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CERTIFICATION REPORT

The certification of the mass concentration of ochratoxin A in red wine

Certified Reference Material ERM®-BD476

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SUMMARY

This report describes the certification of one red wine material intended for the use of ochratoxin A (OTA) determination in food. Detailed information are given regarding the preparation of the material, the homogeneity and stability studies, the used analytical methods and the results of the certification study. The certified value and the uncertainty are:

	Certified value	**Uncertainty**
Compound	Mass concentration in µg L^{-1}	
Ochratoxin A	**0.52**	**± 0.11**

The value given represents the unweighted mean value of four independent results. The uncertainty given for this value represents the estimated expanded uncertainty U_{CRM} with a coverage factor of $k = 2$, corresponding to a level of confidence of about 95% as defined in the Guide to the Expression of Uncertainty in Measurement, ISO (1995).

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BAM	Federal Institute for Materials Research and Testing
CI	Chemical ionisation
CRM	Certified Reference Material
EI	Electron impact
ERM	European Reference Material
ESI	Electro-Spray-Ionisation
FD	Fluorescence detection
GC	Gas chromatography
GUM	Guide to the Expression of Uncertainty in Measurement
IAC	Immunoaffinity column clean-up
ILC	Interlaboratory comparison study
ISO	International Organization of Standardization
ISTD	Internal Standard
LC	Liquid chromatography
LoQ	Limit of quantification
MRM	Multiple reaction monitoring
MS	Mass spectrometry
OTA	Ochratoxin A
PBS	Phosphate Buffered Saline
SIM	Single ion monitoring
VIM	International Vocabulary of Metrology

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1 Introduction

Ochratoxin A (OTA) is a mycotoxin that is produced mainly by *Aspergillus* spp. and *Penicillium* spp.. It was classified as a potential carcinogen of class 2 by the International Agency for Research on Cancer (IARC) and has a number of toxic effects in mammals such as nephrotoxicity and hepatotoxicity [Müller et al. 2003]. OTA can occur in a large variety of food such as cereals, beans, ground nuts, spices, dried fruits, coffee, beer and wine [Gonzalez-Peñas et al. 2004]. Because of carry-over effects it can also be found in meat, especially in kidneys, from animals fed with contaminated feed [Franck 1984]. Exposure to OTA is a common and serious problem in food safety. Limit values for OTA in a number of foodstuffs have been established in the European Regulation EC 1881/2006 and EC 105/2010. It is therefore essential to develop and validate analytical methods for the determination of OTA in different foodstuffs that are reliable and capable to detect OTA within those limit values.

Food and feed reference materials and especially certified reference materials (CRM) are a versatile tool in the verification of the accuracy of analytical measurements. They can be used for the measurement uncertainty estimation, to assess the traceability of the analytical results, or the calibration of analytical instruments.

The reference material ERM-BD476 was produced for the purpose of quality assurance and quality control for the determination of OTA in wine. The material was prepared from naturally contaminated red wine sampled from commercial sources intended for human consumption.

A total number of 15 laboratories were selected based on documented experience and proficiency and invited to participate in an interlaboratory comparison study to support the in-house certification of the candidate material prepared at BAM (Federal Institute for Materials Research and Testing). Following internationally accepted procedures the certified mass concentration of OTA, its uncertainty and the shelf life were evaluated.

2 Production of the candidate material

2.1 Preparation of the candidate material

About 11 litres (15 bottles) of a commercially available red wine were purchased in the retail market. The whole wine was filtered using a 240 mm MN 612 ¼ paper filter by Macherey-Nagel. The filtered material was stored in a refrigerator for three months before further processing. Then again the wine was filtered through a glass-micro-phase-filter (GF/A 110 mm) by Whatman Schleicher & Schuell. The filtrate was collected and homogenised in a

20 L glass flask with pipe. Wine samples were manually filled (gravimetrically controlled) in amber glass bottles that were purged with argon prior to filling.

A total of 205 units were bottled containing (49.5 ± 1.0) g corresponding to (51.1 ± 1.0) mL. The bottles were sealed with screw caps with Teflon inserts and numbered in the order of leaving the bottling process. The whole batch was stored at 4 °C after bottling was finished.

Tab. 1: Matrix characterisation

Measurand	Value	Method
Ethanol content	(11.04 ± 0.02) mL/100 mL	GC-MS
pH-value	3.4 ± 0.2	Glass electrode / pH-meter

2.2 Analytical method

Different methods are available for the determination of OTA. The method we used is a LC-MS/MS method using a $^{13}C_{20}$-OTA as internal standard (ISTD).

