Certification Report

Certified Reference Material

ERM®-BC715

Zearalenone in maize germ oil

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Summary

This report describes the preparation, characterisation and certification of the certified reference material ERM®-BC715.
The certified reference material (CRM) is available as refined maize germ oil from a commercial source contaminated with the mycotoxin zearalenone (ZEN). ERM®-BC715 is intended to be used for performance control and validation of analytical methods for the determination of ZEN in refined maize oils. This CRM may also be applicable for other similar matrices (vegetable edible oils).

The following mass fraction has been certified:

Zearalenone in maize germ oil

Measurand[1)]	Certified value[2)]	Uncertainty[3)]
	Mass fraction in µg kg^{-1}	
Zearalenone[4)]	362	22

1) Zearalenone determined using sample preparation, instrumental separation (HPLC) and mass spectrometric detection as specified in section 3 of the certification report.

2) The value given represents the unweighted mean value of 20 ampoule mean values analysed by BAM. The certified value is traceable to the International System of Units (SI) via an unbroken chain of calibrations to the pure analyte.

3) Estimated expanded uncertainty U with a coverage factor of $k = 2$, corresponding to a level of confidence of approximately 95%, as defined in the Guide to the expression of uncertainty in measurement (GUM), ISO/IEC Guide 98-3 (2008). Uncertainty contributions arising from characterisation as well as from homogeneity and stability testing were taken into account.

4) CAS number: 17924-92-4

Content

List of abbreviations

(if not explained elsewhere)

ACN	Acetonitrile
ANOVA	Analysis of Variance
CH	Cyclohexane
CoA	Certificate of Analysis
CRM	Certified reference material
EFSA	European Food Safety Authority
ERM	European Reference Materials
ESI	Electro-Spray Ionisation
FLD	Fluorescence detection
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
ESI-MS/MS	Electrospray ionisation tandem mass spectrometry
ID	Inner diameter
ILC	Interlaboratory comparison study
ISO	International Organization of Standardization
k	Coverage factor
KFT	Karl-Fischer titration
MeOH	Methanol
MRM	Multiple Reaction Monitoring
SI	International System of Units
SIDA	Stable isotope dilution analysis
ZEN	Zearalenone

1. Introduction

Food safety and consumer protection have gained an increased importance in the last decade. Contaminations with moulds and mycotoxins may occur during the whole production chain of a food product ("from the field to the fork"). Due to serious toxic effects caused by mycotoxins, the surveillance, determination and reduction of these compounds in food and feed is subject to the work of legislative bodies, industry and chemical laboratories.

Mycotoxins are secondary fungal metabolites partially associated with human and animal diseases. Zearalenone (ZEN; Fig. 1) is an estrogenic mycotoxin that contaminates cereal crops worldwide. Grains like wheat, barley, oats, sorghum, and particularly maize are frequently contaminated. ZEN is biosynthesised by several *Fusarium* species including *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium cerealis*, and *Fusarium equiseti*. In vitro and in vivo studies demonstrate ZEN to be estrogenic, hepatotoxic, immunotoxic, and carcinogenic. Humans and animals are exposed to ZEN by ingestion of contaminated food and feed [1, 2]. Previous findings suggest that maize germ oils provide a significant source of ZEN [3, 4]. The EFSA confirmed that vegetable oils present "an important contribution to the zearalenone exposure" in an evaluation on the risks of ZEN [5]. Consequently, the European Union (EU) introduced a maximum level of ZEN mass fraction in refined maize germ oil (400 µg kg^{-1}) in 2006 [6]. Although this maximum level applies, there is no certified reference material for the analysis of ZEN in edible oils available to date.

Therefore, matrix-matched reference materials and especially certified reference materials (CRM) are required to verify the accuracy of analytical measurements. In the framework of an ERM® project, a new certified reference material for zearalenone (ZEN) in a refined maize germ oil (ERM®-BC715) was developed at BAM. The produced ERM®-BC715 is intended to be used for performance control and validation of analytical methods for the determination of ZEN in refined maize oils and similar vegetable edible oils.

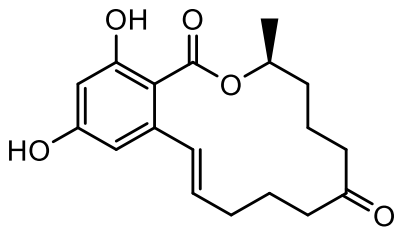

Fig. 1: Zearalenone (ZEN)

In order to support BAM's in-house certification of the candidate reference material, 13 laboratories were selected based on documented competence and invited to participate in an interlaboratory comparison study (ILC). This report describes the preparation, characterisation and certification of ERM®-BC715. Certification of reference material ERM®-BC715 was carried out based on ISO 17034 [7] and the relevant ISO-Guides [8, 9].

2. Production of the candidate material

2.1 Procurement and preparation of candidate material

Based on the European maximum level of 400 µg kg^{-1} for the ZEN mass fraction in refined maize germ oil, the aim was to produce a refined maize germ reference material in a targeted range of 300 – 400 µg kg^{-1}. Criteria to procure a suitable candidate material were *i)* oil from a commercial source and *ii)* oil revealing a natural ZEN contamination. Therefore, a survey of refined maize germ oils from German retail markets was conducted. Based on previous experiences of BAM-Division 1.7 regarding ZEN contamination of maize germ oils, moderate up to high levels of ZEN could be expected in commercial maize germ oil samples. The procured samples were combined in a flask (5150 mL) and thoroughly mixed. Because the yellow mixture was clear, a filtration step was not necessary. An initial analysis revealed a ZEN content of 346 µg kg^{-1} confirming the suitability of the oil mixture regarding the targeted ZEN content of the reference material.

