$: mean square among bottles from an ANOVA
n: average number of replicates per bottle

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

$$u^*_{bb} = \frac{s_{wb}}{\sqrt{n}} \sqrt[4]{\frac{2}{v_{MSwithin}}}$$

$v_{MSwithin}$: degrees of freedom of MS_{within}

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-BB130

	Value
Average[1]	0.216
RSD [%]	3.256
MS_{within}	0.000041
$MS_{between}$	0.000079
s_{wb} [%]	2.947
s_{bb} [%]	1.440
u^*_{bb} [%]	0.749
u_{bb} [%]	**1.440**

[1] Average CAP content in µg/kg

6.1 Minimum sample intake

The minimum sample intake is 5 g of reconstituted material (corresponding to 1.25 g of powder). Homogeneity and stability studies were performed using 5 g of material after reconstitution, proving that the samples are homogeneous at least at this level.

7 Stability studies

7.1 Short-term stability study

A four weeks isochronous study [14] was performed to evaluate stability of ERM-BB130 during transport. Twenty samples were selected from the produced batch using a random stratified sample picking scheme. Samples were dispatched to the testing laboratory on dry ice.

Samples were stored at +4 °C, +18 °C, and +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 analysed in quadruplicate under intermediate precision conditions in the order predefined at IRMM (randomised sample order) using the same LC-MS/MS method as for the homogeneity study.

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 as described for the homogeneity study on page 11. The slope was highly significant for a storage temperature of 60 ºC, whereas no significant slopes were found for storage temperatures of 4 ºC and 18 ºC. 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 under cooled conditions which guarantee that the sample temperature does not exceed 18 ºC.

Table 3. Evaluation of the short-term stability study

Statistical parameter	4 ºC	18 ºC	60 ºC
Slope (b) [%/week]	0.20	0.33	-12.58
$\|b\|/s_b$	0.39	0.80	21.23
Statistical significance (95% conf. interval) [1]	No	No	Yes
u_{sts} [%/week]	0.51	0.41	7.29

[1] $t_{0.05;30}$= 2.042

7.2 Long-term stability study

A twelve months isochronous study [14] was performed to evaluate the stability of ERM-BB130 during storage. The chosen study duration was a compromise between obtaining suitable data for sound statistics, and considering existing stability data from BCR-445.

Twenty-one 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, the samples were transferred to -70 °C until analysis. Samples were dispatched on dry ice and kept at -20 ºC in the laboratory until analysis. Samples were analysed in triplicate under intermediate precision conditions in the order predefined at IRMM (randomised sample order) using the same LC-MS/MS method as for the homogeneity study.

Data (Annex C) were checked for single and double outliers by applying the Grubbs test at confidence levels of 95% and 99%, respectively. One outlier was detected (95% and 99% confidence level); in-depth data inspection showed a considerably smaller peak area for the internal standard 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.

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 on page 11.

Finally, the uncertainty of stability u_{lts} [15] 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. Evaluation of the long-term stability study

Statistical parameter	-20 ºC	4 ºC
Slope (b) [%/year]	-1.37	4.62
$\|b\|/s_b$	0.92	1.86
Statistical significance (95% conf. interval) [1]	No	No
u_{lts} [%/2 years]	2.972	5.146

[1] $t_{0.05;34}$= 2.032

At both tested temperatures, no significant slopes at the 95% level of confidence were detected, demonstrating stability of the material under these conditions. Nevertheless, -20 ºC was chosen as the storage temperature for the batch.

8 Characterisation

8.1 Design of the study

The decision was made to restrict the analytical methods for the characterisation of the reference material to GC-MS and LC-MS/MS methods, the by far predominantly used techniques nowadays. These methods exhibit the necessary selectivity and sensitivity, and allow to reliably detect, confirm, and quantify chloramphenicol in food matrices at a concentration level of 0.3 µg/kg (MRPL), thus meeting the requirements of 2002/657/EC [9].

Sixteen laboratories were selected based on the following criteria: validated methods were an indispensable requirement for participation; an accredited method was considered an asset. All laboratories operated accredited methods, except of laboratory 4, where only the sample preparation part was accredited at the time of the measurements. The laboratories had to prove their measurement capabilities and had to demonstrate previous experience in chloramphenicol analysis in comparable matrices.

A common calibrant (pure substance) was purchased from Sigma-Aldrich (Bornem, BE), and distributed by IRMM. Prior to calibrant dispatch to the laboratories, the purity as indicated by the supplier was assessed by a set of methods, which included liquid chromatography with diode array detection (LC-DAD), coulometric Karl Fischer titration (KFT), 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 CAP in the material preparation. The purity as indicated by the supplier was confirmed.

