Bureau International des Poids et Mesures

Calibrant Assessment Guideline: Aflatoxin B1 [CAG-01]

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1. Scope

This document has been prepared to provide guidance on the preparation and value assignment of a calibration solution of aflatoxin B_1 (AfB_1) in the mass fraction range of $2-50 mg \cdot kg^{-1}$. The calibration solution is prepared by gravimetric dilution of a gravimetrically prepared stock solution having known AfB_1 content and it is intended for use as a primary calibrator for AfB_1 analysis.

The information summarized in the document was obtained as part of the BIPM Metrology for Safe Food and Feed Programme for capacity building and knowledge transfer on the production and characterization of reference materials for mycotoxin analysis.

2. Introduction

In collaboration with the National Institute of Metrology, China (NIM) and the National Metrology Institute of South Africa (NMISA), the BIPM initiated in 2016 a Capacity Building and Knowledge Transfer program for Metrology for Safe Food and Feed in Developing Economies. [1] This project is designed to allow NMIs to work together to strengthen the worldwide mycotoxin metrology infrastructure; provide knowledge transfer to scientists developing capabilities in this area and to enable NMIs in developing regions to provide calibrants, matrix reference materials and proficiency test samples that support testing activities and laboratory services for mycotoxin analysis within their countries.

Calibration solutions prepared from well characterized, high purity compounds are the source of metrological traceability of most routine organic analysis results. The preparation and characterization of these solutions is therefore essential within the measurement infrastructure that supports the delivery of reliable results. It is particularly challenging in the case of the provision of standards to underpin mycotoxin testing in developing economies due to stringent export / import regulations, challenging logistics and high costs.

Aflatoxins are generally produced by fungi of the genus *Aspergillus* that have access either pre- or post-harvest to grain and nut crops in environmental conditions of relatively high temperatures and humidity. Frequently contaminated food products include dried figs, hazelnuts, groundnuts, chili peppers, pistachio and almond. [2] Aflatoxin B_1, among the four major types of aflatoxins, is the most toxic and the most potent carcinogen in humans and animals. Chronic dietary exposure to aflatoxins, mostly occurring in developing countries, results in hepatotoxicity, genotoxicity, immune suppression and malnutrition. [2] [3] [4]

The present guideline summarizes methods that can be used for the preparation and characterization of AfB_1 calibration solutions. The method development and validation studies carried out within the BIPM MMCBKT program are the basis for the results and procedures described herein. The document is intended to be of use to other metrology institutes and reference measurement service providers needing to prepare and characterize their own AfB_1 primary calibrants to use to underpin the metrological traceability of results. Stock and calibration solutions were prepared from an AfB_1 source material. For the MMCBKT programme that material was value assigned in-house for purity. Generic methods for the characterization of AfB_1 pure materials are described in a separate purity evaluation guideline (PEG). [5] The AfB_1 solutions prepared gravimetrically from the MMCBKT material were value assigned and dispensed in amber glass ampoules. A range of analytical methods were developed to quantify the mass fraction content of AfB_1 and related structure impurities in solution in order to evaluate the homogeneity and stability of the materials, as well as to verify the gravimetrically assigned AfB_1 solution mass fraction content value.

3. Properties of Aflatoxin B_1

3.1. Hazards identification

The substance poses high potential risks for human health if handled inappropriately. It is acutely toxic by inhalation, in contact with skin and if swallowed. Exposure to AfB_1 may cause cancer and heritable genetic damage.

DISCLAIMER: The safety recommendations given in this section are based on literature reported best practice and are not verified by the BIPM.

3.1.1. Protective measures

Avoid breathing of dust, vapours, mist or gas. Wear full-face particulate filtering respirator type N100 (US) or type P3 (EN 143) respirator cartridges when working with the solid material. Wear protective gloves, goggles and clothing. Take special care to avoid skin exposure if handling solutions and work in adequately ventilated areas. Wash hands thoroughly after handling.

3.1.2. Emergency procedures

General advice	Immediately call a POISON CENTER or doctor/physician. Show this safety data sheet to the doctor in attendance. Move out of dangerous area.
If inhaled	Move person into fresh air. If not breathing give artificial respiration. Consult a physician.
In case of skin contact	Wash off with soap and plenty of water. Consult a physician.
In case of eye contact	Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.
If swallowed	Immediately call a POISON CENTRE or doctor/physician. Never give anything by mouth to an unconscious person. Rinse mouth with water.

3.1.3. Spillage / Projections

Contain spillage and then collect by wet-brushing and place in container for disposal. Keep in suitable, closed containers for disposal according to local regulations.

3.2. Physical and chemical properties

Common Name:	**Aflatoxin B_1**
Synonyms:	(6αR -cis)-Aflatoxin B_1; 6-Methoxydifurocoumarone; Aflatoxin B; AfB_1.
CAS Registry Number:	1162-65-8

Molecular Formula:	$C_{17}H_{12}O_6$
Molar Mass:	$312.27 g \cdot mol^{-1}$
Monoisotopic mass:	312.063
Melting point:	268−269°C (decomposition) [6]
Appearance:	Colourless crystalline powder
Solubility:	Insoluble in non-polar solvents. Slightly soluble in water ($10–20 \mu g \cdot mL^{-1}$). Freely soluble in moderately polar organic solvents (e.g. methanol, acetonitrile, dimethyl sulfoxide, acetone, ethanol, etc.) [6]
UV maxima:	223nm ($\varepsilon = 22\,100$), 265nm ($\varepsilon = 12\,400$), 360nm ($\varepsilon = 21\,800$) [ε in CH_3OH]. [7]

3.3. Structure

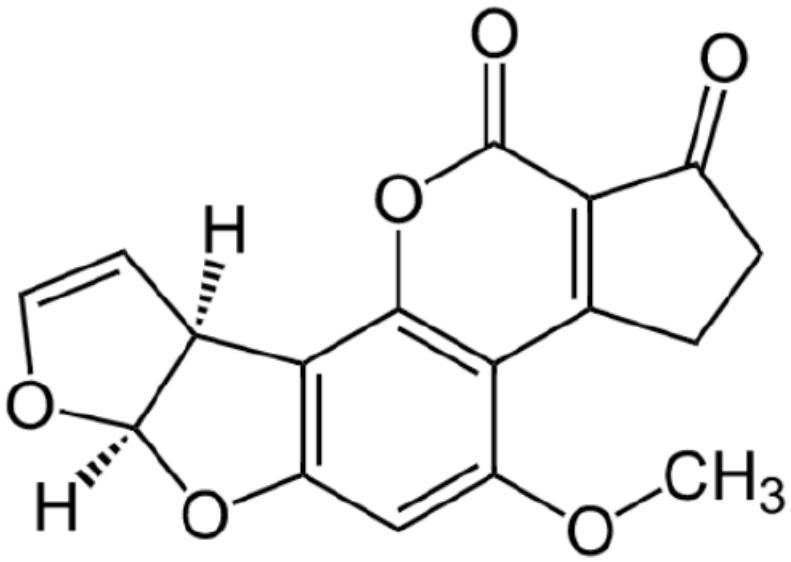


Figure 1 — Chemical structure of Aflatoxin B_1.

4. Methods for the characterization of aflatoxin B_1 solutions

This section of the Guideline describes the methods developed during the BIPM MMCBKT program for the characterization of the aflatoxin B_1 stock and calibration solutions prepared from the source AfB_1 material. The methods are the basis for the stability and homogeneity studies and for the analytical confirmation of the AfB_1 mass fraction value assigned gravimetrically.

DISCLAIMER: Commercial instruments, software and materials are identified in this document in order to describe some procedures. This does not imply a recommendation or endorsement by the BIPM nor does it imply that any of the instruments, equipment and materials identified are necessarily the best available for the purpose.

4.1. AfB_1 and related structure impurities analysis by LC-DAD-MS/MS

A method based on liquid chromatography coupled to diode array detection and tandem mass spectrometry was developed for the quantification of related structure impurities in the AfB_1 source material (BIPM ref. OGO.193a). Details on the method development and validation are described in the separate purity evaluation guideline. [5] Briefly, original standards were purchased for impurities aflatoxin B_2, B_{2a}, Q_1, P_1, G_1, G_2, M_1, M_2 and aflatoxin B_1 8,9-dihydrodiol (DIOL) (Figure 2, see p. 12). Their purity was assessed by LC-DAD at 223nm using the main peak area relative to the total integration across the chromatographic separation. The impurity standards were used to optimize the chromatographic elution and the MS/MS detection parameters that are reported below. The method was validated in-house for the performance characteristics of linearity, precision and limits of detection and quantification.

4.1.1. Materials

- Acetonitrile. HPLC gradient grade (HiPerSolv Chromanorm, VWR)
- Methanol. HPLC gradient grade (HiPerSolv Chromanorm, VWR)
- Ultrapure water (Milli-Q)
- Aflatoxin B_1 stock (BIPM ref. OGP.030) and calibration (BIPM ref. OGP.029) solutions.
- Impurity standards: AfB_2, AfB_{2a}, AfP_1, AfQ_1 and DIOL (First Standard via NIM China).

4.1.2. Sample preparation

Ampoules of the stock or calibration solution were opened and 1mL of solution was transferred to glass injection vials and placed in the autosampler at 4°C for immediate analysis.

4.1.3. Instrumentation

Liquid chromatography system Agilent 1100 HPLC equipped with a diode array detector (DAD) and coupled to a Sciex 4000 Qtrap mass spectrometry detector.

4.1.4. Liquid chromatography parameters

Column:	Phenomenex Kinetex EVO C_{18} 100Å, (250 × 4.6mm, 2.6μm) (OGLC.65)	
Column temperature:	25°C	
Mobile phase:	1. H_2O Milli Q	
	2. Acetonitrile : Methanol = 50:50 (v/v)	
Operation mode:	Gradient (inclusive cleaning gradient)	
Solvent gradient:	Time (min)	Mobile phase A
	0.0	30%
	30	90%
	31	100%
	32	100%
	34	30%
	40	30%
Flow rate:	$0.6mL \cdot min^{-1}$	
Injection volume:	10μL	
Duration:	40min	

To avoid contamination of the sensitive MS instrument by the high content of the main AfB_1 compound, the mobile phase was diverted to waste during the elution window of AfB_1 so it was measured in the DAD detector but it did not reach the MS.

