CERTIFICATION REPORT

The certification of the mass fraction of aflatoxins B1 and G1 in paprika powder: ERM®-BD286

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Abstract

This report describes the production of ERM®-BD286, which is a matrix material certified for the mass fraction of aflatoxins B1 and G1 in paprika powder. This material was produced following ISO 17034:2016 and is certified in accordance with ISO Guide 35:2017.
The starting material consisted of two naturally contaminated batches of paprika powder. Both batches were thoroughly sieved, cryogenically milled, and homogenised before they were mixed in equal quantities. After homogenisation and sieving the powder was manually filled in amber glass bottles. The bottles were packed in aluminised plastic sachets that were heat sealed. The sachets were sterilised by γ-irradiation.
Between-unit homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2017. Within-unit homogeneity was quantified to determine the minimum sample intake.
The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2005. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.
Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties related to possible inhomogeneity, instability and characterisation.
The material is intended for the quality control and assessment of method performance. As with any reference material, it can be used for establishing control charts or validation studies. The certified reference material is available in amber glass bottles packed in aluminised plastic sachets each containing at least 100 g of paprika powder. The minimum amount of sample to be used is 10 g.

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Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Summary

This report describes the production of ERM®-BD286, which is a matrix material certified for the mass fraction of aflatoxins B_1 and G_1 in paprika powder. This material was produced following ISO 17034:2016 [1] and is certified in accordance with ISO Guide 35:2017 [2].

The starting material consisted of two naturally contaminated batches of paprika powder. Both batches were thoroughly sieved, cryogenically milled, and homogenised before they were mixed in equal quantities. After homogenisation and sieving the powder was manually filled in amber glass bottles. The bottles were packed in aluminised plastic sachets that were heat sealed. The sachets were sterilised by γ-irradiation.

Between-unit homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2017 [2]. Within-unit homogeneity was quantified to determine the minimum sample intake.

The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2005 [3]. Technically invalid results were removed but no outlier was eliminated on statistical grounds only.

Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible inhomogeneity, instability and characterisation.

The material is intended for the quality control and assessment of method performance. As with any reference material, it can be used for establishing control charts or validation studies. The certified reference material is available in amber glass bottles packed in aluminised plastic sachets each containing at least 100 g of paprika powder. The minimum amount of sample to be used is 10 g.

The following values were assigned:

	Mass Fraction	
	Certified value [2] [µg/kg]	Uncertainty [3] [µg/kg]
Aflatoxin B_1 (AFB_1) [1]	3.72	0.29
Aflatoxin G_1 (AFG_1) [1]	2.4	0.6

[1] As obtained by analytical procedures based on immunoaffinity clean-up, separation by high-performance liquid chromatography and subsequent quantification by fluorescence detection.

[2] Certified values are values that fulfil the highest standards of accuracy and represent the unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory. The certified value and its uncertainty are traceable to the International System of units (SI).

[3] The uncertainty is the expanded uncertainty of the certified value with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

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Glossary

ACN	Acetonitrile
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFLs	Aflatoxins
ANOVA	Analysis of variance
AOAC	AOAC International (formerly Association of Official Analytical Chemists)
CRM	Certified reference material
DSC	Differential scanning calorimetry
EC	European Commission
ELISA	Enzyme-linked immunosorbent assay
ERM®	Trademark of European Reference Materials
EU	European Union
GC	Gas chromatography
GC-ECD	Gas chromatography-electron capture detection
GC-FID	Gas chromatography-flame ionisation detection
GC-MS	Gas chromatography-mass spectrometry
GUM	Guide to the Expression of Uncertainty in Measurements
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography-diode array detection
HPLC-FLD	High performance liquid chromatography-fluorescence detection
HPLC-UV	High-performance liquid chromatography-ultraviolet detection
HPLC-MS	High-performance liquid chromatography-mass spectrometry
IAC	Immunoaffinity column
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
KFT	Karl Fischer titration
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
$MS_{between}$	Mean of squares between-unit from an ANOVA

MS_{within}	Mean of squares within-unit from an ANOVA
n	Number of replicates per unit
N	Number of samples (units) analysed
n.a.	Not applicable
NMR	Nuclear magnetic resonance spectrometry
OTA	Ochratoxin A
p	Number of datasets used for value assignment
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
RSD	Relative standard deviation
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added when appropriate
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
SI	International System of Units
s_{meas}	Standard deviation of measurement data; an additional index "rel" is added as appropriate
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation; an additional index "rel" is added as appropriate
T	Temperature
t	Time
t_i	Time point for each replicate
t_{sl}	Proposed shelf life
t_{tt}	Proposed transport time
TLC	Thin layer chromatography
u	Standard uncertainty
U	Expanded uncertainty
u^*_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability/intermediate precision select as appropriate; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{Δ}	Combined standard uncertainty of measurement result and certified value

u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{meas}	Standard measurement uncertainty
U_{meas}	Expanded measurement uncertainty
u_{rec}	Standard uncertainty related to possible between-unit inhomogeneity modelled as rectangular distribution; an additional index "rel" is added as appropriate
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
UV	Ultraviolet
Δ_{meas}	Absolute difference between mean measured value and the certified value
$\nu_{s,meas}$	Degrees of freedom for the determination of the standard deviation s_{meas}
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}
X_{10}	Particle diameter corresponding to 10 % of the cumulative undersize distribution (here by volume)
X_{50}	Median particle diameter corresponding to 50th percentile of the cumulative undersize distribution (here by volume)
X_{90}	Particle diameter corresponding to 90 % of the cumulative undersize distribution (here by volume)
$\bar{y}$	Mean of all results

1 Introduction

1.1 Background

Mycotoxins are secondary metabolites of moulds. These toxic metabolites occur as contaminants in a wide range of food and animal feed from plant origin and can pose a potential risk to human and animal health [5, 6].

The most dangerous are aflatoxins (AFLs), among which the most frequent are aflatoxin B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1, AFG_2). As they are considered to be genotoxic and carcinogenic, it is essential to limit the total content of aflatoxins (e.g. sum of AFB_1, AFB_2, AFG_1 and AFG_2) as well as the AFB_1 content alone in food, being the most toxic compound [6, 7]. European Union (EU) legislation stipulates that food containing a level of contaminant that is unacceptable from a public health viewpoint – in particular at a toxicological level – cannot be put on the market [8]. Since many contaminants are naturally occurring, it would be impossible to impose a total ban on these substances. Instead, the best course of action to protect public health is to ensure that these substances are kept at levels as low as reasonably achievable (ALARA principle) and are determined on the basis of sound scientific evidence. Maximum levels of aflatoxins are laid down in Commission Regulation (EC) No 1881/2006 [9, 10]. One of the major contributors to the aflatoxin exposure are spices from dried fruits of *Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika). The maximum tolerable limits for them are set at 10 µg/kg for total AFLs (sum of AFB_1, AFB_2, AFG_1 and AFG_2) and at 5.0 µg/kg for AFB_1.

Provisions for methods of sampling and analysis for the official control of mycotoxins including aflatoxins are laid down in Commission Regulation (EC) No 401/2006 [11]. Laboratories may select any method, provided the selected method meets the predefined performance criteria laid down in point 4 of Annex II to Regulation (EC) 401/2006. Various analytical methods can be applied for the analysis of aflatoxins in food and feed, such as (i) conventional methods such as high performance liquid chromatography (HPLC) with different detection modes (ultraviolet detection (UV), fluorescence detection (FLD), mass spectrometry (MS)), as well as gas chromatography with various detection modes (flame ionisation detection (FID), electron capture detection (ECD) or MS) (ii) multicomponent methods (liquid chromatography with tandem mass spectrometry (LC-MS/MS)), and (iii) screening methods (immunochemical methods, e.g. enzyme-linked immunosorbent assay (ELISA)). However, it seems to be that HPLC-FLD, in combination with immunoaffinity chromatography for a previous sample clean-up, still remains the method of choice. The other methods above are not widely used for routine analysis of specific aflatoxins in food and feed matrices including spices.

Furthermore, Commission Regulation (EC) No 401/2006 [11] recommends to use fully validated confirmatory methods (i.e. methods validated by collaborative trials for relevant material matrices) where appropriate and available, such as EN 14123:2007 [12] or AOAC Official Method No 999.07 [13]. Other suitable validated confirmatory methods (e.g. methods validated in-house on relevant matrices belonging to the commodity group of interest) may also be used provided they fulfil the requested performance criteria. Independent from the choice, laboratories must be able to provide the evidence that the method of analysis used does comply with the established performance criteria. Hence, it is critical to any quality assurance programme to have a reliable option to verify testing procedures, methods, and equipment performance. In order to provide the analytical laboratories with the necessary tools for adequate quality assurance and quality control during the analysis of aflatoxins in

foodstuffs, suitable certified reference materials (CRMs) are necessary. According to ISO/IEC 17025:2017 [14] the use of reference materials and the participation in proficiency testing schemes are essential tools for assuring and controlling the quality of analytical data. They are needed to contribute to the harmonisation of reliable analytical results, and thus to the proper implementation of EU legislation. Yet, for aflatoxin analysis in paprika, access to matrix CRMs is currently limited.

ERM-BD286 is certified for the mass fraction of AFB_1 and AFG_1 in paprika powder. Additional material information is provided for the mass fraction of AFB_2, AFG_2, and ochratoxin A. The provision of ERM-BD286 increases the comparability and reliability of measurement results between laboratories, allowing them to prove their competences.

1.2 Choice of the material

Aflatoxins are mainly produced by three *Aspergillus* spp., i.e. *A. flavus, A. parasiticus and A. nominus*. It is generally considered that *A. flavus* produces mainly AFB_1 and AFB_2 whereas *A. parasiticus* produces AFB_1, AFB_2, AFG_1 and AFG_2 [6, 15, 16]. Since *Aspergillus* spp. toxins quite often appear simultaneously in spices like paprika powder the Joint Research Centre (JRC) decided to certify the mass fraction of individual aflatoxins in paprika powder. Paprika can be found across a large range of foodstuffs e.g. as a seasoning spice. The chosen base material consisted of two naturally contaminated batches (A and B) of paprika powder. The mass fraction of AFB_1 in the final processed material was expected to correspond to approximately 70-80 % of the maximum permitted level in spices [9, 10].

1.3 Design of the CRM project

The initial aim of the project was to certify simultaneously aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) and ochratoxin A (OTA) in paprika powder. The certification of AFB_2, AFG_2, and ochratoxin A could not be realised due to technical reasons. However, some additional material information is provided for them, giving merely information about these properties that may be of interest for the user.

The certification of the material was based on its stability, homogeneity and characterisation studies. The material characterisation study was performed by an intercomparison of the results obtained by expert laboratories. They were selected based on criteria that comprised both technical competence and quality management aspects.

