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Detection of aflatoxins in meat by modified HPLC method

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Abstract

An analytical method for the determination of aflatoxins in meat was developed. Samples prepared by an extraction with dichloromethane followed by a solid phase cleanup and then fluorescence detection. Separation of the four aflatoxins(B1,G1,B2 and G2) with higher selectivity and sensitivity, and within reasonable limits of retention time, was performed. The isocratic mobile phase of water/methanol/acetonitrile (60/20/20) was used at a column temperature of 30C,with flow rate 1ml/minute and injection volume 20µl. Optimization of samples preparation and analytical conditions gave recoveries in the range of 80–90 % at spike levels of 5-40 ug/kg, and relative standard deviations (RSDs) lower than 1% in all the cases. Additionally, the limit of detection (LOD) (5ug/kg) and the limit of quantification (LOQ) (10 ug/kg) of these aflatoxins are within the legal limits set by the European Union **(EU,2006).**

Key words: Aflatoxins, validation,HPLC.

Introduction

Mycotoxins are natural secondary metabolism products of mould fungi, which are part of the foodstuff contaminants as a result of their toxic effect **(Dance et al.,2003**). The worldwide contamination of foods and feeds with mycotoxins is a significant problem. Aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins, and ergot alkaloids are the mycotoxins of greatest agro-economic importance. Some molds are capable of producing more than one mycotoxin and some mycotoxins are produced by more than one fungal species. Often more than one mycotoxin is found on a contaminated substrate. Mycotoxins occur more frequently in areas with a hot and humid climate, favourable for the growth of molds, they can also be found in temperate zones. Exposure to mycotoxins is mostly by ingestion, but also occurs by the dermal and inhalation routes. The diseases caused by exposure to mycotoxins are known as mycotoxicoses. However, mycotoxicoses often remain unrecognized by medical professionals, except when large numbers of people are involved. Factors influencing the presence of mycotoxins in foods or feeds include environmental conditions related to storage that can be controlled. Other extrinsic factors such as climate or intrinsic factors such as fungal strain specificity, strain

variation, and instability of toxigenic properties are more difficult to control. Mycotoxins have various acute and chronic effects on humans and animals (especially monogastrics) depending on species and susceptibility of an animal within a species. The economic impact of mycotoxins include loss of human and animal life, increased health care and veterinary care costs, reduced livestock production, disposal of contaminated foods and feeds, and investment in research and applications to reduce severity of the mycotoxin problem. Although efforts have continued internationally to set guidelines to control mycotoxins, practical measures have not been adequately implemented**.(Zain.2011)**

Aflatoxins are a group of highly toxic and carcinogenic substances, which occur naturally, and can be found in food substances. They are produced mostly by the fungal species *Aspergillus flavus, A. parasiticus*, and the less common *A. nomius*. Out of 20 aflatoxin types so far identified, only aflatoxin B1, B2, G1, and G2 are known to occur naturally, the B and G classes depicting the blue and green fluorescence emitted by their metabolites under ultraviolet (UV) light, and the sub-type 1 and 2 refers to the major and minor compounds respectively. *A. flavus* produces the type B aflatoxin only, while the other two fungal species produce both types of aflatoxins B and G. Aflatoxins occur naturally in food substances such as cereals, oilseeds and spices, as well as in animal feeds, and can colonize these substances either during the growth period or in storage. It remains a formidable task to accurately determine and identify these toxins in food substances at the trace concentration levels, owing in part to their complex sample matrix. **(Tav_car-Kalcher et al., 2007)**

Aflatoxin contamination has been linked to increased mortality in farm animals and thus significantly lowers the value of grains as an animal feed and as an export commodity. Milk products can also serve as an indirect source of aflatoxin. When cows consume aflatoxin-contaminated feeds, they metabolically biotransform aflatoxin B1 into a hydroxylated form called aflatoxin M1 **(Van Egmond, 1989)**. Aflatoxin is associated with both toxicity and carcinogenicity in human and animal populations. The diseases caused by aflatoxin consumption are loosely called aflatoxicoses. Acute aflatoxicosis results in death; chronic aflatoxicosis results in cancer, immune suppression, and other "slow" pathological conditions. The liver is the primary target organ, with liver damage occurring when poultry, fish, rodents, and nonhuman primates are fed aflatoxin B1. There are substantial differences in species susceptibility. Moreover, within a given species, the magnitude of the response is influenced by age, sex, weight, diet, exposure to infectious agents, and the presence of other mycotoxins and pharmacologically active substances. Thousands of studies on aflatoxin toxicity have been conducted, mostly concerning laboratory models or agriculturally important species **(Cullen and Newberne, 1994).**

