Economical Appraisal of Total Aflatoxin Level in the Poultry Feeds by Fourier Transform Infrared Spectroscopy

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Summary: Single-bounce attenuated total reflectance (SB-ATR) Fourier transform infrared (FTIR) spectroscopy has been used for the quantitative determination of total aflatoxins in the broiler poultry feed. An FTIR calibration spanning the range 1-70 µg/L aflatoxin standards in (70:30, v/v) methanol-water solvent system based on partial least square (PLS) model, developed by relating mid IR region between 3755-950 cm^{-1}. The excellent coefficient of determination (R^2 = 0.998) was achieved with 1.49 relative mean square error of calibration (RMSEC). Aflatoxins from each of eight poultry feeds was extracted and the determined by the widely used commercially available Enzyme-linked Immunosorbent Assay (ELISA) procedure and the SB-ATR/FTIR method. The SB-ATR/FTIR aflatoxins predictions were related to those determined by the ELISA method by linear regression, producing an R value of 0.989 and a SD of ± 2.80 µg/L. The result of the study clearly indicated that FT-IR spectroscopy due to its rapidity and simplicity along with data manipulation by advance computer software could be effectively used for routine determination of aflatoxins present in the poultry feeds at very low level.

Keywords: Poultry feed, aflatoxins, FT-IR, enzyme-linked Immunosorbent assay (ELISA).

Introduction

Aflatoxins are naturally-occurring mycotoxins which contain a coumarin and a double-furan rings. Afltoxins are produced by various species of *Aspergillus*, particularly *A. flavus* and *A. parasiticus*. These micro fungi grow rapidly when foods/feeds containing high moisture content stored at elevated temperature [1]. Aflatoxins are reported as carcinogenic, teratogenic and mutagenic that could be isolated from a wide variety of agricultural products [2]. International Agency for Research on Cancer (IARC) has placed aflatoxins on its list of probable human carcinogens [3]. Presently, aflatoxins are regulated in many countries and have established their own legal limits with diverse levels ranging from 5 - 20 µg/kg for the poultry feeds [4]. Therefore, there is strong need for the development of simple method for the quick, economical and accurate analysis of aflatoxins in the poultry feeds for the industrial as well as commercial laboratories. Several analytical methods of aflatoxins analysis in cereal grains have been developed by ACC International and the United States Department of Agriculture (USDA) [5]. Most of the developed methods involve two steps, including isolation of aflatoxins from the matrix (which is a slow extraction process requiring large amount of organic solvents) and quantification by different analytical techniques (which is generally very laborious and expensive) [6]. For the quantification of aflatoxins thin layer chromatography [7], ultra- high- pressure liquid chromatography [8], high performance thin layer chromatography [9], high performance liquid chromatography [10, 11], liquid chromatography coupled with mass spectrometry [12-14] super critical fluid chromatography [15], Thermospray Mass Spectrometry, [16] immunochemical methods like ELISA [17], flow system coupled capillary electrophoresis [18] Immunochromatographic strip [19] and surface plasmon resonance [20] have been already reported. At present, enzyme linked immunosorbent assay (ELISA) is widely used due to simple and rapid analysis but it is also tedious, very expensive and is not so precise [21].

Since last few decades, FT-IR spectroscopy is gaining much popularity as a simple, economical, rapid and nondestructive technique for quantification of aflatoxins [22-27]. The innovative IR group of the National Centre of Excellence in Analytical Chemistry has already developed very sensitive method on FT-IR for the determination of free fatty acids in poultry feed lipids, main fatty acid classes and *trans* fatty acids in edible oil [28-30].This study is aimed to develop a rapid, simple and cheap analytical method for the screening of aflatoxins in the poultry feeds by using Fourier transform infrared (FTIR) spectroscopy and its evaluation with respect to ELISA method.

Results and Discussion

Aflatoxin Detection by ELISA

ELISA analysis was performed according to the instructions of the Neogen Veratox aflatoxin procedure**.** A calibration of equation was generated

by plotting optical density versus aflatoxins standard concentration. From regression equation, a negative correlation coefficient (-0.998) was observed, when the concentration of aflatoxin standards plotted versus their optical densities. The following regression equation obtained was used to assess the unknown concentration of total aflatoxins in the poultry feed samples as shown in Fig. 1.