2.2 Bottling of the candidate material

Immediately before bottling, the maize germ oil mixture was purged with argon for 5 hours to remove residual oxygen. Afterwards, a total number of 569 units of ERM®-BC715 was produced by filling a volume of 9 mL oil in 10 mL amber glass ampoules using a burette. Prior to filling, the empty ampoules were flushed with argon to exclude oxygen. Ampoules were sealed immediately after filling. The ampoules were labelled with general information and specific numbers according to their filling sequence. The whole batch was stored at -20 °C in a freezer.

3. Analytical method

All analyses within this ERM® project (homogeneity, stability, certification) were carried out at BAM according to the accredited in-house procedure BAM-1.7-PV006 [10]. This method is based on high performance liquid chromatography (HPLC) hyphenated to negative electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) using a stable isotope dilution analysis (SIDA) approach.

Sample preparation

In a 15 mL centrifuge tube (Falcon™) the isotopically labelled internal standard [$^{13}C_{18}$]-ZEN solution (Biopure™, Tulln, Austria) is weighed in and the solvent is evaporated at 50 °C in a light nitrogen stream. After cooling to room temperature 0.5 mL oil sample is weighed in, 0.5 mL n-hexane is added, and the mixture is vortexed. 5 mL of the extraction solution (methanol/water, 9/1, v/v) are added, and the tube is well closed. ZEN is extracted on a horizontal shaker for 30 min at 400 min^{-1}. The sample is centrifuged at room temperature at 2.400 U min^{-1} (1.378 *g*) for 10 min. An aliquot of 1 mL from the upper, methanolic layer containing ZEN is transferred into a HPLC vial and evaporated to dryness at 50 °C in a nitrogen stream. The residue is reconstituted in 0.4 mL HPLC eluent (ACN/water, 38/62, v/v), and is dissolved using ultrasound and vortex mixer followed by HPLC-MS/MS analysis. The instrumental measurement conditions for HPLC and MS are summarised in Table 1.

Tab. 1: Parameters of the HPLC-MS/MS system for analysis of zearalenone in ERM®-BC715

	Instrument / Measurement conditions
HPLC	
Instrument	Agilent 1200
Column	Phenomenex® Gemini® NX C_{18} column (150 x 2 mm, particle size 3 µm) coupled to a Gemini® C_{18} guard column (2.0 × 4.0 mm)
Mobile phase (Eluent)	A = water, containing 0.1% formic acid B = acetonitrile (ACN), containing 0.1% formic acid
Gradient program	

Time (min)	Flow rate (µL min^{-1})	Eluent A (%)	Eluent B (%)
0	300	62.0	38.0
15	300	62.0	38.0
15.1	400	5.0	95.0
19.0	400	5.0	95.0
19.1	300	62.0	38.0
27.0	300	62.0	38.0

Oven temperature	50 °C
Injection volume	10 µL
MS/MS detection	
Mass spectrometer	Applied Biosystems API 4000 QTRAP®
Ionisation	ESI negative(-)
Ion source temperature	500 °C
Modus	Multiple reaction monitoring (MRM)

The mass transitions (MRM mode) given in Table 2 were monitored and used for ZEN quantification:

Tab. 2: MRM transitions for the analyses of native and isotopically labelled zearalenone

Compound	MRM transition (m/z)	Dwell time (ms)	DP (V)	CE (eV)	CXP (V)
ZEN	317.1 $[M-H]^-$ → 131.1[a]	50	-80	-42	-8
	317.1 $[M-H]^-$ → 175.0[b]	50	-80	-40	-18
$[^{13}C_{18}]$-ZEN	335.2 $[M-H]^-$ → 140.2	50	-80	-42	-7

DP: Declustering potential; CE: Collision energy; CXP: Collision cell exit potential;
[a]) quantifier transition
[b]) qualifier transition

Six-point calibrations using a certified ZEN standard (Biopure™, Tulln, Austria) as calibrant and ^{13}C-ZEN internal standard (area ratios measured) were used for quantification. Each calibration solution was freshly prepared by weighing. The calibration function for ZEN was assumed to be linear and obtained by regression analysis.

4. Homogeneity study

It could be expected that ZEN, a nonpolar substance (K_{ow} = -3.66), is dissolved in the nonpolar oil matrix (triglycerides) with a satisfactory level of homogeneity. For further quantitative demonstration, 20 units of the candidate material were selected equidistantly from the whole batch of the 569 units (ampoules) in the order of bottling. The selected units were processed five times each according to the analytical method described in Section 3. All resulting extracts (20 x 5 = 100) were analysed by HPLC-MS/MS in a randomised manner under repeatability conditions in that all extracts were quantified against one calibration. The displayed results in Fig. 2 show that no trend was observed regarding the ampoule filling order (and also no trend was detected regarding the sequence order). All measurement results of the homogeneity study are summarised in Annex A.