This study aims at developing a reliable technique to test the presence of aflatoxins in meat. The main objective of this technique is the extraction of meat samples using HPLC-FLD analysis with derivatization.

Material and Method

Chemicals and materials

Total aflatoxins standards were purchased from Sigma (St. Louis, MO, USA) with purity of 99%; standard stock solutions were prepared in acetonitrile according to the Association of Official Analytical Chemists (AOAC) method (IARC, 2002). A working solution prepared in acetonitrile and stored at -20 0C in amber glass vials over a period of 12 months. The external standard solutions used for the calibration curve for the HPLC experiments were prepared by further dilution of the working solution with acetonitrile. HPLC-grade acetonitrile and methanol used for the mobile phase were purchased from Merck (Darmstadt, Germany), whereas analytical grade acetonitrile , methanol and dichloromethane,hexane,acetone and petroleum ether used for extraction were purchased from Fischer scientific (Leicestershire, UK).Water: De-ionized water or HPLC grade water A 0.45 mm disposable membrane filter was purchased from Cronus Filter (UK). A monohydrous citric acid was purchased from Merck (Darmstadt, Germany).

HPLC analysis

Agilent Series 1050 quaternary gradient pump, Series 1050 auto sampler, Series 1050 FLD detector, and HPLC 2D Chemstation software (Hewlett-Packard, Les Ulis, France). The chromatographic separation was performed with a reversed-phase column (Extend-C18, Zorbax column, 4.6 mm i.d., 250 mm, 5 µm, Agilent Co.). The meat extracts were analyzed isocratically using 60:20:20 water/methanol/acetonitrile mixture as the mobile phase. The column temperature adjusted at 30 0C at a flow rate of 1.0 mL/min to achieve the optimum resolution of the aflatoxins. The injection volume was maintained at 20 µL for both the sample and standard solutions.

Sample preparation

Sample recovery was investigated with 2 g of the blank meat samples with four different fortification levels; 0.5 mL of aflatoxin mixed standards were spiked at 10 ,20 and 40 µg/kg of the standard mix. The spiked samples were left for 30 minutes in the dark at room temperature then start the extraction process.

Add 200 µl of 20% citric acid,mixed well then 4 ml of dichloromethane were added ,shaked well on a shaker for about 30 minutes. The mixture was filtered by whatmann filter paper and the filterated materials were evaporated by nitogen evaporator at 40 ^{0}C.The dried

matrix was eluted by 1ml hexane then SPE(Solid Phase Extraction) step(in the original method ,packing the column with silica gel). 2ml hexane,6 ml petroleum ether and 12 ml acetonitril were added to SPE cartridge for conditioning it, then apply the sample extract. The elution by 5ml of dichloromethane: acetone in the ratio (4:1).Aflatoxins were obtained after evaporation of the organic solvent and start the derivatization step.**(Kalantari et al.,1999)** in which no derivatisation step and detection was by UV detector at 244nm.

