Bundesanstalt für Materialforschung und -prüfung

European Reference Materials

ausverkauft / out of stock

CERTIFICATION REPORT

The certification of the mass fraction of ochratoxin A (OTA) in ground roasted coffee

Certified Reference Material ERM®-BD475

M. Koch, W. Bremser, R. Krüger, C. Quast, I. Nehls

Berlin, October 2010

Contact information

BAM Federal Institute for Materials Research and Testing

Department: Analytical Chemistry; Reference Materials

12200 Berlin, Germany

http://www.bam.de

http://www.erm-crm.org

http://www.virm.net

Sales

Email: sales.crm@bam.de

Internet: www.webshop.bam.de

SUMMARY

This report describes the certification of one ground roasted coffee 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 fraction in µg kg^{-1}	
Ochratoxin A	**6.0**	**± 0.6**

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	Immuno-affinity column clean-up
ILC	Interlaboratory comparison study
ISO	International Organization of Standardization
ISTD	Internal Standard
LC	Liquid chromatography
MRM	Multiple Reaction Mode
MS	Mass spectrometry
OTA	Ochratoxin A
PBS	Phosphate Buffered Saline
SIM	Single ion monitoring
VIM	International Vocabulary of Metrology

CONTENTS

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-BD 475 was produced for the purpose of quality assurance and quality control for the determination of OTA in roasted coffee. The material was prepared from ground roasted coffee sampled from commercial sources intended for human consumption and was spiked with OTA.

A total number of 16 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 fraction of OTA, its uncertainty and the shelf life were evaluated.

2 Production of the candidate material

2.1 Preparation of the candidate material

The bulk material (9 kg) was a commercially available ground roasted coffee. It was ground again to obtain a final particle size smaller than 0.5 mm. This material was homogenised with a drum hoop mixer for 6 h.

Approximately 800 g of the homogenised material was then spiked with OTA in methanol using a rotary evaporator for homogenisation and evaporation of methanol. The spiked material was then dried for four days at room temperature in the dark and the OTA-content

was measured. Then 600 g of the spiked coffee were mixed with about 7.6 kg of non-contaminated coffee for 6 h using a drum hoop mixer to obtain a final OTA-content of about 5 µg kg^{-1}.

Further homogenisation and bottling was done using a version of the so-called “cross riffling” procedure. A total of 128 units were bottled in 250 mL amber glass bottles containing (64 ± 1) g sealed with screw caps with Teflon inserts and numbered in the order of leaving the bottling process. The whole batch has been stored at -20 °C since bottling was finished.

Tab 1: Matrix characterisation

Measurand	Value	Method
Particle size range	< 500 µm	Dry sieving
Water content	(4.8 ± 0.1) %	Coulometric Karl-Fischer-Titration
C,H,N-Analysis	w(C) = (49.8 ± 0.46) %	Catalytic combustion
	w(H) = (7.00 ± 0.08) %	
	w(N) = (2.37 ± 0.03) %	

2.2 Analytical method

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

Sample preparation

Approximately 15 g of homogenised sample was weighed into a 250 mL conical flask and spiked with about 1.2 g of ISTD-solution (100 ng g^{-1}). The sample was suspended in 60 mL water/methanol (80+20; v+v) and extracted for 30 min by shaking using a horizontal mixer. The suspension was filtered in another conical flask. Then, 4 mL of the extract were transferred into a beaker (50 mL) using a pipette and 12 mL of PBS-buffer were added. A pH value between 7.0 and 7.5 was adjusted using sodium hydroxide (0.1 mol L^{-1}).

The diluted and neutralised extract was then applied onto an immunoaffinity column (IAC) for clean-up. After washing the OTA-loaded column with 20 mL sodium acetate solution containing 0.025 % Tween 20 (Sigma-Aldrich) and three times 2 mL sodium acetate solution, OTA was eluted using four-times 0.5 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 HPLC-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 and 70% B (A = water/0.2 % acetic acid; B = methanol/0.2 % acetic acid) 0.5 mL min^{-1}, 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 $^{13}C_{20}$-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 coffee 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.

