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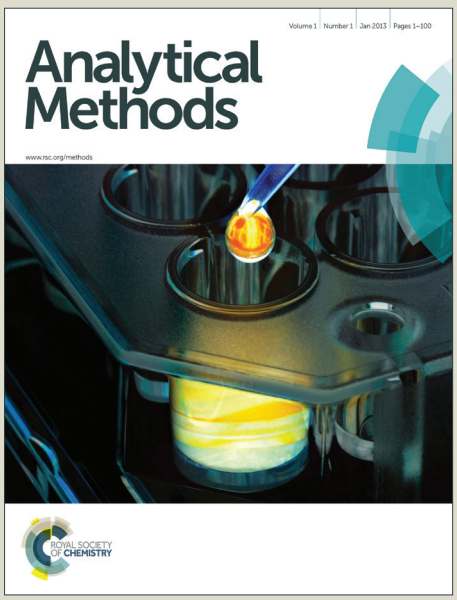

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Ultra-high performance liquid chromatography tandem mass spectrometry for simultaneous analysis of aflatoxins B1, G1, B2, G2, zearalenone and its metabolites in eggs using a QuEChERS-based extraction procedure

Yonggang Li[a,b], Sheng Wen[b,*] , Ziliang Chen[b], Zhidong Xiao[a], Meihu Ma[a,*]
([a]National Research and Development Center for Egg Processing, College of Food Science and Technology, Huazhong Agricultural University, Wuhan, R.P. China，[b]Hubei Provincial Centre for Disease Control and Prevention, Wuhan, 430079)

*Correspondence Author
Phone: +86 27 87283177
Fax: +86 27 87283177
Email: mameihuhn@163.com

*The author contributed equally to this work.

Abstract

A reliable method for the simultaneous determination of aflatoxins B1, G1, B2, G2, Zearalenone and its metabolites α-Zearalanol， β-Zearalanol was developed. Mycotoxins have been extracted from eggs using a QuEChERS-based extraction procedure (Quick, Easy, Cheap, Effective, Rugged and Safe) followed by applying C18 and PSA further clean-up step, then detected by ultra high performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS) using an electro spray-ionization interface (ESI) in both positive and negative ion modes. Matrix-matched calibration was used for quantification in order to reduce the matrix effect. Validation of the method was carried out in eggs by recovery experiments. Recoveries of the spiked samples were in the range between 84.8 and 114.6% with intra-day relative standard deviation, lower than 20.7% and inter-day relative standard deviation lower than 18.2%. Limits of quantification (LOQ) ranged from 3.0–15.0 μg kg^{-1}. The developed method was successfully applied to the analysis of eggs and positive sample which contaminated by AFB1 was detected.

Keywords: UPLC-MS/MS; aflatoxins; zearalenone; metabolites; QuEChERS; eggs

1 Introduction

Aflatoxins B1, G1, B2 and G2 (AFB1, AFG1, AFB2 and AFG2) are fungal secondary toxic metabolites produced by A*spergillus flavus*, A. *parasiticus* and A. *nomiu*[1] whose structures are shown in Figure1. Letters 'B' and 'G' means blue and green fluorescence colors generated by them under UV light and numbers 1 and 2 show major and minor compounds, respectively[2]. The event appeared in 1960 that 100000 turkey poults died was caused by the ingestion of peanut contaminated with Aflatoxins[3]. These compounds are toxic and pose a health hazard to humans and animals such as the production of several hormonal disorders or immunsuppression to the induction of carcinogenic, teratogenic or mutagenic activities. Among them, Aflatoxin B1 can cause liver and kidney toxicity in several species and is most prominently known as a potent liver carcinogen in humans and animals, which is considered the most toxic[4]. Their order of toxicity is B1 > G1 > B2 > G2. The International Agency for Research on Cancer (IARC) has classified aflatoxin B1, G1, B2 and G2 in group 1 as human carcinogens[1]. Zearalenone(ZON) is macrocyclic lactone derivatives of resorcinol acid which can be produce by several *Fusarium* moulds, mainly *F. graminearum, F. culmorum, F. equiseti* and *F. crookwellense*[5], it exhibits distinct estrogenic and anabolic properties in several animal species, resulting in changes in genital organs and in reproductive problems although its acute toxicity is relatively low. Currently, the International Agency for Research on Cancer classifies zearalenone as a 2A carcinogen, the highest possible classification when categorical human epidemiology is absent[4]. In vivo, zearalenone is transformed into