Derivatization: Add 200 µl of hexane on dried extract of aflatoxins,50 µl TFA(trifloroacetic acid) then vortex for 1 minute, add 1.95ml of acetonitrile:water in ratio(1:9) ,vortex for 5 minutes then wait for 5 minutes for separation the solvents to two layers. Take the upper layer and filtered by acrodisc filter 0.45µm into HPLC vials. Inject 20µl into HPLC.(**AOAC;1995).**

Optimized Validated Method

High performance liquid chromatography method with multiple solvents, was used for separation, and quantification of the selected aflatoxins and internal standard, and during each injection analysis, the in-line regeneration of the column was applied. The optimization process was conducted to have a full simultaneous determination of the analytes and the internal standard, by parallel recording of the FLD signals for compound,which was excitation at 360nm and emission at 440 nm. The optimized method contains a very short input with organic solvents to reduce the retention time for the analyte, all these till the limit of stable and reproducible results for good resolution of sharp peaks

Selection of elution mode

Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also better performing in terms of efficiency, stability and reproducibility. Here, C18 250 × 4.6 mm column of 25µm particle packing was selected for separation of total Aflatoxins. Isocratic mode was chosen due to simplicity in application and robustness with respect to longer column stability.

The mobile phase should be sufficiently transparent at the wavelength of detection. The mobile phase was selected on the basis of best separation, peak purity index, peak symmetry, theoretical plate . So, numbers of trial were taken(such as water:acetonitrile:methanol(30:45:25)according to.**(Kalantari et al.,1999),**Also;with other percentage(65:10:25) according to**(Sirhan et al.,2011)**. After number of trial Water: Acetonitrile: Methanol (60:20:20) was selected.

Results and discussion:

Retention times of the analytes are shown on the recorded chromatograms, and they were: for aflatoxin B1 tR =5.1 min, for aflatoxin G1 tR =6.379 min , for aflatoxin B2 tR =

9.269 min and for aflatoxin G2 tR =12.604 min. The lower limits of quantification LOQ for total aflatoxins was 10 ug/kg. The precision of this method determined as RSD≤ 1%. The recovery was ranged from 80% to 90%.

VALIDATION APPROACH

Validation of analytical method shall be done to establish by laboratory studies, that the performance of the method meet the requirement for the intended analytical application. The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines.

Specificity:

Specificity of an analytical method is ability to measure specifically the analyte of interest without interferences from blank and spiked samples.**(Figure2)**

Check for interference from blank: Diluent was used as blank. Standard and sample were prepared as per test procedure. Check for the interference of blank and peaks with the analyte peak and calculate % interference of blank peaks interferes with analyte peak against the standard peak area.

Linearity:

The linearity for total Aflatoxins were assessed by analysis of spiked samples in range of 10-40 µg/Kg respectively, in term of slope, intercept and correlation co-efficient value. The graph of peak area obtained verses respective concentration was plotted.The LOQs for all aflatoxins are lower than 20µg/kg, which is below the maximum permitted level for aflatoxins under the US food safety regulations include a limit of 20 µg/kg for total aflatoxins (B1, B2, G1 and G2) in all foods except milk and a limit of 0.5 µg/kg for M1 in milk. Higher limits apply in animal feeds**.(Lawley,2013)**

Precision:

Method precision for assay was established by determining the assay of seven sample preparations under same conditions. Three replicates of sample were prepared at sample concentration by one analyst and analyzed on same day. Sample concentration by one analyst and analyzed on same day. Intraday precision was performed by standard five times and measuring the area of drugs at same day with time interval. Inter day precision was performed by standard Five times and measuring the area of drugs at different day interval.(Table 2,3,4).

Accuracy:

Accuracy was determined over the range of 50%, 100% and 200% of the sample concentration. Calculated amount of total Aflatoxins was added in spiked sample to attain 50%, 100% and 200% of sample concentration. Each spiked sample was prepared in triplicate at each level and injected. The chromatograms were recorded and from the peak area of drug, % recovery was calculated from regression equation as shown in (Table 5).

Limit of Detection (LOD) and Limit of Quantification (LOQ):

The limit of Detection (LOD) and limit of Quantification (LOQ) of the developed method for total Aflatoxins were determined by injecting progressively known concentrations of the standard solutions using the developed HPLC method. The LOD is the smallest concentration of the analyte that gives a measurable response at signal to noise ratio of 3:1 and 10:1, respectively**. (Indian Pharmacopeia,2007)**

RESULTS AND DISCUSSION

RP-HPLC method developed for simultaneously estimation of total Aflatoxins in Injection Dose. Developed RP-HPLC method was validated according to ICH guideline. RP-HPLC method has shown adequate separation for of total Aflatoxins. Separation was achieved on Inertsil C18 (250 x 4.6mm) 5 µm column by using: Water: Acetonitrile :Methanol (60:20:20) as a mobile phase at a flow rate of 1.0 mL/min, and FLD detection was carried out at excitation at 360nm and emission at 440 nm.**(Figure1).**

In the present study the specificity of the method was determined by assessing interference from the spiked samples & diluents. There were no other co eluting, interfering peaks from excipients, impurities found and the method was specific for estimation of total Aflatoxins.

