Available online at www.jpsscientificpublications.com
Volume – 1; Issue - 1; Year – 2015; Page: 73 – 80

Indo – Asian Journal of Multidisciplinary Research (IAJMR)
ISSN: 2454-1370

METHOD VALIDATION AND UNCERTAINTY REPORT FOR THE DETERMINATION AFLATOXINS IN MELON SEEDS

B.F. Ogunbanwo[1], G.O. Adegoke[2], S.A. Denloye[1], G. T. Adebiyi[1], C. Nnaama[1], L. Agholo[1] and C. Nwachukwu[1]

[1]*National Agency for Food and Drugs Administration and Control (NAFDAC)*
[2]*Department of Food Technology, University of Ibadan, Ibadan, Nigeria*.

Abstract

A method for Aflatoxins analysis in melon seeds based on the AOAC method for the extraction of aflatoxins in melon seeds was validated. The scope of validation and the criteria determined included limit of detection, limit of quantization, linearity of calibration, recovery, precision of the method was determined from repeatability and intermediated reproducibility studies. The uncertainty of the method was calculated from the data generated. The Aflatoxins G2, G1, B2 and B1 eluted with retention times of about 4.58, 4.93, 5.89 and 6.37 min with no significant interference for the limited number of samples analyzed. The LOD was determined to be about 0.24 ng AFB1/g, 0.10 ng AFB2/g, 0.38 ng AFG1/g and 0.38 ng AFG2/g with signal to noise ratio at 3 to1. LOQ was estimated to be 0.39 ng/g for AFB1, 0.17 ng/g for AFB2, 0.63 ng/g for AFG1 and 0.63 ng/g for AFG2 using the three times signal noise to peak height for aflatoxins. The precision and the bias of the method was 0.097 and 1.326 respectively while the expanded uncertainty was 0.242.

Key words: Validation, LOD, LOQ, Performance characteristics, Measurement uncertainty, Aflatoxins B1, B2, G1 and G2 and Egusi.

1.Introduction

The quality and comparability of the analytical results generated by laboratories for enforcement, compliance and for the creation of data for risk assessment purposes must be achieved by using quality assurance systems and specifically by applying methods that have been validated according to procedures and that meet defined performance criteria, and ensures traceability (Bratinova *et al*., 2009). The primary purpose of validating a method of analysis is to show that the method is fit for its intended purpose (AOAC, 2002). Validation is the process of demonstrating or confirming the performance characteristics of a method of analysis. The method of analysis is the detailed set of directions, from the preparation of the test sample to the reporting of the results that must be followed exactly for the results to be accepted for the stated purpose. The performance characteristics of a method of analysis are the functional qualities and the statistical measures of the degree of reliability exhibited by the method under specified operating conditions (AOAC, 2002).

Scope of validation: Analysis of aflatoxins in melon seeds using Hitachi high performance liquid chromatography with immunoaffinity clean up column, and fluorescence detector with post column derivatization based on AOAC's method for the

Corresponding author*: **B.F. Ogunbanwo
Tel.: +234-8086758506
E-mail: oluwabamiwo.b@nafdac.gov.ng
Received: 27.02.2015; *Revised*: 14.03.2015;
Accepted: 23.03.2015.

© 2015 Published by JPS Scientific Publications Ltd. All rights reserved

analysis of aflatoxins in peanuts, pistachio and fig paste (AOAC, 2005).

Criteria: The criteria for the validation are: Limit of Detection (LOD), Limit of Quantitation (LOQ), linearity of the calibration, recovery, precision obtained from repeatability and intermediate reproducibility (single laboratory) and the uncertainty of the method which indicates the degree of reliability of using the method.

2. Materials and Methods

2.1. Materials

Egusi was purchased from Oshodi market in Lagos Nigeria, all chemicals used are of HPLC and analytical grade, Aflastar immunoaffinity columns (Romer Labs), aflatoxin reference standard was purchased from Sigma Aldrich and Biopure, Hiachi HPLC with reversed phase columns (Thermo scientific Aclaim 120 and Waters spherisorb), calibrated Satorious analytical balance, thermometers, class A pipettes and volumetric flasks.

2.2. Methods

2.2.1. Sample Preparation

Fifty (± 0.1) g test portions were weighed into 250 ml Erlenmeyer flasks, 5 g of sodium chloride and 300 mL of extraction solution were added. The flasks were stoppered, shake with hand and shaken on a horizontal shaker for 30 minutes. The extract was filtered using a folded filter paper. Ten mL of filtrate was diluted with 60 mL of PBS solution and mixed thoroughly. The extract was cleaned up by applying 50 ml of the solution to the immunoaffinity column, washed with 20 mL water and eluted with 3 mL methanol. 200 µL of the eluate was injected into the chromatographic apparatus. Post column derivatization using electrochemically generated Bromine (Kobra cell) set at 100^{o}A was used to enhance the detection of Aflatoxins B1and G1.