its major metabolites (α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), and according to report, the estrogenic activity of α-ZEL is higher than for the parent compound [6, 7]. More recently, it was demonstrated that a further reduction of α-zearalenol and β-zearalenol may occur in several animal species such as deer, goats, sheep, cattle, and horses forming into α-zearalanol (zeranol) (α-ZAL) and β-zearalanol (taleranol) (β-ZAL) that showed in Figure 2[7, 8]. These metabolites have been detected in urine and bile. It has been demonstrated that zearalenone and its metabolites may cause carcinogenesis or teratogenesis in some species, but further research is needed. Further research is also needed with regard to human toxicity.

Many methods have been published for the detection and quantification of these compounds due to their potential threat to humans and animals. The enzyme linked immunosorbent assay (ELISA) is widely used for the fast screening analysis of mycotoxins[9, 10], however, unavoidable false-positive results restrict its further application. Thin layer chromatography (TLC)[11, 12] as confirmatory quantification method is simple, rapid and economical but its application have been limited because of unsatisfied accuracy of quantification and poor separation. LC coupled to fluorescence detectors (FLD)[9, 13-17] or UV-diode array detectors (DAD)[18] with pre-column derivatization or post-column derivatization was commonly used for the detection of AFs and achieved good results. But derivatization procedure takes a long time which make the high throughput impossible. As for zearalenone and its metabolites, HPLC methods using a fluorescence detector can be used as sufficiently sensitive and efficient in terms of separation due to their strong native fluorescent

activity [19, 20]. In the recent years, HPLC coupled to mass spectrometry (MS), or preferably to tandem mass spectrometry (MS/MS)[21-24] had become the best choice for the determination of these compounds because of its advantages of high selectivity and sensitivity, time-saving and the accuracy of quantification and confirmation especially the application of UPLC substantially reduce the detection time. In addition, these reported methods involved all kinds of matrix such as cereals, feeds, bee honey, medicinal herbs, Chinese medicines, edible oils, chicken liver, peanuts, broiler meat, chilli, rice, breast milk, beer, tea. Although so many methods were reported for the analysis of these compounds in various matrix, many of them analyze aflatoxins independently of zearalenone and its metabolites. At present, there is almost no study to analyze them simultaneously especially α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) in eggs. One method detected aflatoxins without zearalenone in eggs have been reported by Antonia Garrido Frenich etc.[25] whose recoveries are low relatively compared with our method, Saqer M. Herzallah[26] have ever published one paper which determinate aflatoxins in eggs, milk, meat and meat products using HPLC fluorescent and UV detectors, but we can't find any data about eggs in the paper. Shahzad Zafar Iqbal etc.[27] have nanlyzed aflatoxins , ochratox in A and zearalenone in chicken meat and eggs applied HPLC equipped with fluorescence detector. The LOQ of this method ranged from 0.15 μg/ml-0.3μg/ml, however, it took a long time for analysis and decreased the analysis efficiency.

Bearing in mind that egg is essential in diet and these mycotoxins may be transfered into eggs after feeding of contaminated hay or corn. It is important to avoid

mycotoxins occurrence which will pose hazard to public health due to their toxicity and assure the safety of this product, however, at present, there is not specific maximum levels legislation of mycotoxin in eggs. For these reasons, the development of a fast, sensitive and reliable method for the simultaneous determination of aflatoxins, zearalenone and its metabolites in eggs was needed urgently.

