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Simultaneous detection of multiple mycotoxins in broiler feeds using a liquid chromatography tandem-mass spectrometry

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ABSTRACT. Mycotoxins are secondary fungal metabolites that are typically present in grain and feed ingredients used for animal feeds. An analytical method using LC-ESI-MS/MS was developed to quantify nine mycotoxins, consisting of aflatoxin B_1 (AFB_1), AFB_2, AFG_1, AFG_2, T-2 toxin, deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA) and ochratoxin A (OTA) in broiler feeds. In total, 100 samples of broiler feeds were collected from poultry farms in Central Thailand. The survey found that AFB_1 and ZEA were the most prevalent mycotoxins in the feed samples at percentages of 93% and 63%, respectively. The limit of detections (LODs) of investigated mycotoxins was 0.20–0.78 *n*g/g. AFB_2, DON, AFG_1, NIV and T-2 toxin were also detectable at low contamination levels with percentages of 20%, 9%, 7%, 5% and 1%, respectively, whereas OTA and AFG_2 were not detected in any of the feed samples. These results suggest that there is a very low level of risk of the exposure to mycotoxins in feeds obtained from broiler farms in Central Thailand.
KEY WORDS: broiler feed, LC-ESI-MS/MS, mycotoxin contamination

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Mycotoxins are toxic secondary metabolites produced by filamentous fungi that are ubiquitous contaminants in agricultural commodities which are present in many feed and foodstuffs. The major genera of mycotoxin-producing fungi include; *Aspergillus, Fusarium* and *Penicillium* that frequently occur in major food crops in the field and result in contamination during storage. Mycotoxins can appear in the food chain, either by being consumed directly by humans or by use as livestock feed. Exposure of animals to mycotoxins can result in mycotoxin accumulation in different organs and tissues, thus entering the human food chain through animal products, such as meat, milk or eggs. The Food and Agricultural Organization of the United Nations (FAO) has estimated that up to 25% of the world's food crops are significantly contaminated with mycotoxins [13]. Poultry are highly susceptible to mycotoxicoses caused by aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON), fumonisins (FB_1 and FB_2), T-2 and HT-2 toxin [11]. The co-occurrence of multiple mycotoxins implies a potential risk of additional or even synergistic toxic effects after consumption of contaminated food or feed commodities [15].

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The negative effects of mycotoxins on chicken performance have been demonstrated in numerous studies. For example, feeding a high level (3.5 mg/kg of feed) of an AF mixture (i.e. 79% AFB_1, 16% AFG_1, 4% AFB_2 and 1% AFG_2) to broilers reduced their body weight and increased their liver and kidney weights [16]. *Fusarium* mycotoxins have been shown to adversely affect poultry. In addition to reduced feed intake and body weight gain, buccal-oral ulceration and plaque formation were observed when 7-day-old chicks were given T-2 toxin (4 or16 mg/kg of feed) or diacetoxyscirpenol (DAS) (4 or 16 mg/kg of feed). Similar effects were also observed in 1-day-old to 3-week-old chicks consuming T-2 toxin at 6 mg/kg of feed and in 24–25-week-old hens consuming DAS at 20 mg/kg of feed. Poultry also are adversely affected by both T-2 and DON, but are very resistant to the estrogenic effects of ZEA [3].

Thailand is located in the tropical zone, where there is a high risk of mycotoxin exposure; however, scientific reports on mycotoxins occurrence are limited. Studies indicated frequent contamination of foodstuff in this region with aflatoxins in peanut [21], deoxynivalenol in wheat products [10] and coincidental contamination with fumonisins and aflatoxins in corn samples [23]. More recent studies revealed frequent contamination of aflatoxins in black glutinous rice, brown rice, white rice, white glutinous rice and wheat flour as well as of zearalenone in corn kernels intended for human consumption [14]. Anukul *et al.* [1] discussed the recent findings of mycotoxins in food and feed, with emphasis on aflatoxins, fumonisins, ochratoxins and zearalenone.