Y = 0.85027 + (-0.00872) * X

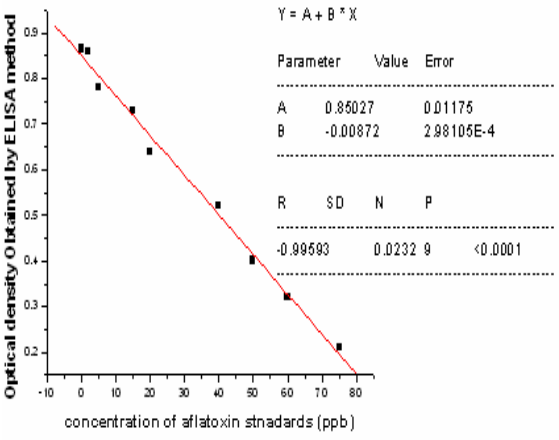

Fig. 1: Plot of the concentration of aflatoxin standards (1, 5, 15, 20, 40, 50, 60 and 75 µg/kg or ppb) versus optical density obtained by ELISA.

Aflatoxins detection by FT-IR

The basic assumption tested was the application of FT-IR for the determination of aflatoxins in the poultry feeds as an alternative mean to the ELISA/AOAC method, with the additional benefits of simplicity, speed and cost-effectiveness. Partial Least Square (PLS) is the chemometric technique of choice for developing a calibration model. The ability of PLS is based on its aptitude to use spectral information from wide spectral regions and to correlate spectral variations with changes in the concentration of a component of interest while concurrently accounting for other spectral contributions that may perturb the spectrum. A PLS calibration model was developed based on the calibration standards. Fig. 2 presents a group mid FT-IR-ATR spectra of the aflatoxins standards dissolved in (70+30, v/v) methanol-water, while Fig. 3 shows the results of PLS calibration achieved through TQ Analyst program. The aflatoxins exhibit characteristic absorption bands at wavelengths 3004–2969 cm^{-1} for CH2, aromatic =CH, –C–H, C=C and phenyls, 1744–1720 cm^{-1} for C=O, 1364–1369 cm^{-1} for methyl adjacent to epoxy ring, 1217–1220 cm^{-1} for in-plane –CH bending of phenyl1035–1037 cm^{-1}) for symmetric stretching of =C–O–C or symmetric bending of phenyl, and 900–902 cm^{-1} for possibly isolated H [32] as shown in Fig. 2