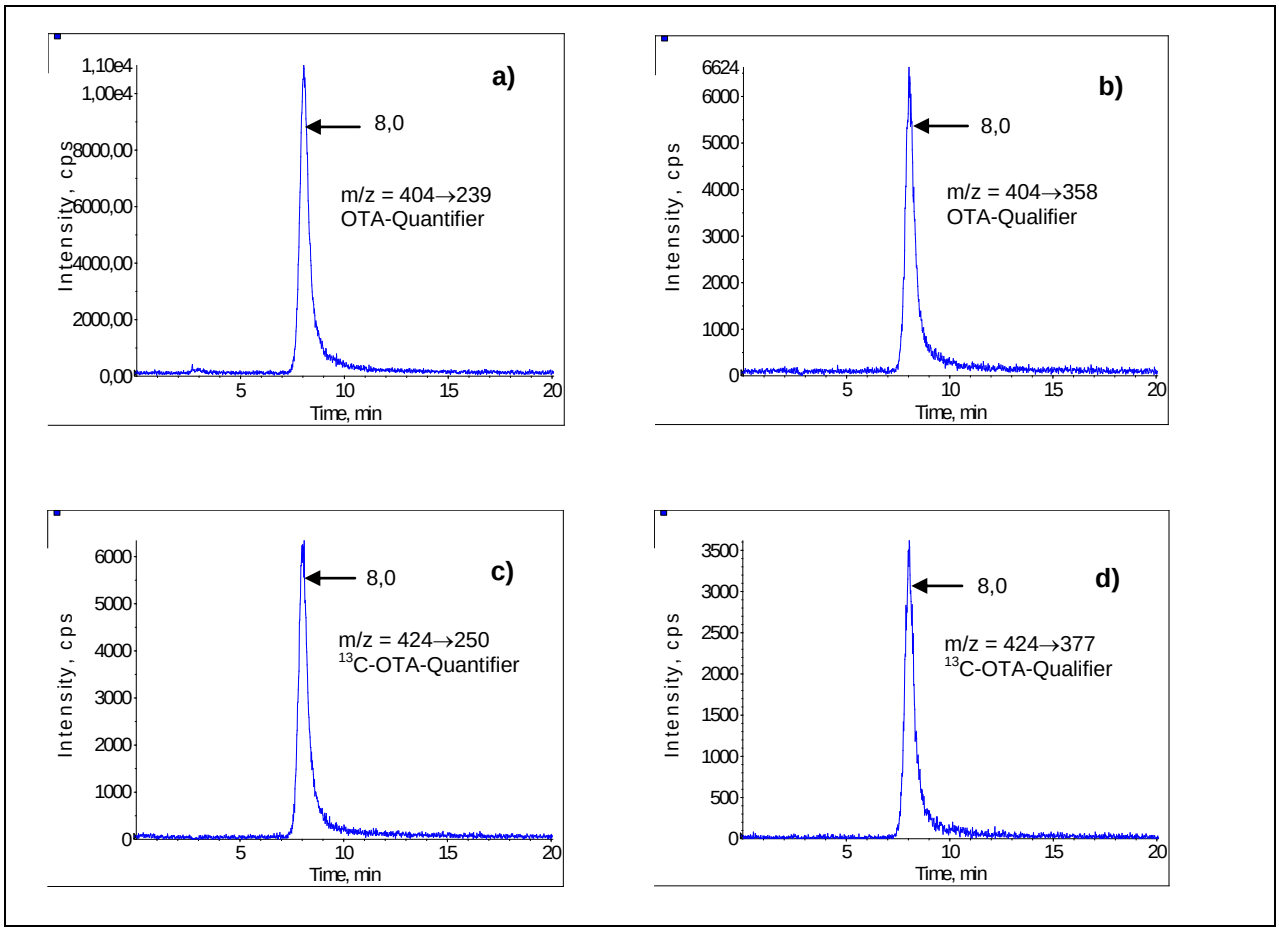

Fig. 1: Typical HPLC-MS/MS chromatogram of a coffee-extract for the following mass transitions (a) m/z = 404→239, (b) m/z = 404→358, (c) m/z = 424→377 and (d) m/z = 424→250