To date, there has been an increase in the number of re-

strictive mycotoxin categories in foods and a reduction in the acceptable permitted levels in foods and feeds defined by the European Union and other developed countries [5, 6]. The analysis of mycotoxins is challenging as they are usually present in low concentrations in complex matrices, and they may occur in various combinations produced by a single or by several fungal species. During the last decade, liquid chromatography tandem mass spectrometry (LC–MS/MS) has been established as a powerful tool for the identification of mycotoxins in food [12]. Furthermore, the simultaneous determination of multi-component mycotoxin contaminants in food and feed was performed by LC-MS/MS [2, 12, 17, 18].

The current study was conducted to develop an analytical method for the simultaneous determination of multiple mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, T-2, DON, NIV, ZEA and OTA) in broiler feeds by using LC-ESI-MS/MS.

MATERIALS AND METHODS

Chemicals and reagents: The analytical standards of the mycotoxins, AFB_1, AFB_2, AFG_1, AFG_2, T-2 toxin, DON, NIV, ZEA and OTA, were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). De-ionized distilled water was produced using the Milli-Q purification system from Millipore, Inc. (Bedford, MA, U.S.A.). HPLC grade acetronitrile and acetic acid were obtained from Burdick & Jackson (Ulsan, Korea), while ammonium acetate was purchased from Ajax Finechem Pty Ltd. (Taren Point, NSW, Australia).

Stock and working standard preparation: Combinations of standard mycotoxin stocks were prepared in methanol at a concentration of 1,000 *n*g/m*l* for the high sensitivity group (AFB_1, AFB_2, AFG_1, OTA and T-2) and for the low sensitivity group (AFG_2, DON, ZEA and NIV).The working standard solutions were prepared by diluting this stock of standard mixture with methanol at different concentration ranges as follows: 1.0–250 *n*g/m*l* for the high sensitivity group and 2.0–500 *n*g/m*l* for the low sensitivity group.

Sampling and sample preparation: In total, 100 broiler feed samples (10 feed samples per each province) were randomly collected from broiler farms in Central Thailand from Bangkok, Pathum Thani, Nakhon Nayok, Nakhon Pathom, Ayutthaya, Saraburi, Lopburi, Supanburi, Chainart, Uthai Thani and Nakhonsawan provinces. In this study, the collected samples were commercial feed that were produced in Thailand. The major ingredients in broiler feed samples include corn, fish meal and soybean. The feed samples were kept at −20°C until analysis.

The feed samples were blended, and then, a 10 g ground feed sample was homogenized with 40 m*l* of the organic extraction solvent mixture (acetronitrile:water:acetic acid, 79:20:1 v/v) by shaking for 60 min. The crude extract was centrifuged at 1,509 *g* for 10 min as described by Soleimany *et al.* [17]. Then, 0.5 m*l* of the supernatant was diluted in 4.5 m*l* of acetronitrile:water:acetic acid, 20:79:1 v/v. The extract was filtered by passing through a 0.22 μm syringe filter and then injected into the unit for LC-MS/MS [17, 18].

LC-MS/MS equipment and parameters: LC analysis was performed using an Agilent 1260 infinity (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, a degasser, a column oven and an auto sampler. The chromatographic separation was performed on a ZORBAX Eclipse Plus RRHT C18 column (50 × 2.1 mm, 1.8 μm particle size). The column was maintained at 40°C. The mobile phase consisted of 5 mM ammonium acetate with 0.2% acetic acid in water (mobile phase A) and 0.2% acetic acid in acetonitrile (mobile phase B). The gradient program of the mobile phase was as follows: 10% B (initial for 1 min), 10–95% B (5 min) and 95% B (10 min). The column was re-equilibrated for 3.5 min between injections. The mobile phase solution was filtered through a 0.22 μm membrane and ultrasonically degassed prior to application. The flow rate was 0.5 m*l*/min, while the injection volume was 10 μl.

