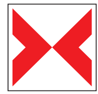

Federal Institute for Materials Research and Testing

CERTIFICATION REPORT

Certification of the mass fractions of T-2 and HT-2 toxin in oat flakes

Certified Reference Material

ERM®-BC720

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I. Summary

This report describes the certification of an oat flakes material intended for the determination of the *Fusarium* mycotoxins T-2 and HT-2 contained. Detailed information is given regarding the preparation of material, homogeneity and stability studies, used analytical methods and results of the certification study. Certified values and respective uncertainties are:

T-2 and HT-2 toxin in oat flakes

Compound [a]	Certified value [b]	Uncertainty [c]
	Mass fraction in $\mu g\ kg^{-1}$	
T-2 toxin	82	4
HT-2 toxin	81	4

[a] T-2 and HT-2 toxin as determined using sample preparation, instrumental separation (HPLC) and mass spectrometric detection as specified on page 9 of this report.

[b] The value given represents the unweighted mean value of 80 results. Certified values are traceable to the SI via an unbroken chain of calibrations to the respective pure analyte.

[c] Estimated expanded uncertainty *U* with a coverage factor of $k = 2$, corresponding to a confidence level of about 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.

II. List of Abbreviations

ACN	Acetonitrile
ANOVA	Analysis of variance
BAM	Federal Institute for Materials Research and Testing
CRM	Certified reference material
DON	Deoxynivalenol
DSM	German Collection of Microorganisms (“Deutsche Sammlung von Mikroorganismen”)
FLD	Fluorescence detection
GC	Gas chromatography
GUM	Guide to the Expression of Uncertainty in Measurement
HPLC	High performance liquid chromatography
HPLC-ESI-MS/MS	High performance liquid chromatography hyphenated to electrospray tandem mass spectrometry
IAC	Immunoaffinity column
ILC	Interlaboratory comparison study
ISO	International Organization of Standardization
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NIV	Nivalenol
SI	International System of Units
SIDA	Stable isotope dilution assay
SPE	Solid phase extraction
ZEN	Zearalenone

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1 Introduction

Contaminations with moulds and mycotoxins may occur during the whole production chain of a food product (e. g. “from the field to the fork”). Due to serious toxic effects caused by mycotoxins, the determination and reduction of these compounds in food and feed is subject to the work of regulators, food business operators and researchers.

Fungi of the genus *Fusarium* are the predominant mycotoxin producers in moderate climate zones. *Fusarium* toxins occur worldwide in a wide variety of foods, particularly in highly consumed cereal based products. The toxicologically - and hence also economically - most important *Fusarium* mycotoxins are zearalenone (ZEN) as well as the type A (T-2 and HT-2 toxin; Table 1 and Figure 1) and type B trichothecenes (deoxynivalenol (DON), nivalenol (NIV)).

Driven by regulatory authorities, extensive consumer protection efforts were made by establishing fast and reliable analytical methods for the determination of the most common *Fusarium* toxins in cereals and derived products. At the same time legally binding maximum levels were introduced for these matrices [European Regulations No. 1881/2006/EC and 1126/2007/EC]. While for DON and ZEN EU maximum levels are already in effect, new levels for T-2 and HT-2 toxin are currently under discussion. To enforce the maximum levels and thus reduce consumer risks, strict controls of food and feed are of prime importance.

For the sum of these reasons, matrix-matched certified reference materials (CRMs) are required to develop and validate analytical methods for the determination of *Fusarium* toxins in different foodstuffs that are reliable and capable to detect the toxins within their legal limits. Furthermore, CRMs can contribute to increase comparability and traceability in trichothecene analysis. In the framework of an ERM® project, a new certified reference material for *Fusarium* toxins in ground oat flakes (ERM®-BC720) was developed at the Federal Institute for Materials Research and Testing (BAM).

The reference material ERM®-BC720 was produced for the purpose of quality assurance and quality control for the determination of T-2 and HT-2 toxin in ground oat flakes. The material was prepared from ground oat flakes sampled from commercial sources intended for human consumption, not naturally contaminated with T-2 and HT-2 toxin. A small portion of the material was inoculated with spores of *Fusarium sporotrichioides* and incubated to obtain a nearly natural contamination with both toxins. The received contaminated material was subsequently analysed and blended with the remaining non-contaminated material.

To support in-house certification of the candidate material prepared at BAM a total number of 24 laboratories were selected based on documented experience and proficiency and invited to participate in an interlaboratory comparison study (ILC).

This report describes the preparation, characterisation and certification of the oat material including homogeneity and stability studies. The certified mass fractions for T-2 and HT-2 toxin, their uncertainties and the shelf lives were evaluated according to internationally accepted procedures.

Table 1: Particulars on T-2 and HT-2 toxin

Trivial name	IUPAC name	CAS number	Chemical formula	Molecular mass (g mol^{-1})
T-2 toxin	(2α,3α,4β,8α)-4,15-bis(acetyloxy)-3-hydroxy-12,13-epoxytrichothec-9-en-8-yl-3-methylbutanoate	21259-20-1	$C_{24}H_{34}O_9$	466.52
HT-2 toxin	15-Acetoxy-3α,4β-dihydroxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene	26934-87-2	$C_{22}H_{32}O_8$	424.48

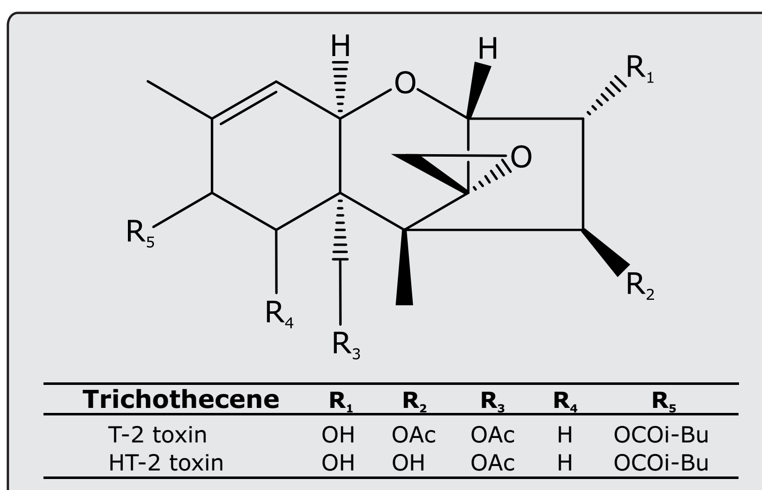

Figure 1: Molecular structure of T-2 and HT-2 toxin

2 Production of the candidate material

2.1 Material preparation

3.0 kg of oat flakes for human consumption (six aliquots, each of 500 g) out of a batch of 25 kg procured from German retail markets in 2010 were inoculated with spores of *Fusarium sporotrichioides* (DSM No.: 62425) and incubated over 3 weeks at 28 °C. After the incubation period, the material was frozen and freeze-dried. Both materials, inoculated/incubated and non-contaminated, were milled with a centrifugal mill (ZM 1000, Retsch® GmbH, Haan, Germany) to obtain a particle size smaller than 1.0 mm and subsequently analysed for their T-2/HT-2 toxin contents. Based on the results, 2,220 g of the inoculated/incubated material were mixed with about 21.98 kg of non-contaminated oat flakes for 20 hours using a drum hoop mixer to obtain final contents of 82 µg kg^{-1} (T-2 toxin) and 81 µg kg^{-1} (HT-2 toxin), respectively. The total quantity was then milled to a final particle size smaller than 0.5 mm and homogenised by means of a drum hoop mixer for 25 hours.

Further homogenisation and bottling of the candidate material were carried out by means of an 8-port rotary sample divider PT 100 (Retsch® GmbH) using the "cross riffling" procedure [van der Veen *et al.*] (Figure 2).