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, Geel, BE
(accredited to ISO 17034 for production of certified reference materials, BELAC No. 268-RM)

2.2 Processing

European Commission, Joint Research Centre, Geel, BE
(accredited to ISO 17034 for production of certified reference materials, BELAC No. 268-RM)

2.3 Homogeneity study

Public Analysts Laboratory, Dublin, IE
(measurements under the scope of ISO/IEC 17025 accreditation, INAB; 099T)

2.4 Stability studies

2.4.1 Short-term stability study

European Commission, Joint Research Centre, Geel, BE
(accredited to ISO 17034 for production of certified reference materials, BELAC No. 268-RM; measurements under the scope of ISO/IEC 17025 accreditation BELAC No. 268-TEST)

2.4.2 Long-term stability study

Public Analysts Laboratory, Dublin, IE
(measurements under the scope of ISO/IEC 17025 accreditation, INAB; 099T)

2.5 Characterisation

Laboratory for Residues and Contaminants, Agricultural Research Centre, Saku, EE
(measurements under the scope of ISO/IEC 17025 accreditation, Estonian Accreditation Centre; L003)

Central Laboratory for Chemical Testing and Control (CLCTC), Sofia, BG
(measurements under the scope of ISO/IEC 17025 accreditation, BAS; 908)

Finnish Customs Laboratory, Espoo, FIN
(measurements under the scope of ISO/IEC 17025 accreditation, FINAS; T006/M23/2013)

Kent Scientific Services, Kent, UK
(measurements under the scope of ISO/IEC 17025 accreditation, UKAS; 1398)

Laboratory of Department of Food and Consumer Articles Research of the National Institute of Hygiene, Warsaw, PL
(measurements under the scope of ISO/IEC 17025 accreditation, Polish Centre for accreditation; AB 509)

LGC – Food and Agriculture, Teddington, UK
(measurements under the scope of ISO/IEC 17025 accreditation, UKAS; 0003)

Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Braunschweig, DE
(measurements under the scope of ISO/IEC 17025 accreditation, DAkkS; D-PL-14378-10-00)

National Institute for Food, Spanish Food Safety and Nutrition Agency (AESAN), Madrid, ES
(measurements under the scope of ISO/IEC 17025 accreditation, ENAC; 178/LE-397)

National Veterinary Institute (SVA), Uppsala, SE
(measurements under the scope of ISO/IEC 17025 accreditation, SWEDAC; 1553)

PhytoLab GmbH & Co. KG, Vestenbergsgreuth, DE
(measurements under the scope of ISO/IEC 17025 accreditation, SAL; SAL-BY G 037-01-05)

Public Analysts Laboratory, Dublin, IE
(measurements under the scope of ISO/IEC 17025 accreditation, INAB; 099T)

SGS Germany GmbH & Co. KG, Hamburg, DE
(measurements under the scope of ISO/IEC 17025 accreditation, DAkks; D-PL-11020-04-00)

Silliker Iberica, Barcelona, ES
(measurements under the scope of ISO/IEC 17025 accreditation, ENAC; 257/LE413)

State Veterinary and Food Institute Košice, Košice, SK
(measurements under the scope of ISO/IEC 17025 accreditation, SNAS; S 239)

Veterinary and Agrochemical Research Centre (CODA-CERVA, now SCIENSANO), Tervuren, BE
(measurements under the scope of ISO/IEC 17025 accreditation, BELAC; 172-TEST)

3 Material processing and process control

3.1 Origin of the starting material

The starting material consisted of two naturally contaminated batches of sweet paprika powder (batch A and B). Batch A was naturally contaminated with aflatoxins (mainly AFB_1 and AFG_1; country of origin - Brazil). Batch B was naturally contaminated mainly with AFB_1 and ochratoxin A (country of origin - Spain).

3.2 Processing

Two batches (A and B) of sweet paprika powder were delivered to the JRC at Geel. Batch A (100 kg), mainly contaminated with AFB_1 and AFG_1, was delivered in 10 bags of 10 kg each. Batch B (100 kg) containing mainly OTA and AFB_1, was delivered in 4 bags of 25 kg each. Upon arrival at the JRC facilities in Geel, both batches were immediately stored at -20 °C until further treatment.

Batch A was sieved on a 500 µm stainless steel sieve (Russel Finex, London, UK). The part of the material that was retained by the sieve (84.2 g) was discarded. The part of the material that passed through the sieve (99.2 kg) was mixed for one hour in a stainless steel drum using a Dynamix CM-500 mixer (WAB, Basel, CH) and then kept at -20 °C in 48 plastic bags by portions of ± 2 kg.

Batch B was sieved using the same conditions as used for batch A (Figure 1). About 96 kg were collected passing through the sieve while 1.5 kg were retained by the sieve. The latter fraction was stored overnight in a metallic drum at liquid nitrogen temperature (-196 °C) prior to cryogenic milling. The powder was milled using a Palla vibrating mill (KHD Humboldt Wedag, Köln, DE) previously cooled to -196 °C with liquid nitrogen. The temperature of the milled product remained below -96 °C during the run. The powder was consequently sieved at room temperature using the set up described above. The part of the material that was retained by the sieve (3.6 g) was discarded. The part of the material that passed through the sieve (1.3 kg) was added to the 96 kg of the first sieving step and mixed for one hour in a Dynamix CM-500 mixer (WAB, Basel, CH). It was then kept at -20 °C in 48 plastic bags by portions of ± 2 kg.

After analyses of the two batches for their individual mycotoxin concentrations and particle size distribution, each batch was transferred into a 200 L HDPE drum for storage. The drums were also stored at -20 °C until further processing. It was decided to create a powder by mixing equal quantities of each batch (mass ratio 50/50). A stainless steel drum of 750 L was first filled with 79.8 kg of batch A. Then 79.8 kg of batch B were transferred into the stainless steel drum. The 159.6 kg of the resulting powder was mixed for one hour using the Dynamix CM-500 mixer. On the following day, the 159.6 kg were mixed again for one hour with the Dynamix CM-500 mixer and collected in 25 L plastic drums. A total of 159 kg were collected. Samples were taken to check the final concentrations of individual mycotoxins present in the material, the water content using Karl Fischer titration (KFT) and the particle size distribution using laser diffraction (Section 3.3). Thereafter the powder was stored at -20 °C until the filling was started.

Filling the paprika powder was performed manually by transferring 103 g of the powder into 280 mL amber glass bottles using a plastic spoon and a stainless steel funnel. It was done under an extraction point to reduce the risk for staff to be contaminated with the toxins. Once filled, all bottles received a PE insert and a screw cap. Thereafter, all bottles were labelled according to fill-order (Figure 2) and packed in aluminised plastic sachets. The sachets were heat sealed and manually labelled using the corresponding bottle number. In

this report the term "unit" is used for each sample item. After filling, the labelled and packed samples were sterilised by γ-irradiation with an average dose of ~10 kGy performed at Synergy Health, Etten-Leur, NL.

Figure 1: Sieving of paprika powder

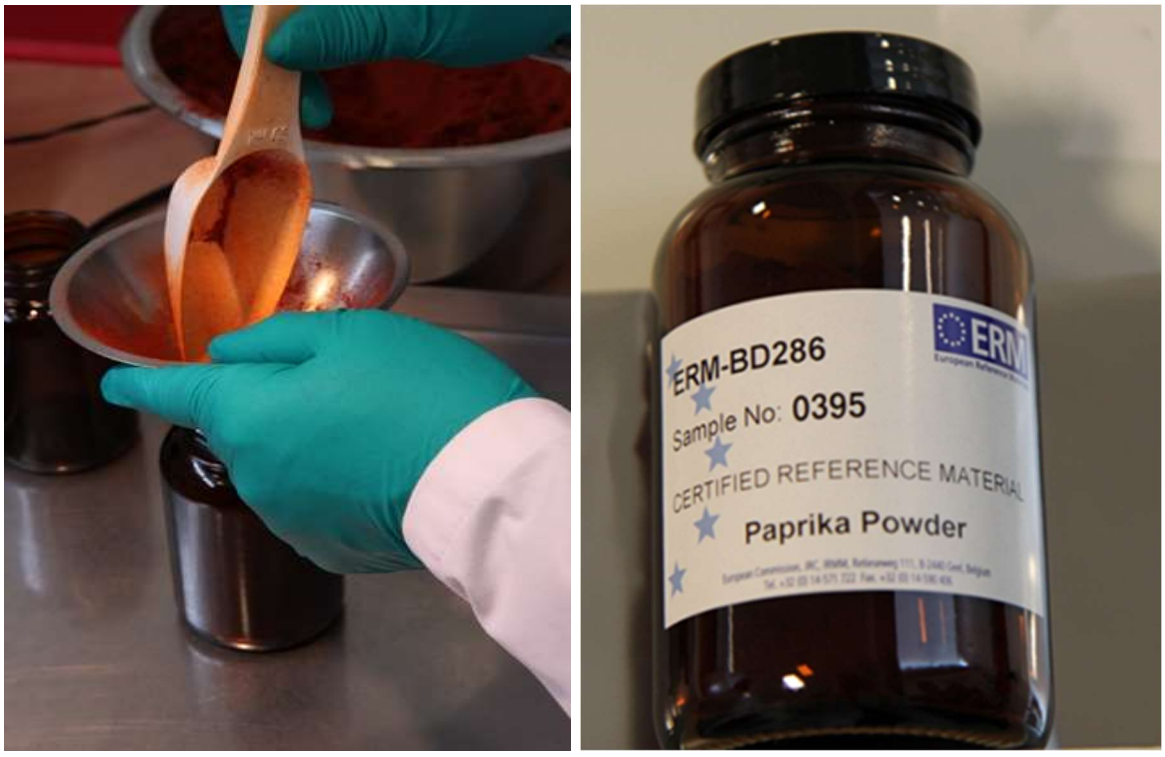

Figure 2: Manual filling of paprika powder and final labelled bottle

3.3 Process control

In this section results obtained on the final processed material are summarised.

The individual mycotoxin mass fractions (AFB_1, AFB_2, AFG_1, AFG_2, and OTA) of ERM-BD286 were analysed by HPLC-FLD on five samples to check the suitability of the material. Even so the levels for AFB_2 and AFG_2 were close to or below the limit of quantification (0.2 µg/kg) respectively, the material was considered to be suitable as the main aflatoxins (AFB_1 and AFG_1) and OTA were present in acceptable levels. The determined level for AFB_1 in the final processed material was close to the predefined target level of 70-80 % of the maximum permitted level in spices.

The water content of ERM-BD286 was measured using volumetric Karl Fischer titration in triplicate using five bottles, resulting in an average value of (6.4 ± 0.9) g/100 g (mean ± U, k = 2). Furthermore, water activity was considered as relevant process control parameter as the amount of free water in food can trigger stability issues. In general, when a matrix is too dry it could be less stable due to oxidation reactions and when it is too wet microbiological processes can degrade the material and possibly the analytes too. The water activity was determined in triplicate on five bottles values, resulting in an average water activity of 0.45. Both outcomes are considered as suitable for this matrix.

Particle size analysis using laser diffraction (Sympatec Helos, Clausthal-Zellerfield, DE) was performed in triplicate on five bottles. Volume-weighted average particle size cumulative (Q3) and density (q3*) distributions, representative for ERM-BD286 are depicted in Figure 3.

