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CERTIFICATION REPORT

The additional certification of the mass fractions of deoxynivalenol and nivalenol in maize: ERM®-BC717

Certified Reference Material ERM®-BC717

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The additional certification of the mass fractions of deoxynivalenol and nivalenol in maize: ERM®-BC717

Certified Reference Material ERM®-BC717

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Disclaimer

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 additional certification of the mass fractions of deoxynivalenol (DON) and nivalenol (NIV) in the already existing material ERM-BC717 (maize powder), which was previously certified for the mass fraction of zearalenone (ZON) (see EUR Report 20782 EN [1]).

The between-unit homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [2]. The within-unit homogeneity was quantified to determine the minimum sample intake.

The material was characterised by an intercomparison among laboratories of demonstrated competence and adhering to ISO/IEC 17025. Technically invalid results were removed, but no outlier was eliminated on statistical grounds only.

The certified values were established by HPLC-UV, LC-MS/MS and GC-MS as independent measurement methods (measurements within the scope of accreditation to ISO/IEC 17025:2005 [3]).

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

The material is intended for quality control. As any reference material, it can also be used for control charts or validation studies. The CRM is available in plastic-aluminium sachets containing at least 60 g of maize powder closed under argon atmosphere. The minimum amount of sample to be used is 10 g for DON and NIV.

The CRM was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

The following values were assigned:

Maize ERM-BC717 Certified property	**Certified mass fraction**	
	Certified value [1] [µg/kg]	**Uncertainty [2] [µg/kg]**
Deoxynivalenol (DON)	673	87
Nivalenol (NIV)	53	10

1) Unweighted mean value of the means of 13 accepted sets of data for DON and 6 accepted sets of data for NIV. Each set being obtained in a different laboratory and/or with a different method of determination. The certified value and its uncertainty are traceable to the International System of Units (SI)

2) The certified uncertainty is the expanded uncertainty 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 [5]

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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.

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Glossary

AOAC	Association of Official Analytical Chemists
ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + b \cdot x$
BCR®	One of the trademarks of CRMs owned by the European Commission; formerly Community Bureau of Reference
CEN	European Committee for Standardization
CI	Confidence interval
CRM	Certified reference material
DON	Deoxynivalenol
EC	European Commission
EN	European norm (standard)
ERM®	Trademark of European Reference Materials
FA	Formic acid
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GUM	Guide to the Expression of Uncertainty in Measurement
HPLC	High performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography-diode array detection
HPLC-UV	High-performance liquid chromatography-ultraviolet detection
HW	Half width
IAC	Immunoaffinity column
ILC	Interlaboratory comparison
IPA	Isopropanol
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
JRC	Joint Research Centre
k	Coverage factor
KFT	Karl Fischer titration
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MeCN	Acetonitrile
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
NaCl	Sodium chloride
n.a.	Not applicable
n.c.	Not calculated
n.d.	Not detectable
NIV	Nivalenol
p	Number of datasets used for value assignment
QA	Quality assurance
QC	Quality control
RASFF	Rapid alert system for food and feed
rel	Index denoting relative figures (uncertainties, etc.)
RM	Reference material
RMP	Reference material producer
RM Unit	Reference Materials Unit of the IRMM
RSD	Relative standard deviation
RSD_r	Relative standard deviation calculated from results generated under repeatability conditions
RSD_R	Relative standard deviation calculated from results generated under reproducibility conditions
RSE	Relative standard error (= RSD / $\sqrt{n}$)
r^2	Coefficient of determination of the linear regression
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added as appropriate
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
SE	Standard error
SI	International System of Units
s_{meas}	Standard deviation of measurement data; an additional index "rel" is added as appropriate
SPE	Solid phase extraction
s_{wb}	Within-unit standard deviation; 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
T	Temperature
t	Time
t_i	Time point for each replicate
$t_{\alpha,df}$	Critical t-value for a t-test, with a level of confidence of $1 - \alpha$ and d_f degrees of freedom
t_{sl}	Proposed shelf life
TLC	Thin layer chromatography
u	Standard uncertainty; an additional index "rel" is added as appropriate
U	Expanded uncertainty; an additional index "rel" is added as appropriate

u^*_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability; 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_c	combined standard uncertainty; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; 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_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
UV	Ultraviolet
ZON	Zearalenone
$\overline{x}$	Arithmetic mean
$\overline{x}_{ns}$	Arithmetic mean of all results of normal stock samples
$\overline{x}_{ref}$	Arithmetic mean of results of reference samples
$\overline{y}$	Mean of all results of the homogeneity study
α	Significance level
Δ_{meas}	Absolute difference between mean measured value and the certified value
$\nu_{s,meas}$	Degrees of freedom for the determination of s_{meas}
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}

1 Introduction

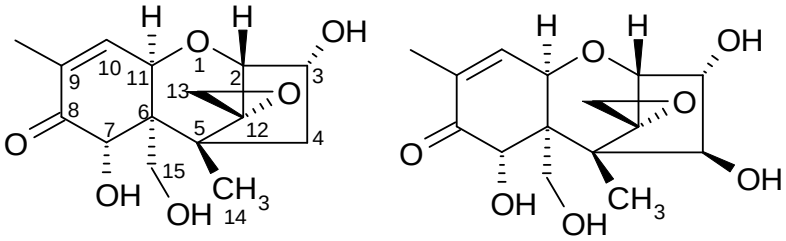
1.1 Background

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 are therefore a potential risk to human and animal health. Contamination of food and feed can appear at two stages: on the field and/or during storage. Moulds infecting food on the field produce different mycotoxins compared to those moulds infecting food during storage [5].

The impact of mycotoxins on agricultural production is massive. The Food and Agriculture Organization of the United Nations (FAO) estimates that 25 % of the world-wide production is affected. For instance, the 2012 annual report of the rapid alert system for food and feed of the European Union (RASFF) also shows that 15 % of all notifications were due to mycotoxin contaminations (about 80 % of them for border rejections which is about 25 % of all border rejections) [6].

Trichothecenes are a very large family of chemically related mycotoxins produced by various species of *Fusarium*, *Myrothecium*, *Trichoderma*, *Trichothecium*, *Cephalosporium*, *Verticimonosporium*, and *Stachybotrys* [7]. They are produced on many different grains like wheat, oat or maize by various *Fusarium* species such as *F. graminearum*, *F. sporotrichioides*, *F. poae* and *F. equiseti*. A variety of *Fusarium* fungi, which are common soil fungi, produce a number of different mycotoxins of the class of trichothecenes (deoxynivalenol (DON) and nivalenol (NIV), T-2 toxin, HT-2 toxin etc.) and some other toxins (zearalenone (ZON) and fumonisins). *Fusarium* fungi are probably the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found on cereals grown in the temperate regions of Europe, America and Asia [7-9].

Trichothecenes belong to tetracyclic sesquiterpene compounds. The most important structural features causing the biological activities of trichothecenes are: the 12,13-epoxy ring, the presence of hydroxyl or acetyl groups at appropriate positions on the trichothecene nucleus and the structure and position of the side-chain [7-9]. Chemical structures and details of DON and NIV are shown in **Figure 1** and **Table 1**.



(a) (b)

Figure 1: Molecular structures of DON (a) and NIV (b)

Table 1: Details on DON and NIV

Trivial name	IUPAC name	CAS number	Chemical formula	Molecular mass [g/mol]
Deoxynivalenol	3*α*,7*α*,15-Trihydroxy-12,13-epoxytrichothec-9-en-8-one	51481-10-8	$C_{15}H_{20}O_6$	296.32
Nivalenol	3*α*,4*β*,7*α*,15-Tetrahydroxy-12,13-epoxytrichothec-9-en-8-one	23282-20-4	$C_{15}H_{20}O_7$	312.32

Trichothecenes can be divided into four types: A, B, C and D. Type A is a group with an oxygen functional group rather than carbonyl at the C_8 position. Examples of this group include the highly toxic HT-2 and T-2 toxins. Type B possess a carbonyl functional group at the C_8 position. The most frequently detected mycotoxins of this group are DON, 3- and 15-acetyldeoxynivalenol and NIV. The less important type C and D trichothecenes are characterised by a second epoxide functional group at $C_{7,8}$ or C_9 (e.g. crotocin) and macrocyclic ring between C_4 and C_{15} (e.g. roridin, verruracin A, statotoxin H) with two ester linkages, respectively [9].

Trichothecenes have a strong impact on the health of animals and humans. The most prominent common effects of trichothecenes at the biochemical and cellular level are [8-14]:

- the strong inhibitory effect on protein synthesis by binding to ribosomes
- the inhibitory effect on RNA and DNA synthesis
- toxic effects on cell membranes

The critical toxicological effects of trichothecenes are the general toxicity and immunotoxicity (leukopenia; reduced antibody production; increased susceptibility to infections) [8-15]. A special feature of DON toxicity is the characteristic induction of vomiting (DON is also called vomitoxin) and feed refusal seen in pigs or delayed gastric emptying and feed refusal observed in rats and mice. The emetic effect is thought to be mediated by affecting serotonergic activity in the central nervous system or via peripheral actions on serotonin receptors [10]. Also growth retardation and reproductive effects are common for all these toxins. These effects are signs of general toxicity in the young / adult animal and in the foetus and can in theory occur via several mechanisms. Although one might hypothesise that common mechanisms could be inhibition of protein synthesis and triggering of apoptosis in different tissues, this has not been shown for all these toxins [10-15].