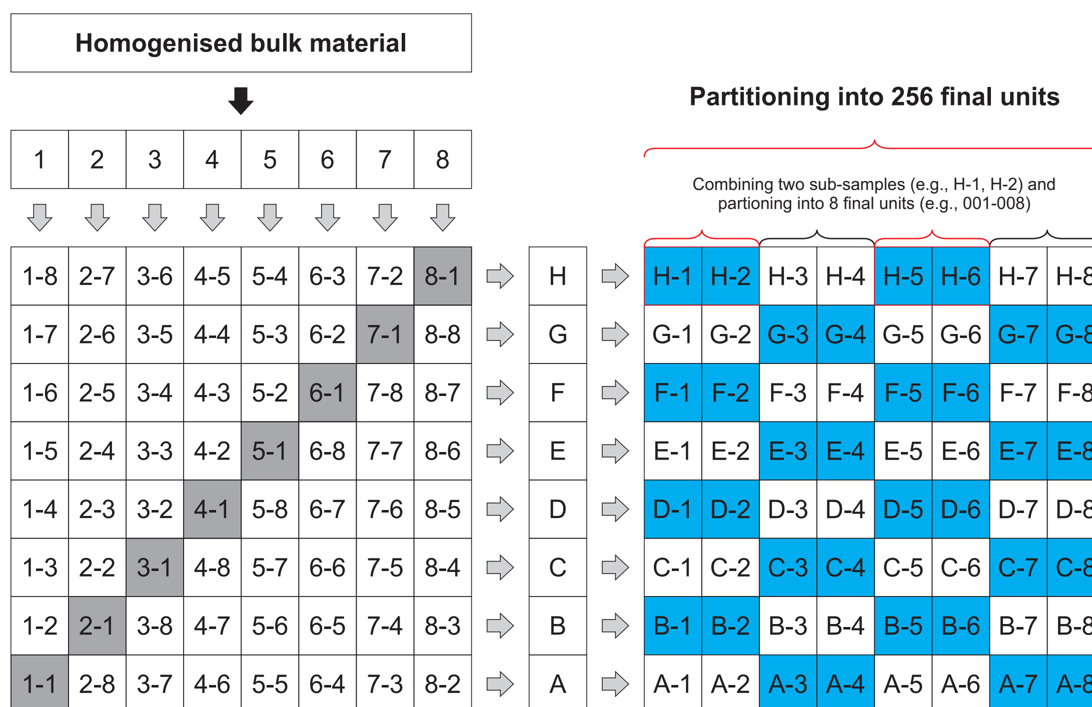

Figure 2: Cross-riffling scheme. The bulk material is divided into 8 sub-samples which are further partitioned and mixed again as depicted. The resulting 8 sub-samples (A-H) are subsequently partitioned by means of a spinning riffler with eight tubes (resulting in 8 x 8 sub-samples) and further divided into 256 sub-samples using a riffler with eight tubes and combining two sub-samples in a finally bottled unit.

After rinsing the 250 ml amber glass bottles with argon to expel oxygen from inside a total of 256 units were bottled containing each (94.2 ± 0.9) g. Bottles were sealed with screw caps containing PTFE-inlays and numbered in the order of leaving the bottling process. Immediately after bottling, the whole batch was stored at -21 °C in a freezer. For secondary

matrix characterisation, two bottles from the batch were selected and analysed by coulometric Karl Fischer titration using a 758 KFD Titrino (Metrohm AG, Herisau, Switzerland) revealing a water content of (8.37 ± 0.05) %. Table 2 summarizes the secondary matrix characterisation.

Table 2: Matrix characterisation of ERM®-BC720

Measurand	Value	Method
Particle size range	< 500 µm	Dry sieving
Water content	(8.37 ± 0.05) %	Coulometric Karl-Fischer-Titration

2.2 Analytical method

Analyses for homogeneity and stability studies as well as for certification purposes were carried out at BAM by high performance liquid chromatography hyphenated to positive electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) based on a stable isotope dilution analysis (SIDA).

Sample preparation

About 1 g of the ground and homogenized oat flakes were weighed into a 15 ml polypropylene centrifugation tube sealed with a screw cap. A 50 µl portion of an internal standard solution containing [$^{13}C_{24}$]-T-2 and [$^{13}C_{22}$]-HT-2 toxin was added to the sample. The first extraction was performed with 7 ml of acetonitrile : water (ACN:H_2O, 80:20, v:v) on an horizontal mixer (300 min^{-1}) for 90 min at room temperature. After extraction, the tubes were centrifuged at room temperature for 10 min (2.400 rpm / 1.288 x g) in a bench top centrifuge Sigma 6K15 (Sigma Laborzentrifugen GmbH, Osterode, Germany). The raw extract was transferred into a previously tared 12 ml screw top vial and evaporated to dryness under a gentle stream of nitrogen using a Reacti-Therm III heating unit at 60 °C and a Reacti-Vap III evaporating unit (Thermo Fisher Scientific Inc., Rockford, USA). After centrifugation, the resulting sample residue was extracted three more times each with 7 ml of extraction solvent for 15 min on a horizontal mixer (300 min^{-1}) at room temperature. After each extraction step, the supernatants were transferred in the screw top vial and treated as described above. The dried residue was weighed and re-dissolved in 1 ml of ACN:H_2O (80:20, v:v). After that, the mixture was vigorously shaken for 10 s on a Vortex shaker ("lab dancer", IKA® Werke GmbH & Co. KG, Staufen, Germany) and then sonicated for 5 min (SONOREX RK 52, Bandelin *electronic* GmbH & Co. KG, Berlin, Germany).

The cloudy suspension was transferred to a 1.5 ml snaplock microtube and centrifuged for 10 min by means of an Eppendorf Minispin plus centrifuge (Eppendorf, Hamburg, Germany) at 14.100 x g to separate the solid particles. The resulting clear supernatant fluid was decanted into a 1.5 ml HPLC vial and stored overnight in a freezer (-21 °C). After thawing 150 µl of the clear supernatant were transferred in a 1.5 ml HPLC vial with a 200 µl microinsert and subsequently analysed by HPLC-ESI-MS/MS (Tables 3 and 4).

Measurement and calibration

Table 3: Parameters of the HPLC-ESI-MS/MS system

	Instrument / Measurement conditions
HPLC	
Instrument	Agilent 1200
Column	Phenomenex® Gemini® C_{18} column (100 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 5 mM of ammonium acetate B = methanol (MeOH), containing 5 mM of ammonium acetate
Gradient program	see below
Flow	0.28 ml min^{-1}
Oven temperature	25 °C
Injection volume	5 µl
MS/MS detection	
Mass spectrometer	Applied Biosystems API 4000 QTRAP®
Ionisation	ESI positive(+)
Ion source temperature	400 °C
Modus	Multiple reaction monitoring (MRM)

Gradient program:

Time (min)	Eluent A (%)	Eluent B (%)
0.5*	75.0	25.0
0.5	75.0	25.0
2.0	38.0	62.0
14.0	35.0	65.0
14.1	75.0	25.0
24.0	75.0	25.0

* Equilibration of the HPLC-System

The following mass transitions were monitored and used for quantification:

Table 4: MRM transitions for native and isotopic mycotoxins

Compound	MRM transition (m/z)	Dwell time (ms)	DP (V)	CE (eV)	CXP (V)
T-2 toxin	484.1 $[M+NH_4]^+$ → 215.1[a]	100	41	27	14
	484.1 $[M+NH_4]^+$ → 305.1[b]	100	41	19	6
$[^{13}C_{24}]$-T-2 toxin	508.3 $[M+NH_4]^+$ → 322.2	100	46	19	8
HT-2 toxin	442.1 $[M+NH_4]^+$ → 215.0[a]	100	36	19	16
	442.1 $[M+NH_4]^+$ → 263.2[b]	100	36	19	6
$[^{13}C_{22}]$-HT-2 toxin	464.1 $[M+NH_4]^+$ → 278.3	100	46	17	6

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

Figure 3 shows a typical HPLC-ESI-MS/MS chromatogram of an oat flake extract for the mass-transitions listed in Table 4. The retention times of the stable isotopically labelled

internal standards, [$^{13}C_{24}$]-T-2 toxin and [$^{13}C_{22}$]-HT-2 toxin, are identical with those of the native compounds (Δt_R: ± 0.05 min).