Sample preparation

Approximately 10 mL of homogenised sample was weighed into a 50 mL conical flask and spiked with approx. 150 µL of ISTD-solution (100 ng/g). The sample was diluted with 10 mL 1 % polyethylene glycol / 5 % sodium-bi-carbonate and well shaken. All the black coloured but clear solutions were applied onto an immunoaffinity column for clean-up. After washing the OTA-loaded column with 10 mL 0.5 % sodium-bi-carbonate / 2.5 % sodium chloride solution (Sigma Aldrich), OTA was eluted using two-times 2.0 mL methanol/glacial acetic acid (98+2; v+v). The eluate was then evaporated to dryness using a water bath and a gentle stream of nitrogen and re-dissolved using 2 mL of methanol / de-ionized water (70+30; v+v). 50 µL of this solution were analysed by LC-MS/MS.

Measurement and calibration

Tab. 2: Parameters of the HPLC-MS/MS system

	Instrument / Measurement conditions
HPLC	
Instrument	Agilent 1100
Column	250 x 3 mm, Particle size 5 µm; Purosphere RP-18
Mobile phase	30 % A 70 % B (A = water/0.2 % acetic acid; B = methanol/0.2 % acetic acid) 0.5 mL/min, isocratic
Oven temperature	40 °C
Injection	Volume: 50 µL, autosampler
Detection	
Mass spectrometer	Applied Biosystems API 4000
Ionisation	ESI^+
Ion source temperature	600 °C
Modus	Multiple reaction monitoring (MRM)

For identification and quantification the following transitions were monitored:

Tab. 3: MRM transitions for OTA and ^{13}C-OTA

Compound	MRM transition	used for:
OTA	$[M+H]^+$ (m/z 404) → $[M\text{-Phenylalanin}]^+$ (m/z 239)	Quantifier
OTA	$[M+H]^+$ (m/z 404) → $[M\text{-HCOOH}]^+$ (m/z 358)	Qualifier
^{13}C-OTA	$[M+H]^+$ (m/z 424) → $[M\text{-Phenylalanin}]^+$ (m/z 250)	Quantifier
^{13}C-OTA	$[M+H]^+$ (m/z 424) → $[M\text{-HCOOH}]^+$ (m/z 377)	Qualifier

Figure 1 shows a typical HPLC-MS/MS chromatogram of a wine extract for the mass-transitions listed in table 3. OTA and the isotope labelled internal standard, $^{13}C_{20}$-OTA, elute at the same retention time at about 8.0 minutes.