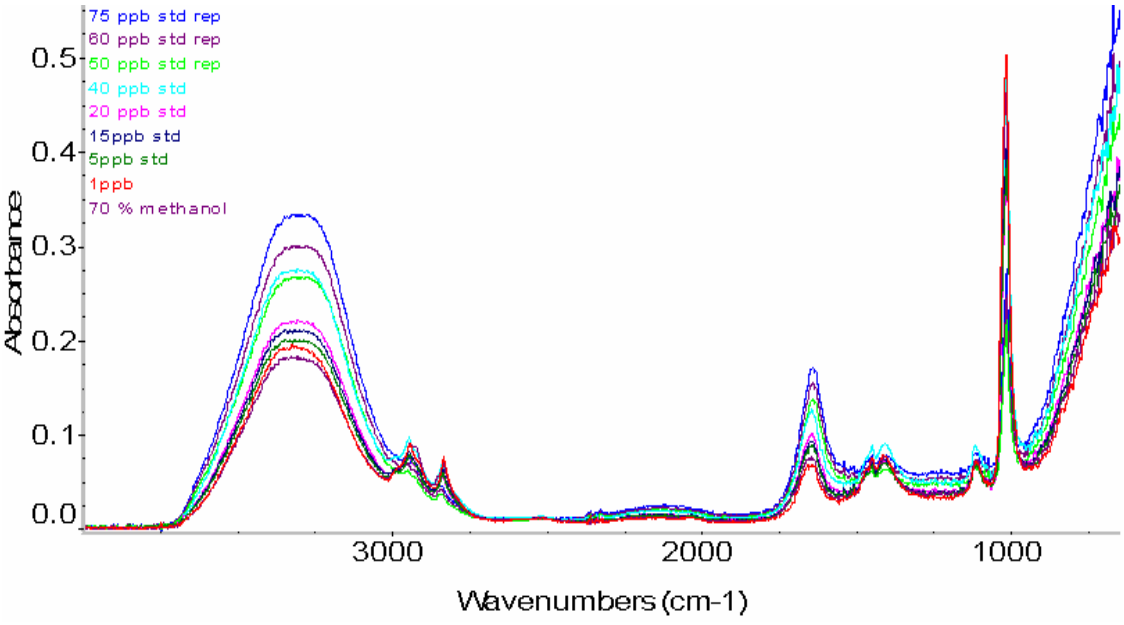

Fig. 2: Mid FT-IR-ATR spectra of aflatoxin standards dissolved in (70:30, v/v) Methanol-water.

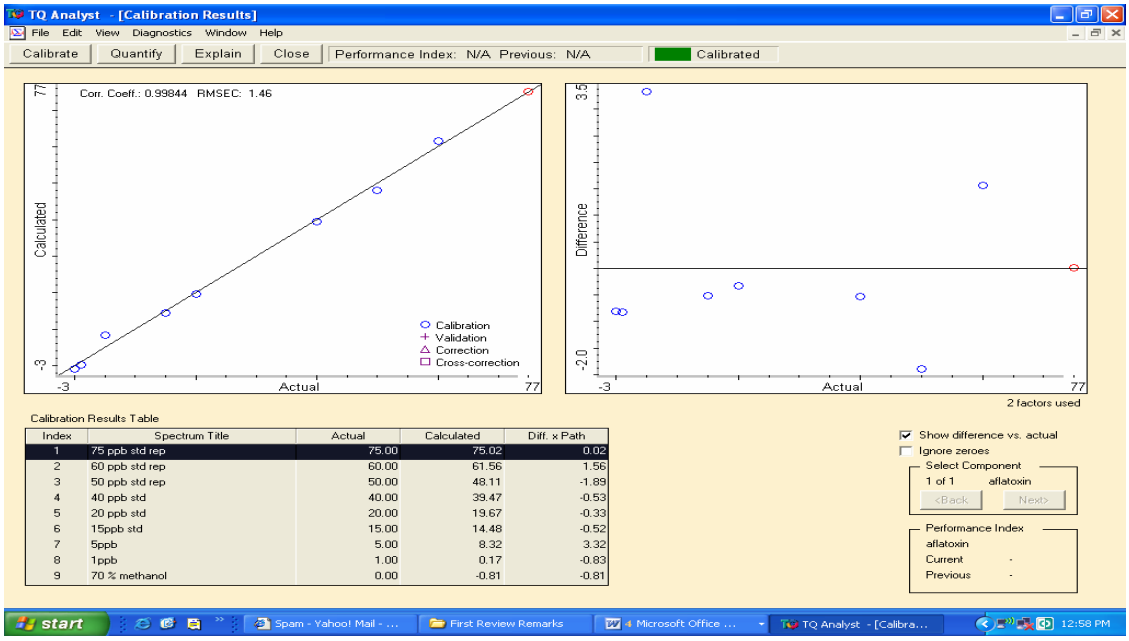

Fig. 3: Calibration of the actual concentration of aflatoxin standards (1, 5, 15, 20, 40, 50, 60 and 75ppb) versus the values obtained by FT-IR.

To calculate the proportionality between the FTIR and ELISA methods, linear regression of the data presented in Fig. 4 was performed, yielding the good relationship when the regression was forced through the origin (Z-reg). The slope of the Z-reg equation is close to the value that one would expect for the better relationship between the methods. Based on the reproducibility, it is obvious that most of the regression error was contributed by the ELISA results. The results clearly indicate that SB-ATR/FTIR analysis can serve as a simple, economical, and accurate alternative to ELISA method for the determination of the total aflatoxins in poultry feed.