Solid phase extraction cartridge or multifunctional column were applied to clean up after extracting to induce the matrix interference in many reported methods, which are expensive and time consuming. Thus, it is necessary to develop generic extraction procedures to make fast, cost-effective and accurate detection possible. Fortunately, in the last few years, several approaches such as the well-known QuEChERS procedure (acronym name for Quick, Easy, Cheap, Effective, Rugged, and Safe), and "dilute and shoot" methodologies[28] have been developed. Especially, QuEChERS has been used for the extraction of pesticides [29, 30] and other compounds such as mycotoxins from various food matrixes [30-32].

In this study, a modified QuEChERS sample extraction method followed by matrix solid phase dispersion cleanup coupled with UPLC–MS/MS has been developed for the simultaneous determination of aflatoxins, zearalenone and its metabolites in eggs. The developed method can be used to determine these compounds in egg samples due to the effectiveness of the extraction procedure and the fast chromatographic analysis.

2 Experimental

2.1. Materials and reagents

Standards for AFs (AFB1, AFB2, AFG1 and AFG2), ZON, α-ZAL and β-ZAL were obtained from Romer labs GmbH (Union, MO). HPLC-grade methanol were obtained from TEDIA (Ohio State，USA）and acetic acid (purity>99.8%) was supplied by CNW Technologies GmbH (European Union). Water was purified using a Milli-Q purification system (resistivity, 18.2 MΩ, Millipore, Bedford, MA, USA). Anhydrous magnesium sulphate and sodium chloride were supplied by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Bondesil-C18 (40μm, 100mg), PSA (Primary secondary amine) (40μm, 100gm) and Graphitized carbon black (GCB) were purchased from Agilent (CA, USA).

Stock standard solutions of individual compounds were prepared in acetonitrile by exact weighing of the compounds and stored at -20 °C, multi-compound working standard solution was prepared by diluting stock solutions with acetonitrile and stored at 4 °C.

2.2. Instruments and apparatus

LC analysis was performed in an Agilent 1200SL Series Rapid Resolution LC System (CA, USA) equipped with a binary pump, degasser, autosampler and column heater. An SB-Aq C18 column (100mm × 3.0 mm, 1.8μm particle size) from Agilent was used for chromatographic separation. MS/MS detection was performed using an Agilent 6460 Triple Quadrupole LC/MS (QQQ) equipped with an ESI source.

Vortex mixer, model HQ-60 was obtained from Beijing North TZ-Biotechnology Development Corp. Ltd. (Beijing, China). Nitrogen evaporator was from Plus Century (Beijing, China). A Centrifugation was performed in centrifuge obtained from

HERMLE Labortechnik GmbH (Stuttgart, Germany).

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile (eluent A) and water (eluent B). The gradient elution program started at 30% of eluent A, keeping constant for 3min and increased linearly up to 90% in 2min, keeping constant for 2min then returned to the initial conditions in 1.5min, and keeping constant for 6.5min in order to equilibrate the column better. The flow-rate was set at 0.25 mL min–1, and column temperature was kept at 40°C. The injection volume was 10 μL.

2.4. MS/MS conditions

The mass spectrometer was connected to the UPLC system via an electrospray ionization (ESI) interface, source parameters were as follows: capillary voltage, 3.5kV; sheath Gas temperature, 320°C; Sheath Gas flow-rates 11 L min-1; Gas temperature 345°C; Gas flow 6L min-1; Nebulizer pressure, 45psi; Nozzle Voltage 500V.

The multiple reaction monitoring mode (MRM) was used for quantification. The parameters of transitions and the applied cone voltages and collision energy of are shown in Table 1.

2.5. Sample preparation

Eggs were homogenized at room temperature through continuous agitation for 5 min, then 2g sample was transformed into a polypropylene centrifuge tube (50mL), 2mL water was added and vortexed for 2min. Subsequently 10mL acetonitrile with 1% acetic acid was added, the mixture was shaken horizontally for 30 min. After that, 4 g anhydrous magnesium sulphate and 1g sodium chloride were added and the

mixture was vortexed for 2 min. After centrifugation at 5000g for 5 min, 1.5mL of the upper layer was taken, PSA and C18 100mg, respectively, were added for the dispersive SPE cleanup. Centrifuging at 5000g for another 5 min, 1mL supernatant was taken and dried with N_2 gas at 40 °C, then redissolved by 500 μL of mixture of acetonitrile and water (1:1, v:v) . After filtering through a 0.22μm nylon filter, the sample was injected into the UPLC–MS/MS system.