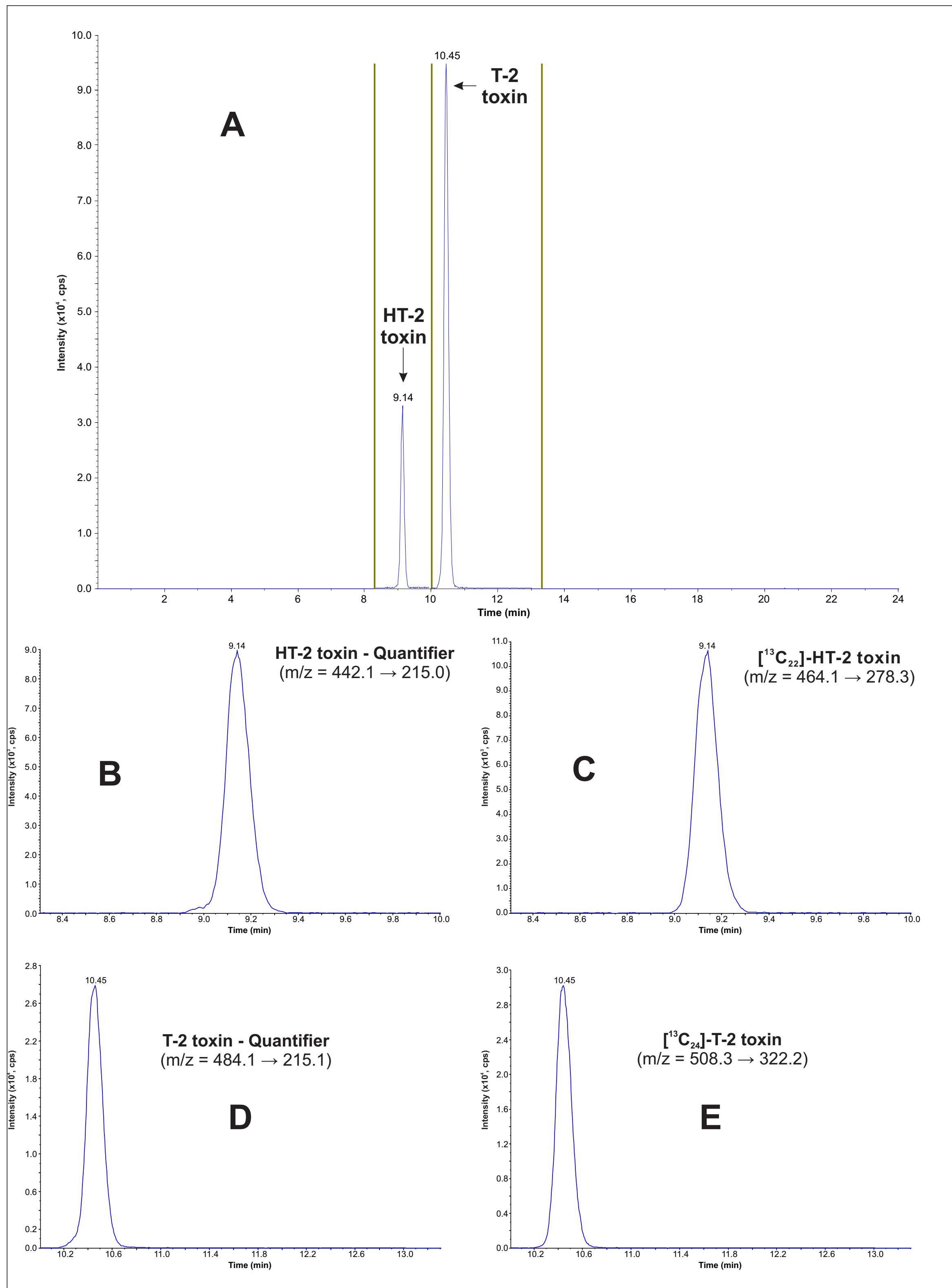

Figure 3: Typical HPLC-ESI-MS/MS total ion chromatogram (**A**) of an oat flake extract and extracted ion chromatograms showing the quantifier mass transitions for native and mass labelled T-2 and HT-2 toxin: **(B)** m/z = 442.1 → 215.0, (**C**) m/z = 464.1 → 278.3, (**D**) m/z = 484.1 → 215.1 and (**E**) m/z = 508.3 → 322.2.

Eight-point calibrations were used for quantification of the measured area ratios. Each calibration solution was freshly prepared by weighing. The calibration functions for T-2 and HT-2 toxin (Figures 4a-b) were assumed to be linear and obtained by regression analysis.

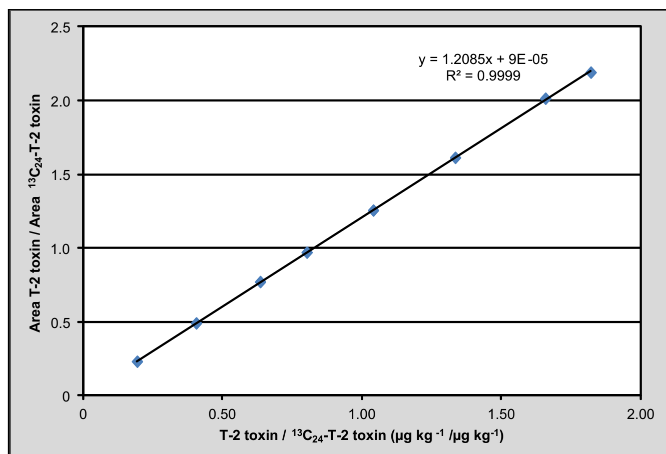

Figure 4a: Linear calibration function for T-2 toxin

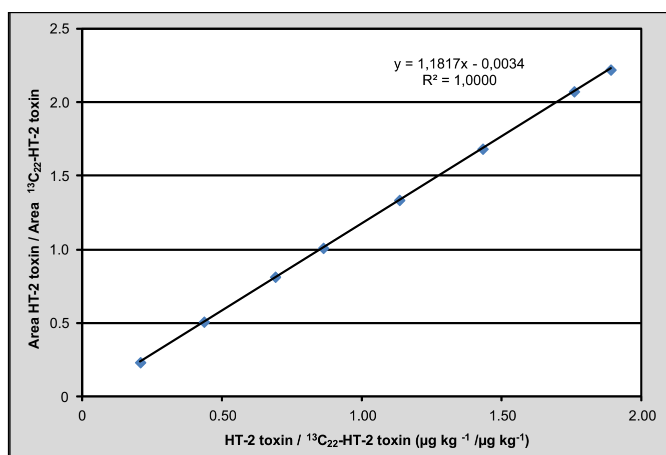

Figure 4b: Linear calibration function for HT-2 toxin

The stable isotopically labelled internal standards, [$^{13}C_{24}$]-T-2 and [$^{13}C_{22}$]-HT-2 toxin, were used for quantification of the respective native compounds. The native mycotoxin calibration standards (T-2 toxin: 99.0 %, HT-2 toxin: 98.8 %) were purchased from Biopure (Tulln, Austria).

2.3 Minimum sample size

The minimum sample intake for one determination should be chosen in a way that no significant heterogeneity within the bottle is to be expected. Homogeneity measurements were successfully evaluated for 1 g sample intake for a single determination. Therefore, a minimum sample intake of 1 g is recommended.

3 Homogeneity study

Based upon thorough batch homogenisation, and the results of preliminary studies, a satisfactory level of sample homogeneity was expected. For further quantitative demonstration, 16 units were selected randomly from the whole set of 256 bottles and analysed four times each according to the analytical method described before (Section 2.2).

All 16 units were extracted and processed once under repeatability conditions followed by the second set of extractions and processed in a randomised manner again under repeatability conditions.

Processed extracts were analysed by HPLC-ESI-MS/MS (MRM mode) under repeatability conditions guaranteeing that all 64 extracts were quantified versus one calibration after randomisation. The ANOVA results are given in Table 5 together with the estimations of the contributions due to the between-bottle inhomogeneity (u_{bb}), assessed according to [ISO Guide 35]. For raw data see Annex A.

Table 5: Analysis of variance (ANOVA) and estimates for uncertainty contribution according to ISO Guide 35

Compound	MS_{among} (µg² kg⁻²)	MS_{within} (µg² kg⁻²)	Test statistic MS_{among} / MS_{within}	Critical value F(*f1, f2; 5%*)	u^*_{bb} (µg kg⁻¹)	u_{bb} (µg kg⁻¹)	u_{bb_rel} (%)
T-2 toxin	8.473	4.812	1.7608	1.8802	0.496	0.957	1.269
HT-2 toxin	14.781	8.543	1.7302	1.8802	0.660	1.249	1.545

For calculation of u_{bb} the following equations were applied:

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

u_{bb}: Inhomogeneity estimate, for MS_{among} > MS_{within}

u^*_{bb}: Inhomogeneity estimate, for MS_{among} < MS_{within}

MS_{among}: Mean of squared deviations between bottles

MS_{within}: Mean of squared deviations within bottles

n: Number of replicate sub-samples per bottle

N: Number of bottles selected for homogeneity study (here N = 16)

Because the test statistic is lower than the critical value, no significant inhomogeneity of the batch was detected. A contribution u_{bb} to the overall uncertainty of the certified reference material was nevertheless derived from the ANOVA results and included in the uncertainty budget of the certified value. For that purpose, the maximum values of u_{bb} and u^*_{bb} have been calculated on the basis of Equations 1 and 2.