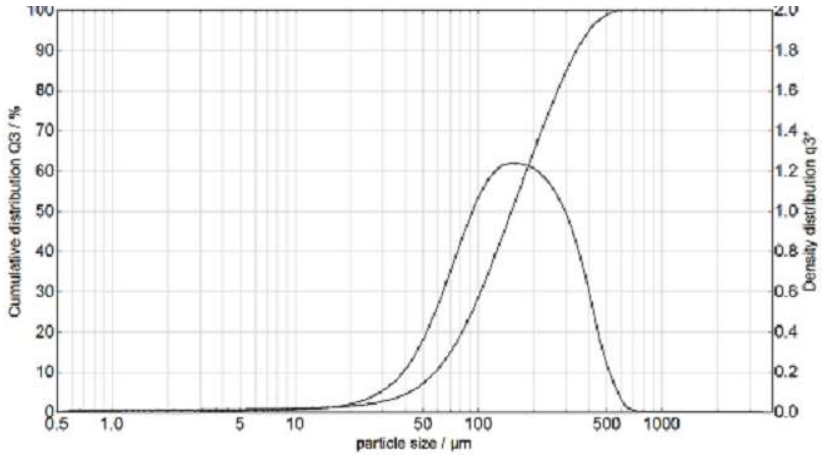

Figure 3: Average particle size distribution curves for ERM-BD286

As an overall assessment of comparability of the volume-weighted particle size distributions between the different units, the average of the deviation for the percentiles X_{10}, X_{50} and X_{90} (X_{10} Particle diameter corresponding to 10 % of the cumulative undersize distribution; X_{50} Median particle diameter corresponding to 50th percentile of the cumulative undersize distribution; X_{90} Particle diameter corresponding to 90 % of the cumulative undersize distribution) from their respective average values can be calculated. Results with an average deviation for X_{10}, X_{50} and X_{90}, below 20 % are considered as acceptable. This quality criterion, based on the experience acquired in the processing sector over many years for many different kinds of powders, is achieved for all tested units.

4 Homogeneity

A key requirement for any reference material aliquoted into units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, but it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO 17034:2016 [1] requires RM producers to quantify the between-unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample intake is respected, but determines the minimum size of an aliquot that is representative for the whole unit. Quantification of within-unit inhomogeneity is therefore necessary to determine the minimum sample intake.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRM are valid for all units of the material, within the stated uncertainties.

Fifteen units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch was divided into several groups (with a similar number of units) and one unit was selected randomly from each group. Three independent samples were taken from each selected unit and analysed by HPLC-FLD (sample intake: 10 g). The measurements were performed under intermediate precision conditions (15 units with three replicates each; spread over 3 days), and in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence. The results are shown as graphs in Annex A. Most of the results for AFB_2 were close to or below the stated limit of quantification (LOQ) of 0.2 µg/kg and all results for AFG_2 were clearly below that value.

For AFB_1 and AFG_1 regression analyses were performed to evaluate potential trends in the analytical sequence (within each of the three analysis days) as well as trends in the filling sequence. For both analytes no significant trends in the filling sequence were visible at a confidence level of 95 %. Furthermore, no trends in the analytical sequence were detected for AFB_1. The only significant trend in the analytical sequence was visible for AFG_1 on day 2, pointing possibly at a signal drift in the analytical system. These instabilities were not detected on the other two measurement days. Moreover, for AFB_1 that was obtained in the same analytical run, no trend was visible. Hence, no correction was applied and the original data used.

As the measurements were performed under intermediate conditions (on three different days), it had to be checked if day-to-day effects are present that could mask the between-unit variation. Significant differences between day means were checked using ANOVA. For both analytes no significant difference between the three days was found at a confidence level of 95 %.

The datasets were assessed for consistency using Grubbs outlier tests at a confidence level of 99 % on the individual results and on the unit means. For AFB_1 no outlying individual results and no outlying unit means were detected at a 99 % confidence interval. For AFG_1 one outlying individual result and one outlying unit mean was detected. Since no technical reason for the outliers could be found, all the data were retained for statistical analysis.

The quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the intermediate precision if the individual samples were representative for the whole unit.

Evaluation by ANOVA requires mean values per unit, which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. Too few data are available for the unit means to make a clear statement of the distribution. Therefore, it was checked visually whether all individual data follow a unimodal distribution using histograms and normal probability plots. Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviations. Finally, all data followed a unimodal distribution.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum inhomogeneity that could be hidden by method repeatability, was calculated as described by Linsinger *et al.* [17]. u^*_{bb} is comparable to the limit of detection (LOD) of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

Method repeatability ($s_{wb,rel}$), between–unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \qquad \textbf{Equation 1}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \qquad \textbf{Equation 2}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}\sqrt[4]{\frac{2}{v_{MSwithin}}}}}{\bar{y}} \qquad \textbf{Equation 3}$$

MS_{within} mean of squares within-unit from an ANOVA

$MS_{between}$ mean of squares between-unit from an ANOVA

$\bar{y}$ mean of all results of the homogeneity study

n number of replicates per unit

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

However, a different approach was adopted for AFG_1 for which one outlying unit mean was detected. In this case between-unit inhomogeneity was modelled as a rectangular distribution limited by the largest outlying unit mean, and the rectangular standard uncertainty of homogeneity was estimated by:

$$u_{rec} = \frac{\left|outlier - \bar{y}\right|}{\sqrt{3} \cdot \bar{y}} \qquad \textbf{Equation 4}$$

$\bar{y}$ mean of all results of the homogeneity study

It should be mentioned that the outlying unit mean is a result of the presence of 1 outlying individual value and does not necessarily reflect the real distribution of this analyte in the material. u_{rec} resulted in 11.39 %. Comparing the outcome of the different approaches the highest uncertainty contribution was due to the outlying unit mean, which is finally used as uncertainty contribution for homogeneity u_{bb}.

The results of the evaluation of the between-unit variation for both analytes are summarised in Table 1. The resulting values from the above equations were converted into relative uncertainties.

Table 1: Results of the homogeneity studies

Measurand	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{rec,rel}$ [%]	$u_{bb,rel}$ [%]
AFB_1	7.35	1.27	2.16	n.a. [1)]	2.16
AFG_1	9.48	3.87	2.78	11.39 [2)]	11.39

[1)] n.a.: not applicable

[2)] from outlying unit mean approach

For AFB_1 the homogeneity study showed neither outlying unit means nor a trend in the filling sequence. Therefore, the between-unit standard deviation s_{bb} can be used as estimate of u_{bb}. As u^*_{bb} sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential inhomogeneity.

One outlying unit mean was found for AFG_1. Taking this extreme value into account, the inhomogeneity quantified as u_{rec} is still sufficiently small to make the material useful. Therefore, u_{rec} was used as estimate of u_{bb}.

The results obtained for AFB_2 and AFG_2 were below the limit of quantification. Hence, they were not considered for the final certification.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used in an analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty.

Homogeneity and stability experiments were performed using a 10 g sample intake. This sample intake gives acceptable repeatability and intermediate precision, demonstrating that the within-unit inhomogeneity no longer contributes to analytical variation at this sample intake. None of the laboratories providing acceptable datasets for the characterisation study were using a sample intake below 10 g.

5 Stability

Time, temperature, light (including ultraviolet radiation), microbial growth and water content were regarded as the most relevant influences on the stability of the material. The water content as determined in section 3 was considered as suitable for the matrix. The sample containers were heat sealed and finally put in plastic-aluminised sachets, preventing the sample from further water loss/uptake. The influence of ultraviolet or visible light was minimised by storing the material in containments which reduces light exposure. In addition, materials are stored in the dark and dispatched in boxes, thus removing any possibility of degradation by light. Additionally the material was sterilised by γ-irradiation to eliminate microbial growth. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the materials to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C can be reached and stability under these conditions must be demonstrated, if the samples are to be transported without any additional cooling.

The stability studies were carried out using an isochronous design [18]. In this approach, samples were stored for a particular length of time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously in the shortest possible time (under intermediate precision conditions).

5.1 Short-term stability study

For the short-term stability study, samples were stored at 4 °C and 18 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -70 °C. Two units per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured by HPLC-FLD and post-column derivatisation with electrochemical bromination. The measurements were performed under intermediate precision conditions (due to the number of measurements that could not be performed on a single day), and a randomised sequence was used to differentiate any potential analytical drift from a trend over storage time.

The data were evaluated individually for each temperature.

Significant trends (95 % confidence level) in the analytical sequence were visible for AFB_1 (day 3) and AFG_1 (day 1), pointing at instability of the analytical system. These instabilities were not detected on the other two measurement days. In both cases, a correction of the analytical sequence trend for these days resulted in an analytical sequence trend over all days. Hence, no corrections were applied and the original data were used, as shown in Annex B.

As the measurements were performed under intermediate precision conditions (different days), it had to be checked if there are significant differences between the day means using ANOVA. This is for the simple reason that day-to-day effects can occur that could mask a potential trend. No significant day-to-day effects were observed at a confidence level of 95 %, neither for AFB_1 nor for AFG_1.

The results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %. Some single outlying results were found for AFB_1 and AFG_1 at 4 °C and 18 °C (Table 2). For AFG_1 at 4 °C no technical reason for the outlier could be found and

consequently the data was retained for statistical analysis. For both analytes one outlier was detected at 18 °C and removed, as they were identified as technical outliers.

In addition, the data were evaluated against storage time, and regression lines of mass fraction versus time were calculated, to test for potential increases/decreases of the measurands due to shipping conditions. The slopes of the regression lines were tested for statistical significance. None of the trends was statistically significant at a 95 % confidence level for any of the temperatures. A tentative removal of the detected outlier for AFG_1 at 4 °C (see above) did not change the outcome of the trend analysis.

The results of the measurements are shown in Annex B. The results of the statistical evaluation of the short-term stability are summarised in Table 2.

Table 2: Results of the short-term stability tests

Measurand	Number of individual outlying results*		Significance of the trend**	
	4 ºC	18 ºC	4 ºC	18 ºC
AFB_1	no	1 technical (removed)	no	no
AFG_1	1 statistical (retained)	1 technical (removed)	no	no

* 99 % confidence level

** 95 % confidence level

A statistical outlier was detected for AFG_1 at 4 °C, and it was retained for the estimation of u_{sts}. None of the trends was statistically significant on a 95 % confidence level for any of the temperatures. The material shall be shipped under cooled conditions to make sure that it is not exceeding 18 °C.

5.2 Long-term stability study

For the long-term stability study, samples were stored at -20 °C for 0, 4, 8, 12, 16, 24 and 60 months. The reference temperature was set to -70 °C. Two samples per storage time were selected using a random stratified sampling scheme. From each unit, three independent replicates were measured by HPLC-FLD. The measurements were performed under intermediate precision conditions (spread over three days; on each day 1 replicate from each unit), in a random sequence to be able to separate any potential analytical drift from a trend over storage time.

The long-term stability data were evaluated individually for each measurand.

Significant trends (95 % confidence level) in the analytical sequence were only visible for AFG_1 on day 3, pointing at instability of the analytical system on that day. This instability was not detected on the other two measurement days. A correction of the analytical sequence trend on day 3 resulted in an analytical sequence trend over all days. Hence, no corrections were applied and the original data obtained on the three days used.

As the measurements were performed under intermediate precision conditions (three different days), it had to be checked if there are significant differences between the day means using ANOVA. This is for the simple reason that day-to-day effects can occur that could mask a potential trend. While no significant day-to-day effect was observed for AFG_1, for AFB_1 a significant day-to-day effect was observed at a confidence level of 95 %. Hence, to check if a trend is masked by the day-to-day effect, a correction was applied by dividing every data point by the respective day mean.

The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. No outlying results were found.

In addition, the data were plotted against storage time and linear regression lines of mass fraction versus time were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected for all analytes at a 95 % confidence level. This was also confirmed for the normalised AFB_1 data (see above). The results of the long-term stability measurements are shown in Annex C.

