Rapport BIPM-2021/01

Purity Evaluation Guideline: Aflatoxin B_1

BIPM PEG-02

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Version 1.0 : February 16th 2021

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1. Scope

This document has been prepared to provide technical guidance and reference data to assist with the establishment of the qualitative identity and quantitative characterization of aflatoxin B_1 (AfB_1) as present in a purified solid material. In particular it is intended for use to assist in the characterization of a Primary Reference Material[1] for AfB_1 that can be used to underpin the metrological traceability of routine testing procedures for the detection of contamination by AfB_1 of food, feedstuffs and primary produce.

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.[2] This project is designed to allow NMIs to work together to strengthen the worldwide mycotoxin metrology infrastructure, to provide knowledge transfer to scientists developing capabilities in this area and to enable NMIs in developing regions to produce calibrants, matrix reference materials and proficiency test samples to support testing and laboratory services for mycotoxin analysis within their countries.

As for all other areas of organic analysis primary reference materials consisting of well characterized, high purity compounds are required for each analyte subject to investigation. These materials are the ultimate source of higher-order metrological traceability for the assigned values of derived calibration solutions, reference materials, proficiency test samples and ultimately the results of routine analysis. Access to pure organic compounds and calibration solutions prepared from these materials is an essential element in the measurement infrastructure supporting the delivery of reliable, comparable results. In the case of mycotoxins purity analysis of source materials involves additional challenges linked to the limited amount of available material and its potential toxicity.

Aflatoxins are a class of mycotoxins 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.[3] 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.[4]

The ability to undertake robust and reliable analysis for contamination of primary produce with AfB_1 and related compounds is required for health and food safety and for trade by countries which produce or consume large quantities of corn grains and wheat.[5]

An essential requirement of the BIPM CBKT project was to obtain and characterize a primary reference material for AfB_1 that could be used subsequently to establish a hierarchy to underpin the metrological traceability[6] of results linked through calibration to this material. This guideline summarizes characterization and purity assignment studies to assess identity and purity of a Primary Reference Material for AfB_1 to deliver the BIPM MMCBKT program and is intended to be of use to other metrology institutes and reference measurement service providers needing to characterize their own primary material for AfB_1 analysis. Reliance was placed on nuclear magnetic resonance spectroscopy (NMR) studies both to confirm the qualitative identity of the main component of the material and to assign the mass fraction content of aflatoxin B_1 it contained.

Due to its relatively complex structure, the assignment by qNMR provided in the first instance an estimate of the total AfB_1 plus related structure impurity content. This initial value needed correction for the related structure impurity content as assigned separately by LC-MS/MS and LC-DAD methods to give the final value for the true AfB_1 content of the material. Additional analyses for the assessment of other potential impurities were undertaken to support the value assigned through the qNMR and LC data.

3. Nomenclature and Ring numbering

Throughout this report the ring numbering and abbreviations[7,8] for the specification of AfB_1 and related compounds are used. The abbreviations and structures for AfB_1 and the primary related aflatoxins are given in Annex 7.1.

The structure of AfB_1 with the standard conventional numbering scheme is shown in Figure 1. A shorthand assignment using designations A-G for each of the eight distinct ^{1}H NMR resonances in AfB_1 was also used in this report and is shown.

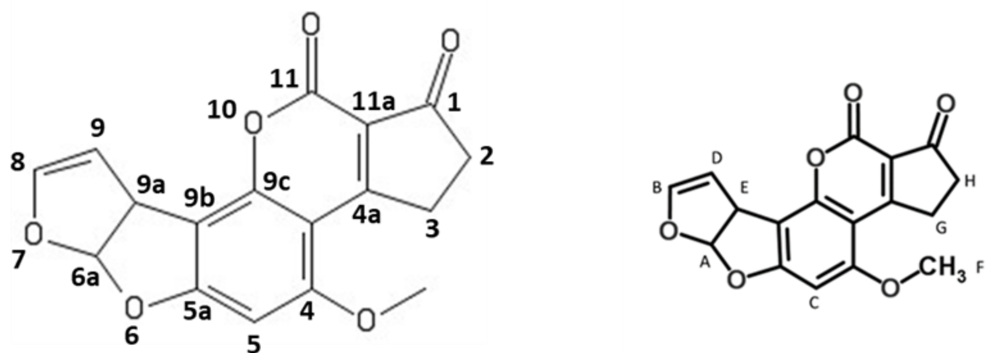


Figure 1: AfB_1 structure with the literature-based numbering scheme (left)[9] and the alphabetical code (right) used in this report to identify ^{1}H assignments.

4. Properties of Aflatoxin B_1

4.1 Hazard Identification

The substance poses high potential risks for human health if handled inappropriately. It is extremely toxic by inhalation, in contact with skin and if swallowed (hazard class 6.1, UN3462).

AfB_1 is believed to be hepatotoxic, carcinogenic and teratogenic.

DISCLAIMER: The safety recommendations given in this section are based on review of literature reports of best practice but have not been verified by the BIPM.

4.1.1 Protective measures

Avoid inhalation 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.

It is advised that pregnant women should avoid handling AfB_1 solutions if possible.

4.1.2 Emergency procedures

General advice: Immediately call a POISON CENTER or doctor/physician. Show this safety information to the doctor in attendance. Move out of dangerous area.

If inhaled: Move 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.

4.1.3 Spillage

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.

4.2 Physical and Chemical Properties

Common Name:	**Aflatoxin B_1**
IUPAC and Chemical Abstracts Names:	2,3,6a*R*,9a*S*-Tetrahydro-4-methoxycyclopenta[c]furo[3',2':4,5] furo[2,3-h]chromen-1,11-dione; [10] 2,3,6a*R*,9a*S*-Tetrahydro-4-methoxycyclopenta[c]furo[3',2':4,5] furo[2,3-h]benzopyran-1,11-dione;[10] 11-Methoxy-6,8,19-trioxapentacyclo [$10.7.0.0^{2,9}.0^{3,7}.0^{13,17}$] nonadeca-1,4,9,11,13(17)-pentaene-16,18-dione[11]
Synonyms:	Aflatoxin B, AfB_1, AFB_1
CAS Registry Numbers:	1162-65-8
Molecular Formula:	$C_{17}H_{12}O_6$
Molar Mass:	312.27 g/mol
Monoisotopic mass:	312.0634
Melting point:	268 °C [12]
Appearance:	Crystals exhibit blue fluorescence[13]
Solubility:	Slightly soluble in water (16 mg/L); progressively more soluble in acetonitrile, CH_2Cl_2, $CHCl_3$, methanol, ethanol, acetone and DMSO.
UV maxima (nm)	EtOH: 223 (ϵ = 25600), 265 (ϵ = 13400), 362 (ϵ = 21800)[13]
FTIR (cm^{-1}, fingerprint)	1731 (C=O), 1655, 1635, 1597, 1557, 1125, 1072, 1040[14]

4.3 Qualitative identification

4.3.1 **NMR Materials and methods**

Chemicals:

- Aflatoxin B_1 (AfB_1); BIPM Reference OGO.193
  Supplier: First Standard, Product No. 1ST7205, Lot ALT603155

NMR Solvents:

- Deuterated chloroform ($CDCl_3$); BIPM Reference OGS.026b
- Acetone-d_6; BIPM Reference OGS.029

Solvents were purchased from a commercial supplier and used without further treatment.

4.3.2 **Sample preparation**

For qualitative NMR analyses sample sizes typically in the range 5 mg - 7 mg of AfB_1 were weighed accurately and made up in 1 mL of deuterated solvent in a glass vial. The sample solution was mixed in a vortex shaker and transferred into NMR tubes (HG-Type: high grade class, 8 inch, 5 mm o.d., with PE caps) using disposable glass pasteur pipettes.

4.3.3 **NMR acquisition parameters**

A JEOL ECS-400 spectrometer operating at 9.4 T (400 MHz for proton) equipped with a direct type automatic tuning (Royal) probe was used for all data acquisition. For qualitative analyses, ^1H spectra were acquired for both solvent blank and the AfB_1 sample using a simple pulse-acquire sequence with the parameters presented in Table 1.

Table 1 - Acquisition parameters for exploratory ^1H analyses.

Parameter	***Value***
Number of Transients	64
Receiver gain	44
Acquisition time (s)	4
Relaxation delay (s)	1.0
Pulse offset (ppm)	7.0
Spectral width (ppm)	20.0
Data points	32768
Temperature (K)	298
Spinning	Off

^{13}C-NMR experiments were conducted using an ordinary power gated sequence (pulse-acquire in ^{13}C channel with proton decoupling both during acquisition and the relaxation delay) using the parameters shown in Table 2.

Table 2 - Acquisition parameters used for ^{13}C analyses.

