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The Analysis of a Feed Component Imported into South Africa for Aflatoxin in Relation to Fungal and Mycotoxin Contamination

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***A*bstract:** Currently there is concern with respect to the occurrence of mycotoxins in feed commodities, which could result in the loss of animal production and danger to consumers. Recent legislation to control the trading of such contaminated materials has been initiated with the result that it is imperative to be able to analyse for mycotoxins in feed commodities, rapidly and with sufficient accuracy to ensure that bulk cargoes of such materials are within set safety limits.

To this end a large batch (800 tonnes) of cotton-seed meal was consigned to a South African feed miller and was sampled according to a protocol devised under the European Union Framework 6 Biotracer programme. These were split and analysed for aflatoxins (AFs) by two laboratories using the VICAM fluorimetry aflatoxin method (VF) and by an high performance liquid chromatography (HPLC) method (HPLC) as part of another study to determine the statistical variation of using composite samples derived from a large bulk cargo (Reiter *et al.,* 2011) .

The results from the HPLC method showed that all the composites were contaminated with aflatoxins (AF) ranging from 24 – 93µg/kg. A comparison of the two analytical methods used, showed that the results compared in terms of trend but in general the Vicam fluorimetry method (VF) gave a higher concentration of aflatoxin B_1 (AFB_1) ranging between 26 – 164µg/kg.

The levels of AF found were in several cases higher than those permitted by current legislation and would not have been allowed into the European Union. The methodology used allowed for reduced sampling and a more rapid method of analysis to assess AF contamination in commodities, subject to further development.

The predominant fungi isolated and identified were *Aspergillus flavus* and *parasiticus*, which are main producer of AFs in the environment.

Keywords: Aflatoxin; Cotton-seed meal; Sampling; Analysis; Feed

Abbreviations

AF(s)	Aflatoxin(s)
AFB1	Aflatoxin B_1
AFB2	Aflatoxin B_2
AFG1	Aflatoxin G_1
AFG2	Aflatoxin G_2
CFU	Colony forming units
DON	Deoxynivalenol
FBs	Fumonisins
HNO3	Nitric Acid
KBr	Potassium bromide
OTA	Ochratoxin A
VF	Vicam fluorometry

1. Introduction

It is now widely accepted that the presence of fungi and mycotoxins in feed materials is not only undesirable but can lead to high costs in unusable commodities and general quality control (CAST, 2003). Whether an agricultural commodity has been infected with a fungus, subsequently produced a mycotoxin is not always easy to discern, particularly if any processing has been carried out. Even if the material is obviously mouldy, the fungus may not be capable of producing a mycotoxin or may not have done so, if the conditions were not appropriate. Another confounding factor is that over 300 toxic fungal metabolites are known (Cole & Cox, 1981) each with different chemistries and physiological effects (Diaz, 2005) and hence requiring different analytical methodologies for their detection and assay. Low levels of these mycotoxins, i.e., µg/kg (parts per billion) level, may not be acutely toxic but can cause chronic poisoning, which may lead to reduced animal production (Krogh, 1989).

Thus the situation facing the feed manufacturer from the point of view of mycotoxin contamination is not an easy one. Feed components must supply at least "energy" (carbohydrate) protein and other nutrients in a balanced diet to enhance animal health and performance but regrettably, both these main feed sources are often contaminated with mycotoxins. Fortunately not all mycotoxins are important in this context and it is now recognised that five such mycotoxins are of high importance. These are: fumonisin (FBs) principally found in maize (Munkvold & Desjardins, 1997); AFs, found in oil seeds and cereal grains (Rustom, 1997); ochratoxins OTs), in cereals; deoxynivalenol (DON) in cereals; and zearalenone (ZEA) in cereals, the cereals generally being those grown in temperate climates such as wheat and barley (Tanaka *et al.*, 1988). These generalisations may not always appertain and other commodities may contain these toxins and further, other mycotoxins, e.g., ergot alkaloids, may be important depending on the commodity and conditions for growth, harvest and storage of the material. Hence certain avenues of approach may be adopted by the millers. It would be obvious to analyse maize for FBs as its presence is very common in this crop but maize can also contain AFs, DON and ZEA (Miller, 2008).

A feed manufacturer sourcing a 100000 tonnes of commodity needs to be certain that the consignment does not contain mycotoxins over a certain limit, which may not only be a question of harmful effects in the final product but also not exceeding legal limits for that toxin. To do this it would be ideal to have a effective sampling system that would give a true and accurate picture of

what mycotoxins are present and analytical methods to complement this sampling scheme. However, neither of these ideal procedures are available at the moment so it is important to develop and refine the methods that already have been used. In addition other factors are involved such as the solid state of the material, i.e., how homogenous is the material? If completely homogenous any sample, will be representative but may not be so for mycotoxin occurrence, if subsequent fungal spoilage has taken place. There are several reviews of these problems and how they may be handled (Anderson, Reiter, Lindqvist, Razzazi-Fazeli, & Häggblom, 2011; Whitaker, 2005).

Most mycotoxins are assayed using HPLC (Stroka, Petz, & Anklam, 2000) where methods specific to the type of mycotoxin being analysed are used. These may be augmented by gas liquid chromatography (for mycotoxins such as the trichothecenes (Schothorst & Jekel, 2001) or more recently advanced mass spectrometry (Razzazi-Fazeli, Rabus, Cecon, & Böhm, 2002). For feed manufacturers the problem with these methods is the time it takes to clear a commodity cargo for use in the feed mill so more rapid methods should be found. An answer to this problem may be the development of enzyme linked immunosorbent assays (ELISA) and other immunochemical methods, which are specific for the mycotoxin to be measured and in theory can be reduced to a simple dip stick type of method (De Saeger & Van Peteghem, 1996). The ultimate aim would be to apply these to composite samples that had been taken and mixed to give a homogenous matrix, from which the mycotoxins could be extracted with a suitable solvent and tested to give a result, which would be predicted to be within a certain concentration range. These, however, depend upon the sampling and mixing regime followed, as well as the test kit used. The main criteria for this approach are that it can determine mycotoxins in a matrix to definite confidence limits, which would tell the manufacturer/miller if the material was below the legal limit or above it and hence tradable or not.