The relative uncertainty of the common calibrant, u_{cal} - in this case identical to the relative uncertainty of the calibrant purity - was calculated from the assessed purity assuming a rectangular distribution; it amounted to 0.145 m/m %.

For the characterisation of ERM-BB130, each laboratory was provided with the following samples:

- 2 units of ERM-BB130
- 1 bottle (ca. 20 g) of blank pork meat powder
- 1 vial of pure chloramphenicol neat substance (ca. 250 mg)

Laboratories were required to apply their validated GC-MS or LC-MS/MS methods and had 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). Measurements had to be performed on two different days with independent calibrations on each day. Each of the two samples had to be measured four times, whereby duplicate measurements for each sample had to be done on both days (example: samples 42 and 317 received; day 1: 42 1^{st} and 2^{nd} sub-sample, 317 1^{st} and 2^{nd} subsample; day 2: 42 3^{rd} and 4^{th} subsample, 317 3^{rd} and 4^{th} subsample). Reconstitution of the samples was prescribed as follows: to 1.25 g powder, 3.75 g of distilled water had to be added. Higher amounts could be used if required by the laboratory's working instruction, whereby the 1:3 m/m ratio of powder to water had to be maintained. The blank pork meat 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.

8.2 Results and technical evaluation

The individual methods employed by the laboratories are summarised in Tables 5 - 8 (sample preparation and calibration; overview LC-MS/MS and GC-MS methods; transitions and ions used for quantification).

Table 5. Methods in the characterisation study – sample preparation and calibration

Lab code	Sample intake [g][1]	Extraction solution	Clean-up	Calibration[2,3]	Internal standard
1	2	Ethyl acetate	Defatting with iso-octane	Matrix-matched (powder)	d_5-CAP (Cambridge Isotope Laboratories)
2	2	Water	LLE (diatomaceous earth) LLE with toluene	Matrix-matched (powder)	$^{37}Cl_2$-CAP (provided by RIVM)
3	4	Ethyl acetate	Defatting with hexane SPE (lipophilic-hydrophilic copolymer)	Neat standards	d_5-CAP (Cambridge Isotope Laboratories)
4	5	Ethyl acetate	SPE (silica)	Matrix-matched (powder)	d_5-CAP (Cambridge Isotope Laboratories)
5	6	McIlvaine buffer[4]/acetonitrile	LLE (tBME/hexane)	Neat standards	d_5-CAP (Cambridge Isotope Laboratories)
6	5	Acetonitrile	Defatting with hexane SPE (reverse-phase) Preparative LC (Ultrasphere ODS C_{18})	Matrix-matched (powder)	d_5-CAP (Dr. Ehrensdorfer)
7	2	Ethyl acetate	Defatting with iso-octane	Matrix-matched (powder)	d_5-CAP (Cambridge Isotope Laboratories)
8	5	Ethylacetate	LLE (petrol ether/ammonium acetate/acetonitrile)	Neat standards	d_5-CAP (Cambridge Isotope Laboratories)
9	3	Sodium chloride and acetonitrile	Defatting with hexane SPE (reverse-phase)	Neat standards	d_5-CAP (Cambridge Isotope Laboratories)
10	3	PBS buffer with β-glucuronidase; sodium chloride and acetonitrile	Defatting with hexane SPE (reverse-phase)	Neat standards	d_5-CAP (Cambridge Isotope Laboratories)
11	1	Acetonitrile	LLE (tBME/hexane)	Matrix-matched (powder)	d_5-CAP (Cambridge Isotope Laboratories)
12	5	Ethyl acetate	Defatting with isooctane/chloroform	Matrix-matched (powder)	d_5-CAP (Cambridge Isotope Laboratories)
13	2	Sodium chloride and acetonitrile	Defatting with hexane SPE (reverse-phase)	Matrix-matched (powder)	d_5-CAP (Cambridge Isotope Laboratories)
14	3	Sodium acetate with β-glucuronidase; ethylacetate	Defatting with isooctane/chlorobutane	Matrix-matched (powder)	d_5-CAP (Dr. Ehrensdorfer)
15	5	Sodium chloride and ethyl acetate	Defatting with hexane SPE (reverse-phase)	Matrix-matched (powder)	d_5-erythro-CAP (Witega)
16	5	Sodium chloride and acetonitrile	-	Neat standards	d_5-CAP (Cambridge Isotope Laboratories)

[1] reconstituted material
[2] powder: blank provided by IRMM
[3] all laboratories used the crystalline pure substance provided by IRMM for calibration
[4] 0.1 M citric acid, 0.1 M Na_2HPO_4, pH 6.0

It can be seen that the laboratory methods varied substantially in terms of employed extraction solution and clean-up procedure. It has to be noted, however, that the typical

duration of the extraction step (addition of extraction solution, agitation, centrifugation) before further sample manipulation (e.g. evaporation, clean-up) was between 10 and 60 min in the laboratories having contributed to establish the certified value.