The mass spectrometer used was a triple quadrupole mass spectrometer (6460 triple, Agilent Technologies) equipped with an electrospray ionization source run in both positive and negative ion modes under the multiple reaction monitoring mode (MRM). The ionization source parameters were optimized as follows: capillary voltage, 3,500 V; gas temperature, 320°C; gas flow rate, 8 *l*/min; and nebulizer, 50 psi. The parameters (for the mass spectrometer) were optimized as shown in Table 1.

Method validation: Validation of the LC-MS/MS method was performed to assess the efficiency of this analytical method by investigating the selectivity, sensitivity, accuracy and precision in accordance with the European Commission regulation for the performance of analytical methods [4].

Linearity: The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample. Linear regression analysis was conducted for the mycotoxin standard mixtures of the AFB_1, AFB_2, AFG_1, AFG_2, OTA, T-2, ZEA, DON and NIV toxins under the optimized LC-MS/MS conditions. Eight-point calibration curves were plotted as the peak area ratio (y) of each mycotoxin against its concentration ($y=mx \pm c$) at the concentration ranges of 1.0–250 *n*g/m*l* for AFB_1, AFB_2, AFG_1, T-2 and OTA; and 2–500 *n*g/m*l* for AFG_2, DON, ZEA and NIV. The linearity was assessed using the correlation coefficient (r^2) (Table 2).

Limit of detection and limit of quantification: The limit of detection (LOD) corresponds to the concentration that will give a signal-to-noise (S/N) ratio of 3:1. The limit of quantification (LOQ) is defined as the concentration of related substance in the sample that will give an S/N ratio of 10:1 (Table 2).

Recovery and precision: Recovery and precision (repeatability, expressed as relative standard deviation (RSD) in%) were determined within-day by analyzing seven replicates containing nine mycotoxins at three different QC levels (Table 3). The inter-day precisions were determined by analyzing QC samples on five different days (one batch per day) (Table 3).

Table 1. MS/MS parameters for the determination of nine mycotoxins

Mycotoxins	Precusor ion (*m/z*)	Product ions (*m/z*)	Collision energy (eV)	Retention time (min)	Fragmentor (eV)	Ionization mode
AFB_1	313.07	285.1	21	5.13	150	ESI^+
	313.07	241.0	35		150	
AFB_2	315.09	287.1	25	4.99	160	ESI^+
	315.09	259.0	29		160	
AFG_1	329.07	311.0	21	4.98	150	ESI^+
	329.07	243.0	25		150	
AFG_2	331.08	313.0	21	4.85	170	ESI^+
	331.08	245.0	29		170	
T-2	489.40	387.3	20	5.6	170	ESI^+
	489.40	245.2	26		170	
OTA	404.00	192.9	48	5.78	130	ESI^+
	404.00	102.1	80		130	
ZEA	319.16	283.0	5	5.94	80	ESI^+
	319.16	187.0	17		80	
DON	355.10	265.1	4	3.5	90	ESI^-
	355.10	59.1	10		90	
NIV	371.10	281.0	4	2.1	80	ESI^-
	371.10	59.1	10		80	

AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; OTA, Ochratoxin A; ZEA, Zearalenone; DON, Deoxynivalenol; NIV, Nivalenol; ESI^+, electrospray ionization in positive mode; ESI^-, electrospray ionization in negative mode.

Table 2. Linearity, limit of detection (LOD) and limit of quantification (LOQ) of the optimized LC-MS/MS method for simultaneous determination of mycotoxins

Mycotoxins	LOD in feed sample (*n*g/g)	LOQ in feed sample (*n*g/g)	Linear range (*n*g/g)	r^2
AFB_1	0.39	1.0	1.0–250	0.9993
AFB_2	0.48	1.0	1.0–250	0.9989
AFG_1	0.20	1.0	1.0–250	0.9995
AFG_2	0.78	2.0	2.0–500	0.9986
T-2	0.39	1.0	1.0–250	0.9963
OTA	0.39	1.0	1.0–250	0.9991
ZEA	0.78	2.0	2.0–500	0.9970
DON	0.78	2.0	2.0–500	0.9989
NIV	0.78	2.0	2.0–500	0.9999

r^2, correlation coefficient.