This study was based on an EU Framework 6 Biotracer programme (Reiter *et al.,* 2011) which investigates the feasibility of using a sampling system that results in a set of composite samples of cottonseed meal, which are then screened for fungi and analysed for the mycotoxin of choice, in this case, AF. In this study sub-samples taken directly from the bulk consignment (Figure 1, Level 2) were taken, milled and split between two participating analytical laboratories. These were analysed for AF in order to assess the presence and level of AFs in the bulk cargo to ensure that composites for the main study would have appropriate levels for a statistical study and two compare results from two different laboratories using different procedure. To investigate whether an immunoaffinity method of AF extraction was comparable with the other methods used, one of laboratories analysed representative composite samples (Figure 1, Level 4) using a VF method in addition to the HPLC method.

2. Materials

Cottonseed meal was obtained from a South African feed manufacturer, sourced from a supplier in Benin West Africa. All chemicals were analytical grade unless otherwise stated.

3. Methodology

3.1 Sampling and Processing of Oil Seed Cake

A cargo of cottonseed meal (800 tonne in 50 kg sacks) was sampled by lance, according to a proposed protocol (Reiter *et al.,* 2011) (Figure 1). In brief, a 800 tonne cargo was divided into 40 x 20 tonne sub batches (Level 1, Figure 1) and of these 20 sub- batches were paired into 2 x 10 sub-batches taken at random from the cargo but with the pairing done from contiguous sub-batches.

This resulted in 10 "A" sub batches and 10 paired B sub-batches (Level 2, Figure1). Each sub-batch was sampled using a lance sampling device to yield 10 x 500 g of each sub-batch. From the samples 100 g was taken after mixing and grinding and these were bulked to give 10 composites of A and B of 1 kg each (Level 3, Figure 1). These samples were split in two (Level 4) and analysed by the two laboratories in as part of a sampling study, which is reported elsewhere (Reiter *et al.,* 2011).

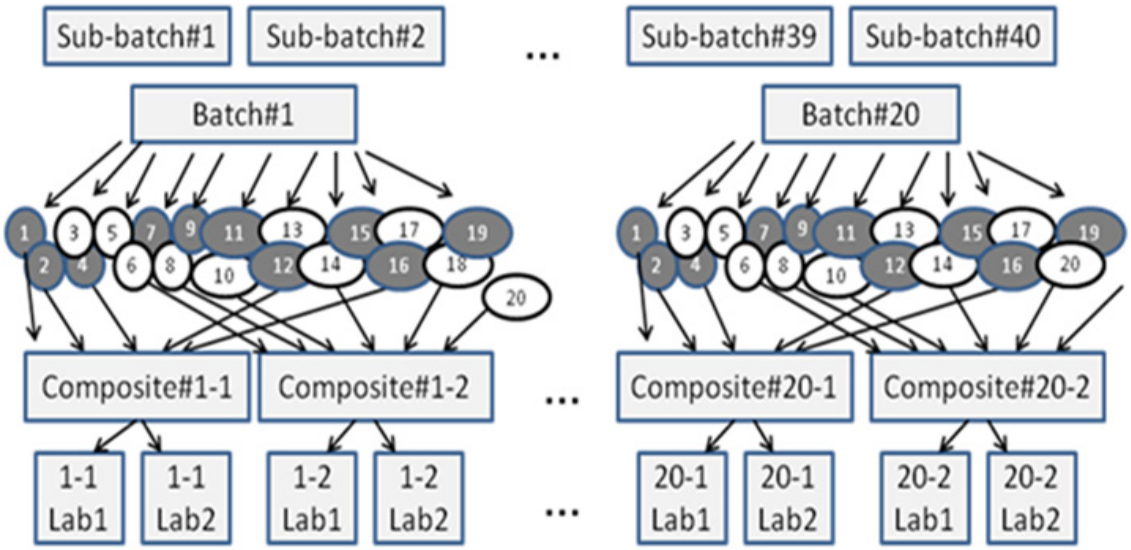

Figure 1.Sampling scheme for Benin cotton seed meal

In addition to this scheme a total of 60 x 20 tonne batches (this included the aforementioned 40x20 tonne samples plus another 20 x 20 tonne batches from another source (Mill 2; Table 2B) in Benin for comparison purposes). Samples from these batches (Level 1) were milled and mixed, numbered as 1-40 (Mill 1, Table 2A) and 2/1-2/20 (Mill 2, Table 2B) and analysed for AF by both laboratories 1 and 2. This was done in an attempted to harmonise the results from both laboratories which were obtained using different extraction/clean-up methods. In addition random samples from Level 4 were analysed using a VICAM fluorimetry and HPLC method in order to evaluate their relative accuracy.

3.2 Fungal Isolation and Identification

Fungi were isolated from cottonseed meal using a serial dilution technique on a restriction agar (Kaufman, Williams, & Sumner, 1963). After counting individual isolates of the various fungi were sub-cultured onto potato dextrose agar and identified by microscopic techniques following the identification keys of Pitt and Hocking (1989) and Klich (2002). No effort was made to identify the aflatoxin producing spp. other than placing them as either *Aspergillus flavus* or *A. parasiticus* "types" within the *Aspergillus* section *flavi*.