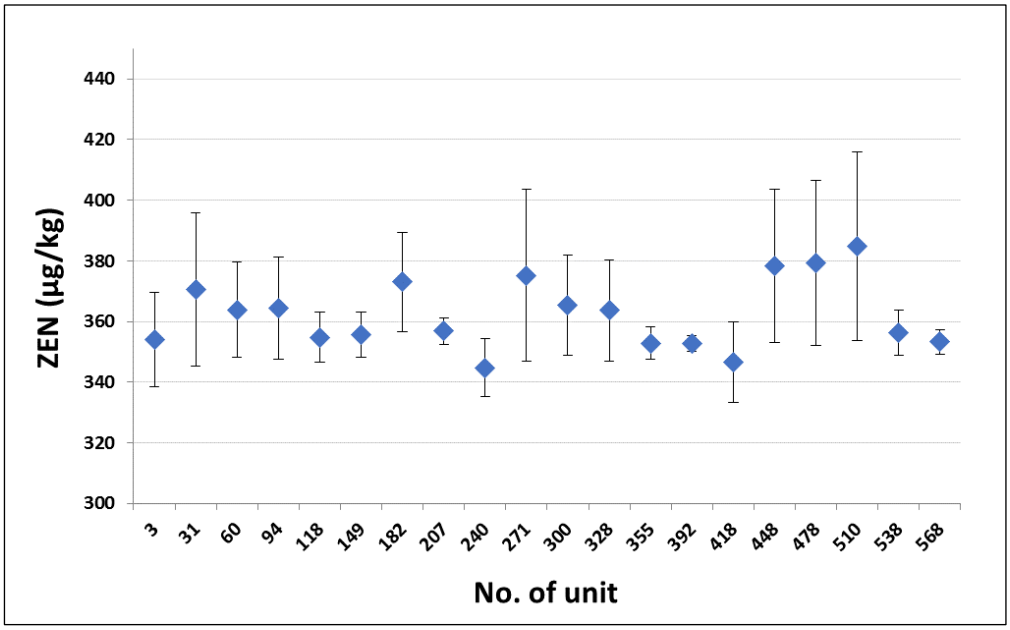

Fig. 2: Homogeneity study of ERM®-BC715 candidate material: Mean values of ZEN of 20 selected units with their standard deviations (n=5) represented by the error bars

The 1-way analysis of variance (ANOVA) results are given in Table 3 together with the estimations of the contributions due to the between-bottle inhomogeneity (u_{bb}), assessed according to [9].

Tab. 3: Analysis of variance (ANOVA) and estimates for uncertainty contribution for the candidate material

Compound	*Mean*[a]	$MS_{between}$[b]	MS_{within}[c]	F_{obs}[d]	F_{crit}[d]	u_{bb}[e]	$u_{bb,r}$[f]
	(µg kg^{-1})	(µg^2 kg^{-2})	(µg^2 kg^{-2})			(µg kg^{-1})	
ZEN	362.326	653.371349	295.264866	2.213	1.718	8.463	0.023357

[a] *Mean of the homogeneity study*
[b] *Mean of squared deviation between units (from 1-way ANOVA)*
[c] *Mean of squared deviation within units (from 1-way ANOVA)*
[d] *Observed F-value (= $MS_{between}/MS_{within}$) and critical F-value*
[e] *Standard uncertainty between the units: Estimate of inhomogeneity contribution to the total uncertainty*
[f] *Relative standard uncertainty between the units (u_{bb}/mean)*

Based on the results the candidate material was considered to be slightly inhomogeneous, but to an extent that can be covered by a corresponding uncertainty, i.e. the intended use as reference material is not affected. A contribution u_{bb} to the overall uncertainty of ERM®-BC715 was derived from the ANOVA results and included in the uncertainty budget of the certified value. For that purpose, the maximum value of u_{bb} and u^*_{bb} has been calculated based on Equations 1 and 2.

$$u_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \qquad \textbf{(1)}$$

$$u^*_{bb} = \sqrt{\frac{MS_{within}}{n}} \cdot \sqrt[4]{\frac{2}{N(n-1)}} \qquad \textbf{(2)}$$

n = number of replicate determinations on each unit (n = 5)
N = number of analysed units (N = 20)

It turned out that u_{bb} (8.463 µg kg^{-1}) was higher than u^*_{bb} (3.056 µg kg^{-1}) and therefore was used as the uncertainty contribution for possible material inhomogeneity (Tab. 3).

Minimum sample intake
The accredited procedure applied for the homogeneity study prescribes a sample intake of 0.5 mL for each analysis. Since the homogeneity study was passed successfully using this scheme, 0.5 mL (corresponding to 0.45 g) of ERM®-BC715 is recommended as minimum sample intake in the CoA.

5. Stability monitoring

5.1 Initial stability study

From earlier experience with organics in various matrices (e.g. ZEN in wheat ERM®-BC600), a temperature-driven deterioration of the ZEN content was to be expected also for this material. Selected units of the candidate material were submitted to an isochronous accelerated ageing [11] at temperatures between +4 °C and +60 °C over periods of 0.25 to 12 months as shown in Table 4. After the respective periods of time, individual units were stored at -20 °C. All units were analyzed for ZEN under repeatability conditions together with reference samples which had been kept at -20 °C since bottling. In addition, two ampoules were analyzed after 12 months of storing at -80 °C, where a degradation of ZEN can reasonably be excluded. For ZEN quantification the accredited procedure SOP BAM-1.7-PV006 was employed. All measurement results are collected in Annex B.

Tab. 4: Accelerated ageing of selected units of ERM®-BC715 (ampoule-no.), exposition temperatures and periods

Ageing time (months)	+4 °C	+23 °C	+40 °C	+60 °C	Remark
0.25	290	037	507	399	*initial short-term study*
0.50	438	329	213	250	
0.75	002	185	071	542	
1	136	474	363	103	
3	255	053	321	---	*initial long-term study*
6	073	497	088	---	
12	155	222	533	---	
24	270	---	---	---	*post certification monitoring*
36	564	---	---	---	
48	004	---	---	---	
60	117	---	---	---	

Data evaluation and expiry date (shelf life) estimation strictly followed the procedures as comprehensively described in [12]: From semi-logarithmic plots of measured single values over time, effective deterioration rates k_{eff} were determined and tested against an *Arrhenius* model describing the temperature dependence of the deterioration rates (Fig. 3).

