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FOOD SCIENCE & TECHNOLOGY | RESEARCH ARTICLE

Analytical method development and monitoring of Aflatoxin B1, B2, G1, G2 and Ochratoxin A in animal feed using HPLC with Fluorescence detector and photochemical reaction device

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Abstract: A sensitive and robust HPLC method with fluorescence detector (FLD), photochemical reaction device (PHRED) was developed to determine Aflatoxins (B1, B2, G1, and G2) and Ochratoxin A in animal feeds. Immunoaffinity columns (IAC) was used to purify the samples. According to monitoring resulted with the established method, aflatoxin and ochratoxin A were found in 44 of 496 domestic commercial samples (8.9% detection rate) and the concentration ranges were 1.76–162.69 μg/kg for Aflatoxin B1, 23.01–45.42 μg/kg for Ochratoxin A in 2015. In 2016, % detection rate was 22.2% (115 of 518 samples) and the concentration ranges are 1.76–43.69 μg/kg for Aflatoxin B1, 3.38–15.80 μg/kg for Ochratoxin A. The developed method was specific and reliable and is suited for the routine analysis of aflatoxin in animal feed.

Subjects: Agriculture and Food; Food Chemistry; Food Analysis

Keywords: Aflatoxin; Ochratoxin A; photochemical reaction device; HPLC-FLD; immunoaffinity column

1. Introduction

Mycotoxins are secondary metabolites that commonly occur during production and storage of agricultural products and have deadly effects on human and livestock. There are various molds that

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PUBLIC INTEREST STATEMENT

Mycotoxins are secondary metabolites that commonly occur during production and storage of agricultural products and have deadly effects on human and livestock. It is detected in various foods worldwide and is considered one of the most dangerous contaminants in food and feed. It is possible that products of domestic animal intake contaminated feed include residue mycotoxins. If human ingest that product, it is expected to be harmful to body. In food industry, there have been great deal of studies on the adverse effects of mycotoxins and their detection and analytical methods. However, relatively few studies have been conducted in feeds industry. Thus, we have established a simultaneous determination method for Aflatoxin, Ochratoxin that are regulated in feed ingredients by the Korean Feed Control Act.

produce mycotoxins, and they may be categorized into hepatotoxin, nephrotoxin, neurotoxin, and immunotoxin as they have different biological effects on different organs. In food industry, there have been great deal of studies on the adverse effects of mycotoxins and their detection and analytical methods. However, relatively few studies have been conducted in feeds industry. In 1960, 100,000 turkeys in England died after being fed with imported feeds that contained peanuts contaminated with molds. Initially, the cause was unknown and the plague was called a "turkey x disease". However, the source was later isolated from A. flavus in peanuts and was termed Aflatoxin (Andrade, Gomes da Silva, & Caldas, 2013). Mycotoxins in feeds not only cause economic problems such as deaths of animals, poor growth and productivity, but also pose threats to humans who consume foods (meat, dairy, eggs) produced from animals fed with contaminated feeds (Araguás, González-Peñas, & De Cerain, 2005; Arroyo-Manzanares, Huertas-Pérez, García-Campaña, & Gámiz-Gracia, 2015).

Aflatoxin is produced by *Aspergillus flavus* and *Aspergillus parasiticus*. It is detected in various foods worldwide and is considered one of the most dangerous contaminants in food and feed. Of the 20 types of aflatoxins identified, 4 types of Aflatoxin B1, B2, G1, and G2 occur widely in nature and are serious contaminants in various food and feed. Aflatoxins are highly toxic and are difficult to remove during food processing as they are very heat stable (Benford, Boyle, Dekant, Fuchs, et al., 2001). Aflatoxin B1 is most common and also most toxic (Benford et al., 2001). As grains, the major source of Aflatoxin contamination, are used as the main ingredients of feed, the risk of Aflatoxin exposure in feed is very high.