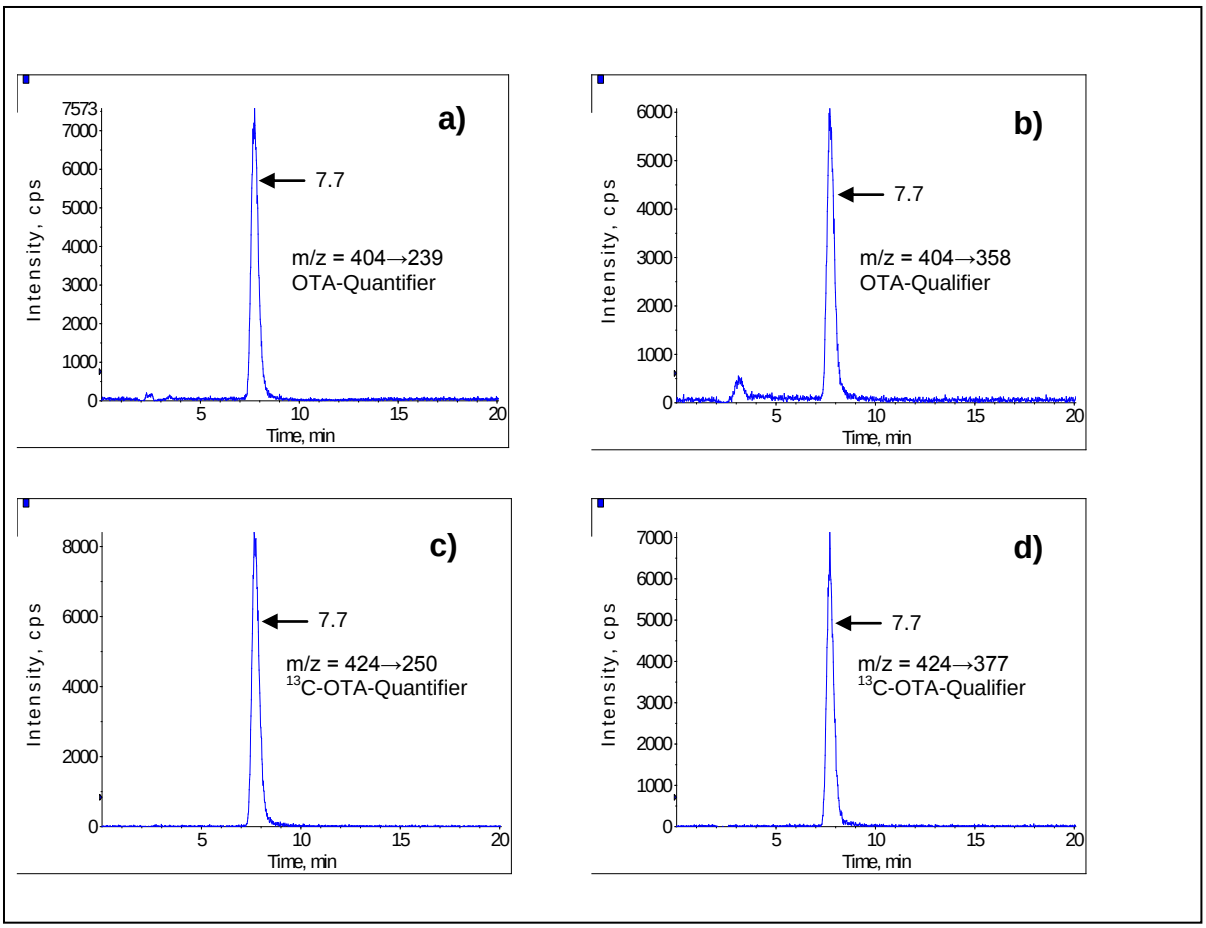

Fig. 1: Typical HPLC-MS/MS chromatogram of a red-wine extract for the following mass transitions (a) m/z = 404→239, (b) m/z = 404→358, (c) m/z = 424→250 and (d) m/z = 424→377

A six-point calibration was used for quantification of the measured area ratios. Each calibration solution was freshly prepared by weighing (range of mass ratios: 0.1 to 1.2 ng OTA / ng ^{13}C-OTA. The calibration function was assumed to be linear and obtained by regression analysis (figure 2).

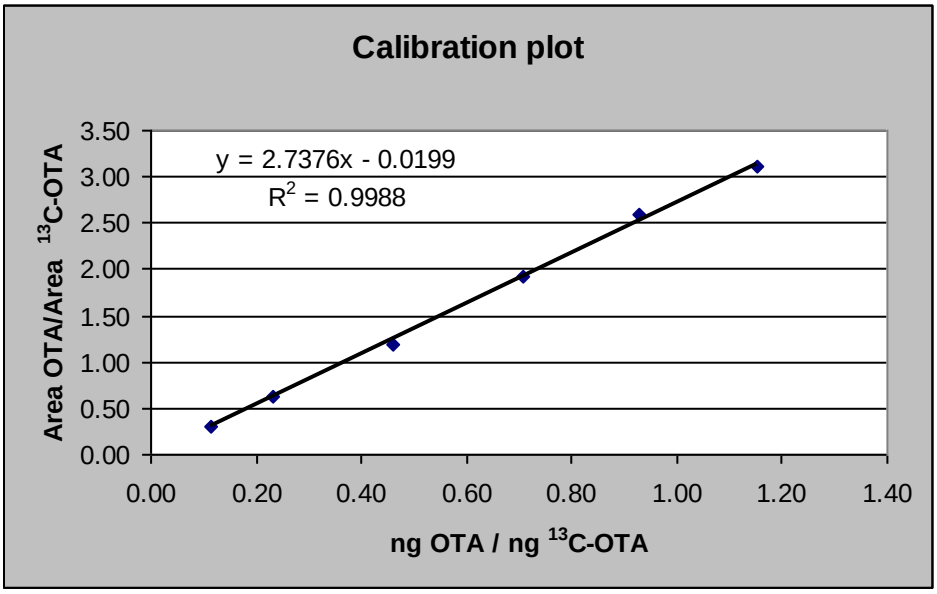

Fig. 2: Calibration function obtained by linear regression analysis

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 10 mL sample intake for a single determination (half amount of the prescribed sample intake given in ISO 14133). Therefore, 10 ml is the intake recommended on the certificate.

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, 8 units were selected randomly from the whole set of 205 bottles, and analysed four times each according to the analytical method described before (chap. 2.2). All 8 units were extracted and processed once under repeatability conditions followed by the second set of extractions and processing in a randomised manner again under repeatability conditions and so on.

Processed extracts were analysed by HPLC-MS/MS under repeatability conditions guaranteeing that all 32 extracts were quantified versus one calibration after randomisation.

Results are given in figure 3 and the ANOVA table (table 4) below. For raw data see Annex A.