Parameter	***Value***
Number of Transients	1024
Receiver gain	50
Acquisition time (s)	1.04
Relaxation delay (s)	2.0
Pulse offset (ppm)	100
Spectral width (ppm)	250
Data points	32768
Temperature (K)	298
Spinning	Off

4.3.4 **1D ^{1}H- and ^{13}C-NMR spectra**

The simple ^{1}H- and ^{13}C-NMR spectra of the AfB_1 material are shown in Figures 2 and 3. The results obtained were consistent with literature assignments.[14,15] Figure 4 shows the attached proton test (APT) ^{13}C-NMR spectrum of AfB_1. Inverted signals correspond to methylene or quaternary carbons and normal signals to methine or methyl carbons.

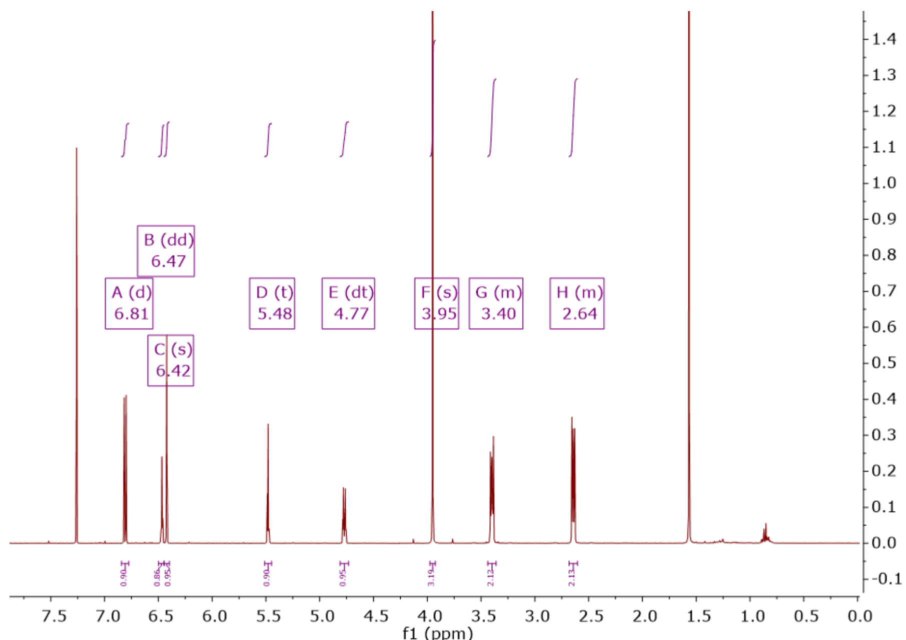


Figure 2 – ^{1}H NMR spectrum of AfB_1 in $CDCl_3$.

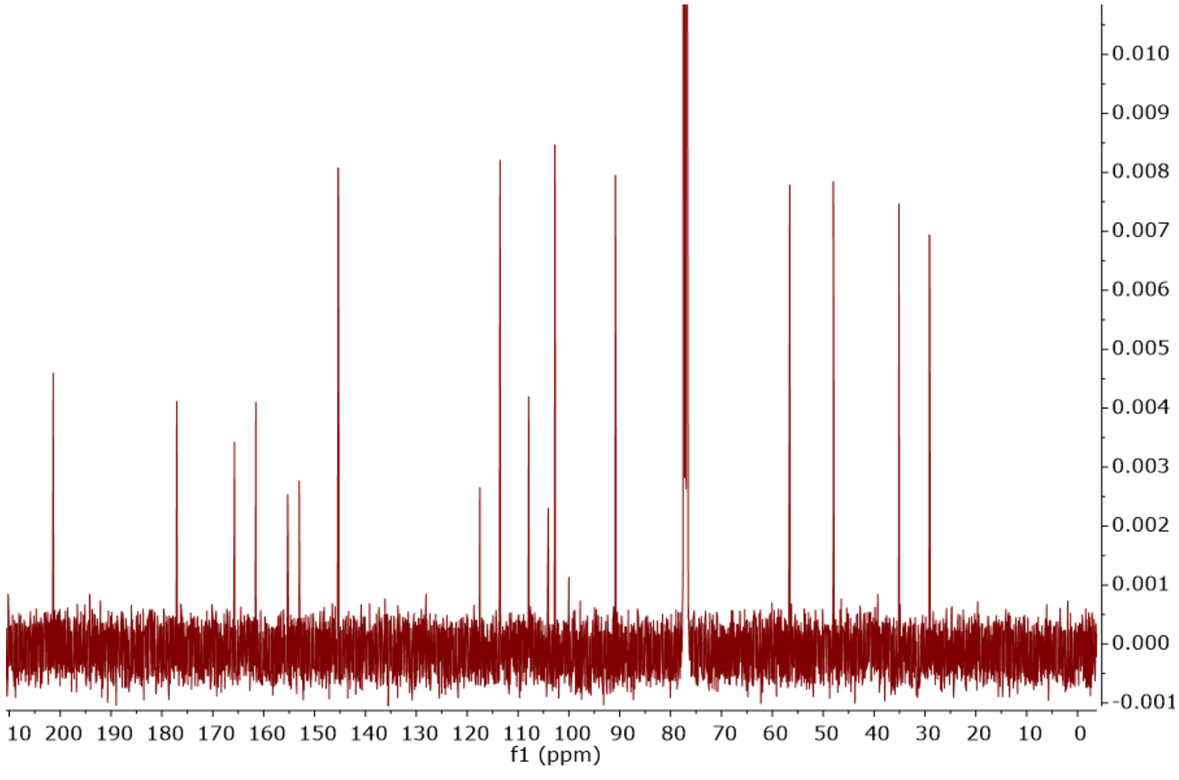


Figure 3 – ^{13}C NMR spectrum of AfB_1 in $CDCl_3$.

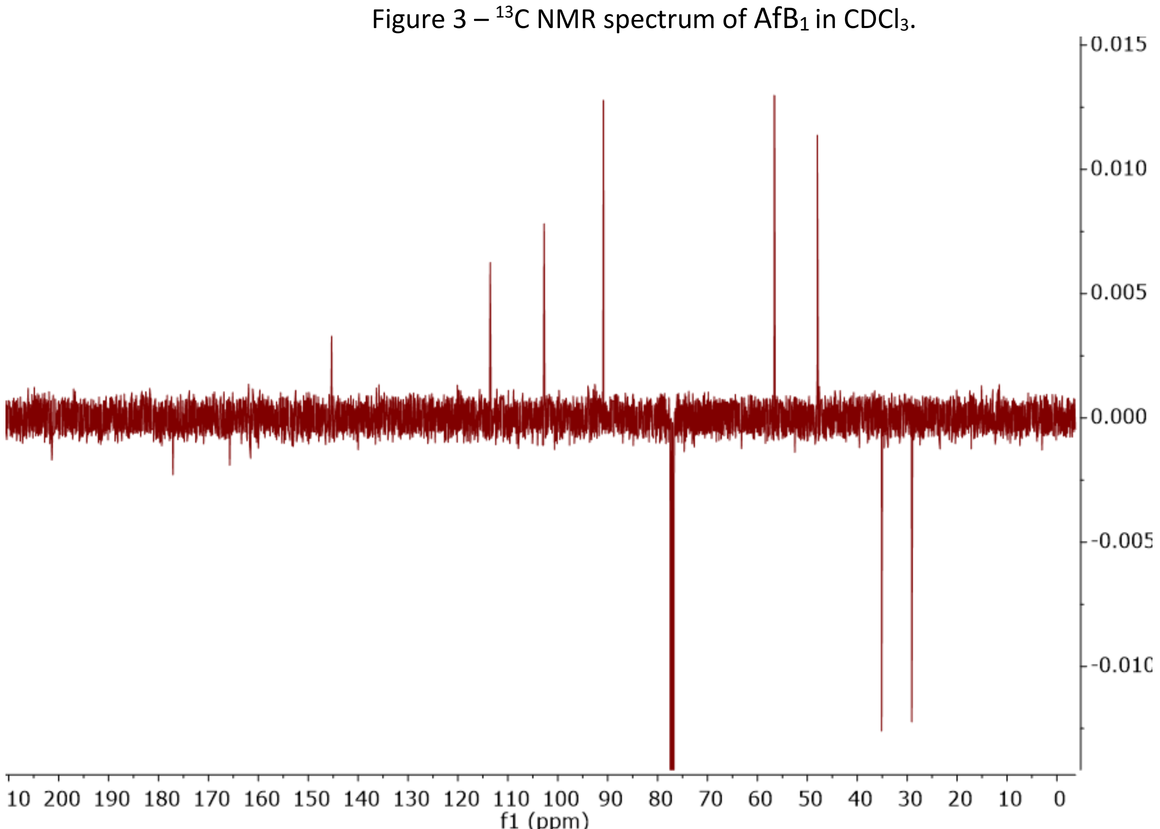


Figure 4 – APT ^{13}C NMR spectrum of AfB_1. Down = C/CH_2; Up = CH/CH_3.