No technically unexplained outliers were observed on 99 % confidence level and none of the trends was statistically significant on a 95 % confidence level for any of the analytes. The material can therefore be stored at -20 °C.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can entirely rule out degradation of materials, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method intermediate precision, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation within an uncertainty to be estimated.

The uncertainties of stability during dispatch and storage were estimated, as described in [19] for each analyte. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum\left(t_i - \bar{t}\right)^2}} \cdot t_{tt} \qquad \textbf{Equation 5}$$

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum\left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \textbf{Equation 6}$$

s_{rel} relative standard deviation of all results of the stability study

t_i time elapsed at time point *i*

$\bar{t}$ mean of all t_i

t_{tt} chosen transport time (1 week at 18 ºC)

t_{sl} chosen shelf life (24 months at -20 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 18 °C studies. The uncertainty describes the possible change during a dispatch at 18 °C lasting for one week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the -20 °C study. The uncertainty contribution describes the possible degradation during 24 months storage at -20 °C.

The results of these evaluations are summarised in Table 3.

Table 3: Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of 18 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of -20 °C and 24 months

Measurand	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
AFB_1	1.14	1.82
AFG_1	1.14	1.47

No significant degradation was observed for transport up to 18 °C. Cooled shipment is therefore necessary.

The material can be stored at -20 °C.

After the certification study, the material will be included in the JRC's regular stability monitoring programme, to control its further stability.

6 Characterisation

The material characterisation is the process of determining the property values of a reference material.

The material characterisation was based on an interlaboratory comparison of expert laboratories, i.e. the mass fractions of the target analytes of the material were determined in different laboratories that applied different measurement procedures (HPLC-FLD and LC-MS/MS) to demonstrate the absence of a measurement bias. This intercomparison approach aims at randomisation of laboratory bias, which reduces the combined uncertainty. However, at the very end all laboratories providing valid datasets used HPLC-FLD for the measurements.

6.1 Selection of participants

Fifteen laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of aflatoxin measurements in paprika by submitting results of intercomparison exercises or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 [3] was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2.5).

6.2 Study setup

Each laboratory received two units of ERM-BD286 and was requested to provide six independent results, three per unit. The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations and measurements had to be spread over three days to ensure intermediate precision conditions. On each day one replicate from each of the two provided units of ERM-BD286 had to be analysed once, but with duplicate injections (the mean of 2 injections was used for the evaluation process). Only results corrected for recovery (provided by each laboratory) were used for evaluation. Laboratories were asked to measure all four aflatoxins (B_1, G_1, B_2, and G_2) even so the results from the homogeneity study had already indicated AFB_2 and AFG_2 levels close to or well below the LOQ. The intention was to use the provided results for additional material information. The same applied for OTA that was excluded from all studies because of technical reasons.

External calibrations were based on dilutions of the provided common calibrants, i.e. one ampoule of ERM-AC057 (AFB_1), one ampoule ERM-AC058 (AFB_2), one ampoule ERM-AC059 (AFG_1), and one ampoule ERM-AC060 (AFG_2) [20]. An independent calibration was performed each measurement day. The certified mass fractions of these common calibrants are based on the gravimetric preparation corrected for the purity of the materials. A multitude of methods were used to assess the purity and the identity of the aflatoxins. Purity and identity were evaluated using ^{1}H and ^{13}C nuclear magnetic resonance spectrometry (NMR), elemental analysis, differential scanning calorimetry (DSC), thin-layer chromatography (TLC), HPLC-DAD, and HPLC-MS/MS. All dilutions were performed gravimetrically on calibrated balances, and the gravimetric values traceable to the International System of Units (SI) were confirmed by UV spectrometric measurements. Uncertainties of the CRMs comprise contributions from purity assessment, long-term stability and the gravimetric preparation [20].

In addition, each laboratory was asked to provide raw results, recovery factors, results corrected for recovery (indicating how the correction was performed), relative standard

deviations, limits of quantification for each measurand, measurement uncertainty (if available), and a few representative chromatograms.

6.3 Methods used

For the extraction of AFLs from paprika powder participants used different extraction solvents and techniques, i.e. mainly methanol/water (MeOH/H_2O) or acetonitrile/water (ACN/H_2O) in different ratios with or without addition of sodium chloride (NaCl) in combination with either shaking or blending. Hence, independency to the extraction method is given. In all cases (accepted data sets) immunoaffinity columns (IAC) were used to clean-up the sample extracts. For the chromatographic separation and determination of AFLs only HPLC-FLD (accepted data sets) were used. Although AFB_1 and AFG_1 are chemically clearly defined analytes, they are operationally defined by IAC clean-up and HPLC-FLD, as independency of the measurands to these steps of the method could not be guaranteed.

All methods used during the characterisation study are summarised in Annex D. The laboratory code (e.g. L01) is a random number and does not correspond to the order of laboratories in Section 2. The lab-method code consists of a number assigned to each laboratory (e.g. L01) and abbreviation of the measurement method used (e.g. HPLC-FLD).

6.4 Evaluation of results

The characterisation study resulted in 15 datasets per analyte. All individual results of the participants, grouped per analyte are displayed in tabular and graphical form in Annex E for AFB_1 and AFG_1. Results obtained for AFB_2, AFG_2, and OTA are displayed in tabular and graphical form in Annex F, solely for additional material information.

6.4.1 Technical evaluation

The obtained data were first checked for compliance with the requested analysis protocol and for their validity based on technical reasons. The following criteria were considered during the evaluation:

- appropriate validation of the measurement procedure according to ISO 17025 [3]
- compliance with the analysis protocol:
    - sample preparations and measurements performed on three days;
    - sample intake not below 10 g;
    - LOQ below or equal to 1 µg/kg for AFLs (B_1, B_2, G_1, G_2)
- absence of values given as below limit of quantification

Based on the above criteria, the following datasets were rejected as not technically valid.

The measurement results for AFB_1 and AFG_1 from laboratory L03, L05, L09 were not used for the evaluation as the sample intake did not comply with the analysis protocol, i.e. below 10 g.

The measurement results for AFB_1 and AFG_1 from laboratory L07 were not used for the evaluation as the laboratory reported a technical problem during the use of the IAC, leading to incorrect recovery results. The lab retracted the results.

The measurement results for AFG_1 from laboratory L10 were not used for the evaluation as the laboratory reported a technical problem, i.e. insufficient separation of AFG_1 peak with matrix interferences. The lab retracted the results.

The measurement results for AFB_1 and AFG_1 from laboratory L13 were not used for the evaluation as the laboratory confirmed that they did not correct for recoveries. The lab retracted the results.

In total five out of 15 datasets were rejected for AFB_1 and six for AFG_1.

6.4.2 Statistical evaluation

The datasets accepted based on technical reasons were tested for normality of dataset means using kurtosis/skewness tests and normal probability plots and were tested for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations (both at a 99 % confidence level). Standard deviations within (s_{within}) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Table 4.

Table 4: Statistical evaluation of the technically accepted datasets for ERM-BD286.

p: number of technically valid datasets

Analyte	*p*	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Mean [µg/kg]	*s* [µg/kg]	$s_{between}$ [µg/kg]	s_{within} [µg/kg]
AFB_1	10	none	none	yes	3.718	0.210	0.185	0.243
AFG_1	9	none	none	yes	2.413	0.149	0.115	0.231

The laboratory means follow normal distributions. None of the data contains outlying means and variances. The datasets are therefore consistent and the mean of the laboratory means is a reliable estimate of the true value.

The uncertainty related to the characterisation (u_{char}) is composed of two uncertainty components, u(I) and u(II) (Equation 7 and Table 5)

- u(I), the uncertainty of the characterisation, which is estimated as the standard error of the mean of laboratory means ($s/\sqrt{p}$)
- u(II), the uncertainty of the common calibrant taken from the certificate of the common calibrant [20].

$$u_{char} = \sqrt{u(I)^2 + u(II)^2}$$ **Equation 7**

Table 5: Uncertainty of characterisation for ERM-BD286

Analyte	p	Mean [µg/kg]	s [µg/kg]	$u(I)_{rel}$ [%]	$u(II)_{rel}$ *[%]*	$u_{(char),\ rel}$ *[%]*	$u_{(char)}$ *[µg/kg]*
AFB_1	10	3.718	0.210	1.78	1.45	2.30	0.085
AFG_1	9	2.413	0.149	2.05	1.72	2.68	0.065

7 Value Assignment

Certified and informative values were assigned.

Certified values are values that fulfil the highest standards of accuracy. Procedures at the JRC, Directorate F require generally pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

Additional material information refers to values that were obtained in the course of the study. For example, results reported from only one or two laboratories or in cases where individual measurement uncertainty is high, would fall under this category.

7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 5 was assigned as certified value for each parameter.

The assigned uncertainty consists of uncertainties relating to potential between-unit inhomogeneity, u_{bb} (Section 4.1), potential degradation during transport, u_{sts}, and long-term storage, u_{lts} (Section 5.3), and characterisation, u_{char} (Section 6.4). These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM, rel}$) with a coverage factor k given as:

$$U_{CRM,rel} = k \cdot \sqrt{u_{bb,rel}^2 + u_{sts,rel}^2 + u_{lts,rel}^2 + u_{char,rel}^2} \qquad \textbf{Equation 8}$$

- u_{char} was estimated as described in Section 6
- u_{bb} was estimated as described in Section 4.1
- u_{sts} and u_{lts} were estimated as described in Section 5.3

Because of the sufficient numbers of the degrees of freedom of the different uncertainty contributions, a coverage factor k = 2 was applied, to obtain the expanded uncertainties. The certified values and their uncertainties are summarised in Table 6.

Table 6: Certified values and their uncertainties for ERM-BD286

Analyte	Certified value [µg/kg]	$u_{char, rel}$ [%]	$u_{bb, rel}$ [%]	$u_{sts, rel}$ [%]	$u_{lts, rel,}$ [%]	$U_{CRM, rel}$ [1)] [%]	U_{CRM} [2)] [µg/kg]
AFB_1	3.72	2.30	2.16	1.14	1.82	7.63	0.29
AFG_1	2.4	2.68	11.39	1.14	1.47	23.69	0.6

[1)] Expanded (k = 2) uncertainty

[2)] Expanded (k = 2) and rounded uncertainty

Additional material information

The data provided in this section should be regarded as informative only on the general composition of the material and cannot be, in any case, used as certified or indicative value.

An additional material information value was assigned for AFB_2 and AFG_2. As the results obtained for AFB_2 and AFG_2 in the homogeneity study were below the limit of quantification, they were excluded from further studies and from the final certification. However, laboratories participating in the characterisation study still provided their outcome for these two analytes to be used for additional material information (Annex F). For the technical and statistical evaluation of the data they were treated in the same way as the certified values.

Due to technical reasons uncertainties derived from homogeneity, stability and characterisation were not achieved for OTA. However, laboratories participating in the characterisation study still provided their outcome for this analyte to be used for additional material information (Annex F). For the technical and statistical evaluation of the data they were treated in the same way as the certified values.

In case of AFB_2 many laboratories reported levels close to or below their limit of quantification. Out of the 15 datasets only three datasets were finally accepted.
In case of AFG_2 most of the laboratories reported levels below their limit of quantification. Only two datasets were finally accepted.
15 laboratories provided datasets for ochratoxin A. The results from six laboratories were not accepted for various reasons, i.e. (i) sample intake too low and hence not compliant with the analysis protocol, (ii) usage of incorrect recoveries, and (ii) retraction of results due to technical problems. Nine datasets were finally accepted.