4.1.5. DAD detection parameters

The absorption wavelengths used for the detection of the main component AfB_1 were 362nm and 263nm (reference 450nm).

4.1.6. MS/MS detection parameters

The 4000 QTRAP was operated in a negative-positive switching electrospray ionization (ESI) mode. The capillary voltage was set at 5500V and the source temperature at 600°C for the positive ESI. For the negative ESI mode, the capillary voltage was −4500V and the source temperature 550°C. Nitrogen was used as the ion source gas, curtain gas and collision gas. The Gas 1 and Gas 2 of the ion source were set at 55 psi and 60 psi, respectively. The curtain gas (CUR) was set at 15 psi. The Collision Gas (CAD) was set at “Mid”. Table 1 lists the optimized transitions and conditions for multiple reaction monitoring (MRM) detection of AfB_1 and its most frequent, structurally related impurities depicted in Figure 2.

Table 1. Transition ions and MS/MS parameters for the detection of AfB_1 and its impurities in MRM mode. Transitions marked with an asterisk were used for quantification purposes.

Compounds	Q1 m/z	Q3 m/z	Time (ms)	DP(V)	CE(V)	EP(V)	CXP(V)
AfB_1	311.3	296*	50	-50	-25	10	10
		283	50	-50	-25	10	10
AfB_2	315.4	287.2*	50	70	38	10	10
		259.1	50	70	38	10	10
AfG_1	327.2	283*	50	-50	-25	10	10
		268	50	-50	-25	10	10
AfG_2	329.2	285*	50	-50	-25	10	10
		242	50	-50	-25	10	10
AfM_1	327.4	312.1*	50	-50	-30	10	10
		299.2	50	-50	-30	10	10
AfM_2	329.3	314.1*	50	-50	-30	10	10
		301.1	50	-50	-30	10	10
AfB_{2a}	329.2	258.1*	50	-50	-30	10	10
		243.2	50	-50	-30	10	10
AfQ_1	327.4	312.2*	50	-50	-25	10	10
		299.1	50	-50	-25	10	10
AfP_1	299.4	271.2*	50	70	40	10	10
		229.2	50	70	40	10	10
DIOL	345.2	283.2*	50	-50	-25	10	10
		327.2	50	-50	-25	10	10

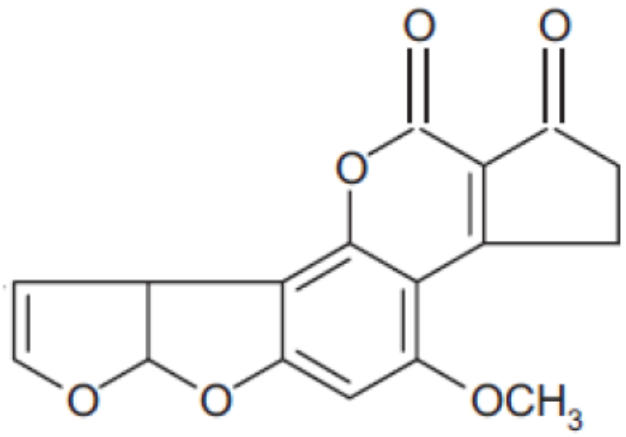


Figure 2-1 — Aflatoxin B_1, $C_{17}H_{12}O_6$; 312.06 Da; P_{kow}: -1.23

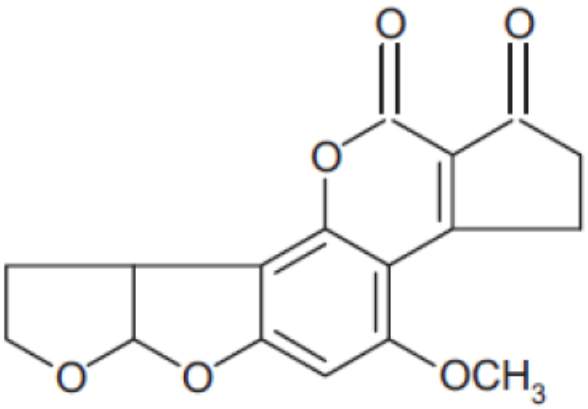


Figure 2-2 — Aflatoxin B_2, $C_{17}H_{14}O_6$; 314.06 Da; P_{kow}: 0.3

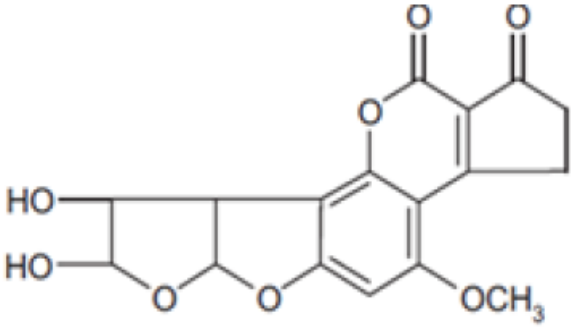


Figure 2-3 — Aflatoxin B_1 8,9-dihydrodiol (DIOL); $C_{17}H_{14}O_8$, MW:346.07 Da;

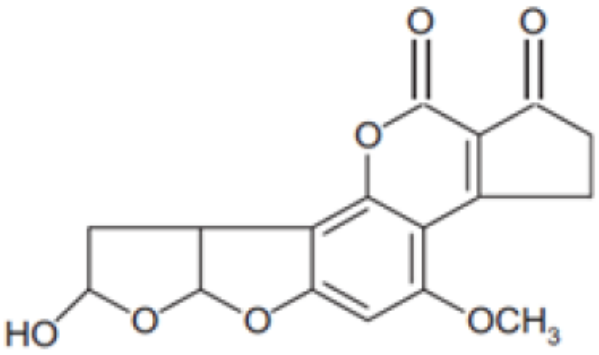


Figure 2-4 — Aflatoxin B_{2a}; $C_{17}H_{14}O_7$, MW:330.07 Da; P_{kow}: 0.09

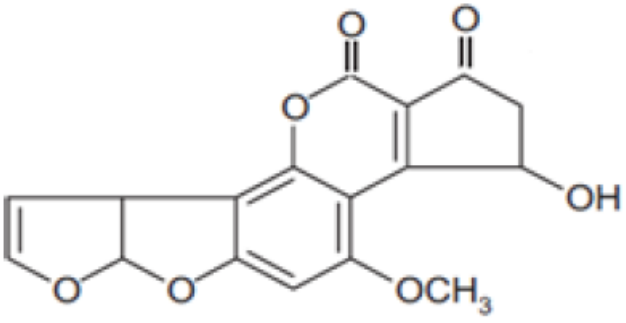


Figure 2-5 — Aflatoxin Q_1, $C_{17}H_{12}O_7$; 328.06 Da; P_{kow}: 0.3

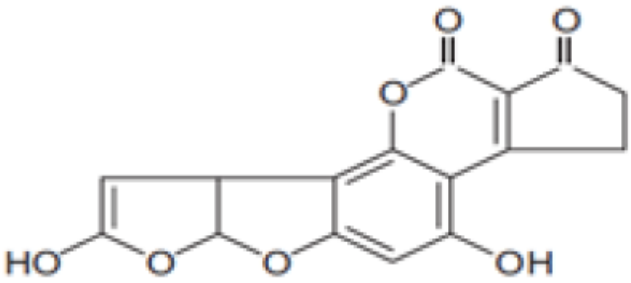


Figure 2-6 — Aflatoxin P_1, $C_{16}H_{10}O_6$; 298.04 Da; P_{kow}: 1.77

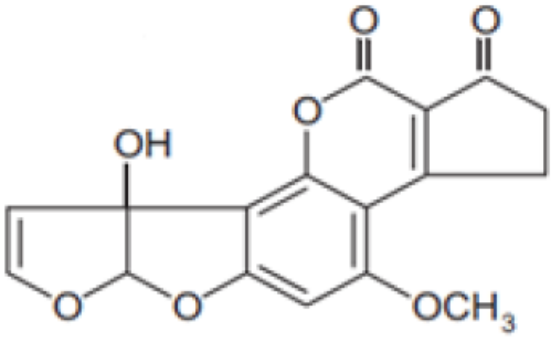


Figure 2-7 — Aflatoxin M_1, $C_{17}H_{12}O_7$; 328.06 Da; P_{kow}: -0.5

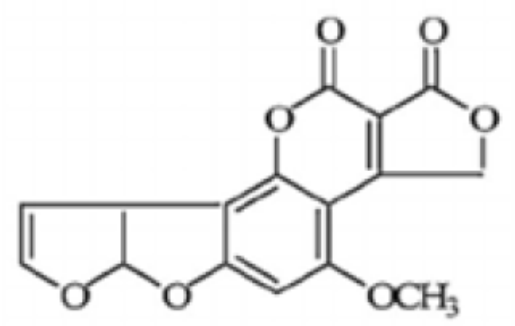


Figure 2-8 — Aflatoxin G_1 , $C_{17}H_{12}O_7$; 328.07 Da; P_{kow}: 0.83

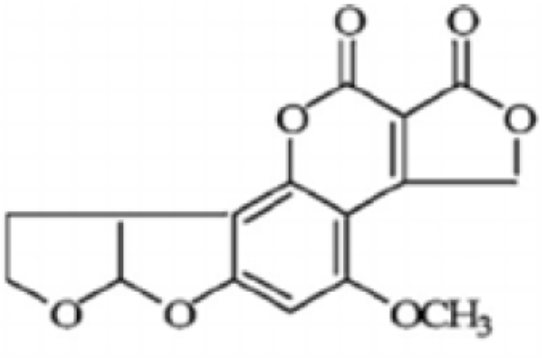


Figure 2-9 — Aflatoxin G_2, $C_{17}H_{14}O_7$; 330.07 Da; P_{kow}: 0.09

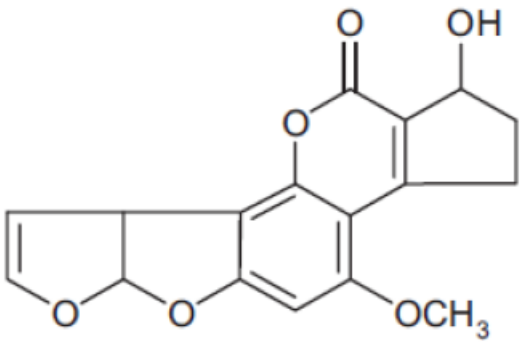


Figure 2-10 — Aflatoxicol, $C_{17}H_{14}O_6$; 314.08 Da; P_{kow}: -1.46

Figure 2 — Chemical structure of Aflatoxin B_1 related impurities optimised for detection by LC-MS/MS.