4.3.5 **2D NMR spectra**

To confirm the identification and assignment of the signals, spectra were acquired of a solution of the material using two-dimensional homonuclear (^{1}H-^{1}H) correlated spectroscopy (COSY) and heteronuclear single-quantum correlation (^{13}C-^{1}H) spectroscopy (HSQC).[16] The 2D-spectra obtained are reproduced as Figure 11 and Figure 12 in Annex 7.2. The peak assignments derived from the combined data are summarized in Table 3. They are consistent with literature assignments[9] and established the identity of the primary component in the material as AfB_1.

Table 3 – ^{1}H and ^{13}C peak assignments for AfB_1 in OGO.193.
^{13}C NMR signals for quaternary carbons marked * were not detected in the APT experiment.

Position	^{1}H-NMR (ppm, integral)	^{13}C-NMR (ppm, APT assignment)	COSY	HSQC (ppm, adjacent ^{1}H signal)
1	-	201.37 – C_q	-	-
2	H (2.64, 2H)	35.11 - CH_2	Couples with G	35.14, H
3	G (3.40, 2H)	29.08 - CH_2	Couples with H	29.05, G
3a	-	177.09 - C_q	-	-
3b	-	104.06*	-	-
4	-	161.54 - C_q	-	-
4-OCH_3	F (3.95, 3H)	56.56 - CH_3	-	56.58, F
5	C (6.42, 1H)	90.85 - CH	-	90.82, C
5a	-	165.73 - C_q	-	-
6a	A (6.81, 1H)	113.54 - CH	Couples with E	113.56, A
8	B (6.47, 1H)	145.31 - CH	Couples with D and E	145.31, B
9	D (5.48, 1H)	102.73 - CH	Couples with B and E	102.88, D
9a	E (4.77, 1H)	47.99 - CH	Couples with A, B and D	47.97, E
9b	-	107.90*	-	-
9c	-	153.01*	-	-
11	-	155.26*	-	-
11a	-	117.50*	-	-

4.3.6 **Residual solvent content by NMR**

The ^{1}H NMR spectrum of the material was examined for signals due to residual solvent.[17] The presence of trace levels of ethanol and dichloromethane were observed but due to their low intensity relative to baseline noise accurate quantification was not possible. It was estimated based on experience that combined residual solvent constituted less than 1 mg/g of the content of the material.

4.3.7 **UV-Vis spectrophotometry**

Scan and fixed wavelength UV-VIS measurements in absorbance mode:

Scan mode:

- Deuterium lamp: on
- Tungsten lamp: on
- Scan from 370 nm to 190 nm
- Data interval: 1.00 nm, scan speed: 267 nm/min
- Slit: 2 nm

Fixed wavelength:

- Deuterium lamp: on
- Tungsten lamp: on
- Wavelengths: 262 nm, 360 nm and 354 nm
- Cycle: 3
- Slit: 1 nm
- Gain: Auto
- Response 0.2s
- No cell changer

Micro-cuvettes containing a minimum volume of 50 µl of solution were used. The reference cell contained spectroscopic grade pure acetonitrile. Autozero was performed at the beginning of the method using pure solvent in the sample cuvette. Three measurements were acquired and averaged for each sample replicate. Temperature was controlled at 20 °C. A representative UV spectrum for a solution with AfB_1 content of 6 µg/g in acetonitrile[18] is reproduced in Figure 5.

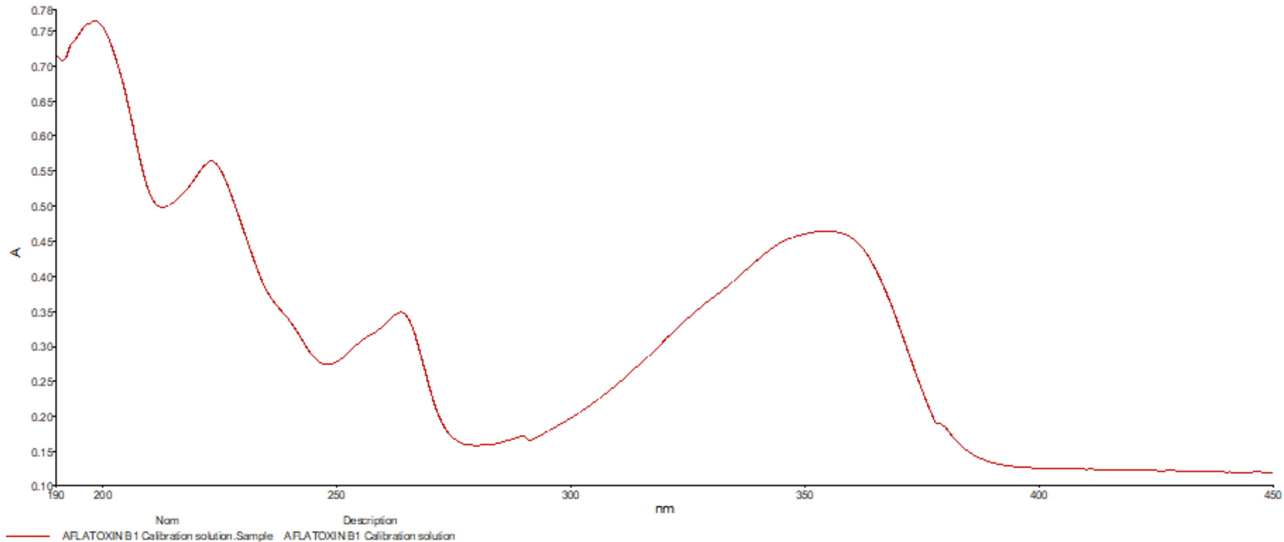


Figure 5: UV-VIS spectrum for AfB_1 (6 µg/g) in acetonitrile.

4.3.8 **Mass spectrometry**

Reference MS data for AfB_1 are available under the entry for "aflatoxin B_1" from open access databases including the European Mass Bank, the Mass Bank of North America and PubChem.

From studies undertaken at the BIPM, the MS parameters in a negative-positive switching electrospray ionization mode were optimised by direct infusion of single LC standards of AfB_1, AfB_2, Af_{DIOL}, AfB_{2a}, AfQ_1 and AfP_1. From the typical overlay chromatogram of multiple reaction monitoring (MRM) transitions, negative ionization mode revealed higher sensitivity compared with the positive ESI mode for AfB_1, Af_{DIOL}, AfB_{2a}, AfQ_1, AfG_1, AfG_2, AfM_1 and AfM_2. MRM periods in positive mode were added in the acquisition method to increase the sensitivity for AfB2 and AfP1. Every measurement was repeated in triplicate to establish optimum MRM parameters of 5500 V for the capillary voltage and 600 °C source temperature for the positive ESI mode and capillary voltage of -4500 V and the source temperature of 550 °C for the negative ESI mode. Nitrogen was used as the ion source gas, curtain gas and collision gas. The Gas 1 and Gas 2 pressures of the ion source were 55 psi and 60 psi, respectively. The curtain gas (CUR) and the Collision Gas (CAD) were set at 15 psi and mid, respectively. Table 4 summarizes the optimized transitions and variable conditions for MRM detection and quantification of AfB_1 and its structurally related impurities.

Table 4: Selected reaction monitoring transitions and MS/MS parameters for aflatoxins

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
Af_{DIOL}	345.2	283.2*	50	-50	-25	10	10
		327.2	50	-50	-25	10	10

* quantification transitions

5. Purity assignment of Aflatoxin B_1

5.1 Introduction

The approach developed during the BIPM MMCBKT program for the purity assignment of the AfB_1 source material used a quantitative NMR (qNMR) measurement[19, 20] to quantify the combined AfB_1 and related structure impurity content. Subsequent correction of the raw qNMR result for the AfB_1-related impurity content, quantified by LC-MS/MS methods, gave the final value for the true AfB_1 mass fraction. This approach has the significant advantage of requiring a much smaller amount of the difficult to obtain solid material than that required for a conventional mass balance purity assignment.

The qualitative identity of the AfB_1 material was established and an estimate of residual solvent impurity content in the material was obtained using the combination of 1D- and 2D-NMR techniques described in Section 4.4.1 – 4.4.5 above.[21] This identification was independently confirmed by determination of the UV-Vis spectrophotometric (4.4.6) and mass spectrometric properties (4.4.7) of the material, which corresponded with reported values.