4 Stability study

4.1 Initial stability study

From experience, a temperature-driven deterioration of the mycotoxin contents had to be expected also for this material. Selected units of the candidate material were submitted to an accelerated ageing at temperatures between 4 and 60 °C over periods of 1 week to 1.5 months (short-term study) and 1 month to 12 months (long-term study) as shown in Table 6. Samples were measured following the so-called isochronous scheme [Lamberty *et. al.*]. After the respective periods of time individual units were stored at -21 °C. All units were analysed for T-2 and HT-2 toxin in quadruplicate using the method described above under repeatability conditions. Annex B shows the raw data of the initial stability study.

Table 6: Accelerated ageing of exposed samples to perform an isochronous stability study

Ageing (months)	Storage temperature (°C)				Remark
	4	23	40	60	
0.25	x	x	x	x	Initial study
0.50	x	x	x	x	
0.75	x	x	x	x	
1	x	x	x	x	
3	x	x	x	x	
6	x	x	x	x	
12	x	x	x	x	
24	x	x			Post-certification monitoring
36	x	x			
48	x	x			
60	x	x			

Data processing and result assessment were carried out in accordance with [Bremser *et al.*] assuming an *Arrhenius* model for the dependence of the reaction rate $k(T)$ on the temperature. The plots of the logarithms of the reaction rate $ln(k_{eff})$ over the inverse temperature for T-2 and HT-2 toxin are given in Figures 6a-b.

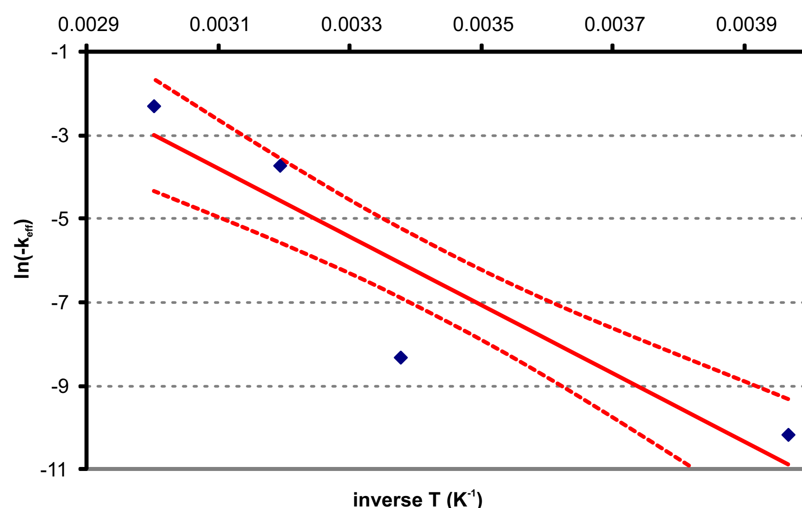

Figure 6a: Effective reaction rate for T-2 toxin in dependence on the inverse temperature (semi-logarithmic plot)

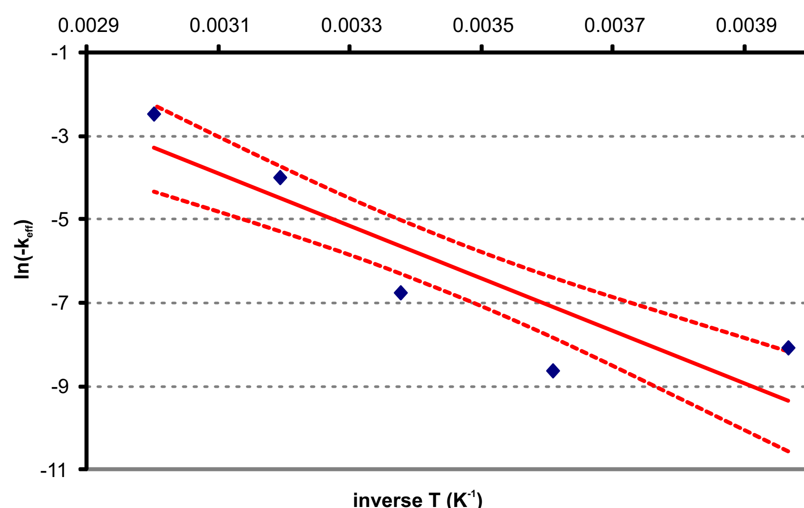

Figure 6b: Effective reaction rate for HT-2 toxin in dependence on the inverse temperature (semi-logarithmic plot)

The graphs contain values for -21 °C since measurements at an even lower storage temperature of -80 °C were available and used as reference. Both temperature dependencies can merely be approximated by a straight line. The corresponding confidence interval for the line is also given in the figure. The estimated activation energies ΔE are 67.9 kJ mol^{-1} (T-2 toxin) and 52.2 kJ mol^{-1} (HT-2 toxin). These values are in acceptable agreement with activation energies determined for a large variety of organic compounds. By using these data and the assumed 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 Figure 6. The results are given in Table 7.

Table 7: Estimation of shelf life of T-2 as well as HT-2 toxin

Temperature (°C)	Expiry (months)	
	T-2 toxin	HT-2 toxin
-21	493	153
4	49	26
23	8	7
40	2	2
60	0	0

Note that (comp. [Bremser *et al.*]) calculations have been carried out with the effective degradation rates as given by the upper confidence limits of graph 6a and 6b, thus, the estimates as given in Table 7 are worst-case. The data table will be updated during post-certification monitoring. Shelf life at a storage temperature of 4 °C is considerable but not quite enough for a desirable minimum shelf life of 5 years. This shelf life can reliably be assumed at a storage temperature of -21 °C for both mycotoxins. However, exposure to temperatures higher than room temperature may reduce the time of validity of ERM®-BC720 drastically. Therefore, a common user-end expiry date of **one year after delivery from**

storage is established provided the sample is stored equal to or lower than -18 °C at the user's site. Transportation/delivery time should be kept at the possible minimum and any exposure to heat should be avoided.

4.2 Post-certification stability monitoring

The first rough estimation of stability will be updated by annual measurements of units stored at -21 °C (reference), 4 °C and 23 °C over the period of availability of the material.

5 Certification study

5.1 Design of the study

The assignment of the certified T-2 and HT-2 toxin mass fractions of the oat flakes reference material based upon an in-house study at BAM using HPLC-ESI-MS/MS analysis including ^{13}C-labelled T-2 and HT-2 toxin as internal standards. For in-house certification purposes four units of the candidate reference material were analysed. From each unit 20 subsamples were taken, resulting in a total of 80 analyses. Simultaneously, an interlaboratory comparison study (ILC) involving 24 expert laboratories was conducted in order to support the in-house certification study at BAM. Each ILC-participant received two units of the candidate reference material (sample_1 and sample_2) for analyses. The measurements had to be performed on three different days (one analysis per day and unit). Information was provided to the laboratories that the T-toxin level of the samples is expected below 250 µg kg^{-1}. For measurement control purposes, two HPLC vials containing T-2 toxin and HT-2 toxin in acetonitrile were dispatched for direct analysis. Results returned to BAM were scrutinised for consistency.

5.2 Participants of supporting ILC

A total number of 24 laboratories (Table 8) were selected to participate in the ILC based on their approved expertise in the field of mycotoxin analysis.