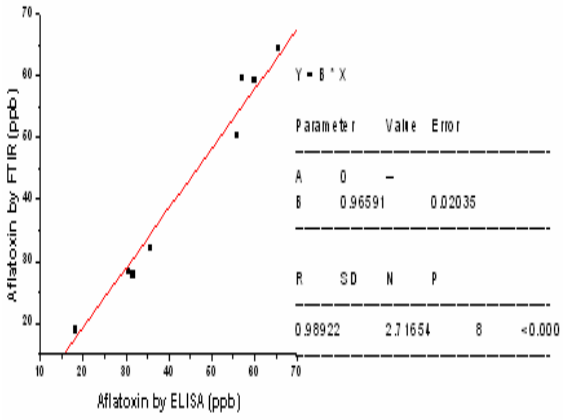

Fig. 4: Plot of predicted aflatoxin obtained by SB-ATR FTIR analysis vs ELISA method.

Detection Limit and Statistical Analysis

The minimum sample peak that could be determined was double as tall as the noise signals and was equal to concentration of 1.5 µg/l. These results were created with high precision (CV = 3.5%). The statistics of aflatoxins recovery tests (Table-1) on the one selected sample revealed high recovery performance (103.5, 93.3 and 91.4 %) with high precision (CV = 3.5, 3.2 and 1.4 %) of the proposed method.

Table-1: Recovery test of aflatoxin (ppb) from poultry feed samples after exogenous addition of known amount of standards

	(A)	(B)	By FT-IR (C)	Recovery[a] (%)	CV[b] (%)	By ELISA (C)	Recovery[a] (%)	CV[b] (%)
1	5	50	57.0 ± 1.4	103.6	3.6	46 ± 3.5	83.6	16.4
2	10	50	56.0 ± 2.2	93.3	6.7	51 ± 4.2	85.0	15.0
3	20	50	64.0 ± 2.5	91.4	8.5	58 ± 4.1	82.0	17.1

(A) Exogenous addition.
(B) Before addition.
(C) After addition.
aRecovery (%)=(C–B)/A x100%.
bCoefficient of variation was obtained from triplicate tests.

Methods Correlations

Eight feed samples were analyzed by the ELISA and FT-IR-ATR method. The results obtained by the both methods are shown in Table-1. Both methods verified that all analyzed samples were found to be contaminated with aflatoxins. Only one poultry feed (PF-6) contained less than 20 µg/kg aflatoxins (18.8 µg/kg) While, other analyzed

samples were found to be contained higher than 20 µg/kg and ranged between 27.0 to 65 µg/kg. No big difference was found in the results of both methods. On the basis standard deviations as shown in Table-2, it can be concluded that results of FT-IR-ATR are more precise as compared to ELISA method.

Table-2: Aflatoxin (ppb) in different broiler poultry feed samples analyzed by ELISA and FT-IR-ATR.

S.No.	Samples	ELISA	FTIR
1	PF-1	31.6 ± 3.2	27.8 ± 0.4
2	PF-2	30.5 ± 4.1	28.3 ± 0.6
3	PF-3	35.6 ± 3.6	32.0 ± 0.4
4	PF-4	65.5 ± 4.4	64.3 ± 0.5
5	PF-5	55.9 ± 2.6	50.3 ± 0.5
6	PF-6	18.1 ± 2.4	18.8 ± 0.3
7	PF-7	60.0 ± 4.2	59.2 ± 0.4
8	PF-8	57.2 ± 2.8	59.6 ±0.4

Experimental

Apparatus, Standards and Reagents

Beckman Coulter Tm (Model AD 340, Austria) was used as ELISA Reader. FTIR used for present study was NICOLET 5700 equipped with a ZnSe crystal, deuterated triglycine sulfate (DTGS) detector (Thermo Nicolet Analytical Instruments, Madison, WI). Veratox quantitative aflatoxin test kit (GIPSA-FGIS 2008-111 Product # 8030) was purchased from Neogen Corporation USA and aflatoxins standard. HPLC grade methanol and n-hexane was used for the extraction of aflatoxins from the poultry feed samples. Standard solution 1, 5, 15, 20, 40, 50, 60 and 75 µg/L (parts per billion ppb) of aflatoxins was prepared in methanol- water (70:30, v/v). These standard solutions were stored in the dark at 4 ^{0}C until further analysis.