4.1.7. Data analysis

Data was evaluated using Analyst 1.6.3 software (SCIEX). Peak integration was verified manually for all samples and standards. Peak areas were extracted for quantification and uncertainty evaluation.

4.2. Total aflatoxin analysis by UV-spectrophotometry

4.2.1. Materials

- Acetonitrile. HPLC gradient grade (HiPerSolv Chromanorm, VWR)
- Ultra-Micro Cell Quartz Cuvette, 10mm light path (Perkin Elmer).
- Aflatoxin B_1 stock (OGP.030) and calibration (OGP.029) solutions.

4.2.2. Sample preparation

Ampoules of the stock or calibration solution were opened and an aliquot of the material was transferred to the cuvette for analysis without further manipulation. Acetonitrile was used in a reference cuvette to perform the instrument auto-zero (blank subtraction).

4.2.3. Instrumentation

Measurements were performed in a PerkinElmer Lambda 650 UV/VIS spectrometer.

4.2.4. UV-spectrophotometry parameters

A wavelength scan measurement method was used for qualitative analysis (i.e. identification of absorption maxima) and a fixed wavelength method to determine the absorbance value of solutions for quantitative analysis.

Wavelength scan method parameters:

- Deuterium lamp: on

- Tungsten lamp: on
- Scan from 370.00nm to 190.00nm
- Data interval: 1.00nm, scan speed: $266.75\text{nm} \cdot \text{min}^{-1}$
- Ordinate mode: A (Absorbance)
- Cycle: 1
- Slit: 2nm
- No cell changer

Fixed wavelength method parameters:

- Deuterium lamp: on
- Tungsten lamp: on
- Wavelengths: 223nm, 263nm and 360nm
- Ordinate mode: A (Absorbance)
- Cycle: 3
- Slit: 1nm
- Gain: Auto
- Response 0.2s
- No cell changer

4.2.5. Data analysis

The typical wavelength spectrum of AfB_1 is represented in Figure 3. The three observed absorption maxima at 223nm, 263nm and 360nm were selected as the fixed wavelengths for the quantitative analysis of AfB_1.

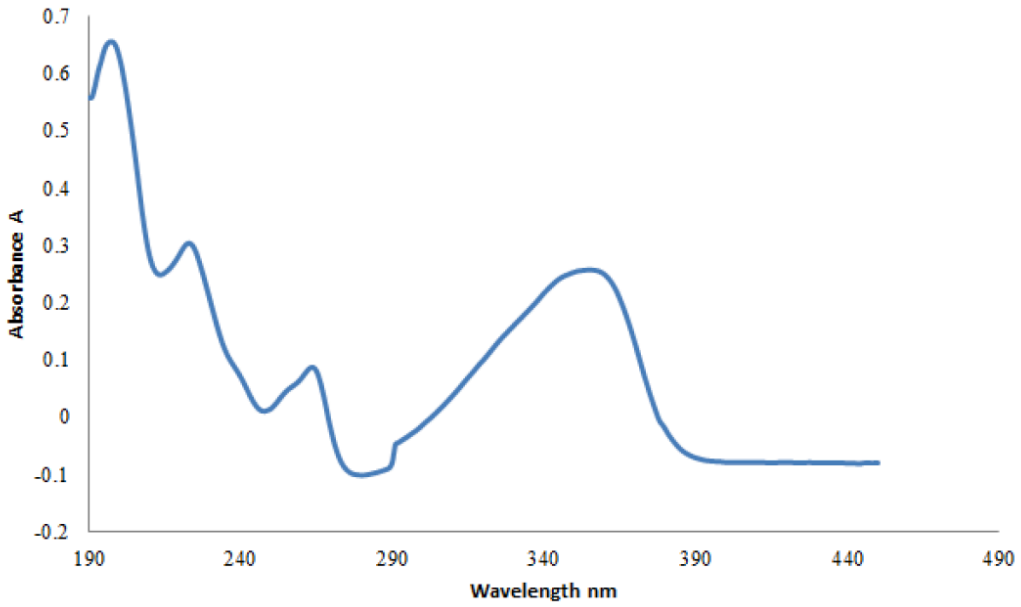


Figure 3 — UV-vis absorption spectrum of AfB_1.

Data were acquired using the Perkin Elmer UV WinLab software and absorbance measurements were extracted for data evaluation.

5. Characterization summary of the aflatoxin B_1 stock solution

5.1. Preparation and value assignment

The aflatoxin B_1 stock solution (OGP.030) was prepared gravimetrically by dissolving about 100mg of AfB_1 powder material (OGP.193a) in 1L of acetonitrile. Mettler Toledo balances MX5 and XP_10002S were used for the weighing of OGP.193a and the final solution mass, respectively. Table 2 demonstrates the preparation of the stock solution and the mass fraction assignment, calculated according to Equation (1). The purity of OGP.193a was determined in-house by quantitative NMR corrected for related structure impurities, as described in the Aflatoxin B_1 purity evaluation guideline. [5]

Table 2. Experimental data corresponding to the preparation of the aflatoxin B_1 stock solution and the calculated mass fraction.

Aflatoxin B_1 Stock solution preparation			
	Weighed mass (m)	Buoyancy (b)	$m \times b$
AfB_1 powder (mg)	102.630	1.000596	102.691
stock solution (g)	779.060	1.001386	780.140
purity $\pm u$ $(\mathrm{mg \cdot g^{-1}})$ *	979.6 ± 2.3		
Mass fraction $(\mu\mathrm{g \cdot g^{-1}})$	**128.95**		

$$w_{\text{stock}} = \frac{m_p \cdot b_p \cdot w_p}{m_{\text{sol}} \cdot b_{\text{sol}}} \tag{1}$$

where

m_p weighed mass of AfB_1 powder

b_p buoyancy correction of powder weighing

w_p purity of AfB_1 powder

m_{sol} weighed mass of stock solution

b_{sol} buoyancy correction of solution weighing

The uncertainties from input quantities in Equation (1) were combined (Equation (2), see p. 19) and the final uncertainty was calculated (Table 3, see p. 19). A minor uncertainty component, $u(V)$, was included to account for the potential solvent loss due to evaporation during sample preparation and weighing. The buoyancy mass correction and its uncertainty were calculated as described by Reichmuth et al. [8]

$$u(w_{\text{stock}}) = w_{\text{stock}} \cdot \sqrt{\left[\frac{u(m_p)}{m_p}\right]^2 + \left[\frac{u(b_p)}{b_p}\right]^2 + \left[\frac{u(w_p)}{w_p}\right]^2 + \left[\frac{u(m_{\text{sol}})}{m_{\text{sol}}}\right]^2 + \left[\frac{u(b_{\text{sol}})}{b_{\text{sol}}}\right]^2 + \left[\frac{u(V)}{V}\right]^2} \quad (2)$$

Table 3. Individual uncertainty components contributing to the final combined uncertainty of the AfB_1 stock solution mass fraction.

Unc. source	$\frac{u(m_p)}{m_p}$	$\frac{u(b_p)}{b_p}$	$\frac{u(w_p)}{w_p}$	$\frac{u(m_{\text{sol}})}{m_{\text{sol}}}$	$\frac{u(b_{\text{sol}})}{b_{\text{sol}}}$	$\frac{u(\text{V})}{\text{V}}$	u_{rel} (%)	$u(w_{\text{stock}})$ $\mu g \cdot g^{-1}$	$u(w_{\text{stock}})$ $\mu g \cdot g^{-1}$ $(k=2)$
Value (%)	0.0037	0.0017	0.23	0.0058	0.0012	0.005	0.235	0.303	0.61

The 1L flask containing the stock solution was agitated thoroughly and about 50mL were used to prepare the calibration solution (Chapter 6, see p. 22). The rest of the stock solution was stored at 4°C until ampouling, which took place within 24h of the preparation. The ampouling process was similar to that of the calibration solution and is described in detail in Section 6.1.

5.2. Stability study

The present section provides a summary of the stock solution stability results. A detailed description of the study design and evaluation is given for the characterization of the calibration solution (Section 6.2, see p. 22). The detected AfB_1 related impurities in the stock solution were AfB_2, AfB_{2a}, AfP_1, AfQ_1 and DIOL. They were measured in the tested ampoules by LC-MS/MS whereas the main component AfB_1 was measured by LC-DAD. In addition, all samples were measured by UV spectrophotometry for total aflatoxin content.

Original impurity standards were used for external calibration of the LC-MS/MS method and the calculated mass fractions were normalized to the reference samples (stored at −20°C). For the main component AfB_1, no calibration was performed so absorbance values were directly normalized to the main peak absorbance of the reference samples. Data were evaluated as a function of the storage time at each of the studied temperatures

A summary of the stability results of the stock solution is presented in Figure 4. Based on the data, it was concluded that shipping conditions should not exceed 22°C and one week transport time. Long-term storage is recommended at the reference temperature (−20°C) given the instability of AfQ_1 and DIOL, which albeit present at concentrations close to the limit of detection, were found to increase over time at higher temperatures.