The method was validated in terms of linearity, precision, accuracy, specificity. The linearity of the proposed method was investigated in the range of 10-40 µg/L of test concentration for total Aflatoxins. Accuracy was determined by recovery study & it was found to be 101.3167. The mean assay (n=3) was 93.95833. The percentage RSD value for the five assay values was 0. 0467.

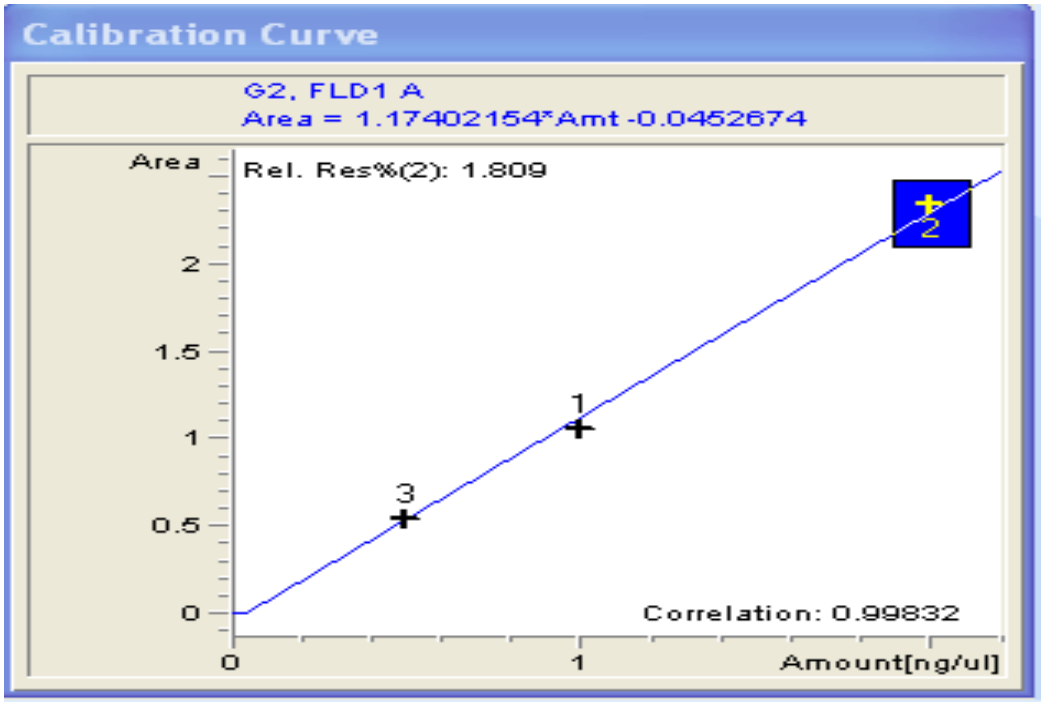

Figure 1: Calibration Curve of Total aflatoxins:

