Development of a sensitive and reliable high performance liquid chromatography method with fluorescence detection for high-throughput analysis of multi-class mycotoxins in Coix seed

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HIGHLIGHTS
• A USLE-IAC-HPLC-PCD-FLD method was developed.
• High-throughput analysis of multi-class mycotoxins in Coix seed was performed.
• Method allowed the determination of the analytes at low µg kg−1 level.
• Samples that tested positive were further confirmed by HPLC-MS/MS.

GRAPHICAL ABSTRACT
A simple and sensitive USE-IAC-HPLC-PCD-FLD method was developed for high-throughput analysis of multi-class mycotoxins in Coix seed. Positive confirmation of detected samples was performed by LC-MS/MS.

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ABSTRACT
As an edible and medicinal plant, Coix seed is readily contaminated by more than one group of mycotoxins resulting in potential risk to human health. A reliable and sensitive method has been developed to determine seven mycotoxins (aflatoxins B1, B2, G1, G2, zearalenone, α-zearalenol, and β-zearalenol) simultaneously in 10 batches of Coix seed marketed in China. The method is based on a rapid ultrasound-assisted solid–liquid extraction (USLE) using methanol/water (80/20) followed by immunoaffinity column (IAC) clean-up, on-line photochemical derivatization (PCD), and high performance liquid chromatography coupled with fluorescence detection (HPLC-FLD). Careful optimization of extraction, clean-up, separation and detection conditions was accomplished to increase sample throughput and to attain rapid separation and sensitive detection. Method validation was performed by analyzing samples spiked at three different concentrations for the seven mycotoxins. Recoveries were from 73.5% to 107.3%, with relative standard deviations (RSDs) lower than 7.7%. The intra- and inter-day precisions, expressed as RSDs, were lower than 4% for all studied analytes. Limits of detection and quantification ranged from 0.01 to 50.2 µg kg−1, and from 0.04 to 125.5 µg kg−1, respectively, which were below the tolerance levels for mycotoxins set by the European Union. Samples that tested positive were further
analyzed by HPLC tandem electrospray ionization mass spectrometry for confirmatory purposes. This is the first application of USLE-IAC-HPLC-PCD-FLD for detecting the occurrence of multi-class mycotoxins in Coix seed.

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1. Introduction

As an edible and medicinal food/drug, Coix seed (Semen coicus, Yi-jiren in Chinese), the seed of Coix lacryma-jobi L. var. meyuan (Romen.) Stapf (family Craminae), has been used not only as a popular food, but also as a common traditional Chinese medicine (TCM) for thousands of years in the treatment of diseases such as cancer metastasis, hypertension, arthritis, asthma, and immunological disorders [1–3]. However, like other foods and TCMS, Coix seed may be subject to contamination by mycotoxins (toxic secondary metabolites produced by toxigenic fungi), such as aflatoxins (mainly AFB1, AFB2, AFG1, AFG2), zearalenone (ZON) and its metabolites α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) [4,5]. In general, these mycotoxins are carcinogenic, mutagenic, teratogenic, and genotoxic posing a significant threat to human and animal health through ingestion. The strong and various toxicities of these mycotoxins have led many countries to set up legislations for their limits in foods and medicinal plants that are intended for human or animal consumption [6–8].

To ensure compliance with current legislation, as well as to assess the toxicological risk, the use of reliable and accurate analytical methods for mycotoxins is necessary, which allow their unambiguous identification and confirmation, as well as accurate quantification at very low concentrations. For this goal, many efforts have been made to improve method sensitivity, selectivity, and accuracy, as well as the robustness of mycotoxin analysis. The same food or medicinal plant matrix might be contaminated by more than one mycotoxin belonging to various groups or classes, however, most of the present analytical methods are based primarily on the determination of a single mycotoxin or multiple mycotoxins belonging to the same group or class [9,10]. Clearly, the simpler methods for simultaneous determination of multi-group or -class mycotoxins are limited in scope. Owing to the different physical and chemical properties of aflatoxins (AFs), ZON and its metabolites, the determination of trace amounts (i.e. ppb) of them in samples represents an extremely challenging task. The analytical task is further compounded by the presence of large quantities of co-extracted compounds and a complex food or medicinal plant matrix. In recent years, high performance liquid chromatography (HPLC) with various detection systems has become the most common approach for the determination of mycotoxins in foodstuffs, feed and medicinal plants [11,12]. In particular, mass spectrometric (MS) detection has become increasingly widespread for the simultaneous determination of multiple mycotoxins in foods or medicinal plants [12–15], but the high cost of such instrumentation, together with the difficulty of selection of an internal standard and the diversity of analyte polarities and their different ionization capabilities, still pose significant practical limitations for many laboratories worldwide. As an alternative to MS detection, fluorescence detection (FLD) remains widely popular because of its great versatility, high sensitivity and selectivity [16–18]. One issue with FLD, however, is that the target mycotoxins may exhibit solvent-dependent quenching. Using aqueous solvent mixtures for reversed-phase chromatography, the fluorescence of AFB1 and AFG1 is significantly quenched. Therefore, a derivatization reaction is normally used for analysis. Although pre- and post-column derivatization procedures [19,20] can overcome this problem, some disadvantages still limit their wide applicability [21]. Thus, an on-line photochemical derivatization (PCD) performed just after the chromatographic column is a satisfactory alternative; this reaction increases significantly the fluorescence signal of AFB1 and AFG1, avoiding the quenching of AFB2 and AFG2 in aqueous solvent, whilst the fluorescence responses of other mycotoxins is unaffected [17,22,23]. In addition, the PCD technique does not need any chemical reagents and additional pumps or electrochemical cells making derivatization quick and easy, and minimizing intervention of the analyst. Moreover, the technique is reproducible, gives good linearity of response, and is easier to implement compared with traditional derivatization procedures.