A six-point calibration was used for quantification of the measured area ratios. Each calibration solution was freshly prepared by weighing (range of mass fractions: 0.1 ng g^{-1} to 1.2 ng g^{-1} OTA and $^{13}C_{20}$-OTA. The calibration function was assumed to be linear and obtained by regression analysis (see fig. 2).

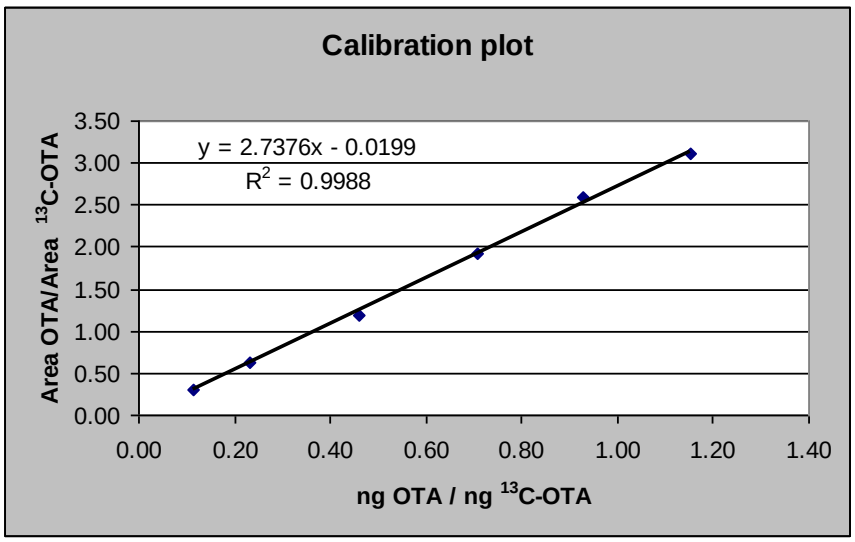

Fig. 2: Calibration function obtained by linear regression analysis

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. Measurements revealed that this is the case with 6 g sample intake for a single determination (obtained from the point of intersection of the tangents in the figure 3 below).

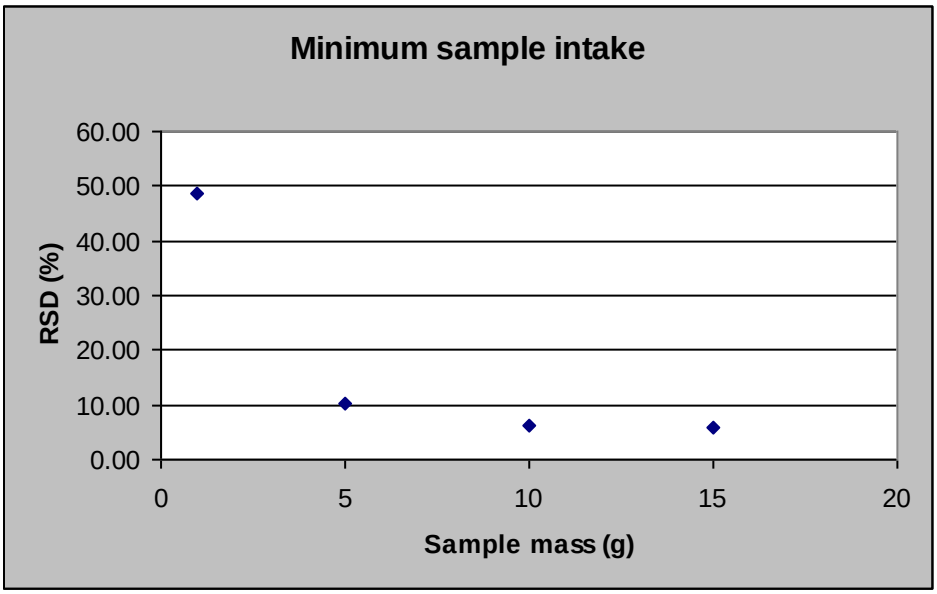

Fig. 3: Minimum sample intake obtained from the point of intersection of the tangents (not displayed)

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 128 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 4 and table 4 (ANOVA) below. For raw data see Annex A.