Some laboratories used neat standard solutions for calibration, whereas other applied matrix-matched calibration. All laboratories applied isotope dilution mass spectrometry, and except laboratory 2, which used $^{37}Cl_2$-labelled CAP, all other laboratories used d_5-CAP as internal standard.

LC-MS/MS methods differed in the type of reversed-phase column used (dimension, particle size), and specific compound-dependent 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 negative ESI mode, and the mass spectrometer was used as a triple quadruple spectrometer in the multiple reaction monitoring (MRM) mode. The transition used for quantification was the same in all 13 laboratories, except for laboratory 16, which used the average of four monitored transitions for quantification.

Table 6. LC-MS/MS methods in the characterisation study – separation and quantification

Lab code	LC column	Solvent system[1]	HPLC system	Mass spectrometer[2,3]
1	SunFire™ C_{18}, 150 x 2.1 mm, 3.5 µm (Waters)	Water/ acetonitrile	Alliance 2695 (Waters)	Quattro Micro API (Waters)
2	XBridge® C_{18}, 150 x 3 mm, 5 µm (Waters)	Ammonia/ acetonitrile	Acquity UPLC (Waters)	Quattro Ultima (Waters)
3	XTerra® C_{18}, 150 x 2.1 mm, 3.5 µm (Waters)	Ammonia/ acetonitrile	Alliance 2695 (Waters)	Quattro Micro (Micromass)
4	Inertsil ODS-3 C_{18}, 150 x 2 mm, 3 µm (GL Sciences Inc.)	Acetic acid/ methanol	HP 1100 (Agilent)	Quattro Ultima (Waters)
5	Luna® C_{18} (2), 50 x 2 mm, 5 µm (Phenomenex)	Ammonium acetate/methanol	HP 1100 (Agilent)	Quattro LC (Waters)
7	Symmetry® C_{18}, 150 x 3.9 mm, 5 µm (Waters)	Ammonium acetate/acetonitrile	HP 1100 (Agilent)	API 4000 (Applied BioSystems)
8	XBridge® C_{18}, 100 x 2.1 mm, 3.5 µm (Waters)	Ammonium acetate/acetonitrile	Alliance 2695 (Waters)	Quattro Micro (Waters)
10	Columbus™ C_{18}, 150 x 2 mm, 5 µm (Phenomenex)	Water/methanol	Alliance 2795 (Waters)	Quattro Ultima (Micromass)
11	Purospher Star C_{18}, 55 x 4 mm, 3 µm (Merck)	Formic acid/ methanol	HP 1100 (Agilent)	Qtrap 4000 (Applied BioSystems)
12	XTerra® MS C_{18}, 100 x 2.1 mm, 3.5 µm (Waters)	Ammonia/ methanol	Alliance 2690 (Waters)	Quattro Ultima (Waters)
14	Luna® C_{18}, 50 x 2 mm, 3 µm (Phenomenex)	Water/methanol	Alliance 2695 (Waters)	Quattro Ultima (Waters)
15	Synergi™ Max-RP C_{18}, 150 x 2 mm, 4 µm (Phenomenex)	Ammonium acetate/methanol	Surveyor (Thermo Finnigan)	TSQ Quantum (Thermo Finnigan)
16	HyPurity C_{18}, 150 x 3 mm, 5 µm (Thermo Scientific)	Formic acid/ acetonitrile	HP 1100 (Agilent)	API 4000 (Applied BioSystems)

[1] gradient elution in all cases except for laboratory 3 (isocratic conditions)
[2] all laboratories operated the ion source on the mass spectrometer in the negative ESI mode
[3] all laboratories used the mass spectrometer as a triple quadrupole instrument, operated in MRM mode

GC-MS methods differed in the type of capillary column used (coating), and GC-specific parameters such as injection type, carrier gas, flow rate, and temperature gradient. Ion sources were exclusively operated in the ECNI mode, and the mass spectrometers were used in the selected ion monitoring (SIM) mode; the same ion was used for quantification in

all 3 laboratories. MS settings however differed among methods (solvent delay time, dwell time). - The individual results as obtained are listed in Annex D.