Aflatoxins were separated by isocratic reverse-phase HPLC at 40°C on a reverse-phase column at a flow rate of 1 mL/min. Aflatoxins were eluted in the order G2, G1, B2 and B1 at retention times of approximately 3.1, 3.4, 3.6 and 4.1(short column 4.6 x 15.0 mm) and 4.58, 4.93, 5.89 and 6.37 (long column 4.6 x 250 mm) minutes respectively and were baseline resolved for both columns. A calibration curve was prepared using the working calibrant solutions described in sections I, II, IV and V (AOAC, 2006).

The calibration range was set at 0.1 ng/mL to 3.6 ng/mL. The calibration curve was verified prior to analysis and the plot was checked for linearity. The coefficient of determination (r^2) obtained for the calibration curve was 0.98 which is above the limit $\geq$0.95. Samples of grounded melon seeds were spiked with aflatoxins for recovery studies. The spiking covered the scope of the analytical method and concentration range (0.5 - 10.0 µg/kg).

2.2.2. Peak Identification

Aliquots of the test solutions were injected into the chromatograph using the same conditions used for the preparation of the calibration graph. Aflatoxin peaks of the test solution were identified by comparing the retention times of the peaks from the test solution with that of the calibrant.

2.2.3. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were calculated from the standards prepared in 60 % methanol. LOD was determined as the signal-to-noise ratio of 3:1 and LOQ as three times LOD (n=3-6).

2.2.4/The linearity of the calibration

The linearity of the calibration was evaluated with the regression curve and percent residuals (Figure - 1).

© 2015 Published by JPS Scientific Publications Ltd. All right reserved

2.2.5. Recovery and repeatability

To evaluate recovery and repeatability, spiked samples at a concentration level of 1 µg/kg and 10 µg/kg (n=15) each were prepared from the melon matrix extracted, cleaned up and detected using the HPLC (Table - 2).

2.2.6. Precision

Repeatability and intermediate reproducibility were determined from spiked samples. To calculate the precision of the method, the relative standard deviation is pooled using equation 1

Calculations: (Pooled Relative Standard Deviations) using

$$\text{RSD pooled} = \frac{\sqrt{(RSD1)^2*(n1-1)+(RSD2)^2*(n2-1)+\cdots\ldots}}{(n1-1)+(n2-1)+\cdots\ldots} \quad (1)$$

2.2.7. Trueness (bias) study

Identification and evaluation of other uncertainty contributions not adequately covered by the precision and trueness studies were carried out. The precision was estimated through spiking studies. The spiking studies covered the scope of the analytical method in terms of the typical sample (grounded melon) and concentration range (0.5 - 10.0 µg/kg).

This gave a value for the standard uncertainty due to run to run variation of the overall analytical process. The bias of the method was calculated at 1 ppb, 4 ppb and 5 ppb. The mean recovery was calculated and "t" test was carried out to determine whether or not the mean recovery was significantly different from 1 (100%).

2.2.8. Uncertainty of the analysis

The stages covered in the quantification of measurement uncertainty such as precision, bias and other sources were summed up to give the uncertainty of the method as follows: Uncertainty = precision + bias + other sources (balances, volumetric apparatus, standard purity etc.) precision (intermediate) (Table - 7) was observed over a period of two months with two analysts, using different batches of chemicals and spiked samples. The bias was observed over a period of one week, with recoveries obtained from samples spiked at 1 - 10 ppb.

Other Sources of Uncertainty

Balances/volumetric measuring devices/ environmental temperature

All balances and the important volumetric measuring devices are calibrated by ISO certified calibration laboratory and checked daily with certified weights.

Precision and recovery studies took into account the influence of the calibration of the different volumetric measuring devices, during the investigation various calibrated volumetric flasks and pipettes were used. The purity of the reference standard was obtained from the manufacturer and included in the uncertainty calculations.

Sample homogeneity

Samples were grounded with the Romer Analytical mill recommended by the AOAC method.