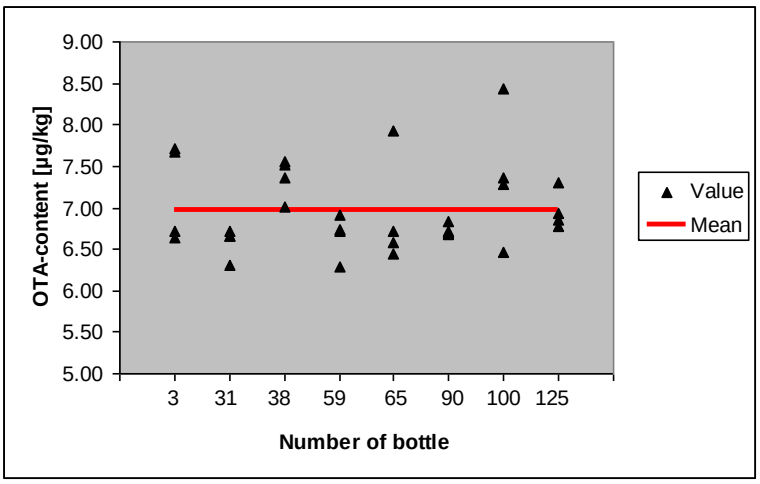

Fig. 4: Measurement results for the homogeneity test

Tab. 4: Analysis of Variance (ANOVA)

Mean Square errors (MS)		degrees of freedom	Test criterion		Critical value	
between bottles	0.386	$f_1 = 7$ [1)]	$\frac{MS_{between}}{MS_{within}}$	1.840	$F(f_1, f_2, 5\%)$	2.42
within bottles	0.210	$f_2 = 24$ [2)]				

1) $f_1 = Z - 1 = 7$
2) $f_2 = Z\,(M - 1) = 24$

Mean Square errors between bottles

$$MS_{between} = \frac{M \cdot \sum_{L=1}^{Z} \left(\bar{x}_L - \bar{\bar{x}}\right)^2}{Z - 1}$$

Mean Square errors within bottles

$$MS_{within} = \frac{\sum_{L=1}^{Z} \sum_{k=1}^{M} \left(x_{L,k} - \bar{x}_L\right)^2}{Z \cdot (M - 1)}$$

$\bar{x}_L$: Mean value of bottle number L

$\bar{\bar{x}}$: Total mean of all bottles

$x_{L,k}$: Value of analysis k in bottle number L

L: Identifier of an individual bottle
Z: Total number of bottles
k: Identifier of an individual analysis
M: Total number of analyses per bottle

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

Standard uncertainty between bottles	µg kg^{-1}	relative in %
u_{bb}	0.21	3.01

$$u_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}}$$

u_{bb}: uncertainty between bottles

$MS_{between}$: Mean square errors between bottles

$MS_{within:}$ Mean square errors within bottles

$n:$ Number of replicate analysis

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.

4 Stability study

4.1 Initial stability study

From experience a temperature-driven deterioration of the OTA content was to be expected also for this material. Selected units of the candidate material were submitted to accelerated ageing at temperatures between 4 °C and 40 °C over periods of 4 weeks 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 study.