The assignment of the "raw" AfB_1 content by qNMR, uncorrected for contributions from related structure impurities, is described below in section 5.2. The development and application of methods for the identification and quantification of the AfB_1-related impurity content of the material by LC-MS/MS and LC-DAD is described in section 5.3. These results were used to correct the "raw" qNMR value for the AfB_1-related impurity content and gave the final assignment of the "true" AfB_1 content of the material.

Supporting analyses undertaken to detect other impurity classes are summarized in section 5.4 and the combination of the data to give the final purity assignment of the material is described in section 5.5. A description of the approach for the purity assignment of AfB_1 described in this document has been published separately.[22]

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

5.2 qNMR [21]

5.2.1 Materials

Chemicals

- Aflatoxin B_1 (AfB_1); BIPM Reference OGO.193
- Supplier: First Standard, Product No. 1ST7205, Lot ALT603155
- Dimethylterephthalate (DMTP); BIPM Reference OGE.022b was used as the qNMR internal standard[23]. The mass fraction content of DMTP in the material was assigned at the BIPM by qNMR measurements using CRMs as internal standard as 999.3 ± 0.8 mg/g (k = 2).

NMR Solvents:

- Acetone-d_6; BIPM Reference OGS.029
- Deuterated chloroform ($CDCl_3$); BIPM Reference OGS.026b

Deuterated solvents were purchased from a commercial supplier and used without further treatment. NMR tubes were HG-Type: high grade class, 8 inch, 5 mm diameter rated for use with 600 MHz spectrometers fitted with PE caps.

5.2.2 qNMR Sample preparation

Gravimetric operations were performed using a Mettler Toledo XP2U ultramicrobalance. Prior to all weighing operations the repeatability of the balance was assessed for suitability to the preparation of qNMR samples by repeat mass determinations of an empty weigh boat. The general recommendations for qNMR sample preparation reported by Yamazaki *et al* [24] were followed.

In the primary study, using deuterated chloroform as solvent, five separate samples were prepared. The individual sample sizes were in the range 5 mg - 8 mg for the AfB_1 material and 2.7 mg to 4.0 mg for the internal standard (DMTP). Each sample was separately weighed into an aluminium weighing boat and in order to avoid contact of the solvent with the metal boat the contents of both were transferred into a common glass vial and each emptied boat was reweighed. The amount of AfB_1 and DMTP transferred into the common vial was determined by difference and this value was used for qNMR calculations. 1 mL of deuterated solvent was added to the vial and the sample solution was mixed in a vortex shaker and checked visually for completeness of dissolution. Approximately 800 µL of this solution was transferred into an NMR tube (HG-Type: high grade class, 8 inch, 5 mm o.d., with PE cap) using a glass pasteur pipette.

5.2.3 Choice of solvent and quantification signals

Three clean integration areas within AfB_1 provided independent quantitative NMR (qNMR) results. The first area selected were the overlapping multiplets due to protons H-5 and H-8 (C and B respectively) at chemical shift of ca. 6.4 ppm, the second area was the region of the multiplet due to proton H-9 (D) at chemical shift of ca. 5.5 ppm and the third area the multiplet from H-9a (E) at chemical shift 4.7 ppm. DMTP was used as the internal standard since its singlet resonance from four magnetically-equivalent aromatic protons at chemical shift 8.1 ppm occurs in a clean region in

the AfB_1 spectrum. The 90-degree pulse calibration was established at 6.05 µs and the longest measured T_1 time constant for the quantified peaks was for the DMTP aromatic peak and was 3.6 s. An FID acquisition time of 4 s followed by a relaxation delay of 56 s between pulses, corresponding to in excess of fifteen times the longest T_1, was applied for quantification studies and an excitation pulse offset of 7.3 ppm, situated roughly midway between the internal standard and AfB_1 signals, was used.

5.2.4 NMR acquisition parameters

A JEOL ECS-400 spectrometer operating at 9.4 T (400 MHz for proton) equipped with a direct type automatic tuning (Royal) probe operating using the Delta software was used for all NMR data acquisition.

The general recommendations for optimizing spectrometer performance, determining the relevant NMR experiment parameters and undertaking a qNMR experiment as described in the BIPM Internal Standard Reference Data report for the use of DMTP for qNMR measurements[23] were followed, with the exception that for this assignment the acquisition was carried out with ^{13}C-decoupling activated to eliminate satellite peaks and simplify the integration process. The final qNMR acquisition parameters used for AfB_1 are summarized in Table 5. A representative section of the NMR spectrum obtained for one sample is reproduced in Annex 7.3, Figure 13.

Table 5 - Acquisition parameters for qNMR.

Parameter	*Value*
AfB1 Sample size (mg)	5 – 8
DMTP Sample size (mg)	2 – 4
Number of Transients	64
Receiver gain	36
Acquisition time (s)	4
Relaxation delay (s)	56
Pulse offset (ppm)	7.3
Spectral width (ppm)	400
Data points	639652
Temperature (K)	298
^{13}C-Decoupling	On
Spinning	Off
Integral ratio (AfB1:DMTP)	0.25 – 0.48

5.2.5 qNMR signal integration

A baseline correction window of eighty times the full width at half maximum (FWHM) was applied to each integrated signal. The integration range used start and end points placed fifty Hertz

beyond the visible edge of each signal. Results from four independent sample mixtures each measured four times were obtained.

5.2.6 Value assignment and measurement uncertainty

Results from five independent sample mixtures each measured five times were obtained and three independent purity assignments were obtained for each replicate using either the signals E (1H, 4.7 ppm), D (1H, 5.5 ppm) or B/C (2H, 6.4 ppm). For each purity determination the assigned value was the overall mean of the twenty-five contributing results. The measurement uncertainty budget is reproduced below in Table 6. The integral ratio is the mean of the five replicate values obtained for each of the five samples. The contributions to the overall standard uncertainty of the assignment are listed in Table 6 and their relative contributions are shown in Figure 6 for the purity assignment against the overlapping signals from protons H-5/H-8 (C/B, Fig. 1) of AfB_1 at around 6.4 ppm. The standard deviation of the mean of the twenty-five determinations of the integral ratio, normalized to take into account the different compositions of the five independent samples, was taken as the standard uncertainty of the repeatability of the integration ratio.

Uncertainty sources	Value	Type	Standard Uncertainty	Sensitivity coefficient	Uncertainty Component
Precision (Integral Ratio)	0.5817	A	0.000124	1685.619902	2.09E-01
AfB_1 Molar Mass	312.272	B	0.00994	3.140201888	3.12E-02
DMTP Molar Mass	194.183	B	0.00589	-5.049856572	2.97E-02
DMTP sample Mass	2.75	B	0.00124	356.5940819	4.42E-01
AfB_1 sample mass	5.24	B	0.00124	-187.0941895	2.32E-01
IS purity	998.9	B	0.25	0.981677912	2.45E-01
				Combined Uncertainty	**5.96-01**
AfB_1 content	**980.6**	**±**	**1.2**	**$mg.g^{-1}$**	

Table 6 – Uncertainty budget for uncorrected AfB_1 purity by qNMR against the AfB_1 H-5 and H-8 ^1H NMR peaks with DMTP as IS in solution in $CDCl_3$.

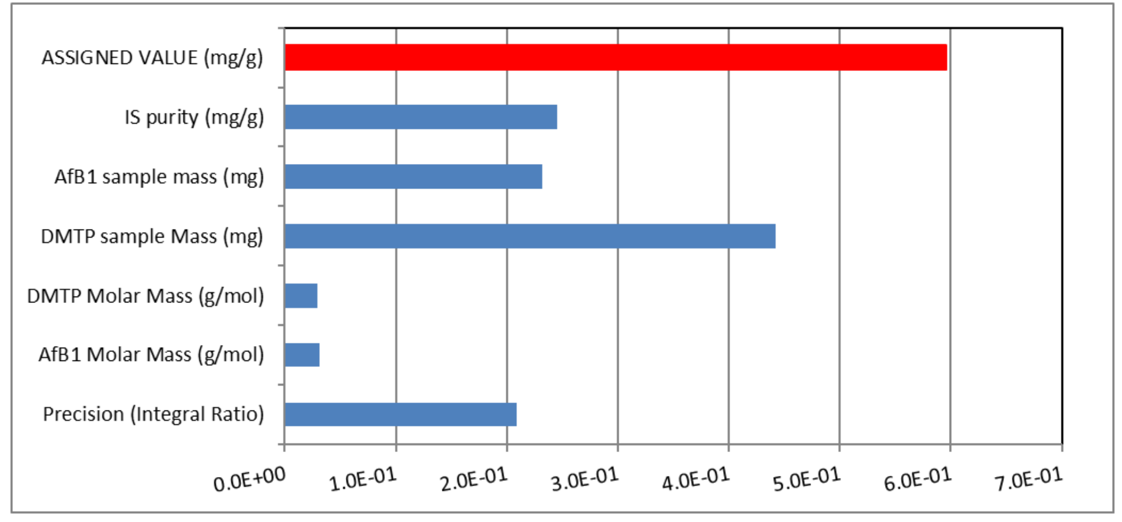


Figure 6 - Relative uncertainty components: AfB_1 assignment against DMTP IS in $CDCl_3$.