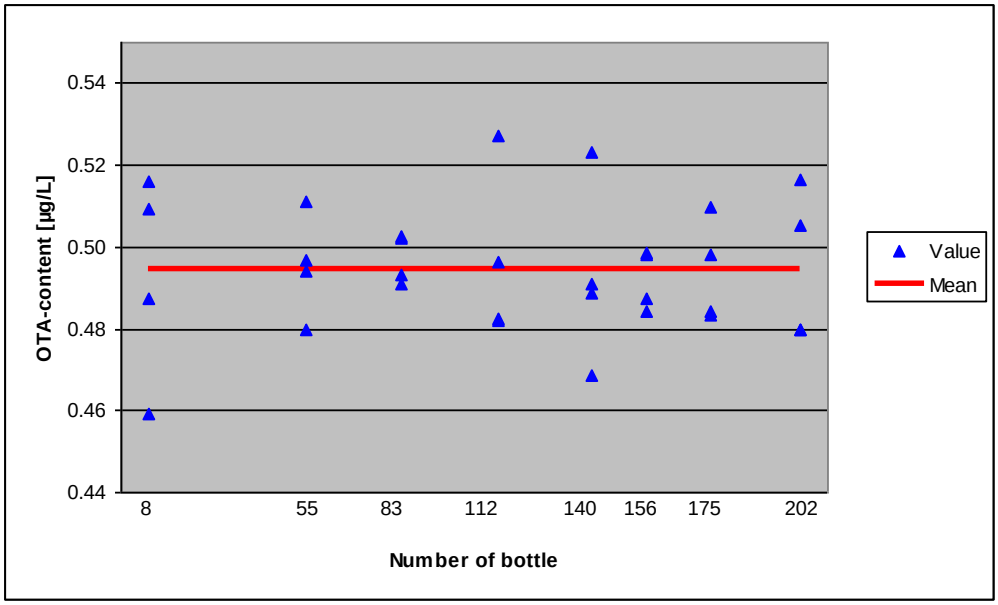

Fig. 3: Measurement results for the homogeneity test

Tab. 4: Analysis of Variance (ANOVA)

Mean sum of squared deviation (MS)		degrees of freedom	Test criterion		Critical value	
between bottles	1.4873 E-05	$f_1 = 7$	$\frac{MS_{between}}{MS_{within}}$	0.0504	$F(f_1, f_2, \alpha)$	2.42
within bottles	2.9538 E-04	$f_2 = 24$				

f_1 = Z – 1 = 7
f_2 = Z (M – 1) = 24
α = 0.05

Because the test criterion is smaller 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 total uncertainty budget of the certified value (table 5).

Tab. 5: Estimate for uncertainty contribution according to ISO Guide 35

Standard uncertainty between bottles	µg/L	relative in %
u_{bb}	0.0046	0.9

$$u_{bb} = \sqrt{\left(\frac{MS_{within}}{n} * \sqrt{\frac{2}{f_2}}\right)}$$

u_{bb}: uncertainty between bottles
MS_{within}: Mean sum of squared deviations within bottles
n: Number of replicate analysis
f_2: degrees of freedom within bottles

4 Stability study

4.1 Initial stability study

From experience a temperature-driven deterioration of the OTA mass concentration was to be expected for this material. Selected units of the candidate material were exposed to accelerated ageing at temperatures between 4 °C and 60 °C over periods of 1 week to 12 months as shown in table 6 to perform a so-called isochronous stability study [Lamberty et al. 1998]. Annex B and C show the raw-data for this studies.

Tab. 6: Accelerated ageing of exposed samples

Ageing [months]	Bottle-No. / Storage temperature				Remark
	4°C	23°C	40°C	60°C	
0.25	32/115	155	138	101	Initial study
0.50	32/115	95	10	205	Initial study
0.75	32/115	167	190	41	Initial study
1	32/115	56	69	124	Initial study
3	71	36	113	-	Initial study
6	11	99	76	-	Initial study
9	111	43	-	-	Initial study
12	34	123/186	-	-	Initial study
24					[1])
36					[1])
48					[1])

[1]) post-certification monitoring

After the respective periods of time the exposed units were stored at 4°C. All units were analysed for OTA using the method described in chapter 2.2 under repeatability conditions together with one or two reference samples which had been kept at 4°C over the whole period of the initial stability study. Two independent extracts were obtained for each exposed sample and reference sample. The extracts of the reference samples were evenly distributed over the whole measurement sequence and measured together with the exposed samples. Sampling points in time were taken after exposure to temperatures of 4°C, 23°C, 40°C and 60°C as given in table 6.

Data processing and result assessment was carried out in accordance with [Bremser et al.] assuming an *Arrhenius* model for the dependence of the reaction rate k(T) on temperature. A plot of the logarithm of the reaction rate ln(k_eff) over the inverse temperature is given in figure 4.