Tab. 6: Accelerated ageing of exposed samples

Ageing [months]	Bottle-No. / Storage temperature			Remark
	4°C	20°C	40°C	
1	36	84	63	Initial study
3	120	30	53	Initial study
6	92	23	12	Initial study
12	45	112	6	Initial study
24	114	96		[1])
36	102	11		[1])
48	60	122		[1])

[1]) post-certification monitoring

After the respective periods of time the exposed units were stored at -20°C. All 12 units were analysed for OTA-content using the method described in chapter 2.2 under repeatability conditions together with 2 reference samples which had been kept at -20°C over the whole period of the initial stability study. Two independent extracts were obtained for each exposed sample and reference sample (total: 12x2 + 2x2 = 28 extracts). 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 3, 6 and 12 months exposure to the temperatures 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 5.

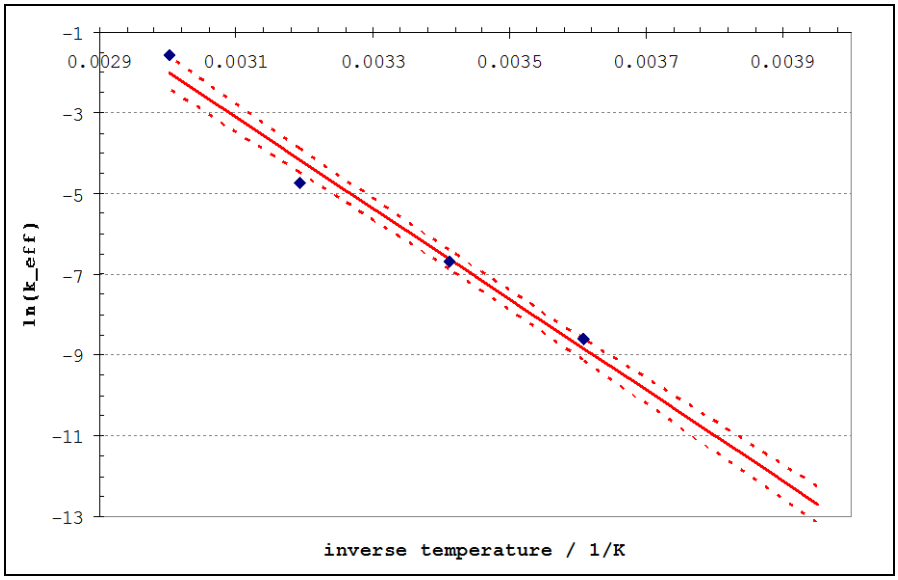

Fig. 5: Effective reaction rate for OTA in dependence on the inverse temperature (semi-logarithmic plot)

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 93.74 kJ/mol, slightly above but still 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 OTA-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 5. The results are given in table 7.

Tab. 7: Estimation of shelf life

Temperature °C	Months	years
-20 °C		1800
4 °C		45.5
20 °C	61.1	5.1
40 °C	5.0	0.4
60 °C	0.5	0

The data table will be updated during post-certification monitoring. Shelf life at a storage temperature of -20°C is considerable, and even at a storage temperature of 4°C the estimated shelf life exceeds the out-of-stock date of the material. A minimum shelf life of 5 years can reliably be assumed. However, exposure to temperatures higher than room temperature may reduce the time of validity of ERM-BD475 drastically. Therefore, a common user-end expiry date of **1 year after delivery from storage** is established provided the sample is stored at 20 °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 and 20°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 fraction of the coffee 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 16 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 content were to be reported on the basis of total mass intake, no dry mass determinations. Results returned to BAM were scrutinised for consistency.

5.2 Participants of supporting ILC

The following 16 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	Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	Oberschleißheim, Germany
2	Bundesinstitut für Risikobewertung	Berlin, Germany
3	Chemisches und Veterinäruntersuchungsamt Sigmaringen	Sigmaringen, Germany
4	CR3-Kaffeeveredlung H. Hermsen GmbH	Bremen, Germany
5	General Chemical State Laboratory, Food Division & Division of Environment	Athens, Greece
6	Kantonales Labor Zürich	Zürich, Switzerland
7	Labor Eurofins Wiertz-Eggert-Jörissen	Hamburg, Germany
8	Landesuntersuchungsamt Rheinland-Pfalz	Trier, Germany
9	Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen	Dresden, Germany
10	Lebensmittelchemisches Institut Köln	Köln, Germany
11	Lebensmittelversuchsanstalt	Vienna, Austria
12	Max Rubner Institut	Detmold, Germany
13	National Food Administration Sweden	Uppsala, Sweden
14	Public Analyst´s Laboratory Dublin	Dublin, Ireland
15	SGS Germany GmbH	Hamburg, Germany
16	Tchibo Manufacturing GmbH & Co KG	Hamburg, 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 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