RESULTS

Optimization of LC-MS/MS conditions: For the multi-mycotoxin method, it was decided to perform simultaneous determination within a single run with detection in positive and negative modes. In both ionization modes, the best sensitivity for all investigated compounds was achieved using 5 mM ammonium acetate in 0.2% acetic acid (mobile phase A) and 0.2% acetic acid in acetronitrile (mobile phase B) as the mobile phase. In addition, nearly all compounds could be separated, except AFG_1 and AFB_2, which eluted at the same retention time (Fig. 1). The differentiation of AFG_1 and AFB_2, the $[M+H]^+$ mass numbers of which are 329.07 and 315.09, respectively, could be achieved by the difference of characteristic parent ions in the multiple reaction monitor (MRM) mode. Furthermore, when considering that the selected product ions were 329.07 >311, 329.07 >243 and 315.09 >287.1, 315.09 >259 for AFG_1 and AFB_2, respectively, the selectivity of daughter ions could not disturb each other.

LC-MS/MS validation method: The linearity and sensitivity results are reported in Table 2. The method exhibited good linearity over the relevant working range, with r^2 values between 0.9963 (for T-2) and 0.9999 (for NIV). The LOD and LOQ values in matrices ranged from 0.20 to 0.78 *n*g/g and from 0.78 to 1.56 *n*g/g, respectively (Table 2). The recoveries were obtained within the range of 81–103% for all nine mycotoxins, as summarized in Table 3.

Occurrence of the mycotoxins in broiler feeds: The 100 broiler feed samples were quantified for the nine mycotoxins studied by simultaneous determination using LC-MS/MS. In summary, 93% of the samples contained AFB_1 at concentrations ranging from 0.47 to 8.52 *n*g/g. The second most prevalent mycotoxin was ZEA found in 63% of the samples at concentrations ranging from 2.22 to 263.51 *n*g/g. The other mycotoxins found to be contaminating feed samples were AFB_2, DON, AFG_1, NIV and T-2 in 20%, 9%, 7%, 5% and 1%, respectively, of samples. In the present study, AFG_2 and OTA were not detectable in broiler feed samples (Table 4). The co-occurrence of mycotoxins was also detected in broiler feeds, with AFB_1 and ZEA in 46 of 100 feed samples, AFB_1 and DON in 9 of 100 feed samples, AFB_1 and NIV in 4 of 100 feed samples, AFB_1, AFB_2 and ZEA in 13 of the 100 feed samples and aflatoxins (AFB_1, AFB_2 and AFG_1) in 4 of the 100 feed samples.

Table 3. Accuracy and precision for mycotoxin determination in optimal LC-MS/MS conditions for spiked broiler feed samples

Mycotoxins	Spiking level (*n*g/g)	Recovery (%)	Intra-day precision (%RSD)	Inter-day precision (%RSD)
AFB_1	2	96	5.46	6.37
	10	98	3.37	5.43
	50	101	3.42	3.89
AFB_2	2	97	6.38	7.98
	10	95	4.43	4.64
	50	98	2.21	3.17
AFG_1	2	92	3.61	3.77
	10	95	4.86	4.22
	50	103	2.70	3.45
AFG_2	10	91	2.59	2.96
	50	96	7.28	7.67
	200	88	5.76	7.11
T-2	2	92	6.41	6.11
	10	87	5.53	5.79
	50	95	3.15	3.48
OTA	2	89	5.13	4.35
	10	85	4.64	3.87
	50	86	3.79	3.82
ZEA	10	93	3.63	4.05
	50	94	5.02	4.69
	200	92	1.78	1.98
DON	10	82	5.46	4.86
	50	84	3.37	3.51
	200	85	3.42	2.89
NIV	10	82	2.51	3.15
	50	81	1.87	2.03
	200	84	2.27	1.89