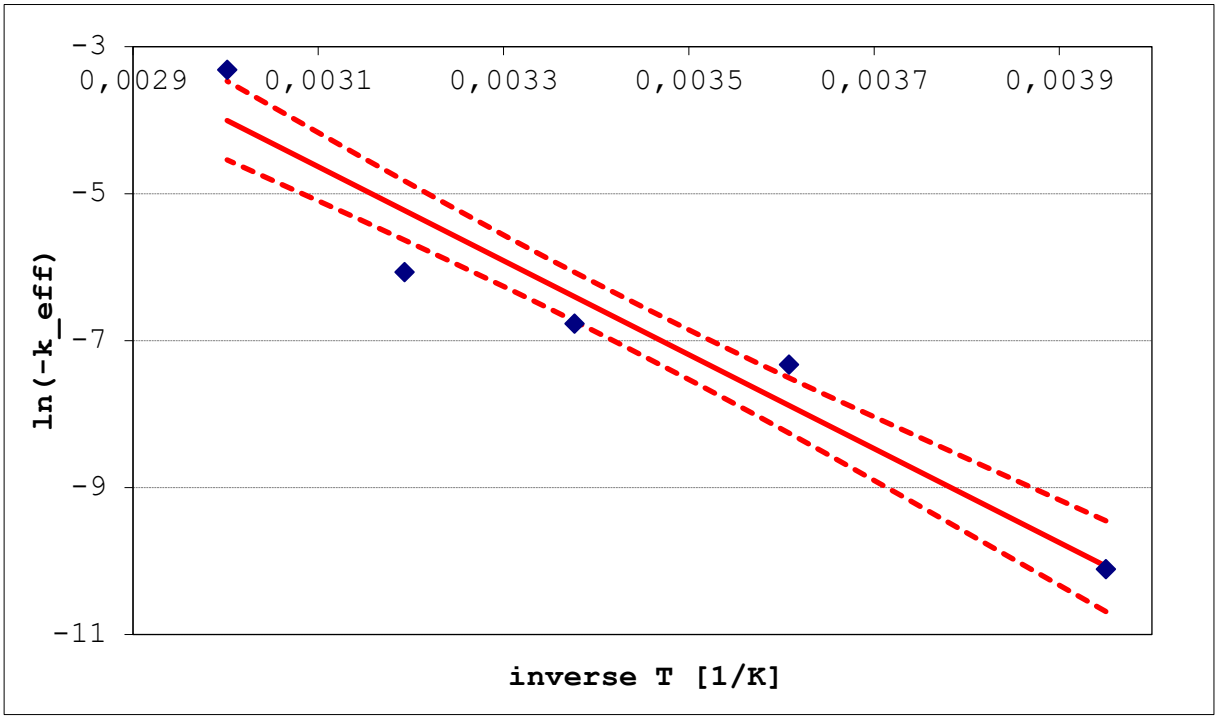

Fig. 3: Effective deterioration rate of ZEN versus inverse temperature for ERM®-BC715

The graph also contains a data point for -20 °C since measurements at an even lower storage temperature of -80 °C were possible and used as reference. Temperature dependence can merely be approximated by a straight line. The corresponding confidence interval for the line is also given in Figure 3. The calculated activation energy ΔE for ZEN (53.2 kJ mol^{-1}) is in an acceptable agreement with activation energies determined for a large variety of organic compounds and is also similar to ΔE (ZEN) resulting from stability study of ERM®-BC600 (ΔE = 58.0 kJ mol^{-1}).

By using these data and the assumed *Arrhenius* model, an estimate can be obtained when degradation will presumably force the mycotoxin content to fall short of the certified lower expanded uncertainty limit. In the sense of a worst-case estimation, these calculations are carried out for the reaction rates at the upper confidence limit of the line as shown in the above figure. Shelf life estimations for ZEN in ERM®-BC715 are stated in Table 5 for different storage temperatures.

Tab. 5: Estimated period in months until which the certified ZEN value of ERM®-BC715 will remain within the expanded uncertainty *U* at different storage temperatures

Temperature (°C)	Expiry (months)
-20	799
4	114
23	27
40	8
60	2

A storage temperature of -20 °C and even +4 °C is sufficient for a desirable minimum shelf life of 5 years. For this reason, an uncertainty contribution due to long-term (in)stability was not considered. Exposure to room temperature or higher than room temperature may reduce the time of validity of ERM®-BC715. Therefore, a common user-end expiry date of two years after delivery from storage is established provided the sample is stored equal or lower than +4 °C at the user's site. Transportation/delivery time should be kept at the possible minimum and any exposure to elevated temperatures should be avoided.

5.2 Post-certification monitoring

The first rough estimation of stability will be updated by annual measurements of units stored at -20 °C (reference) and +4 °C, over the period of availability of the material.

6. Certification study

6.1 Design of the study

The assignment of the certified ZEN mass fraction of ERM®-BC715 is based on an in-house study using HPLC-ESI-MS/MS analysis including certified ZEN standard (BiopureTM, Tulln, Austria) and ^{13}C-labelled ZEN as internal standard (see section 3). For in-house certification, 20 units of the candidate reference material were analysed at BAM. From each unit 5 subsamples were taken, resulting in a total of 20x5=100 analyses. For this purpose, the data form the homogeneity study were used. Simultaneously, an interlaboratory comparison study (ILC) involving 13 expert laboratories was conducted in order to support and to confirm the in-house certification data produced at BAM.