Table 8: Participants of the interlaboratory comparison study for certification of ERM®-BC720

Laboratory	City, Country	Compound	
		T-2 toxin	HT-2 toxin
AGES Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH	Linz, Austria	x	x
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	Oberschleißheim, Germany	x	x
Chemisches und Veterinäruntersuchungsamt Rheinland	Leverkusen, Germany	x	x
chemlab GmbH	Bensheim, Germany	x	x
Coop	Pratteln, Switzerland	x	x
Eurofins Analytik GmbH Wiertz-Eggert-Jörissen	Hamburg, Germany	x	x
Food GmbH	Jena, Germany	x	x
Institut Kirchhoff Berlin GmbH	Berlin, Germany	x	x
Kantonales Laboratorium Thurgau	Frauenfeld, Switzerland	x	x

Laboratory	City, Country	Compound	
		T-2 toxin	HT-2 toxin
Landesamt für Landwirtschaft, Lebensmittel-sicherheit und Fischerei Mecklenburg-Vorpommern	Rostock, Germany	x	x
Landesamt für Verbraucherschutz Sachsen-Anhalt	Halle (Saale), Germany	x	x
Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer	Speyer, Germany	x	x
Landwirtschaftliches Technologiezentrum Augustenberg	Karlsruhe, Germany	x	x
Lebensmittelversuchsanstalt	Wien, Austria	x	x
LUFA NORD-WEST	Hameln, Germany	x	x
LUFA-ITL GmbH	Kiel, Germany	x	x
Max Rubner Institut	Detmold, Germany	x	x
Niedersächsisches Landesamt für Verbraucher-schutz und Lebensmittelsicherheit	Stade, Germany	x	x
R-Biopharm AG	Darmstadt, Germany	x	
SGS Germany GmbH. Laboratory Service Hamburg	Hamburg, Germany	x	x
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft	Leipzig, Germany	x	x
Staatliches Veterinäruntersuchungsamt Arnsberg	Arnsberg, Germany	x	x
Stadt Bochum, Chemisches Untersuchungsamt	Bochum, Germany	x	x
Thüringer Landesanstalt für Landwirtschaft	Jena, Germany	x	x

5.3 Methods used by ILC-participants

The participants of the ILC applied methods of their own choice with own calibration standards of known purities and with various sample intakes. The reported sample intake was in the range between 2.5 g and 25 g, whereas 52 % of the participants used a sample weight of 10 g. Predominant extraction method for both compounds was shaking in combination with acetonitrile : water mixtures (e. g. 84:16, v:v). Sample preparation, generally including dilution of extract, clean-up and derivatisation steps, was handled in different ways. As clean-up methods, solid phase extraction (SPE) or immunoaffinity columns (IAC) were mostly used to purify the extracts for T-2 and HT-2. In some cases (HPLC-MS/MS using internal standards) the use of a clean-up procedure was completely omitted.

For separation of the purified extract, mostly HPLC but also gas chromatography (GC) was applied. Different types of detectors (HPLC: FLD, MS, MS/MS; GC: MS) were used for T-2 and HT-2 toxin depending on sample preparation and separation technique. HPLC with fluorescence detection (FLD) provides high sensitivity, selectivity and repeatability of measurements, but it is not applicable to the detection of T-2 and HT-2 toxin trichothecenes, owing to the lack of fluorophore groups in their chemical structure. The possibility of using HPLC-FLD for the determination of T-2 and HT-2 toxin requires therefore suitable derivatisation reagents. As a fluorescent-labeling reagent for T-2 and HT-2 1-anthroylnitrile or anthracene-9-carbonyl cyanide were used within the ILC. Laboratories using GC-MS for the

T-toxin analysis require a derivatisation to increase the volatility and thermal stability of both compounds. For this purpose, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) plus 1 % trimethylchlorosilane (TMCS) as catalyst or trifluoroacetic anhydride (TFAA) was preferably used. The HPLC-MS/MS is suited for a sensitive and selective measurement of both compounds and was frequently used by participants (68 %).

Table 9: Extraction and determination methods used in the ILC

Entry	Analytical method	Extraction method	Extraction solvent	Clean-up method
A	HPLC-MS/MS	Shaking	ACN:H_2O	SPE
B	HPLC-MS/MS	Shaking	ACN:H_2O (84:16, v:v)	-
C	HPLC-MS/MS	Shaking	ACN	Dispersive method
Da[a]	GC-MS	Stirring	ACN:H_2O (84:16, v:v)	SPE + IAC
Db[a]	HPLC-MS/MS	Stirring	ACN:H_2O (84:16, v:v)	-
E	HPLC-MS/MS	Shaking	ACN:H_2O	Dispersive method
F	GC-MS	Shaking	ACN:H_2O (84:16, v:v)	SPE
G	HPLC-FLD	Shaking	MeOH:H_2O (90:10, v:v)	IAC
H	HPLC-MS/MS	Shaking	ACN:H_2O (80:20, v:v)	SPE
I	HPLC-MS/MS	PFE[b]	ACN:H_2O	-
J	HPLC-MS/MS	Shaking	ACN:H_2O	-
K	HPLC-MS/MS	Shaking	ACN:H_2O (84:16, v:v)	SPE
L	HPLC-MS/MS	Shaking	ACN:H_2O	SPE
M	HPLC-FLD	Ultra-Turrax® agitation	MeOH:H_2O (90:10. v:v)	IAC
N	HPLC-MS/MS	Shaking and sonication	ACN:H_2O (84:16, v:v)	-
O[c]	ELISA	Shaking	MeOH:H_2O (70:30. v:v)	-
P	HPLC-FLD	Shaking	ACN:H_2O	SPE
Q	HPLC-MS/MS	Shaking	ACN:H_2O (84:16, v:v)	SPE
R	GC-MS	Shaking	MeOH:H_2O	IAC
S	HPLC-MS/MS	Ultra-Turrax® agitation	MeOH:H_2O (80:20, v:v)	IAC
T	HPLC-MS/MS	Shaking	ACN:H_2O	SPE
U	HPLC-MS/MS	Shaking and sonication	ACN:H_2O	SPE
V	GC-MS	Stirring	ACN:H_2O	SPE
W	HPLC-MS/MS	Shaking	ACN:H_2O (84:16, v:v)	SPE
X	HPLC-MS/MS	Shaking and Ultra-Turrax® agitation	ACN:H_2O:HAc (79:20:1,. v:v:v)	-

[a] One laboratory submitted two sets of results obtained with different analytical procedures; [b] PFE – pressurised fluid extraction; [c] Laboratory reported results only for T-2 toxin

5.4 Evaluation of ILC results

The submitted results of the supporting ILC were technically and statistically evaluated in accordance with ISO Guide 35, ISO 13528, and the specific requirements of the ERM agreement (for detailed information see: http://www.erm-crm.org).

Figure 7 depicts (in a Youden plot arrangement) the results of the laboratories, namely their findings on the control solutions for T-2 and HT-2, and their values obtained for the unknown sample against the determinations for the control solutions. All values are normalised against the gravimetric value (control solutions) or the value assigned based on the in-house study (unknown sample).