Note that the contribution from the gravimetric operations and the purity of the internal standard are as important to the overall uncertainty of the purity assignment as the precision of the integral ratio determination.

The results of the three separate purity determinations obtained from using each of the integration signals are shown in Table 7 and plotted in Figure 7.

Table 7 – "Combined" AfB$_1$ purity values by NMR with their expanded uncertainties.

Comparison of the results for Aflatoxin B$_1$ Purity						
Solvent	**IS**	**AfB$_1$ peak (ppm)**	**IS peak (ppm)**	**Content (mg/g)**	**U_{95} (mg/g)**	**RSD (%)**
Chloroform-D	DMTP	4.7	8.1	982.3	1.4	0.19
Chloroform-D	DMTP	5.5	8.1	981.0	1.2	0.18
Chloroform-D	DMTP	6.4	8.1	980.6	1.2	0.10

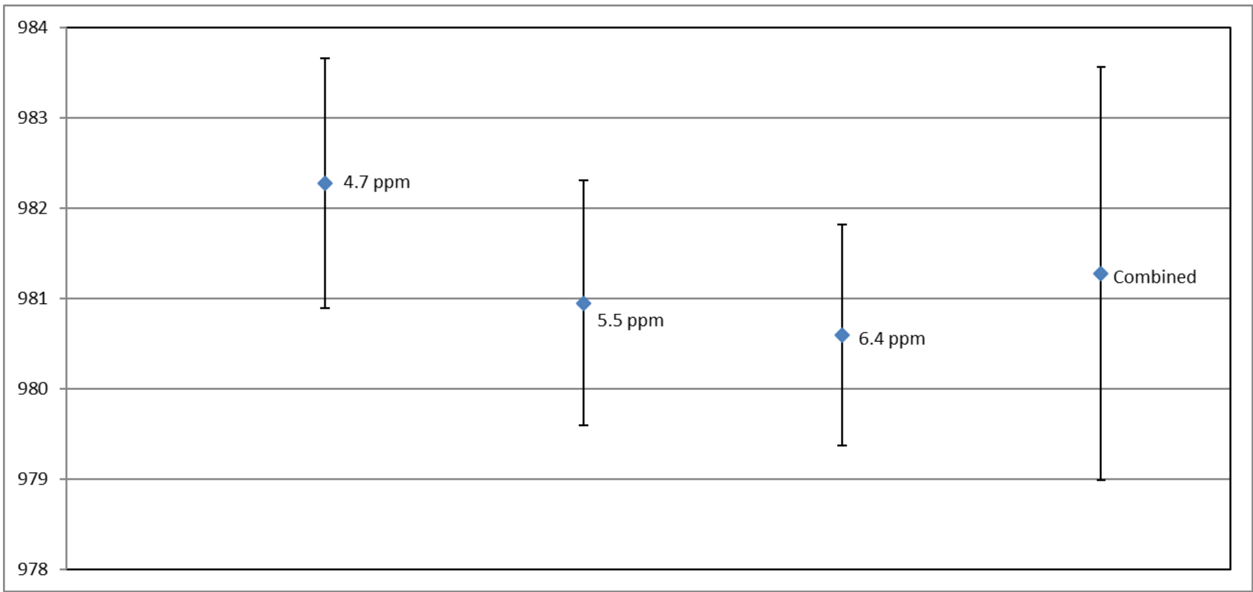


Figure 7 – Comparison of the qNMR values obtained for AfB$_1$ using different analyte peaks for integration.

The final qNMR estimate, which as noted earlier provides a measure of the total AfB$_1$ plus related structure impurity content, was assigned as 981.3 ± 2.3 mg/g. This value is the mean of the three separate values with a relative standard uncertainty calculated as the quadratic combination of the relative standard uncertainty of each contributing assignment. The qNMR assignment was repeated independently using a single sample freshly prepared by another operator and with acetone-d_6 as solvent. The assignments obtained using this limited qNMR characterization were 985.7 ± 3.4 mg/g against H-9a (E, 4.8 ppm) and 980.5 ± 3.6 mg/g against the H-9 (D, 5.5 ppm). These were consistent with the assigned value obtained from the main study.[25]

5.3 Related structure impurities by LC-DAD and LC-MS/MS

5.3.1 Apparatus

LC-DAD-MS/MS. The liquid chromatography (LC) system consisted of an Agilent (Massy, France) 1100 series micro vacuum degasser, binary pump, thermostatted standard autosampler, thermostatted column compartment and diode array detector (DAD). An Applied Biosystems (Courtaboeuf, France) 4000 Qtrap hybrid tandem mass spectrometer (MS/MS) was coupled to the LC system employing a Sciex (Concord, ON, Canada) TurboIonSpray (TIS) source and a Valco (VICI, Schenkon, Switzerland) 10-position valve. A direct flow injection device from Harvard Apparatus (Holliston, MA, United States) was used for optimisation by direct injection.

5.3.2 Materials

Aflatoxin B_1 (AfB_1). BIPM Reference OGO.193a

Supplier: First Standard Aflatoxin B1 No. 1ST7205, Lot ALT603155

- Aflatoxin B_2 (AfB_2) BIPM Reference OGO.189a
  Supplier: First Standard, Product No. 1ST7206-100A, Lot ALT602201
- Aflatoxin B_{2a} (AfB_{2a}) BIPM Reference OGO.210a
  Supplier: First Standard, Product No. 1ST7205, Lot ALT603155
- Aflatoxin G_1 (AfG_1) BIPM Reference OGO.190a
  Supplier: First Standard, Product No. 1ST7207-100A, Lot ALT602198
- Aflatoxin G_2 (AfG_2) BIPM Reference OGO.191a
  Supplier: First Standard, Product No. 1ST7208-100A, Lot ALT602199
- Aflatoxin M_1 (AfM_1) BIPM Reference OGO.181a
  Supplier: First Standard, Product No. 1ST7209-100A, Lot LZ106697
- Aflatoxin M_2 (AfM_2) BIPM Reference OGO.181a
  Supplier: First Standard, Product No. 1ST7210-10A, Lot LZT106717
- Aflatoxin Q_1 (AfQ_1) BIPM Reference OGO.213a
  Supplier: First Standard, Product No. 1ST9196, Lot FS1603746
- Aflatoxin P_1 (AfP_1) BIPM Reference OGO.212a
  Supplier: First Standard, Product No. 1ST001445, Lot FS1603748
- Aflatoxin B_1 diol (Af_{DIOL}) BIPM Reference OGO.211a
  Supplier: First Standard, Product No. 1ST7298, Lot LZ017082-2

Pure water was obtained from a MilliQ RiOs gradient ultrapure device (Molsheim, France).

Methanol (MeOH) was HiPerSolv CHROMANORM from VWR (Fontenay-sous-Bois, France).

Acetonitrile (ACN) was HiPerSolv CHROMANORM from VWR (Fontenay-sous-Bois, France).

5.3.3 HPLC parameters

An LC-DAD-MS/MS method was implemented for the detection and quantitative

determination of aflatoxin related structure impurities in AfB_1 material including aflatoxin B_2 (AfB_2), aflatoxin B_1 8,9-dihydrodiol (Af_{DIOL}), aflatoxin B_{2a} (AfB_{2a}), aflatoxin Q_1 (AfQ_1), aflatoxin G_1 (AfG_1), aflatoxin G_2 (AfG_2) and aflatoxin P_1 (AfP_1). The method was validated for the usual performance characteristics (linearity, repeatability, limits of detection, intermediate precision, etc.) and was assessed for the quantification of the AfB1 and its main related structure impurities. Calibration curves of AfB_2, Af_{DIOL}, AfB_{2a}, AfQ_1, AfP_1 and AFB_1 were constructed by use of corresponding standard solutions. A multi-component calibrant mixture was prepared containing AfB_2, Af_{DIOL}, AfB_{2a}, AfQ_1 and AfP_1 for use in quantification. An AfB_1 single calibrant was prepared separately to obtain performance characteristics and for use as a calibration standard to quantify related-structure unidentified impurities. Chromatographic separation was performed at 25 °C using a Kinetex EVO C18 100Å, (250 x 4.6 mm, 2.6 µm) column from Phenomenex (Le Pecq, France).

