International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(5): 1920-1925

Received: 07-07-2019
Accepted: 09-08-2019

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Comparative study of immunoaffinity column clean-up and modified quechers using HPLC-FLD with post-column derivatization for determination of aflatoxins in rice

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Abstract
A comparative study was carried out between two different sample extraction techniques including Immunoaffinity column (IAC) clean-up and modified QuEChERS method for determination of Aflatoxins (AFB1, AFB2, AFG1 & AFG2) in rice. These methods were validated using high performance liquid chromatography (HPLC) with post-column derivatization (i.e. bromine derivatization) in a KOBRA® cell using water: methanol (40:60 v/v), with addition of KBr and HNO_3 as mobile phase, followed by fluorescence detection at excitation/emission wavelengths of 360/455 nm. The recoveries of Aflatoxins were in the range of 79.3-112.3% for both the sample extraction techniques, with relative standard deviation (% RSD) lower than 20%. The limits of detection and quantification were found to be 0.25µg/kg and 0.5µg/kg, respectively for both the methods. Both sample extraction techniques performed well, but the proposed modified QuEChERS approach was more time saving, cost-effective and easy for analysis of aflatoxins, while achieving excellent sensitivity.

Keywords: Aflatoxins, method validations, QuEChERS, IAC, HPLC-FLD (KOBRA® cell)

Introduction
Rice is the most important food crop of the developing world and the staple food of more than half of the world's population [1]. Aflatoxins are one of many natural occurring mycotoxins that are found in soils, foods, humans, and animals. Aflatoxins are produced in areas where climatic conditions are favorable to fungal growth. Aflatoxin exposure that can negatively affect human health, food security and economic trade [2]. Aflatoxin contamination is a global phenomenon, but generally crops in tropical and subtropical areas are more susceptible to contamination than those in temperate regions [3].

Aflatoxins are classified by the International Agency for Research on Cancer in 2012 as Group 1 carcinogens (i.e. carcinogenic to humans; IARC, 2014) [4]. The major aflatoxins are B1, B2, G1, and G2, which can poison the body through respiratory, mucous or cutaneous routes, resulting in over activation of the inflammatory response [5]. Aflatoxin B1, the most common one, is considered as one of the most potent carcinogen [6]. Commission Regulation (EU) No 165/2010 published on February 27, 2010 amends Regulation (EC) No 1881/2006 [7], to assure proper consumer protection, which has established their maximum allowable legal limits in the lower µg/kg range (2µg/kg for Aflatoxin B1 and 4µg/kg for total Aflatoxins) for food (Cereals and products derived from cereals).

Determination of aflatoxins concentration in food and feeds stuff is very important. However, due to their low concentration in foods and feedstuff, analytical methods for detection and quantification of aflatoxins have to be specific, sensitive and simple to carry out. There are different techniques of analysis for Aflatoxins. Many methods have been developed for the analysis of aflatoxins, including Thin layer chromatography (TLC), Solid-Liquid extraction (SLE), Solid Phase extraction (SPE), Enzyme linked immunosorbent assay (ELISA), Radio Immunoassay (RIA), Immunoaffinity chromatography, High performance liquid chromatography (HPLC) and Liquid Chromatography Mass Spectrometry (LC/MS/MS). Each of these methods has several advantages and limitations in aflatoxins analysis. In general, HPLC-FLD and HPLC-MS/MS represent the most widespread analytical techniques for quantitative purpose and also offer significant advantages over other techniques since they provide good sensitivity and detection of trace level of toxins [8-11].

Furthermore, selection of an appropriate sample extraction technique is a crucial step for the analysis of Aflatoxins from the sample for qualitative and quantitative determination. The common technique is immunoaffinity column (IAC) clean-up [12]. It is based on the principle of binding between an antibody and antigen to separate and purify the aflatoxins from the matrices. A key component of this approach is the immunoaffinity sample-preparation column containing a gel suspension of monoclonal antibody covalently attached to a solid support [13]. The aflatoxins are recovered using methanol which breaks the bond between the antibody and the aflatoxins. These methods have some disadvantages including use of large amount of organic solvents, long time consumption and expensive SPE cartridges and IAC columns [14] A disadvantage is the slow constant column flow rate, which is tedious when carried out manually and can be a source of variable recoveries when not properly controlled (Patey *et al*., 1991) [15]. The other technique used is less intensive and straight forward sample preparation technique, such as QuEChERS (quick, easy, cheap, effective, rugged and safe). Due to great flexibility of QuEChERS, different modification may be introduced to the procedure to ensure its robustness, even for residue analysis in complex matrixes [16]. The advantage of this method is that it is simple and reduces time consumption [17].

