Standard Operating Procedure

Detection of aflatoxins B1,B2, G1, G2 and M1 in milk, edible vegetable oil and animal feed

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1. INTRODUCTION

Aflatoxins are a group oftoxic secondary metabolites produced by severalspecies of fungi such as *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Aflatoxins B1, B2, G1 and G2 are the majornaturally occurring aflatoxins and are known to contaminate a variety of agricultural products during their growth, harvest and storage. Aflatoxin M1,which is the 4-hydroxylated metabolite of aflatoxin B1,is excreted in milk of mammals that have ingested aflatoxin B1-contaminatedfeed. Aflatoxins are carcinogenic, teratogenic and mutagenic to humans and animals, and hence,aflatoxin contamination in food/feed remains a global food safety issue that has forced regulatory authorities in many countries to limit the aflatoxin levels in food/feed.These aflatoxins in milk, feed/ingredients and vegetable oils can be determined quantitativelyusing HPLC-FLD method.

2. SCOPE

This standard Operating Procedure (SOP) describes the procedure for screening of aflatoxins M1, B1, B2, G1 and G2 in milk, and aflatoxins B1, B2, G1 and G2 in edible vegetable oil and animal feed / ingredients,using High-Performance Liquid Chromatography-Fluorescence Detection method (HPLC-FLD).

3. OBJECTIVE/PURPOSE

To ensure the accuracy, quality and reproducibility of the procedure / protocol.

4. DEFINITIONS AND ABBREVIATIONS

4.1 AFB1:Aflatoxin B1

4.2 AFB2: Aflatoxin B2

4.3 AFG1: Aflatoxin G1

4.4 AFG2: Aflatoxin G2

4.5 AFM1: Aflatoxin M1

4.6 HPLC: High-Performance Liquid Chromatography

4.7 FLD: Fluorescence Detection/Detector

4.8 IAC: Immunoaffinity Column

4.9 WS: Working Standard

4.10 CS: Calibration Standard

4.11 ppb: parts per billion

5. PRINCIPLE OF THE METHOD

Samples are prepared by mixing with an extraction solution [salt and methanol/water (for feed and vegetable oil) or salt (for milk)] and blendingat high speed (feed and vegetable oil) or by centrifuging (milk). Coarse particulate sample solidsand precipitatesare removed from the sample extract bytwo separate filtration steps.Sample extract is then cleaned up using monoclonal antibody-based affinity chromatography. The immunoaffinity column is bound with antibodies specific to aflatoxins B1, B2, G1, G2 and M1, and when the extract is applied to the column, aflatoxins in the extract bind to antibodies on the column.Then impurities in the column are removed by

several washing steps. Aflatoxins bound to antibodies areelutedby passing methanol (for feed and vegetable oil)or methanol/water (for milk) through the column, and detected by HPLC-FLD system set at 360 nm (excitation) and 450 nm (emission).

6. SAFETY CONSIDERATIONS& PRECAUTIONS

The chemicals use in this procedure can be highly toxic and may constitute a serious health risk for the laboratory workers. Further, aflatoxins are proven carcinogens.Suitable gloves must be worn whenchemicals/ aflatoxin standards are handled, and the work shall be performed in a fume hood at all possible time.

7. MATERIALS AND EQUIPMENT

7.1 WATER

Unless stated otherwise, use only distilled-deionised water or water of equivalent purity.