Some moulds that produce trichothecene mycotoxins, such as *Stachybotrys chartarum*, can grow in damp indoor environments. It has been found that macrocyclic trichothecenes produced by *Stachybotrys chartarum* can become airborne and thus contribute to health problems among building occupants [16].

When it comes to animal and human food, type B trichothecenes (e.g. DON, NIV, 3- and 15-acetyldeoxynivalenol) are of special interest because they are more often found in cereals and cereal derived foodstuffs in Europe than other mycotoxins. For instance, DON is of concern as it is the most prevalent trichothecene in Europe [8-12, 17]. Several surveys demonstrate that the most prevalent trichothecene mycotoxins are the type B-trichothecenes DON, NIV, 3- and 15-acetyldeoxynivalenol and the type A-trichothecenes HT-2 and T-2 toxins, which can mainly be found on maize, oats, barley and wheat. Durum wheat, which is used nearly exclusively for the production of pasta, is especially susceptible to *Fusarium* infection and often shows high levels of DON contamination. In European agricultural commodities, type A-trichothecenes occur less frequently and at lower mean concentrations compared to DON. The simultaneous occurrence of DON with other *Fusarium* mycotoxins, mainly type B-trichothecenes and zearalenone, has been reported for various agricultural commodities [18]. These findings were underpinned by a recent large-scale European study on occurrence of *Fusarium* toxins (trichothecenes, fumonisins and zearalenone) and dietary intake by the European population [19]. In the frame of this study, 12 European countries provided about 35,000 results covering 12 different trichothecenes. The study demonstrated that 57 % and 20 % of the samples were positive for DON and T-2 toxin, respectively. A high frequency of DON was found in maize (89 %) and wheat (61 %). In addition, a world-wide survey of DON and NIV, on 500 agricultural samples, from 19 countries, reports that about 40-50 % of the total samples were positive for these mycotoxins. Average contents of 292 µg/kg for DON and 267 µg/kg for NIV could be monitored [20]. In contrast to the numerous legal regulations for aflatoxins in food and feed at both national and international levels [21], only a few maximum tolerated levels exist for type B-trichothecenes, especially for DON — the most prevalent trichothecene. Maximum levels

for certain *Fusarium* toxins, among them DON and ZON, have been introduced in the European Union since 2005 (Commission Regulation EC/856/2005) [22]. Currently, the maximum permitted level for *Fusarium* mycotoxin – DON in maize ranges from 750 to 1750 µg/kg, depending on the processing stage and the intended use (Commission Regulation EC/1881/2006) [23]. As regards legislation, there are currently no maximum levels for NIV in food and feed. Hence, accurate and reliable methods for the determination of the most common trichothecenes in cereals and cereal-based food and feed are required.

Various analytical methods for the determination of trichothecenes in food and feed have been developed and published over the years [9]. Due to the fact that the trichothecenes, which occur naturally in cereals, are a group of closely related compounds, analytical methods are usually intended to determine more than one single trichothecene. However, analytical methods usually differ in extraction, clean-up and final determination, depending on which group of trichothecenes (type A or B) is to be analysed. They can be divided into the more polar type B-trichothecenes carrying a keto group at the C_8 position and substances of the less polar type A group, which contain no keto functional group at the C_8 position and have generally fewer free hydroxyl groups as mentioned above [9]. Analytical methods routinely used, especially by enforcement laboratories for the implementation of regulations, must be subject to validation and have to be checked against certified reference materials (CRMs), in order to demonstrate that the method provides comparable, accurate and traceable results. This is crucial in consideration of legal actions and trade specifications as well as for monitoring and risk-assessment studies [9, 24].

For trichothecenes, there are currently only two standardised methods published in the Official Methods of Analysis of AOAC [25]. One method (986.17) is based on the principle of thin layer chromatography (TLC) and another method (986.18) was developed and validated on the principle of gas chromatography (GC). However, both methods are not capable of supporting current European regulations due to lack of sensitivity. Currently, there are two European Committee for Standardisation (CEN) standard methods, which satisfy EU regulations: one for DON determination in animal feed (EN 15791:2009) and one for DON determination in cereals and cereal based food (15891:2010) by HPLC-UV with immunoaffinity column clean-up, respectively [26,27]. No standardised method exists for the simultaneous determination of DON, NIV or T-2 and HT-2 toxins in food; however, it is anticipated that a standard method at least in cereals, cereal products and cereal-based foods will be adopted in the forthcoming years, but it may take several years until we see any standard methods for DON, NIV or T-2 and HT-2 in food or feed. Up to now, there is also no common European standardised method available for simultaneous analysis of DON and NIV in maize. Though, a specific CEN Committee (Technical Committee 275, Working Group 5 'Biotoxins') has already established minimum requirements for the performance characteristics that mycotoxin methods should meet, depending on the level of contamination. For DON and NIV, analytical methods intended to be used for concentrations of more than 100 µg/kg, are required to have a recovery in the range of 100 % and relative within-laboratory standard deviation (RSD_r) and relative between-laboratory standard deviation (RSD_R) values of less than 20 % and 40 %, respectively [28].

As mentioned above, various analytical methods for the determination of trichothecenes in food and feed have been developed and published over the years [9]. However, there is a need for method validation in order to guarantee reliable results in terms of comparability and traceability. Validated methods can then serve as confirmatory methods to identify the mycotoxin(s) and its quantity present in a sample. In general, the objective of a method validation is to demonstrate that a defined analytical system (specific matrix, various steps) produces accurate results for a given property. An in-house validation study of a single analytical method usually investigates in detail the applicability of a range of matrices by testing its compliance to various acceptance criteria (e.g. within-lab and within/between-day precision) for spiked and naturally contaminated materials. In addition, its accuracy for a

range of matrices, by comparing it with an already validated method or a CRM, has to be investigated [9, 24].

Any validated method that has been adopted by a standardisation body such as CEN or ISO is recognised as being an official method for the purpose of enforcement or international trade. In addition, it must be pointed out that in Europe each method standardised by CEN legally supersedes equivalent methods at the national level. Standards usually include a detailed analytical protocol, validated performance characteristics and statistical summaries of the interlaboratory studies [9].

The frequent contamination of food and feed with trichothecene mycotoxins like DON and NIV, the high consumption of these products, and the potential risk associated herewith, has led to an increasing public awareness and therefore to the establishment of measures to control trichothecene contamination. The analytical difficulty and the economic importance of controlling trichothecenes in food and feed support the need for validated/standardised methods and therefore a clear need for CRMs to support EU legislation.

1.2 Selection of the material

The Institute for Reference Materials and Measurements (IRMM) in cooperation with a number of expert laboratories in Europe has developed a set of certified reference materials with certified values for *Fusarium* toxins. Since *Fusarium* toxins (like DON, NIV, ZON, etc.) quite often appear in maize simultaneously (which was also found in this case), the IRMM decided to certify additionally the mass fractions of DON (mass fraction of DON corresponds to approximately 40 % of the maximum permitted level in maize [23] and NIV (no regulation on maximum permitted level) in the existing material ERM-BC717, which was previously certified for the mass fraction of ZON (certified mass fraction of ZON corresponds to approximately 80 % of the maximum permitted level) [1, 23].

1.3 Design of the project

The certification of the material was based on its stability, homogeneity and characterisation studies. Expert laboratories were selected based on the following criteria (according to ISO Guide 34 [29]): validated methods were an obligatory requirement for participation; ISO/IEC 17025 accreditation of the laboratory for this method was considered an asset. In addition, laboratories had to prove their measurement capabilities and had to demonstrate previous experience in DON and NIV analysis (e.g. successful participation in recent proficiency tests).