All data were recovery-corrected intrinsically as in all cases the isotopically labelled internal standard was added at the beginning of the sample preparation. For quantification, labs either directly used the output of the validated instrument software (calibration line calculated by regression analysis), or copied the obtained areas from the instrument software into a validated excel sheet for further calculation of the analyte concentrations in the samples.

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

Lab code	GC column	Injection type	Detector	GC system and mass spectrometer[1,2]
6	DB5-MS, 30 m x 0.25 mm x 0.25 µm (Agilent)	Splitless	LRMS	HP6890 (Hewlett Packard), MSD 5975 (Agilent)
9	ZB5-MS, 30 m x 0.25 mm x 0.25 µm (Phenomenex)	Splitless	HRMS	HP6890 (Hewlett Packard), 95 XP (Thermo Finnigan MAT)
13	HP1-MS, 30 m x 0.25 mm x 0.25 µm (Agilent)	Cool on column	LRMS	HP6890 (Hewlett Packard), MSD 5973N (Agilent)

[1] all laboratories operated the mass spectrometer in the ECNI mode, with methane as moderating gas
[2] all laboratories used the mass spectrometer in the SIM detection mode

Table 8. Methods in the characterization study - MRM transitions[1] (LC-MS/MS methods) and ions[1] (GC-MS methods) used for quantification and confirmation

Lab code	Transition/ion for quantification[1]	Transition/ion for confirmation	Lab code	Transition/ion for quantification[1]	Transition/ion for confirmation
1	321>152	321>257	9	466	468
2	321>152	321>194	10	321>152	321>257 321>152
3	321>152	321>257 321>194	11	321>152	321>194
4	321>152	321>257	12	321>152	321>257
5	321>152	321>257 323>152	13	466	376 378 468
6	466	376 378 468	14	321>152	321>257
7	321>152	321>257 323>152	15	321>152	321>257
8	321>152	321>257	16[2]	321>152 321>194 321>257 323>152	

[1] Values represent the parent (molecular) ion and the daughter ion (MRM transitions in LC-MS/MS methods), except for laboratories 6, 10 and 13 (selected ions, SIM mode, GC-MS methods)
[2] quantification: average of values obtained with the 4 indicated transitions

After receipt of the data sets, the results were subjected to technical evaluation. The obtained data set from laboratory 15 was rejected as it did not meet the specified quality requirements of IRMM.

The results from laboratories 10 and 14 (methods which stipulated a β-glucuronidase digestion step) are presented in Table 9 (details in annex D).

Table 9. Summary of results for methods including a β-glucuronidase digestion step

Parameter	
Number data sets	2
Number of replicate measurements	16
Laboratory 10; mean ± *s* [µg/kg][1]	0.269 ± 0.032
Laboratory 14; mean ± *s* [µg/kg][1]	0.236 ± 0.023

[1] obtained with 8 independent measurements over 2 days, see Annex D1 for details

These appear to be not significantly different from those obtained with methods lacking the β-glucuronidase digestion step (Table 10, annex D).

It has to be noted that the digestion step itself is usually poorly characterised (e.g. completeness of digestion not verified) and varies substantially among procedures (enzyme source, enzyme activity ("unit"), reaction buffer, temperature, digestion time, etc.). Due to the lack of transparency of the digestion step, and the fact that only two data sets were submitted which included this enzymatic digestion step, it could not be determined how significant the fraction of CAP released by β-glucuronidase treatment versus CAP present in the material as such (non-conjugated form) is.

In any case, the measurand of the two groups of methods - either applying or not applying β-glucuronidase treatment - is different. Therefore it was decided not to include the data from laboratories 10 and 14 for calculating the certified value.

Consequently, laboratories operating methods which foresee a β-glucuronidase treatment for CAP quantification in meat cannot refer to the certified value.

In total, 104 results from 13 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. Laboratory 2 was flagged as outlier of variance. This is mainly due to the fact that a large part of the uncertainty on their result is captured by the standard deviation, whereas standard uncertainties of other laboratories usually are much larger than the standard deviations. The result was therefore retained.

The results of the statistical tests of the finally considered data for ERM-BB130 are summarized in Table 10. It shall be noted that the mean of means (certified value) hold for the reconstituted material.