Internal quality control

Initial Calibration Verification (ICV) & Continuing Calibration Verification (CCV) were used to check that the response of the analytical process to the analyte was stable. Calibrations prepared with Sigma aflatoxin standards were verified at the middle point by comparison with calibrants prepared from Biopure aflatoxin standards (ICV). Same standard concentrations were used for continuing verification at the end of the run sequence and documented as the CCV. The primary standard used is expected to be within 90 - 110% of the concentration of ICV and CCV, this is a check on the concentration of the standard used for the

© 2015 Published by JPS Scientific Publications Ltd. All right reserved

analysis. It is expected that the primary standard and the ICV must be from two different sources. A condition that must be met during the method validation and continuous use of the method was as stated in Table – 1.

Method Comparison

This was done by comparing the recoveries obtained using the validated method with that obtained using the reference method and performing a "t" test to compare the mean values obtained. The repeatability standard deviation, reproducibility standard deviation and repeatability limit and reproducibility limit were also calculated and compared for the two methods.

Result Calculation

Quantitative determination was carried out by the integration of the peak area. The content of Aflatoxins (B1. B2, G1 and G2) in the test portion was determined in ng/g, from the formula given below:

$$\text{Aflatoxin } \{ng/g\} = k \frac{*\text{Solvent}* (A+PBS)* \text{Final vol.}}{W * \text{Aliquot} * (\text{A Extract})*\text{Elution}} \quad (2)$$

Where:

k = nanogram of Aflatoxin calculated from linear regression (from the calibration curve)

Solvent = extraction solvent (60 mL)

Aliquot = taken from the extract (10mL)

Aliquot + PBS = 70mL

Elution = Volume of methanol for aflatoxin elution from column

Final vol. = final volume achieved after elution from IAC (4.5 mL)

W = sample test portion taken for analysis (10 g)

A Extract = aliquot of the extract passed through the IAC (50 mL)

3. Results and Discussion

Method Performance

The total run time was 5.0 minutes with the aflatoxins eluting in the order G2, G1, B2 and B1 at 3.19, 3.58, 3.90 and 4.459 minutes respectively. The Thermoscientific column (Acclaim 120) gave complete baseline resolution; the waters spherisorb analytical column did not give complete baseline resolution for G1 and B2. The result of performance of the method is as stated below.

Limit of detection and quantitation (LOD and LOQ)

The limit of detection (LOD) was estimated to be 0.24 ng AFB1/g, 0.10 ng AFB2/g, 0.38 ng AFG1/g and 0.38 ng AFG2/g with signal to noise ratio at 3 to1. The limit of quantitation (LOQ) was estimated to be 0.39 ng/g for AFB1, 0.17 ng/g for AFB2, 0.63 ng/g for AFG1 and 0.63 ng/g for AFG2 using the three times signal noise to peak height for aflatoxins.

Recovery

The result of melon samples spiked at 0.5 ppb, 1 ppb, 5 ppb and 10 ppb were within 70% -110 % thus fulfilling the criteria for acceptable recoveries as shown in Table - 2. The repeatability standard deviation, relative standard deviation, and reproducibility standard deviation obtained 4.97 at 1 µg/kg and 11.58 at 10 µg/kg spiking levels were all within acceptable limits of 20% for recoveries within this concentration range (Table - 2). The precision is therefore acceptable for a single laboratory method validation.

Precision

The intermediate reproducibility (precision) result obtained (Figure - 3) was 0.097, was incorporated into the uncertainty of the analysis.

Trueness of the method (Bias study)

Using the result of the recoveries obtained at 0.5, 5.0 and 10ug/kg the overall mean obtained was 91.38% (0.9138) with a standard uncertainty value

© 2015 Published by JPS Scientific Publications Ltd. All right reserved

of 0.065, for n=26 t test result was t = 1.326 and tc = 1.708 (degree of freedom = 25). This indicated that there is no significant difference (t calculated is below t critical) (Table - 4).

Uncertainty of analyzing aflatoxins in melon seeds using AOAC method 999.07 (2005)

The uncertainty of the method is presented was calculated from the intermediate precision of the method (Table - 3) and the bias of the method (Table - 4). The highest source of uncertainty was from the intermediate precision, followed by the bias of the method. The lowest contributor to the uncertainty of the analysis was obtained from the purity of the standard, reference standards are expected to be as close as possible to one hundred per cent purity, with a certificate indicating that the accuracy is traceable. The total expanded uncertainty value obtained was ±24.20% (Figure - 2).

From this expanded uncertainty, the true value of aflatoxin results obtained using the method for the analysis of aflatoxin B1 in melon seeds lies within ± 24.20% of the reported value 95% of the time. When result is to be corrected for recovery, the actual result reported will be Mean ± 24.2%.