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE (accredited to ISO Guide 34 for production of certified reference materials by BELAC; 268-RM)

2.2 Processing

The material was processed by Spain's National Food and Nutrition Centre (CNA), Madrid, ES (accredited to ISO 17025 by ENAC; 178/LE 397) (see details in EUR Report 20782 EN [1])

2.3 Homogeneity study

Analyses for homogeneity studies were performed by the Dutch Organization for Applied Scientific Research (TNO), Zeist, NL (accredited to ISO 17025 by RvA; L027)

2.4 Stability study

Analyses for short-term stability studies were performed by the Dutch Organization for Applied Scientific Research (TNO), Zeist, NL (accredited to ISO 17025 by RvA; L027)

Analyses for long-term stability studies were performed by the Eurofins WEJ Contaminants GmbH, Hamburg, DE (accredited to ISO 17025 by DAkkS; D-PL-14602-01-00)

2.5 Characterisation

Central Laboratory for Chemical testing and Control (CLCTC), Sofia, BG (accredited to ISO 17025 by BAS; 908)

Federal Agency for the Safety of the Food Chain (FAVV), Federal Laboratory for Food Safety (FLVVM), Tervuren, BE (accredited to ISO 17025 by BELAC; 014-Test)

General State Laboratory, Directorate of Environment, Athens, GR (accredited to ISO 17025 by ESYD; 72-2)

General State Laboratory, E'Chemical Service of Athens, Athens, GR (accredited to ISO 17025 by ESYD; 142-3)

National Food and Veterinary Risk Assessment Institute, Vilnius, LT (accredited to ISO 17025 by DAkkS; D-PL-14028-01-00)

National Health Laboratory, Division of Food Control, Luxembourg, LU (accredited to ISO 17025 by OLAS; 1/002)

National Veterinary Institute (SVA), Uppsala, SE (accredited to ISO 17025 by SWEDAC; 1553)

Public Analysts Laboratory, Dublin, IE (accredited to ISO 17025 by INAB; 099T)

State Veterinary and Food Institute Kosice, Kosice, SK (accredited to ISO 17025 by SNAS; S-239)

The Food and Environment Research Agency (FERA), York, UK (accredited to ISO 17025 by UKAS; 050)

University of Ljubljana, National Veterinary Institute, Ljubljana, SI (accredited to ISO 17025 by SA; LP-021)

Veterinary and Agrochemical Research Centre (CODA-CERVA), Tervuren, BE (accredited to ISO 17025 by BELAC; 172-Test)

3 Material processing and process control

3.1 Origin of the starting material

The existing material (ERM-BC717) certified for the mass fraction of *Fusarium* mycotoxin – zearalenone (ZON) was used for the additional certification process (see EUR Report 20782 EN [1] for details on starting material).

3.2 Processing

Since, the existing material (ERM-BC717) already packed in aluminium-laminated plastic sachets (approx. 60 g each), was used for the additional certification, no further processing was required. Processing of ERM-BC717 is described in EUR Report 20782 EN [1].

3.3 Process control

The processing control of ERM-BC717 is described in EUR Report 20782 EN [1].

4 Homogeneity

A key requirement for any reference material is the equivalence between the various units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value. In contrast to that it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 requires RM producers to quantify the between unit variation [29]. 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.

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 uncertainty.

The study was performed using 20 units, which were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch (2400 units) was divided into 20 groups (with a similar number of 120 units) and one unit was selected randomly from each group. Three independent samples were taken from each selected unit and analysed by LC-MS/MS (sample intake: 10 g). The measurements were performed under intermediate precision conditions (10 units with 3 replicates each per day over 2 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 in **Table A1 (Annex A)**.

Regression analyses (within each of the two days) as well as F- and t-tests (both at 95 % confidence level) were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trends in the filling sequence were visible for both – DON and NIV. A significant trend (at 95 % and 99 % confidence level) in the analytical sequence was visible for DON (day 1 and day 2) and NIV (day 1), pointing at a signal drift in the analytical system.

The correction of biases, even if they are statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value [30]. Correction of trends is therefore expected to improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. As the analytical sequence and the unit numbers were not correlated, trends significant on at least a 95 % confidence level were corrected as shown below:

$$x_{i\_corr} = x_i - b \cdot i \qquad \textbf{Equation 1}$$

x_{i_corr} corrected result

x_i measured result

b slope of linear regression line

i position of result in analytical sequence

Analytical sequence trends were corrected for each day separately. In order to see if there is still a significant difference in variances and day means, corrected results were tested with F- and t-tests (both at 95 % confidence level). To remove day-to-day effects all corrected data were normalised as shown below:

$$x_{i\_norm} = \frac{x_{i\_corr}}{\bar{x}_{day}} \cdot \bar{\bar{x}}$$ **Equation 2**

x_{i_norm} normalised result

$\bar{x}_{day}$ day mean

$\bar{\bar{x}}$ mean of all results

The trend-corrected and normalised datasets (DON and NIV) were tested for consistency using the Grubbs outlier tests on a confidence level of 99 % on the individual results and the unit means. No outliers could be detected.

Quantification of between-unit inhomogeneity was accomplished by analysis of variance (ANOVA). ANOVA can separate the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the method repeatability, if the individual samples are representative for the whole unit.

Evaluation by ANOVA requires unit means, which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. Distribution of the unit means was visually tested 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. The results of all statistical evaluations are given in **Table 2**.

Table 2: Results of the statistical evaluation of the homogeneity studies at 95 % and 99 % confidence levels

Analyte	Trends (before correction)		Outliers		Distribution	
	Analytical sequence	Filling sequence	Individual results	Unit means	Individual results	Unit means
DON	yes [1)]	no	none	none	unimodal	unimodal
NIV	yes [1)]	no	none	none	unimodal	unimodal

1) on both, 95 % and 99 % confidence levels

One has to bear in mind that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and 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.* [30]. u^*_{bb} is comparable to the limit of detection 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:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$ **Equation 3**

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$$ **Equation 4**

$$u^{*}_{\mathrm{bb,rel}} = \frac{\sqrt{\frac{\mathrm{MS}_{\mathrm{within}}}{n}}\sqrt[4]{\frac{2}{v_{\mathrm{MSwithin}}}}}{\bar{y}}$$ **Equation 5**

$\mathrm{MS}_{\mathrm{within}}$	mean square within a unit from an ANOVA
$\mathrm{MS}_{\mathrm{between}}$	mean squares between-unit from an ANOVA
$\bar{y}$	mean of all results of the homogeneity study
n	mean number of replicates per unit
v_{MSwithin}	degrees of freedom of $\mathrm{MS}_{\mathrm{within}}$

The results of the evaluation of the between-unit variation are summarised in **Table 3**. The resulting values from the above equations were converted into relative uncertainties. In both cases, the uncertainty contribution for homogeneity was determined by the method repeatability.

Table 3: Results of the homogeneity study

Analyte	$s_{\mathrm{wb,rel}}$ [%]	$s_{\mathrm{bb,rel}}$ [%]	$u^{*}_{\mathrm{bb,rel}}$ [%]	$u_{\mathrm{bb,rel}}$ [%]
DON	4.74	n.c. [1)]	1.29	1.29 [2)]
NIV	10.5	n.c. [1)]	2.86	2.86 [2)]

1) n.c.: cannot be calculated as $\mathrm{MS}_{\mathrm{between}} < \mathrm{MS}_{\mathrm{within}}$

2) higher value of $u^{*}_{\mathrm{bb,rel}}$ or $s_{\mathrm{bb,rel}}$ was taken as contribution of heterogeneity

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore, the between-unit standard deviation 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.

4.2 Within-unit homogeneity and minimum sample intake

The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus can be used in an analysis. Sample sizes equal or above the minimum sample intake guarantee the certified value within its stated uncertainty.

All measurements contributing to establish certified values for this reference material (homogeneity, stability, and characterisation) used 10 g sample intake. Therefore, this sample intake is considered the minimum sample intake (no measurement results are available which would confirm validity of the uncertainty values for sample intakes lower than 10 g).

5 Stability

Time, temperature, radiation and water content were regarded as the most relevant influences on stability of the materials. The influence of ultraviolet or visible radiation was minimised by the choice of the containment, which eliminates most of the incoming light. In addition, materials are stored and dispatched in the dark, thus eliminating practically the possibility of degradation by radiation. The water content was adjusted to an optimum during processing. Additionally, the material was sterilised by γ-irradiation treatment to eliminate microbial growth. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish conditions for storage (long-term stability) as well as conditions for dispatch to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C could be reached and stability under these conditions must be demonstrated if transport at ambient temperature will be applied.

The stability studies were carried out using an isochronous design [31]. In that approach, samples are stored for a certain time at different temperature conditions. Afterwards, the samples are moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples are analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

5.1 Short-term stability study

For the short-term stability study, samples were stored at 4 °C, 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -20 °C. Two units per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured. The laboratory employed their in-house method based on LC-MS/MS. The measurements were performed under intermediate precision conditions (8 units with three replicates per day over 2 days), and in a randomised sequence to be able to separate a potential analytical drift from a trend over storage time.

Regression analyses were performed to evaluate potential trends in the analytical sequence A significant trend (at 95 % and 99 % confidence level) in the analytical sequence was visible for DON (day 1) and NIV (day 2), pointing at a signal drift in the analytical system. Therefore, all obtained results were corrected for analytical trend using **Equation 1** (Section 4.1) and then all corrected results were normalised using **Equation 2** (Section 4.1) to remove day-to-day effects. Normalised results were screened for outliers using the Grubbs test. No outliers for NIV at all tested temperatures were detected. One outlier was detected for DON at 18 °C by single Grubbs test at 99 % level of confidence. For DON no technical reason could be found for the statistical outlier, therefore it was retained for the estimation of u_{sts}.

Furthermore, the data were evaluated against storage time and regression lines of mass fraction versus time were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to shipping conditions). A significant slope at 95 % and 99 % levels of confidence was detected for DON at 60 °C in the short-term study.