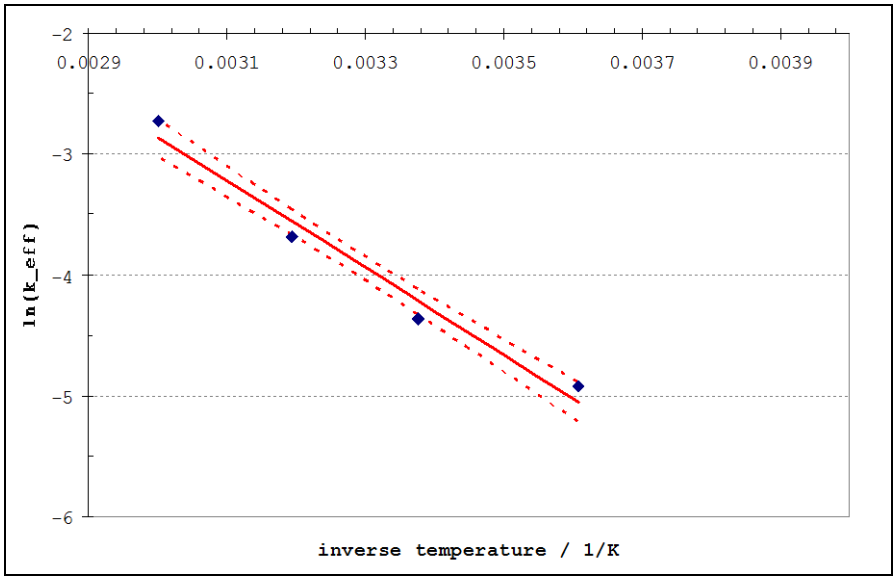

Fig. 4: Effective reaction rate for ochratoxin A in dependence on the inverse temperature (semi-logarithmic plot)

As obvious from the graph, the temperature dependence can indeed be approximated by a straight line. The corresponding confidence interval for the line is also given in the figure. The estimated activation energy ΔE is 30.0 kJ/mol, significantly smaller than the activation energy for ochratoxin A in a coffee matrix which was, however, in good agreement with activation energies determined for a large variety of organic compounds. The discrepancy is most probably caused by the difficulties of assessing very small changes of a sub-ppb value at low stress levels, in particular at the 4 °C level, leading to an overestimation of degradation effects.

By using these data and the assumed model, an estimate can be obtained when degradation will presumably force the OTA mass concentration 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 4. The results are given in table 7. Bearing in mind the rather overestimated degradation influence, these values most likely underestimate the real shelf life.

Tab. 7: Estimation of shelf life

Temperature °C	Months
4 °C	35.2
20 °C	16.3
40 °C	8.4
60 °C	4.0

The data table will be updated during post-certification monitoring. Shelf life at a storage temperature of 4 °C is considerable. A minimum shelf life of 36 months can reliably be assumed. However, exposure to temperatures higher than 4 °C may reduce the time of validity of ERM-BD476 drastically. Therefore, an expiry date of **1 year after delivery from storage** is established provided the sample is stored at 4 °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 further measurements of units stored at 4 °C over the period of availability of the material. The first post-certification measurements will be conducted according to the information given in table 6.

5 Certification study

5.1 Design of the study

The assignment of the certified OTA mass concentration of the wine reference material based upon an in-house study at BAM using HPLC-MS/MS analysis including ^{13}C-labelled OTA as internal standard. Simultaneously, an interlaboratory comparison study (ILC) involving 15 expert laboratories was conducted in order to support the in-house certification study at BAM.

For in-house certification purposes as well as for ILC two units of the candidate reference material (sample_1 and sample_2) were to be analysed by each laboratory in duplicate. An information was provided to the laboratories that the OTA level of the samples is expected in the range of the legally established limit for wine to ensure – as far as technically feasible – comparable analytical conditions.

In addition, each participant received one unit of the candidate reference material for determination of the overall method recovery. Results for the OTA concentration were to be reported on the basis of total mass intake. Results returned to BAM were scrutinised for consistency.

5.2 Participants of the supporting ILC

The following 15 participants of the ILC (table 8) were selected based on their experiences in the field of mycotoxin (OTA) analysis.