To further confirm the identities of the isolated fungi, isolates were sub-cultured on malt extract agar (MEA) for 2-3 days at 25 °C. The mycelia was harvested from the MEA cultures and transferred into a 0.5 ml sterile screw-cap vial containing 200 µl of ringer solution, freeze-dried and stored at − 40 °C until analysed. PCR and DNA sequencing were performed at the Inqaba Biotechnological Industries (Pty) Ltd, Pretoria, South Africa for fungal identification. Accordingly the freeze dried samples were kept at room temperature for about 3 hr and genomic DNA extracted

using a fungal/bacterial DNA extraction kit (Zymo Research Corporation, Southern California, USA) according to the manufacturer's recommendations. The PCR primers, including the forward primer, ITS-1(TCCGTAGGTGAACCTGCGG) and reverse primer ITS-4(TCCTCCGCTTCTTGCTGC) were used to amplify the internal transcribed spacer (ITS) region of the extracted fungal DNA. PCR was done with Lucigen EconoTaq Plus Green (Lucigen Corporation, Middleton, Wisconsin, USA) according to the manufacture's specifications and further subjected to an ABI 9700 PCR system. An automated DNA sequencing was performed using the ABI3500XL sequencer, while the sequencing reaction included the ABIV3.1 big dye kit (Applied Biosystems Foster City, California, USA) as well as the same primers originally used for PCR (ITS-1 and ITS-4). The ITS sequences of the fungal species obtained were blasted alongside other sequences of fungi available in the literature (Altschul *et al.*, 1997).

3.3 Aflatoxin Analysis

The analytical methods for the AF in cottonseed meal were performed in two separate laboratories, those at the Food, Environment & Health Research Group, Faculty of Health Sciences, University of Johannesburg, Johannesburg, South Africa (Laboratory 1); and the Department of Veterinary Public Health, Institute of Nutrition, University of Veterinary Medicine, Vienna, Austria (Laboratory 2).

3.3.1 *Laboratory 1*

A VICAM fluorometer (VF) procedure for cottonseed meal was used to extract aflatoxins from the samples. This was performed in duplicate with one extract preserved for quantification of the toxins via VF and the other on HPLC. To this effect a 50g sample was mixed with 10 g salt (NaCl) and 200 ml methanol (MeOH)/water (80:20v/v) and for blended for 5 min. Ten ml of the mixture were filtered, diluted with 40 ml of purified water and filtered through a glass microfiber. Ten ml of the diluted filtrate was passed completely through an Aflatest immuno affinity column (VICAM®/Waters SA) (with a flow rate of about 50 drops/min). The column was washed with 2 x 10ml of distilled water and the analytes eluted with 1ml of MeOH.

For the quantitation of AFs by VF, 1 ml of Aflatest developer was added to the eluate and read after 60 seconds on the VICAM fluorimeter. Using the duplicate extract for the HPLC no developer was added and 100 µl of the obtained elute was injected on the HPLC. The HPLC instrument used was a Shimadzu Corporation (Kyoto, Japan) LC-20AB liquid chromatograph equipped with CBM-20A communication bus module, LC-20AB degasser, CTO-20A column oven, with Waters Symmetry C18 column (250 mm long, 4.6 mm internal diameter) SIL-20A auto sampler, RF-10AxL fluorescence detector (set at 362 nm for excitation and 440nm emission wavelengths) RISD-10A refractive index detector and SPD-M20A photodiode detector linked to LC solutions version 1.22 Software Release. This was incorporated with a Coring cell (Coring Systems, Germany) for electrochemical derivatisation of aflatoxin B_1 (AFB_1) and aflatoxin B_1 (AFG_1). The mobile phase consisted of MeOH/acetonitrile (ACN)/water (20/20/60v/v/v) containing 119 mg of potassium bromide (KBr) and 100 µl of 65% nitric acid (HNO_3). The elution sequence was AFs G_2, G_1, B_2, and B_1. Recoveries of AFs was done by spiking cottonseed meal that was shown to be free from AFs with AF standards to give three different concentrations, followed by HPLC analysis as described. Results were adjusted for the recoveries.

3.3.2 *Laboratory 2*

Samples of cotton seed meal (25 g) were extracted with 100 ml MeOH/H_2O (80/20v/v) using a magnetic stirrer, followed by Ultraturrax for 2 min. After filtration through a prefolded filter (595 ½; Ø185 mm; Schleicher & Schuell, Dassel, Germany) an aliquot (4 ml) was diluted with 28 mL

PBS and applied onto the immunoaffinity columns (AflaCLEAN^{TM}, LC-Tech, Dorfen, Germany) with a flow rate of one droplet every second. A washing step was carried out using 2x5 mL PBS followed by 2x10 ml H_2O. Residual water was removed under a gentle vacuum. Finally the AFs were eluted with 2x1 ml MeOH accompanied by back-flushing. The eluates were evaporated to dryness and the residue was dissolved in 3ml mobile phase. An aliquot was injected in the HPLC system.