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 4**.

Table 4: Results of the short-term stability tests

Analyte	Number of individual outlying results			Significance of trend on 95 % and 99 % confidence levels		
	4 °C	18 °C	60 °C	4 °C	18 °C	60 °C
DON	none	one [1)]	none	no	no	yes [2)]
NIV	none	none	none	no	no	no

1) at 99 % confidence level

2) at 95 % and 99 % confidence levels

As degradation could be observed for DON at 60 °C in the short-term study, it was concluded that special precautions regarding temperature control during shipment are necessary. Shipment with cooling elements is recommended.

5.2 Long-term stability study

For the long-term stability study, samples were stored at 4 °C for 0, 60, 84 and 103 months. The reference temperature was set to -20 °C. Two samples per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured. The laboratory employed their in-house method based on LC-MS/MS. The measurements were performed over 3 days (8 units with three replicates per day). A replicate of each unit was analysed in duplicate (duplicate injection) on each day. The measurements were performed in a random sequence to be able to separate any potential analytical drift from a trend over storage time.

No analytical drift was observed at 95 % and 99 % confidence levels for each day. Values from each day were compared to evaluate if a day drift was present. t-test was performed and at 95 % confidence level, no difference between the day mean values was observed. The results were screened for outliers using the Grubbs test. No outliers for both measurands were detected.

Furthermore, data were plotted against storage time and linear regression lines of mass fraction versus time were calculated. The slope of the regression lines was tested for statistical significance (loss/increase due to storage conditions). The results of the long-term stability measurements are shown in **Annex C**. The results of the statistical evaluation of the long-term stability study are summarised in **Table 5**.

Table 5: Results of the long-term stability tests

Analyte	Number of individual outlying results	Significance of trend at 95 % and 99 % confidence levels
	4 °C	4 °C
DON	none	no
NIV	none	no

No outliers were observed and no statistically significant trends at 95 % and 99 % confidence levels were detected. The material can therefore be stored at 4 °C ± 3 °C.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can rule out degradation of materials completely, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method repeatability, i.e. to estimate the uncertainty of stability. This means, even under ideal conditions, the outcome of a stability study can only be "degradation is 0 ± x % per time".

Uncertainties of stability during dispatch and storage were estimated as described in [32] for each measurand. For this approach, the uncertainty of the linear regression line with a slope

of zero is calculated. The uncertainty contributions u_{sts} and u_{lts} are calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot t_{tt}$$ **Equation 6**

$$u_{lts,rel} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot t_{sl}$$ **Equation 7**

RSD relative standard deviation of all results of the stability study

x_i result at time point *i*

$\bar{x}$ mean results for all time points

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

t_{sl} chosen shelf life (160 months at 4 °C)

The following uncertainties were estimated:

$u_{sts,rel}$ uncertainty of degradation during dispatch, estimated from the 18 °C study describing the possible change during dispatch at 18 °C for 1 week

$u_{lts,rel}$ uncertainty of stability during storage, estimated from the 4 °C study describing the possible degradation during storage at 4 °C for 160 months

The results of these evaluations are summarised in **Table 6**.

Table 6: Estimated uncertainties of stability during dispatch and storage ($u_{sts,rel}$ and $u_{lts,rel}$)

Analyte	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
DON	0.98	4.45
NIV	1.82	4.84

After the certification campaign, the material will be subjected to IRMM's regular stability monitoring programme to control its further stability.

6 Characterisation

The material characterisation is the process of determining the property value(s) of a reference material. It was based on an intercomparison of expert laboratories, i.e. the mass fractions of both mycotoxins in the material were determined in different laboratories that applied different measurement procedures to demonstrate the absence of a measurement bias. This approach aims at randomisation of laboratory bias, which reduces the combined uncertainty.

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 mycotoxin measurements in relevant matrices by submitting results for intercomparison exercises or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 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 was provided with the following samples:

- 2 units of ERM-BC717
- 2 ampoules of the common calibrant deoxynivalenol in acetonitrile, IRMM-315
- 2 ampoules of the common calibrant nivalenol in acetonitrile, IRMM-316

External calibrations were based on dilutions of the provided common calibrants. A new calibration had to be performed on each day.

Each laboratory was requested to provide 6 independent results per unit for each measurand. In addition, each laboratory was asked to provide:

- raw results
- recovery factor
- results corrected for recovery (indicating how correction was performed)
- relative standard deviations
- limits of quantification for each measurand
- a few representative chromatograms.

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 two provided units of ERM-BC717 had to be analysed once, but with duplicate injections (the mean of 2 injections was used in evaluation process). Only results corrected for recovery (provided by each laboratory) were used for evaluation.

6.3 Methods used

Four extraction methods with three different quantification steps were used to characterise the material. The combination of results from methods based on completely different principles mitigates undetected method bias.

All methods used during the certification study are summarised in **Annex D**. The laboratory code is a random code 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. L1) and abbreviation of the measurement method used (e.g. HPLC-UV).

6.4 Evaluation of results

The characterisation campaign resulted in 13 accepted datasets for DON (15 laboratories invited) and 6 accepted datasets for NIV (8 laboratories invited). All accepted individual results of the participants, grouped per measurand are displayed in tabular and graphical forms in **Annex E**.

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
- compliance with the analysis protocol:
  - sample preparations and measurements performed on at least 3 days;
  - duplicate injections;
  - sample intake of 10 g;
  - LOQ is below or equal to 200 µg/kg for DON and 30 µg/kg for NIV

Since one of the participating laboratories failed to follow pre-defined analytical specifications, based on the criteria above, one data set for DON and one for NIV were rejected as technically not valid. Another laboratory could not provide any results for DON and NIV due to technical problems.

6.4.2 Statistical evaluation

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

The laboratory means followed normal distributions. The datasets were therefore consistent and the mean of laboratory means is a good estimate of the true value. Standard deviations between laboratories were considerably larger than the standard deviation within laboratories, showing that confidence intervals of replicate measurements are unsuitable as estimate of measurement uncertainty. The uncertainty related to the characterisation is estimated as a combination of uncertainties, which are exclusively laboratory-dependent $u(I)$ (estimated as the standard error of the mean of laboratory means) and uncertainties that are common to all laboratories participating in the certification $u(II)$ (is the uncertainty of the common calibrant taken from the certificate of the common calibrant) (**equation 8**).

$$u_{\text{char}} = \sqrt{u(I)^2 + u(II)^2}$$ **Equation 8**

The results of these evaluations are shown in **Table 7**.

Table 7: Statistical evaluation of the technically accepted datasets for ERM-BC717; 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]	u_{char} [µg/kg]	$u_{\text{char,rel}}$ [%]
DON	13	none	yes	yes	672.52	90.40	89.44	30.07	29.82	4.43
NIV	6	none	none	yes	53.42	8.97	8.81	4.16	3.86	7.23

The statistical evaluation flagged laboratory L11 as outlying variance for DON at 99 % confidence level. No outliers according to other tests were detected. This merely reflects the fact that different methods in the hands of different analysts have different intrinsic variability. As all measurement methods were found technically sound, all results were retained. It should be borne in mind that the methods used in the characterisation are methods routinely applied for measuring DON and NIV in maize. The agreement of results from different methods demonstrates that the processing did not affect any properties relevant for these methods and that ERM-BC717 behaves like a sample as met in routine testing.

7 Value Assignment

Certified values were assigned to DON and NIV. Certified values are values that fulfil the highest standards of accuracy. Procedures at IRMM 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.

The unweighted mean of the means of the accepted datasets as shown in **Table 8** was assigned as certified value for each measurand.

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

$$U_{CRM,rel} = k \cdot \sqrt{u_{char,rel}^2 + u_{bb,rel}^2 + u_{sts,rel}^2 + u_{lts,rel}^2} \qquad \textbf{Equation 9}$$

$u_{char,rel}$ was estimated as described in Section 6

$u_{bb,rel}$ was estimated as described in Section 4.1

$u_{sts,rel}$ was estimated as described in Section 5.3

$u_{lts,rel}$ was 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 8**.

Table 8: Certified values and their uncertainties for ERM-BC717

Analyte	Certified value [µg/kg]	$u_{char,rel}$ [%]	$u_{bb,rel}$ [%]	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]	$U_{CRM,rel}$ [%]	U_{CRM} [µg/kg] 1)
DON	673	4.4	1.3	1.0	4.5	13.0	87
NIV	53	7.3	2.9	1.9	4.9	19.0	10

1) Expanded rounded uncertainty ($k = 2$)

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

DON and NIV in maize powder are method-defined measurands and can only be obtained by following the procedures used by laboratories participated in this study (e.g. extraction, clean-up and detection is the same or similar to the ones used by the participants of this study). The assigned values are therefore operationally defined by method.

Quantity value

Only validated methods were used for the determination of the assigned values. The values assigned to common calibrants is traceable to the SI, as described in this report, and all relevant input parameters were calibrated. The individual results are therefore traceable to the SI, as it is also confirmed by the agreement among the technically accepted datasets. As the assigned values are combinations of agreeing results individually traceable to the SI, the assigned quantity values themselves are traceable to the SI as well.