Tab. 8: Participants of the ILC

No.	Laboratory	City/Country
1	Analytec Labor für Lebensmitteluntersuchung	Freilassing, Germany
2	Bundesinstitut für Risikobewertung	Berlin, Germany
3	Chemisches und Veterinäruntersuchungsamt Sigmaringen	Sigmaringen, Germany
4	Food GmbH	Jena, Germany
5	General Chemical State Laboratory	Athens, Greece
6	Görtler Analytical Service GmbH	Vaterstetten, Germany
7	Institut für Lebensmittel, Arzneimittel und Tierseuchen (ILAT)	Berlin, Germany
8	Labor Dr. Scheller GmbH	Augsburg, Germany
9	Labor Eurofins Wiertz-Eggert-Jörissen	Hamburg, Germany
10	Landesuntersuchungsamt Rheinland-Pfalz	Trier, Germany
11	Lebensmittelversuchsanstalt	Vienna, Austria
12	Max Rubner Institut	Detmold, Germany
13	Public Analyst´s Laboratory Dublin	Dublin, Ireland
14	SGS Germany GmbH	Hamburg, Germany
15	UIS Umweltinstitut synlab GmbH	Stuttgart, Germany

The participant laboratories applied methods of their own choice which in all cases included an IAC after the extraction step. All laboratories used HPLC for separation of the purified extract and fluorescence or MS/MS detection combined with an external calibration.

5.3 Evaluation of results and certified values

The results of the certification study were evaluated in accordance with ISO GUIDE 35 and the specific requirements of the ERM agreement (For detailed information see: www.erm-crm.org/ermcrm).

5.3.1 Technical evaluation of ILC

Data of laboratory 14 reporting all replicate measurements for all samples below their LoQ (0.03 µg/L) had to be removed for technical reasons.

From the *Youden* plot it could be seen that most of the laboratories were able to treat the two samples identically (judged by the orthogonal distances from the diagonal). Two laboratories (No. 4 and No. 10) revealed quite significant bias from the rest (judged by the distance of the orthogonal projection root point from the bivariate mean).

Since the standard outlier tests however did not identify laboratories no. 4 and no. 10 as outlying (only 10 is a straggler in the Nalimov test), data from these laboratories were retained for further processing.

5.3.2 Statistical evaluation

After removal of laboratory 14 for technical reasons, the data set as shown in table 10 was used for further statistical processing.

Tab. 10: Accepted laboratory data sets of ILC. The BAM values (highlighted) were **not** taken into account for ILC-evaluation.

Lab-No.	01	02	03	04	05	06	07	08	09	10	11	12	13	15	BAM
Values[1)]	0.26	0.31	0.43	0.29	0.49	0.48	0.49	0.63	0.41	1.1	0.46	0.79	0.42	0.53	0.45
(µg L^{-1})	0.25	0.42	0.72	0.32	0.47	0.50	0.47	0.65	0.44	1.0	0.63	0.69	0.42	0.53	0.48
	0.28	0.32	0.72	0.28	0.50	0.49	0.50	0.65	0.40	1.1	0.53	0.64	0.42	0.51	0.46
	0.26	0.36	0.67	0.28	0.48	0.50	0.50	0.62	0.43	1.0	0.53	0.58	0.43	0.52	0.46
recovery (%)	84.0	77.9	88.1	105	91.5	89.5	88.5	92.3	67.5	99.9	87	73.8	88.4	69.5	89.4
mean value[2)] (µg L^{-1})	0.31	0.45	0.72	0.28	0.53	0.55	0.55	0.69	0.62	1.05	0.62	0.91	0.48	0.75	0.52

[1]) The single values of each laboratory are not corrected for recovery.

[2]) The mean value of each laboratory is corrected for recovery.

The determined OTA recovery rates displayed in table 10 fulfil the requirement specified in EC 401/2006 (i.e. recovery rates between 50 % and 120 % for an OTA-content < 1 µg/kg).

Further statistical analysis was carried out within which the following statistical parameters were calculated:

- the mean of laboratory means
- the standard deviation of the distribution of laboratory means, and the standard deviation of the mean of laboratory means
- the confidence interval of the mean of laboratory means at the 95 % confidence level

The following statistical tests were carried out (at significance levels of 0.05 and 0.01):

- Cochran test for the identification of outliers with respect to laboratory variance
- Grubbs test for the identification of outliers with respect to the mean
- Dixon and Nalimov test for the verification of possible outlier indications
- Kolmogorov-Smirnov Test (Lilliefors version) for the normality test
- Test for skewness and kurtosis

The results of the calculations and tests for a data evaluation based upon the laboratory means are given in table 11.