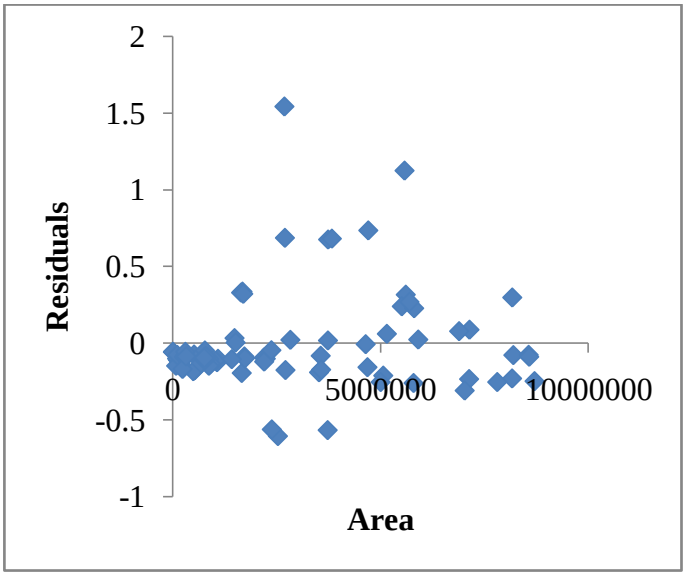
Method Comparison

The result of the data obtained in the method validation (Table - 5) indicated that standard deviation and repeatability limit were 0.14 at 1 ug/kg and 1.44 at 10 ug/kg and reproducibility standard deviation and reproducibility limit were 5.69 at 1 ppb and 10.42 at 10 ppb respectively. Values obtained for the recoveries were between 76.35% and 101.70% and repeatability standard deviations were 0.05 at 1 ppb, 0.51 at 10 ppb with relative standard deviations were 5.69 and 10.42 respectively. The values were within the expected values (> 20) of AOAC acceptable criteria stated in method 999.07 2005.

Statement on fitness for purpose

The method is therefore fit for the intended purpose of analyzing aflatoxins in melon seeds.

Figure - 1: Figure - 1: Residual plot from 12 calibration curves



Criteria for Acceptance of Validation Results

Table - 1: Criteria for method validation and Internal Quality Control

Quality Controls Tests	Expected
System Suitability Checks	Standard should be baseline resolved
Blank sample analysis value	Must be less than the determined LOD
Solvent blank	To demonstrate low system background
Accuracy (Spike sample recovery)	70 - 110%
Initial run sequence with Initial Calibration Verification (ICV)	90 - 110%
Calibration Check Standard (Continuing Calibration Verification (CCV))	90 - 110%
Recovery repeatability (RSD_r)	20 % for RSD_r
Recovery reproducibility (RSD_R)	30 % for RSD_R

© 2015 Published by JPS Scientific Publications Ltd. All right reserved

Table - 2: Recovery study results of samples spiked at 1 and 10ppb (Aflatoxin B1)

The linearity of the calibration	The linearity of the calibration		
	1 ppb (µg/kg)	10 ppb (µg/kg)	Expected range
1	106.00	109.65	70-110%
2	97.20	102.12	
3	96.10	89.41	
4	97.40	100.39	
5	91.50	76.59	
6	91.70	95.01	
7	93.53	77.6	
8		93.67	
Mean	96.21	93.06	
Sd	4.97	11.58	
RSD	0.052	0.12	
Rsd^2	0.0027	0.0155	
n-1	6	7	

n=15

Recoveries were all within the acceptable range of 70-110% while the standard deviations (4.97 and 11.58) are within the expected range of 10-20.

Table – 3: Precision (Intermediate Reproducibility)

Analysts	Conc. {ng/g}	Recovery {%}	Average Recovery (%)	RSD (%)
Anal 1	0.99	99.70	100.58±5.72	5.69
	1.02	101.70		
	0.94	93.53		
	1.07	107.4		
Range	0.94-1.07			
Mean	1.01	100.58		
Anal 2	7.65	76.35	81.28±8.47	10.42
	7.69	76.90		
	9.39	93.94		
	7.79	77.93		
Mean	8.47	81.28		
Range	7.65-9.35			

Precision of the method for n=15

Precision

$$= \frac{\sqrt{(0.0027)^2 * (6) + (0.0155)^2 * (7)}}{(6) + (7)}$$

$= 0.097$

Precision of the method is estimated at 0.0.097

© 2015 Published by JPS Scientific Publications Ltd. All right reserved

Table - 4: Result of Trueness (Bias study)