8.2 Commutability

8.2.1 Background

Many measurement procedures include one or more steps, which are selecting specific (or specific groups) of measurands from the sample for the subsequent steps of the whole measurement process. Often the complete identity of these 'intermediate measurands' is not fully known or taken into account. Therefore, it is difficult to mimic all the 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 expressing this concept. For instance, the CSLI Guideline C-53A [33] 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 a reference material and for representative samples of the type intended to be measured."*

The commutability of a CRM defines its fitness for use and, thus, is a crucial characteristic in case of the application of different measurement methods. When commutability of a CRM is not established in such cases, 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. For instance, CRMs intended to be used to establish or verify metrological traceability of routine measurement procedures must be commutable for the routine measurement procedures for which they are intended to be used.

8.2.2 The present material

ERM-BC717 was produced from a naturally grown maize material by milling and mixing. The analytical behaviour is assumed to be the same as for a routine sample of maize powder. One has to bear in mind that the extractability of DON and NIV from this certified reference material may be different to the extractability from a sample as milled in the user's laboratory. For samples other than maize powder the commutability has to be re-assessed.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

DON and NIV are toxic substances, therefore they should be also handled with extreme caution. The sachets should be used only by personnel who are trained in the safe handling and use of the contents.

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

9.2 Storage conditions

The material shall be stored at +4 ± 3 °C in the dark. Care shall be taken to avoid change of the moisture content once the units are open, as the material might be hygroscopic. The user is reminded to close sachets immediately after taking a sub-sample.

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

9.3 Preparation and use of the material

Units should be allowed to warm to ambient temperature before opening to avoid water condensation. The contents should be thoroughly mixed before sub-samples are taken. The maize should be weighted out immediately after opening the sachets and the concentrations of DON and NIV calculated based on this weight.

9.4 Minimum sample intakes

The minimum sample intakes representative for DON and NIV is 10 g.

9.5 Use of the certified values

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 also be used for 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 [34]).

For assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is described here in brief:

- Calculate the absolute difference between the 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 $\Delta_{meas} \leq U_\Delta$ no significant difference between the measurement result and the certified value exists at a confidence level of about 95 %

Use in quality control charts

The materials can be used for quality control charts. Different CRM units will give the same result as inhomogeneity was included in the uncertainties of the certified values.

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Annexes

Annex A: Results of the homogeneity measurements

Table A1: Homogeneity study results (not corrected for recovery) for DON and NIV corrected for analytical trends, normalised and sorted by unit number

Result no.	Unit no.	Replicate no.	DON [µg/kg]	NIV [µg/kg]	Result no.	Unit no.	Replicate no.	DON [µg/kg]	NIV [µg/kg]
1	113	1	633.0	39.0	31	200	1	608.0	44.9
20	113	2	675.3	50.4	50	200	2	673.9	48.9
21	113	3	670.6	44.0	51	200	3	649.7	50.3
2	436	1	601.9	39.7	32	660	1	569.7	46.8
19	436	2	634.3	47.6	49	660	2	620.7	46.5
23	436	3	662.5	39.3	53	660	3	564.6	41.7
3	714	1	630.6	44.7	33	771	1	576.2	36.1
18	714	2	586.5	45.1	48	771	2	594.2	43.9
25	714	3	615.3	45.4	55	771	3	625.7	45.3
4	801	1	630.6	41.9	34	856	1	587.0	53.1
17	801	2	600.8	45.8	47	856	2	605.5	46.7
27	801	3	592.2	34.9	57	856	3	581.2	46.0
5	940	1	593.7	45.4	35	1171	1	619.6	49.7
16	940	2	572.1	46.7	46	1171	2	637.3	40.8
29	940	3	604.3	41.7	59	1171	3	633.9	42.8
6	1175	1	653.5	41.2	36	1250	1	684.5	38.0
15	1175	2	635.3	43.3	45	1250	2	656.2	35.4
30	1175	3	644.5	42.4	60	1250	3	619.0	43.8
7	1260	1	622.5	46.7	37	1921	1	649.6	51.6
14	1260	2	618.7	48.3	44	1921	2	609.7	40.6
28	1260	3	637.4	34.8	58	1921	3	608.4	46.8
8	2042	1	638.5	36.6	38	2077	1	627.7	38.0
13	2042	2	636.6	53.8	43	2077	2	671.1	38.5
26	2042	3	592.4	43.6	56	2077	3	623.0	43.0
9	2095	1	660.5	41.4	39	2180	1	641.9	42.4
12	2095	2	619.0	38.4	42	2180	2	657.3	37.6
24	2095	3	620.0	47.8	54	2180	3	612.0	41.0
10	2224	1	593.0	48.2	40	2302	1	631.4	46.0
11	2224	2	605.8	42.9	41	2302	2	627.7	44.9
22	2224	3	624.6	50.1	52	2302	3	639.1	40.0

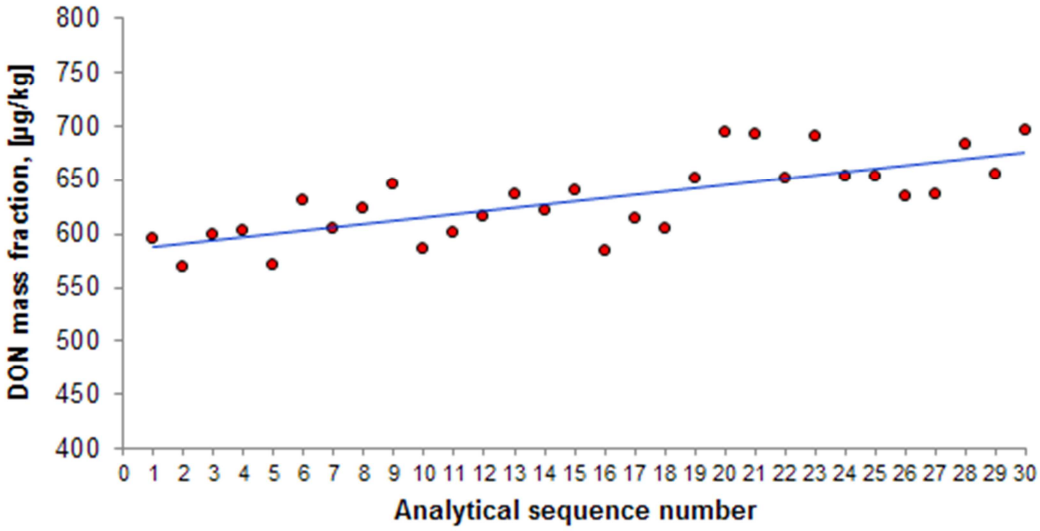

Figure A1: Analytical trend (DON; day 1; non-normalised data); 10 samples (out of 20 randomly selected sachets) with 3 sub-samples (extraction replicates) were measured on the first day of analysis (N = 10, n = 3).

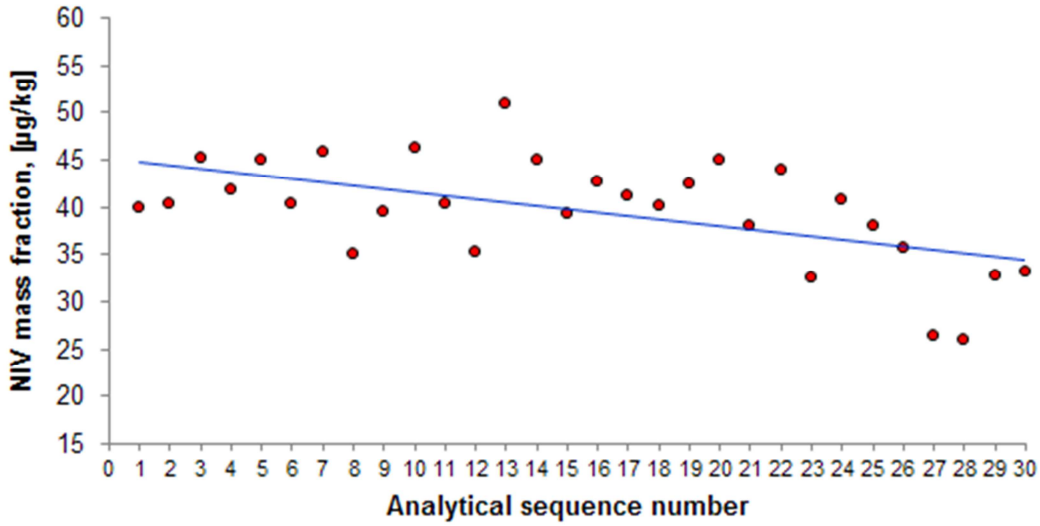

Figure A2: Analytical trend (NIV; day 1; non-normalised data); 10 samples (out of 20 randomly selected sachets) with 3 sub-samples (extraction replicates) were measured on the first day of analysis (N = 10, n = 3).