Table 10. Summary of statistical evaluation for ERM-BB130

Parameter	
Number of data sets	13
Number of replicate measurements	104
Mean of means [µg/kg]	0.230
Relative standard deviation of mean of means [%]	11.39
Relative standard error of mean of means [%]	3.16
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)	Yes (lab 2)
Lab variances homogeneous? (Bartlett test)	No
Distribution of means normal? (Skewness & kurtosis, normal probability plot)	Yes

α = 0.05 unless stated otherwise

9 Certified values and uncertainties

The certified value for ERM-BB130 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, and the characterisation study. The contribution from the common calibrant (purity) is negligible compared to the other uncertainties and therefore not included in the calculation of the combined uncertainty. 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:

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

Table 11 summarizes the individual uncertainty contributions and the resulting expanded uncertainties, and indicates the certified values and their uncertainties after rounding.

Table 11. Certified value and uncertainty for ERM-BB130

Parameter	
u_{bb} [%]	1.440
u_{lts} [%][1)]	2.972
u_{char} [%]	3.158
$u_{CRM,\ rel}$ [%]	4.569
$U_{CRM,rel}$ (k=2) [%]	9.139
Certified value [µg/kg]	**0.230**
U_{CRM} (k=2) [µg/kg]	**0.021**

[1)] Shelf life 24 months

10 Metrological traceability

The measurement results for assigning a chloramphenicol mass fraction value to the material were obtained by employing methods with different sample preparation procedures (from extraction with organic solvent without any clean-up, up to extensive sample preparation involving β-glucuronidase treatment, defatting step and solid-phase extraction). Therefore, independence of the results from the sample preparation part can be concluded.

GC-MS and LC-MS/MS methodologies were used for analyte separation and quantification, thereby defining the measurand. 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 negative electro-spray ionisation and utilised the instruments in the triple quadrupole configuration by applying tandem mass spectrometry in the multiple reaction monitoring mode. The same transition (parent ion, daughter ion) was used for quantification except for one laboratory. 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.). As for the applied GC-MS methods, different derivatisation procedures were applied as the final step of sample preparation. The chromatographic part differed in the type of carrier gas, flow rate, temperature gradient, injection type and amount, columns used (stationary phase). Mass spectrometry parts exclusively used electron-capture negative ionisation, and the mass spectrometers were operated in the selected ion monitoring mode. MS settings (solvent delay time, dwell time, all ions monitored) however differed among methods.

The certified value is traceable to the common calibrant used. The common calibrant (pure crystalline substance) was purchased and provided by IRMM. The documented purity was verified by a set of methods including LC-DAD, KFT, DSC, total ash determination according to European Pharmacopoeia 6.0, TG-FTIR, LC-TOF, and qNMR.

Consequently, the certified mass fraction for CAP is traceable to the International System of Units (SI).

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 1.25 ± 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 3.75 ± 0.01 g of distilled water to the powder.
- In case the working instruction of the laboratory's method foresees a higher sample intake than 5 g of reconstituted material, the 1:3 m/m ratio of powder to distilled water has to be maintained.
- Mix to a homogeneous sample, for instance by vortexing the powder-water mixture for at least 1 min at maximum speed. Proceed with the sample preparation as foreseen in the laboratory's working instruction without unnecessary delay.

11.3 Extraction step in sample preparation procedure

- Please note that after reconstitution, the typical duration of the extraction step (addition of extraction solution, agitation, centrifugation) before further sample manipulation (e.g. evaporation, clean-up) was between 10 and 60 minutes in the laboratories contributing to the certified value. Any unnecessary delay during extraction (e.g. leaving the sample in the extraction solution at room temperature for a non-controlled time span) shall be avoided.

11.4 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 by Linsinger [16]. 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 %.

11.5 Storage conditions

The materials should be stored at a temperature of -20 ± 2 °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

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Annex A. Homogeneity data

Table A1. Results of the homogeneity study

Bottle number	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Replicate 3 (µg/kg)	Replicate 4 (µg/kg)
72	0.224	0.226	0.229	0.220
245	0.220	0.220	0.221	0.216
353	0.211	0.216	0.208	0.223
508	0.220	0.207	0.212	0.208
612	0.207	0.220	0.228	0.212
725	0.220	0.215	0.213	0.218
888	0.204	0.221	0.211	0.205
1017	0.222	0.213	0.208	0.206
1103	0.211	0.214	0.217	0.211
1238	0.212	0.214	0.218	0.234