The HPLC analysis of the AFs was carried out using isocratic elution, where the HPLC system consisted of a pump (LC9A, Shimadzu, Tokyo Japan) connected to an autosampler with a 100µl loop (AS-2000^{TM}, Merck-Hitachi, Tokyo, Japan) and a column heater (Jetstream 2 plus, TECHLAB, Erkerode, Germany). A guard column (Bischoff chromatography Lichrospher 100RP 18E 5µm – 20x4mm; Leonberg, Germany) was placed between the sample injection and the analytical column (Bischoff chromatography Lichrospher 100_RP 18E 5µm – 250x4mm; Leonberg, Germany). The signal enhancement was performed using a Kobra cell from R-Biopharm Rhône LTD (Glasgow, Scotland) and the detection was obtained by a Waters 474 fluorescence detector (MA, USA) with an excitation wavelength of 365 nm and emission wavelength of 440 nm, the signals were developed with Stratos® (Polymer laboratories, Version 4.5, Shropshire, UK).

The isocratic mobile phase, consisted of H_2O/MeOH/ACN (620/220/160 v/v/v) where 119 mg of KBr and 350 µL of 4M HNO_3 were added to 1 L, the flow rate was 1 ml min^{-1} . Using this mobile phase retention times of 8.7, 10.64, 12.3 and 16.3 min were registered for the AFs G_2, G_1, B_2 and B_1.

3.3.3 *Statistical Analysis*

Linear regression was done using Microsoft Office Excel 2007

4. Results and Discussion

The results of the fungal screening and confirmation of specie identity in the composite samples are given in Table 1. In general there was a heavy fungal contamination $<1x10^6$ CFU/g which was supported by the observation that many of the samples showed evidence of fungal growth. The principle fungi isolated were *Aspergillus flavus* and *A. parasiticus*, (*Aspergillus* section *flavi*) which were present in all of the samples screened. This was not unexpected, as these fungi do predominate in cotton oil seed (Cotty & Lee 1989) and are widely distributed throughout Benin, where the cotton seed originated (Cardwell & Cotty, 2002). Although other aflatoxin producing spp. of *Aspergillus* are now recognised (Varga, Frisvad, & Samson, 2009) some of which occur in Benin (Cotty & Cardwell, 1999) no effort was made to identify them in these samples, as this was not within the scope of this study. Other fungal spp. were found but not consistently throughout the samples, although the presence of *Aspergillus ochraceus* in some of the composites (7) gave rise to concerns of possible contamination of these by OTA (Pardo, Sanchis, Ramos, & Marin, 2006). The high levels of *A. flavus* suggested that the samples may be contaminated with the aflatoxin B series in addition to the G in the case of *A. parasiticus* (and other less well known producers) (Frisvad, Skouboe, & Samson, 2005). In other reports on cotton seed and soils used to grow this commodity, *A flavus* has been the main AF producer present (Mazen, El-Kady, & Saber, 1990) with *A. parasiticus* being there at lower levels (Cotty, 1997). Whether our observation indicate a different mycoflora and hence inocula in Benin in comparison with other regions or not is a matter for further investigation. It was not possible to find out where and when the heavy fungal contamination occurred as they were received in this state and it can only be speculated that the inocula was already present in the cottonseed meal and this proliferated during the course of the voyage from Benin (West Africa) to South Africa.

Table 1. Results of fungal screening of oil seed cake composite samples

Sample*	Fungus Isolate	CFU	PCR Result**
1-1	*Aspergillus flavus*	$7x10^6$	Confirmed
(3+17)	*Aspergillus ochraceus*	$2x10^6$	ND
	Aspergillus parasiticus	$4x10^6$	Confirmed
	Fusarium verticillioides	$2x10^6$	Confirmed
1-6	*Aspergillus flavus*	$5x10^6$	Confirmed
(10+19)	*Aspergillus parasiticus*	$5x10^6$	Confirmed
1-7	*Aspergillus flavus*	$5x10^6$	Confirmed
(8+32)	*Aspergillus parasiticus*	$4x10^6$	Confirmed
1-8	*Aspergillus flavus*	$6x10^6$	Confirmed
(28+57)	*Aspergillus parasiticus*	$5x10^6$	Confirmed
	Fusarium solani	$2x10^6$	Confirmed
1-10	*Aspergillus flavus*	$6x10^6$	Confirmed
(12+18)	*Aspergillus ochraceus*	$2x10^6$	ND
	Aspergillus parasiticus	$3x10^6$	Confirmed
	Aspergillus restrictus	$2x10^6$	Confirmed
1-12	*Aspergillus flavus*	$8x10^6$	Confirmed
(7+15)	*Aspergillus ochraceus*	$3x10^6$	ND
	Aspergillus parasiticus	$4x10^6$	Confirmed
	Aspergillus restrictus	$3x10^6$	Confirmed
1-15	*Aspergillus flavus*	$7x10^6$	Confirmed
(54+59)	*Aspergillus parasiticus*	$5x10^6$	Confirmed
1-20	*Aspergillus flavus*	$8x10^6$	Confirmed
(16+31)	*Aspergillus parasiticus*	$3x10^6$	Confirmed
	Aspergillus penicillioides	$2x10^6$	Confirmed
2-1	*Aspergillus flavus*	$8x10^6$	Confirmed
(17+3)	*Aspergillus ochraceus*	$3x10^6$	ND
	Aspergillus parasiticus	$6x10^6$	Confirmed
	Fusarium verticillioides	$7x10^6$	Confirmed
2-2	*Aspergillus flavus*	$7x10^6$	Confirmed
(20+29)	*Aspergillus parasiticus*	$4x10^6$	Confirmed
2-3	*Aspergillus flavus*	$7x10^6$	Confirmed
(6+11)	*Aspergillus ochraceus*	$3x10^6$	ND
	Aspergillus parasiticus	$7x10^6$	Confirmed
2-6	*Aspergillus flavus*	$6x10^6$	Confirmed
(10+19)	*Aspergillus parasiticus*	$7x10^6$	Confirmed
2-7	*Aspergillus flavus*	$5x10^6$	Confirmed
(8+32)	*Aspergillus parasiticus*	$3x10^6$	Confirmed
	Penicillium citrinum	$2x10^6$	Confirmed
	Penicillium griseofulvum	$2x10^6$	Confirmed
2-8	*Aspergillus penicillioides*	$2x10^6$	Confirmed
(28+57)	*Aspergillus flavus*	$5x10^6$	Confirmed
	Aspergillus parasiticus	$4x10^6$	Confirmed
2-10	*Aspergillus flavus*	$4x10^6$	Confirmed
(12+18)	*Aspergillus parasiticus*	$3x10^6$	Confirmed
	Aspergillus restrictus	$3x10^6$	Confirmed
	Penicillium spp.	$6x10^6$	Confirmed
2-12	*Aspergillus flavus*	$7x10^6$	Confirmed
(7+15)	*Aspergillus parasiticus*	$6x10^6$	Confirmed
	Aspergillus restrictus	$2x10^6$	Confirmed
2-15	*Aspergillus flavus*	$6x10^6$	Confirmed
(54+59)	*Aspergillus ochraceus*	$2x10^6$	Confirmed
	Aspergillus parasiticus	$4x10^6$	Confirmed
	Eurotium amstelodami	$2x10^6$	Confirmed
2-20	*Aspergillus flavus*	$7x10^6$	Confirmed
(16+31)	*Aspergillus ochraceus*	$2x10^6$	ND
	Aspergillus parasiticus	$3x10^6$	Confirmed
	Aspergillus penicillioides	$3x10^6$	Confirmed