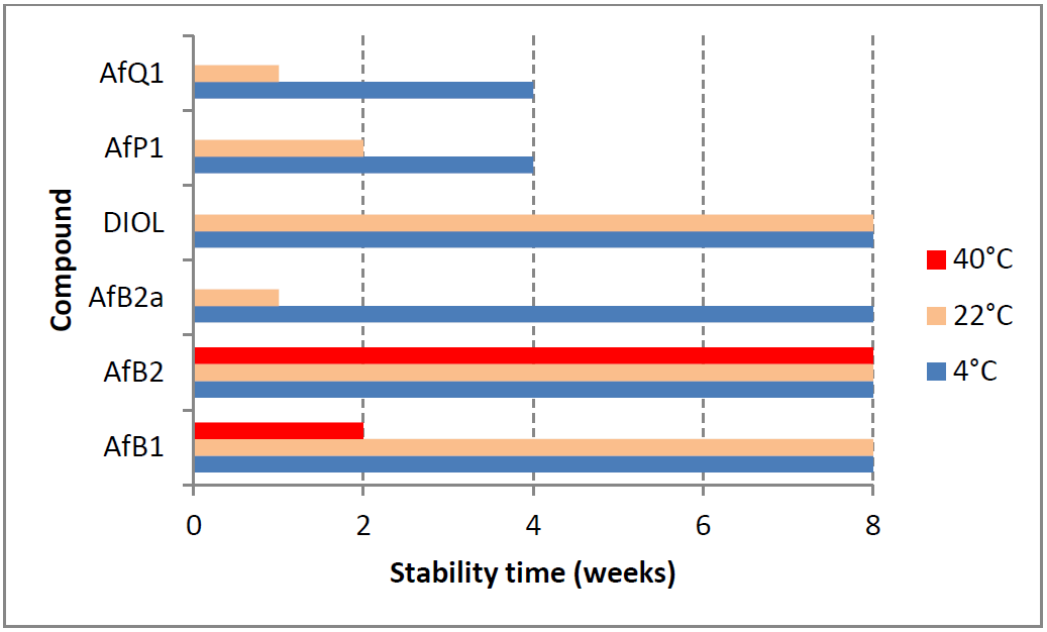


Figure 4 — Summary of the stability results for aflatoxin B_1 and detected related impurities in the AfB_1 stock solution. Bars represent the amount of time the indicated compound was found stable at the tested temperature.

5.3. Homogeneity study and combined uncertainty

The homogeneity study for the AfB_1 stock solution is analogous to that of the calibration solution, which is discussed in detail in Section 6.3. The present discussion is therefore limited to a summary of the results. AfB_1 and its impurities in the selected homogeneity samples were measured by LC-DAD and LC-MS/MS, respectively.

Homogeneity evaluation was done by single factor ANOVA, allowing for the separation of the variation associated with the method (s_{wb}) from the actual variation between ampoules (s_{bb}), which is an estimate of the uncertainty associated to batch heterogeneity. This uncertainty was 0.24 %, 2.62 % and 4.68 % for AfB_1 and the two major impurities AfB_2 and B_{2a}, respectively (Table 4, see p. 20). Other impurities had associated larger uncertainties due to being present at concentrations near the limit of detection of the method.

Table 4. Homogeneity results of the AfB_1 stock solution.

	AfB_1	AfB_2	B_{2a}	DIOL	AfQ_1	AfP_1
N (df)	29	29	29	29	29	29
s_{wb} (%)	0.62	8.08	8.23	11.15	6.96	6.90
s_{bb} (%)	0.24	- [(a)]	4.68	6.70	11.82	2.65
$u*_{bb}$ (%)	0.20	2.62	2.67	3.62	2.26	2.24
u_{bb} (%) or s_{bb} (%) [(b)]	0.24	2.62	4.68	6.70	11.82	2.65
F	1.44	0.84	1.97	2.08	9.66	1.44
F_{crit}	2.39	2.39	2.39	2.39	2.39	2.39

(*a*) Not calculable because $MS_{between} < MS_{within}$

(*b*) Highest value ($u*_{bb}$ or s_{bb}) was taken as uncertainty estimate for potential inhomogeneity. See Section 6.3 for detailed explanation.

The homogeneity uncertainty contribution for the main component AfB_1, u_{bb}, was combined with the uncertainty from the gravimetric value assignment – see $u(w_{stock})$ in Section 5.1 – to produce a final estimate of the mass fraction uncertainty of the batch (Table 5, see p. 21).

Table 5. Combination of the uncertainty from the gravimetric value assignment and the uncertainty from between-ampoule homogeneity to estimate the final uncertainty of the AfB_1 mass fraction in the batch of the stock solution.

$u(w_{stock})_{rel}$ (%)	u_{bb} (%)	$u(comb)_{rel}$ (%)	w_{stock} $\mu g \cdot g^{-1}$	U(comb) $\mu g \cdot g^{-1}$ (k=2)
0.235	0.237	0.33	128.95	0.86

6. Preparation and characterization of the aflatoxin B_1 calibration solution

6.1. Preparation and ampouling

The aflatoxin B_1 calibration solution (BIPM reference: OGP.029) was prepared by gravimetric dilution of 50mL of the stock solution with acetonitrile to a final volume of 1L. The solution was stored at 4°C until ampouling, which took place within 24h of the preparation. A 500mL bottle and 1–10mL bottle-top dispenser (Dispensette, Brand GMBH) were rinsed twice with the calibration solution and a stainless steel flat tip syringe needle was fitted at the outlet of the dispenser to ensure that all solution is discharged at the bottom of the ampoule.

10mL glass ampoules were selected for a filling volume of 4mL to ensure that sufficient head space remains above the liquid and therefore minimize the risk of accidental ignition of the solvent during the sealing process. An Ampoulmatic (Bioscience Inc) system connected to propane and oxygen cylinders was used to ampoule the batch. The flow of both gases was adjusted so as to produce a bright blue flame at the neck of the ampoules.

The ampoules were filled with 4mL of OGP.029, one at a time, to minimize the impact of evaporation of acetonitrile. A refrigerant (Jelt Refroidisseur 5320) was sprayed onto the lower portion of the ampoule before being placed in the ampouling carousel to further reduce the ignition risk. After flame sealing, ampoules were allowed to cool down at room temperature in an upright position.

To test for possible leaks, ampoules were placed into a vacuum drying oven (Haraeus) in an upright position and vacuum (50 mbar aprox.) was applied for at least 4 hours. The ampoules then remained in the sealed oven overnight, after which they were visually inspected for changes in the solution levels. Leaking ampoules were recorded and discarded while the rest of the batch was stored at –20°C.

6.2. Stability study

6.2.1. Study design

Short-term stability studies consider the impact of temperature and time to simulate potential transport conditions and/or storage conditions. Any significant influence of light, UV-radiation, moisture, etc. is excluded provided that the storage facilities and transport/ packaging conditions are appropriate.

The stability study of OGP.029 followed an isochronous design [9] with a reference temperature of –20°C and study temperatures of 4°C, 22°C and 40°C and storage in the dark. Selected sample units were transferred from study temperatures to the reference temperature every two weeks until the end of the eight-week study.

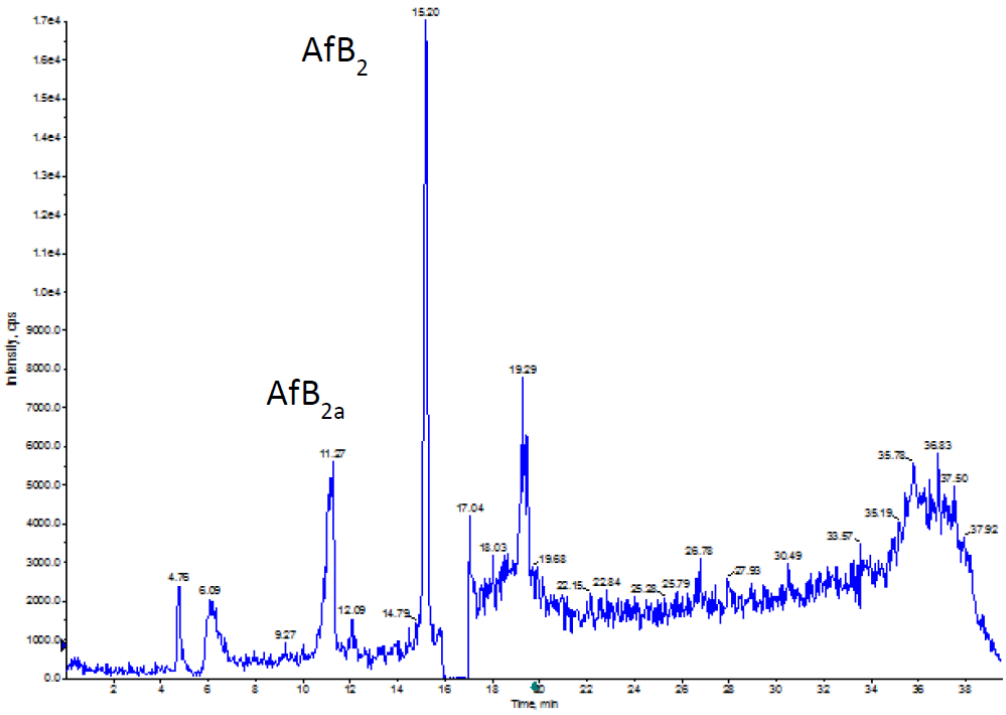
The sample units were selected using a random stratified sampling scheme from each of the quartiles of the approximately 200-unit batch. The study was composed of three units at the reference temperature and twelve units at each of the study temperatures, requiring 39 samples in total (Table 6, see p. 23).

Table 6. Temperatures, time points and sample units selected for the stability study of OGP.029. Units between parentheses were kept as back-up.