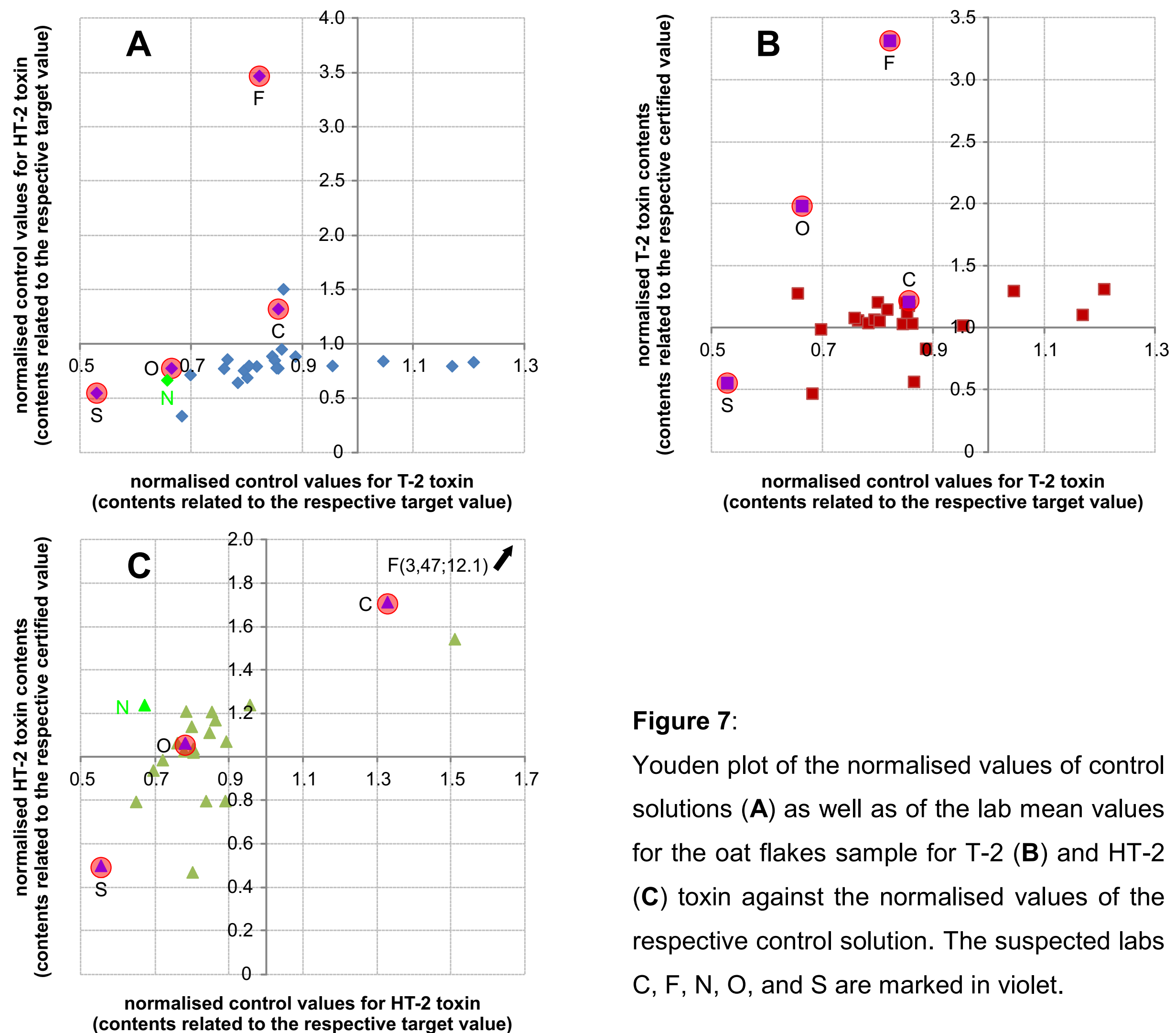

Figure 7:
Youden plot of the normalised values of control solutions (**A**) as well as of the lab mean values for the oat flakes sample for T-2 (**B**) and HT-2 (**C**) toxin against the normalised values of the respective control solution. The suspected labs C, F, N, O, and S are marked in violet.

On the basis of a thorough inspection of the data provided by the laboratories, five data sets have been deleted on technical grounds. This refers to:

- laboratory C which reveals large differences in-between the control solutions and in-between the unknowns
- laboratory F which reveals maximum deviation at the high end for the control solution HT-2 and for both T-2 and HT-2 in the unknowns
- laboratory O is low on all control solutions but high on the unknowns, in particular T-2
- laboratory N which is similarly, and even slightly worse, underperforming as laboratory O for both control solutions
- laboratory S which reveals low values for all control solutions and all unknowns.

The accepted data sets together with the corresponding unweighted means are given in Table 10.

Table 10: Accepted laboratory data sets of ILC for T-2 and HT-2 toxin

T-2 toxin							
Entry	**Values[a] (µg kg^{-1})**						**Mean (µg kg^{-1})**
A	108.90	102.80	105.40	109.90	109.40	104.40	106.80
B	82.00	82.20	77.60	82.80	83.30	81.00	81.48
Da	92.30	83.60	87.00	90.50	81.00	89.90	87.38
Db	94.50	88.80	74.20	84.10	88.90	79.50	85.00
E	79.10	88.70	89.40	82.60	89.00	84.90	85.62
G	80.30	78.60	97.00	75.70	79.50	92.80	83.98
H	100.00	98.00	92.00	94.00	107.00	105.00	99.33
I	73.60	64.00	68.50	66.30	69.10	70.40	68.65
J	42.00	36.00	-	43.00	35.00	-	39.00
K	96.90	86.70	109.20	86.70	87.80	100.00	94.55
L	91.90	89.80	87.60	86.50	85.70	85.90	87.90
M	51.80	81.80	9.00	50.60	36.40	51.30	46.82
P	95.60	95.50	99.00	96.60	95.70	100.00	97.07
Q	98.20	104.60	101.90	93.30	95.30	100.60	98.98
R	91.30	90.00	88.70	91.90	91.10	93.10	91.02
T	95.30	100.30	124.10	80.10	117.30	130.80	107.98
U	83.50	90.30	88.30	83.50	89.30	86.40	86.88
V	88.30	86.20	81.90	91.50	87.20	76.60	85.28
W	89.00	81.00	92.00	90.00	85.00	97.00	89.00
X	99.00	83.00	93.00	90.00	96.80	92.80	92.43
						Mean of laboratory means:	**85.76**
HT-2 toxin							
Entry	**Values[a] (µg kg^{-1})**						**Mean (µg kg^{-1})**
A	92.40	88.70	86.80	89.80	95.10	87.50	90.05
B	84.50	73.20	77.60	88.60	80.00	75.20	79.85
Da	96.50	91.70	98.50	95.40	88.80	97.50	94.73
Db	95.70	92.70	83.30	83.80	85.80	79.20	86.75
E	66.40	61.30	68.60	70.10	61.80	57.90	64.35
G	94.40	74.30	77.10	90.00	89.50	78.40	83.95
H	77.00	86.00	78.00	56.00	73.00	86.00	76.00
I	68.10	60.90	66.50	66.10	60.70	65.60	64.65
J	72.00	77.00	-	76.00	76.00	-	75.25
K	94.70	84.20	96.80	89.50	94.70	93.70	92.27
L	86.70	88.70	83.60	88.90	88.30	80.90	86.18
M	118.80	253.60	18.90	143.10	72.20	142.10	124.78
P	85.30	86.70	86.60	85.10	87.60	85.50	86.13
Q	109.20	98.70	92.90	100.50	91.80	93.40	97.75
R	61.00	34.20	35.90	54.60	25.00	18.20	38.15
T	66.20	41.70	91.70	51.30	48.80	88.00	64.62
U	86.80	79.20	78.30	84.00	87.70	80.20	82.70
V	102.00	99.00	107.90	100.00	95.00	98.00	100.32
W	86.00	82.00	75.00	81.00	90.00	85.00	83.17
X	136.80	51.30	88.90	99.60	115.70	95.30	97.93
						Mean of laboratory means:	**83.48**

[a] Single values of each laboratory are corrected for recovery and purity of the individual calibration standards (purity was considered by the laboratories).

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 and the assigned value x_2 according to:

$$E_n = \frac{|x_1 - x_2|}{2\sqrt{s_{ILC}^2 + u_c^2}}$$

(with s_{ILC}: standard deviation of the mean of accepted laboratory means in the ILC, u_c : uncertainty of the assigned value, and the factor 2 converts the standard uncertainties in the denominator into expanded uncertainties). The resulting E_n criteria were determined to be 0.494 for T-2 toxin and 0.348 for HT-2 toxin, respectively. Therefore, the outcome of the ILC is fully consistent with the in-house certification results based on the SIDA using HPLC-MS/MS at BAM. The mean values of 80 results were determined to be 82 µg kg^{-1} (T-2 toxin) and 81 µg kg^{-1} (HT-2 toxin), respectively (Annex C).

5.5 Certified values and uncertainty budget

The combined uncertainty is calculated based on the data of the in-house certification study according to Equation 3:

$$u_c^2 = u_x^2 + u_{bb}^2 + u_{lts}^2 + u_{cal}^2 + u_{pur}^2 + u_{handling}^2 \qquad \textbf{(3)}$$

The results are given in Table 11.

Table 11: Uncertainty contributions for calculation of the combined uncertainty

Uncertainty contribution		**T-2 toxin**		**HT-2 toxin**	
		%	**µg kg^{-1}**	**%**	**µg kg^{-1}**
Uncertainty of characterisation (standard deviation of the mean[a])	u_x	0.64	0.52	0.69	0.56
Contribution from a possibly undetected inhomogeneity	u_{bb}	1.27	1.04	1.55	1.25
Contribution from long-term stability (sufficiently stable for shelf lives up to 5 years)	u_{lts}	0.00	0.00	0.00	0.00
Calibration uncertainty	u_{cal}	0.98	0.81	0.84	0.68
Uncertainty of the purity of used native calibration standard[b]	u_{pur}	0.90	0.74	1.10	0.89
Contribution from handling of samples (weighing, volumetric operations, aliquoting internal standard)	$u_{handling}$	1.00	0.82	1.00	0.81
Total	$\boldsymbol{u_c}$	**2.19**	**1.79**	**2.40**	**1.94**

[a] The mean value standard deviation is calculated from the four unit (bottle) means (divided by $\sqrt{4}$).
[b] At the same time traceability contribution.

