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Comparison of ELISA and HPLC techniques in determination of AFB_1 in feedstuff

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ABSTRACT

This study aimed to determine the aflatoxin B_1 (AFB_1) levels in feed-stuff samples. For this purpose, 90 animal feed samples including barley, wheat bran, wheat pulp, canola meal, safflower meal, cottonseed meal and sunflower meal were randomly obtained from retail stores in Mashhad-Iran and analyzed by both enzyme linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC) techniques. Results showed presence of AFB_1 in 33 (36.67%) and 10 (11.11%) samples analyzed by HPLC and ELISA techniques, respectively. The average±SD concentration of AFB_1 in the samples was 2.21±2.25 and 10.76±0.86 µg/kg for HPLC and ELISA techniques, respectively. The range of contamination reported 0.34-5.81 and 9.5-12.6 µg/kg for the mentioned techniques respectively. ELISA showed reliability and a high correlation with HPLC of 0.93 indicating its potential for aflatoxin screening in animal feed samples. However, sensitivity and specificity of HPLC was higher than ELISA method.

KEYWORDS

Feedstuff;
AFB_1;
HPLC;
ELISA.

INTRODUCTION

Aflatoxins, a class of mycotoxins, are highly toxic, carcinogenic, mutagenic and teratogenic compounds generally produced by some competent mould strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. Aflatoxin B_1 (AFB_1) is the most predominant of aflatoxins in food and feed; and has been reported to be the most powerful natural carcinogen in mammals[1,3]. Typical materials that are susceptible to aflatoxin contamination include maize and other cereals such as wheat and rice, groundnuts and other nuts such as pistachios and Brazil nuts, cottonseed, copra and spices[18]. A direct relationship has been observed between the amount of Aflatoxin M_1 (AFM_1) in milk and AFB_1 consurnption via feedstuffs. The conversion rate of ingested AFB_1 into AFM_1 is highly variable, ranging from 0.3% to 6.2%[2,7]. Therefore, it is necessary to monitor amount of AFB_1 in animal feed. Different techniques are used in determining aflatoxin such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC) and immunochemical methods such as enzyme linked immunosorbent assay (ELISA). HPLC is ideal and more useful than the other methods in terms of specificity and sensitivity, and ELISA not only require costly instru-

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mentation but also is rapid, simple, specific, sensitive and can be used to analyze a large number of samples simultaneously and require no sample clean-up[8,21]. In this study, an indirect competitive ELISA technique was used and validated for aflatoxin screening in animal feed samples and its performance was compared with that of HPLC.

MATERIAL AND METHODS

Materials

Ninety samples of animal feed were purchased from retail stores in Mashhad, Iran. Samples included barley, wheat bran, wheat pulp, canola meal, safflower meal, cottonseed meal and sunflower meal.

Chemicals and reagents

AFB_1 standard solution was prepared from Sigma (Germany) with purity of 98%; standard stock solutions were prepared in acetonitrile according to the AOAC method[5]. All solvents used for the experiments (methanol, acetonitrile and deionized water) were HPLC grade supplied by Merck. Aflatest immunoaffinity columns (IAC) were purchased from VICAM Co. HPLC column (C_{18}) was used.

Sample preparation

All the samples were grinded with miller and collected in a plastic bag. Fifty g of the ground samples was taken for analysis.

ELISA determination

Fifty grams of ground samples were extracted with 250 ml of 70% methanol by mixing vigorously on a magnetic stirrer for 3 minutes. After filtering the extract through a Whatman No.1 filter, the extract was diluted 1:1 with phosphate-buffered saline (PBS). Aflatoxin analysis was developed using a competitive ELISA commercial Kit for AFB_1[15] . According to Europroxima Aflatoxin B_1 (Art No.5121) test kit manual, 50 µl aflatoxin standard solutions and 50 µl prepared test samples were added into separate wells of microtiter plate, in duplicate. Then, 25 µl of the diluted conjugate (Aflatoxin-HRP) and 25 µl of the antibody solution were added to each well, mixed gently and incubated for 1 h at 37°C in the dark. The liquid was then removed completely from the wells, the each well was washed with rinsing buffer. The washing procedure was repeated for three times in ELISA washer (ELX 50, Bio-Tek Inst., USA). After the washing step, 100 µl substrate solution was added to each well and incubated for 30 min at room temperature in the dark. Finally, 100 µl of the stop solution was added to each well and the absorbance was measured at 450 nm in ELISA plate reader (ELX 808, Bio-Tek Inst.,USA).