Temperature	Time (weeks)	Units
–20°C (reference temperature)	n.a.	020,127,(074)
4°C dark	2	048,111,(067)
	4	023,174,(118)
	6	012,157,(100)
	8	026,163,(071)
22°C dark	2	004,191,(104)
	4	007,150,(080)
	6	018,182,(144)
	8	036,185,(113)
40°C dark	2	035,159,(115)
	4	028,189,(081)
	6	042,179,(110)
	8	010,138,(064)

6.2.2. Stability study measurements

Two samples of each time point and temperature conditions were measured under repeatability conditions (same day and run) in a randomised manner using the LC-DAD method for AfB_1 and the LC-MS/MS method for the related structure impurities. Ampoules were vortexed before opening and two aliquots of 0.5mL were transferred into separate injection vials to have duplicate measurements of each sample (4 measurements for each condition). Representative TIC and DAD chromatograms of OGP.029 samples are shown in Figure 5.



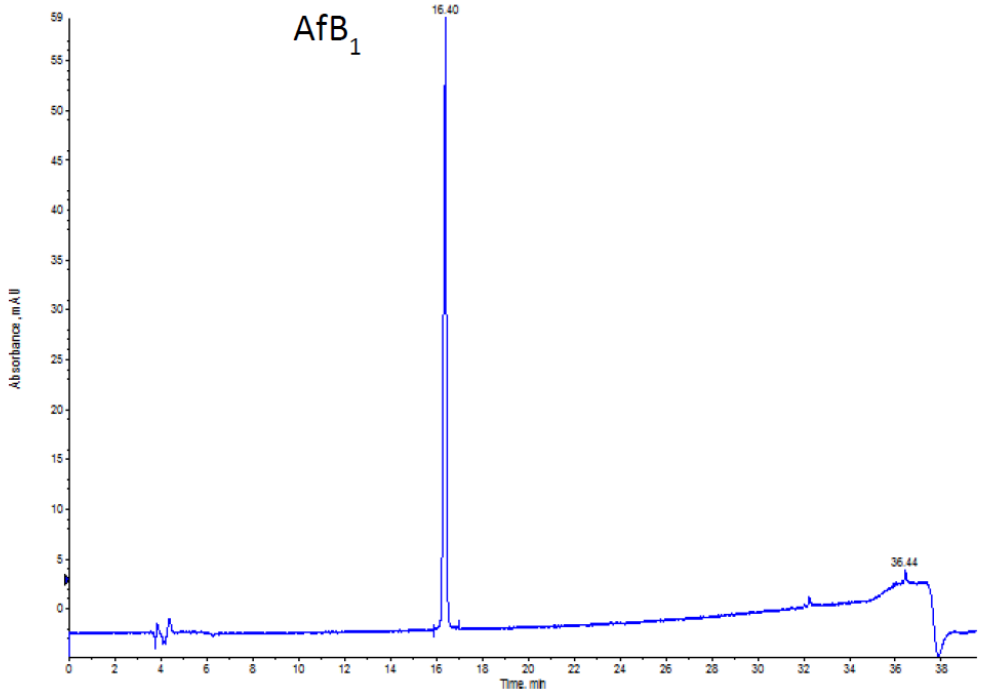
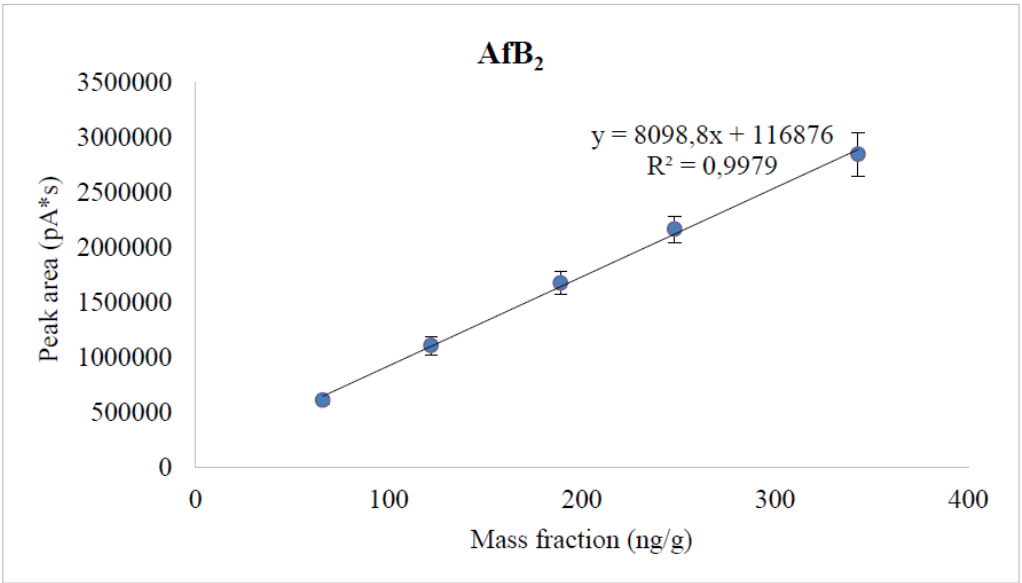


Figure 5 — Total ion chromatogram (top) and DAD 362nm chromatogram (bottom) of a representative sample of OGP.029.

The only two structure-related impurities found in OGP.029 above the limit of detection of the LC-MS/MS method were AfB_2 and AfB_{2a}. Four to five standard calibration solutions containing AfB_2 and AfB_{2a} standards in the ranges $4.3-21.4 ng \cdot g^{-1}$ and $9.5-652 ng \cdot g^{-1}$, respectively, were prepared to quantify these impurities by external calibration (Figure 6, see p. 24). Triplicate injections per standard level were spread over the analytical sequence. For the main component AfB_1, no calibration was performed but instead the peak areas from the LC-DAD chromatograms were directly evaluated.



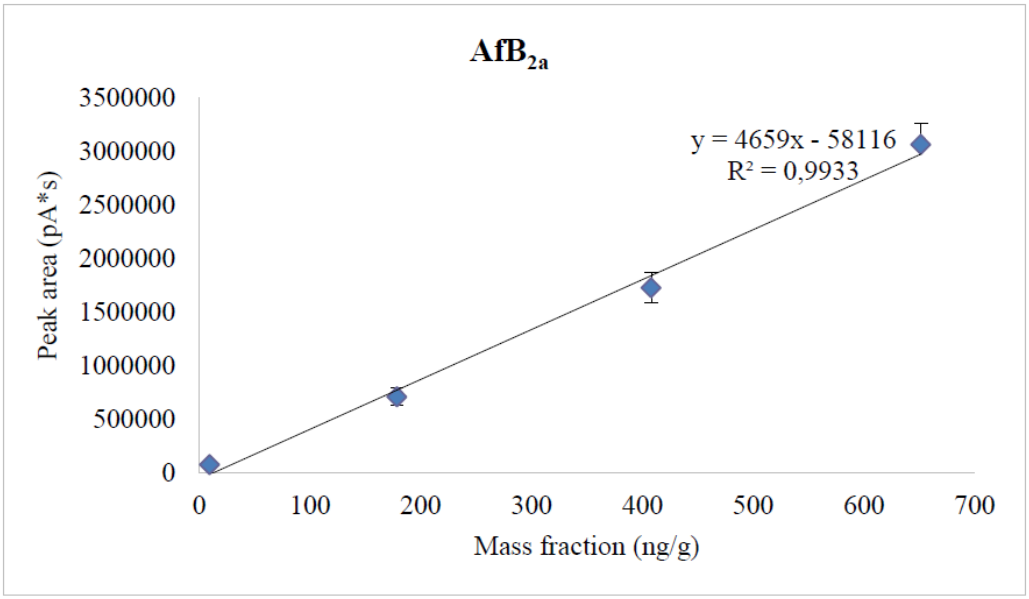


Figure 6 — External calibration functions for the quantification of structure-related impurities AfB_2 (top) and AfB_{2a} (bottom) in OGP.029.

6.2.3. Stability data evaluation

Calculated mass fraction values of impurities AfB_2 and AfB_{2a} and peak area values of the main component AfB_1 were normalized to the average values of the reference samples (stored at −20°C) to render results comparable. Statistical outliers were only removed in case of known technical reasons. As a first evaluation step, normalized data were plotted according to the injection sequence to discard any potential analytical drift. The slopes of the fitted regression lines were not significant (t-test) at the 95% confidence level (Figure 7, see p. 26).