*The numbers in parenthesis column 1 are the numbers of the sub-batches (Table 2) used to make the composites as per Figure 1. Numbers not in parenthesis commencing with a 1, e.g., 1-1 correspond to 'A' samples and those with a 2, e.g. 2-1 correspond to 'B' samples.

ND = Not determined; **Confirmed = Identity of the fungus was confirmed by gene probing

The results of the AF analysis of both sets of samples taken at Level 1, i.e., mill 1 (Table 2A) and mill 2 (Table 2B) and the statistically based composites (20 samples) (Table 3) showed that for most of them that the VF method (laboratory 1) gave higher concentrations of total AF than the HPLC/VICAM method (laboratory 1) with both having higher values than for the HPLC alone (laboratory 2). On taking the ratios of the amounts of total AF present for the sub-sample bulk (Table 2A) i.e., the result obtained by Lab 1 for the VF method being divided by those for VICAM measured using HPLC, ranged from 1.03 (sample 58) to 1.75 (sample 12) with 5 samples having a ratio <1 (lowest 0.89) (Table 2A); and 1.14 (sample 46) to 2.28 (sample 44) for mill 2 with 1 (47) having a ratio of <1 (0.92) (Table 2B).

Table 2A. Aflatoxin analysis of the composites of the ten samples taken from the origin 60 x 20Tonnes batches for Mill 1

	Lab1						Lab2
	VICAM	High performance liquid chromatography					
Sample	Total AF (µg/kg)	AFB1 (µg/kg)	AFB2 (µg/kg)	AFG1 (µg/kg)	AFG2 (µg/kg)	Total AF (µg/kg)	Total AF (µg/kg)
2	146	59.6	2.6	35.0	2.6	99.8	85.0
3	58	31.6	2.1	9.1	2.5	45.3	38.2
4	96	39.3	1.9	28.0	2.1	71.3	43.4
6	112	48.3	1.6	37.0	1.9	88.7	49.2
7	130	58.5	1.4	29.3	1.3	90.5	67.9
8	112	46.8	2.0	24.3	1.9	75.0	51.0
10	142	55.8	1.1	31.5	1.9	90.3	62.6
11	100	35.9	1.8	12.4	1.8.	51.9	48.2
12	114	41.3	1.5	20.2	2.2	65.1	57.8
13	100	39.5	2.0	23.6	1.4	66.5	45.7
14	96	35.3	1.2	25.6	1.8	63.9	87.0
15	100	43.3	1.4	29.1	3.4	77.1	93.6
16	45	26.6	2.5	18.5	0.9	48.4	34.4
17	82	36.4	2.2	19.5	4.2	62.3	57.2
18	84	35.9	1.9	23.7	3.2	64.7	59.1
19	96	36.6	1.6	24.8	6.3	69.3	42.3
20	78	25.1	1.3	16.5	2.1	45.0	43.9
21	68	25.8	1.6	15.7	2.0	45.1	53.6
22	44	40.5	1.5	11.4	1.4	34.8	36.9
23	56	21.6	1.4	9.6	1.2	33.8	23.3
24	36	14.1	1.2	5.2	1.8	22.3	29.9
25	58	24.5	1.4	14.5	1.5	41.9	26.5
26	116	53.6	1.8	32.1	1.0	88.5	39.9
27	96	38.2	1.8	19.8	2.0	61.8	34.1
28	96	43.0	2.2	26.1	1.7	73.0	32.6
29	76	29.8	2.8	6.5	2.8	41.9	37.2
30	82	35.3	3.5	13.3	2.8	54.9	38.5
31	116	52.5	1.8	21.9	4.0	80.2	36.5
32	104	40.2	1.3	21.4	5.0	67.9	41.9
33	164	71.7	1.4	25.8	4.4	103.3	40.1
34	36	21.5	1.2	9.9	4.0	36.6	24.7
35	80	39.5	2.0	12.5	3.6	57.6	36.0
51	42	15.4	2.7	5.3	3.4	26.8	40.6
53	56	31.6	1.8	27.6	1.6	62.6	24.5
54	82	43.3	2.5	19.2	2.6	67.6	42.3
55	76	39.3	1.8	22.3	2.1	65.4	26.6
56	50	39.3	1.8	19.1	2.1	62.3	46.3
57	60	30.1	1.5	17.6	1.6	51,3	25.9
58	64	36.4	3.1	19.5	3.2	62.2	54.0
59	48	30.3	3.3	16.9	2.6	53.1	46.3