From the *Youden* plot (lab mean sample_2 vs. lab mean sample_1) it could be seen that most of the laboratories were able to treat the two samples identically (judged by an orthogonal distances from the diagonal). Two laboratories (No. 4 and No. 16) revealed quite

significant bias from the rest (judged by the distance of the orthogonal projection root point from the bivariate mean).

A double-outlier (one high, one low) Grubbs test according to ISO 10723:1995 on the root-point distances from the bivariate mean gave not sufficient justification for exclusion of the corresponding mean values of laboratory 04 (high) and laboratory 16 (low).

Laboratory 04 reported the lowest recovery rate (60.5 %) of all participants but the highest contents in the samples. The recovery was lower than the minimum requirement specified in EC 401/2006 (i.e. recovery rates between 70 % and 110 % for an OTA-content of 1 to 10 µg/kg). Therefore, laboratory 04 had to be excluded from the study for technical reasons. Beside the fact, that laboratory 16 reported the lowest OTA-content for the samples it was obviously unable to process both samples in the same manner. This was tested using the E_n criterion on the difference between the sample means x_1 and x_2 according to:

$$E_n = \frac{|x_1 - x_2|}{\sqrt{s_r^2 + u_{bb}^2}}$$

where the uncertainty in the denominator consists of a contribution representing the individual average repeatability of the laboratory and an allowance for possible inhomogeneities between the two samples calculated using the estimate obtained in chapter 3. The critical value of the E_n criterion is two.
While all other labs were able to obtain, within their individual repeatabilities, consistent values for sample_1 and sample_2, the E_n value for laboratory 16 reaches 6.01 indicating certain problems in the control of the analytical procedure. Although no further investigations into the reasons for this discrepancy have been conducted, it seems justifiable to exclude, for technical reasons, the sample value of this particular laboratory 16 from further processing.

5.3.2 Statistical evaluation of ILC

After removal of laboratories 04 and 16 for technical reasons, the data set as shown in table 9 was used for further statistical processing.

Tab. 9: Accepted laboratory data sets of ILC. The BAM values (highlighted) were **not** taken into account for ILC-evaluation.

Lab	01	02	03	05	06	07	08	09	10	11	12	13	14	15	BAM
Values[1] (µg kg^{-1})	4.91	5.12	6.70	5.30	3.60	5.30	4.36	5.50	5.40	5.50	3.40	6.50	5.30	4.70	6.01
	4.95	5.54	7.40	5.50	3.60	6.20	4.77	5.60	5.30	5.40	3.60	7.10	3.90	5.10	6.10
	4.72	5.86	7.20	5.50	3.60	5.90	4.40	5.80	5.00	5.50	4.00	5.10	4.50	4.60	5.73
	4.78	5.84	7.00	5.50	3.90	5.50	4.95	5.80	5.00	5.60	3.90	6.80	4.60	5.20	5.97
recovery (%)	72.4	94.7	102.9	89.8	83.0	90.0	90.5	92.0	86.4	85.8	80.5	88.5	84.2	82.0	100.0
mean value[2] (µg kg^{-1})	6.69	5.91	6.88	6.07	4.43	6.36	5.10	6.17	5.99	6.41	4.63	7.21	5.43	5.98	5.95

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

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

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

and 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 the table 10 below.