HPLC determination

In brief 5 g of homogenized sample was extracted with 0.5 g NaCl and 30 ml methanol: water, (2:8) by high-speed blender (in fatty samples n-Hexan was added in order to remove fat) and then filtered through a Wathman filter paper No. 4. Five milliliters of extract was diluted with 95 ml of phosphate buffered saline (PBS, pH 7.4). The immunoaffinity column was conditioned with 10 ml of PBS and 50 ml of the diluted filtrate were applied to the column at a flow rate of 3 ml/min. After the clean-up step the column was washed with 20 ml of water and air was forced through the column prior to eluate aflatoxins by applying 1.75 ml of methanol. The eluate was diluted with 3.25 ml of water to give a total volume of 4.50 ml and 100 µl of eluate was injected onto HPLC system. The mobile phase consisted of acetonitrile:methanol:water (17:29:54, v/v/v) with a flow rate of 1 ml/min. AF was quantitated by reverse-phase HPLC and fluorescence detector[6,19,20]. The AFB_1 was detected at the excitation and emission wavelengths of 365 and 435 nm, respectively. The employed column was a C_{18} 150 * 4.6 mm, 5 µm.

Statistical analysis

Results are presented as means±SD. ANOVA was also used in the general linear model procedure in SPSS.16. Variable means for measurements showing significant differences in the ANOVA were compared using the least significant difference procedure. Values were judged to be significantly different if $P < 0.05$. All experiments were carried out as triplicates.

RESULT AND DISCUSSION

TABLE 1 shows among a total of 90 samples, the incidence of AFB_1 by ELISA method, was 11.11% (10

samples) within the range of 9.5-12.6 µg/kg. In comparison, the number of contaminated samples by HPLC technique was 33 (36.67%) in extent of 0.34-5.81 µg/kg. Yet the mean contamination level in all samples was much lower than maximum acceptable level (20 µg/kg) of AFB_1 determined by Iranian standard organization[5], and because of low level of AFB_1 contamination in feedstuff, the incidence of AFM_1 contamination in pasteurized milk samples in north east of Iran,was reported low by Mohamadi sani et al. (2011)[9]. In another study reported by Mohamadi sani et al. (2012), AFT levels in rice samples were found lower than the maximum tolerable limit for total AF as stated in the EU regulation[10].

TABLE 1 : AFB_1 contamination in animal feed samples using ELISA and HPLC method

Mycotoxin	Method	Positive samples (%)	range (µg/kg)	Mean±SD[a] (µg/kg)
AFB_1	HPLC	33(36.67%)	0.34-5.81	2.21±2.25
AFB_1	ELISA	10(11.11%)	9.5-12.6	10.76± 0.86

[a]:Standard deviation

The findings demonstrate that the values of AFB_1 contamination by ELISA method were higher than HPLC technique ones. Rodrý´guez-Cervantes et al. (2012) analyzed 30 samples of animal feed using HPLC and ELISA methods for AFB_1 contamination. According to their results, AFB_1 was detected in 63.3% of the samples by ELISA technique, but no positive sample was shown by HPLC method[15].

However, our findings showed the ELISA method could not detect low concentrations of AFB_1; on the other hand, concentration of 0.51 µg/kg of AFB_1 on HPLC assay did not detect by ELISA technique. In another hand, at higher concentrations of AFB_1, the results of ELISA technique were higher than HPLC results (Figure 1). This particular issue has been in discussion fairly often and in different studies, reporting higher values obtained by ELISA kits than those obtained in HPLC analysis[16].

According to Figure 1, AFB_1 contamination was detected by HPLC in cottonseed and sunflower meals but ELISA method showed the contamination in only cottonseed meal samples. The mean AFB_1 concentration of cottonseed meal reported by HPLC and ELISA techniques at 5.98 and 3.47 µg/kg respectively. Statistical evaluation showed significant difference between the mean concentrations of AFB_1 in cottonseed meal samples by ELISA and HPLC method ($p<0.05$).

According to the results of Pirestani et al. (2011) AFB_1 concentration in different feedstuff including alfalfa, straw, rapeseed, cottonseed, corn silage and soybean meal detected by HPLC technique as: 0.38, 0.39, 1.54, 34.96, 0.45 and 0.65 µg/kg, respectively[12]. It is clear from the data given that cottonseed had highest level of contamination even higher than maximum tolerated level which confirms our results.