Aflatoxins fluoresce naturally under UV light (i.e. B's fluoresce blue and G's fluoresce green) with the subscripts relating to their relative chromatographic mobility. Aflatoxin B1 and G1 do not fluoresce naturally at high degree and must be derivatized using iodine or bromine. During derivatization the chemical structures of Aflatoxins B1 and G1 are changed to a more fluorescent form, increasing the fluorescent signal in each case for detection by HPLC. Several chromatographic methods based on RP-HPLC and fluorescence detection (FLD) with chemical pre- or post-column derivatization of analytes has been developed [18].

The aim of this study is focused on the comparison of two different sample extraction techniques including Immunoaffinity column (IAC) clean-up and modified QuEChERS for determination of Aflatoxins (AFB1, AFB2, AFG1 and AFG2) in rice. The proposed modified QuEChERS method involves solvent extraction using acetonitrile, followed by a salting-out step of the analytes into the acetonitrile phase and then a purification based on a quick dispersive SPE. The detection of aflatoxins (AFB1, AFB2, AFG1, AFG2) for both extraction techniques is done by post column derivatization method of bromination by an electrochemical cell (KOBRA® cell) with potassium bromide dissolved in an acidified mobile phase for determination of aflatoxins with high accuracy and excellent sensitivity. The method aims for a fast, environmentally friendly, and low cost detection of Aflatoxins in rice.

Materials and Methods

Chemicals and reagents

Reference standards of four solid Aflatoxin standards (B1, B2, G1 and G2) were purchased from Sigma. Acetonitrile (Qualigens) and Methanol (S d fine) used were of HPLC-grade. Deionized water (Millipore-Advantage A10) and phosphate-buffered saline (pH 7.4) were used for the preparation of standard and sample solutions. Easi-Extract® Aflatoxin columns were used for Immunoaffinity column clean-up. Magnesium sulfate heptahydrate (Agilent), sodium chloride (Merck), Sodium citrate dibasic sesquihydrate (Sigma-Aldrich), Sodium citrate tribasic dihydrate (Sigma-Aldrich), Carbon 18 (Agilent), Potassium bromide (Rankem) and Nitric acid (Fischer Chemical) were used in the present study.

Preparation of stock and working standard solutions

Standard stock solutions of all four aflatoxins (B1, B2, G1 and G2) of concentration 500mg/kg were prepared individually in methanol. Mixed working standard solutions of aflatoxins were prepared from stock standard solutions in in the desired linearity range of 0.5-10µg/kg using 50% methanol in case of Immunoaffinity Column (IAC) clean-up method and 50% acetonitrile in case of modified QuEChERS method. Prepared Stock and working standard solutions of Aflatoxins were stored in cool and dark place.

Extraction procedure

Organic rice was purchased from the market and ground into powder for homogenization.

Immunoaffinity column clean-up

About 5g of the prepared sample was weighed into 50 mL capacity PTFE centrifuge tube and extracted with 20mL of 80% methanol (v/v) followed by the addition of 1.2g NaCl. The above solution was vigorously shaken for 2-5 minutes on vortex followed by centrifugation @4000rpm for 10 minutes. 2mL of the supernatant was diluted to 20mL with PBS buffer and loaded onto IAC column. Washing was given with 20mL PBS Buffer and elution was done with 1mL methanol & then 1mL of water prior to injection on HPLC.