ND = not determined; AFB1 = aflatoxin B_1; AFB2 = Aflatoxin B_2; AFG1 = Aflatoxin G_1; AFG2 = Aflatoxin G_2

Table 2B. Aflatoxin analysis of the composites of the ten samples taken from the origin 10 x 20Tonnes batches from Mill 2

	Lab1						Lab2
	VICAM	High performance liquid chromatography					
Sample	Total AF (µg/kg)	AFB1 (µg/kg)	AFB2 (µg/kg)	AFG1 (µg/kg)	AFG2 (µg/kg)	Total AF (µg/kg)	Total AF (µg/kg)
1	110	61.8	0.8	26.2	0.5	89.3	72.3
5	104	45.2	1.8	30.1	2.0	79.1	52.3
9	110	48.5	1.6	15.6	2.3	68.0	67.5
36	108	38.1	1.4	21.5	3.2	64.2	31.6
37	64	25.2	0.7	16.8	2.8	45.5	43.3
38	116	59.5	0.9	19.5	1.8	81.7	33.7
39	138	59.9	2.0	31.5	2.4	95.8	37.5
40	116	41.8	2.5	28.9	2.9	76.1	50.8
41	108	46.4	1.9	21.3	2.5	72.1	35.6
42	84	26.2	2.4	14.8	3.0	46.4	33.7
43	70	28.5	2.2	5.3	3.3	39.3	43.2
44	66	16.7	1.8	8.2	2.2	28.9	33.2
45	70	23.5	2.2	7.2	2.8	35.7	38.5
46	46	21.3	1.3	16.1	1.6	40.3	27.6
47	64	38.5	1.4	27.5	2.1	69.5	26.0
48	120	46.5	2.2	27.7	3.5	79.9	43.3
49	76	27.5	3.2	27.5	3.6	61.8	34.7
50	84	29.4	3.4	11.6	3.3	47.7	45.3
52	84	46.5	2.6	18.7	1.9	69.7	ND
60	26	16.2	2.3	9.5	0.7	28.7	ND

ND = not determined; AFB1 = aflatoxin B_1; AFB2 = Aflatoxin B_2; AFG1 = Aflatoxin G_1; AFG2 = Aflatoxin G_2

Table 3. Aflatoxin (µg/kg) analysis of selected cotton oil seed cake composite samples – Laboratory 1 (Vicam) and Laboratory 2 (HPLC) As per Figure 1

	Total Aflatoxin (µg/kg)	
Sample	Laboratory 1	Laboratory 2
1-1	26	31.3
2-1	28	38.7
1-2	42	28.9
2-2	40	32.7
1-3	42	36.3
2-3	64	31.9
1-6	45	22.4
2-6	36	34.3
1-7	52	40.3
2-7	67	28.3
1-8	40	26.5
2-8	51	25.3
1-10	57	29.9
2-10	56	33.1
1-12	73	34.2
2-12	77	39.2
1-15	65	23.4
2-15	59	32.6
1-20	37	27.3
2-20	35	29.0

On finding these ratio for the VICAM/HPLC method versus the HPLC method (laboratory 2) they ranged from 1.03(sample 20) to 2.57 (samples 33 & 53) with 7 results being lower than a ratio of 1 (sample 14 being the lowest at 0.74) (Table 2A). The reason for this consistent discrepancy is not clear but the VF method gives a total aflatoxin content based on the addition of a fluorescence developer that does not take cognisance of the fluorescence of the individual aflatoxins, as do the methods based on HPLC, which measure each aflatoxin separately. Further it is claimed that the VICAM immuno-affinity columns, being specific for aflatoxins, give a better recovery of aflatoxin than other clean ups. The question is: can the VF method be used as a more rapid screen for the presence of AF in cotton seed meal to yield a practically useful result for millers? An analysis of the correlation between analytic results from the same lot revealed apart from the systematic differences there was a strong correlation between the results obtained with VICAM-HPLC and VICAM alone when applied on the same extract at laboratory 1 (VICAM Fluorometer = 1.349 HPCL-VICAM + 3.06; $R^2 = 0.7591$, SE coefficient = 0.0998) (Figure 2A).