On the other hand, because of the differences in the physicochemical properties of the multi-class mycotoxins, as well as the complexity of the sample matrices, the development of appropriate optimization methods for the co-extraction and clean-up of the mycotoxins is required. Recently, many extraction methods, such as solid-phase extraction (SPE), solid-phase microextraction, liquid–liquid extraction have been developed for this purpose [24–26]. However, these methods tend to be based on sophisticated instrumentation and require multiple sample processing steps. Ultrasound-assisted solid–liquid extraction (USLE) based on an ultrasonic bath, a common laboratory tool can offer an efficient and cost-effective extraction and enrichment of multi-mycotoxins in a single sample. Moreover, the approach can provide a high level of automation and offer extraction in a short time with low-solvent consumption [27]. A further crucial step in methodology is the sample clean-up step. A multifunctional column such as a Mycosep column, or an immunoaffinity column (IAC), has been used for the clean-up of multi-mycotoxins in many foods and medicinal plant matrices [24–26,28]. However, the clean-up efficiency of the different columns for Coix seed has not been compared; in addition, the question as to which type of column is more suited for a clean-up of this complex matrix has not been addressed.

With regard to the important edible and medicinal values of Coix seed, together with the fact that the seeds are readily contaminated by more than one group of mycotoxins, there is an urgent need to develop a sensitive and accurate analytical method for simultaneous multi-class analysis of mycotoxins to properly assess risk to human health and to formulate corresponding standards to ensure the safe use of this valuable edible and medicinal food/drug. Nevertheless, to the best of the authors' knowledge, no data are available on the co-occurrence of multi-class mycotoxins in Coix seed in China; in addition, regulatory methods or standards are not in place in the Chinese Pharmacopoeia.

The aim of this work is to develop a sensitive and reliable HPLC-FLD method for high-throughput analysis of multi-class mycotoxins including AFs (AFB1, AFB2, AFG1, AFG2), ZON, α-ZOL, and β-ZOL (Table 1) in Coix seed samples after USL extraction, IAC clean-up, and on-line post-column PCD. Positive confirmation of the seven investigated mycotoxins in these samples was carried out using HPLC-MS/MS in multiple reaction monitoring (MRM) mode.

2. Experimental

2.1. Materials and reagents

A mixed standard solution of AFs consisting of 1.0 μg AFB1, 0.3 μg AFB2, 1 μg AFG1, and 0.3 μg AFG2 in 1.0 mL of methanol was purchased from Supelco (Bellefonte, PA, USA). Stock solution of ZON (1.0 mg mL⁻¹ in methanol) was supplied by Alexis (Lausen, Switzerland). Standards of α-ZOL and β-ZOL were purchased from
Table 1
Chemical structures and main mold producers of the investigated mycotoxins.

<table>
<thead>
<tr>
<th>Main mold producer</th>
<th>Mycotoxin</th>
<th>Chemical structure</th>
<th>Empirical formula</th>
<th>Molecular weight (g mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus</td>
<td>Aflatoxin B₁(AFB₁)</td>
<td>![Structure Image]</td>
<td>C₁₂H₁₂O₆</td>
<td>312.27</td>
</tr>
<tr>
<td></td>
<td>Aflatoxin B₂(AFB₂)</td>
<td>![Structure Image]</td>
<td>C₁₂H₁₄O₇</td>
<td>314.29</td>
</tr>
<tr>
<td></td>
<td>Aflatoxin G₁(AFG₁)</td>
<td>![Structure Image]</td>
<td>C₁₂H₁₂O₇</td>
<td>328.27</td>
</tr>
<tr>
<td></td>
<td>Aflatoxin G₂(AFG₂)</td>
<td>![Structure Image]</td>
<td>C₁₂H₁₄O₇</td>
<td>330.29</td>
</tr>
<tr>
<td>Fusarium</td>
<td>Zearalenone (ZON)</td>
<td>![Structure Image]</td>
<td>C₁₀H₁₄O₆</td>
<td>318.36</td>
</tr>
<tr>
<td></td>
<td>α-zearalenol (α-ZOL)</td>
<td>![Structure Image]</td>
<td>C₁₀H₁₂O₄</td>
<td>320.38</td>
</tr>
<tr>
<td></td>
<td>β-zearalenol (β-ZOL)</td>
<td>![Structure Image]</td>
<td>C₁₀H₁₂O₄</td>
<td>320.38</td>
</tr>
</tbody>
</table>

Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile were purchased from Burdick & Jackson (Morris, NJ, USA). Formic acid (purity > 98%) was supplied by MREDA (Beijing, China). Water was double distilled. Other reagents and chemicals were all analytical grade and provided by Beijing Chemical Works (Beijing, China). Phosphate buffer saline (PBS) was prepared by dissolving 8.0 g NaCl, 2.9 g Na₂HPO₄, 0.24 g KH₂PO₄ and 0.2 g KCl in 1000.0 mL water (pH was adjusted to 7.0).