Ochratoxin is a toxin mainly produced by several molds that belong to *Penicillium verrucosum* and *Aspergillus ochraceus*. Ochtratoxin producing molds are widely distributed throughout the world and are detected in various foods such as grains, cereal-derived products, dried fruits, spices, coffee, beer, and wine (Blesa, Berrada, Soriano, Molto, & Manes, 2004; Castegnaro & Wild, 1995; Cole, 1986; Duarte, Lino, & Pena, 2012; Ferracane et al., 2007; Garcia-Villanova, Cordón, González Paramás, Aparicio, & Garcia Rosales, 2004). There has also been a report that high levels of these toxins have been detected in feed (González, Juan, Soriano, Moltó, & Mañes, 2006). Ochratoxin A is a secondary metabolite produced by *Aspergillus ochraceus* mold. It was first discovered in South African maize in 1965. It is nephrotoxic and is presumed the source of Balkan endemic nephropathy (Hernández Hierro, Garcia-Villanova, Rodríguez Torrero, & Toruño Fonseca, 2008). Ochratoxin A is highly nephrotoxic and is the nephritis inducing agent in pigs and chickens. In addition, it suppresses immunity of livestocks and also inhibits the intestinal absorption of nutrients causing poor growth and disease resistance.

As Aflatoxin and Ochratoxin A (AO) is toxic contaminants in food and feed, a multi-faceted research is needed. Individual analyses on AO have been reported (Khayoon et al., 2010; Lopez-Garcia, Mallmann, & Pineiro, 2008; Midio, Campos, & Sabino, 2001; Monaci, Tantillo, & Palmisano, 2004; Muscarella et al., 2009; *Validation of analytical procedures,* 2005) and simultaneous analyses of these two toxins using immunoaffinity columns (IAC) and high performance liquid chromatography (HPLC) have also been reported for food such as milk, peppers, and olive oil (Pena, Cerejo, Lino, & Silveira, 2005; Ren et al., 2007; Solfrizzo, Avantaggiato, & Visconti, 1998; Streit et al., 2012; Thompson, Ellison, & Wood, 2002). However, research on the simultaneous determination of these two toxins in feed is mainly conducted by LC-MS/MS methods (Detection of aflatoxins, 2012) and even these are not yet done extensively. The provisions of CODEX limit the levels to 105 μg/kg or lower in Total Mixed Ratio (TMR) and 40 μg/kg or lower in feed ingredients. In the EU, the levels are regulated to be lower than 10 and 30 μg/kg, respectively. In the Korean Feed Control Act, Aflatoxin levels (the sum of B1, B2, G1 and G2) are regulated to be lower than 10–20 μg/kg in TMR, 50 μg/kg in feed ingredients; Ochratoxin A levels are set to be lower than 200 μg/kg for both TMR and feed ingredients. Currently, AO are determined individually in accordance with the regulations and it requires much effort in terms of time and costs. Simultaneous determination methods mainly use LC-MS/MS methods, but a method using HPLC is more economical in terms of maintenance and supply costs, and also has an advantage as instrument operation is also relatively easier. Thus, we have established a

simultaneous determination method for AO and investigated the contamination of feed in domestic distribution using the established determination methods.

2. Experimental

2.1. Samples

Feed samples were collected from the domestic market of Korea. In 2015, 365 TMR samples, 131 feed ingredient samples were collected and in 2016, 407 TMR samples and 111 feed ingredient samples. TMR samples are for cattles (beef cattles and dairy cows), pigs, chickens, ducks, dogs, and cats; feed ingredients are grain byproducts (broken grains and brans), meals (proteins), and fibers.

2.2. Standards and chemicals

Standards for Total Aflatoxin (G2: G1: B1: B2 = 0.10: 0.38: 0.10: 0.41 μg/kg) and Ochratoxin A used in the experiments were purchased by Romer Labs (Tulln, Ausrtria). The standards were diluted with acetonitrile to 7 different concentrations and used as the working standard to calibrate. Acetonitrile and methanol were HPLC grade by Merck (Billerica, MA, USA) and other solvents were up to analytical purity grade. Water was produced using Mili-Q water purification system Rios/Elix (Millpore Bedford, MA, USA).

2.3. Analytical instruments and devices

The HPLC instrument used in the experiments is NANOSPACE SI-2 (Shiseido, Tokyo, Japan) and is equipped with Fluorescence detector (FLD) and photochemical reaction device (PHRED) (Aura industries, NY, USA) used. The columns used are CAPCELL PAK C_{18} UG120 (4.6 × 250 mm, 5 μm, Shiseido, Tokyo, Japan). Immunoaffinity columns (IAC) used for purification of the samples are AflaOchra Test WB (VICAM, Milford, MA, USA) In the last phase of the pretreatment, syringe filter (25 mm, 0.2 μm nylon membrane Agela Technologies, DE, USA) was used.