7.2 CHEMICALS

Unless stated otherwise, use only the reagents of recognized analytical grade

7.2.1 Methanol (CH_3OH) (HPLC grade)

7.2.2 Acetonitrile (CH_3CN) (HPLC Grade)

7.2.3 Salt (NaCl) (Analytical grade)

7.3 STANDARD SUBSTANCES

7.3.1 AFB1: aflatoxin B1 at 2000 ppb (Fermentek Ltd., Jerusalem,Israel)

7.3.2 AFB2: aflatoxin B2 at 500 ppb (Fermentek Ltd., Jerusalem,Israel)

7.3.3 AFG1: aflatoxin G1 2000 ppb (Fermentek Ltd., Jerusalem,Israel)

7.3.4 AFG2: aflatoxin G2500 ppb (Fermentek Ltd., Jerusalem,Israel)

7.3.5 AFM1: aflatoxin M1 at 500 ppb (Fermentek Ltd., Jerusalem,Israel)

7.4 EQUIPMENT / APPARATUS

7.4.1 Analytical balance (Range 0.0000g–15.0000 g, Last digit 0.0001g, OHAUS)

7.4.2 Filtering device for solvents and water

7.4.3 Phenex™ Nylon Filter Membranes, 0.20 µm (Phenomemex®)

7.4.4 Laboratory blender (Waring)

7.4.5 Grinder (Moulinex®)

7.4.6 Laboratory centrifuge (Centurion scientific Ltd.)

7.4.7 Vortex mixer (Velp)

7.4.8 Fluted filter papers, 24 cm, (Vicam)

7.4.9 Glass microfiber filter papers, 1.5 µm (Vicam)

7.4.10 Measuring cylinder, 25 ml, 50 ml 100 ml, 250 ml

7.4.11 Volumetric flask, 50 ml

7.4.12 Stopwatch

7.4.13 Glass beakers

7.4.14 Conical flasks

7.4.15 Glass funnels

7.4.16 Polypropylene tubes with screw cap, 15 ml and 50 ml

7.4.17 Sterile disposable syringes, 10 ml, 20 ml and 50 ml

7.4.18 18G syringe needles

7.4.19 Micropipettes,0.5–10µl, 2–20µl, 10–100µl, 100–1000µl, 500–5000µl (Eppendorf)

7.4.20 Immunoaffinity column (AflaTest®, Vicam)

7.4.21 Vacuum Pump (Cole Parmer)

7.4.22 Extraction manifold with a vacuum chamber, rack and stopcocks (Agilent)

7.4.23 Amber colour HPLC vials

7.4.24 Glass HPLC vialinserts with bottom polymer feet, 250 µl

7.4.25 HPLC-FLD system (Agilent 1100 series) consisting of a

7.4.25.1 Degasser (G1379A)

7.4.25.2 Quaternary pump(G1311A)

7.4.25.3 Autosampler (G1313A)

7.4.25.4 Column oven(G1316A)

7.4.25.5 Fluorescence Detector(Agilent 1200 series, G1321A)

7.4.25.6 Column: Phenomenex Luna C18 (2) 100A , 4.6 × 250 mm, particle size 5 µm

7.4.25.7 Software: ChemStation for LC 3D (Rev. A. 10.02) software

8. SOLUTIONS

8.1 Methanol/water (10/90, v/v)

Mix 10 ml of methanol (7.2.1) with 90 ml of water (7.1)

8.2 Methanol/water (60/40, v/v)

Mix 60ml of methanol (7.2.1) with 40 ml of water (7.1)

8.3 Methanol/water (80/20, v/v)

Mix 80 ml of methanol (7.2.1) with 20 ml of water (7.1)

8.4 Mobile phase A

Purified water (7.1)filtered through a 0.20-µm nylon membrane filter (7.4.3).

8.5 Mobile phase B

Acetonitrile(7.2.2) filtered througha 0.20-µm nylon membrane filter (7.4.3)

8.6 Mobile phase C

Methanol (7.2.1) filtered through a 0.20-µm nylon membrane filter (7.4.3)

9. PROCEDURE

9.1 TEST PORTION

9.1.1 Milk

From each fluid milk sample, measure 50 ml using a 50-ml volumetric flask (7.4.11) into a 50-ml polypropylene tube (7.4.16).

9.1.2 Vegetable oil

From each vegetable oil sample, weigh 25 ± 0.05 g into a 50-ml polypropylene tube (7.4.16).