The AflaZearal Test™ immunoaffinity columns and GF/A glass microfiber filter (1.5 μm) were all purchased from VICAM (Waterworn, MA, USA). Sample C₁₈-SPE columns were purchased from Agilent Technologies (Little Falls, DE, USA). Mycosep226 columns were supplied by Romer Labs (Union, MO, USA). Bond Elut Mycotoxin-SPE and MF160 columns were obtained from Varian Inc., (Palo Alto, CA, USA). Sepax universal columns were supplied from Sepax Technologies, Inc (Little Falls, DE, USA). Waters HLB columns were purchased from Waters (Milford, MA, USA). A photochemical reactor with a mercury lamp (λ = 254 nm) and a knitted reactor coil of 0.74 mL (15 mm × 0.25 mm) was bought from AURA Industries (New York, NY, USA). A KQ-500 ultrasonic clean bath (50 × 30 × 35 cm) was purchased from Kunshan Ultrasonic Instrument Co. Ltd (Jiashu, China).

2.2 Instrumentation and conditions

2.2.1 HPLC-FLD equipment and conditions

HPLC-FLD analysis was performed on a Shimadzu LC-20AT HPLC system (Shimadzu, Kyoto, Japan) consisting of three LC-20 AT pumps, a SIL-20 A autosampler, and an RF-10AXL fluorescence detector, equipped with a post-column PCD reactor. Chromatographic separation was achieved on a Prontosil Kromasilus 100-5-C₁₈ column (250 mm × 4.6 mm, 5 μm, Bischoff, Germany) and the column temperature was kept at 30 °C. The mobile phase consisted of (A) methanol and (B) water at a flow rate of 1.0 mL min⁻¹, freshly prepared every day. The optimized gradient elution procedure was as follows: 0–12.0 min (50–68%, A), 12–25 min (68%, A). The injection volume was 50.0 μL. The maximum emission wavelength of the PCD reactor was set at 254 nm. The eluate was monitored by FLD with an excitation wavelength of 360 nm and an emission wavelength of 440 nm for the first 12 min of the run, and then at an excitation wavelength of 280 nm and an emission wavelength of 440 nm. The system was interfaced via LC solution ChemStation software (Shimadzu, Kyoto, Japan) to a personal computer for instrument control and data acquisition and processing.

2.2.2 HPLC-MS/MS equipment and operating conditions

HPLC-MS/MS confirmation was carried out on a QTrap® 5500 MS/MS system from Applied Biosystems (Foster City, CA, USA) equipped with an electrospray ionization (ESI) interface and a NANO SI-2 LC system comprising a NANOSPACE SI-2 3202 pump and a NANO F5100 autosampler from Shimadzu Technologies (Shimadzu, Kyoto, Japan). Analyst software, version 1.5.1 (Applied Biosystems, Foster City, CA, USA), was used to control the LC-MS/MS system and for data acquisition and processing. The mycotoxins, in samples that tested positive, were separated on a Phenomenex Luna 5 μ C₁₈(2) 100 A column (50 mm × 2.0 mm, 5 μm;
Phenomenex, Torrance, CA, USA). The mobile phase consisted of (A) acetonitrile (containing 0.1% formic acid) and (B) water (containing 0.1% formic acid) delivered at a flow rate of 0.4 mL min\(^{-1}\) and the injection volume was 10 \mu L. The gradient procedure was as follows: 0.01–1.50 min, 5% A; 1.50–2.50 min, 5%–95% A; 2.50–5.00 min, 95% A; 5.00–5.01 min, 95%–5% A; 5.01–8.00 min, 5% A. A two-phase switching valve was used to divert the pre-eluent from entering the ion source. The mass spectrometer was operated in the positive and negative ESI modes with MRM at unit mass resolution. Nitrogen was used as the nebulizer (GS1), heater (GS2) and curtain (CUR) gases as well as the collision activation dissociation (CAD) gas. Three precursor-to-product ion transitions were simultaneously monitored at \(m/z\) 313.0 → 285.0, \(m/z\) 313.0 → 269.1 and \(m/z\) 313.0 → 241.2 for AFB\(_1\), and \(m/z\) 317.0 → 175.1, \(m/z\) 317.0 → 160.1 and \(m/z\) 317.0 → 131.1 for ZON. The optimum parameters used for ionization source were set as follows: GS1: 55.0 psi, GS2: 65.0 psi, CUR 30.0 psi, and CAD: medium; source temperature: 500 °C; ion spray (IS) voltage: +4500 V for the positive ion mode and -4500 V for the negative ion mode. For AFB\(_1\): the declustering potential (DP) was 120 V for \(m/z\) 313.0 → 285.0, \(m/z\) 313.0 → 269.1, and \(m/z\) 313.0 → 241.2; collision energies (CE) were 33 eV for \(m/z\) 313.0 → 285.0, 42 eV for \(m/z\) 313.0 → 269.1, and 53 eV for \(m/z\) 313.0 → 241.2, while collision cell exit positive potentials (CXP) were all 22 V for the three precursor-to-product ion transitions. For ZON, the DP was -100 V for \(m/z\) 317.0 → 175.1, \(m/z\) 317.0 → 160.1, and \(m/z\) 317.0 → 131.1; CE were -30 eV for \(m/z\) 317.0 → 175.1, -40 eV for \(m/z\) 317.0 → 160.1, and -40 eV for \(m/z\) 317.0 → 131.1, while CXP were all -10 V for the three precursor-to-product ion transitions.