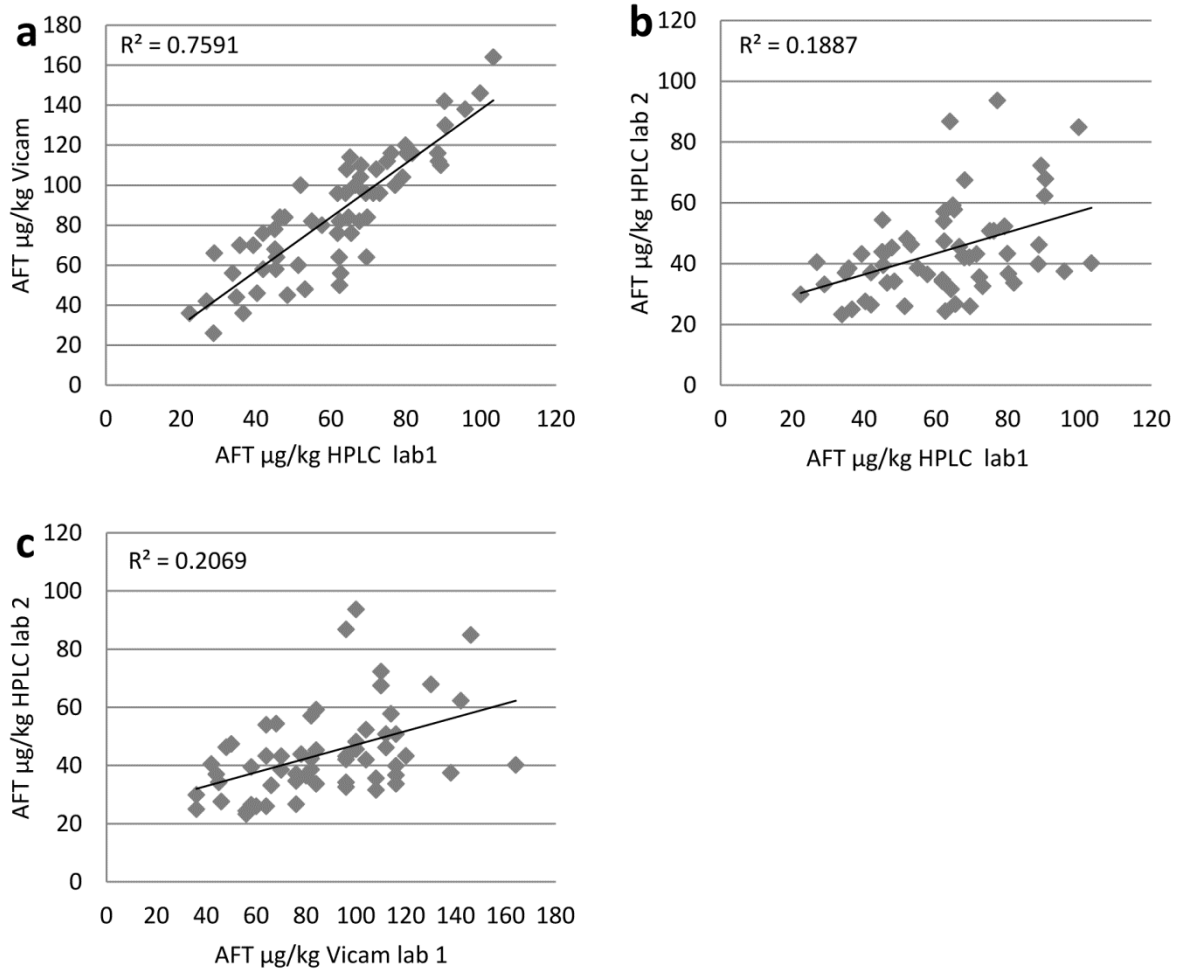

Figure 2. Results from analysis for total aflatoxin (AFT)

In contrast, the correlation between analytic results obtained in both laboratories was poor (R^2 = 0.1887 for total aflatoxin) (Figure 2B). Similarly the VICAM results for Lab 1 were poorly correlated with those for Lab 2 (Figure 2C). In a previous study at laboratory 2 the variation between repeated HPLC analyses on the same extract corresponded to a relative expanded uncertainty (2* x sd) of approximately 1% (Reiter *et al.* 2011) whereas the variation between extract from different subsamples from 20 different bulk samples from the same study the correlation in analytical results for total aflatoxin between extracts from the same bulk sample was R^2=0.295 (Figure 3) corresponding to a relative expanded uncertainty of approximately 20%. Considering the good correlation between VF and VICAM-HPLC applied to the same extract and the fact that the correlation between results from the two laboratories was poorer than could be expected based on the results from repeated extractions at laboratory 2 it is possible that the extraction method used at laboratory 1 may have introduced additional uncertainty.

The lack of correlation between the two laboratories is, however, a cause for concern. Discrepancies in laboratory proficiency assessments are not unknown (e.g., Bao *et al.,* 2009) particularly where more complex matrices are being analysed. Where accreditation and reliability is to be maintained, further re-evaluation is required. Currently laboratory 1 is undergoing an accreditation process and the method and results for cotton seed meal analysis will be part of this programme, in an attempt to pin point the reasons for lack of correlation with laboratory 2, which is certified (ISO 9001:2008). Clearly the results given in this paper underscore the necessity for accreditation where difficult analytes like mycotoxins are being analysed in complexed substrates.

The total aflatoxin result from VF correlated strongest with the results for AFB_1 and AFG_1 from VICAM-HPLC (Appendix Figure 4. a, b) whereas the correlation with AFB_2 and AFG_2 (Appendix Figure 4. c, d) which were present at low levels was very poor reflecting the fact that AFB_1 is the dominant contaminant.

Figure 2A shows correlation between results for Vicam and HPLC when applied to the same extraction from the same analytical sample. Regression results m=1-349, SE=0.0998, b=3.06, SE=6.43; Figure 2B shows correlation between analytical results with HPLC from laboratory 1 and 2. Regression results, m=0.346, SE=0.0958, b= 22.63, SE=6.21 or m=0.6796, SE=0.0312 when b is fixed at 0. Figure 2C shows correlation between Vicam results from laboratory 1 and HPLC of laboratory 2. Regression results m=0.236, SE=0.0617. b=23.49, SE=5.68.