9.1.3 Feed

Grind the feed sampleusing the grinder (7.4.5),andweigh 50 ± 0.05 g of the ground sample.

9.2 NEGATIVE CONTROL

9.2.1 Milk

A blank milk sample containing noAFM1, AFB1, AFB2, AFG1 and AFG2. Measure 50 ml of blank milk sample in to a 50-ml polypropylene tube (7.4.16). Continue with the procedure 9.5and further.

9.2.2 Vegetable oil

A blank vegetable oilsample containing no AFB1, AFB2, AFG1 and AFG2. Weigh 25± 0.05 gof blank vegetable oilsample into a 50-ml polypropylene tube (7.4.16). Continue with the procedure 9.5 and further.

9.2.3 Feed

A blank feed sample containing no AFB1,AFB2, AFG1 and AFG2. Weigh 50 ± 0.05 g of ground blank feed sample. Continue with the procedure9.5 and further.

9.3 RECOVERY CONTROL / QUALITY CONTROL SAMPLE

Note: Let fortified samples stand for at least 30 minutes before continuing with the rest of the procedure

9.3.1 Milk

A blank sample fortified with a known amount of AFM1, AFB1, AFB2, AFG1 and AFG2. Measure 50 ml of blank fluid milk into a 50-ml polypropylene tube. Pipette and discard 0.5 ml of the sample. Fortify the blank sample to yield 0.5 µg/l (0.5 ppb) concentration of all five aflatoxins by adding 500µl of the below described working solution (WS1),and continue with the procedure 9.5 and further.

9.3.1.1 Preparation of WS 1

To prepare the aflatoxin mixture to fortify the positive control, pipette out 100 µl of AFM1, AFB2, and AFG2, and 25 µl of AFB1 and AFG1, and mix with 650 µl of methanol.

9.3.2 Vegetable oil

A blank sample fortified with a known amount of AFB1, AFB2, AFG1 and AFG2. Weigh 24. 75± 0.05 g of blank vegetable oil into a 50-ml polypropylene tube.Fortify the blank sample to yield 2 µg/kg (2 ppb) concentration of all four aflatoxins by adding 250 µl of the below described working solution (WS 2),and continue with the procedure 9.5and further

9.3.2.1 Preparation of WS 2

To prepare the aflatoxin mixture tofortify the positive control, mix 50 µl of AFB1 and AFG1, and 200 µl of AFB2 and AFG2.

9.3.3 Feed

A blank sample fortified with a known amount of AFB1, AFB2, AFG1 and AFG2. Weigh 49.50 ± 0.05 g of ground blank feed sample.Fortify the blank sample to yield 2 µg/kg (2 ppb) concentration of all four aflatoxins by adding 500 µl of the above described working solution (WS 2) (9.3.2.1)and continue with the procedure 9.5 and further.

9.4 CALIBRATION STANDARDS

9.4.1 Preparation of mixed standard containing AFM1, AFB1, AFB2, AFG1 and AFG2 at 50 µg/l (50 ppb) (WS 3)

Pipette out 150 µl of AFM1, AFB2, and AFG2, and 37.5 µl of AFB1 and AFG1, and mix with 975 µl of mobile phase to prepare a mixture of standards with 50 µg/l of each aflatoxin (WS 3).

Prepare the rest of the calibration curve standards according to Table 1 using the mixed calibration standard (WS 3) prepared above (9.4.1).

Table 1:Preparation of calibration standard samples

Calibration Standard name	Calibration standard concentration (ppb)	Preparation
CS 1	20	Mix 600 µl of WS 3 (9.4.1) with 900 µl of mobile phase.
CS 2	10	Mix 600 µl of CS1 with 600 µl of mobile phase
CS 3	5	Mix 500 µl of CS2 with 500 µl of the mobile phase.
CS 4	3	Mix 450 µl of CS3 with 300 µl of mobile phase
CS 5	1	Mix 200 µL of CS4 with 400 µl of mobile phase
CS 6	0.5	Mix 200 µl of CS5 with 200 µl of mobile phase
CS 7	0.25	Mix 100 µl of CS6 with 100 µl of mobile phase