Tab. 11: Statistical parameters of the accepted data sets of ILC

OTA mass concentration [µg/L]							
Value	**SD_{char}**	**u_{char}**	**CI**	**TI**	**Data sets**		**Pooling**
0.609	0.211	0.056	0.122	0.636	14		No

Scheffé	Bartlett	Outlier α = 0.01 (0.05)				Normality	Skewness/ Kurtosis
	α = 0.01	Cochran	Grubbs E	Grubbs D	Nalimov	α = 0.01	
No	Inhom	3(3)	- (-)	- (-)	- (12)	normal	normal

u_{char} Uncertainty of the characterisation step (standard deviation of the mean of means)
CI Confidence interval of the mean of means at a 0.05 significance level
TI Tolerance interval of the mean of means at a 95 % confidence level

The main features are as follows:

- *Scheffé*- and *Snedecor*-F-Test: Data sets differ significantly.
- *Bartlett*-Test: Variances are inhomogeneous (at a significance level of 0.01).
- *Cochran*-Test: One outlier detected (3) (significance level 0.01).
- *Dixon*-, *Grubbs*- und *Nalimov*-Test: Laboratory means do not contain outliers (signifycance level 0.01). Laboratory 10 is a straggler in the Nalimov test.
- *Kolmogorov-Smirnov* and *Skewness/Kurtosis*-Test: Based on the available data, the hypothesis of normality cannot be rejected.

Based on the outcome of the above tests, no further laboratory was excluded from contributing to the certification study result. Participants of the ILC used different methods or implementations for extraction, IAC clean-up, HPLC and detection. Obviously there was no good reason for assuming that the single values measured by the different laboratories would belong to a common population. Single measurement results cannot be pooled, and therefore the mean of laboratory means of w(OTA) = 0.61 µg L^{-1} was considered an appropriate estimate for the OTA mass concentration of the reference material.

The outcome of the ILC is consistently to the in-house certification results based on the SIDA (stable isotope dilution analysis) using HPLC-MS/MS at BAM. The mean value of four independent OTA results was determined to 0.52 µg L^{-1} (table 10).

5.3.3 Uncertainty budget and certified values

The combined uncertainty is calculated according to equation (1):

$$u_{c,r}^2 = u_{w,r}^2 + u_{cal,r}^2 + u_{13c,r}^2 + u_{pur,r}^2 + u_{rep,r}^2 + u_{ec,r}^2 + u_{rec,r}^2 + u_{bb,r}^2 + u_{lts,r}^2 + u_{trc,r}^2 \quad \textbf{(1)}$$

where the index *r* refers to the corresponding relative uncertainties. The results are given in table 12.

Tab. 12: Uncertainty contributions for calculation of the combined uncertainty

Uncertainty contribution from …		**rel. value**	**Remarks**
weighing	u_w	0.01039732	assessed using balance control chart
calibration	u_{cal}	0.01879578	estimate for mean of four replicate determinations (standard scheme for certification)
^{13}C aliquoting	u_{13c}	0.00059522	uncertainty of the aliquoted amount of the isotope spike, not contained in the general estimate for weighing
purity native standard	u_{pur}	0.005	as assessed by NMR (certified Biopure standard)
reproducibility	u_{rep}	0.04442835	accounts for variability in the sample preparation (weighing, clean-up, bulk-up, peak integration, etc.), plus day-to-day variations
extraction completeness	u_{ec}	0	included in extraction efficiency
recovery	u_{rec}	0.02876484	referring to an R = 0.894, determined from recovery experiment
homogeneity	u_{bb}	0.00933376	from homogeneity study
(in)stability	u_{lts}	0	for a minimum shelf life of 36 months
commutability	u_{trc}	0.08859027	half of the difference between BAM value and mean of the ILC (without BAM)
Total (rel.)	$u_{c,r}$	0.1059405	according to eq. 1
Total	u_c	0.05480702	µg L^{-1}

The final certified values for the CRM are given in table 13 where the coverage factor for the expanded uncertainty is $k = 2$. The value and the expanded uncertainty are rounded according to the recommendations of ISO GUM:1995 and are given with respect to raw sample mass.

Tab. 13: Certified OTA mass concentration of ERM-BD476 (sample-mass basis)

CRM	OTA mass concentration in µg L^{-1}		
	Certified value	Uncertainty of the certified value	Expanded uncertainty of the certified value
ERM-BD476	**0.52**	**0.05**	**0.11**

5.3.4 Traceability

Traceability of the certified values was directly established to stated references of the pure mycotoxin using the BAM certification method – stable isotope dilution analysis (SIDA) using ^{13}C-isotopic labelled internal standard for HPLC-MS/MS measurement. These measurements took traceability from pure reference substance (OTA: 99.5+ %; Biopure, Tulln, Austria) with a purity independently confirmed by UV absorption measurements.

The certified values were confirmed within their stated uncertainties by a supporting interlaboratory comparison of 15 participating laboratories, all using their duly validated and calibrated methods.

6 Information on the proper use of ERM-BD476

6.1 Shelf life

From the initial stability study a preliminary shelf life of 36 months 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 12 months beginning with the dispatch of the material from BAM. The validity of this information will be maintained by the post-certification monitoring.