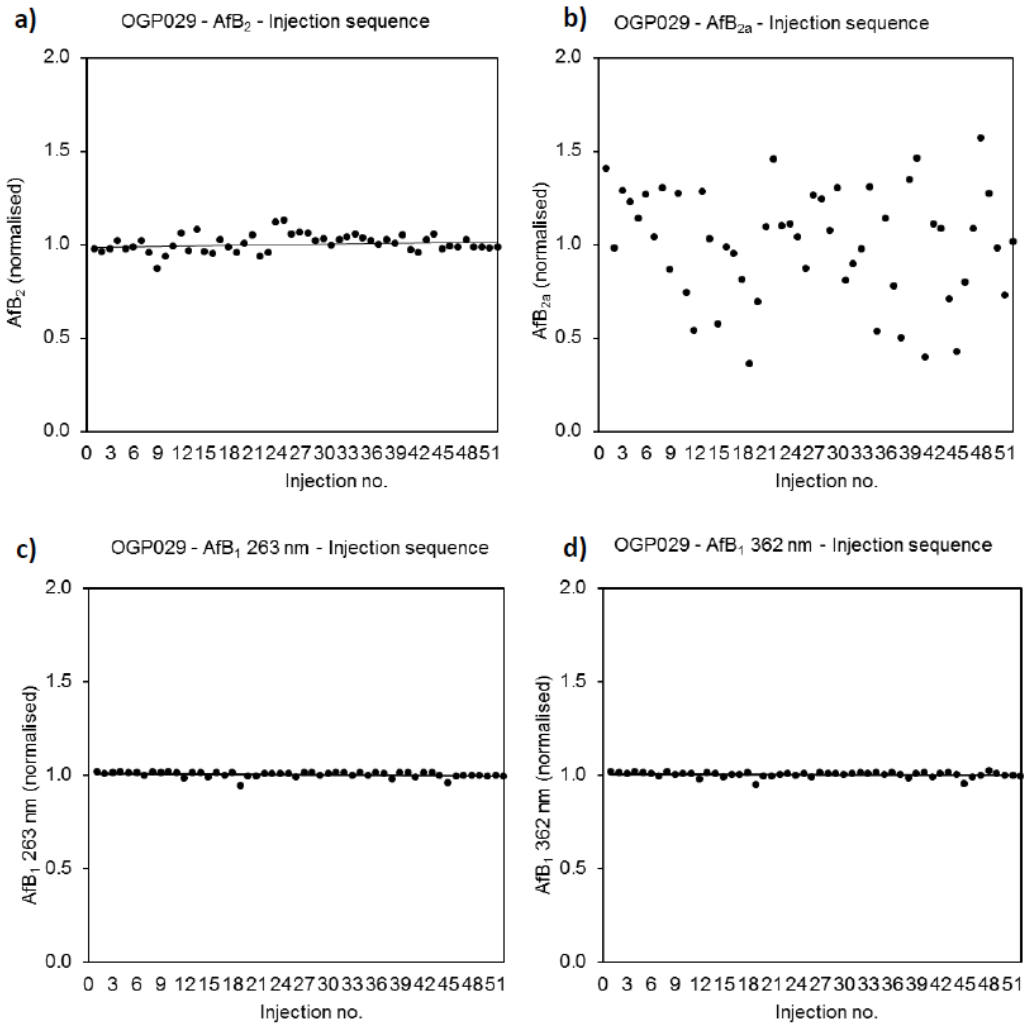


Figure 7 — OGP.029 stability data analysis to identify potential trends in the analytical sequence. Data correspond to normalized mass fractions of AfB_2 (a) and AfB_{2a} (b) impurities and normalized peak areas of the main compound AfB_1 as detected by LC-DAD at 263nm **(c) and** 362nm **(d).**

For each temperature, regression lines of the normalized values versus storage time were calculated. The fitted regression model was tested for overall significance (loss/increase due to storage) using an F-test (95% confidence level). The stability results of the main component and the impurities at each of the studied temperatures are shown in Figure 8.

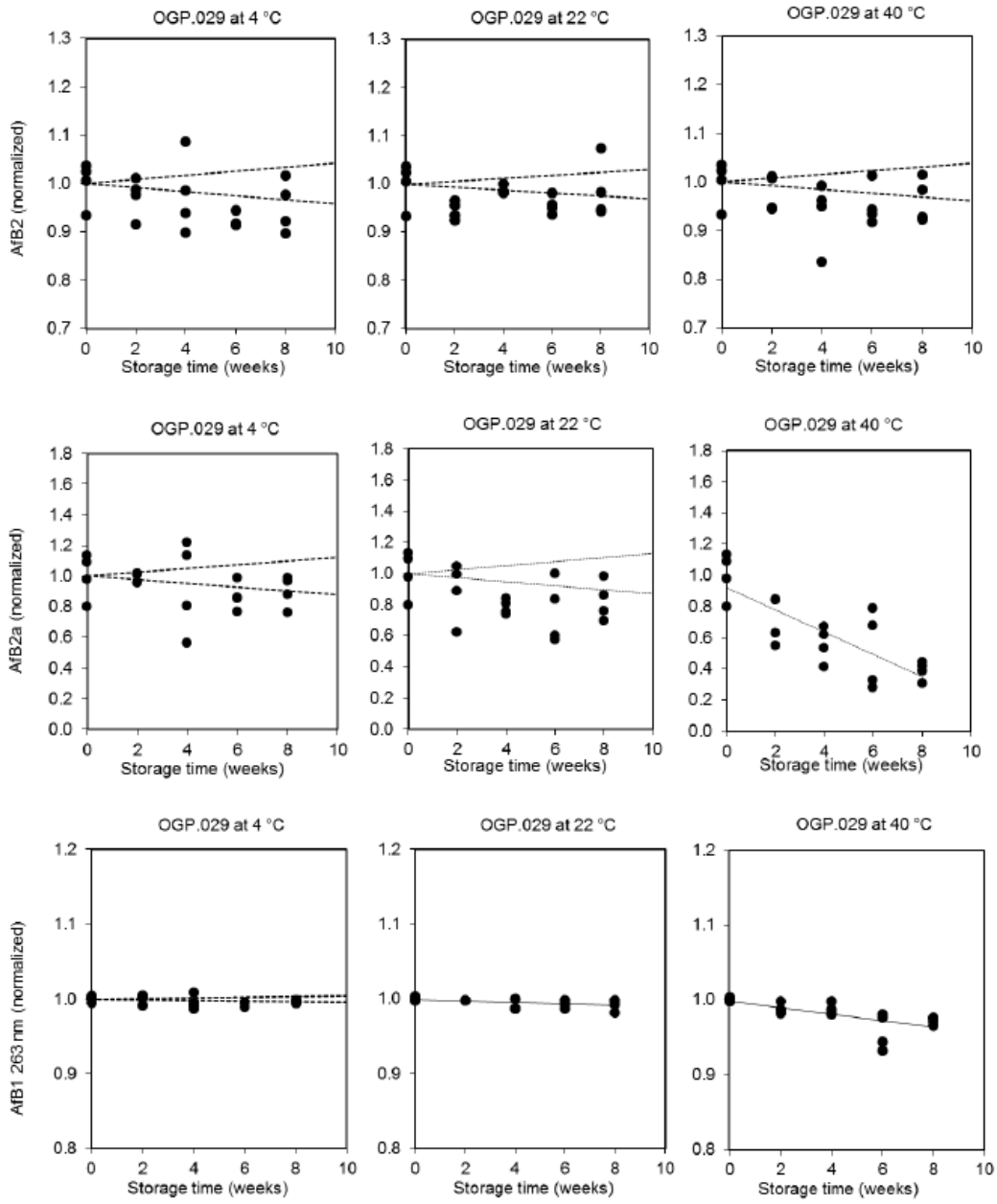


Figure 8 — Stability results of OGP.029 for the main component AfB_1 and impurities at the three studied temperatures. Data correspond to normalized mass fractions of AfB_2 (top) and AfB_{2a} (middle) impurities and normalized peak areas of the main compound AfB_1 as detected by LC-DAD at 263nm **(bottom). AfB_1 data acquired at** 362nm **were similar to those at** 263nm **(not shown). Dotted lines represent stability-associated uncertainty intervals of the normalized values as a function of the storage time. Single lines are fitted where the stability trend was found to be significant at the 95% confidence level.**

At 4°C, all studied compounds in OGP.029 were stable for 8 weeks, as evidenced by the absence of a significant trend (F-test, 95% confidence level). At 22°C, a small declining trend was observed for the main component AfB_1 over the studied period although the changes were considered negligible at 2 weeks. At 40°C, both the main component and the AfB_{2a} impurity degraded significantly over the 8-week period.

In conclusion, the OGP.029 calibration solution can be shipped safely in the dark at 22°C if the transport time does not exceed 2 weeks. In addition, storage at 4°C for a period of up to 8 weeks does not result in significant changes in composition.

6.3. Homogeneity study

6.3.1. Study design

Homogeneity between ampoules is evaluated to ensure that the assigned value of the calibration solution is valid for all units of the material, within the stated uncertainty. It is therefore necessary to determine this between-unit variation and incorporate it in a combined uncertainty estimate.

Ten ampoules were selected from the OGP.029 batch following a randomly stratified sampling scheme. They were measured under repeatability conditions using UV-spectrophotometry for total aflatoxin content and LC-DAD-MS/MS for AfB_1 and the detected structure-related impurities AfB_2 and AfB_{2a}.

6.3.2. Homogeneity study measurements

The selected ampoules were allowed to equilibrate at room temperature were vortexed before opening. They were analyzed in a random order to ensure that any trends in the ampouling process could be distinguished from potential trends in the analytical sequence.

Three aliquots per ampoule were measured consecutively by UV-spectrophotometry using the fixed wavelength method at 223, 265 and 360nm. Triplicate measurements of each aliquot gave rise to a total of 9 measurements per ampoule and wavelength. Another three aliquots ($> 500\mu L$) were transferred into glass injection vials and stored at $-20°C$ until LC-DAD-MS/MS analysis.

6.3.3. Homogeneity data evaluation

Absorbance and peak area values were normalized with respect to the average result for each of the studied compounds. Statistical outliers were only removed in case of known technical reasons. Linear regression functions were calculated for the normalized values arranged in ampouling and analysis order. The slopes of the lines were tested for significance at a 95 % confidence level to discard the presence of trends. Figure 9 shows the UV-360nm aflatoxin measurements displayed according to the order of analysis and of ampouling. The dispersion of measurement results across the analytical sequence implied no need for trend correction and this variability is encompassed by the uncertainty associated to homogeneity.