9.5 SAMPLEEXTRACTIONANDDILUTION

9.5.1 Milk

Add 1 ± 0.05 g of NaCl (7.2.3)to the test portion (unknown sample, negative control and recovery / quality control samples). Close the caps tightlyand mix wellby vortexing or shaking vigorously by hand. Centrifuge the samples at 2000gfor 10 minutes. After the centrifugation, poke a hole into the bottom of the polypropylene tube, using an 18G syringe needle, and carefully remove the bottom layer of the milk (i.e., skim portion, ~30 ml)into a 50-ml syringe for analysis without disturbing the top fat layer. Filter the skim sample through a

fluted filter paper(7.4.8) and collect the filtrate in a clean glass container. Then, filter the above filtered skim milk sample through a 1.5-µm glass microfiber filter (7.4.9), andcollect the filtrate into a 50-ml polypropylene tube oraglass container. Proceed to9.6 and further.

9.5.2 Vegetable Oil

Place the test portion(unknown sample, negative control and recovery / quality control samples) in a clean blender jar (7.4.4).Add 5 ± 0.05 g of NaCl (7.2.3) and 125 ml of methanol/water (60/40)(8.2) into the blender jar. Close the blender jar tightly and blend at high speed (~ 22,000 rpm) for 1 minute. Remove the cover from jar and filter the extract through a fluted filter paper (7.4.8) into a glass container.

Pipette out20 ml of the filtered extract into a 50-ml polypropylene tube. Add 20 ml of purified water (7.1) to the tube to dilute the extract, and mix well by shaking with hand. Filter the diluted extract through a 1.5-µm glass microfiber (7.4.9)filter into a clean glass container or a 50-ml polypropylene tube. Proceed to9.6and further.

9.5.3 Feed

Place the test portion(unknown sample, negative control and recovery / quality control samples) in a clean blender jar (7.4.4).Add 5 ± 0.05 g of NaCl (7.2.3) and 100 ml of methanol/water (80/20) (8.3) to the blender jar. Close the blender jar tightly and blend at high speed (~22,000 rpm) for 1 minute. Remove the cover from jar and filter the extract through a fluted filter paper(7.4.8).Collect filtrate into a clean glass container and discard the supernatant.

Pipette out 10 ml of the filtered extract into a 50-ml polypropylene tube. Add 40 ml purified water (7.1)to the tube and dilute the extract and mix

well by shaking with hand. Filter the diluted extract through 1.5-µm glass microfiber filter (7.4.9) into a clean container or a 50-ml polypropylene tube. Proceed to9.6 and further.

9.6 IMMUNOAFFINITYCOLUMNCHROMATOGRAPHY

Remove the top cap from the AflaTest®IAC (7.4.20),and cut bottom 1/8 inch off the end of the cap with a sharp blade or a scissor.Using this top cap as a coupling, attach the syringe barrel to the IAC.Pipette out or pour the desired volume of extract or diluted extract into the syringe barrel, and then remove the bottom cap from the IAC.Achieve the required flow rate (given for each matrix below) through the column, using gravity, a vacuum pump (7.4.21) or by pressing the plunger as necessary.

9.6.1 Milk

Pass 10 ml of filtered skim milk completely though the AflaTest® IAC(7.4.20) at a rate of about 1 drop/second until air comes through column. Remove column from the loading syringe barrel, and fill column headspace with methanol/water (10:90) (8.1) solution. Attach a second syringe barrel to the column,and fill it with 10 ml of methanol/water (10:90) solution. Pass the methanol/water (10:90) through the column at a rate of about 1–2 drop/second. Repeat the previous step once more until air comes through the column. Elute the column with 1 ml of methanol/water (80/20) (8.3) at a rate of 1 drop/second or slower and collect the eluate. Double dilute the eluate with purified water (7.1) and transfer to an HPLC vial (7.4.23).