The chromatographic conditions used for the separation of the compounds were:

Column:	Phenomenex Kinetex EVO C_{18} 100Å,(250 x 4.6 mm, 2.6 µm) (OGLC.65)	
Column temperature:	25 °C	
Detector:	Qtrap, UV lamp and visible lamp required	
Detection wavelength:	**360 nm (reference wavelength 360 nm)**	
Mobile phase:	A) Acetonitrile:Methanol = 50:50 (v/v) B) H_2O Milli Q	
Operation mode:	Gradient (inclusive of cleaning gradient)	
Solvent gradient:	Time (min)	Mobile phase A content
	0.0	30%
	30	90%
	31	100%
	32	100%
	34	30%
	40	30%
Flow rate:	0.6 mL/min	
Injection Mode:	Standard	
Injection volume:	10 µL	
Duration:	40 min	

By applying the optimised conditions, AfB_1, AfB_2, Af_{DIOL}, AfB_{2a}, AfQ_1 and AfP_1 eluted at retention times (RT) of 16.2, 15.3, 9.2, 11.3, 12.3 and 14.1 min respectively.

5.3.4 LC-MS/MS results

The optimized MS/MS parameters for each compound had been identified as described in

Section 4.4.7 and were applied to the elution window of each compound under these HPLC conditions. The TIC obtained for a standard mixture containing each of AfB_1, AfB_2, Af_{DIOL}, AfB_{2a}, AfQ_1 and AfP_1 at ca. 100 $ng.g^{-1}$ is shown in Figure 8. The inserted figures show the corresponding ionization responses using ESI negative and ESI positive modes only.

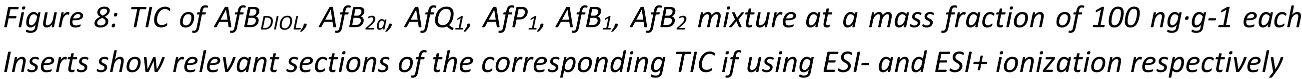
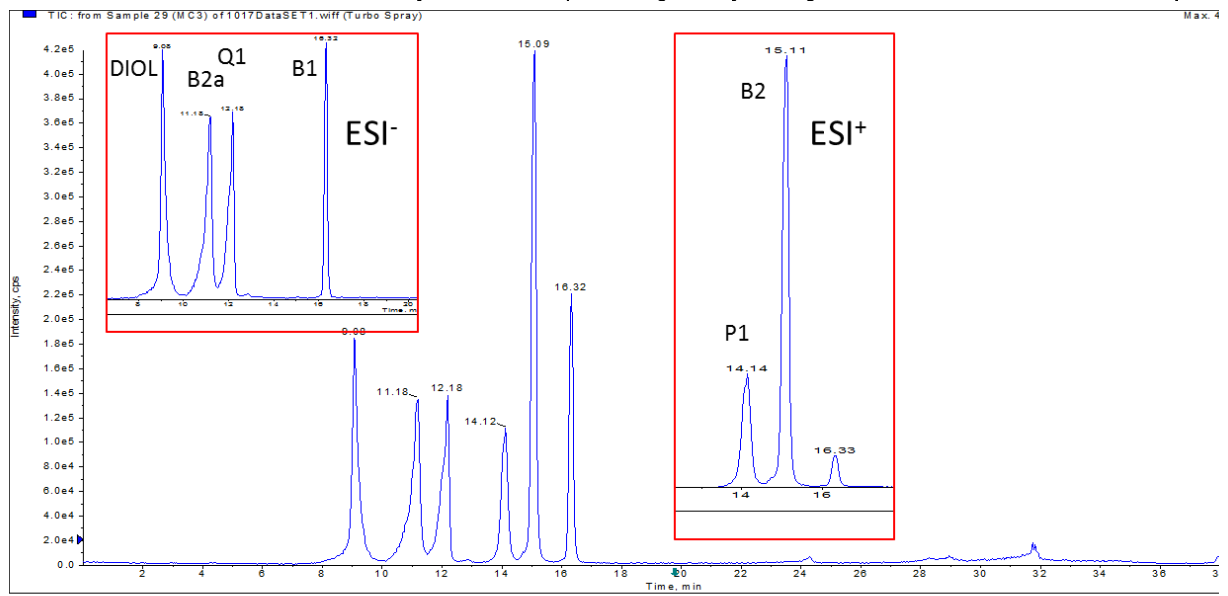


Figure 8: TIC of AfB_{DIOL}, AfB_{2a}, AfQ_1, AfP_1, AfB_1, AfB_2 mixture at a mass fraction of 100 ng·g-1 each Inserts show relevant sections of the corresponding TIC if using ESI- and ESI+ ionization respectively



Preliminary LC-MS/MS analysis of a solution of the AfB_1 material at a concentration of 2000 µg/g identified the presence of two major impurities. LC-MS/MS in MS3-IDA-EPI mode was used to identity these impurities. The LC conditions were the same as listed above. For subsequent quantification of impurities a solution of approximate 100 µg/g of AfB_1 was prepared. The LC method was slightly changed to avoid contamination of the highly sensitive LC-MS/MS instrument by the high content of AfB_1 component. After chromatographic separation the mobile phase was switched to the waste position to cut out the major component's peak and to be able to detect the minor impurities in the AfB_1 material. Figure 9 shows the total ion chromatogram of the AfB_1 material recorded in MS3-IDA-EPI mode under these conditions. Two impurities, AfB_2 and AfB_{2a} were present and eluted at about 15.2 min. and 11.3 min respectively. Figure 10 shows the corresponding total wavelength chromatogram recorded before switching the major component AfB_1 to waste. The presence of the AfB_2 and AfB_{2a} impurities were confirmed by comparison of retention time and mass spectral data with those obtained for authentic standards of each material.

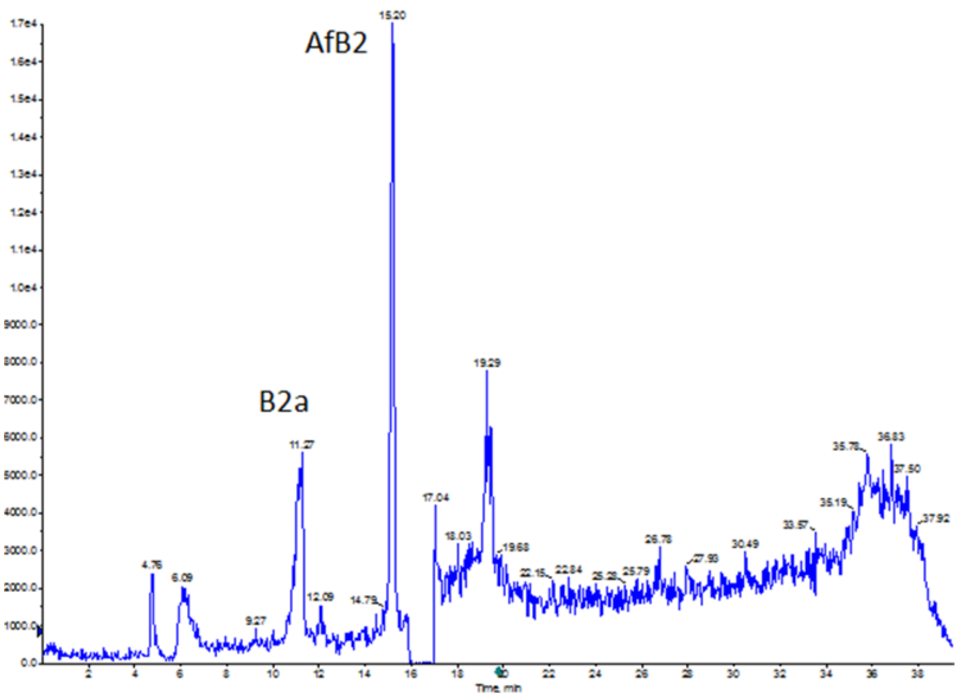


Figure 9: TIC of the AfB$_1$ sample at 100 µg·g-1 (main component peak cut off to waste)