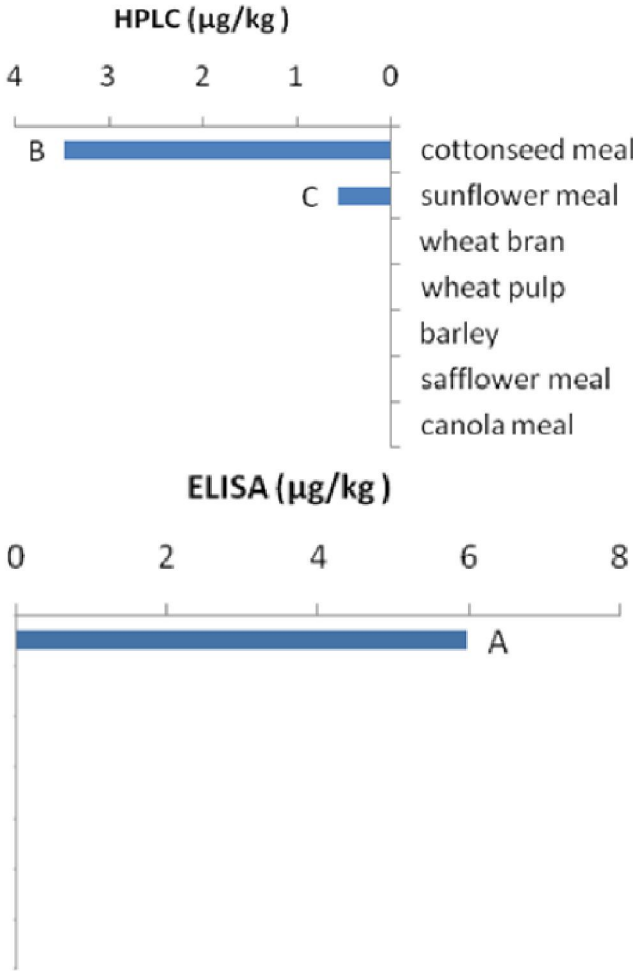

Figure 1 : Incidence of AFB_1 in different animal feed samples

TABLE 2 indicates that the range of contamination to AFB_1 in cottonseed meal samples by ELISA and HPLC methods is 9.5-12.6 and 0.46-5.81 µg/kg, respectively, and all the samples were contaminated according to HPLC analysis. Yet, ELISA had not been able to detect AFB_1 completely.

Limit of detection (LOD) and limit of quantitation (LOQ) for both analytical parameters in applied methods were calculated from the mean value of ten deter-

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minations of a blank feedstuff samples plus two- and ten-fold standard deviation, respectively[13].

Out of 90 feedstuff samples, 26 of samples were reported contaminated to AFB_1 in the range of 1-20 µg/kg by ELISA and HPLC methods (TABLE3).

The ELISA method recoveries were determined at 5 different levels (three replicates per concentration level) by the standard solutions of concentration from 6.25 to 100 ppt AFB_1 to find calibration standard curve. Recovery measurements for HPLC technique were also carried out by spiking noncontaminated samples with 20 µg/kg of AFB_1 in triplicate. (TABLE 4).

TABLE 2 : Range and percentage of AFB_1 contamination in animal feed samples using ELISA and HPLC

Sample category	Method	Number of sample	Number of contaminated sample	Percentage of contaminated sample	Contamination range (µg/kg)
Cottonseed meal	ELISA	18	10	55.56%	9.5-12.6
Cottonseed meal	HPLC	18	18	100%	0.46-5.81
Sunflower meal	ELISA	28	ND[b]	0%	-
Sunflower meal	HPLC	28	13	46.43%	0.34-0.9
Wheat bran	ELISA	12	ND	0%	-
Wheat bran	HPLC	12	ND	0%	-
Wheat pulp	ELISA	16	ND	0%	-
Wheat pulp	HPLC	16	ND	0%	-
barley	ELISA	9	ND	0%	-
barley	HPLC	9	ND	0%	-
Safflower meal	ELISA	5	ND	0%	-
Safflower meal	HPLC	5	ND	0%	-
Canola meal	ELISA	2	ND	0%	-
Canola meal	HPLC	2	ND	0%	-

[b]: Not Detected

TABLE 3 : Numbers of samples in different range of AFB_1

Sample matrix	Numbers of samples in the range mg kg $^{-1}$				
	Method	ND	0.1 - 0.5	0.5 - 1.0	1.0 - 20.0
Cottonseed meal	ELISA	8	-	-	10
Cottonseed meal	HPLC	-	1	1	16
Sunflower meal	ELISA	28	-	-	-
Sunflower meal	HPLC	15	4	9	-
Wheat bran	ELISA	12	-	-	-
Wheat bran	HPLC	12	-	-	-
Wheat pulp	ELISA	16	-	-	-
Wheat pulp	HPLC	16	-	-	-
barley	ELISA	9	-	-	-
barley	HPLC	9	-	-	-
Safflower meal	ELISA	5	-	-	-
Safflower meal	HPLC	5	-	-	-
Canola meal	ELISA	2	-	-	-
Canola meal	HPLC	2	-	-	-