2.3. Standard solutions

A stock solution of ZON was diluted with methanol to yield a concentration of 0.1 mg mL\(^{-1}\). The concentrations of α-ZOL and β-ZOL standard solutions, which were prepared in methanol, were 0.5 mg mL\(^{-1}\). A series of multi-compound working solutions were prepared by combining suitable aliquots of each individual standard stock solution and diluting them with appropriate amounts of methanol/water (50/50, v/v). These solutions were kept at -20 °C and renewed weekly.

2.4. Sample preparation

2.4.1. Collection

One Coix seed sample was collected from Yunnan province, China. A further nine Coix seeds were purchased randomly from local supermarkets (Beijing, China) with their origins being Guizhou, Fujian, and Yunnan provinces, China. All samples were ground to a homogenous size by a mill and sized through a 40-mesh sieve before mycotoxin extraction.

2.4.2. Extraction and cleanup

Multi-class mycotoxins were extracted from test samples using an ultrasound-assisted solid–liquid extraction procedure. Briefly, the procedure was as follows: 5.0 g of each ground sample was weighed in a conical flask (50.0 mL) and 1.0 g NaCl and 25.0 mL of a methanol/water (80/20, v/v) solution were added and the mixture was extracted by ultrasonication in an ultrasonic clean bath at 500 W for 20 min and then filtered through the GF/A glass microfiber filter.

Following the manufacturer’s instruction sheet, 10.0 mL of the filtrate was mixed with 40.0 mL PBS solution (pH 7.0), and the solution was filtered through the GF/A glass microfiber filter, again. 25.0 mL of the filtrate (equivalent to 1 g test portion) was then cleaned up using an IAC. The IACs were equilibrated at room temperature for at least 15 min before use. Then, 10.0 mL of water was added to rinse the IA column and the investigated toxins were finally eluted with 3.0 mL of MeOH into a dark flask. The eluate was evaporated under a stream of N\(_2\) at 45 °C and was re-dissolved in 1.0 mL of methanol/water (50/50, v/v). The solution containing the investigated mycotoxins was vortexed for 30 s and filtered through a 0.45-μm filter. 50.0 μL of the filtrate was then injected into the HPLC-FLD system and mycotoxins peaks were identified in the test solution by comparing retention times with those for a mixed standard solution.

2.5. Analytical measurement

Quantization of the multi-class mycotoxins in Coix seed was performed by measuring the peak area responses in the HPLC-FLD at each mycotoxin retention time and comparing them with the linear equation of the calibration graph. The peak area (response, y-axis) of each mycotoxin was plotted against the concentration (ng mL\(^{-1}\), x-axis) as \(y = mx + n\). The slope (m) and Y-intercept (n) were determined. The content of each mycotoxin in the test sample was calculated as follows:

\[
\text{Content of mycotoxin (μg kg}^{-1}\text{)} = \left( \frac{R - n}{m} \right) \times \frac{V}{W} \times f \tag{1}
\]

where \(R\) is the test solution peak area, \(V\) is the volume (mL) of the injected test solution, and \(W\) is the weight of the test sample (1 g) that passed through the IAC, and \(f\) is the dilution factor (f = 0.2 when \(V = 5.0\ mL\)).

3. Results and discussion

This work aimed to develop and validate a simple, sensitive and reliable method for high-throughput determination of multi-class mycotoxins in Coix seed by HPLC-FLD after USLE, IAC cleanup and on-line post-column PCD.

3.1. Method establishment and development

3.1.1. Optimization of the extraction procedure

For the accurate determination of multi-class mycotoxins, the extraction and clean-up procedure of tested samples is a critical step. As a convenient, economic and fast method for extraction of mycotoxins without the need of specialized instrumentation, USLE was applied to reduce sample handling time and increase sample throughput. Therefore, before chromatographic determination, the USLE procedure was optimized.

First, different solvents including methanol, acetonitrile, water, a mixture of several ratios of methanol and acetonitrile aqueous solution was checked to identify the most efficient extraction solvent. Best results were obtained when methanol was used as an extractant because this yielded improved signal responses for most of the mycotoxins. Although some published studies have recommended a mixture of different ratios of acetonitrile/water [28,30], this mixed system was not used in this study on account of the higher toxicity of acetonitrile than methanol. In addition, the responses decreased considerably when the percentage of water increased. For all the mycotoxins, the most intense fluorescence responses were obtained when methanol/water (80/20, v/v) was used as the extraction solvent.