9.6.2 Vegetable oil

Pass 5 ml of filtered diluted extract of oil through the AflaTest®IAC (7.4.20) at a rate of about 1 drop/second until air comes through column. Then pass 10 ml of purified water (7.1) through the column at a rate of about 1–2 drop/second. Pass another 10 ml of purified water (1–2 drop/second) until air comes through the column. Elute the column with 1 ml of HPLC grade methanol (7.2.1) at a rate of about 1 drop/second or slower and collect the eluate. Double dilute the eluate with purified water (7.1) and transfer to an HPLC vial (7.4.23).

9.6.3 Feed

Pass 10 ml of filtered diluted extract of feed through the AflaTest® IAC (7.4.20) at a rate of about 1 drop/second until air comes through column. Then pass 10 ml of purified water (7.1) through the column at a rate of about 1–2 drop/second. Pass another 10 ml of purifiedwater (1–2 drop/second)until air comes through the column. Elute the column with 1 ml of HPLC grade methanol (7.2.1) at a rate of about 1 drop/second or slower and collect the eluate. Double dilute the eluate with purified water and transfer to an HPLC vial (7.4.23).

9.7 HPLC-FLDANALYSIS

Flow rate: 1ml/min

Injection volume: 10 µl

Total run time: 22min

Excitation wave length: 360nm , emission wavelength: 450 nm

Mobile Phase: water/methanol/acetonitrile (50/30/20, v/v/v)

Table 2: Mobile phase gradient

Time (minutes)	Mobile phase A % (8.4)	Mobile phase B % (8.5)	Mobile phaseC % (8.6)
0	50.0	20.0	30.0
12	50.0	20.0	30.0
13	20.0	20.0	60.0
14	20.0	20.0	60.0
15	50.0	20.0	30.0
22	50.0	20.0	30.0

9.8 CALCULATION OF AFLATOXIN CONCENTRATION IN A SAMPLE

The concentration of a particular aflatoxin in milk, vegetable oil and feed samples will be calculated using the respective calibration curve.

9.9 INTERPRETATION OF TEST RESULTS

Any HPLC run is considered valid if the following results are achieved.

1. The calibration curves of eachaflatoxinshall give a coefficient of determination / correlation coefficient (R^2)greater than or equal to 0.95.

2. The recovery controls or quality control samples shall yield recoveries in the following range.

Matrix	Analyte	Spike level	Recovery range

		(ppb)	(%)
Milk	AFM1	< 1	50–120
	AFB1	< 1	50–120
	AFB2	< 1	50–120
	AFG1	< 1	50–120
	AFG2	< 1	50–120
Vegetable oil / animal feed	AFB1	1–10	70–120
	AFB2	1–10	70–120
	AFG1	1–10	70–120
	AFG2	1–10	70–120*

*For AFG2 in vegetable oil, acceptable range of recovery: 50–120%

If the above two conditions are true, the test samples can be interpreted as follows.

Positive samples:

1. Milk samples with corresponding HPLC-FLD peak areafor AFM1, B1, B2, G1 and G2above 0.5µg/l level

2. Vegetable oil samples with corresponding HPLC-FLD peak area for AFB1 above 1 µg/kg **and / or** peak area for total aflatoxin level (AFB1, AFB2, AFG1, and AFG2) above 4 µg/kg

3. Feed samples with corresponding HPLC-FLC peak area for AFB1 above 1 µg/kg

Any other result shall be reported as negative.

10. SCHEMATIC DIAGRAM OF THE HPLC PROCEDURE

10.1. Milk

Test Sample

1. Measure 50 ml of milk sample into a 50-ml polypropylene tube

Sample Extraction

1. Add 1 ± 0.05 g of NaCl to sample and mix well
2. Centrifuge sample at 2000g for 10 minutes
3. Remove the bottom skim layer, using an 18G needle
4. Filter the skim sample through fluted filter paper and collect filtrate.
5. Filter the filtered skim sample through 1.5-µm glass microfiber filter and collect filtrate.