DISCUSSION

The results illustrated that AFB_1 and ZEA were the most common of the contaminants in broiler feed. On the other hand, detection ratios of DON and NIV, which are *Fusarium* toxins, are very low due to the compositions of such feed samples. Accordingly, processing and other ingredients, such as corn, soybean and fish meal, can also play a role regarding AFB_1 and ZEA contaminations in broiler feed samples. The levels of mycotoxin contamination in the samples were below the regulatory limits, which corresponded to the U.S. Food and Drug Administration advisory level (FDA). These results suggest that risk of the exposure to mycotoxins in completed feeds obtained from broiler farms in Central Thailand is very low. On the other hand, low concentrations of mycotoxin contaminants, such as aflatoxins, trichothecenes and zearalenone, can cause a reduction in growth performance in pigs and broilers [8]. Moreover, the co-contamination of mycotoxins usually occurs in food or feeds, which can increase the potency of toxicity to the animals. Poor management of farm animals, especially with regard to farm feed storage and farm feeding, may have a major influence on the level of mycotoxin contamination in broiler feeds and relate to increased mycotoxin production [24]. The result of the present study corresponded well with the study of Streit *et al.* [19], since they also surveyed the contamination of mycotoxins in finished feed samples, with 2,743 samples analyzed over a period of 8 years (2004–2011). They found that Fumonisins, DON, ZEA, AF and OTA were the most prevalent in finished feed samples in South-East Asia. Khatoon *et al.* [9] also reported that the contamination of NIV and DON was found with relatively low level in broiler feeds in Pakistan. However, an UHPLC-MS/MS-based method for the determination of 191 mycotoxins and other fungal metabolites was developed [20]. They reported that UHPLC-MS/MS allowed better separation of the analytes from the matrix. Regarding the method validation, the recovery levels were slightly better than those reported by Ren *et al.* [11] (76.2–102%), Yibadatihan *et al.* [22] (81–112%) and Soleimany *et al.* [17] (76.8–108.4%). The intra- and inter-day precision values were in the range of 1.8–7.3% and 1.9–7.7%, respectively. All the recovery and precision results were in agreement with performance criteria recommended by European Commission Regulation No. 401/2006 /EC [7].

In summary, an analytical method was developed for the simultaneous determination of nine mycotoxins in broiler feeds by using LC-MS/MS. This method can detect mycotoxins within a single run. The analytical method was demonstrated to be an excellent tool for unambiguous identifica-