Next, different volumes of methanol/water (80/20, v/v) were checked to optimize the extraction solvent volume using the USLE procedure. As is well known, better extraction efficiencies and recoveries can be obtained when using high volumes of extraction solvent. For relatively large volumes, however, this became time consuming, on account of the time needed to evaporate the extract to dryness, hence, a volume of 25.0 mL was selected for sample extraction. Finally, the extraction time for the USLE procedure was investigated. It was found that the peak areas responses for the
targeted mycotoxins increased with extraction time up to 20 min, and then remained constant, therefore, 20 min was selected as the optimum extraction time.

3.1.2. Effect of salt addition on mycotoxin extraction

In general, addition of electrolytes to the sample solution can decrease the solubility of solutes in the aqueous phase and increase their distribution into the organic phase or onto an adsorbent according to the salting-out effect. This process would result in a reduction of mutual miscibility of the two liquids, and also would disrupt the weak intermolecular forces between the organic molecules or nonelectrolytes and water due to the hydration of electrolytes [31]. Here, the effect of salt on extraction efficiency was studied by adding NaCl, in the range of 0.1–2.0 g, to Coix seed solution. The results showed that addition of 1.0 g NaCl to the solution gave the best improvement in extraction efficiency.

3.1.3. Optimization of clean-up procedure

Coix seed, an edible and medicinal food/drug, is a complex matrix of many chemical components. These components, such as lipids, proteins, amino acids, would interfere with the analyte fluorescence signals when using HPLC-FLD for analysis. In an attempt to achieve an optimum sample clean-up, seven commercially used clean-up columns including Agilent SampliQ C18-SPE column, Romer Mycosep226 column, Sepax universal column, Waters HLB column, Bond Elut Mycotoxin-SPE column, MF160 column, and AflaZearal Test™ immunoaffinity were investigated for the clean-up and enrichment of the target mycotoxins. It can be seen in Fig. 1A–G that many interfering peaks appeared at the retention times of the seven investigated mycotoxins or that the chromatograms were not sufficiently clear to permit accurate analysis. These results illustrated that the clean-up efficiency for the above columns was not satisfactory. In contrast, as shown in Fig. 1H for the AflaZearal Test™ IAC, few interfering peaks were registered indicating a high selectivity and specificity and satisfactory clean-up efficiency for the Coix seed samples.

3.1.4. Optimization of derivatization method

On account of solvent-dependent quenching of AFB1 and AFG1 in aqueous solvents, derivatization of the two AFs is necessary when using FLD. The ideal derivatization technique should be easy to perform with simple laboratory procedures and achieve a significant enhancement in the fluorescence responses of AFB1 and AFG1, while not affecting the responses of other mycotoxins. The on-line PCD method is a satisfactory alternative in comparison with other derivatization techniques including pre-column derivatization using a series of reagents. Here, on-line post-column PCD of mycotoxins was achieved by adding a photochemical reactor between the chromatographic column and the fluorescence detector.
detector. After passing through the post-column derivatization device, AFB₁ and AFG₁ formed AFB₂ₐ (derivative of AFB₁) and AFG₂ₐ (derivative of AFG₁)[24]. Typical chromatograms for a mixed standard solution and a spiked Coix seed sample, shown in Fig. 2, indicated good baseline resolution for the seven mycotoxins and demonstrated that high sensitivity was achieved for AFG₂, AFG₁, AFB₂, AFB₁, β-ZOL, α-ZOL, and ZON at corresponding retention times of 7.4, 8.3, 9.7, 10.8, 17.1, 20.6, and 22.2 min.

3.1.5. Optimization of HPLC-FLD conditions

Several variables involved in the chromatographic process and in FLD were evaluated and optimized to reduce analysis time, increase sensitivity, and provide good peak shape.

First, the Prontosil Kromasil 100-5-C₁₈ column gave satisfactory results with respect to effective separation of the seven mycotoxins by HPLC within a short analysis time, i.e., <23 min, which was comparable to other chromatographic methods applied for separation of these compounds[32,33].

Owing to the chemical diversity of the investigated mycotoxins, a suitable composition of the mobile phase must be selected. In general, the reported mobile phase consists of a combination of water and an organic solvent (methanol or acetonitrile). From an evaluation of organic solvents for the mobile phase, methanol was observed to be a more suitable solvent for the separation of all analytes in providing better sensitivity, as well as having lower toxicity than acetonitrile. Then, a series of water/methanol mixtures as the mobile phase was studied, in the range of 20–80% methanol. It was found that good peak shape, high sensitivity, and short analysis time were obtained with increasing the percentage of methanol in the initial composition. However, when the percentage of methanol exceeded 60%, the baseline shift was significant. Taking account of the trade-off between separation efficiency and sensitivity, 50% methanol was selected as the initial composition of the mobile phase. Moreover, in order to get the effective separation of the seven mycotoxins, gradient elution procedure was used and optimized as outlined previously.