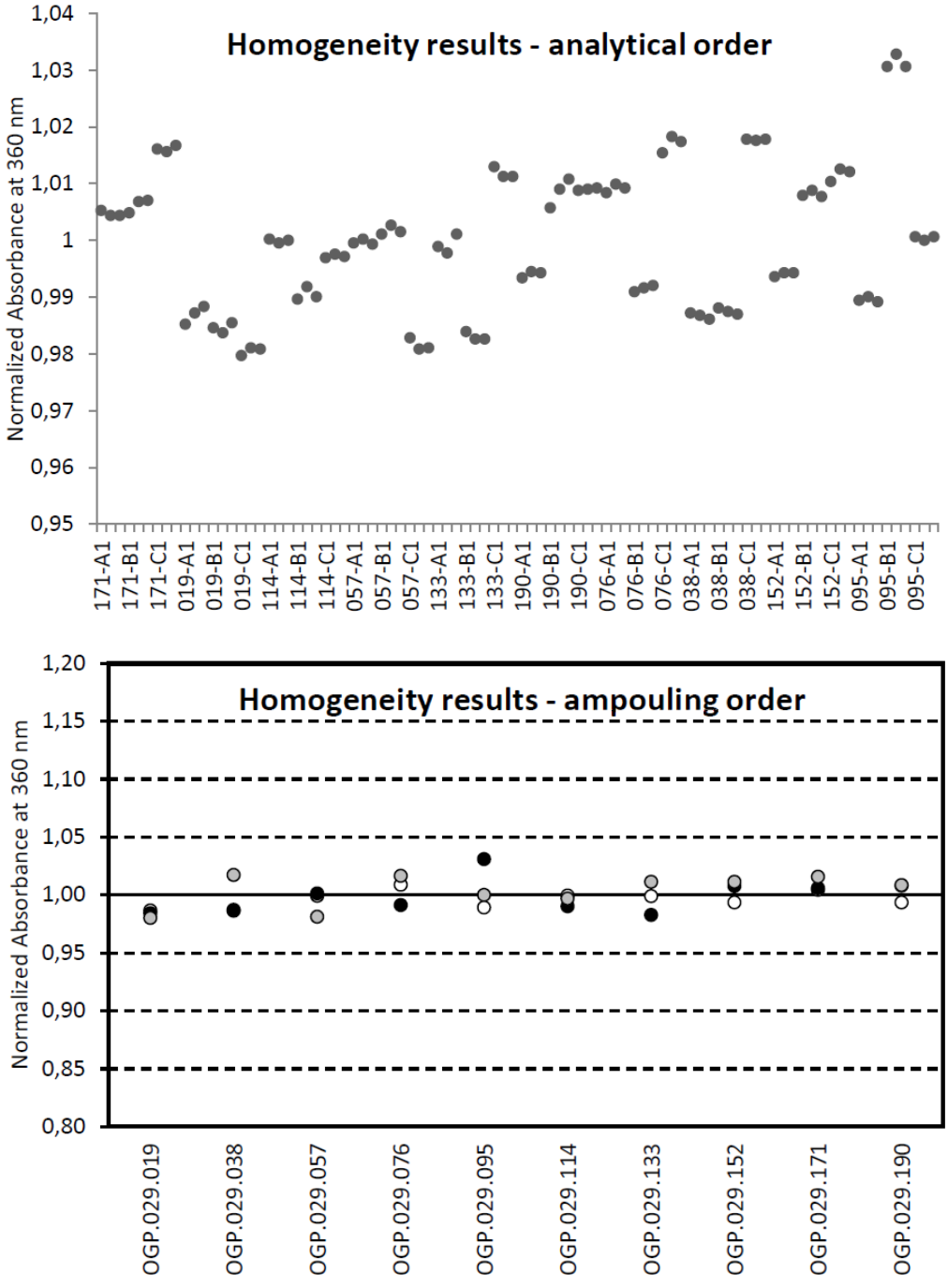


Figure 9 — Homogeneity results of OGP.029 as determined by UV-spectrophotometry at 360nm **plotted according to the analysis (top) or ampouling (bottom) order.**

Homogeneity samples results for the main compound AfB_1 and related impurities AfB_2 and AfB_{2a} obtained by LC-DAD-MS/MS are shown in Figure 10 as normalized peak areas.

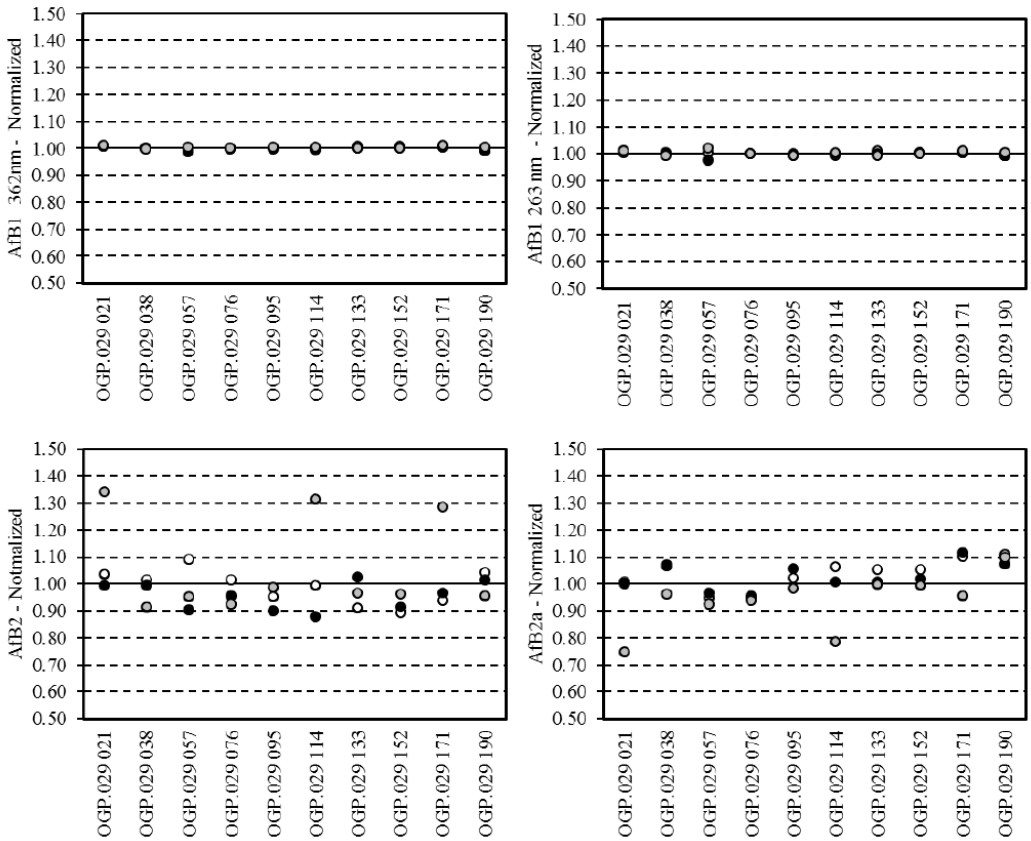


Figure 10 — Homogeneity results of OGP.029 calibration solution as determined by LC-DAD-MS/MS for AfB_1 (362nm **and** 263nm **detection, top) and related impurities AfB_2 and AfB_{2a} (MS/MS detection, bottom).**

Quantification of between-unit heterogeneity was done by analysis of variance (ANOVA), which allows for the separation of the variation between ampoules (s_{bb}) from that associated with the method repeatability (s_{wb}). These variances are calculated as follows:

$$s_{bb}^2 = \frac{MS_{btw} - MS_{with}}{n} \tag{3}$$

$$s_{wb}^2 = MS_{with} \tag{4}$$

where MS_{btw} and MS_{with} are the mean sums of squares between- and within-units obtained by the ANOVA evaluation and n is the number of replicates per ampoule ($n = 3$).

The standard deviation between the sample units is used as the estimator for the between-units variability. The measurement variation sets a lower limit to this estimator reflected in MS_{btw} being smaller than MS_{with}. This does not imply that the material is perfectly homogeneous, but only shows that the study set-up was not adequate to detect evidence of heterogeneity. In this case, the maximum heterogeneity that could be hidden by the intrinsic variability of the method, $u*_{bb}$, is calculated according to the equation below: [10]

$$u_{bb}* = \sqrt{\frac{MS_w}{n}} \cdot \sqrt[4]{\frac{2}{p(n-1)}} \tag{5}$$

where p is the number of measured ampoules ($p = 10$) and n is the number of measurement replicates per ampoule ($n = 3$).

The final uncertainty from homogeneity (u_{bb}) is estimated as s_{bb} or $u*_{bb}$, depending on which of these is larger. This uncertainty is presented in Table 7 for every measured compound using the LC-DAD-MS/MS method. The F-test at the 95% confidence level did not detect significant differences between ampoules for any of the studied compounds.

Table 7. Homogeneity uncertainty results of OGP.029 from data generated by LC-DAD-MS/MS

	AfB_1 362nm	AfB_2	AfB_{2a}
N (df)	29	29	29
s_{wb} (%)	0.51	12.13	7.65
s_{bb} (%)	0.32	- (a)	3.55
$u*_{bb}$ (%)	0.17	3.94	2.48
u_{bb} (%) or s_{bb} (%) (b)	0.32	3.94	3.55
F	2.15	0.81	1.65
F_{crit}	2.39	2.39	2.39

(a) Not calculable because $MS_{btw} < MS_{with}$

(b) Higher value ($u*_{bb}$ or s_{bb}) was taken as uncertainty estimate for potential inhomogeneity

Homogeneity results obtained by UV-spectrophotometry at the 3 measured wavelengths are shown in Table 8. They confirm the findings of the chromatographic method and therefore the AfB_1 calibration solution can be regarded as homogeneous.

Table 8. Homogeneity uncertainty results of OGP.029 from data generated by UV-spectrophotometry at the three measured wavelengths 223**,** 265 **and** 360nm**.**

	223nm	265nm	360nm
N (df)	29	29	29
s_{wb} (%)	1.21	0.86	1.16
s_{bb} (%)	0.81	0.23	- (a)
$u*_{bb}$ (%)	0.39	0.28	0.38
u_{bb} (%) or s_{bb} (%) (b)	0.81	0.28	0.38
F	2.33	1.21	0.36
F_{crit}	2.393	2.393	2.393

(a) Not calculable because $MS_{btw} < MS_{with}$

(b) Higher value ($u*_{bb}$ or s_{bb}) was taken as uncertainty estimate for potential inhomogeneity

6.4. Mass fraction value assignment and uncertainty

The preparation of the calibration solution and the mass fraction assignment, w_{cal}, are shown in Table 9. Mettler Toledo balance $XP_1$0002S was used for all mass determinations.

Table 9. Experimental data corresponding to the preparation of the aflatoxin B_1 calibration solution and the calculated mass fraction.