All participants of the ILC received two ampoules of ERM®-BC715 and were requested to analyse each ampoule in three replicates. For measurement control purposes, two control solutions (containing ZEN in acetonitrile with unknown concentration for the participants) were dispatched for direct analysis. The control solutions were gravimetrically prepared at BAM ("1" = 50.60 ng mL^{-1} and "2" = 180.72 ng mL^{-1}). Results returned to BAM were scrutinised for consistency.

6.2 Participants in the supporting ILC

A total number of 13 laboratories (Table 6) were selected to participate in the ILC based on their approved expertise in the field of mycotoxin analysis.

Tab. 6: Participants of the interlaboratory comparison study for ERM®-BC715 (in alphabetical order)

Laboratory	City, Country
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	Oberschleißheim
Bioanalytik Weihenstephan, Zentralinstitut f. Ernährungs- u. Lebensmittelforschung	München (DE)
Chemisches Labor Dr. Wirts + Partner Sachverständigen GmbH	Hannover (DE)
Chemisches und Veterinäruntersuchungsamt Rheinland	Leverkusen (DE)
Chemisches und Veterinäruntersuchungsamt Westfalen	Bochum (DE)
Coop	Pratteln (CH)
Food GmbH	Jena (DE)
Institut Kirchhoff Berlin GmbH	Berlin (DE)
Landeslabor Berlin-Brandenburg	Berlin (DE)
LUFA-ITL GmbH	Kiel (DE)
Multilab Hamburg Bergedorf GC, SGS Germany GmbH	Hamburg (DE)
Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit	Braunschweig (DE)
Österreichische Agentur für Gesundheit und Ernährungssicherheit	Linz (AT)

6.3 Analytical methods used by ILC participants

The participants of the ILC applied methods of their own choice all using a certified ZEN calibration standard provided by BAM. The reported sample intake was in the range between 1 g and 7.7 g (Table 7). The predominant ZEN extraction method was L/L partition by shaking using acetonitrile/water or methanol/water mixtures (after dilution with n-hexane). Some laboratories used alkaline extraction mixtures to improve the solubility of non-polar ZEN by deprotonation of the phenolic groups. If a clean-up step was applied (5 out of 13 labs), immunoaffinity column (IAC) was the method of choice to purify the extracts (4 out of 5 labs). Instrumental analysis was uniformly done by HPLC using either fluorescence detection (9 labs) or MS/MS detection (4 labs).

Tab. 7: Extraction, clean-up and determination methods used in the ILC for ERM®-BC715

Lab code	Sample (g)	Extraction method	Extraction solvent	Clean-up	Instrumental method	Internal standard
A	2.5	shaking	ACN:water	no	HPLC-MS/MS	[$^{13}C_{18}$]-ZEN
B	1	shaking	ACN:water	no	HPLC-MS/MS	No
C	1.5	shaking	MeOH/water	no	HPLC-FLD	No
D	1-2	shaking	MeOH/water	no	HPLC-FLD	No
E	1	shaking	MeOH/water	IAC	HPLC-FLD	No
F	2	shaking	MeOH/water	no	HPLC-FLD	No
G	2	shaking	MeOH/water	IAC	HPLC-FLD	No
H	1.5	shaking	ACN:water	IAC	HPLC-FLD	No
I	7.7	shaking	ACN:water	IAC	HPLC-FLD	No
J	2	shaking	MeOH/water	no	HPLC-FLD	No
K	1	GPC	CH/EtOAc	no	HPLC-MS/MS	[$^{13}C_{18}$]-ZEN
L	2.5	shaking	ACN:water	SPE	HPLC-MS/MS	[$^{13}C_{18}$]-ZEN
M	2	shaking	MeOH/water	no	HPLC-FLD	No

6.4 Evaluation of ILC results

Figure 4 depicts (in a Youden plot arrangement) the results of the laboratories, namely their findings on both control solutions for ZEN in acetonitrile, and their values obtained for the unknown oil sample. The values of the control solutions measured by the laboratories were normalised against the target

value of the control solutions resulting from the gravimetric preparation at BAM-1.7 (control solution 1 = 50.60 ng mL^{-1} and control solution 2 = 180.72 ng mL^{-1}). The ZEN content of the unknown oil sample was normalised against the value assigned by BAM's in-house study (362.326 µg kg^{-1}).