Column chromatography

1. Pass 10 ml of filtered skim milk through IAC (1 drop/s) until air comes through the column
2. Remove the loading syringe barrel from column
3. Fill column headspace with methanol/water (10.90)
4. Attach new syringe barrel to column and pass 10 ml of methanol/water (10:90) through IAC (1–2 drop/s)
5. Pass another 10 ml of methanol/water (10/90) until air comes through
6. Elute the column by passing 1 ml of methanol/water (80:20) (1 drop/s or slower) through IAC
7. Double dilute the eluate with purified water and transfer to HPLC vial

10.2. Vegetable oil

Test Sample	1.Weigh 25 ± 0.05 g of vegetable oil into a 50-ml polypropylene tube
Sample extraction	1. Place test sample in the blender jar. 2. Add 5 ± 0.05 g of NaCl 3. Add 125 ml of methanol/water (60/40). 4. Blend at high speed (~22,000 rpm) for 1 minute. 5. Pour extract into a fluted filter paper and collect filtrate 6. Discard the supernatant.
Extract dilution	1. Pipette 20 ml of filtered extract into a clean vessel 2. Add 20 ml of purified water and mix well 3. Filter through 1.5-µm glass microfiber filter in to a clean vessel
Column chromatography	1. Pass 5 ml of filtered diluted extract through IAC (1 drop/s) until air comes through 2. Pass 10 ml of purified water through IAC (1–2 drop/s) 3. Pass another 10 ml of purified water until air comes through IAC 4. Elute column by passing 1 ml of methanol though IAC (1 drop/s or slower) 5. Double dilute the eluate with purified water and transfer to HPLC vial

10.3 Feed

Test Sample	1. Grind the feed/ingredient sample and weigh 50 ± 0.05 g of ground sample
Sample extraction	1. Place test sample in the blender jar. 2. Add 5 ± 0.05 g of NaCl 3. Add 100 ml of methanol/water (80/20). 4. Blend at high speed (~22,000 rpm) for 1 minute. 5. Pour extract into a fluted filter paper and collect extract. 6. Discard the supernatant.
Extract Dilution	1. Pipette 10 ml of filtered extract into a clean vessel. 2. Add 40 ml of purified water and mix well. 3. Filter through 1.5-µm glass microfiber filter into a clean vessel
Column chromatography	1. Pass 10 ml of filtered diluted extract through IAC (1 drop/s) until air comes through. 2. Pass 10 ml of purified water (1–2 drops/s) through the IAC. 3. Repeat previous step until air comes through the IAC. 4. Elute aflatoxins by passing 1 ml of methanol through IAC (1 drop/s or slower) 5. Double dilute the eluate with purified water and transfer to HPLC vial

11. REPEATABILITY, REPRODUCIBILITY, SELECTIVITY AND UNCERTAINTY CALCULATION

Precision of the HPLC-FLD method was considered at two level: repeatability (intra-assay) and intermediate precision (within laboratory). They were expressed as %RSD values.

Chromatographic selectivity of the AFM1, AFB1, AFB2, AFG1 and AFG2 was determined by calculating the peak resolution between adjacent peaks, using the following equation

$$Rs = \frac{RT2 - RT1}{0.85(W2 + W1)}$$

Where, *Rs = peak resolution*

RT2 = retention time of peak 2

RT1 = retention time of peak 1

W1 =width of peak 1 at half peak height

W2 =width of peak 2 at half peak height

Most validation guidelines recommend peak resolution above 1.5 since measuring peak areas accurately become difficult when resolution drops below 1.2.