Tab. 10: Statistical parameters of the accepted data sets of ILC

OTA content [µg kg^{-1}]							
Value	**SD**	**u_{char}**	**CI**	**TI**	**Data sets**		**Pooling**
5.95	0.808	0.216	0.466	2.433	14		No

Scheffé	Bartlett	Outlier α = 0.01 (0.05)				Normality	Skewness/ Kurtosis
	α = 0.01	Cochran (0.01)	Grubbs E	Grubbs D	Nalimov	α = 0.01	
No	Inhom	13(13)	- (-)	- (-)	- (6)	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 95/95 % confidence level

The main features are as follows:

- *Scheffé*- and *Snedecor*-F-Test: Data sets differ significantly.
- *Bartlett*-Test: Variances are inhomogeneous (at the significance level of 0.01).
- *Cochran*-Test: One outlier detected (significance level 0.01).
- *Dixon*-, *Grubbs*- und *Nalimov*-Test: Laboratory means do not contain outliers (significance level 0.01). The suspected earlier laboratory 04 is a straggler in the Nalimov test but not identified as such by the other tests. The laboratory mean is therefore retained.
- *Kolmogorov-Smirnov* and *Skewness/Kurtosis*-Test: Based on the available data, the hypothesis of normality cannot be rejected.

Participants of the ILC used different methods or implementations for extraction, IAC clean-up, HPLC and fluorescence 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) = 5.95 µg kg^{-1} was considered an appropriate estimate for the OTA mass fraction 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 5.95 µg kg-1 (table 9).

5.3.3 Uncertainty budget and certified values

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

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

where the index r refers to the corresponding relative uncertainties. The results are given in table 11.

Tab. 11: Uncertainty contributions for calculation of the combined uncertainty

Uncertainty contribution from …		rel. value	Remarks
weighing	u_w	0.010397	assessed using balance control chart
calibration	u_{cal}	0.018796	estimate for mean of four replicate determinations (standard scheme for certification)
^{13}C aliquoting	u_{13c}	0.000102	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}	0.031141	accounts for variability in the sample preparation (weighing, clean-up, bulk-up, peak integration, etc.), plus day-to-day variations
extraction completeness	u_{ec}	0.000000	OTA in coffee is a spiked material, completeness accounted for in the estimate for the recovery
recovery	u_{rec}	0.011248	referring to an R = 1 assumption
homogeneity	u_{bb}	0.030110	from homogeneity study
(in)stability	u_{lts}	0	from stability study, sufficiently stable for shelf lives up to 5 years (very conservative estimate)
commutability	u_{trc}	0.000462	half of the difference between BAM value and mean of the ILC (without BAM)
Total (rel.)	$u_{c,r}$	0.049895	according to eq. 1
Total	u_c	0.297002	µg kg^{-1}

The final certified values for the CRM are given in table 12 where the coverage factor for the expanded uncertainty is $k = 2$. The value and the expanded uncertainty are rounded according to the recommendations of the Guide to the Expression of Uncertainty in Measurement [GUM 1995] 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).

Tab. 12: Certified OTA content of ERM-BD475 (sample-mass basis)

CRM	OTA mass fraction in µg kg^{-1}		
	Certified value	Uncertainty	Expanded uncertainty
ERM-BD475	**6.0**	**0.3**	**0.6**

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 16 participating laboratories, all using their duly validated and calibrated methods.

6 Information on the proper use of ERM-BD475

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 -20 °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 20 °C. Before withdrawing a sub-sample the bottle should be allowed to reach room temperature and mixed thoroughly. Thereafter, the bottle must be closed tightly and stored at a temperature equal to or lower than 20 °C. The water content remains stable when the material is treated as described.

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 the Federal Institute for Materials Research and Testing (BAM) 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.

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

Franck, B. (1984)
Mycotoxine aus Schimmelpilzen – Waffen ungebetener Tischgenossen von Mensch und Tier: Strukturen, Wirkungen, Biosynthese und Schutzmöglichkeiten, *Angew. Chemie*, 96, 462-474.