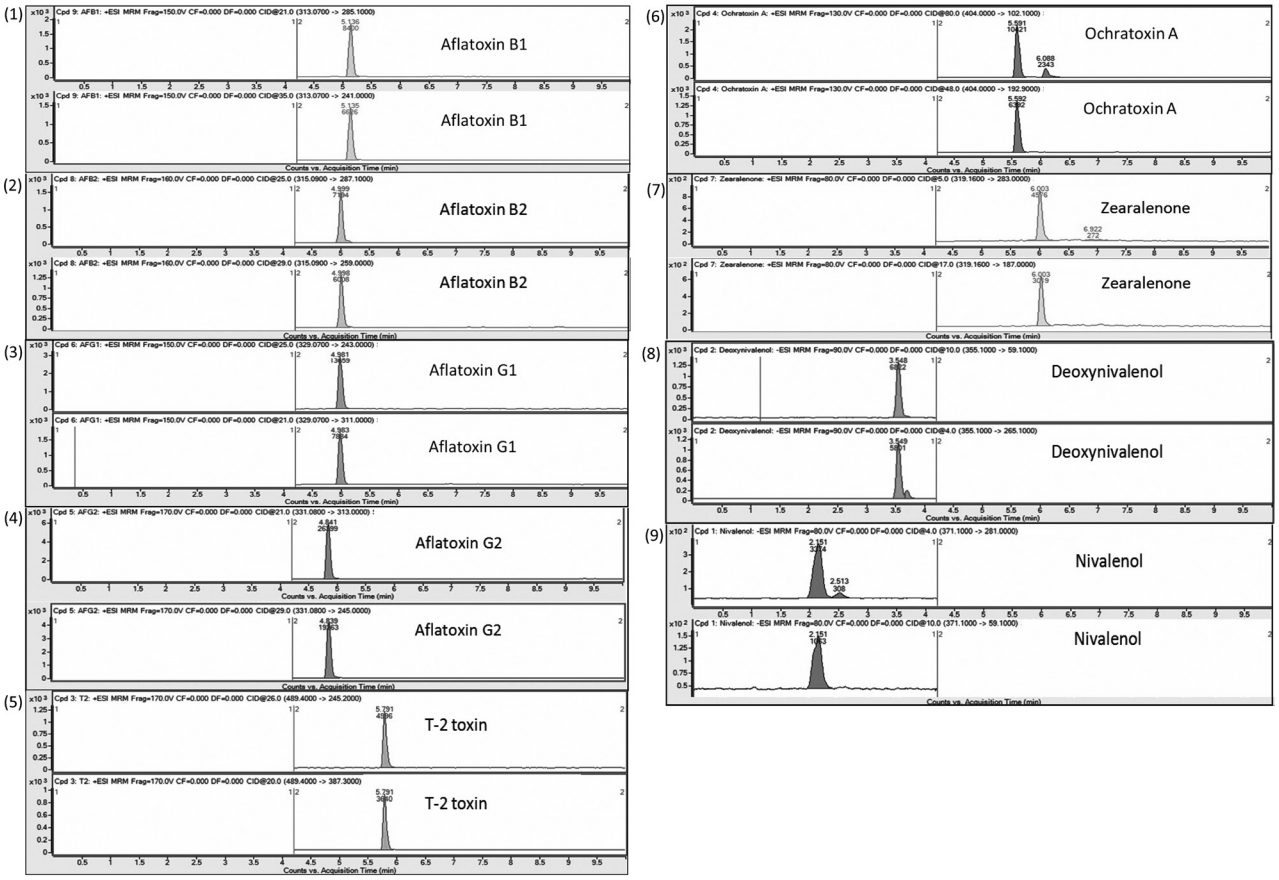

Fig. 1. LC-MS/MS extracted ion chromatogram of the spiked broiler feed sample for mycotoxins under optimized conditions of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, T-2 toxin, ochratoxin A, zearalenone, deoxynivalenol and nivalenol. Two mass transitions per each analyte were determined for quantification and identification. Upper chromatogram for each toxin represents the quantifier transition ion, whereas lower chromatogram represents the qualifier transition ion.

Table 4. Occurrence of mycotoxins in broiler feeds (total of 100 samples)

Mycotoxins	Percentage of positive samples	Range of concentration level of contaminations (*n*g/g)	Mean of concentration (*n*g/g)
AFB_1	93	0.47–8.52	2.02
AFB_2	20	0.79–3.30	1.87
AFG_1	7	0.66–1.89	1.30
AFG_2	ND	ND	ND
T-2	1	1.15	1.15
OTA	ND	ND	ND
ZEA	63	2.22–263.51	84.27
DON	9	33.58–60.81	45.05
NIV	5	12.75–35.83	27.26

Mean of Concentration (*n*g/g) is the mean of the positive samples. ND=Not detected (lower than LOD).

tion of mycotoxins using the retention time, accurate mass of precursor and product ions. Furthermore, this is the first study to present data on multi-toxin occurrence in completed feeds obtained from broiler farms in Thailand using LC-ESI-MS/MS. The results of mycotoxin contamination in broiler feeds showed that aflatoxins are the most common present mycotoxin (mainly AFB_1) and can co-occur with other mycotoxins (AFB_1 and ZEA). This evidence highlighted that the production of mycotoxins differs according to the climatic conditions especially as Thailand is located in the tropical zone and the climatic conditions which are suitable for mycotoxin production. Nevertheless, the levels of mycotoxin contamination in the feeds were below the U.S. Food and Drug Administration advisory levels (FDA). These results

also suggest that the risk level of exposure to mycotoxins in completed feeds obtained from brolier farms in Central Thailand is very low due to the low level contamination.

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