6.2 Transport, storage and use

Due to the proved stability of the reference material a cooled dispatch is not necessary. On receiving, the bottle is to be stored at a temperature equal to or lower than 4 °C (freezing has to be avoided). Before taking a sub-sample for analysis the bottle has to have reached ambient temperature. Thereafter, the bottle must be closed tightly and stored at a temperature equal to or lower than 4 °C (freezing has to be avoided).

6.3 Safety instructions

No hazardous effect is to be expected when the material is used under conditions usually adopted for the analysis of foodstuff matrices moderately contaminated with OTA. 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.

6.4 Legal notice

Neither BAM Federal Institute for Materials Research and Testing nor any person acting on their behalf make 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

Biopure Referenzsubstanzen GmbH, Tulln, Austria (2004)
Certificate of Analysis for ochratoxin A; Lot #: 03093Z; white crystals of ochratoxin A.

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. 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.

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8 Annexes

Annex A: Raw data for homogeneity test

	OTA mass concentration (µg/L)						
Bottle-No.	a	b	c	d	Average	Standard deviation	%
8	0.49	0.46	0.51	0.52	0.4930	0.0257	5.21
55	0.51	0.49	0.48	0.50	0.4955	0.0128	2.58
83	0.49	0.49	0.50	0.50	0.4973	0.0060	1.21
112	0.48	0.50	0.48	0.53	0.4971	0.0210	4.23
140	0.47	0.49	0.52	0.49	0.4930	0.0225	4.57
156	0.50	0.49	0.48	0.50	0.4922	0.0073	1.49
175	0.51	0.48	0.48	0.50	0.4939	0.0127	2.56
202	0.48	0.51	0.52	0.48	0.4954	0.0185	3.74
Average					**0.4947**		
Standard deviation					**0.0019**		
RSD %					**0.39**		

1) Homogeneity test was carried out using HPLC-MS/MS-measurement with an $^{13}C_{20}$-OTA isotopic standard after IAC-clean-up.

Annex B: Data (OTA mass concentration) from short-term stability study

Reference: 4 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/L	µg/L	µg/L	µg/L
Value 1 (bottle 32)	0.5017	0.5017	0.5017	0.5017
Value 2 (bottle 32)	0.5575	0.5575	0.5575	0.5575
Value 3 (bottle 32)	0.5023	0.5023	0.5023	0.5023
Value 1 (bottle 115)	0.5272	0.5272	0.5272	0.5272
Value 2 (bottle 115)	0.5087	0.5087	0.5087	0.5087
Value 3 (bottle 115)	0.5459	0.5459	0.5459	0.5459

23 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/L	µg/L	µg/L	µg/L
Value 1	0.5049	0.5673	0.5449	0.5294
Value 2	0.5163	0.5511	0.5731	0.5605

40 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/L	µg/L	µg/L	µg/L
Value 1	0.5382	0.5744	0.5152	0.5537
Value 2	0.5148	0.5650	0.5687	0.4730

60 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/L	µg/L	µg/L	µg/L
Value 1	0.4842	0.5499	0.5326	0.4823
Value 2	0.5128	0.5714	0.5897	0.5542

1) Short term stability measurements were carried out isochronous using HPLC-MS/MS and an $^{13}C_{20}$-OTA isotopic standard after IAC-clean-up

Annex C: Data (OTA mass concentration) from long-term stability study

Reference: 4 °C	3 Months	6 Months	9 Months	12 Months
	µg/L	µg/L	µg/L	µg/L
Value 1	0.543	0.493	0.435	0.579
Value 2	0.526	0.438	0.480	0.566
Value 3	0.525	0.411	0.498	0.535
Value 4				0.567

23 °C	3 Months	6 Months	9 Months	12 Months
	µg/L	µg/L	µg/L	µg/L
Value 1	0.345	0.417	0.398	0.520
Value 2	0.467	0.416	0.427	0.525
Value 3	0.531	0.412	0.412	0.528

40 °C	3 Months	6 Months	9 Months	12 Months
	µg/L	µg/L	µg/L	µg/L
Value 1	0.516	0.660	-	-
Value 2	0.520	0.742	-	-
Value 3	0.496	0.659	-	-

1) Long term stability measurement was not carried out isochronously due to the fact that it was not known whether a reference temperature of 4 °C is sufficient.
2) Sampling and measurements were always carried out from both, samples stored at elevated temperature and a reference sample stored at 4 °C.
3) Measurements of the 3, 6 and 9 month samples were carried out using IAC-clean-up and HPLC-FLD, whereas 12 months samples were measured by LC-MS/MS using a $^{13}C_{20}$-OTA isotope standard.