The calibration uncertainty u_{cal} is the uncertainty of a typical determination in the centre of the analytical range, for a typical calibration curve as shown in Figures 4a and 4b. It is

calculated from the uncertainties of intercept (u_{ic}) and slope (u_{sl}) of the line, the covariance between them, and the uncertainty of the measured response r (residual scatter of the calibration curve) according to:

$$u_{cal}^2 = \frac{u_r^2 + u_{ic}^2}{sl^2} + \frac{(r - ic)^2}{sl^4} \cdot u_{sl}^2 + \frac{r - ic}{sl^3} \cdot cov(ic, sl)$$

with r standing for the response, ic the intercept, and sl the slope of the line.

The uncertainty from handling is a combined, rather worst-case, estimate for all gravimetric and volumetric sample handling procedures.

The final certified values for ERM®-BC720 are summarised in Table 12 together with the expanded uncertainty U_{ERM} calculated based on a coverage factor k = 2. The values and the expanded uncertainties are rounded according to the recommendations of the Guide to the Expression of Uncertainty in Measurement [ISO Guide 98] and are given with respect to raw sample mass.

The water content was seen to remain stable if the material is handled according to the instructions in the certificate (see also Clause 6).

Table 12: Certified mass fractions of ERM®-BC720

Compound	Mass fraction (µg kg^{-1})		
	Certified value	**Uncertainty**	**Expanded uncertainty**
T-2 toxin	82	2	4
HT-2 toxin	81	2	4

5.6 Traceability

Beside the fact that all laboratories, which provided accepted data, used validated and calibrated methods, traceability of the certified values was directly established to stated references of the pure mycotoxins using the BAM certification method – stable isotope dilution analysis using ^{13}C-isotopically labelled internal standard for HPLC-MS/MS measurement. These measurements derived traceability from calibration with pure reference substances (T-2 toxin: 99.0 %, HT-2 toxin: 98.8 %; Biopure) with purities independently confirmed by UV-absorption and HPLC-MS (scan mode; ESI+/-) measurements. The certified values for the mass fractions of T-2 and HT-2 toxin are traceable via the common, certified calibrants used. Mass fractions of the common, certified calibrants are certified for T-2 and HT-2 toxin in acetonitrile. The certified values of the calibrants are traceable to the International System of Units (SI), as stated on the respective certificate, due to the gravimetric preparation employed. Therefore, the mass fractions of both toxins in the CRM are traceable to the SI.

6 Information on the proper use of ERM®-BC720

6.1 Shelf life

From the initial stability study, a considerably large shelf life well above a period of 5 years at a storage temperature of -21 °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 12 months beginning with the dispatch of the material from BAM. The validity of this information will be maintained by post-certification monitoring.

6.2 Transport and storage conditions

Due to the proved stability of the reference material a cooled dispatch is not necessary during transport. On receiving, the bottle has to be stored at a temperature equal to or lower than -18 °C. Before withdrawing a sub-sample, the bottle should be allowed to reach room temperature and be mixed thoroughly. Thereafter, the bottle must be closed tightly and stored at a temperature equal to or lower than -18 °C. The water content remains stable when the material is treated as described. 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.

6.3 Use of the material

This material is intended to be used for performance control and validation purposes. Samples should be allowed to equilibrate to ambient temperature (e. g. overnight) before opening to avoid water condensation. The content of the bottle used should be thoroughly mixed before sub-samples of at least 1 g are taken. The oat flakes flour should be weighed out immediately after opening the bottle and the mass fractions of the toxins have to be calculated based on this mass.

6.4 Safety instructions

The usual laboratory safety precautions apply. 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 T-2 and HT-2 toxin. Although the mycotoxin content in the sample is at trace levels, any use other than the intended of the content of the bottles should be avoided. Personnel handling of the material must adequately be trained and follow regular laboratory safety precautions. It is strongly recommended to handle and dispose of the reference material in accordance with the guidelines for hazardous materials legally in force at the site of end use and disposal.

6.5 Legal notice

Neither the Federal Institute for Materials Research and Testing (BAM) nor any person acting on their behalf makes any warranty or representation, express or implied, that the use of any information, material, apparatus, method or process disclosed in this document may not

infringe privately owned rights, or assume any liability with respect to the use of, or damages resulting from the use of any information, material, apparatus, method or process disclosed in this document.

7 References

Bremser, W.; Becker, R.; Kipphardt. H.; Lehnik-Habrink, P.; Panne, U.; Töpfer, A. (2006): Stability testing in an integrated scheme. Accreditation and Quality Assurance, 11(10): 489-495.

European Regulation No. 1126/2007/EC (2007)
Commission regulation from 28.09.2007, amending regulation (EC) No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products.

European Regulation No. 1881/2006/EC (2006)
Commission regulation from 19.12.2006, setting maximum levels for certain contaminants in foodstuffs.

ISO/IEC Guide 98-3 (2008)
Uncertainty of measurement - Part 3: Guide to the expression of uncertainty in measurement.

ISO/REMCO, ISO Guide 35 (2006):
Reference materials - General and statistical principles for certification.

Lamberty, A.; Schimmel, H.; Pauwels, J. (1998):
The study of the stability of reference materials by isochronus measurements. *Fres. J. Anal. Chem*.. 360: 359-361.

van der Veen, A.M.H.; Nater, D.A.G. (1993):
Sample preparation from bulk samples: an overview. *Fuel Process Technol*. 36: 1–7.

ISO 13528:2005
Statistical methods for use in proficiency testing by interlaboratory comparisons.

8 Annexes

Annex A: Raw data of homogeneity testing for T-2 and HT-2 toxin in ERM®-BC720

	T-2 toxin content (µg kg^{-1})						
Bottle-No.	**1**	**2**	**3**	**4**	**Mean**	**SD**	**RSD (%)**
15	78.18	74.90	76.90	76.15	76.53	1.38	1.80
33	70.95	71.76	76.95	72.70	73.09	2.67	3.65
50	74.97	74.21	77.86	78.42	76.37	2.08	2.73
60	74.39	73.76	73.45	70.44	73.01	1.76	2.41
77	71.90	71.22	75.21	77.69	74.01	3.01	4.07
99	78.41	75.92	76.98	74.76	76.52	1.55	2.03
110	77.95	73.55	72.72	76.86	75.27	2.53	3.36
128	72.79	74.93	71.17	78.38	74.32	3.12	4.19
139	76.84	75.84	77.08	77.41	76.79	0.68	0.88
145	73.76	78.01	76.07	76.93	76.19	1.81	2.37
172	73.64	76.81	69.78	74.13	73.59	2.90	3.94
191	72.91	74.47	76.41	76.41	75.05	1.69	2.26
210	79.00	76.90	78.49	75.83	77.56	1.46	1.88
222	74.00	76.51	76.58	76.27	75.84	1.24	1.63
237	77.78	75.00	71.85	74.13	74.69	2.45	3.28
256	77.67	73.23	77.23	80.19	77.08	2.88	3.74
					75.37	**2.38**	**3.16**

	HT-2 toxin content (µg kg^{-1})						
Bottle-No.	**1**	**2**	**3**	**4**	**Mean**	**SD**	**RSD (%)**
15	83.55	82.04	82.62	80.97	82.30	1.08	1.31
33	75.90	78.08	82.54	80.64	79.29	2.91	3.66
50	79.95	79.95	84.61	85.19	82.42	2.87	3.48
60	77.36	79.10	76.89	75.38	77.18	1.53	1.99
77	77.75	76.16	78.98	84.18	79.27	3.47	4.38
99	83.45	82.92	82.89	80.34	82.40	1.40	1.70
110	86.46	77.19	76.59	83.37	80.90	4.81	5.94
128	76.36	79.26	74.99	85.59	79.05	4.71	5.96
139	83.44	83.32	83.78	81.53	83.02	1.01	1.22
145	77.83	82.86	81.87	88.23	82.70	4.28	5.18
172	77.06	81.33	74.19	78.05	77.66	2.95	3.79
191	78.46	80.22	82.59	82.91	81.04	2.10	2.59
210	83.56	82.13	83.80	82.09	82.90	0.91	1.10
222	78.83	83.55	80.89	82.44	81.43	2.05	2.52
237	80.56	79.59	77.08	80.66	79.47	1.67	2.10
256	81.85	77.70	81.92	87.71	82.29	4.12	5.00
					80.83	**3.17**	**3.92**

Annex B: Raw data of stability testing for ERM®-BC720. The mass fractions of T-2 and HT-2 toxin are given in µg kg^{-1}.