González- Peñas, E; Leache, C.; Viscarret, M. et al. (2004)
Determination of ochratoxin A in wine using liquid phase microextraction combined with liquid chromatography with fluorescence detection, *J. Chromatography A*, 1025, 163-168.

International Organization for Standardization - ISO (1995):
Guide to the Expression of Uncertainty in Measurement (GUM). ISBN 92-67-10188-9, Reprinted (1995).

ISO 10723 (1995)

Natural gas – Performance evaluation of on-line analytical systems, Annex B: Statistical tests and methods.

ISO Guide 99 (2007):
International vocabulary of metrology (VIM) – basic and general concepts and associated terms.

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

Josephs, R.D.; Krska R.; et al. (2003):
Preparation of a calibrant as Certified Reference Material for the determination of the fusarium mycotoxin Zearalenone. *J. AOAC Int*., 86, 50-60.

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.

Müller, G.; Rosner, H.; Rohrmann, B. et al. (2003)
Effects of the mycotoxine ochratoxin A and some of its metabolites on the human cell line THP-1, *Toxicology*, 184, 69-82.

8 Annexes

Annex A: Raw data for homogeneity test

	OTA-content (µg/kg)						
Bottle-No.	a	b	c	d	Average	Standard deviation	%
3	7.67	7.71	6.63	6.71	7.18	0.59	8.22
31	6.65	6.31	6.65	6.72	6.58	0.18	2.81
38	7.37	7.01	7.52	7.56	7.37	0.25	3.40
59	6.71	6.73	6.92	6.29	6.66	0.27	3.99
65	6.45	6.58	6.72	7.92	6.92	0.68	9.79
90	6.84	6.72	6.68	6.69	6.73	0.07	1.09
100	7.36	7.28	8.43	6.47	7.39	0.80	10.89
125	6.86	6.93	7.30	6.78	6.97	0.23	3.30
Average					**6.97**		
Standard deviation							
%					**5.4**		

1) Homogeneity test was carried out using HPLC-MS/MS-measurement with OTB as internal standard after IAC-clean-up.

Annex B: Data (OTA content in µg/kg) from short-term stability measurement

Reference: -20 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/kg	µg/kg	µg/kg	µg/kg
Value 1	5.748	5.748	5.748	5.748
Value 2	6.467	6.467	6.467	6.467
Value 3	6.586	6.586	6.586	6.586
Value 4	7.086	7.086	7.086	7.086

4 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/kg	µg/kg	µg/kg	µg/kg
Value 1	5.503	6.406	6.001	6.388
Value 2	6.513	6.480	6.112	6.200

20 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/kg	µg/kg	µg/kg	µg/kg
Value 1	5.744	6.090	6.398	6.165
Value 2	6.577	6.167	6.306	6.704

40 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/kg	µg/kg	µg/kg	µg/kg
Value 1	6.373	5.483	6.264	6.302
Value 2	5.799	6.537	6.044	6.112

60 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/kg	µg/kg	µg/kg	µg/kg
Value 1	5.430	5.644	4.747	5.179
Value 2	6.253	6.201	5.491	5.510

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 content in µg/kg) from long-term stability measurement

Reference: -20 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/kg	µg/kg	µg/kg	µg/kg
Value 1	6.171	6.171	6.171	6.171
Value 2	6.204	6.204	6.204	6.204
Value 3	6.180	6.180	6.180	6.180
Value 4	5.985	5.985	5.985	5.985

4 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/kg	µg/kg	µg/kg	µg/kg
Value 1	6.252	6.634	5.954	5.752
Value 2	6.195	6.185	6.240	6.201

20 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/kg	µg/kg	µg/kg	µg/kg
Value 1	6.584	5.606	6.012	6.056
Value 2	6.092	5.881	5.914	5.955

40 °C	1 Week	2 Weeks	3 Weeks	4 Weeks
	µg/kg	µg/kg	µg/kg	µg/kg
Value 1	5.714	5.756	5.478	5.410
Value 2	5.954	5.593	5.472	5.521

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