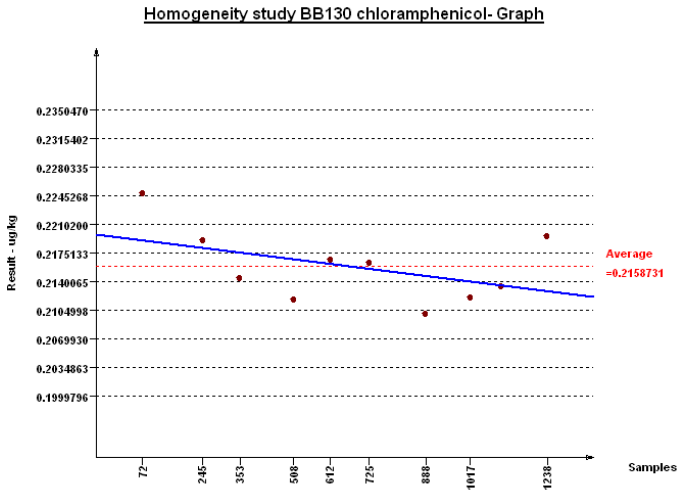

Figure A1. Homogeneity of CAP in ERM-BB130. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of quadruplicate measurements.

Annex B. Short-term stability data

Table B1. Results of the short-term stability study

Time (weeks)	4 ºC	18 ºC	60 ºC
0	0.215	0.215	0.215
0	0.213	0.213	0.213
0	0.209	0.209	0.209
0	0.231	0.231	0.231
0	0.224	0.224	0.224
0	0.220	0.220	0.220
0	0.212	0.212	0.212
0	0.224	0.224	0.224
1	0.216	0.200	0.199
1	0.224	0.217	0.195
1	0.219	0.210	0.196
1	0.208	0.223	0.191
1	0.222	0.218	0.210
1	0.210	0.209	0.191
1	0.231	0.221	0.199
1	0.203	0.211	0.187
2	0.206	0.215	0.177
2	0.229	0.208	0.176
2	0.204	0.215	0.146
2	0.224	0.221	0.168
2	0.226	0.225	0.166
2	0.216	0.221	0.180
2	0.240	0.214	0.167
2	0.229	0.231	0.180
4	0.210	0.216	0.130
4	0.223	0.214	0.128
4	0.212	0.222	0.130
4	0.212	0.214	0.123
4	0.229	0.210	0.141
4	0.214	0.230	0.120
4	0.234	0.228	0.144
4	0.220	0.223	0.117

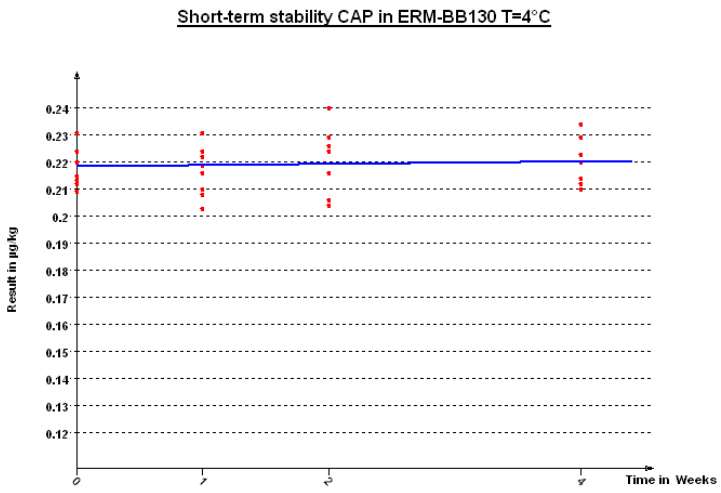

Figure B1. Short-term stability for CAP at 4 ºC.

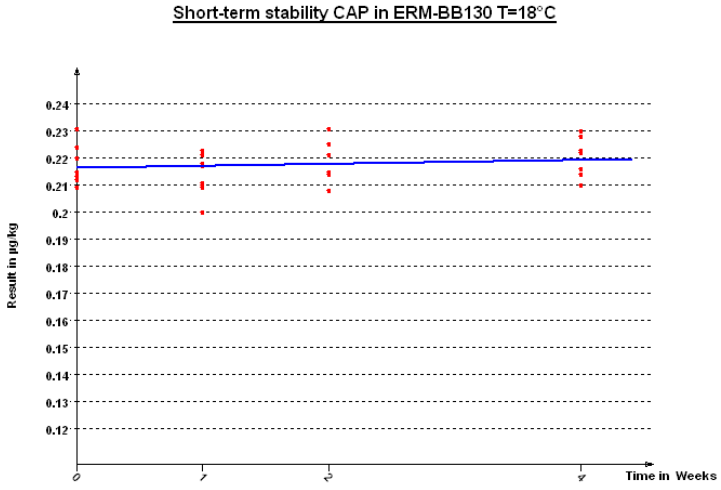

Figure B2. Short-term stability study for CAPat 18 ºC.