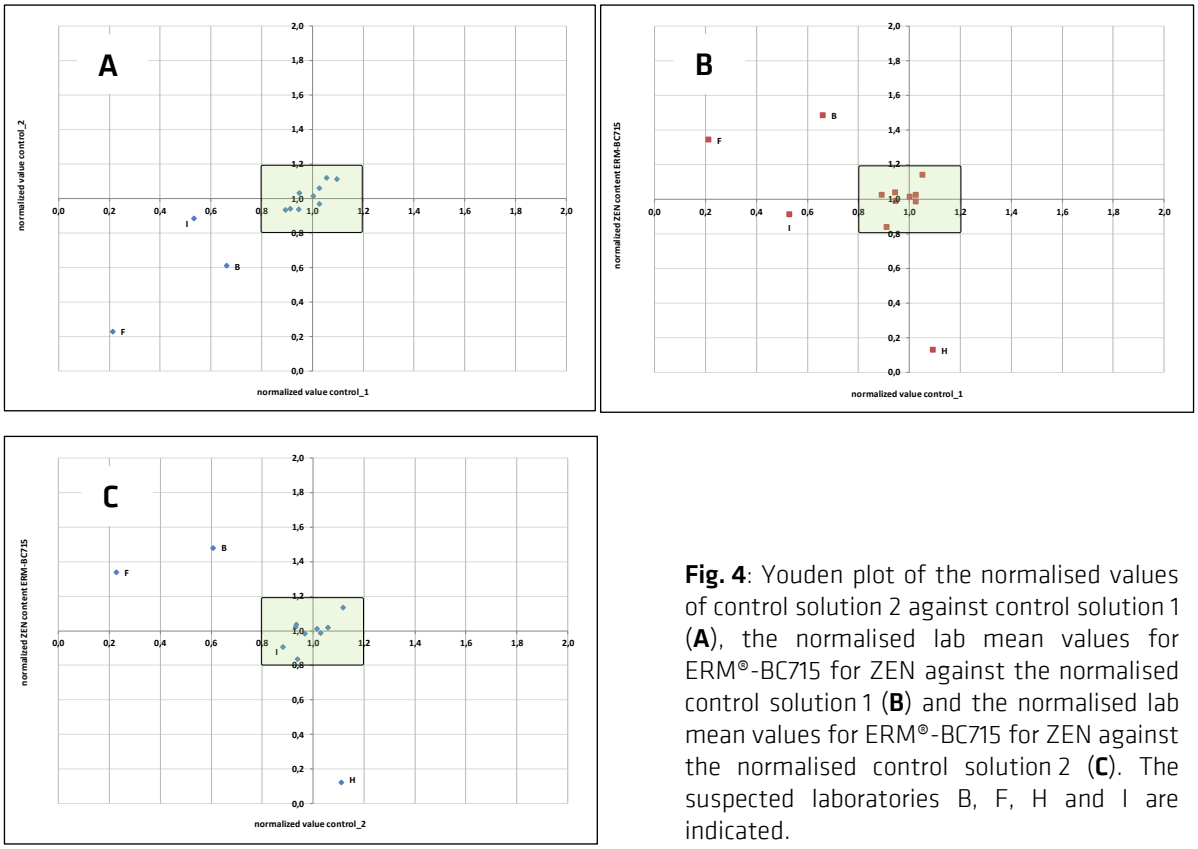

Fig. 4: Youden plot of the normalised values of control solution 2 against control solution 1 (**A**), the normalised lab mean values for ERM®-BC715 for ZEN against the normalised control solution 1 (**B**) and the normalised lab mean values for ERM®-BC715 for ZEN against the normalised control solution 2 (**C**). The suspected laboratories B, F, H and I are indicated.

Based on a thorough inspection of the data provided by the laboratories, all data sets within 80% to 120% for both control solutions and the ERM®-BC715 candidate were retained (9 labs; green box in Fig. 4). Four out of 13 labs were excluded from further evaluation due to technical reasons. This refers to:

Lab B: low on both control solutions (66%, 61%), but high for ERM®-BC715 (148%)
Lab F: very low on both control solutions (21%, 23%)
Lab H: very low for ERM®-BC715 (12%)
Lab I: low on first control solution (53%)

According to EC Regulation 401/2006 [13] the ZEN recovery of official control methods should fall within 70-120%. Eight out of 9 labs fulfilled this requirement. One lab (G) reported a ZEN recovery of 55%. However, both control solutions (100%, 102%) and the value for ERM®-BC715 (101% after correction by recovery) showed very good results, so Lab G was retained. The accepted data sets together with the corresponding lab means are given in Table 8.

Tab. 8: Accepted laboratory data sets of ILC for ZEN in maize germ oil (ERM®-BC715)

Lab code	Zearalenone (ZEN) in ERM®-BC715, values[a] (µg kg^{-1})						Lab mean (µg kg^{-1})
A	367.0	369.0	374.0	367.0	374.0	371.0	370.3
C	302.3	310.0	298.7	299.5	308.3	296.7	302.6
D	379.7	355.8	382.0	386.7	382.5	365.7	375.4
E	371.1	359.0	343.4	350.6	342.2	371.1	356.2
G	379.5	367.5	342.9	392.7	361.8	352.9	366.2
J	304.1	284.0	301.9	304.6	300.2	275.6	295.1
K	376.0	368.0	363.0	373.0	368.0	369.0	369.5
L	347.7	363.7	366.9	356.8	356.1	354.6	357.6
M	408.1	421.2	418.9	409.6	408.7	404.0	411.8
					Mean of laboratory means:		**356.083**

[a]) Values are corrected for recovery

The conformity of the ILC result and the assigned value was tested using the (amended) E_n criterion on the difference between the overall laboratory mean x_1 (356.083 µg kg^{-1})and the assigned value x_2 (362.326 µg kg^{-1})according to Equation 3:

$$E_n = \frac{|x_1 - x_2|}{2\sqrt{s_{ILC}^2 + u_c^2}} \qquad \textbf{(3)}$$

(with s_{ILC}: standard deviation of the mean of accepted laboratory means in the ILC (12.10 µg kg^{-1}), u_c : uncertainty of the assigned value (11.02 µg kg^{-1}), and the factor 2 converts the standard uncertainties in the denominator into expanded uncertainties). The resulting E_n criterion was determined to be 0.191. Conclusion: Because the E_n criterion is lower than the critical value (E_n=2), the outcome of the ILC is fully consistent with the in-house certification results based on the SIDA using HPLC-MS/MS at BAM.