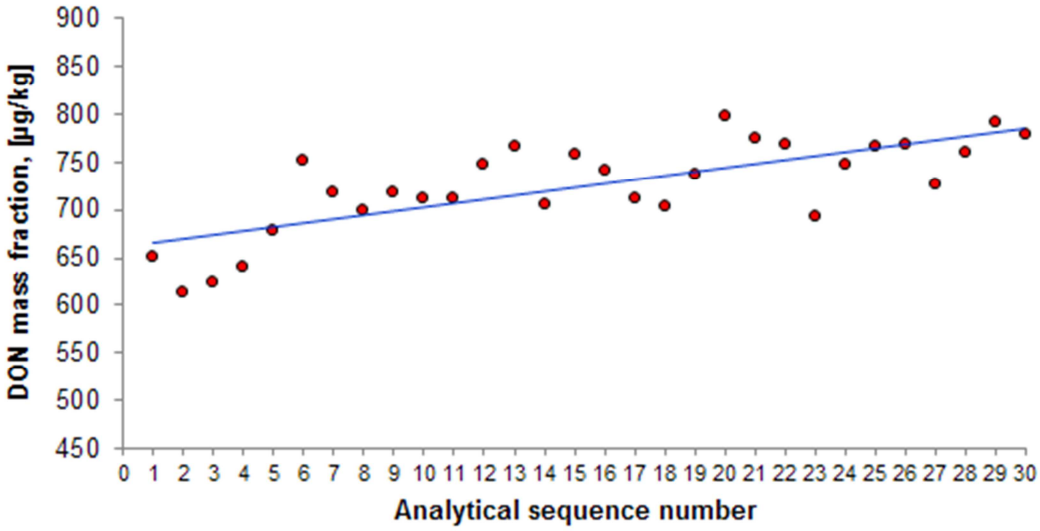

Figure A3: Analytical trend (DON; day 2; non-normalised data); 10 samples (out of 20 randomly selected sachets) with 3 sub-samples (extraction replicates) were measured on the second day of analysis (N = 10, n = 3).

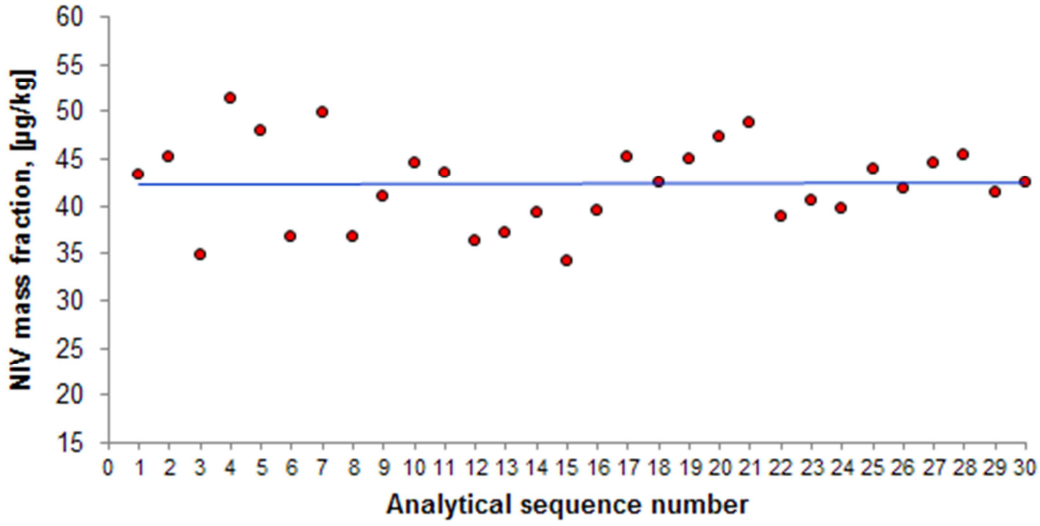

Figure A4: Analytical trend absence (NIV; day 2; non-normalised data); 10 samples (out of 20 randomly selected sachets) with 3 sub-samples (extraction replicates) were measured on the second day of analysis (N = 10, n = 3).

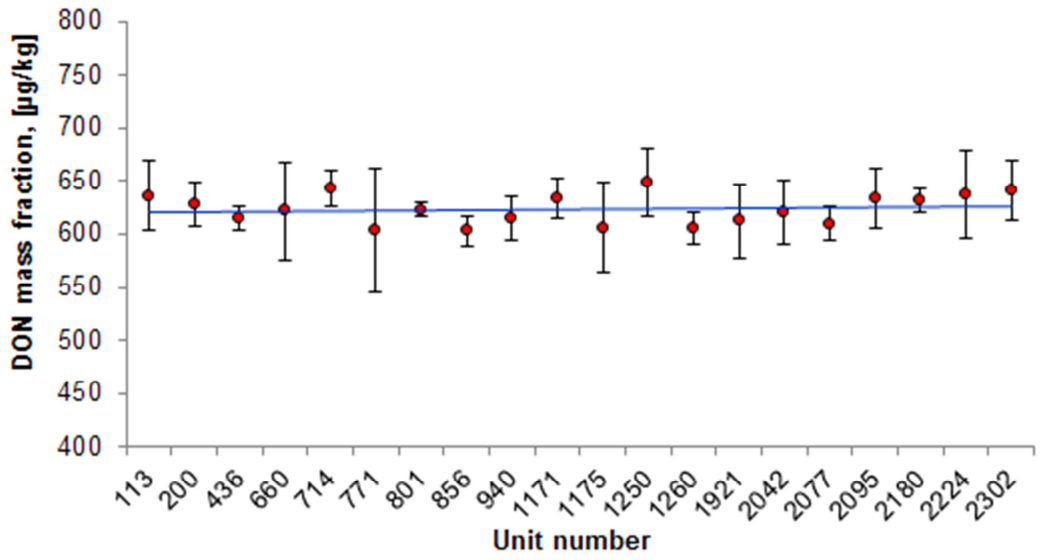

Figure A5: Filling trend absence (DON; normalised data); 3 samples (extraction replicates) were measured from each of 20 randomly selected sachets (N = 20, n = 3); error bars represents ± s of 3 replicates

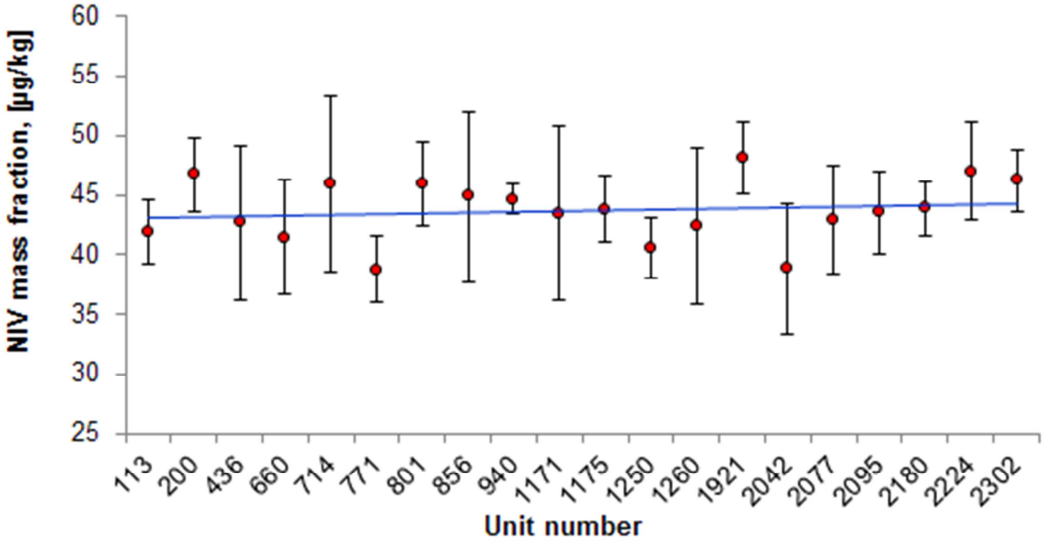

Figure A6: Filling trend absence (NIV; normalised data); 3 samples (extraction replicates) were measured from each of 20 randomly selected sachets (N = 20, n = 3); error bars represents ± s of 3 replicates

Annex B: Results of short-term stability measurements

Table B1: Short-term stability studies results (not corrected for recovery) for DON and NIV corrected for analytical trends, normalised and sorted by testing temperature and time