Modified quechers method

About 2g of the sample was weighed into 50mL capacity PTFE centrifuge tube and soaked in 10mL of deionized water for 30 minutes to improve the extraction efficiency. Sample was extracted with 10mL of acetonitrile and a salt mixture (4g anhydrous MgSO4, 1g NaCl, 1g sodium citrate dibasic sesquihydrate and 0.5g of sodium citrate tribasic dehydrate) for 10 minutes with vigorous shaking using multitube vortexer followed by centrifugation at 5000rpm for 10 minutes. Dispersive clean-up was given to 5mL of supernatant organic layer with 900 mg of anhydrous $MgSO_4$ and 300 mg of C18 in another 15mL capacity PTFE centrifuge tube followed by vortex for 2 minutes and centrifugation at 10000rpm for 5 minutes. Solution was passed through 0.45µm PTFE membrane filter and 1mL of the aliquot was evaporated to dryness under a stream of nitrogen gas. Final volume was reconstituted in 1mL of 1:1 ratio of ACN: Water (v/v) prior to injection on HPLC.

Instrumentation and conditions

Analysis was carried out on HPLC-Agilent 1200 Technologies system equipped with a fluorescence detector and a Prominence autosampler was used for the analysis. R-Biopharm electrochemical derivatization kit, including KOBRA® cell, was employed for derivatization. The HPLC operating conditions are mentioned in Table 1.

Table-1: HPLC Operating Conditions

Derivatization	KOBRA® Cell at 100µA Setting
Column	Zorbax Eclipse Plus C18 (4.6mm x 150mm); 5µm
Column Temperature	40 °C
Flow rate	1mL/min
Mobile Phase (60:40) Isocratic	Water : MeOH (Containing 119 mg KBr and 350 µL 4M HNO3)
Flow Rate	1mL/min.
Detection (λ)	Ex: 360nm, Em: 455nm
Injection	20µL
Elution Order	G2, G1, B2, B1

Results

Method validation

Both the Immunoaffinity column (IAC) and QuEChERS extraction techniques were evaluated for their performance characteristics based on the validation parameters such as Specificity, Linearity, Accuracy and Precision (Repeatability and Ruggedness).

Specificity

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that are expected to be present in the sample matrix. Chromatograms of blank and spiked samples analyzed for aflatoxins, ensured that there were no interferences in the retention time of the target analyte.

Linearity

Linearity indicates the ability to produce results that are directly proportional to the concentration of the analyte in sample. In this study, five concentration levels of aflatoxin standard solutions ranging from 0.5 to 10.0 µg/kg were analyzed to evaluate the linearity of the calibration curves by plotting the peak areas which were used as the analytical signal response versus concentration. The calibration curves obtained were linear within the range and showed good regressions (correlation coefficients (r^2) > 0.99). The linearity data for both the methods is shown in Table 2 whereas Linearity graphs are shown in Figure 2 and 3.

Table 2: Linearity data for Aflatoxins: B1, B2, G1 and G2 in rice

Conc.	Area (IAC)				Area (QuEChERS)			
(µg/kg)	AFB1	AFB2	AFG1	AFG2	AFB1	AFB2	AFG1	AFG2
0.5	150492	92744	133166	162137	95542	81189	117107	162707
1.0	269758	183193	255677	312310	170924	144367	179882	277313
2.0	551113	370329	494739	652294	389264	291927	396478	603355
5.0	1411000	940579	1265080	1600117	955587	716958	931030	1328035
10.0	2628925	1772616	2391733	2924408	1866197	1430818	1764309	2477647