Next, other parameters for HPLC separation such as flow rate (0.5, 1.0, and 1.5 mL min⁻¹), column temperature (20, 30, and 40 °C) and injection volume (10, 30, 50, and 70 μL) were studied. The optimum conditions were as follows: 0.35 mL min⁻¹ as flow rate, 30 °C as column temperature, and 70 μL as injection volume.

Because of the different chemical structures and properties of AFs and ZON and its metabolites (Table 1), the excitation and emission wavelengths, as well as the fluorescence responses of the analytes, might alter, especially after on-line post-column PCD. Therefore, the optimum excitation and emission wavelengths for the derivatized products of the seven mycotoxins should be identified to achieve maximum detection sensitivity. According to the excitation and emission wavelength scans (Fig. S1), the optimal excitation and emission wavelengths during the first 12 min of the chromatographic run were 360 nm and 440 nm for the AFs, and after 12 min were 280 nm and 440 nm for ZON and its two metabolites.

3.2. Method in-house validation

Validation of the method was assessed in terms of selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and repeatability.

3.2.1. Selectivity

The selectivity of the method was evaluated using IAC for sample clean-up. Under the above-optimized chromatographic conditions, the chromatograms obtained from the analysis of the mixed standard solution and a spiked Coix seed sample with 5 ng mL⁻¹ of AFB₁ and AFG₁, 1.5 ng mL⁻¹ of AFB₂ and AFG₂, 60 ng mL⁻¹ of ZON and α-ZOL, and 300 ng mL⁻¹ of β-ZOL were shown in Fig. 2 and indicate an effective separation of the seven mycotoxins in less than 23 min. No interfering peaks were observed at the retention times of AFG₂ (7.4 min), AFG₁ (8.3 min), AFB₂ (9.7 min), AFB₁ (10.8 min), β-ZOL (17.1 min), α-ZOL (20.6 min), and ZON (22.2 min). These results clearly demonstrated the good selectivity of the established HPLC-PCD-FLD method for the seven analytes.

3.2.2. Linearity

As a check on the linearity of the developed method, a series of multi-compound mixed working solutions including 0.1–50.0 ng mL⁻¹ for AFB₁, 0.03–15.0 ng mL⁻¹ for AFB₂, 0.1–50.0 ng mL⁻¹ for AFG₁, 0.03–15.0 ng mL⁻¹ for AFG₂, 15.0–1000.0 ng mL⁻¹ for ZON and α-ZOL, and 75.0–5000.0 ng mL⁻¹ for β-ZOL were prepared by diluting each individual standard solution with methanol/water (50/50, v/v), followed by derivatization, and then injection into the HPLC unit (in triplicate for each concentration) whereupon the respective peak area signal responses were recorded. The linearity was determined using linear regression analysis by plotting peak area response (y) versus concentration (x) for each mycotoxin and calculating the correlation coefficient (r) for the line of best fit. The linear equation, correlation coefficient, and linear range are listed in Table 2. Good linearity was found for all the mycotoxins within their corresponding linear ranges, with all r values higher than 0.999.

3.2.3. LODs and LOQs

LODs and LOQs, which were used to evaluate method sensitivity for each mycotoxin, were determined as the lowest concentration of the mycotoxins that produce chromatographic peaks with signal-to-noise ratios of 3 and 10, respectively. As listed in Table 2, relatively low LODs and LOQs were obtained for the AFs (0.01–0.11 μg kg⁻¹ for LODs and 0.04–0.32 μg kg⁻¹ for LOQs), whereas the LODs and LOQs for ZON, α-ZOL and β-ZOL were bigger...
than 11.7 and 29.3 μg kg⁻¹, respectively. The LOQs, in all the cases, were all lower than the maximum residue limits established by the European Union [6,7], confirming the suitability of the established method for the determination of trace concentrations of the target mycotoxins.

### 3.2.4. Precision

The precision of the established method was studied by measuring intra- and inter-day reproducibility by performing a repeat analysis of a single mixed working solution at 5.0 ng mL⁻¹ for AFB₁ and AFG₁, 1.5 ng mL⁻¹ for AFB₂ and AFG₂, 60.0 ng mL⁻¹ for ZON and α-ZOL, and 300 ng mL⁻¹ for β-ZOL. The reproducibility measurements were expressed as the relative standard deviation (RSD) of peak area response for the seven analytes. The intra-day reproducibility of the assay was determined by six consecutive injections of working solutions (50 μL) on the same day, while the inter-day variability of the assay was determined from six injections of standard solutions (50 μL) on six different days. The RSDs for peak area response for intra- and inter-day reproducibility were all lower than 4.0%, which were well below the values recommended by the European Union. This result testifies to the very good precision for the established method.