2.4. Sample pretreatment

Before pretreatment, samples are ground evenly and kept in the refrigerator. For simultaneous AO determination, 25 g of samples are homogenized in 100 ml of 70% methanol for 30 min and then it is filtered with whatman No. 4. 10 ml of the filtrate is placed in a 100 ml flask and 40 ml of PBS (phosphate buffer saline) is mixed in the flask. Then 20 ml of the filtrate is place in the AflaOchra IAC and is passed through at 1 drop/sec. Then 10 ml of PBS and distilled water is passed through at the same speed. The residues remaining in the column is extracted using a suction pump and is eluted with 1 ml each of methanol and distilled water (*Standards and Specifications of Animal Feeds,* 2017).

2.5. Conditions of analyses

The wavelength conditions of the FLD for Aflatoxin was set to be 360 nm for the excitation wavelength, 450 nm for the emission wavelength; for Ochratoxin A, the excitation wavelength was set at 330 nm, the emission wavelength at 460 nm. Mobile phase A consisted of acetonitrile: methanol: distilled water (1:3:6, v/v), mobile phase B consisted of acetonitrile: 0.1% phosphoric acid (1:1, v/v). The initial conditions are 100% A for 12, 13 min at 5% A, 95% B, from 23.1 min at 100% A. The flow rate was 1.2 ml/minute and the injection volume was 20 μl. The temperature of the column was 50°C.

2.6. Method validation

Method validation was performed according to guidelines set by International Conference on Harmonization (Visconti, Pascale, & Centonze, 2000) and International Union of Pure and Applied Chemistry (Wang et al., 2013). The method was evaluated in terms of linearity, sensitivity, selectivity and precision.

In order to measure the accuracy of AO simultaneous determination methods, we have added mixed standard solutions to the Association of American Feed Control Officials (AAFCO, Champaign, Illinois, USA) samples such that the concentrations of Total Aflatoxin (the sum of G2, G1, B2, B1) and

Ochratoxin A are 10, 20, 50 μg/kg. In addition, Food Analysis Performance Assessment Scheme (FAPAS) Certified Reference Material (CRM), which is cereal based animal feed sample used Aflatoxin Standard (#34012) and Ochratoxin A (#22140) the contamination level was G2: G1: B2: B1 = 2.05: 2.79: 3.57: 5.72 and 33.66 μg/kg.

3. Results and discussion

3.1. Method validation

The mixed standard solution of AO was varied at 7 different concentrations to confirm the linearity (Bae, Ham, Jeong, Kim, & Kim, 2015; Kim, 2016; Kim, Jeong, Park, Kim, & Kim, 2016; Kim et al., 2015). The calibration curve was obtained by comparing the peak area versus 7 level concentrations. Each mixed standard solution was repeatedly injected 3 times to calculate the regression value (Bae et al., 2015; Kim, 2016; Kim et al., 2015, 2016). The results show that external standard calibration can be applied for the quantitative purposes. The coefficients of correlation (r^2) were all greater than 0.999. The range of AO was 1.73–68.1 μg/kg and detailed results on the regression value can be seen in Table 1.

The sensitivity of the developed methods was tested by the limits of detection (LOD) and limits of quantification (LOQ). Under current chromatographic conditions, LOD and LOQ were calculated on the basis of the response and slope of each regression equation at signal-to-noise ratios (S/N) of 3:1 and 10:1. LOD values ranged from 0.15 to 0.90 μg/kg, while the LOQ values ranged from 0.50 to 3.00 μg/kg. The results show in Table 1.

The selectivity was evaluated by the presence or the absence of inhibiting materials in the chromatographic windows of the results. As seen in Figure 1, 5 peaks are isolated well. The detection time for each peak was 7.6, 8.5, 9.6, 11.1, and 22.1 min. All substances were detected with good resolution and narrow symmetric peaks within 25 min. Thus 5 AO substances showed satisfactory peak isolation in the HPLC system.

The precision of the developed method was determined by measuring intra- and inter-day precisions. For intra-day precision test, the mixed standard solution was analyzed 6 times in a day, and for inter-day precision test, the analysis was repeated three times in a day for three consecutive days. The precision is expressed as a percentage of relative standard deviations (% RSD). The % RSD of intra- and inter-day was 0.8–5.1. This shows the accuracy of the proposed method. These validation data show that the proposed AO simultaneous determination method exhibits good linearity, sensitivity, selectivity and precision.