11.1 Milk

11.1.1 Repeatability and Intermediate precision

Analyte	Day	Repeatability (%RSD)	Intermediate precision (%RSD)
AFM1	1	9.8	10.8
	2	12.9	
AFB1	1	9.6	8.5
	2	5.6	
AFB2	1	9.8	9.5
	2	8.6	
AFG1	1	8.3	8.6
	2	8.8	
AFG2	1	10.0	10.4
	2	11.0	

11.1.2 Selectivity

Peaks	Peak resolution
AFM1-AFG2	1.74
AFG2-AFG1	2.52
AFG1-AFB2	2.51
AFB2-AFB1	3.00

11.1.3 Uncertainty calculation

	AFM1	AFG2	AFG1	AFB2	AFB1
Avg. Conc. Of spiked samples at 0.5 ppb	0.454	0.464	0.495	0.483	0.462
STDEV (spikes samples at 0.5 ppb)	0.048	0.074	0.072	0.045	0.033
Sample Size	8.0	8.0	8.0	8.0	8.0
Standard Uncertainty	0.02	0.03	0.03	0.02	0.01
Degrees of Freedom	7.0	7.0	7.0	7.0	7.0
95% t-Value	2.365	2.365	2.365	2.365	2.365
95% Confidence Deviation	0.04	0.06	0.06	0.04	0.03
95% Confidence Interval (Min)	0.41	0.40	0.43	0.45	0.43
95% Confidence Interval (Max)	0.49	0.53	0.56	0.52	0.49

11.2 Vegetable oil

11.2.1 Repeatability and Intermediate precision

Analyte	Day	Repeatability (%RSD)	Intermediate precision (%RSD)
AFB1	1	8.15	14.8
	2	18.8	
	3	14.8	
AFB2	1	15.1	14.3
	2	10.2	
	3	17.8	
AFG1	1	15.6	14.9
	2	13.7	
	3	15.7	
AFG2	1	18.7	15.4
	2	6.38	
	3	16.0	

11.2.2 Selectivity

Peaks	Peak resolution
AFG2-AFG1	2.45
AFG1-AFB2	2.43
AFB2-AFB1	2.95

11.2.3. Uncertainty calculation

	AFG2	AFG1	AFB2	AFB1
Avg. Conc. Of spiked samples at 2 ppb	0.9	1.9	1.8	1.8
STDEV (spikes samples at 2 ppb)	0.2	0.3	0.3	0.3
Sample Size	9	9	9	9
Standard Uncertainty	0.06	0.09	0.09	0.08
Degrees of Freedom	8	8	8	8
95% t-Value	2.306	2.306	2.306	2.306
95% Confidence Deviation	0.14	0.20	0.21	0.19
95% Confidence Interval (Min)	0.71	1.67	1.63	1.56
95% Confidence Interval (Max)	1.00	2.06	2.05	1.95

11.3 Feed

11.3.1 Repeatability

The precision of the HPLC-FLD method was considered in terms of repeatability. For AFB1, AFB2, AFG1 and AFG2 %RSD values were 3.5%, 3.7%, 5.4% and 2.2%, respectively.

11.3.2. Selectivity

Peaks	**Peak resolution**
AFG2-AFG1	2.45
AFG1-AFB2	2.43
AFB2-AFB1	2.95

11.3.3. Uncertainty calculation

	AFG2	**AFG1**	**AFB2**	**AFB1**
Avg. Conc. of spiked samples at 2 ppb	1.72	1.91	1.50	1.22
STDEV (spikes samples at 2 ppb)	0.0	0.1	0.1	0.0
Sample Size	3.0	3.0	3.0	3.0
Standard Uncertainty	0.02	0.06	0.03	0.02
Degrees of Freedom	2.0	2.0	2.0	2.0
95% t-Value	4.303	4.303	4.303	4.303
95% Confidence Deviation	0.09	0.26	0.14	0.11
95% Confidence Interval (Min)	1.62	1.65	1.36	1.12
95% Confidence Interval (Max)	1.81	2.17	1.64	1.33

12. REFERENCES

AOAC Official Methods of Analysis. (2012). Appendix F: Guidelines for standard method performance requirements.