### 3.2.5. Accuracy

Accuracy was evaluated through recovery experiments. For this, one Coix seed sample was selected at random, and aliquots (n=9) of this sample were spiked with mixed standard solution at a high concentration (10.0 μg kg⁻¹ for AFB₁ and AFG₁, 3.0 μg kg⁻¹ for AFB₂ and AFG₂, 300.0 μg kg⁻¹ ng mL⁻¹ for ZON, 120.0 μg kg⁻¹ for α-ZOL, and 500.0 μg kg⁻¹ for β-ZOL), a medium concentration level (5.0 μg kg⁻¹ for AFB₁ and AFG₁, 1.5 μg kg⁻¹ for AFB₂ and AFG₂, 60.0 μg kg⁻¹ ng mL⁻¹ for ZON, 60.0 μg kg⁻¹ for α-ZOL, and 300.0 μg kg⁻¹ for β-ZOL) and a low concentration level (1.0 μg kg⁻¹ for AFB₁ and AFG₁, 0.3 μg kg⁻¹ for AFB₂ and AFG₂, 30.0 μg kg⁻¹ ng mL⁻¹ for ZON, 30.0 μg kg⁻¹ for α-ZOL, and 150.0 μg kg⁻¹ for β-ZOL), respectively. The spiked samples were extracted, cleaned-up, derivatized, and analyzed by HPLC-FLD, as previously. The recovery values (%), calculated by the following equation, have been summarized in Table 3, together with the corresponding RSDs.

\[
\text{Recovery} = 100 \times \frac{\text{measured amount} - \text{original amount}}{\text{spiked amount}} \quad (2)
\]

It can be seen that for the AFs the average recoveries were from 87.4% to 107.3% with RSDs lower than 7.7% and for ZON and its two metabolites values were from 73.5% to 101.5% with RSDs lower than 7.2%. The results were in compliance with the requirements of the European Union [6,7].

### 3.2.6. Repeatability

Method repeatability was also determined by recovery studies. Six portions of the same Coix seed sample, selected at random, and each weighing 5 g, were spiked with 5.0 μg kg⁻¹ of AFB₁ and AFG₁, 1.5 μg kg⁻¹ of AFB₂ and AFG₂, 60.0 μg kg⁻¹ of ZON and α-ZOL, and 300.0 μg kg⁻¹ of β-ZOL. The samples were then processed as above. For HPLC analysis, samples were injected in triplicate. The average recoveries were from 91.2% to 105.7% for AFs with RSDs lower than 5.9%, and recovery values for ZON and its two metabolites were

### Table 3

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Original amount (μg kg⁻¹)</th>
<th>Spiked amount (μg kg⁻¹)</th>
<th>Measured amount (μg kg⁻¹)</th>
<th>Average recovery (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>AFB₁</td>
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<td>1.04</td>
<td>1.01</td>
<td>1.04</td>
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<tr>
<td></td>
<td>0.11</td>
<td>5</td>
<td>4.70</td>
<td>4.65</td>
<td>4.09</td>
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<tr>
<td></td>
<td>0.11</td>
<td>10</td>
<td>9.13</td>
<td>9.03</td>
<td>8.71</td>
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<tr>
<td>AFB₂</td>
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<td>1.27</td>
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<td>3</td>
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<td>9.06</td>
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<tr>
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<td>10</td>
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<td>28.92</td>
<td>28.54</td>
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<td>AFG₂</td>
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<td>2.74</td>
<td>2.71</td>
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<tr>
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<td>29.54</td>
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</tr>
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<td>300</td>
<td>30</td>
<td>219.75</td>
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<tr>
<td>α-ZOL</td>
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<tr>
<td>β-ZOL</td>
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<td>30</td>
<td>312.69</td>
<td>289.69</td>
<td>292.31</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>500</td>
<td>474.41</td>
<td>481.87</td>
<td>454.76</td>
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</table>

<sup>a</sup> Recovery (%) = 100 × (measured amount – original amount)/spiked amount.

<sup>b</sup> Not contained.
Table 4
Contamination levels of the investigated mycotoxins in test Coix seed samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Origin</th>
<th>Content (µg kg⁻¹)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>AFB₁</td>
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<tr>
<td>1</td>
<td>Guizhou province</td>
<td>0.10</td>
</tr>
<tr>
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<td>Fujian province</td>
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</tr>
<tr>
<td>3</td>
<td>Guizhou province</td>
<td>0.14</td>
</tr>
<tr>
<td>4</td>
<td>Fujian province</td>
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</tr>
<tr>
<td>5</td>
<td>Fujian province</td>
<td>0.11</td>
</tr>
<tr>
<td>6</td>
<td>Yunnan province</td>
<td>0.10</td>
</tr>
<tr>
<td>7</td>
<td>Guizhou province</td>
<td>0.12</td>
</tr>
<tr>
<td>8</td>
<td>Guizhou province</td>
<td>0.11</td>
</tr>
<tr>
<td>9</td>
<td>Fujian province</td>
<td>0.12</td>
</tr>
<tr>
<td>10</td>
<td>Yunnan province</td>
<td>&lt;LOQ</td>
</tr>
</tbody>
</table>

* Not detected.

from 97.1% to 103.4% with RSDs lower than 8.5%, indicating good method repeatability.

In summary, regardless of the high complexity of the tested matrices, the established HPLC-FLD method was satisfactory with regard to selectivity, sensitivity, precision, accuracy and repeatability for the simultaneous multi-class determination of AFs and ZON and its metabolites in Coix seed samples after USLE and IAC clean-up.