In order to measure the accuracy, the mixed standard solutions were added to AFFCO samples at 10, 20, 50 μg/kg concentrations and the analyses were repeated three times with the results of 78.1–94.4%. This corresponds to the recommended recovery rate of 70–110% by JECFA (2016). The

Table 1. Validation parameters of the developed method

Compound	Linear range (μg/kg)	r^2	LOD (μg/kg)	LOQ (μg/kg)	Rs	Asymm.	Precisions (% RSD)	
							Intra-day (n = 6)	Inter-day (n = 9)
Aflatoxin G2	1.73–17.3	0.9998	0.39	1.29	–	0.95	1.4	0.8
Aflatoxin G1	6.30–63.0	0.9997	0.84	2.77	1.55	1.04	4.8	4.1
Aflatoxin B2	1.74–17.4	0.9998	0.20	0.66	1.48	1.01	4.0	3.2
Aflatoxin B1	6.81–68.1	0.9999	0.53	1.75	2.07	0.94	5.1	4.9
Ochratoxin A	5.02–50.2	0.9999	1.01	3.33	17.4	0.95	2.0	1.8

Notes: r^2: Coefficients of correlation; LOD: Limit of detection; LOQ: Limit of quantification; Rs: Resolution; Asymm: Asymmetry.

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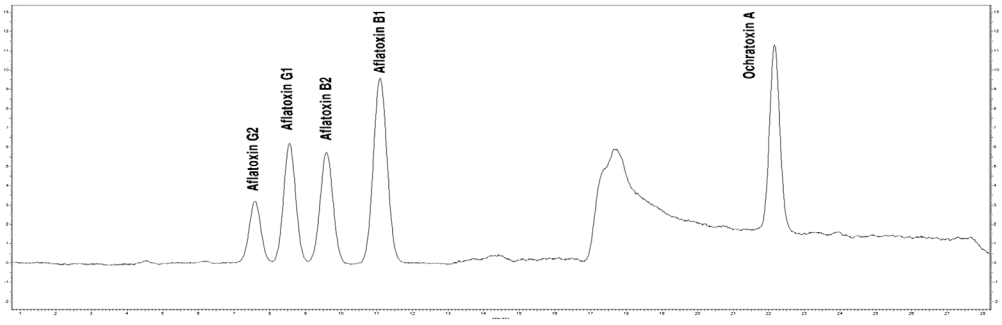

Figure 1. HPLC chromatogram of Aflatoxins and Ochratoxin A by established conditions.

Table 2. Recovery of Aflatoxin and Ochratoxin A in AFFCO samples

Compounds	Recovery (%) (*n* = 3)		
	10 µg/kg	20 µg/kg	50 µg/kg
Aflatoxin B1	91.3	94.3	87.0
Aflatoxin G1	82.2	84.4	81.1
Aflatoxin B2	85.1	94.4	92.4
Aflatoxin G2	79.4	78.1	80.4
Ochratoxin A	90.2	82.3	91.3

CRM of FAPAS results have the median values of 2.05, 2.79, 3.57, 5.72 µg/kg for Aflatoxin G2, G1, B2, B1 and 33.66 µg/kg for Ochratoxin A. In this study, Aflatoxin values are 1.89, 2.64, 3.41, 5.01 µg/kg and are deviations from the CRM median range from 87.6% to 95.5% and Ochratoxin A value of 32.1 µg/kg is 95% of the CRM median. Detailed data are shown in Table 2.

3.2. Monitoring of Aflatoxin and Ochratoxin A in feeds

The results of 2015 and 2016 analyses of feed distributed nationwide are shown in Table 3. In 2015, Aflatoxin or Ochratoxin A was detected in 44 out of 496 samples to result in 8.9% detection rate. In TMR, these toxins were detected in two samples (1.5%) for cattle, 5 samples (6.3%), 56 samples (32.1%) for chicken, 1 sample (16.7%) for ducks, 8 samples (10.3%) for dogs. None was detected in TMR for cats. In feed ingredients, toxins were detected in 4 grain byproduct samples (4.9%), 5 samples (16.1%) in meals (proteins), 1 sample (5.3%) in fibers. Ochratoxin A was only detected in TMR for pigs. The detected concentration range is 1.76–162.69 µg/kg for Aflatoxin B1, 23.01–45.42 µg/kg for Ochratoxin A. Aflatoxin B2, and G1 were detected in some of the feed samples, but the concentration was lower than LOQ and G2 was not detected at all. According to the national regulations, the sum of Aflatoxin B1, B2, G1, and G2 concentrations of Aflatoxin must be lower than 10–20 µg/kg in TMR and 50 µg/kg in feed ingredients. Thus, one sample of meals (proteins) that had 162.69 µg/kg does not meet the standards, and other feeds with toxin detection were within the limits of the national standards.