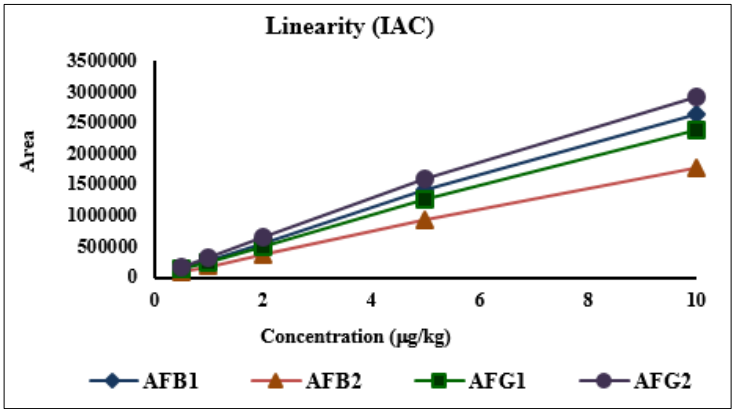

Fig 2: Linearity graph of Aflatoxins by IAC

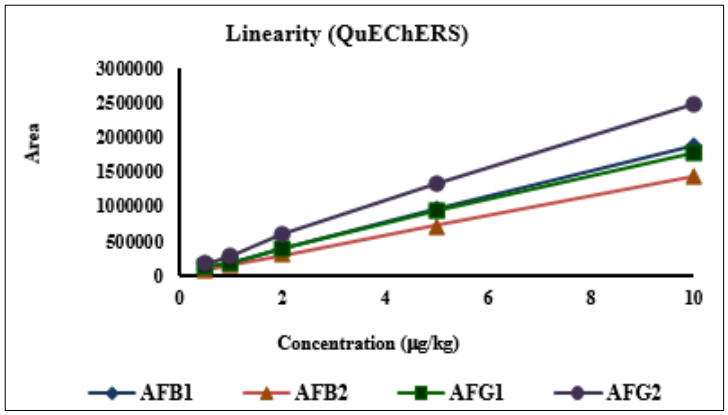

Fig 3: Linearity graph of Aflatoxins by QuEChERS

Limit of detection (LOD) and limit of quantification (LOQ)

The sensitivity of the method was expressed as LOD and LOQ. The limits of detection (LOD) and quantification (LOQ) were found by adding decreasing concentrations of standard solution containing the four aflatoxins in the samples, and then subjected to extraction and quantification, up to the lowest detectable concentration (LOD) and the lowest quantifiable concentration (LOQ), under suitable conditions of repeatability [19] (n = 5, RSD < 20%). The limits of detection and quantification found were 0.25µg/kg and 0.5µg/kg, respectively for both the extraction methods.

Accuracy

Typically, accuracy is represented and determined by recovery studies. The accuracy of the extraction techniques used was evaluated by spiking the samples at three fortification levels i.e. 0.5, 1.0, and 2.5 µg/kg with 6 replicates at each level. The relative standard deviation (% RSD) for all spiked levels were found lower than 20%. The recovery data for both the methods is as shown in Table 3.

Table 3: Recovery data for Aflatoxins: B1, B2, G1 and G2 in rice

Compound name	Fortification Level (µg/kg)	Mean Recovery (µg/kg)		% Recovery ± SD	
		IAC	QuEChERS	IAC	QuEChERS
AFB1	0.5	0.56	0.54	112.3 ± 5.0	107.8 ± 7.5
	1.0	1.06	1.08	106.5 ± 2.5	107.6 ± 6.1
	2.5	2.29	2.35	91.6 ± 4.4	94.0 ± 2.6
AFB2	0.5	0.53	0.54	105.9 ± 6.9	108.0 ± 3.5
	1.0	0.84	1.07	83.9 ± 4.9	107.3 ± 6.4
	2.5	1.98	2.22	79.3 ± 4.4	88.7 ± 3.9
AFG1	0.5	0.53	0.53	106.9 ± 7.0	106.4 ± 5.0
	1.0	0.93	1.01	92.6 ± 3.1	101.4 ± 6.4
	2.5	1.99	2.24	79.5 ± 2.0	89.5 ± 2.8
AFG2	0.5	0.49	0.49	98.6 ± 5.8	98.6 ± 5.0
	1.0	0.93	0.87	92.8 ± 3.1	87.1 ± 4.6
	2.5	2.00	2.00	80.0 ± 2.1	79.8 ± 1.1