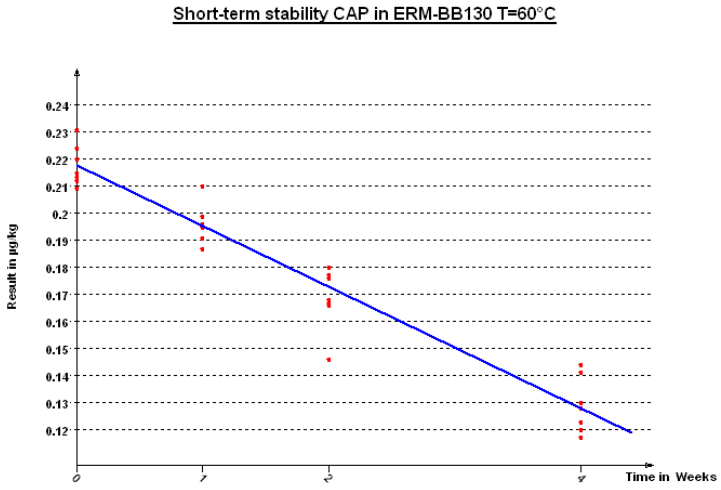

Figure B3. Short-term stability study for CAPat 60 ºC.

Annex C. Long-term stability data

Table C1. Results of the long-term stability study

Time (months)	4 ºC	-20 ºC
0	0.166	0.166
0	0.171	0.171
0	0.172	0.172
0	0.172	0.172
0	0.165	0.165
0	0.166	0.166
0	0.169	0.169
0	0.174	0.174
0	0.168	0.168
4	0.183	0.182
4	0.172	0.181
4	0.180	0.166
4	0.167	0.170
4	0.165	0.162
4	0.162	0.174
4	0.167	0.164
4	0.163	0.166
4	0.162	0.162
8	0.180	0.177
8	0.179	0.179
8	0.161	0.169
8	0.167	0.168
8	0.171	0.168
8	0.172	0.172
8	0.172	0.163
8	0.158	0.162
8	0.177	0.166
12	0.186	0.169
12	0.175	0.178
12	0.176	0.170
12	0.174	0.163
12	0.173	0.166
12	0.169	0.165
12	0.215	0.164
12	0.168	0.159
12	0.161	0.167

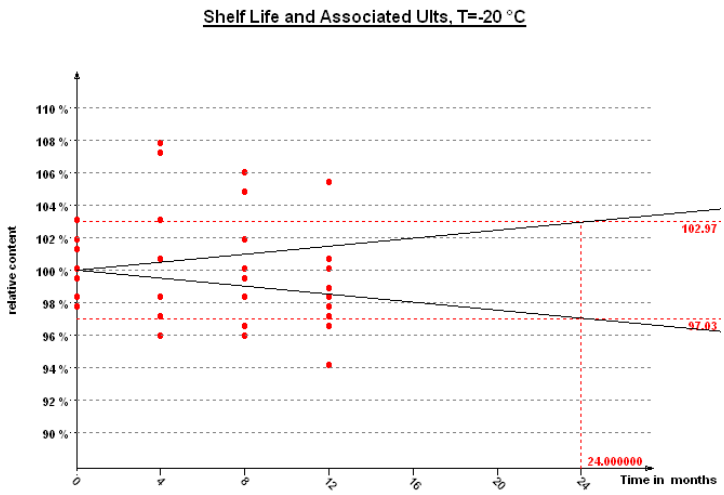

Figure C1. Long-term stability for CAP at -20 ºC with associated u_{lts} for storage period of 24 months

Annex D. Characterisation data

Table D1. Results of the characterisation measurements for chloramphenicol. 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.