3.3. Application to real samples

Because of the health implications of mycotoxins to humans and animals, the European Union has set a legal maximum limit of 5.0 µg kg⁻¹ for AFB₁ and 10.0 µg kg⁻¹ for the sum of AFB₁, AFB₂, AFG₁, and AFG₂ in foods, spices, and some TCMs [6], and 20.0–400.0 µg kg⁻¹ for ZON in grains and foods [34]. In China, a maximum limit of 60.0 µg kg⁻¹ for ZON has been set for grains, wheat, and maize [8]. Toward this goal, many research institutes and laboratories have or are trying to develop advanced analytical methods for monitoring the occurrence and contamination levels of AFs and ZON in various matrices. However, according to the Chinese Pharmacopoeia and other governmental documents, regulatory methods and standards on the co-occurrence of multi-class mycotoxins in some edible and medicinal foods/drugs are lacking. To test the suitability of the developed method for the simultaneous determination of multi-class mycotoxins, 10 batches of Coix seed samples were purchased from local supermarkets and samples were subjected to the USLE-IAC-HPLC-PCD-FLD method. The results, presented in Fig. 3 and Table 4, indicate that many samples tested positive. For instance, it can be seen that all the 10 (100%, 10/10) batches of Coix seed samples were contaminated by AFB₁ with concentrations in the range of 0.10–0.18 µg kg⁻¹. However, as shown in Fig. 4A, these contamination levels were not more than the maximum limit of 5.0 µg kg⁻¹ set by the European Union. On the other hand, 7 (70%, 7/10) batches of Coix seed samples tested positive to ZON with concentrations of 44.71–931.07 µg kg⁻¹. The histograms in Fig. 4B illustrated 6 out of the 7 samples that tested positive were contaminated by ZON with the contamination levels exceeding the maximum limit of 60.0 µg kg⁻¹ set by China. The high occurrence and contamination level of AFB₁ and ZON does highlight the need to be more vigilant in our daily life when consuming Coix seed and its related products. Further studies are in progress to clarify possible relationships between the mycotoxin contamination levels and the Coix seed sample matrix, as well as seed origin and processing methods.
3.4. Method confirmation by HPLC-MS/MS

Although AFB₁ and ZON have been detected in Coix seed samples, the accuracy and reliability should be further confirmed to exclude false-positive results. Here, the occurrence of AFB₁ and ZON in positive samples that tested positive was further confirmed by HPLC-ESI-MS/MS analysis. Three pairs of precursor-to-product ion transitions of m/z 313.0 → 285.0, m/z 313.0 → 269.1, and m/z 313.0 → 241.2 for AFB₁, and m/z 317.0 → 175.1, m/z 317.0 → 160.1, and m/z 317.0 → 131.1 for ZON were selected to confirm their presence. From the MRM chromatograms of AFB₁ and ZON standards and the positively tested samples (Fig. 52), it can be seen that the retention times of AFB₁ (3.44 min) and ZON (3.65 min) in the positively tested samples were consistent with those in standard, hence confirming the presence of AFB₁ and ZON in the Coix seed samples.

4. Conclusions

The diversity of mycotoxins has led to the requirement for high-throughput analytical methods for multi-class mycotoxins rather than a method for a single mycotoxin or even multiple mycotoxins belonging to the same group or class. In this study, a simple, sensitive, and reliable USLE-IAC-HPLC-PCD-FLD method has been developed and successfully applied to simultaneous determination of multi-class mycotoxins in 10 batches of edible and medicinal Coix seed samples marketed in China. The use of USLE technology afforded a simple and rapid extraction of mycotoxins from test samples without the need of specialized instrumentation. In comparison to other clean-up columns and methods, the IAC was preferred because of its high selectivity and specificity providing satisfactory clean-up efficiency for a complex matrix. Using a simple derivatization technique, the on-line post-column PCD allowed a large increase in the fluorescence yield for some mycotoxins, thus avoiding the quenching in aqueous solvents.

The USLE-IAC-HPLC-PCD-FLD method permitted the simultaneous determination of 7 mycotoxins at low μg kg⁻¹ level, meeting the requirements of statutory bodies such as the European Union. Although AFG₁, AFB₁, AFG₂, α-ZOL and β-ZOL were not detected in the 10 Coix seed samples, AFB₁ was measured in all samples investigated and ZON in 6 samples exceeded the maximum limit set by China. The results were confirmed by HPLC-ESI-MS/MS to be accurate and reliable.

In conclusion, this is to the best of our knowledge, the first paper describing the high-throughput analysis of multi-class mycotoxins in Coix seed by USLE-IAC-HPLC-PCD-FLD method. This approach offers many advantages over current practices including simple extraction, satisfactory clean-up, rapid separation, and sensitive detection. There is scope for applying the method to different types of mycotoxins in diverse biological and botanical samples, thus producing valuable data for preliminary surveillance investigation and, in so doing, makes a contribution to decreasing public concerns regarding mycotoxin contamination in the food chain.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jaca.2013.08.042.

References