6.5 Certified value and uncertainty budget

The assigned (=certified) mass fraction of ZEN in ERM®-BC715 was taken from the results of the homogeneity measurements, i.e. the unweighted mean value of 20 ampoule mean values as displayed in Annex A. Since 5 subsamples were analysed per unit, the certified value was derived from a total of 20x5=100 analyses resulting in 362.326 µg kg^{-1}.
The combined uncertainty is calculated based on the data of the in-house certification study according to Equation 4. The results are given in Table 9.

$$u_c^2 = u_x^2 + u_{bb}^2 + u_{lts}^2 + u_{cal}^2 + u_{pur}^2 + u_{handling}^2 \qquad \textbf{(4)}$$

Tab. 9: Uncertainty contributions for calculation of the combined uncertainty

Uncertainty contribution		µg kg^{-1}
Uncertainty of characterisation	u_x	4.615
Contribution from a possibly undetected inhomogeneity	u_{bb}	8.463
Contribution from long-term stability (sufficiently stable for shelf life of 5 years, no MU contribution required)	u_{lts}	0
Calibration uncertainty	u_{cal}	3.268
Uncertainty of the purity of used native calibration standard[a]	u_{pur}	2.174
Contribution from handling of samples: weighing, volumetric operations, aliquoting internal standard (pragmatic 1%)	$u_{handling}$	3.623
Total	u_c	**11.02**

[a]) At the same time traceability contribution: 0.6% of certified standard concentration

The calibration uncertainty u_{cal} is the uncertainty of a typical determination in the centre of a typical calibration curve. The uncertainty from handling $u_{handling}$ is a combined, rather worst-case, estimate for all gravimetric and volumetric sample handling procedures. The uncertainty contribution u_x is calculated from characterisation (= homogeneity) study based on Equation 5:

$$u_x = \sqrt{\left(\frac{s}{\sqrt{N}}\right)^2 + \frac{\sum_i^N s_i^2}{N \cdot n}} \qquad \textbf{(5)}$$

with:
s = standard deviation of the 20 individual mean values of the analysed ampoules
N = number of ampoules in homogeneity/characterisation study, N=20
n = number of replicates in homogeneity study, n=5
$\mathbf{s_i}$ = standard deviation of 5 replicates of ampoule *i*

The certified mass fraction of ZEN in ERM®-BC715 is given in Table 10 together with the expanded uncertainty calculated based on a coverage factor *k*=2. The certified value and the expanded uncertainty are rounded according to the recommendations of the Guide to the Expression of Uncertainty in Measurement [14] and are given with respect to raw sample mass.

Tab. 10: Certified mass fraction of ZEN in ERM®-BC715

Compound	Mass fraction (µg kg^{-1})		
	Certified value	Uncertainty	Expanded uncertainty
Zearalenone (ZEN)	362	11	22

Rounding: Intermediate results were not rounded; rounding was done for expanded uncertainty, not for combined uncertainty. The same number of significant figures is stated for the certified value and its uncertainty.

6.6 Metrological traceability

Traceability of the certified value is directly established using HPLC-MS/MS stable isotope dilution analysis applying a certified ZEN standard (100.4 ± 0.6 µg mL^{-1}, Biopure, Tulln, Austria) as calibrant and [$^{13}C_{18}$]-isotopically labelled ZEN as internal standard. The certified mass fraction of ZEN in ERM®-BC715 is traceable via the certified calibrant used. The certified value of the calibrant is traceable to the International System of Units (SI), as stated on the respective certificate, due to the gravimetric preparation employed. Therefore, the mass fraction of ZEN in ERM®-BC715 is traceable to the SI.

6.7 Commutability

ERM®-BC715 was produced from a commercial source intended for human consumption without changes of the matrix. Therefore, the analytical behavior is the same as for a routine sample of refined maize germ oil. For samples other than refined maize germ oil, the commutability must be re-assessed.

7. Information on the proper use of ERM®-BC715

7.1 Shelf life

From the initial stability study, a considerably large shelf life well above a period of 5 years at a storage temperature of +4 °C was estimated. Since the dispatch to the end user may occur at any time during this period, the certified properties will be valid for 24 months beginning with the dispatch of the material from BAM. The validity of this information will be maintained by post-certification monitoring.

7.2 Transport and storage

Due to the proven stability ERM®-BC715 can be shipped at ambient temperature. On receiving, the ampoule must be stored at a temperature equal to or lower than +4 °C.

7.3 Instructions for use

This material is intended to be used for performance control and validation of analytical methods for the determination of ZEN in refined maize oils. Before initial opening, the ampoule should reach room temperature and must be shaken thoroughly. The stability of the reference material is not affected by short periods of handling at ambient temperature during transport and use. However, BAM cannot be held responsible for any alteration of the material occurring during handling and storage at the customer's premises, especially of opened samples. Exposure to sunlight may cause the isomerisation of natural zearalenone (*trans*-isomer) to its *cis*-isomer. Therefore, the material is filled in an amber glass ampoule and should to be protected from sunlight.

7.4 Safety instructions

The usual laboratory safety precautions have to be applied. No hazardous effects are to be expected when the material is used under conditions usually adopted for the analysis of foodstuff matrices low or moderately contaminated with zearalenone. Although the mycotoxin content in the sample is at trace levels, any use other than the intended one should be avoided. The personnel handling the material must be trained adequately and follow the regular safety precautions of the laboratory. It is strongly recommended that the reference material is handled and disposed of in accordance with the guidelines for hazardous materials legally in force at the site of end use and disposal.