Correlation between ELISA and HPLC assays

In this study ELISA technique was conducted because of simplicity of installation and performance in laboratories with limited possibilities. The validation of ELISA technique for AFB_1 content was determined in order to compare its reliability with that of HPLC, a well-established technique for aflatoxin determination. Overall correlations of HPLC versus ELISA for AFB_1

TABLE 4 : validation of AFB_1 determination by HPLC method

sample	Added (µg/kg)	RSD%	Recovery%
Cottonseed meal	20	2.59	86
	NC[c]	61.25	-[d]
Sunflower meal	20	3.15	78
	NC	24.63	-
Wheat bran	20	1.38	82
	NC	-	-
Wheat pulp	20	2.76	80
	NC	-	-
barley	20	1.75	76
	NC	-	-
Safflower meal	20	2.7	70
	NC	-	-
Canola meal	20	1.92	84
	NC	-	-

[c]: naturally contaminated.; [d]:statistical parameters not calculated; levels were below limits of detection.

are illustrated in Figure 2. There was good correlation between two techniques. The correlation coefficient (r) was 0.93 and r^2 was 0.86.

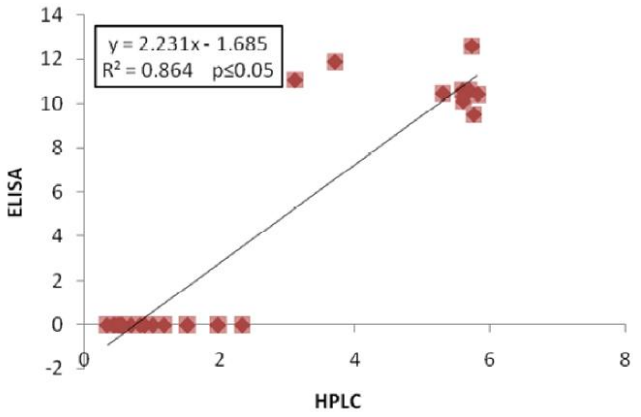

Figure 2 : Comparison of the analysis of AFB_1 from feedstuff samples between ELISA and HPLC (n=32)

Park et al. (2002) compared ELISA and HPLC methods for the analysis of AFB_1, fumonisin B and ochratoxin A in barley and corn foods and reported correlation factors ranging between 0.81-0.87[11]. Razzazi-Fazeli et al. (2004) reported better correlations between ELISA and HPLC could be obtained at low concentration ranges of AFB_1[14]. According to Pleadin et al. (2012) there was a high concordance of ELISA and TLC method for detection of deoxynivalenol (DON) as well as ELISA and HPLC methods for detection of zearalenone (ZEA) in maize samples[13]. Rossi et al. (2012) revealed the standardized indirect competetive ELISA method in poultry feed samples showed reliability and a high correlation with HPLC of 0.97 (broiler feed) and 0.98 (laying hen feed) indicating its potential for aflatoxin screening in poultry feed samples[17].

CONCLUSION

The data obtained from this monitoring showed AFB_1 levels below the Iranian standard organization acceptable limits for livestock consumption. This study showed that sensitivity and specificity of HPLC system is more than ELISA method. HPLC system determined the concentration of AFB_1 with more sensitivity. Meanwhile, ELISA method is cheaper and easier to use than HPLC system. Since ELISA method showed a good correlation with HPLC, and because of simplicity, rapidity, reliability and cost-effectivity of this technique, it can be used in routine screening of AFB_1 contamination in feed stuff, but the result of ELISA method should be confirmed by HPLC technique.

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The authors declare that they have no conflict of interest.

REFERENCES

[1] H.Aycicek, A.Aksoy, S.Saygi; Determination of aflatoxin levels in some dairy and food products which consumed in Ankara, Turkey. Food Control, **16**, 263-266 (**2005**).

[2] E.E.Creppy; Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicology Lett ers, **127**, 19-28 (**2002**).

[3] L.Hussain, J.Anwar, M.A.Munawar, M.R.Asi; Variation of levels of aflatoxin M, in raw milk from different localities in the central areas of Punjab, Pakistan. Food Control, **19**, 1126-1129 **(2008)**.

[4] IARC; Monograph on the evaluation of carcinogenic risk to human. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene: Summary of data reported and evaluation., **82**, (2002).