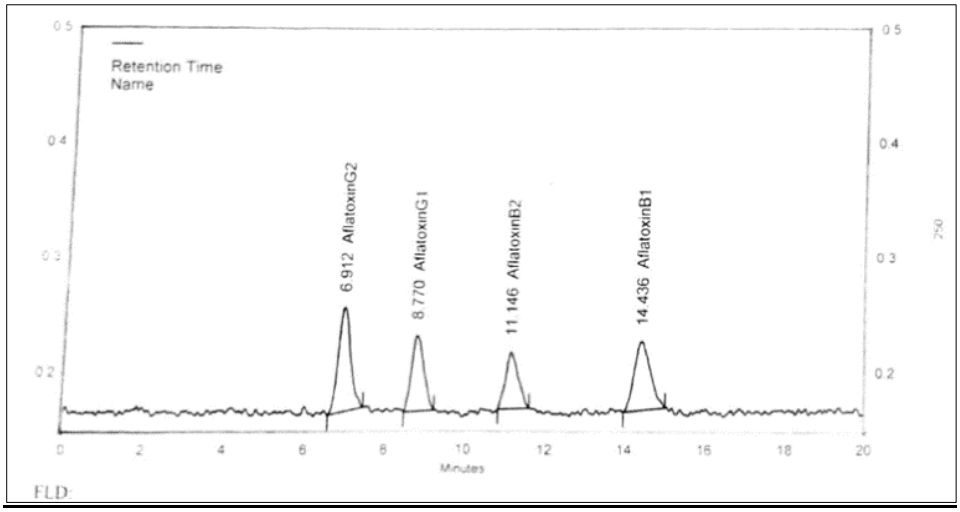

Fig 4: HPLC Chromatogram (IAC) of Organic Rice spiked with Aflatoxins at 1.0 µg/kg

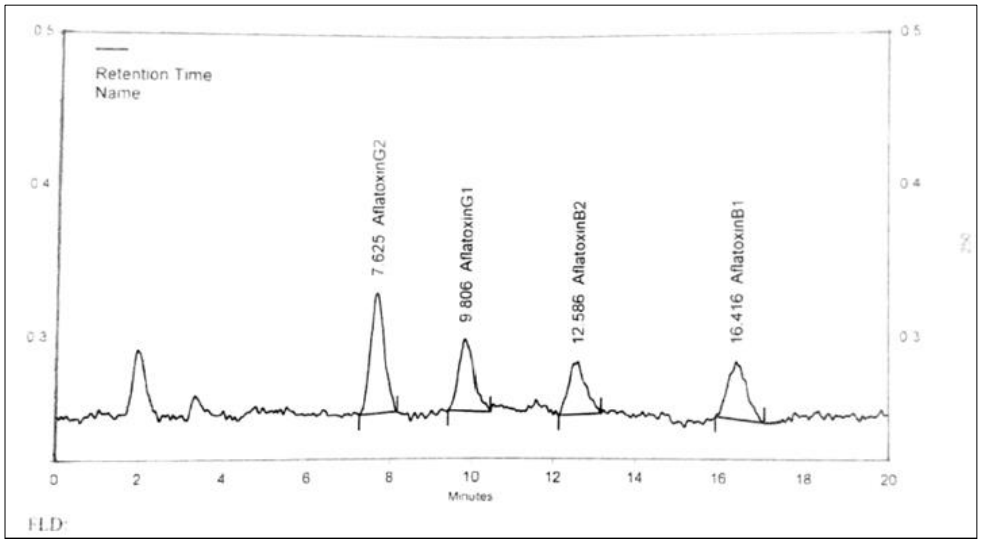

Fig 5: HPLC Chromatogram (QuEChERS) of Organic Rice spiked with Aflatoxins at 1.0 µg/kg

Precision

Precision in terms of repeatability (Intra-day precision) and ruggedness (Intermediate precision) was evaluated at three concentration levels i.e. 0.5, 1.0 and 2.5µg/kg with six replicates at each level. The acceptance criterion found was within 20% relative standard deviation (% RSD). The repeatability and ruggedness data for both the methods are shown in Table 4 and 5.

Table 4: Repeatability data for Aflatoxins: B1, B2, G1 and G2 in rice

Compound name	Fortification Level (µg/kg)	Mean Recovery (µg/kg)		% Recovery ± SD	
		IAC	QuEChERS	IAC	QuEChERS
AFB1	0.5	0.57	0.51	114.0 ± 3.5	102.8 ± 6.1
	1.0	1.06	1.05	106.3 ± 4.9	105.4 ± 4.7
	2.5	2.43	2.18	97.1 ± 4.0	87.3 ± 2.7
AFB2	0.5	0.52	0.55	105.0 ± 7.2	109.2 ± 3.5
	1.0	0.95	1.00	94.6 ± 5.8	99.7 ± 4.7
	2.5	2.13	2.14	85.3 ± 2.0	85.5 ± 1.1
AFG1	0.5	0.53	0.56	106.2 ± 7.3	111.4 ± 2.7
	1.0	0.9	1.05	90.4 ± 3.2	104.9 ± 4.9
	2.5	2.02	2.09	80.8 ± 2.8	83.4 ± 2.0
AFG2	0.5	0.51	0.49	102.1 ± 5.8	97.1 ± 2.7
	1.0	0.9	0.94	90.2 ± 2.3	93.5 ± 1.1
	2.5	2.06	2.06	82.3 ± 2.8	82.4 ± 0.8