Samples Collection and Extraction

Eight broiler feed samples were collected from different feed mills located in Hyderabad and Karachi. Sample preparation and extraction procedure for determination of aflatoxins by ELISA was performed according to the instructions given on the test kit manual [31]. Extraction of Aflatoxins for FTIR analysis was done by according [23] with slight modifications. The feed samples were ground using grinding machine (Haan, Germany), and sieved through 0.75 mm mesh. Some 5 g of ground feed sample was extracted with 25 ml of methanol-water (70:30, v/v) in 100ml conical flask. Ten ml of Hexane and 0.4mg sodium chloride were added to the flask for the defatting the sample. The contents of conical flask were vibrated vigorously with mechanical shaker for about 30 minutes. The upper hexane layer, containing interfering impurities, was discarded and methanol-water layer containing aflatoxins were filtered through a whatman#1 filter paper and filtrate was analyzed by FT-IR

Analysis of Aflatoxins by ELISA

ELISA analysis was performed according to the instructions of the Neogen Veratox aflatoxin procedure. Kits and extracts were brought to ambient temperature before analysis. Concentration of total aflatoxins in parts per billion (µg /L) was recorded by a 650 nm filter ELISA reader that was calibrated using aflatoxin standards.

Analysis of Aflatoxin by FT-IR

All spectra were obtained using a Thermo Nicolet 5700 FT-IR spectrometer equipped with a ZnSe crystal, deuterated triglycine sulfate (DTGS) detector, controlled by OMNIC software. All spectra were collected by co addition of 32 scans at resolution of 4 cm^{-1} in the range of 4000-650cm^{-1}. FT-IR spectra of aflatoxin standards and extracted aflatoxins from poultry feed samples by methanol-water (70:30, v/v) were obtained by placing only 20 µl of solution on the ATR cell.

Method Accuracy

Accuracy of the method was assessed by the recovery test. A recovery study was conducted by spiking feed samples with three levels of aflatoxins (5, 10 and 20 µg/kg) added to the sample already contained 50µg/kg. Fore each spiking level was prepared in triplicate. After the samples were extracted as described above, they were analyzed by the ELISA and FTIR method.

Detection Limit

To establish the limit of detection (LOD) of proposed method, the selected peak height was measured at low concentrations of standards, until the aflatoxins related signal vanished. The analysis at the minimum concentration which produced considerable signal was repeated eleven times, with independent measures. The limit of detection (LOD), defined as the smallest concentration from which it is possible to deduce the presence of the analyte, was calculated by applying the formula:

$$LOD = 3 \times SD \times C / M$$

where: SD is the standard deviation; C is the analyte concentration and M is the mean area.

Validation of FT-IR Method

Data acquired from FT-IR-ATR spectra were calculated by means of the standard curve of the

aflatoxin standards. The peak heights of standards were plotted against their known concentrations, and the slope of standard curve was calculated using method of least squares due to the linear relationship. From the calibration leave one out was applied for the cross validation that shows the excellent results as shown in Fig. 3. Furthermore, recovery efficiency (RE) was also evaluated by the ratio of aflatoxin recovered to the aflatoxin content (µg) added. The calculation was carried out by the following equation:

$$R\ (\%) = (C–B/A)\ x100$$

where R is the aflatoxin content recovered (%); A is added amount of aflatoxin; B is actual concentration present in the poultry feed samples: C is sum of actual and added amount in parts per billion. The CV (%) of the data set was calculated and used as relative standard deviation (RSD, %)

Conclusion

The results of this study demonstrate that there are considerable advantages in terms of speed, precision, and accuracy to be gained from the use of SB-ATR/FTIR spectroscopy for the quick and economical determination of total aflatoxin level in the poultry feeds. Owing to its high variation in replicates, the ELISA method was found to be suitable only as a screening method. To record the FTIR spectrum of aflatoxins, after the extraction from the poultry feeds, needs less than 2 min per sample; consequently, it is possible to analyze hundreds of poultry feed samples daily. Another advantage over ELISA method is that only single drop (20 µl) is sufficient to record the FT-IR spectra. Therefore, quick determination of total aflatoxin by ATR-FT-IR spectroscopy is a feasible method for the process and quality control laboratories in the poultry feed industries.

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