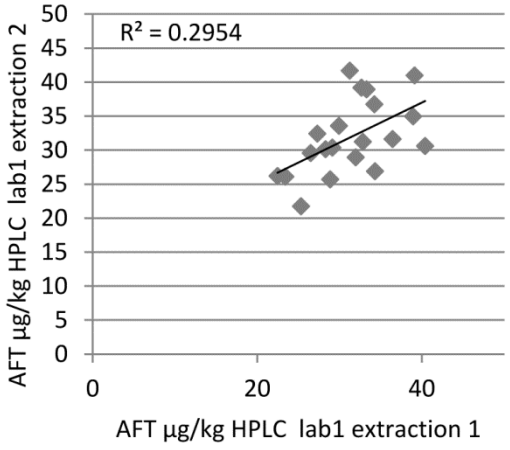

Figure 3. Correlation between HPLC results for total aflatoxin between repeated extractions at laboratory 2. Regression results, m=0.5861, SE=0.213, b=13.5, SE=6.77

From the original 60 batches of 20 tonnes 2 x 10 samples, 40 were used to make the composites and were from company 1 in Benin. The remaining 20 x 20tonne batches were from mill 2 and were included for AF analytical comparison. The results (Table 2B) were similar in level and range of AF present, the presence of the G AFs indicating that these samples were also infected with *A. parasiticus*. As both mills produced cotton seed meal with similar contamination of aflatoxin it seems reasonable to suppose that this commodity being produced in Benin is unsatisfactory for trading purposes in some countries in terms of certain legislation directed towards this product (e.g. the European Union, 20 μg/kg (20ppb) AFB_1 in cotton seed meal (Directive 2002/32/EC 2002)).

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Appendix

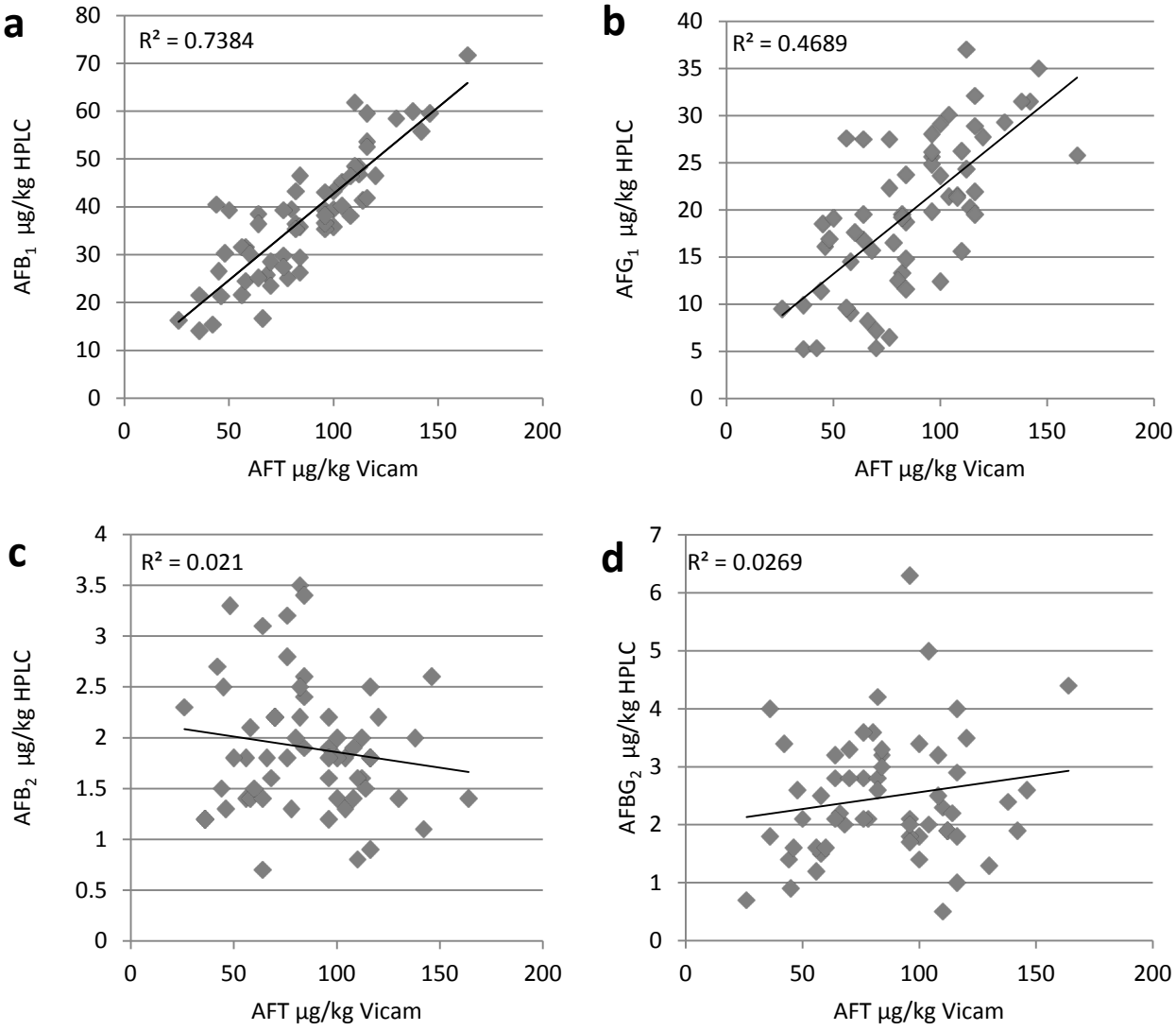

Figure 4. Correlation between total aflatoxin by Vicam and individual aflatoxin species by Vicam-HPLC

AFT = Total aflatoxin. Regression results. A: m=0.236, SE 0.0617 b=23.49 SE 5.68. B: m=0.1827 SE 0.0255 b=4.068 SE 2.33 C: m=-0.003056 SE 0.00274 b=2.165 SE 0.249 D: Regression line m= 0.00580 SE 0.00458 b=1.97 SE 0.417.