Table 5: Ruggedness data for Aflatoxins: B1, B2, G1 and G2 in rice

Compound name	Fortification Level (µg/kg)	Mean Recovery (µg/kg)		% Recovery ± SD	
		IAC	QuEChERS	IAC	QuEChERS
AFB1	0.5	0.55	0.53	111.0 ± 6.3	106.1 ± 2.3
	1.0	0.99	0.89	98.5 ± 3.8	88.9 ± 6.7
	2.5	2.42	2.05	96.8 ± 3.3	82.2 ± 1.2
AFB2	0.5	0.53	0.56	105.2 ± 4.7	111.0 ± 6.1
	1.0	0.89	1.04	89.1 ± 4.9	104.4 ± 6.5
	2.5	2.14	2.08	85.5 ± 1.8	83.0 ± 2.0
AFG1	0.5	0.49	0.54	98.4 ± 5.6	108.4 ± 4.2
	1.0	0.83	0.99	83.2 ± 6.4	99.0 ± 6.3
	2.5	1.99	2.12	79.5 ± 3.1	84.7 ± 1.4
AFG2	0.5	0.51	0.54	102.2 ± 3.0	108.9 ± 4.4
	1.0	0.86	0.9	85.8 ± 2.3	90.2 ± 3.4
	2.5	2.04	2.03	81.6 ± 1.6	81.2 ± 1.0

Discussion

For the determination of aflatoxins, the sample extraction (pretreatment) has a crucial impact on the accuracy of the results, especially when complex matrices such as cereals and nuts are analyzed for the very low levels of aflatoxins [20]. In the present work, we have compared the two extraction techniques i.e. Immunoaffinity column clean-up and modified QuEChERS technique for determination and quantification of aflatoxins for future application. Good analytical results were obtained, including good linearity, specificity, accuracy and precision (Intra and Inter day). The analytical limits (LOD and LOQ) for both the methods were same i.e. 0.25µg/kg and 0.5µg/kg, respectively. Both the sample extraction methods were performed well with average recovery values in the range of 91.6-112.3% for AFB1, 79.3-108.0% for AFB2, 79.5-106.9% for AFG1 and 79.8-98.6% for AFG2.

The detection of aflatoxins by HPLC-FLD with post column derivatization by bromination in a KOBRA™ cell achieved lower MRLs than the limits of EU for all four aflatoxins in rice. Also when bromine post-column derivatization was used, no background peaks were observed in rice samples.

Conclusion

In this comparative study of two different sample extraction techniques, Immunoaffinity column (IAC) clean-up and modified QuEChERS, results completely fulfilled the performance criteria fixed by Regulation (EC) 1881/2006 of the Commission of the European Union. In recent years, the use of IACs in the cleanup step of aflatoxin analysis provides a number of advantages over the conventional chemical methods but to reduce the sample handling and contaminant waste, modified QuEChERS method is recommended as an alternative to the expensive and time-consuming method of Immunoaffinity columns for the extraction of aflatoxins in rice. The recommended modified QuEChERS method allows simple, fast, easy, inexpensive and rapid determination of aflatoxins compared to Immunoaffinity column clean-up, using HPLC-FLD achieving higher sensitivity and selectivity with post-column derivatization by bromination in KOBRA™ cell. Moreover, the process of concentrating and re-dissolving in suitable solvent increases the sensitivity of the method making the modified QuEChERS to be the method of choice for routine extraction of aflatoxins.

Acknowledgement

The authors are thankful to the Department of Analytical Science Division, Shriram Institute for Industrial Research, Delhi for providing the facilities for this research work.

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