No. of replicates	% Recoveries at three different Spiking levels		
	1.00 (µg/kg)	5.00 (µg/kg)	10.00 (µg/kg)
1	106.00	60.04	109.65
2	97.20	43.09	102.12
3	96.10	42.74	89.41
4	97.40	48.63	100.39
5	91.50	169.72	95.01
6	91.70	168.72	95.01
7	93.53	47.18	77.60
8			93.67
9			76.52
10			93.15
11			76.90
12			93.94
Mean	96.20	88.62	90.41
Sd	4.97	57.25	11.24
RSD	0.052	0.65	0.12
Overall mean	91.38		
Overall sd	30.83		
Overall rsd	0.34		
R_m	0.9138		
N	26		

R_m = Observed/Expected) spiked amount = 91.38/100 = 0.9138

Standard uncertainty (Ux) = (rsd)/ sqrt(n) = $0.34/\sqrt{26} = 0.065$

At degree of freedom of 25, t = 1.326 and tc=1.7081. No significant difference as t calculated is below t critical. Therefore relative standard uncertainty can be calculated from standard uncertainty and there is no need to correct for recoveries.

Table - 5: Method comparison

Parameter	Values obtained	Expected
		>0.95
Linear calibration range (≥ 0.95)	0.98	
Spiking level	1.0ng/g	10ng/g
Number of analysts	2	2
Number of samples	4	4
Mean values (ng/g)	1.005	9.91
Repeatability standard deviation Sr	0.05	0.51
Relative standard deviation RSD	5.42	5.15
Repeatability limit r{r=2.8*Sr)	0.14	1.44
Recovery %	101	81.3
Intermediate Reproducibility standard deviation Sr	5.69	10.42

When analysis is carried out the absolute difference between two values obtained must not be more than 0.14 µg/kg at about 1 ppb and 1.44 µg/kg at 10 ppb under repeatability conditions.

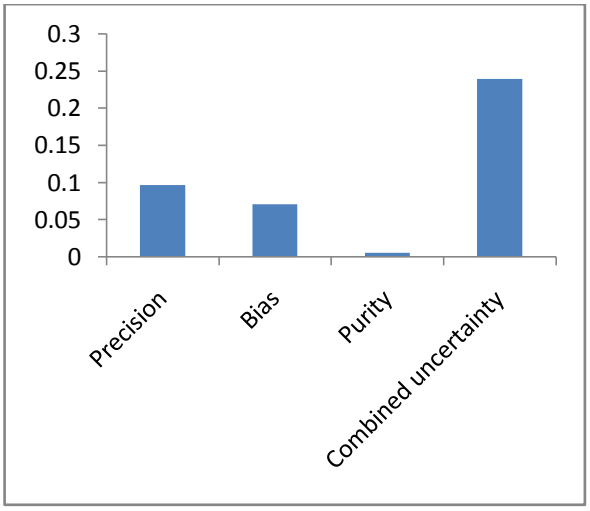


Figure - 2: Histogram of Level of Uncertainty

Combined uncertainty = $\sqrt{(0.097)^2 + (0.071)^2 + (0.006)^2}$ 0.121

Expanded uncertainty of the method = (2* 0.121) = 0.242

© 2015 Published by JPS Scientific Publications Ltd. All right reserved

4. References

1) AOAC (2005). Determination of aflatoxins in rice, using High Performance Liquid Chromatography Method with Immunoaffinity and post column derivatization.
2) Barwick V. J. and Elliso S L R (2000) VAM Project 3.2.1 Development and Harmonisation of Measurement Uncertainty Principles Part (d): Protocol for uncertainty evaluation from validation data
3) Bratinova S., Raffael B., Simoneau C. (2009) Guidelines for performance criteria and validation procedures of analytical methods used in controls of food contact materials.
4) EURACHEM / CITAC Guide Quantifying Uncertainty in Analytical Measurement, Second Edition http://www.measurementuncertainty.org/
5) Harmonized Guidelines for Single Laboratory Validation of Methods of Analysis (IUPAC Technical Report), International Union of Pure and Applied Chemistry Pure Appl. Chem., Vol. 74, No. 5, pp. 835–855.
6) Ng W., Mankotia M., Pantazopoulos P. and Neil R. J. (2005) Method Validation & Uncertainty Report Determination of Ochratoxin A in Cereals and Pasta using Immunoaffinity Column Clean Up and HPLC with Fluorescence Detection.
7) Thienpont L. (2007) Method validation with Confidence Dietmar@sttconsulting.com

© 2015 Published by JPS Scientific Publications Ltd. All right reserved