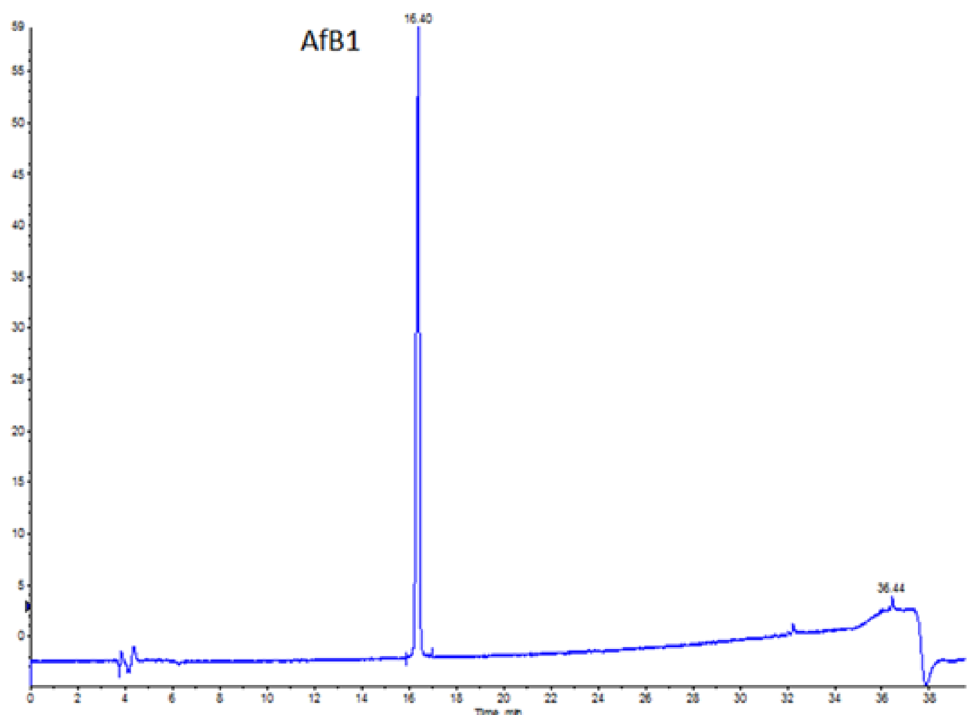


Figure 10: Total wavelength chromatogram of the AfB$_1$ material

Table 8 The result of identification of impurities in AfB_1 material with QTRAP-MS

Compound	Impurity 1	Impurity 2	Impurity 3	Impurity 4	Impurity 5
Rt (min)	9.19	11.31	12.11	13.9	15.2
Precursor ions	344.9	329	327	297	313
Fragment ions	327	285	312	283	296
	312	269	299	277	285
	283	258	283	212	255
	268	243	268		242
Prediction of probable compounds according to the parent ions and series fragment ions					
Molecular formula	$C_{17}H_{14}O_8$	$C_{17}H_{14}O_7$	$C_{17}H_{12}O_7$	$C_{16}H_{10}O_6$	$C_{17}H_{14}O_6$
Molecular weight (Da)	346.07	330.07	328.06	298.04	314.06
Compound	DIOL	B2a	Q1	P1	B2
Structure					

A selected reaction monitoring (SRM) method was set up for the quantification of the two major impurities in the AfB_1 sample. The precursor and fragment ions of the two impurities AfB_2 and AfB_{2a} and certain other AfB_1-related compounds are listed in Table 8. The method has been in-house validated using authentic standards for AfB_1, AfB_2, AfB_{2a}, Af_{DIOL}, AfP_1 and AfQ_1. The AfB_2 and AfB_{2a} were determined to be present in the AfB_1 material at mass fraction values and corresponding expanded uncertainties (k = 2) of 1.16 ± 0.12 mg/g (AfB_2) and 0.52 ± 0.02 mg/g (AfB_{2a}) respectively.

5.4 Water content by Karl Fischer Titration[26]

Water content measurements by coulometric Karl Fischer titration were carried out on the OGO.193a AfB1 material. The challenges with handling AfB_1 in solid form due to its toxicity and fears of contaminating equipment meant that a method based on the use of a heated oven to release water from the material was precluded. A protocol was implemented to avoid as much as possible exposure to the material in powder form.

Individual samples were weighted into a tared GC vial and the vial was sealed as soon as the gravimetric measurement was completed. Solvent (Anhydrous acetonitrile > 99.9%) was added via syrine into the sealed vial immediately prior to the measurement and the vial was weighed. After dissolution of the AfB_1 solid the bulk of the resulting solution was withdrawn by syringe and injected directly into the coulometric titration cell containing the KFT reagent. By measuring the mass changes of the vial and the syringe before and after transfer of the AfB1 solution it was possible to calaculate the mass of AfB1 introduced into the titration cell.

A series of "blank" measurements were obtained by injection of the solvent only to establish the background level of water introduced in the injection process and a reference material solution of nominal water content 103 ± 3 µg/g water in hexane was used to validate the sensitivity of the measurement process.

The injection of five separate samples of AfB1 in solution in acetonitrile, each containing ca 1 mg of solid, gave results that were not statistically different from the results obtained using blank solvent. On the basis of these results it was determined that the material did not contain a quantifiable level (< 0.1 mg/g) of water.

5.5 Final AfB_1 Purity assignment

The "raw" qNMR value for the AfB_1 mass fraction in the material had been estimated by qNMR as described at 981.3 ± 2.3 mg/g. This value was corrected for the total related structure impurity contributions (1.68 ± 0.13 mg/g) determined by LC-MS/MS to give the final assigned value for the "true" AfB_1 content of 979.6 ± 2.3 mg/g.

The other components present in the material were assigned as:

Minor component	Mass fraction and uncertainty (mg/g)	Measurement Method(s)	Verification Method
AfB_2	1.16 ± 0.12	LC-MS/MS	LC-UV
AfB_{2a}	0.52 ± 0.02	LC-MS/MS	LC-UV
VOC (CH_2Cl_2, EtOH)*	< 1 mg/g	qNMR	
Unidentified aliphatic impurities (lipid, etc)	18.7 ± 2.4	qNMR (by difference)	LC-CAD

* VOC identifications were based on ^{1}H-NMR and are qualitative only.

The unidentified impurity visible in the upfield region of the NMR spectrum of the material in the area 0.8 – 1.0 ppm appears to be primarily aliphatic in nature and is probably lipidic residue from

the harvesting and purification of the aflatoxin from its biological source material. The assigned value was obtained by difference from the upper purity limit of 1000 mg/g and the combined mass fraction of AfB_1 and related structure impurities as determined by qNMR, given that there was no evidence of other significant impurities present in the material.

6. Acknowledgements

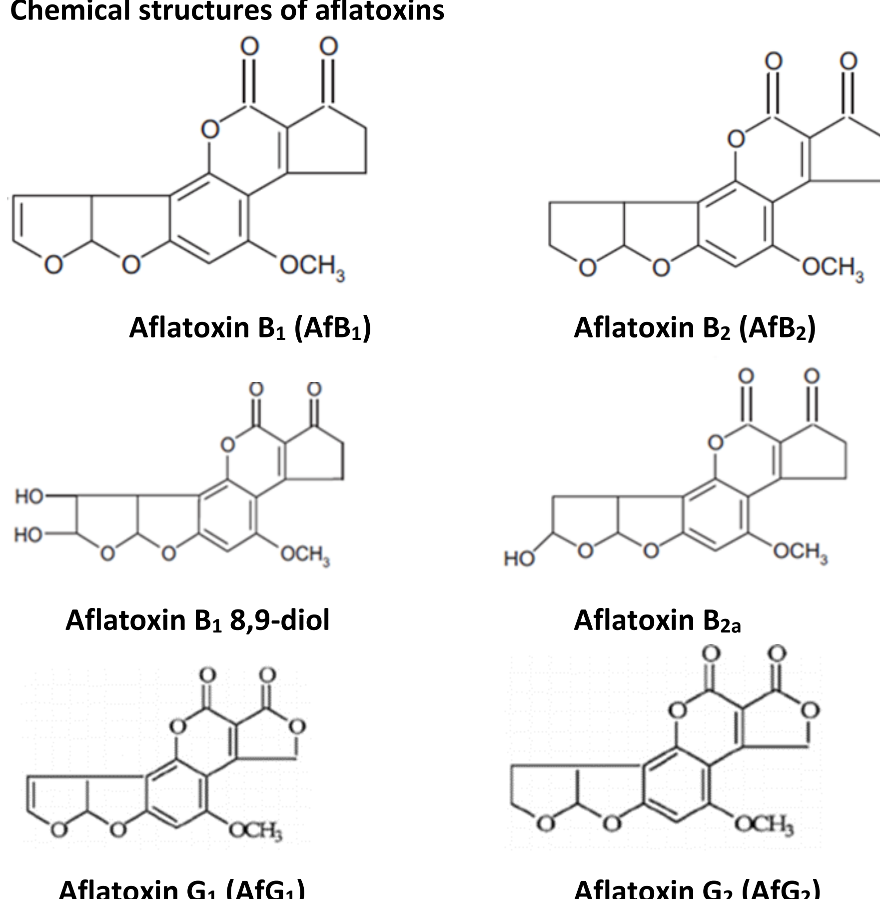
All NMR and LC studies were carried out by the co-authors of this document in the course of secondments at the BIPM. The support of the parent institution for each scientist to the BIPM work programme is gratefully acknowledged.

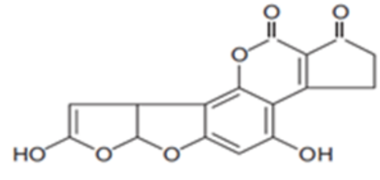
The authors would like to thank the National Key R&D Program of China for funding support (No. 2016YFE026500).