[5] ISIRI; Maximum Tolerated Limits of Mycotoxins in Foods and Feeds. National Standard No. 5925, **(2002)**.

[6] ISIRI; Foodstuffs, Determination of Aflatoxins by HPLC and Clean up by IAC. National Standard No. 6872, **(2004)**.

[7] B.Kabak, I.Var; Factors affecting the removal of aflatoxin M, from food model by Lactobacillus and Bifidobacterium strains. Environ. Sci. Health B, **43**, 617-624 **(2008)**.

[8] R.Krska, P.Schubert-Ullrich, A.Molinelli, M.Sulyok, S.Macdonald, C.Crews; Mycotoxin analysis: An update. Food Addit. Contam, **25(2)**, 152–163 **(2008)**.

[9] A.Mohamadi sani, H.Nikpooyan; Determination of aflatoxin M_1 in milk by high-performance liquid chromatography in Mashhad (north east of Iran). Toxicol. Ind. Health, **4**, 334-338 **(2011)**.

[10] A.Mohamadi sani, E.Gholampour Azizi, E.Ataye Salehi; Reduction of aflatoxin in rice by different cooking methods. Toxicol. Ind. Health Published online Sept. 27, doi:10.1177/0748233712462466, **(2012)**.

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[11] J.W.Park, E.K.Kim, D.H.Shon, Y.B.Kim; Natural co-occurrence of aflatoxin B_1, fumonisin B_1 and ochratoxin A in barley and corn foods from Korea. Food Addit. Contam, **19(11)**, 1073-1080 **(2002)**.

[12] A.Pirestani, S.N.Tabatabaei, M.H.Fazeli, M.Antikchi, M.Baabaei; Comparison of HPLC and ELISA for Determination of Aflatoxin Concentration in the Milk and Feeds of Dairy Cattle. Res. Agr. Sci, **7(1)**, 71-78 **(2011)**.

[13] J.Pleadin, M.Sokolovic, N.Persi, M.Zadravec, V.Jaki, A.Vulic; Contamination of maize with deoxynivalenol and zearalenone in Croatia. Food Control, **28**, 94-98 **(2012)**.

[14] E.Razzazi-Fazelii, C.T.Noviandi, S.Porasuphatana, A.Agus, J.Bohm; A survey of aflatoxin B~ and total aflatoxin contamination in baby food, peanut and corn products sold at retail in Indonesia analysed by ELISA and HPLC. Mycotoxin Res., **20**, 51-58 **(2004)**.

[15] C.H.Rodrý´guez-Cervantes, A.J.Ramos, M.L.Robledo-Marenco, V.Sanchis, S.Marý´n, M.I.Giro´n-Pe´rez; Determination of aflatoxin and fumonisin levels through ELISA and HPLC, on tilapia feed in Nayarit, Mexico. Food Agric Immunol, 1-10 **(2012)**.

[16] J.Rojas-Jaimes; Evaluation of the level of contamination by the ''ochratoxine'' mycotoxine in samples of Capsicum annuum ''Paprika'' using ELISA and high Performance liquid chromatography ''HPLC''. Revista Horizonte Me´dico, **8**, 48-52 **(2008)**.

[17] C.N.Rossi, C.R.Takabayashi, M.A.Ono, G.H.Saito, E.N.Itano, O.Kawamura, E.Y.Hirooka, E.Y.Sataque Ono; Immunoassay based on monoclonal antibody for aflatoxin detection in poultry feed. Food Chem, **132**, 2211–2216 **(2012)**.

[18] G.S.Shephard; Aflatoxin and Food Safety: Recent African Perspectives J. Toxicol., Toxin Rev., **22(2-3)**, 267-286 **(2003)**.

[19] J.Stroka, E.Anklam, U.Jörissen, J.Gilbert; Immunoaffinity column clean-up with liquid chromatography post column bromination for determination of aflatoxins in peanut butter, pistachio paste, fig paste and paprika powder: collaborative study. J. AOAC Int., **83**, 320-340 **(2000)**.

[20] J.Stroka, C.Von Holst, E.Anklam; Immunoaffinity column clean-up with liquid chromatography using post column bromination for determination of aflatoxin B_1 in cattle feed: collaborative study.. J. AOAC Int., **86(6)**, 1179–1186 **(2003)**.

[21] Z.Zheng, C.W.Humphrey, R.S.King, J.L.Richard; Validation of an ELISA test kit for the detection of total aflatoxins in grain and grain products by comparison with HPLC. Mycopathologia, **159**, 255–263 **(2005)**.