CAP mass fraction in ERM-BB130 [µg/kg]								
Lab code	Day1/1	Day1/2	Day1/3	Day1/4	Day2/1	Day2/2	Day2/3	Day2/4
1	0.220	0.220	0.240	0.250	0.250	0.230	0.250	0.250
2	0.232	0.295	0.311	0.230	0.240	0.234	0.257	0.261
3	0.260	0.240	0.260	0.250	0.270	0.260	0.260	0.250
4	0.206	0.189	0.191	0.207	0.219	0.228	0.231	0.232
5	0.224	0.227	0.231	0.218	0.231	0.226	0.228	0.228
6	0.216	0.218	0.222	0.216	0.226	0.231	0.236	0.228
7	0.301	0.317	0.311	0.269	0.282	0.297	0.287	0.251
8	0.240	0.200	0.240	0.230	0.210	0.220	0.230	0.220
9	0.215	0.231	0.233	0.213	0.217	0.225	0.224	0.217
10[1]	0.250	0.230	0.240	0.240	0.290	0.290	0.310	0.300
11	0.195	0.221	0.197	0.212	0.217	0.236	0.233	0.239
12	0.193	0.189	0.197	0.204	0.194	0.190	0.196	0.192
13	0.210	0.190	0.190	0.210	0.190	0.190	0.180	0.190
14[1]	0.200	0.220	0.220	0.260	0.250	0.240	0.230	0.270
15[2]	0.340	0.350	0.340	0.310	0.260	0.280	0.260	0.260
16	0.230	0.210	0.210	0.240	0.230	0.230	0.220	0.250

[1] laboratories with β-glucuronidase digestion step in sample preparation; not considered for calculation of certified value (see pages 18 and 19 for details)
[2] data set rejected for technical reason (see page 18)

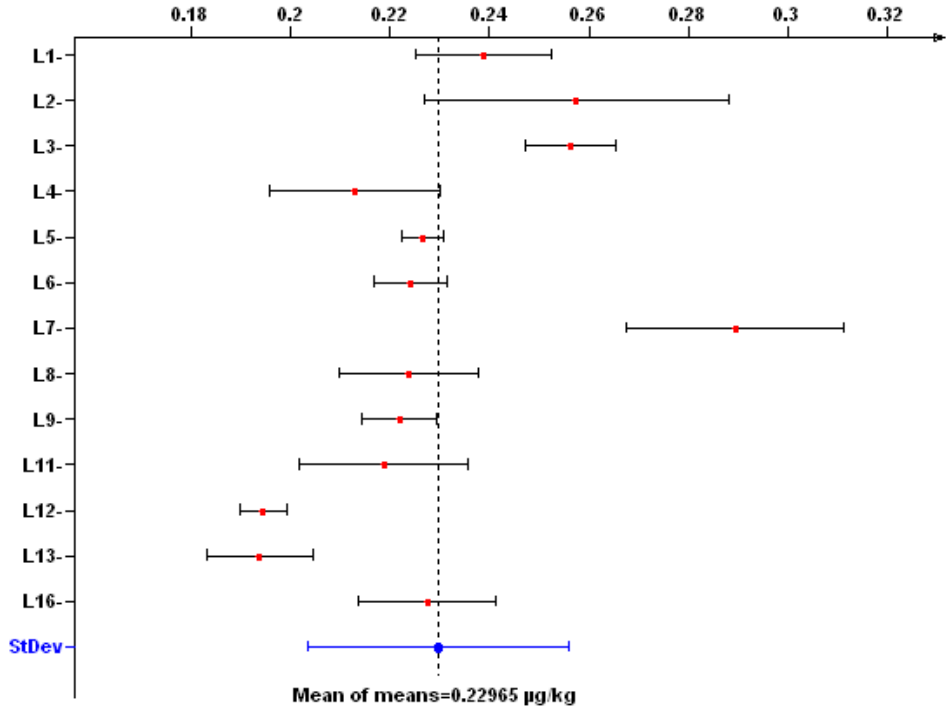

Figure D1. Laboratory means, mean of means and their standard deviations for CAP

European Commission

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Abstract

This report describes the preparation of the pork meat matrix reference material ERM-BB130 and the certification of the content (mass fraction) of chloramphenicol.

The preparation and processing of the material, homogeneity and stability studies, and the characterisation are described hereafter and the results are discussed. Uncertainties were calculated in compliance with ISO Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM) [1] and include uncertainties due to possible heterogeneity, instability, and characterisation. The certified value is listed below:

Measurand in the reconstituted material	Certified value [1)] [µg/kg]	Uncertainty [2)] [µg/kg]	Number of accepted sets of results
Chloramphenicol	0.230	0.021	13

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

The assigned value and its uncertainty is based on a minimum sample intake of 5 g reconstituted material (corresponding to 1.25 g powder).

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