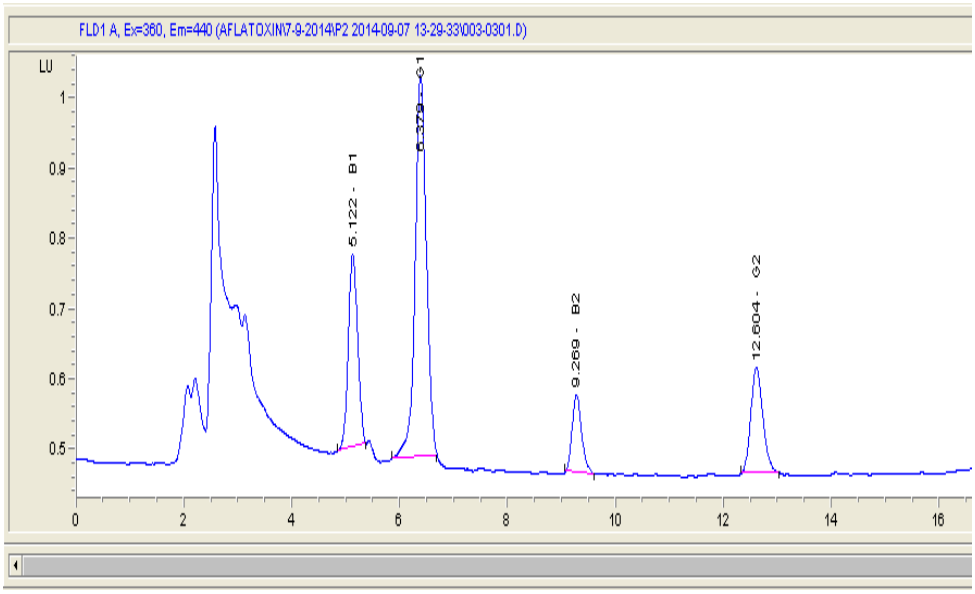

Figure 2:Chromatogram of total aflatoxins concentration (40µg/kg):

Table (1): series a Linearity data for Total Aflatoxins:

Serial No.	Concentration(µg/kg)	Concentration readings(n=3)
1	10	10.13±0.3
2	20	17.46±0.65
3	40	37.5±0.82

Table (2): Intraday precision data for estimation of total Aflatoxins:

Serial No.	Concentration(µg/kg)	Concentration readings(n=3)	RSD%
1	10	10.13±0.3	0.032
2	20	17.46±0.65	0.03725
3	40	37.5±0.82	0.022

Table (3): Interday precision data for estimation of Total Aflatoxins:

Serial No.	Concentration(µg/kg)	Concentration readings(n=3)	RSD%
1	10	9.48±0.47	0.049
2	20	17.9±0.46	0.0256
3	40	38.05±1.2	0.03

Table(4): Repeatability data for Total Aflatoxins:

Conc.(µg/kg)	Concentration readings	Conc.Mean±SD(n=5)	RSD%
10	10.5	9.79±0.56	0.058
	9.89		
	10.005		
	9.58		
	8.97		

Table 5: Accuracy data for Total Aflatoxins:

Conc.level	Amount spiked	Amount recovered	Recovery%	%Mean Recovery±SD
50	10	10.5	105	101.32±3.24
	10	9.89	98.9	
	10	10.005	100.05	
100	20	16.8	84	87.33±3.25
	20	18.1	90.5	
	20	17.5	87.5	
200	40	38.45	96.13	93.75±2.06
	40	36.99	92.48	
	40	37.06	92.65	

Conclusion:

The validated method showed that a simple High Performance liquid chromatograph as modular system is capable to handle such a complex simultaneous determination of

mycotoxins, and the quantification limits, although a FLD detector for multiple channels was used, have reached the GLP(Good Laboratory Practice) requirements. The procedure for method optimization included the liquid-liquid extraction process and is settled longer to allow more samples to be analyzed and to check the status of the column after the washing stage for each injection. The excipients of the commercial sample analyzed did not interfere in the analysis, which proved the specificity of the method for these drugs. The proposed method involves direct quantification of both the components. By adopting an isocratic mobile phase of water/methanol/acetonitrile (60:20:20) at a column temperature of 30 ^{0}C, the separation of the four (4) aflatoxins was possible with higher selectivity and sensitivity in an adequate retention time (for aflatoxin B1 tR =5.1 min, for aflatoxin G1 tR =6.379 min , for aflatoxin B2 tR = 9.269 min and for aflatoxin G2

TR =12.604 min). In addition, it was found that the wavelength setting at 360 nm excitation and 440 nm emission combination could be used as the optimum wavelength for all aflatoxins. Hence, the developed HPLC method can be conveniently adopted for the routine quality control analysis in the combination formulation.

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