T-2 toxin	Storage temperature (°C)					
Time (months)	-80	-21	4	23	40	60
0.25			*	73.87	74.65	69.55
0.25			72.58	73.26	73.18	70.92
0.25			72.48	74.42	73.46	70.83
0.25			72.75	73.54	74.06	72.14
0.5			73.36	75.23	74.16	68.72
0.5			73.31	73.90	73.65	67.94
0.5			75.30	73.83	72.90	69.76
0.5			74.01	74.41	71.78	71.89
0.75			74.86	75.51	75.51	68.60
0.75			73.76	77.10	72.47	68.77
0.75			73.76	74.44	70.58	65.38
0.75			74.94	76.14	71.05	63.51
1		76.74	78.01	70.68	71.13	63.13
1		75.77	74.28	72.07	71.52	59.51
1		76.04	73.85	75.07	74.45	60.46
1		74.04	73.21	74.24	69.89	64.45
3			78.92	77.33	73.65	49.35
3			78.22	77.61	71.48	48.76
3			75.14	75.46	72.49	47.27
3			77.25	76.30	72.83	47.76
6			76.98	78.92	65.51	33.97
6			78.93	78.22	66.71	33.17
6			75.54	75.14	65.04	33.16
6			78.74	77.25	67.18	32.87
9			75.97	72.93	59.01	29.16
9			75.90	73.39	59.02	30.58
9			79.14	74.53	59.75	30.43
9			75.70	75.86	59.85	29.98
12	76.97	76.91	76.07	74.57	56.49	21.64
12	75.47	76.78	78.28	76.10	55.59	21.42
12	77.00	75.25	73.87	75.58	56.94	22.00
12	75.89	77.83	78.94	75.34	55.76	22.01

* - The point at 4 °C for T-2 toxin is missing since the data as measured suggested a deterioration rate smaller than zero (i.e. an increase of the T-2 toxin content) which is fully counterintuitive with respect to all other results of the study.

HT-2 toxin	Storage temperature (°C)					
Time (months)	-80	-21	4	23	40	60
0.25			74.75	82.92	83.52	79.70
0.25			81.74	81.54	81.62	81.26
0.25			83.03	84.21	83.68	84.90
0.25			82.33	83.77	84.80	81.01
0.5			81.00	82.90	83.70	74.24
0.5			82.65	83.43	83.38	77.88
0.5			84.02	82.93	83.32	80.71
0.5			81.23	83.59	84.28	80.73
0.75			84.52	83.35	83.21	79.27
0.75			82.32	84.71	82.88	77.41
0.75			85.04	84.03	81.43	75.82
0.75			84.66	86.01	80.70	71.41
1		84.58	85.13	78.37	80.58	73.39
1		84.28	83.27	82.67	81.47	67.10
1		84.69	84.72	84.06	84.24	71.37
1		84.40	82.81	83.97	81.95	76.79
3			80.88	81.75	75.07	53.39
3			83.08	84.04	77.73	54.52
3			82.04	86.97	78.61	57.37
3			84.08	83.53	78.05	55.84
6			81.14	82.72	72.85	40.39
6			82.71	83.90	76.69	39.58
6			82.85	83.01	74.05	41.34
6			84.30	84.97	75.62	41.38
9			83.68	82.77	68.35	37.31
9			83.36	80.80	73.05	39.22
9			84.95	81.79	69.45	38.72
9			82.95	84.41	72.00	39.04
12	81.52	83.18	82.27	82.63	67.10	28.82
12	84.46	83.39	83.02	83.90	67.67	30.36
12	80.35	82.74	82.65	84.87	66.42	30.94
12	84.09	82.46	83.64	80.53	65.82	31.30

Annex C: Results of characterisation measurements for T-2 and HT-2 toxin.

T-2 toxin content (µg kg^{-1})								
Bottle-No.	**39**		**52**		**126**		**243**	
#	**run A**	**run B**	**run A**	**run B**	**run A**	**run B**	**run A**	**run B**
1	81.671	81.050	80.296	80.998	82.533	82.461	78.946	77.553
2	75.007	74.863	81.630	82.386	84.923	82.497	77.379	77.521
3	80.491	78.428	78.849	78.868	78.821	78.390	81.627	83.458
4	80.683	82.373	80.614	82.907	81.816	83.025	82.049	81.580
5	80.080	80.176	82.295	85.305	76.964	76.236	81.330	82.983
6	82.581	82.802	81.719	82.802	83.519	84.910	83.490	85.893
7	84.737	84.290	82.286	82.592	80.214	79.719	85.544	86.442
8	83.130	82.870	85.572	88.089	78.652	82.868	82.525	84.231
9	81.235	83.463	78.278	81.256	73.735	72.976	84.441	83.967
10	82.708	84.770	76.805	77.485	78.453	77.195	79.809	79.572
11	80.370	-	83.373	83.806	77.886	79.299	84.250	81.244
12	85.970	83.119	82.621	81.670	80.661	82.190	84.046	81.621
13	78.387	-	82.465	81.921	80.892	81.049	84.505	83.513
14	86.607	85.316	86.316	84.022	81.955	80.624	80.963	84.321
15	80.699	82.165	81.706	83.087	82.986	82.343	84.927	86.479
16	81.472	83.148	83.066	83.513	76.874	77.842	83.425	81.824
17	83.726	82.419	81.163	83.125	77.680	79.907	82.597	83.664
18	81.771	81.795	85.652	84.532	84.143	82.157	83.915	83.698
19	81.780	82.029	82.167	85.556	81.161	81.608	83.869	82.522
20	82.624	86.128	82.482	81.781	85.742	85.286	83.092	85.003
Mean	**81.92**							

HT-2 toxin content (µg kg^{-1})								
Bottle-No.	**39**		**52**		**126**		**243**	
#	**run A**	**run B**	**run A**	**run B**	**run A**	**run B**	**run A**	**run B**
1	80.776	84.371	79.946	79.906	82.888	81.932	77.479	78.087
2	73.643	73.481	81.346	82.497	83.329	80.719	75.697	73.683
3	76.365	78.011	75.758	78.475	77.998	77.923	79.145	81.249
4	79.752	80.209	78.237	78.827	83.389	84.985	79.547	80.513
5	80.862	81.130	82.662	81.597	74.813	73.231	81.035	80.385
6	82.338	80.567	79.758	79.910	84.221	84.164	81.331	83.217
7	78.407	79.884	81.137	83.262	80.516	79.930	81.912	83.643
8	82.421	84.710	86.266	85.460	82.856	85.170	82.937	85.298
9	81.187	83.898	82.035	83.025	80.653	80.825	77.353	77.093
10	76.972	77.785	76.962	77.956	82.657	85.642	80.600	82.518
11	82.998	82.794	81.440	80.398	79.423	80.419	79.031	77.897
12	78.239	79.928	77.580	77.408	79.417	82.251	82.367	82.327
13	80.972	82.492	77.181	78.453	76.323	77.929	79.691	81.888
14	80.640	80.834	77.621	79.501	78.754	83.419	85.392	84.164
15	80.452	85.013	78.890	85.583	84.244	85.742	84.454	86.099
16	78.950	79.702	79.772	79.298	76.175	75.922	80.984	78.788
17	76.550	79.346	79.705	82.030	77.594	78.289	80.613	79.051
18	80.657	79.138	81.584	82.204	83.664	84.916	80.881	85.572
19	80.291	82.050	80.372	82.077	78.795	81.848	79.262	82.202
20	82.880	84.334	81.573	84.553	82.230	83.617	81.104	83.009
Mean	**80.71**							