The measurement ranges of the obtained mean values from the accepted datasets are shown in Table 7.

Table 7: Measurement ranges of mean values from accepted datasets for AFB_2, AFG_2, and OTA

Analyte	Values [1)] [µg/kg]
AFB_2 [2)]	0.20 – 0.23
AFG_2 [3)]	0.08 – 0.13
OTA [4)]	10.38 – 13.61

1) These values are derived from datasets obtained in different laboratories and are stated without an uncertainty and give merely information about these properties that may be of interest for the user.

2) These values are derived from three individual datasets, showing the measurement range of the obtained mean values from these datasets.

3) These values are derived from two individual datasets, showing the measurement range of the obtained mean values from these datasets.

4) These values are derived from nine individual datasets, showing the measurement range of the obtained mean values from these datasets.

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

AFB_1 and AFG_1 are chemically clearly defined analytes. However, as all participants were using the same measurement principles (apart from the extraction method) the analytes are considered as method-defined measurands and can only be obtained by following the analytical procedures used by laboratories participating in the characterisation study. The measurands are therefore operationally defined by immunoaffinity clean-up, separation by high-performance liquid chromatography and subsequent quantification by fluorescence detection.

Quantity value

Only validated methods were used for the determination of the assigned values. The certified values assigned to the common calibrants are traceable to the International System of units (SI), as described in this report and all relevant input parameters were SI-traceably calibrated. All technically accepted datasets are therefore traceable to the same reference, namely the SI. This traceability to the same reference is also confirmed by the agreement of results within their respective uncertainties. As the assigned values are combinations of agreeing results individually traceable to SI, the assigned quantity values themselves are traceable to the SI as well.

8.2 Commutability

Many measurement procedures include one or more steps which select specific (or specific groups of) analytes from the sample for the subsequent whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions that define this concept. For instance, the CLSI Guideline C53-A [21] recommends the use of the following definition for the term *commutability*:

> "The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and is therefore a crucial characteristic when applying different measurement methods. When the commutability of a CRM is not established, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrant.

ERM-BD286 was produced from a naturally grown paprika material (naturally contaminated) by milling and mixing. The analytical behaviour will be the same as for a routine sample of paprika powder. Nevertheless, one has to bear in mind that the extractability of aflatoxins from this CRM can be different to the extractability from a sample as milled in the user's laboratory due to a different particle size. For samples other than paprika powder the commutability has to be assessed.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

Aflatoxins are genotoxic and carcinogenic substances [7]; therefore, they should be handled with extreme caution. The sample bottles should be used only by personnel who are trained in the safe handling and use of hazardous substances.

Normal safety precautions should be followed. In particular the following: the sachet should be opened inside a safety cabinet. Normal laboratory safety wear including protective clothing (laboratory coat), dust mask, safety glasses and gloves should be worn.

9.2 Storage conditions

The material should be stored at (-20 ± 5) °C in the dark. Care should be taken to avoid any change of the moisture content once the units are open, as the material can be hygroscopic. The user should close any unit immediately after taking a sample.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially for opened units.

9.3 Preparation and use of the material

The units shall be allowed to warm to ambient temperature before opening to avoid water uptake by condensation. The contents should be thoroughly mixed before sub-samples are taken. Care shall be taken to avoid any change of the moisture content once the units are open, as the material can be hygroscopic. The user is reminded to close a unit immediately after taking a sub-sample. The material should be weighted out immediately after opening a bottle and the concentrations of aflatoxins calculated based on the recorded weight.

9.4 Minimum sample intake

The minimum sample intake, representative for all aflatoxins, is 10 g.

9.5 Use of the certified value

The main purpose of this material is to assess method performance, i.e. for checking accuracy of analytical results. As any reference material, it can be used for establishing control charts or validation studies.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, https://crm.jrc.ec.europa.eu/ [22].

When assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).

- Combine the 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 ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %
- If $\Delta_{meas} \leq U_{\Delta}$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

Use in quality control charts

The material can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

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11 References

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Annexes

Annex A: Results of homogeneity measurements

Annex B: Results of the short-term stability measurements

Annex C: Results of the long-term stability measurements

Annex D: Summary of methods used in the characterisation study

Annex E: Results of the characterisation study

Annex F: Additional material information

Annex A: Results of the homogeneity measurements

Data points represent data as reported by the laboratory

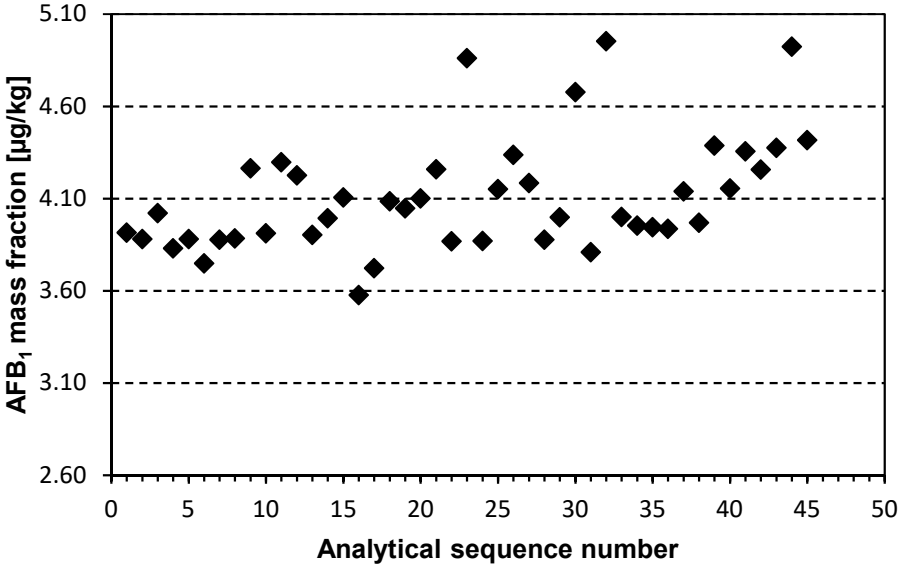

Figure A1: Individual measurement replicates for AFB_1 mass fraction in the order of measurement.

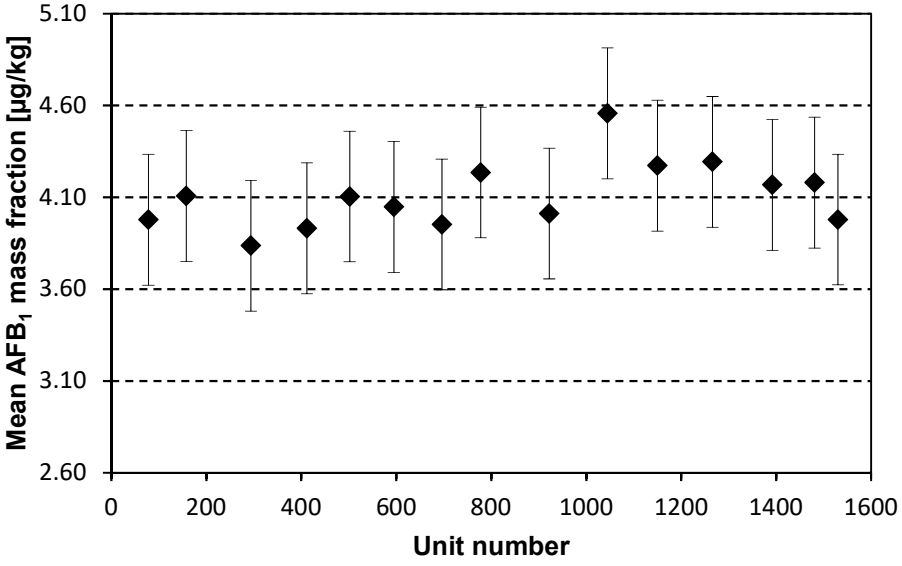

Figure A2: Unit means for AFB_1 mass fraction, against unit number. Vertical bars are a 95 % confidence interval derived from s_{wb} from ANOVA for all units of the homogeneity study.

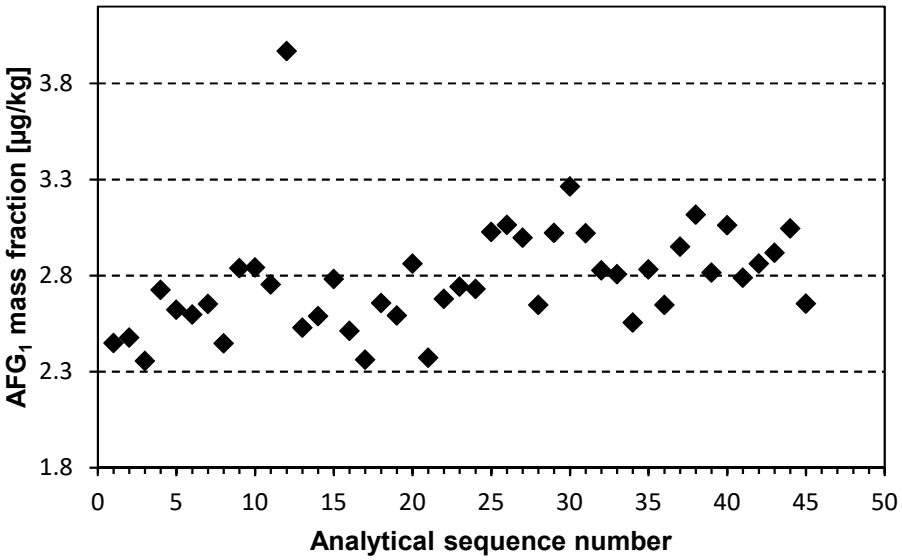

Figure A3: Individual measurement replicates for AFG_1 mass fraction in the order of measurement.

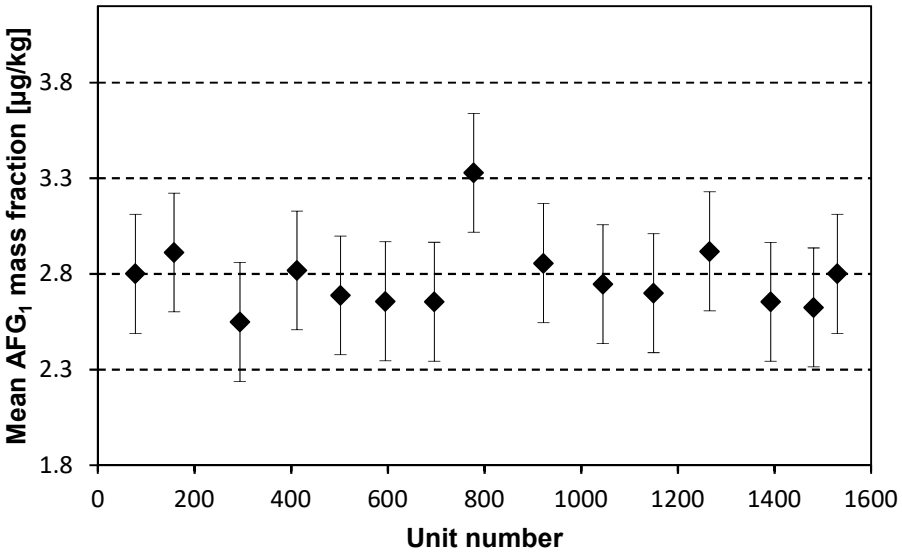

Figure A4: Unit means for AFG_1 mass fraction, against unit number. Vertical bars are a 95 % confidence interval derived from s_{wb} from ANOVA for all units of the homogeneity study.