In 2016, 22.2% detection rate was obtained with contamination with these two toxins in 115 of 518 samples. In TMR for cattle, 37 samples (33.6%), for pigs 11 samples (12.0%), for chicken 10 samples (11.9%), for ducks 2 samples (14.3%), for dogs 14 samples (18.4%), for cats 2 samples (6.5%) are shown to have been contaminated. In feed ingredients, 12 samples (24.5%) of the grain byproducts, 5 samples (16.1%) of the meals (proteins), 2 samples (12.5%) of the fibers were shown to have been contaminated. The concentration ranges are 1.76–43.69 µg/kg for Aflatoxin B1, 3.38–15.80 µg/kg for Ochratoxin A. As in 2015, Aflatoxin B2 and G1 were detected in some of the feeds, but with the concentrations below LOQ. Aflatoxin G2 was not detected at all. One sample of grain

Table 3. Monitoring of Aaflatoxin and Ochratoxin A in feed

Year	Feed type		No.	No. of detected	Concentration (μg/kg)				
					Aflatoxin G2	Aflatoxin G1	Aflatoxin B2	Aflatoxin B1	Ochratoxin A
2015	Compound feeds	Cattle feeds	132	2	–	–	–	4.62–5.92	–
		Pig feeds	79	5	–	–	–	1.98–3.57	23.01–45.42
		Chicken feeds	56	18	–	N.D.	N.D.	1.90–14.67	–
		Duck feeds	6	1	–	–	–	2.68	–
		Dog feeds	78	8	–	N.D.	N.D.	1.89–14.17	–
		Cat feeds	14	0	–	–	–	–	–
	Single ingredient	By-products of grains	81	4	–	–	–	1.76–18.42	–
		Vegetable proteins	31	5	–	–	N.D.	1.98–162.69	–
		Fibrous feeds	19	1	–	–	–	8.88	–
2016	Compound feeds	Cattle feeds	110	37	–	N.D.	N.D.	2.40–15.91	3.52–15.80
		Pig feeds	92	11	–	–	–	1.85–1.87	3.40–12.65
		Chicken feeds	84	10	–	–	–	1.82–2.90	20.5
		Duck feeds	14	2	–	–	–	2.46–2.47	–
		Dog feeds	76	14	–	–	N.D.	2.40–5.68	3.82–8.10
		Cat feeds	31	2	–	–	–	2.05–4.58	–
	Single ingredient	By-products of grains	49	12	–	N.D.	N.D.	1.80–43.69	11.80–12.28
		Vegetable proteins	46	25	–	N.D.	N.D.	1.76–16.06	3.38–3.40
		Fibrous feeds	16	2	–	–	–	3.31–4.56	–

Notes: No.: Number of total tested sample; –: Not Detected; N.D: Lower than LOD.

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byproduct (cottonseed hulls) had a detection level of 43.69 μg/kg and did not meet the standards. Other feeds that were contaminated all had levels below regulations.

In 2015, Ochratoxin A was only detected in feed for pigs, but it was detected in feeds for different animals including cattle, pigs, chicken and dogs, albeit at levels lower than the regulation. However, the occurence of Ochratoxin A is increasing and continuous management is necessary.

4. Conclusion

Both of the feeds deemed unfit for higher than regulation level detection of AO are imported raw materials and this suggest the need for special management plans as a great percentage of the feed is sourced from imported raw materials. The studies on monitoring of AO conducted in 2015 and 2016 show that the mycotoxin contamination in Korea is generally at a safe level that meets the national regulation. However, Aflatoxin B1 contamination is observed in all types of feed and sometimes is contaminated at a very high level. Ochratoxin A is also shown to be contaminated in various types of feed.

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Competing Interests
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