Dr. Bruno Garrido wishes to acknowledge funding for his secondment from the Brazilian Ministry of Education under the Coordination for the Improvement of Higher Education Personnel (CAPES) post-doctoral scholarship programme (process: 99999.007374/2015-01).

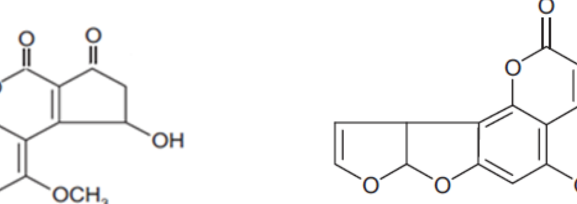
7. Annexes

7.1 Chemical structures of aflatoxins





Aflatoxin M_1 (AfM_1) **Aflatoxin P_1 (AfP_1)**



Aflatoxin Q_1 (AfQ_1) **Aflatoxicol**

7.2 2D-NMR of AfB_1

7.2.1 COSY

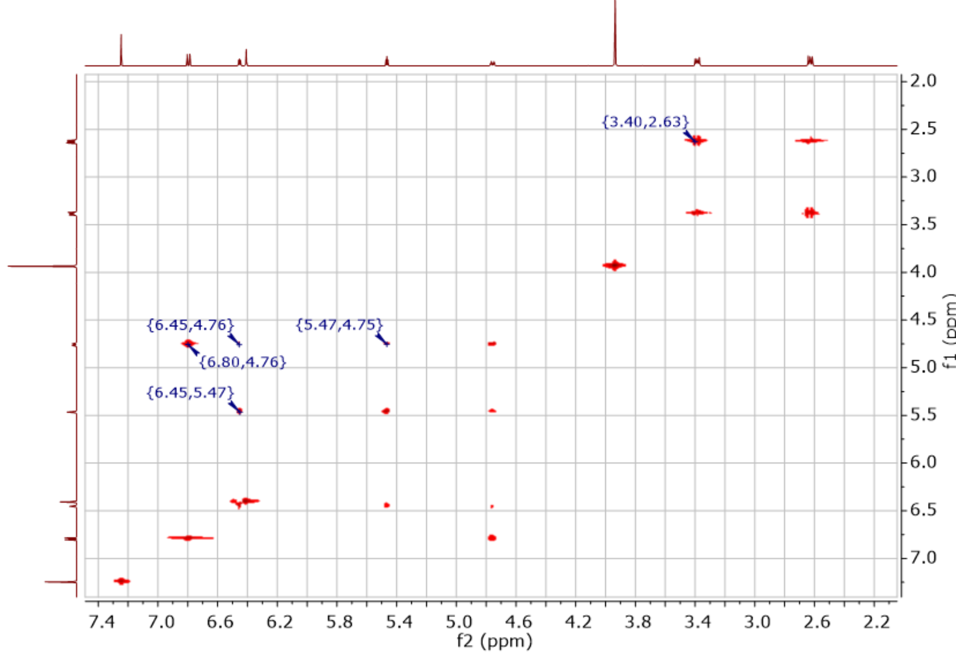


Figure 11 – COSY spectrum of AfB_1

7.2.2 HSQC

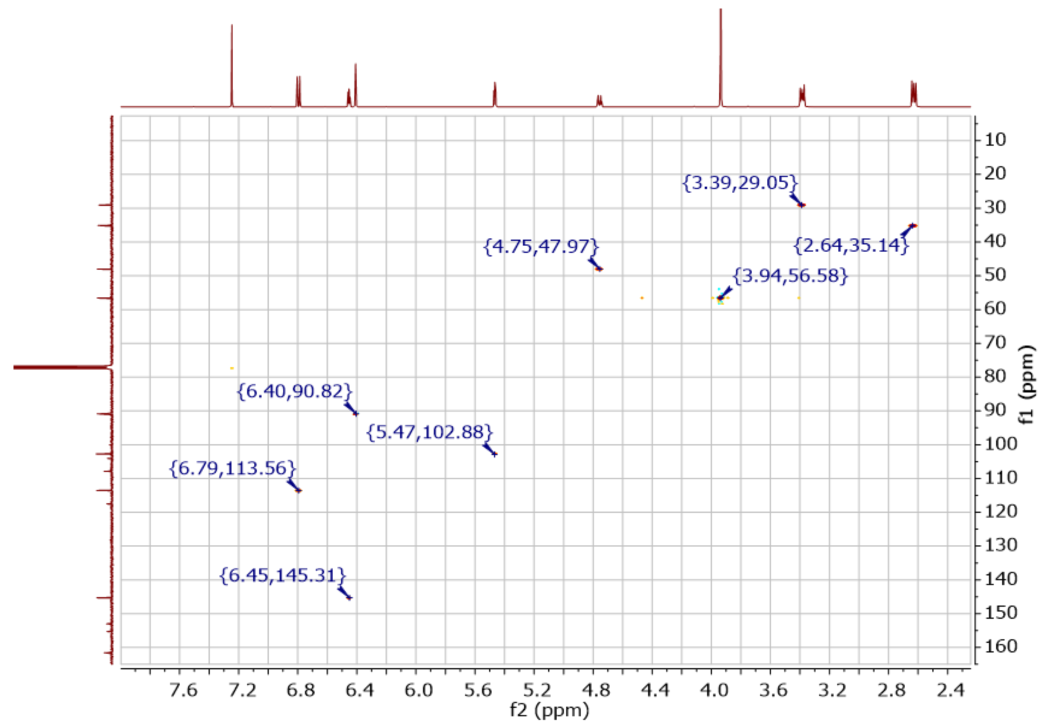


Figure 12 – HSQC spectrum of AfB_1

7.2.3 qNMR

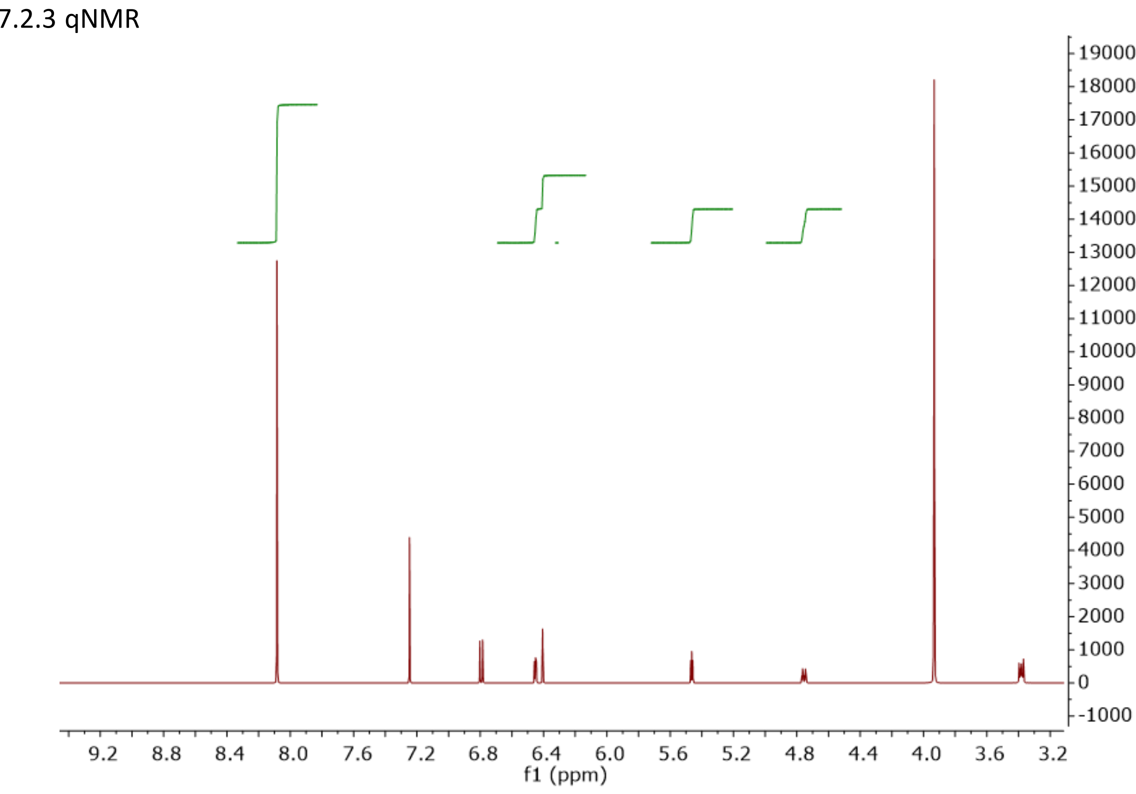


Figure 13 - ^{1}H qNMR spectrum of AfB_1 and DMTP in $CDCl_3$.

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