Annex B: Results of the short-term stability measurements

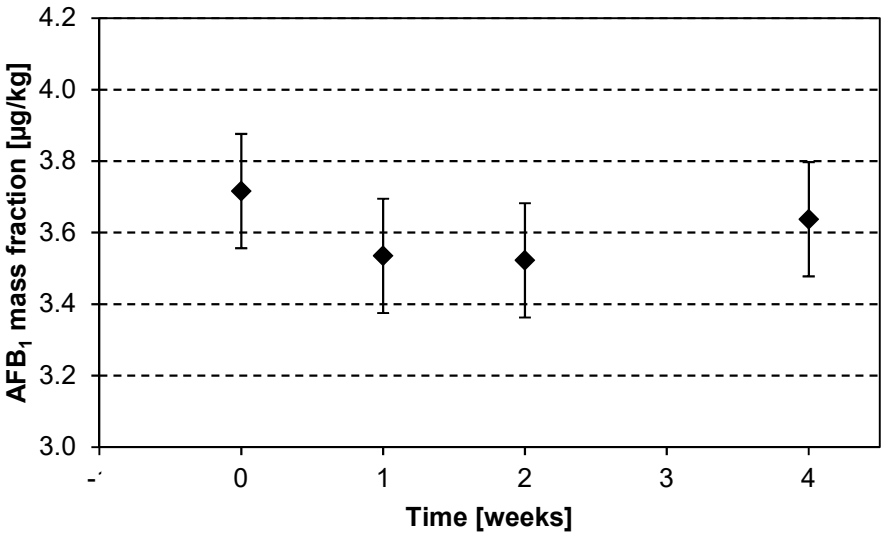

Figure B1: AFB_1 mass fraction means measured after storage at 4 °C at each time-point. Vertical bars represent the 95 % confidence interval of the mean, based on the within-group standard deviation as obtained by single-factor ANOVA.

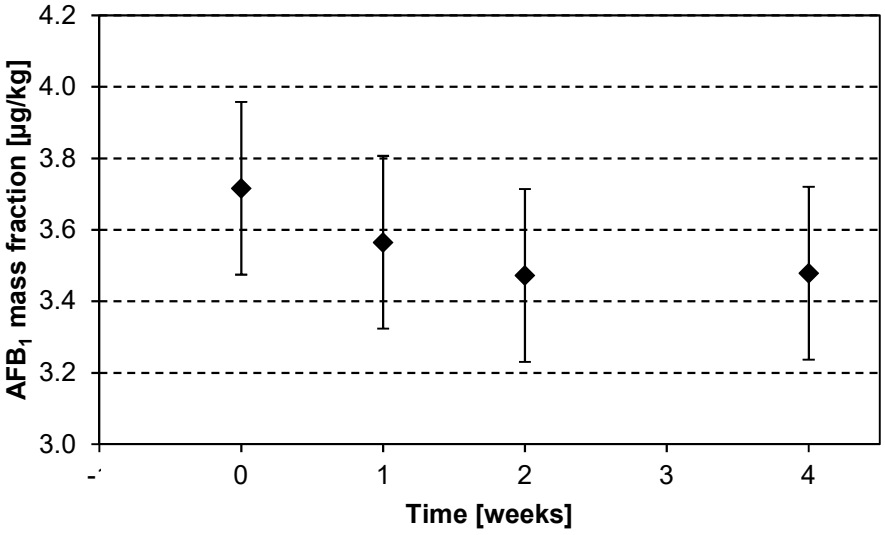

Figure B2: AFB_1 mass fraction means measured after storage at 18 °C at each time-point. Vertical bars represent the 95 % confidence interval of the mean, based on the within-group standard deviation as obtained by single-factor ANOVA.

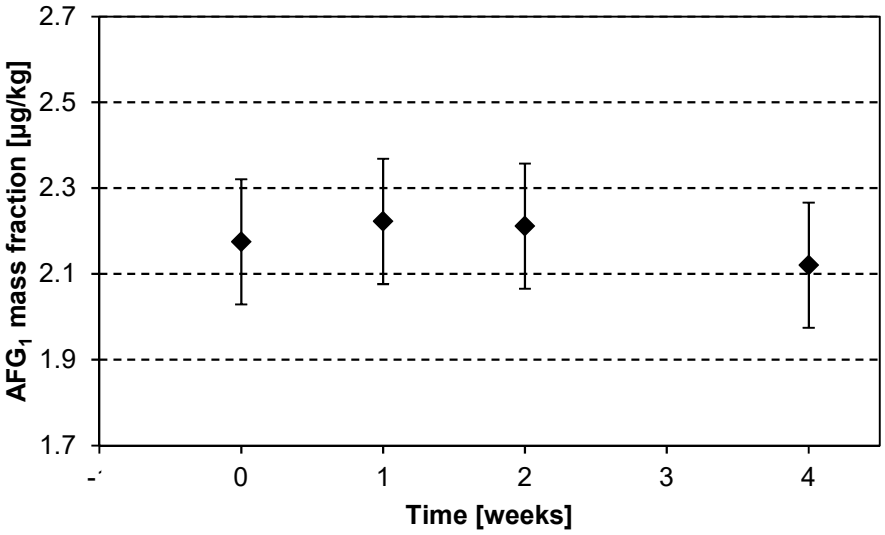

Figure B3: AFG_1 mass fraction means measured after storage at 4 °C at each time-point. Vertical bars represent the 95 % confidence interval of the mean, based on the within-group standard deviation as obtained by single-factor ANOVA.

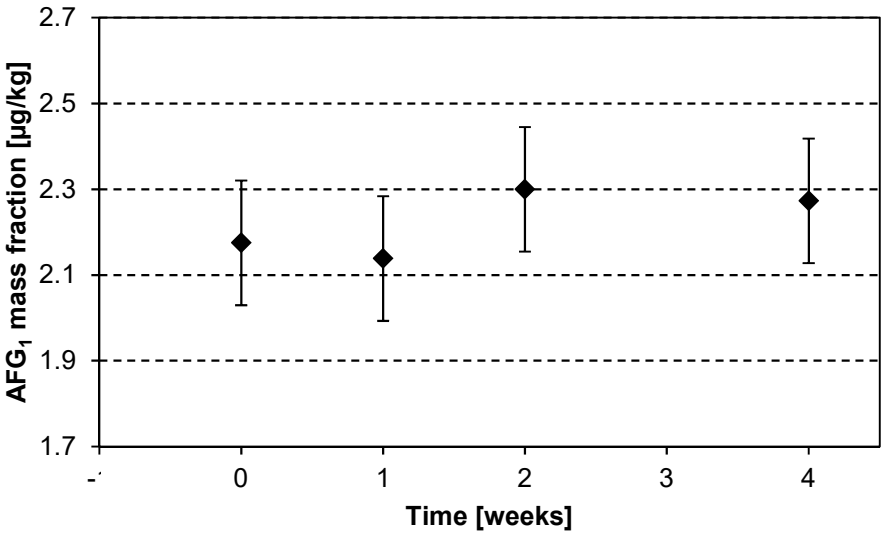

Figure B4: AFG_1 mass fraction means measured after storage at 18 °C at each time-point. Vertical bars represent the 95 % confidence interval of the mean, based on the within-group standard deviation as obtained by single-factor ANOVA.

Annex C: Results of the long-term stability measurements after storage at -20 °C

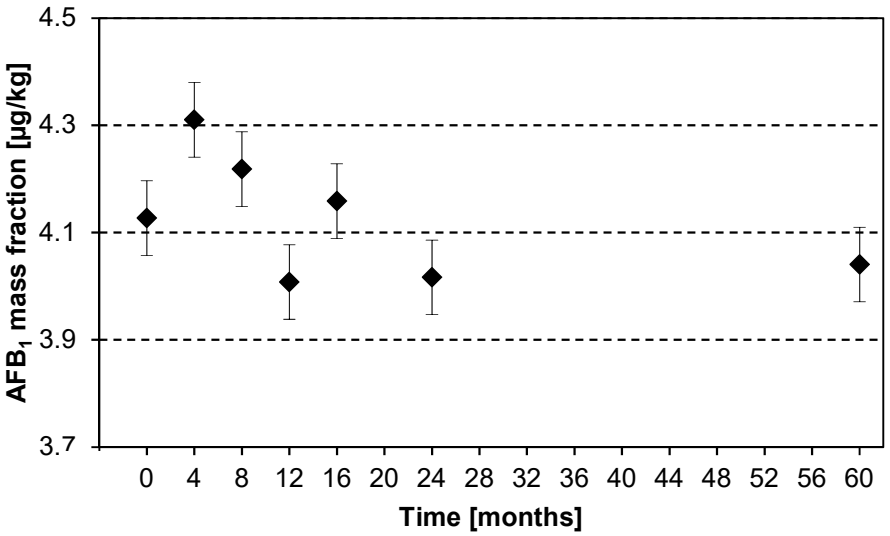

Figure C1: AFB_1 mass fraction means measured at each time-point. Vertical bars represent the 95 % confidence interval of the mean, based on the within-group standard deviation as obtained by single-factor ANOVA.

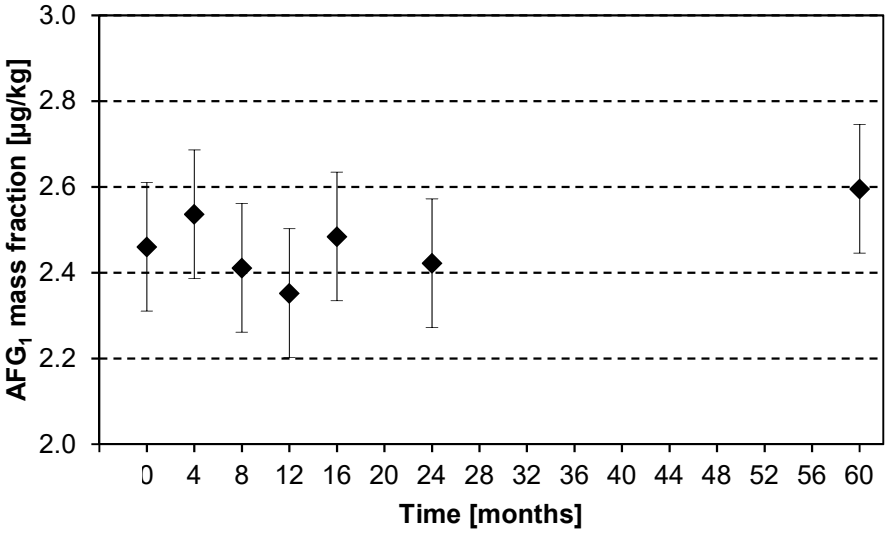

Figure C1: AFG_1 mass fraction means measured at each time-point. Vertical bars represent the 95 % confidence interval of the mean, based on the within-group standard deviation as obtained by single-factor ANOVA.