7.5 Legal notice

Neither BAM, its contractors nor any legal person acting on their behalf:

(a) make any warranty or representation, express or implied, that the use of any information, material, apparatus, method or process disclosed in this document does not infringe any privately-owned intellectual property rights; or

(b) assume any liability with respect to, or for damages resulting from the use of any information, material, apparatus, method or process disclosed in this document save for loss or damage arising solely and directly from the negligence of BAM.

8. Information on and purchase of the CRM

The certified reference material ERM®BC-715 is supplied by

Bundesanstalt für Materialforschung und -prüfung (BAM)

Department 1 – Analytical Chemistry; Reference Materials
Division 1.7 - Organic Trace and Food Analysis
Richard-Willstätter-Str. 11, D-12489 Berlin, Germany
Phone: +49 (0)30 - 8104 2061
Fax: +49 (0)30 – 8104 72061
E-Mail: sales.crm@bam.de

Each unit of ERM®BC715 will be distributed together with a CoA containing the certified value and its uncertainty, a material description and instructions for use, storage and safety.

Information on certified reference materials can be obtained from BAM homepage https://www.bam.de and BAM-webshop www.webshop.bam.de.

9. References

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[13] European Regulation No. 401/2006/EC (2006) Commission regulation from 23.02.2006, laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

[14] ISO/IEC Guide 98-3 (2008), Uncertainty of measurement - Part 3: Guide to the expression of uncertainty in measurement (GUM:1995) ISO, Geneva, Switzerland.

10. Annexes

Annex A: Raw data of homogeneity testing for ZEN in ERM®-BC715

Ampoule No.	ZEN mass fraction (µg kg^{-1})							RSD (%)
	1	2	3	4	5	Mean	SD	
3	356.183	361.123	346.169	373.840	332.594	353.982	15.562	4.396
31	398.227	347.057	341.834	390.337	375.443	370.580	25.289	6.824
60	376.480	348.218	382.765	349.116	362.578	363.832	15.656	4.303
94	381.169	348.169	361.008	348.707	382.943	364.399	16.928	4.646
118	353.654	347.344	368.249	349.664	355.265	354.835	8.130	2.291
149	353.056	348.805	367.431	351.110	358.121	355.705	7.401	2.081
182	355.844	356.598	387.725	374.705	390.397	373.054	16.475	4.416
207	356.261	356.948	352.432	363.841	354.579	356.812	4.298	1.205
240	346.913	330.891	344.918	343.509	357.728	344.792	9.575	2.777
271	358.772	354.652	382.459	358.082	422.107	375.214	28.452	7.583
300	356.042	352.221	391.393	371.967	355.411	365.407	16.430	4.496
328	393.169	353.091	354.911	355.985	361.026	363.637	16.770	4.612
355	356.966	346.549	349.056	352.120	359.713	352.881	5.447	1.544
392	353.304	349.218	352.571	351.590	356.370	352.611	2.606	0.739
418	329.681	364.609	347.972	351.780	339.078	346.624	13.190	3.805
448	364.171	362.405	367.912	422.870	375.004	378.472	25.286	6.681
478	399.768	414.643	372.339	351.231	358.423	379.281	27.112	7.148
510	373.135	369.611	379.343	439.670	362.837	384.919	31.183	8.101
538	361.923	365.057	347.991	356.291	349.886	356.229	7.390	2.075
568	353.171	357.824	346.998	352.360	355.908	353.252	4.119	1.166
						362.326	**14.865**	**4.044**

Annex B: Raw data of stability testing for ZEN in ERM®-BC715; ZEN mass fractions are given in µg kg^{-1}

Time (months)	Storage temperature (°C)					
	-80	-20	+4	+23	+40	+60
0		360.147	360.147	360.147	360.147	360.147
0		367.265	367.265	367.265	367.265	367.265
0		361.083	361.083	361.083	361.083	361.083
0		376.269	376.269	376.269	376.269	376.269
0.25			360.668	352.389	367.875	352.266
0.25			358.450	350.833	376.286	360.238
0.25			358.644	355.579	360.940	359.874
0.25			356.622	356.019	369.450	352.186
0.5				353.392	350.763	361.964
0.5			358.533	359.499	356.482	365.620
0.5			358.945	366.426	355.760	359.213
0.5			358.122	360.621	349.003	365.450
0.75			364.272	357.688	358.558	349.060
0.75			363.162	353.902	359.816	348.410
0.75			360.696	357.760	354.942	343.408
0.75			352.784	363.615	360.818	343.015
1		358.693	358.980	363.858	364.103	358.326
1		356.934	362.786	359.963	364.404	351.819
1		359.748	357.174	358.548	360.828	357.837
1		364.984	346.455	357.895	360.204	351.967
3			358.460	359.518	354.922	
3			361.492	351.021	349.554	
3			360.601	369.190	365.943	
3			362.146	366.387	360.134	
6			359.913	346.604	366.085	
6			359.605	355.093	358.922	
6			363.991	362.922	358.910	
6			365.033	357.130	356.425	
9			358.612	356.366	348.320	
9			362.045	357.212	351.921	
9			360.472	355.746	357.407	
9			363.893	356.797	353.932	
12	360.147	357.501	354.518	352.595	352.516	
12	367.265	356.672	354.180	359.430	353.976	
12	361.083	363.296	360.781	365.945	358.443	
12	376.269	368.878	353.143	361.792	353.238	