Time points [weeks]	Testing temperatures / mass fraction [µg/kg]					
	DON			NIV		
	4 °C	18 °C	60 °C	4 °C	18 °C	60 °C
0	727.3	727.3	727.3	74.3	74.3	74.3
0	576.8	576.8	576.8	53.5	53.5	53.5
0	599.1	599.1	599.1	57.0	57.0	57.0
0	565.6	565.6	565.6	58.9	58.9	58.9
0	619.7	619.7	619.7	58.6	58.6	58.6
0	634.7	634.7	634.7	54.6	54.6	54.6
1	680.5	539.0	508.2	57.4	45.4	46.7
1	562.7	547.0	509.9	53.0	50.5	48.6
1	633.7	595.2	541.7	63.5	54.0	60.3
1	609.7	615.9	547.0	52.6	70.7	52.8
1	601.0	607.9	503.5	64.9	61.0	52.0
1	585.5	604.8	506.5	59.8	60.4	49.1
2	691.8	533.9	477.1	66.0	42.8	52.6
2	566.5	550.5	466.8	59.6	61.4	68.2
2	635.3	604.9	493.1	56.5	60.3	43.7
2	618.2	649.4	462.9	54.0	51.9	55.2
2	624.6	590.7	457.7	72.8	53.1	49.2
2	586.0	605.6	441.6	50.1	52.1	57.7
4	603.0	552.5	431.0	48.5	51.5	64.3
4	593.6	580.3	439.2	49.5	71.5	69.0
4	635.2	633.3	449.0	45.5	63.9	74.1
4	641.5	634.8	413.3	55.9	55.2	62.3
4	616.5	583.9	384.2	68.5	56.0	50.0
4	615.8	631.6	398.0	48.8	56.7	68.1

Annex C: Results of long-term stability measurements

Table C1: Long-term stability studies results (corrected for recovery) for DON and NIV performed at 4 °C

Time points [months]	Mass fraction [µg/kg]	
	DON	**NIV**
0	586.5	48.5
0	645.0	51.0
0	660.0	50.0
0	594.5	46.0
0	637.0	51.5
0	600.0	46.5
60	664.5	53.0
60	667.5	49.0
60	705.0	48.0
60	590.0	45.0
60	638.5	53.0
60	621.0	56.0
84	655.0	46.5
84	631.5	49.0
84	642.0	53.0
84	601.0	50.5
84	605.0	47.5
84	645.5	51.5
103	615.5	53.0
103	623.5	50.5
103	595.0	49.0
103	560.5	48.0
103	652.5	46.0
103	598.0	46.5

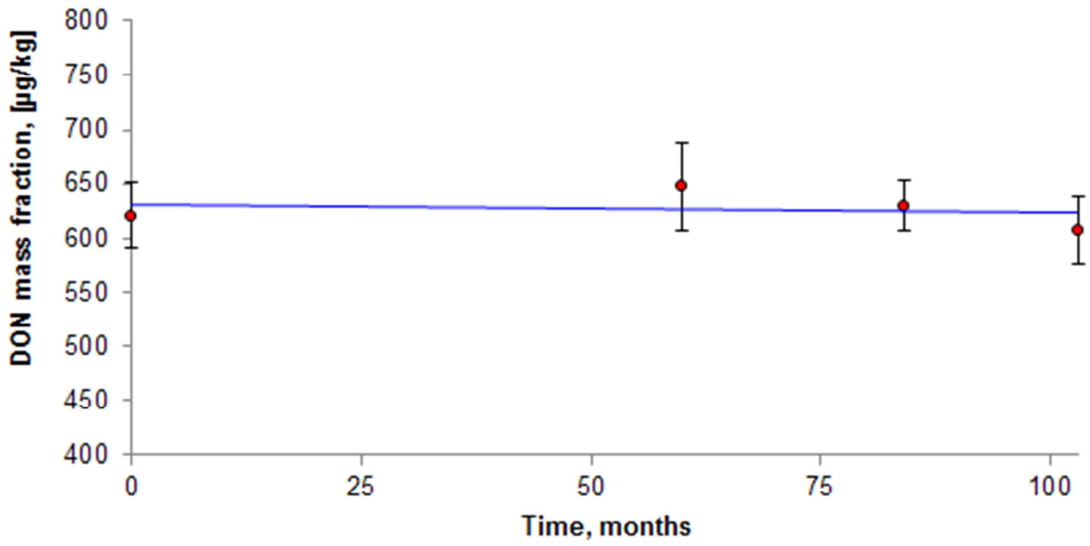

Figure C1: DON measurement results obtained for ERM-BC717 during long-term stability study; for each storage time, 3 samples (extraction replicates) were measured from each of the 2 randomly selected sachets (N = 2, n = 3); error bars represent ± s of the mean of 6 replicates derived from 2 sachets (3 replicates per sachet) as mentioned before

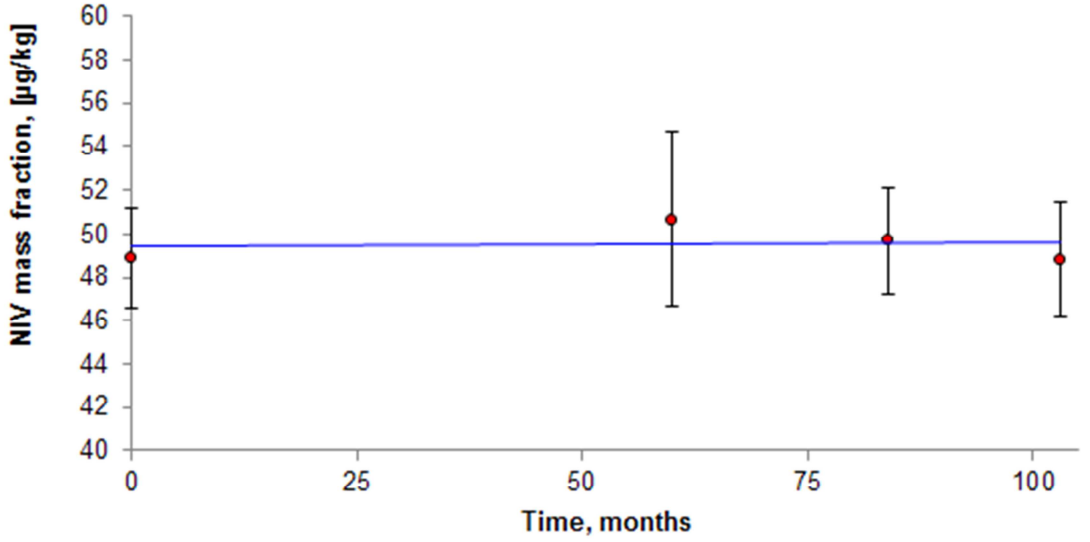

Figure C2: NIV measurement results obtained for ERM-BC717 during long-term stability study; for each storage time, 3 samples (extraction replicates) were measured from each of the 2 randomly selected sachets (N = 2, n = 3); error bars represent ± s of the mean of 6 replicates derived from 2 sachets (3 replicates per sachet) as mentioned before

Annex D: Summary of methods used in the characterisation study

Table D1: Summary of methods used in the characterisation study of DON

Laboratory code	Detection method used	Extraction			Clean-up method	Stated by the laboratory		
		Solvent	Type	Time [min]		Reproducibility [%]	Mean recovery [%]	LOQ [µg/kg]
L1	HPLC-UV	H_2O	shaking	120	IAC	21	86	150
L2	HPLC-UV	H_2O	blending	2	IAC	15	78-88	125
L3	HPLC-UV	H_2O	blending	2	IAC	7	95	50
L4	HPLC-UV	H_2O	shaking	120	IAC	20	102	100
L5	HPLC-UV	H_2O	shaking	30	IAC	12	65-100	6,8
L6	HPLC-UV	MeCN/H_2O (84/16)	shaking	60	filtration, MultiSep 227, IAC	15	78	100
L7	GC-MS	MeCN/H_2O (84/16)	shaking	60	SPE-MycoSep	18	84-100	100
L8	LC-MS/MS	H_2O/MeCN/IPA/FA +1 g NaCl (19/40/40/1)	shaking	60	QuEChERS	40	85	15
L9	LC-MS/MS	H_2O	blending	3	IAC	20	89	30
L10	LC-MS/MS	H_2O	shaking	60	IAC	10	93-120	30
L11	LC-MS/MS	H_2O	blending	3	IAC	8	85-90	10
L12	LC-MS/MS	MeCN/H_2O/FA (74/25/1)	shaking	60	filtration, MultiSep 226	10	91	120
L13	LC-MS/MS	MeCN/H_2O (84/16)	shaking	120	SPE-MycoSep	28	98	110

Table D2: Summary of methods used in the characterisation study of NIV

Laboratory code	Detection method used	Extraction			Clean-up method	Stated by the laboratory		
		Solvent	Type	Time [min]		Reproducibility [%]	Mean recovery [%]	LOQ [µg/kg]
L1	HPLC-UV	H_2O	shaking	30	IAC	15	95-120	6.7
L2	GC-MS	MeCN/H_2O (84/16)	shaking	60	SPE-MycoSep	18	78-96	20
L3	LC-MS/MS	H_2O/MeCN/IPA/FA +1 g NaCl (19/40/40/1)	shaking	60	QuEChERS	40	85	2.0
L4	LC-MS/MS	H_2O	blending	3	IAC	20	77	15
L5	LC-MS/MS	H_2O	shaking	60	IAC	10	95	25
L6	LC-MS/MS	MeCN/H_2O (84/16)	shaking	120	SPE-MycoSep	28	79	20