Annex D: Summary of methods used in the characterisation study

Table D1: Summary of methods used in the characterisation study of aflatoxins (B_1 and G_1 as reported by laboratories)

Laboratory code-method	Extraction			Clean-up method	Analytical column	Stated by the laboratory		
	Solvent	Type	Time [min]			Reproducibility [%]	Mean recovery [%]	LOQ [µg/kg]
L01-HPLC-FLD	MeOH/H_2O 8:2	blending	2	IAC	Waters Spherisorb ODS2, 250 x 4.6 mm, 5 µm	2-8	70-81	0.1
L02-HPLC-FLD	ACN/H_2O 8:2	shaking	45	IAC	GraceSmart RP-C18, 150 x 4.6 mm, 5 µm	7-11	106-124	0.2
L03-HPLC-FLD [1)]	*MeOH/H_2O 8:2*	*blending*	*2*	*IAC*	*Waters C18 Spherisorb ODS2, 250 x 4.6 mm, 5 µm*	*4-8*	*92-99*	*0.4*
L04-HPLC-FLD	MeOH/H_2O 8:2 +NaCl	shaking	30	IAC	Waters Symmetry C18, 250 x 4.6 mm, 5 µm	4	80-83	0.1-0.4
L05-LC-MS/MS [1)]	*Toluene/ACN/AcAc 75:24:1*	*shaking*	*60*	*NO*	*C18 Waters, 100 x 2.1 mm, 1.8 µm*	*15*	*89*	*0.5-1*
L06-HPLC-FLD	MeOH/H_2O 7:3	blending	2	IAC	Zorbax Eclipse XDB-C18, 250 x 4.6 mm, 5 µm	13-17	72-102	0.3-0.6
L07-HPLC-FLD [2)]	*MeOH/H_2O 8:2*	*blending*	*3*	*IAC*	*Intersil ODS2, 150 x 4.6 mm, 5 µm*	*1-2*	*93-99*	*0.2*
L08-HPLC-FLD	MeOH/H_2O 8:2	shaking	45	IAC	Spherisorb ODS1, 150 x 4.0 mm, 5 µm	2-10	80-98	0.1
L09-HPLC-FLD [1)]	*ACN/H_2O 6:4*	*shaking*	*30*	*IAC*	*Phenomenex C18, 150 x 4.6 mm, 5 µm*	*2-10*	*74-100*	*0.1*
L10-HPLC-FLD [3)]	ACN/H_2O 3:2	shaking	45	IAC	Eclipse XDB-C18, 150 x 4.6 mm, 5 µm	13	65-100	0.3-0.4
L11-HPLC-FLD	ACN/H_2O 6:4	blending	1	IAC	C18, 150 x 4.6 mm, 4 µm	9-27	60-90	0.3-0.5
L12-HPLC-FLD	MeOH/H_2O 8:2	blending	3	IAC	Kinetex C18, 100 x 4.6 mm, 2.6 µm	2-10	76-86	0.2
L13-LC-MS/MS [2)]	*ACN/H_2O+NaCl*	*shaking*	*45*	*IAC*	*Restek DB AQ C18, 100 x 2.1 mm, 1.9 µm*	*6-12*	*93-107*	*0.2*
L14-HPLC-FLD	MeOH/H_2O 8:2	shaking	60	IAC	RP-C18 WatersNovaPak, 300 x 3.9 mm, 4 µm	8-10	90-91	1.0
L15-HPLC-FLD	MeOH/H_2O 8:2	blending	2	IAC	Waters C18 Spherisorb ODS2, 250 x 4.6 mm, 5 µm	11-18	59-94	0.2-0.5

[1)] all datasets excluded as sample intake was below 10 g; [2)] all datasets excluded due to technical issue; [3)] AFG_1 dataset excluded due to technical issue;

Annex E: Results of the characterisation measurements

Table E1: Mass fractions of AFB_1 in ERM-BD286 as reported by each individual lab

Laboratory code	replicate 1 [µg/kg]	replicate 2 [µg/kg]	replicate 3 [µg/kg]	replicate 4 [µg/kg]	replicate 5 [mg/kg]	replicate 6 [µg/kg]	mean [µg/kg]	RSD [%]
L01	3.56	4.29	3.75	3.63	3.99	3.56	3.80	7.66
L02	3.71	4.38	4.09	4.02	3.83	4.38	4.07	6.80
L04	3.498	3.672	3.906	3.805	3.843	3.661	3.731	4.00
L06	3.114	3.698	3.769	3.461	3.957	3.740	3.623	8.16
L08	4.11	3.76	4.11	4.08	3.92	3.92	3.98	3.54
L10	3.80	3.54	3.50	2.79	3.70	3.80	3.52	10.80
L11	3.017	3.192	3.557	3.248	3.709	3.391	3.352	7.54
L12	3.4	3.6	3.6	3.7	3.8	3.6	3.6	3.68
L14	3.45167	3.87167	3.5650	3.84056	3.80834	3.70889	3.70769	4.52
L15	3.7	3.5	4.0	4.0	3.6	3.9	3.8	5.65
Results not used for characterisation								
L03	*3.99*	*3.72*	*4.04*	*3.80*	*3.79*	*3.86*	*3.87*	*3.21*
L05	*4.5*	*3.1*	*3.5*	*5.6*	*4.4*	*5.0*	*4.4*	*21.31*
L07	*2.75*	*2.64*	*2.79*	*2.92*	*2.53*	*2.53*	*2.69*	*5.76*
L09	*5.30*	*5.29*	*5.64*	*5.39*	*6.29*	*5.00*	*5.49*	*8.11*
L13	*2.88*	*2.51*	*2.40*	*2.68*	*2.15*	*2.44*	*2.51*	*9.96*

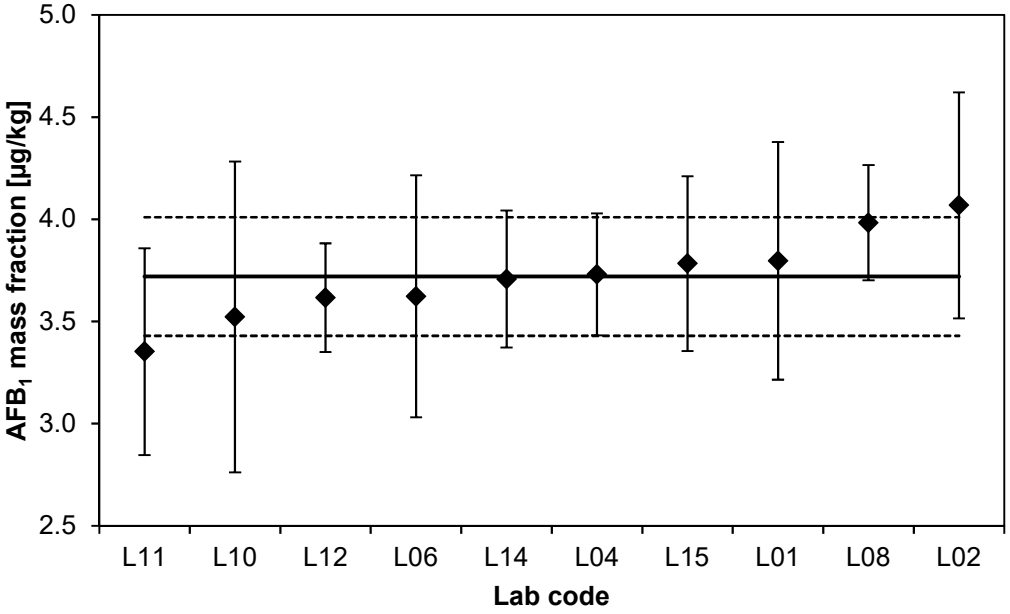

Figure E1: Results of the characterisation study for the mass fraction of AFB_1 in paprika powder (continuous line: certified value; dashed line: expanded uncertainty with k = 2; error bars: expanded measurement uncertainty).

Table E2: Mass fractions of AFG_1 in ERM-BD286 as reported by each individual lab

Laboratory code	replicate 1 [µg/kg]	replicate 2 [µg/kg]	replicate 3 [µg/kg]	replicate 4 [µg/kg]	replicate 5 [mg/kg]	replicate 6 [µg/kg]	mean [µg/kg]	RSD [%]
L01	2.24	2.19	2.13	2.02	2.23	2.10	2.15	3.94
L02	2.09	2.64	2.45	2.13	2.21	2.58	2.35	10.12
L04	2.417	2.469	2.758	2.216	2.400	2.307	2.428	7.62
L06	1.712	2.727	2.581	1.993	2.325	2.540	2.313	16.84
L08	2.45	2.44	2.31	2.84	2.82	2.33	2.53	9.40
L11	1.898	2.134	2.387	2.215	2.578	2.572	2.297	11.60
L12	2.7	2.5	2.6	2.8	2.4	2.6	2.6	5.44
L14	2.4907	2.4022	2.5951	2.5198	2.2588	2.5132	2.4633	4.78
L15	2.7	2.4	3.0	2.4	2.3	2.7	2.6	10.22
Results not used for characterisation								
L03	*2.66*	*2.74*	*2.45*	*2.30*	*2.63*	*2.57*	*2.56*	*6.23*
L05	*3.3*	*2.3*	*2.7*	*2.8*	*2.2*	*2.4*	*2.6*	*15.55*
L07	*1.89*	*1.92*	*1.89*	*2.07*	*2.09*	*1.79*	*1.94*	*5.97*
L09	*2.93*	*2.93*	*2.96*	*2.43*	*2.46*	*2.77*	*2.75*	*8.85*
L10	*3.15*	*3.30*	*3.06*	*2.79*	*4.06*	*3.21*	*3.26*	*13.13*
L13	*1.63*	*1.40*	*1.49*	*1.73*	*1.19*	*1.41*	*1.48*	*12.88*

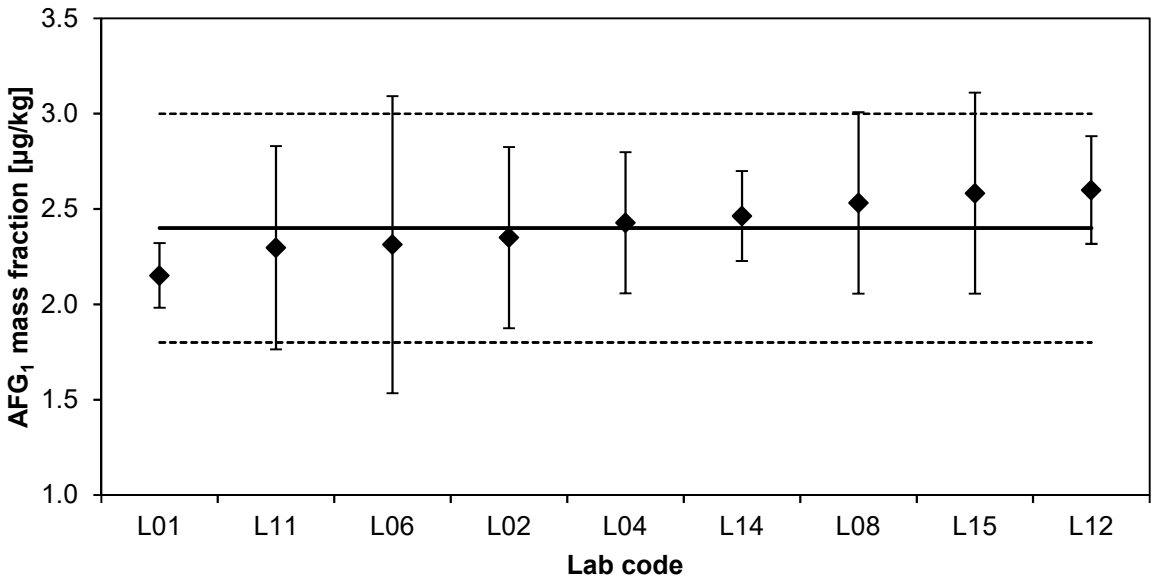

Figure E2: Results of the characterisation study for the mass fraction of AFG_1 in paprika powder (continuous line: certified value; dashed line: expanded uncertainty with k = 2; error bars: expanded measurement uncertainty)