Aflatoxin B_1 calibration solution preparation			
	Weighed mass (m)	Buoyancy (b)	$m \times b$
AfB_1 stock sol. (mg)	39.02	1.001386	39.074
Calibration sol. (g)	779.640	1.001386	780.721
w("stock") ± u ($mg \cdot g^{-1}$)	128.95 ± 0.30 [a]		
w_{cal} ($\mu g \cdot g^{-1}$)	6.454		

(*a*) The uncertainty of the stock solution mass fraction does not comprise any homogeneity contribution since the bulk stock solution (prior to ampouling) was used as source material.

The AfB_1 mass fraction of OGP.029, calculated according to Equation (6), was $6.45 \mu g \cdot g^{-1}$. The associated uncertainty was calculated by considering the input quantities and related uncertainties represented in the Ishikawa diagram of Figure 11.

$$w_{cal} = \frac{m_{stock} \cdot b_{stock} \cdot w_{stock}}{m_{sol} \cdot b_{sol}} \quad (6)$$

where

m_{stock} weighed mass of AfB_1 stock solution

b_{stock} buoyancy correction of stock solution weighing

w_{stock} AfB_1 mass fraction of the stock solution

m_{sol} weighed mass of calibration solution OGP.029

b_{sol} buoyancy correction of calibration solution weighing

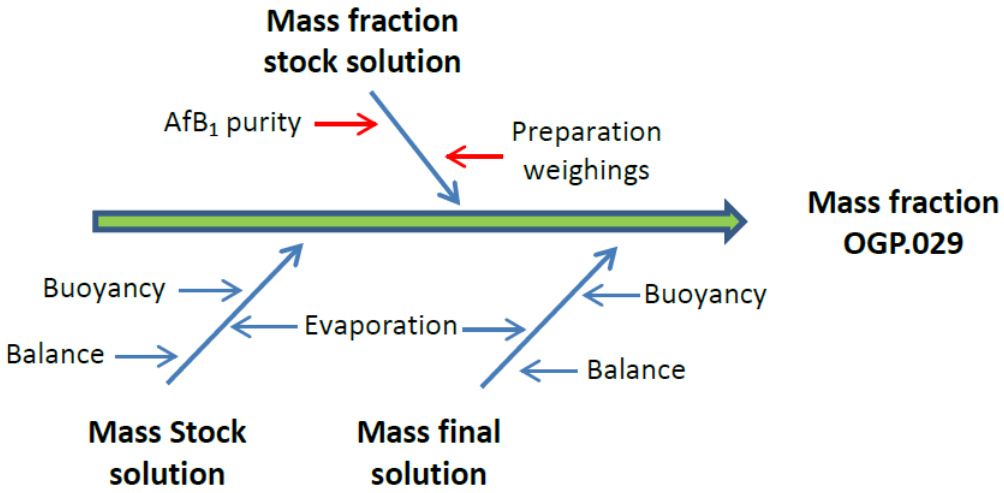


Figure 11 — Ishikawa diagram indicating the input quantities contributing to the final uncertainty of the AfB_1 mass fraction of the calibration solution OGP.029.

The standard uncertainties of the input quantities of Figure 11 were combined (Equation (7), see p. 33) to produce the uncertainty of the calibration solution mass fraction, $u(w_{cal})$ (Table 10, see p. 33). The uncertainty of the stock solution already comprises the purity of the source material and the weighing operations, as described in Section 5.1. The evaporation uncertainty, $u(V)$, accounts for potential solvent losses during the weighing of the stock solution and of the final solution. The buoyancy mass correction and its uncertainty were calculated as described by Reichmuth et al. [8]

$$u(w_{cal}) = w_{cal} \cdot \sqrt{\left[\frac{u(m_{stock})}{m_{stock}}\right]^2 + \left[\frac{u(b_{stock})}{b_{stock}}\right]^2 + \left[\frac{u(w_{stock})}{w_{stock}}\right]^2 + \left[\frac{u(m_{sol})}{m_{sol}}\right]^2 + \left[\frac{u(b_{sol})}{b_{sol}}\right]^2 + 2 \cdot \left[\frac{u(V)}{V}\right]^2} \qquad (7)$$

Table 10. Individual uncertainty components contributing to the final combined uncertainty of OGP.029 mass fraction.

Unc. source	$\frac{u(m_{stock})}{m_{stock}}$	$\frac{u(b_{stock})}{b_{stock}}$	$\frac{u(w_{stock})}{w_{stock}}$	$\frac{u(m_{sol})}{m_{sol}}$	$\frac{u(b_{sol})}{b_{sol}}$	$\frac{u(V)}{V}$	u_{rel} (%)	$u(w_{stock})$ $\mu g \cdot g^{-1}$	$u(w_{stock})$ $\mu g \cdot g^{-1}$ $(k=2)$
Value (%)	0.0466	0.0012	0.235	0.0028	0.0012	0.005	0.240	0.015	0.031

The uncertainty $u(w_{cal})$ corresponding to the gravimetric value assignment was combined with the homogeneity uncertainty contribution for the main component AfB_1, $u_{bb} = 0.32\%$ (Table 7, Section 6.3, see p. 28) to produce a final estimate of the mass fraction uncertainty of the batch (Table 11, see p. 33).

Table 11. Combination of the uncertainty from the gravimetric value assignment and the uncertainty from between-ampoule homogeneity to estimate the final uncertainty of the AfB_1 mass fraction in the batch of the calibration solution OGP.029.

$u(w_{cal})_{rel}$ (%)	u_{bb} (%)	$u(comb)_{rel}$ (%)	w_{cal} $\mu g \cdot g^{-1}$	$U(comb)$ $\mu g \cdot g^{-1}$ $(k=2)$

0.240	0.316	0.397	6.454	0.051

The aflatoxin B_1 mass fraction value and associated expanded uncertainty (k=2) of the calibration solution batch was $6.454 \pm 0.051 \mu g \cdot g^{-1}$.

6.5. Mass fraction value verification by analytical methods

The AfB_1 mass fraction value assigned gravimetrically to the calibration solution OGP.029 can be verified by an independent analytical method to gain additional confidence in the certified value. Both the LC-DAD and the UV-spectrophotometry methods described in Chapter 4 can be used for this purpose. Ideally, a different AfB_1 calibrant of certified purity should be used for calibration so that results are completely independent. In the absence of such calibrant, a partially independent calibration solution could be prepared from the same original source material.

Figure 12 shows the verification of three different AfB_1 calibration solution batches prepared in accordance with this guideline. The values assigned gravimetrically were compared to the analytical values obtained using the LC-DAD and the UV-spectrophotometry methods calibrated externally with a semi-independent AfB_1 standard. The agreement between the pairs of methods values is conveniently assessed using the degrees of equivalence (DoE):

$$\mathrm{DoE} = w(cal)_{meth} - w(cal)_{grav} \tag{8}$$

where $w(cal)_{meth}$ and $w(cal)_{grav}$ are the mass fractions calculated using the analytical and the gravimetric methods, respectively.

The standard uncertainties of the gravimetric (including the homogeneity component) and analytical values add in quadrature to yield the combined uncertainty of the DoE value. The expanded uncertainty bars (k=2) crossing zero indicate the agreement of the analytical measurements (LC-DAD or UV-spectrophotometry) with the gravimetrically assigned values at an approximately 95% confidence level.

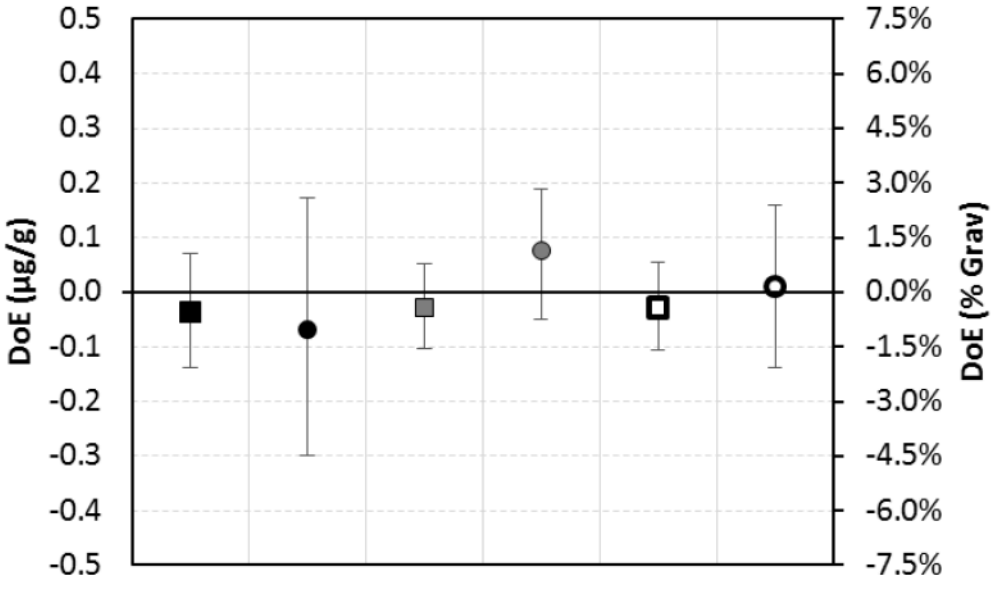


Figure 12 — Degrees of equivalence (DoE) between the gravimetrically assigned values of three different AfB_1 calibration solution batches (shown in different

colors) and the analytical values obtained by LC-DAD (squares) and UV spectrophotometry (circles). Bars represent expanded uncertainties of the DoE values (k=2).

7. Acknowledgements

The chromatography and spectrophotometry methods used in this study were developed by the co-authors of this document in the course of secondments at the BIPM. MM-CBKT participants Lucía Casas (LATU), Vanesa Morales (INM) and Rachel Torkhani (INRAP) are acknowledged for their contribution to the comparison results of different AfB_1 calibration solution batches. The support of the parent institution of each scientist in making them available for secondment to the BIPM is gratefully acknowledged.

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Document Control

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