2.6. Method validation

Linearity, trueness, precision, limit of detection (LOD) and limit of quantification (LOQ) were applied for evaluating the applicability of optimized method.

Because of the matrix effects, linearity was tested using matrix-matched calibration curves by spiking blank extract eggs.

Trueness and precision (intra-day and inter-day) were studied by spiking blank eggs at three fortification levels which showed in table 3 by analyzing six replicates at each concentration. Inter-day precision was evaluated by analyzing six spiked samples on three separate days. Limits of quantification (LOQ) and limits of detections (LOD) were determined as the amount of analyte for which signal-to-noise ratios (S/N) were equal than 10 and 3 respectively.

3 Results and discussion

3.1. Optimization of UPLC-MS/MS conditions

ESI positive and negative ion modes were evaluated, observing that the best response for aflatoxins was achieved working in positive ESI mode; meanwhile, ZON exhibited good signal response and little interference in negative mode, despite

reports published by Sofie Monbaliu et al. [33]and F. Soleimany et al.[34] showed that ZON was better detected in positive mode. Futhermore, although ammonium acetate [24, 35, 36] was used in some studies, nice peak shapes and best separation of ZON, α-ZAL and β-ZAL were obtained in our paper when water and acetonitrile were selected as mobile phase which showed in figure 3.

However, it is not possible to avoid the co-elution of some of the targeted mycotoxins in the chromatographic separation system, such as AFB2 and AFG1. Due to their similar molecular structure and physicochemical property, thus it is hard to separated them. However, the selective detection power of MS/MS can facilitate their identification. Full scan mode displayed that $[M + H]^+$ mass numbers of which are 315.2 and 329.2 in turn. Furthermore, when considering that the selected daughter ions were 315.2 > 287.1, 315.2 > 259.1 and 329.2 > 311.1, 329.2 > 243.1 for AFB2 and AFG1, respectively, the selectivity of daughter ions could not disturb each other.

3.2. Sample pretreatment optimization

3.2.1. Selection of extraction solvent

In many reported mycotoxin detection methods, the critical and complicated extraction and clean-up procedure is used, especially when complex matrix was analyzed. To reduce sample handling and increase throughput, a simple extraction QuEChERS-based procedure was chosen before chromatographic determination.

Bearing in mind that the characters of egg matrix, that is containing more than 70% water, pure acetonitrile was evaluated as the extraction solvent despite that conventional extraction procedures of mycotoxins from different samples use a mixture of acetonitrile/water(84/16,v/v)[35], acetonitrile/water(80/20,v/v)[37,38] or methanol/water(80/20,v/v)[25]. According to the previous reports[3, 37, 39] we tried to add

acid in acetontrile, expecting a better results. Consequently, it can be observed that the best results were obtained when the acetic acid was added, allowing the extraction of compounds with suitable recoveries, on the contraty, without acetic acid addition, matrix enhancement effect is obvious. The results with or without acetic acid addition was shown in figure 3.

3.2.2. Optimization of clean-up procedure

In some reported methods, crude extracts were injected into UPLC-MS/MS directly for detection without any clean-up procedure[1, 33, 34, 36, 39]. A clean-up procedure after the extraction was necessary considering that some interfering compounds could be co-extracted, interfering with mycotoxin detection and reducing the lifetime of the column. Conventional SPE cartridges such as OASIS HLB[1]or C18[33], Mycosep 226 or 228 Multifunctional cartridges [35] and immunoaffinity column[23, 40, 41] were used according to some reported papers, although they made samples purified, they are time-consuming and expensive. In our paper, dispersive solid phase extraction utilizing sorbents was employed to reduce the co-elution of matrix. According to previous reports[42-44], PSA、C18 and GCB sorbents were evaluted in order to obtain the best results. Unfortunately, due to the addtion of GCB sorbent, the recoveries of AFs were decreased apparently, hence GCB was not applicable in our procedure. Figure 4 showed the impact on recoveries with the addtion of GCB sorbent.