Annex F: Additional material information

Table F1: Mass fractions of AFB_2 in ERM-BD286 as reported by each individual lab

Laboratory code	replicate 1 [µg/kg]	replicate 2 [µg/kg]	replicate 3 [µg/kg]	replicate 4 [µg/kg]	replicate 5 [mg/kg]	replicate 6 [µg/kg]	mean [µg/kg]
L01	0.21	0.24	0.20	0.22	0.22	0.19	0.21
L04	0.178	0.188	0.197	0.218	0.198	0.207	0.198
L08	0.24	0.22	0.23	0.25	0.22	0.23	0.23
Results not used for additional material information							
L02	*0.16*	*0.18*	*0.16*	*0.16*	*0.17*	*0.20*	*0.17*
L03	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	
L05	*0.5*	*0.8*	*0.9*	*0.6*	*0.7*	*0.7*	*0.7*
L06	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	
L07	*<0.2*	*<0.2*	*<0.2*	*<0.2*	*<0.2*	*<0.2*	
L09	*0.24*	*0.21*	*0.21*	*0.24*	*0.23*	*0.23*	*0.23*
L10	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	
L11	*0.126*	*0.137*	*0.143*	*0.154*	*0.174*	*0.16*	*0.149*
L12	*<0.4*	*<0.4*	*<0.4*	*<0.4*	*<0.4*	*<0.4*	
L13	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	
L14	*0.1835*	*0.2341*	*0.2*	*0.1676*	*0.2061*	*0.1835*	*0.1958*
L15	*<0.2*	*<0.2*	*<0.2*	*<0.2*	*<0.2*	*<0.2*	

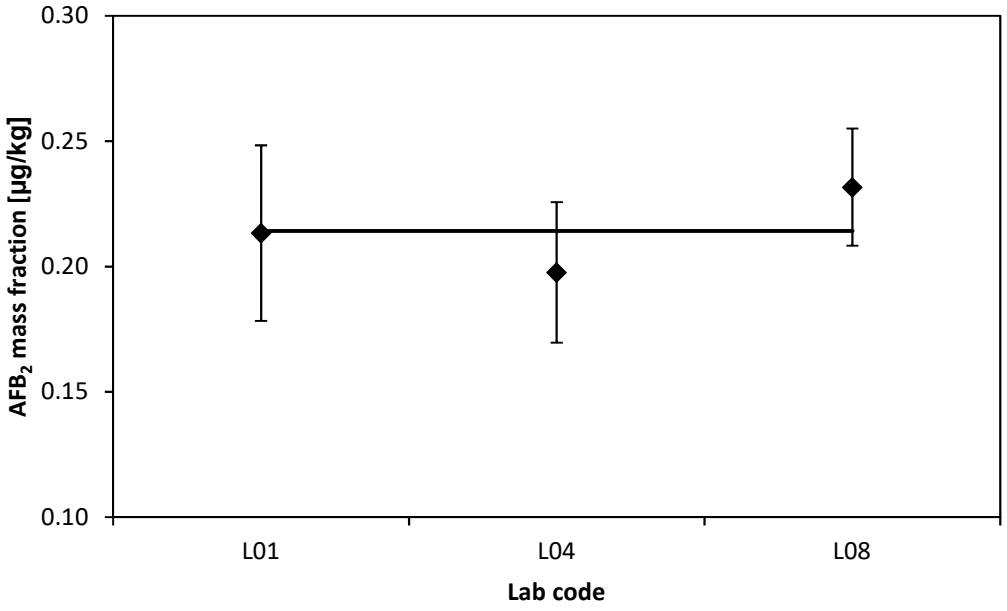

Figure F1: Results for the mass fraction of AFB_2 in paprika powder (continuous line: weighted mean value of means of the three accepted sets of data; error bars: expanded measurement uncertainty).

Table F2: Mass fractions of AFG_2 in ERM-BD286 as reported by each individual lab

Laboratory code	replicate 1 [µg/kg]	replicate 2 [µg/kg]	replicate 3 [µg/kg]	replicate 4 [µg/kg]	replicate 5 [mg/kg]	replicate 6 [µg/kg]	mean [µg/kg]
L04	0.086	0.095	0.079	0.076	0.076	0.071	0.081
L08	0.13	0.12	0.12	0.13	0.12	0.13	0.13
Results not used for additional material information							
L01	*<ND*	*<ND*	*<ND*	*<ND*	*<ND*	*<ND*	
L02	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	
L03	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	
L05	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	
L06	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	
L07	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	
L09	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	
L10	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	
L11	*0*	*0*	*0*	*0*	*0*	*0*	
L12	*<0.4*	*<0.4*	*<0.4*	*<0.4*	*<0.4*	*<0.4*	
L13	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	
L14	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	
L15	*<0.2*	*<0.2*	*<0.2*	*<0.2*	*<0.2*	*<0.2*	

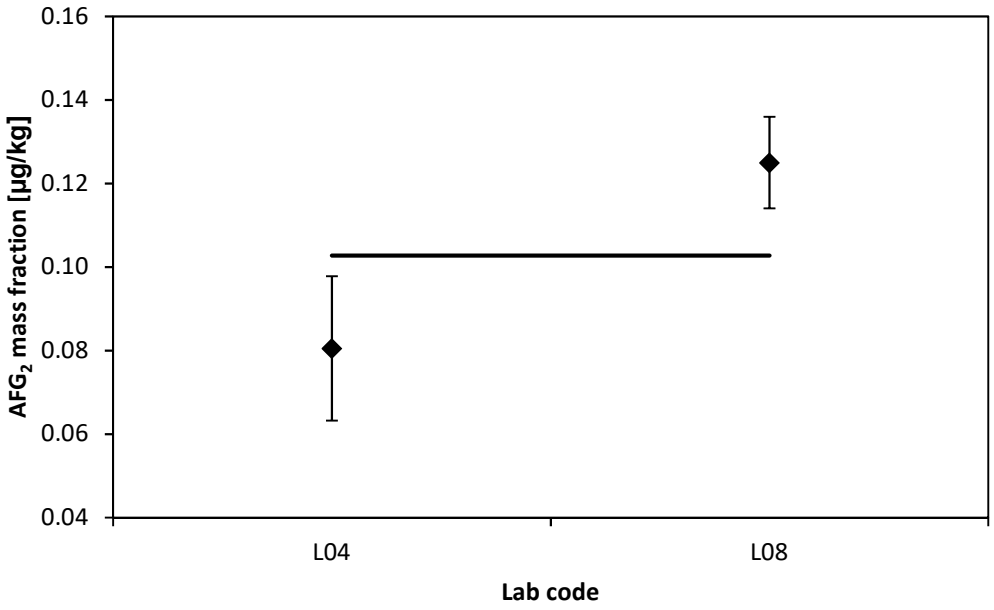

Figure F2: Results for the mass fraction of AFG_2 in paprika powder (continuous line: weighted mean value of means of the two accepted sets of data; error bars: expanded measurement uncertainty).

Table F3: Mass fractions of ochratoxin A in ERM-BD286 as reported by each individual lab

Laboratory code	replicate 1 [µg/kg]	replicate 2 [µg/kg]	replicate 3 [µg/kg]	replicate 4 [µg/kg]	replicate 5 [mg/kg]	replicate 6 [µg/kg]	mean [µg/kg]
L01	15.81	14.12	13.08	13.47	13.29	11.88	13.61
L02	9.84	10.98	10.78	10.21	10.31	10.49	10.44
L03	14.10	12.00	12.91	14.13	12.91	12.28	13.06
L04	10.44	10.28	11.34	9.72	9.45	11.03	10.38
L06	11.717	12.036	10.854	10.870	11.700	10.826	11.334
L10	10.54	10.08	10.22	10.59	10.04	10.80	10.38
L11	12.134	11.916	10.471	13.33	12.629	10.958	11.906
L12	12.138	11.723	10.932	11.257	11.928	11.3	11.547
L14	11.81758	11.58846	11.6824	11.62143	11.14176	11.87858	11.62171
Results not used for additional material information							
L05	*11.718*	*12.036*	*10.854*	*10.871*	*11.700*	*10.826*	*11.334*
L07	*7.12*	*7.78*	*3.89*	*7.36*	*7.09*	*4.64*	*6.31*
L08	*10.56*	*10.24*	*10.37*	*10.58*	*10.71*	*10.60*	*10.51*
L09	*14.47*	*11.83*	*12.59*	*12.36*	*11.19*	*11.68*	*12.35*
L13	*8.29*	*6.47*	*6.94*	*7.58*	*4.92*	*6.75*	*6.83*
L15	*9.6*	*12.3*	*11.0*	*12.2*	*12.0*	*10.8*	*11.3*

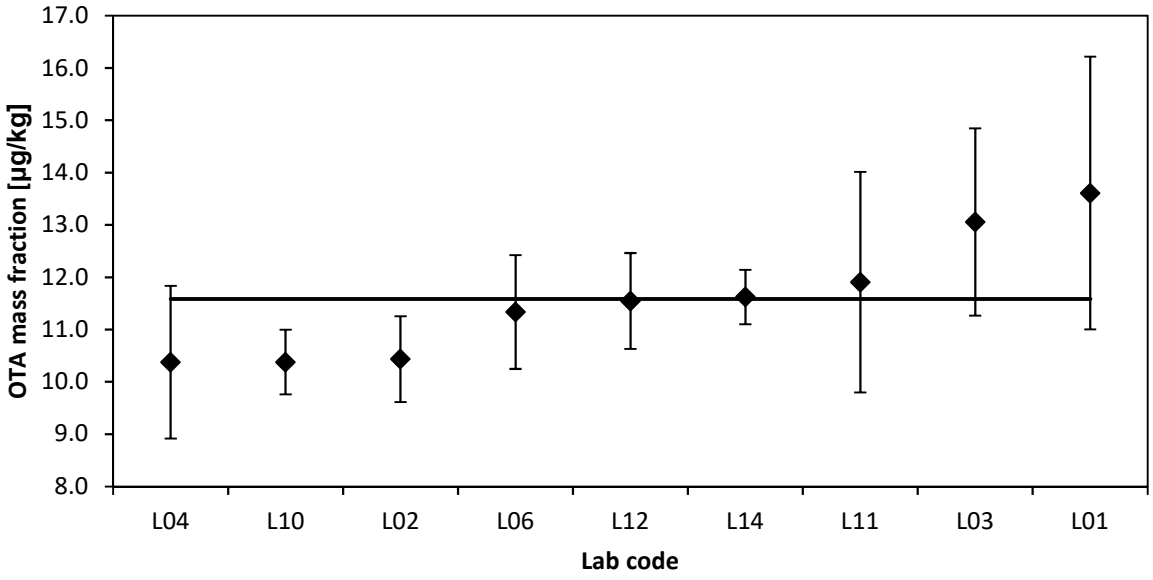

Figure F3: Results for the mass fraction of ochratoxin A in paprika powder (continuous line: weighted mean value of means of the accepted sets of data; error bars: expanded measurement uncertainty).

European Commission

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