Annex E: Results of the characterisation measurements

Table E1: Individual results of DON characterisation study

Laboratory code	Method used	Mean	*s*	*SE*	H.W. CI (95%)	Sample no. * / result, [µg/kg]					
						#1	#2	#3	#4	#5	#6
L1	HPLC-UV	543.0	37.9	15.5	39.8	534.6	546.3	506.1	613.1	543.3	514.3
L2	HPLC-UV	740.6	29.7	12.1	31.1	771.3	729.8	723.6	784.7	718.8	715.5
L3	HPLC-UV	832.3	34.8	14.2	36.5	849.5	867.7	788.8	849.2	850.6	787.8
L4	HPLC-UV	653.9	11.2	4.6	11.8	649.9	640.8	652.8	660.2	647.0	672.7
L5	HPLC-UV	554.8	27.0	11.0	28.3	559.7	511.0	551.6	544.1	590.6	571.8
L6	HPLC-UV	727.4	18.5	7.5	19.4	745.6	719.3	729.5	723.3	748.4	698.3
L7	GC-MS	654.7	19.6	8.0	20.5	658.8	646.7	634.1	680.2	634.6	673.6
L8	LC-MS/MS	528.4	28.6	11.7	30.0	538.8	542.4	478.8	532.4	562.4	515.9
L9	LC-MS/MS	718.1	35.1	14.3	36.9	692.3	715.9	665.6	760.7	747.2	727.1
L10	LC-MS/MS	644.1	19.1	7.8	20.0	657.6	655.9	638.1	651.7	607.8	653.5
L11	LC-MS/MS	768.0	63.7	26.0	66.8	765.6	649.3	773.5	815.9	829.6	774.2
L12	LC-MS/MS	676.8	16.7	6.8	17.5	704.9	687.9	672.1	659.0	671.0	666.2
L13	LC-MS/MS	700.7	39.0	15.9	40.9	701.1	654.9	749.7	729.7	654.9	713.6

* each result is a mean calculated by IRMM from raw data (corrected for recovery by each laboratory itself) of duplicate injections provided by the participants (non-rounded data)

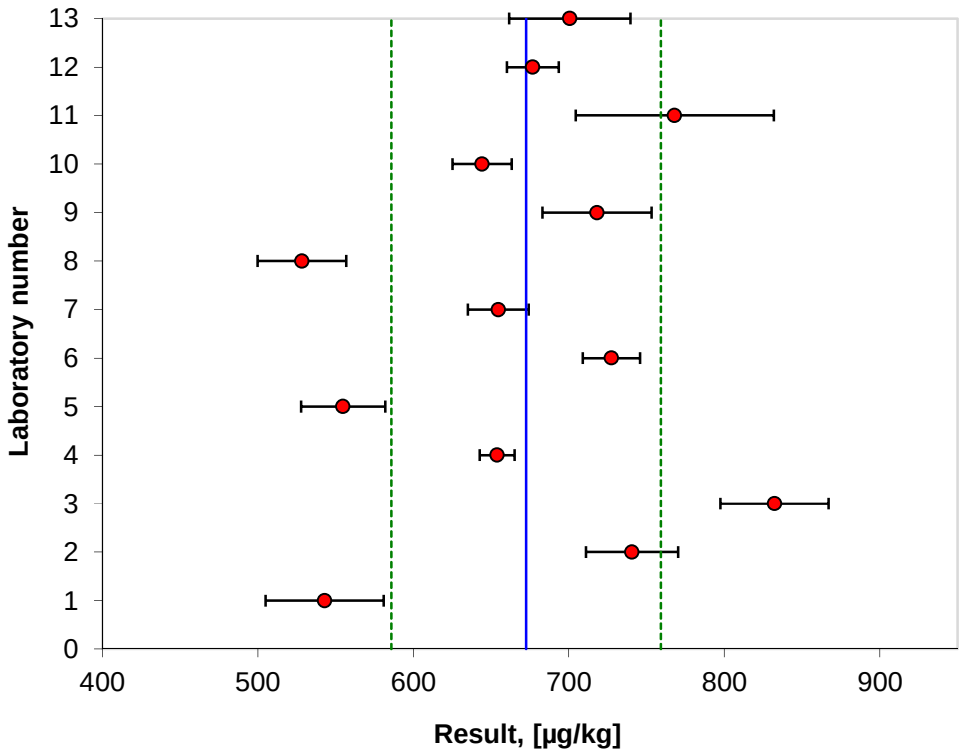

Figure E1: Laboratory means and their standard deviations (± *s*) for certification of ERM-BC717 of DON mass fraction in maize powder. Blue line (in the middle) represents mean of means, green lines (dotted) represents expanded uncertainty range (U_{CRM}; k = 2)

Table E2: Individual results of NIV characterisation study

Laboratory code	Method used	Mean	*s*	*SE*	H.W. CI (95 %)	Sample no. * / result, [µg/kg]					
						#1	#2	#3	#4	#5	#6
L1	HPLC-UV	56.6	5.1	2.1	5.3	49.3	56.1	52.1	60.4	59.5	62.2
L2	GC-MS	46.2	3.8	1.5	4.0	40.0	51.3	47.8	44.4	46.5	47.1
L3	LC-MS/MS	62.1	3.4	1.4	3.5	65.8	60.1	56.7	62.9	65.1	61.9
L4	LC-MS/MS	64.9	4.4	1.8	4.6	61.2	61.6	65.0	72.1	61.7	68.0
L5	LC-MS/MS	46.2	4.5	1.8	4.7	54.2	44.9	42.7	44.4	42.5	48.6
L6	LC-MS/MS	44.5	3.6	1.5	3.8	47.7	41.4	46.5	48.4	39.3	43.7

* each result is a mean calculated by IRMM from raw data (corrected for recovery by each laboratory itself) of duplicate injections provided by the participants (non-rounded data)

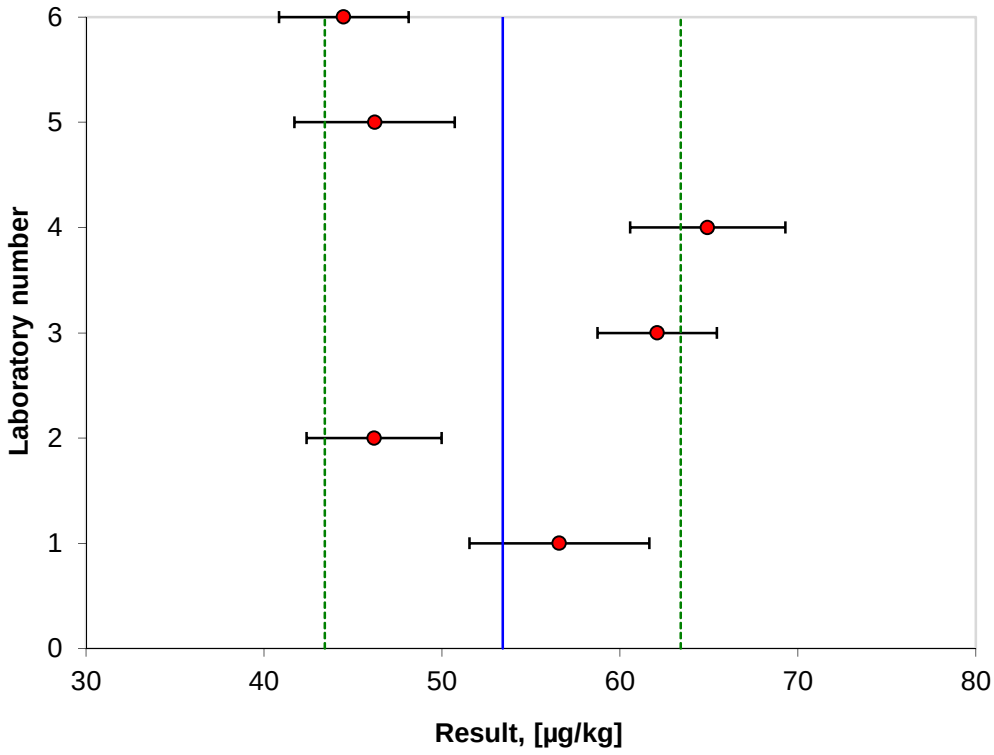

Figure E2: Laboratory means and their standard deviations (± *s*) for certification of ERM-BC717 of NIV mass fraction in maize powder. Blue line (in the middle) represents mean of means, green lines (dotted) represents expanded uncertainty range (U_{CRM}; k = 2)

European Commission

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Abstract
This report describes the additional certification of the mass fractions of deoxynivalenol (DON) and nivalenol (NIV) in the already existing material ERM-BC717 (maize powder), which was previously certified for the mass fraction of zearalenone (ZON). The between-unit homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006. The within-unit homogeneity was quantified to determine the minimum sample intake. The material was characterised by an intercomparison among laboratories of demonstrated competence and adhering to ISO/IEC 17025. The certified values were established by HPLC UV, LC MS/MS and GC MS as independent measurement methods (measurements within the scope of accreditation to ISO/IEC 17025:2005. Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties related to possible inhomogeneity and instability as well as to characterisation. The material is intended for quality control. As any reference material, it can also be used for control charts or validation studies.

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