3.3. Matrix effect

The presence of matrix components can affect the ionization efficiency of

analytes, leading to either suppression or enhancement of the signal. This may be caused by competition between the analyte and co-elution for the available charge, or because of the influence of matrix components on the release of ions from the electrospray droplets to the gas phase. To evaluate the matrix effect, the slope ratios of the matrix-matched calibration were compared to the standard calibration in pure solvent, which is referred to as the signal suppression/enhancement (SSE) value, was calculated for each analyte. Usually, when the SSE value was between 0.8 and 1.2, signal suppression or enhancement effect was considered acceptable, and the values outside this range indicated a strong matrix effect, which may influence the quantification results. Fig. 5 shows slope ratios matrix/solvent for each analyte. It can be observed that matrix suppresses the response for ZON, however, for others, there are almost no signal suppression indicating that our pretreatment procedure is perfect.

3.4. Validation

3.4.1. Linearity

Calibration curves were constructed with concentration sequences of 2, 4, 8, 20, 40 ng ml-1 for AFB1 and AFG1, 0.6, 1.2, 2.4, 6, 12 ng ml-1 for AFB2 and AFG2, and 2.5, 5,10, 20, 50 ng ml-1 for ZON, 3.75、7.5、15、30、75 ng ml^{-1} for α-ZAL and 4.1875、8.375、16.75、33.5、83.75 ng ml^{-1} for β-ZAL. Correlation coefficients (r) were greater than 0.9900. Table 2 shows these results, which demonstrates that the method can be used to monitor these analyte residues eggs.

3.4. 2. Trueness and precision

Recoveries, intra-day precision and inter-day precision for each compound at low,

intermediate and high concentration levels were performed by adding standard to analyte-free egg samples. The mean recoveries and coefficients of variation of target compounds are shown in Table 3. The RSDs of the intra-day precision were in the range 3.1–20.7% and the RSDs of inter-day precision were in the range 7.4–18.2%. Figure 6 shows the MRM chromatograms of blank samples fortified with target analytes. All these data revealed that the established method had an acceptable result.

3.4.3. Limit of detection and limit of quantification

The detection (quantitation) limits were determined by successive analyses of chromatographic extracts of egg spiked samples with decreasing amounts of every mycotoxin standard until a signal-to-noise ratio of 3 : 1 (10 : 1) was reached. The LOD and LOQ of AFB1and AFB2 are 1.0 and 3.0 μg kg^{-1}, 5.0 and 15 μg kg^{-1} for AFG1, 2.0 and 6.0 μg kg^{-1} for AFG2 and ZON, 1.5 and 5.0 μg kg^{-1} for α-ZAL and β-ZAL respectively, which are shown in Table 3.

3.4.4. Application to real samples

The develop method was applied to 40 egg samples collected from markets. As a result, aflatoxin B1 were detected with the amount of 3.1 μg kg^{-1} in one sample which showed in figure 7, indicating the method we set up was suitable for the detection of analytes.

4 Conclusions

A new method based on QuEChERS extraction procedure and UPLC–MS/MS detection was developed for the simultaneous determination of AFB1, AFB2, ABG1, AFG2、ZON, α-ZAL and β-ZAL in egg. Acetonitrile acidified with acetic acid was

used as extractant followed by PSA and C18 sorbent clean-up step, then detected by UPLC-MS/MS. The method gives quantitative results for the assayed mycotoxins, providing good validation parameters in terms of linearity, trueness, precision and LOQ. Finally, this method was applied to real samples and positive sample was found. This method is simple, fast and high throughput which could be applied for regular monitoring of mycotoxins in eggs by routine.

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