Development of a novel validated method for aflatoxin analysis on edible bird nest

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Abstract. Edible bird nest (EBN) is an excellent export commodity from Indonesia and South East Asia countries. To keep the quality assurance of EBN, aflatoxin analysis should be conducted to check its biological hazard. This research was conducted to develop a rapid, sensitive and validated method to detect aflatoxin contamination in EBN. This study showed that in-house AF.2 IAC containing AF.2 monoclonal antibody can be used for EBN sample clean-up. Validation methods were measured using parameters which were recovery (86.5-96.3% for AFB1, 87.8-98.5% for AFB2, 82.8-100.5 for AFG1, 79.8-94.3% for AFG2), intra-day repeatability RSD (≤ 6.4% for all aflatoxins), linearity (≥ 0.99997 for all aflatoxins), limit of detection (0.26 ng/g for AFB1, 0.04 ng/g AFB 2, 0.26 ng/g for AFG1, 0.03 ng/g for AFG2) and limit of quantification (0.79 ng/g for AFB 1, 0.13 ng/g AFB2, 0.81 ng/g for AFG1, 0.09 ng/g for AFG2). These results showed that both recovery test of spiked PBS and recovery test of 3-level aflatoxin concentration spiked IBN were good, thus in-house AF.2 IAC can be used to analyze aflatoxins in EBN. Based on validation parameters such as recovery test, intra-day repeatability, linearity, LOD, and LOQ which had been tested, our method can be used for validated method for aflatoxin analysis in EBN. Further analysis of aflatoxins in edible bird nest will be reported separately.

1. Introduction
Edible bird nest is an exotic nest made from the saliva of a swiftlet (Aerodramus fuciphaga). The history of eating edible bird nest is unclear but it is believed that consumption of edible bird nest began in the Tang Dynasty (AD 618-907), and edible bird nest was a delicacy sometimes offered as a tribute to the emperor. By the sixteenth century, the edible bird nest was an important ingredient for both Chinese cuisine and pharmacy [1]. Edible bird nest is one of the most expensive food ingredients in the world, and it is sometimes called as caviar from East. A study reported that unprocessed edible bird nest contains some structures such as mites, mite eggsheells, faecal pellets, feather strands, arthropods, bacteria, and yeast or fungal spores [2]. Furthermore, the fungal structures found on the surface of the raw edible bird nest included yeast and hyphae and various kind of fungal spores. The study also showed that even in the commercial edible bird nest fungal structures including hyphae, yeast, and fungal spores were found. This finding gives us a question about the probability of a presence of fungal metabolite or mycotoxin in EBN.
In the term of food safety, aflatoxin analysis is one of food safety analysis and frequently conducted to some feed and food commodities in Indonesia such as poultry feed, maize, bran in various level of contamination [3–7]. Nowadays, the requirement for food safety is being a concern on food industry in many countries, including Indonesia. Concerning the big amount of exported edible bird nest from Indonesia, there is a requisite to guarantee product safety. To meet the requirement, a method of aflatoxin analysis for edible bird nest should be developed. Aflatoxin is a very stable compound and may resist quite severe processes like roasting, baking, extrusion plus baking [8], thus aflatoxin analysis should be conducted even in processed edible bird nests. In the scientific publication, there is still no information about aflatoxin investigation on edible bird nest, especially the method for aflatoxin analysis on EBN. This issue invites the authors to develop a standard method for aflatoxin analysis on EBN.

2. Materials and methods

2.1. Edible bird nest preparation

A piece of uncleaned edible bird was ground using for 2 minutes. Ground EBN was loaded into plastic seal and stored in 4°C refrigerator.

2.2. Aflatoxin extraction and in-house IAC clean-up

An amount of 0.2 g NaCl was added to 0.5 g edible bird nest sample powder, then 6 mL of methanol:water (MeOH:H2O, 70:30, v/v) was added to extract aflatoxin. Edible bird nest sample extract was cleaned-up with in-house immunoaffinity column (IAC). Firstly, in-house IAC was equilibrated with 10 mL of PBS. Ten milliliters of EBN sample extract were loaded through the IAC. Impurities from the EBN sample extract were washed with 5 mL of PBS and 5 mL of Milli-Q water. Aflatoxins bound to antibody-gel were eluted with 2 mL of methanol and 2 mL of Milli-Q water. The yield of elution was centrifuged at 15,000 rpm for 10 minutes prior to HPLC analysis. Finally, 100 µL of centrifuged solution were inputted into HPLC vial for analysis.

2.3. High performance liquid chromatography

Calibration curve was constructed by diluting AFs standard solution in methanol:water (MeOH:H2O, 50:50, v/v) into 3 level concentration (0.65 ng/mL, 0.3125 ng/mL and 0.0625 ng/mL). A hundred microliter of diluted AFs standard solution and/or sample solution was inputted into HPLC vial. Twenty microliters of diluted AFs standard solution or sample solution was automatically injected into HPLC column (Shimadzu Shim-pack XR-ODS 100x3.0). Mobile phase for HPLC analysis was water:methanol:acetonitrile (H2O:MeOH:CH3CN, 60:30:10, v/v/v) with 0.4 mL/min flow rate and pressure of the column was 21.0 MPa. Oven temperature of HPLC machine was 50°C. Excitation and emission wavelengths were 365 nm and 450 nm, respectively. Validation of analytical procedures was evaluated by calculating precision/repeatability, linearity, the limit of detection and limit of quantification.

3. Results and discussion

3.1. Edible bird nest preparation

Indonesian edible bird nests (IBNs) were used for the development of aflatoxin analysis in edible bird nest. Edible bird nests used in this experiment were uncleaned EBNs which contain bird feather, small piece of wood, and/or bird’s dropping [2]. The EBN were ground to make EBN powder. Grinding process will convert the EBN into small pieces and it will be easier to be used for extraction. Ground EBN was stored in 4°C refrigerator and it keeps the EBN sample fresh and extends its shelf life.
3.2. Aflatoxin extraction and in-house IAC clean-up
Extraction of aflatoxin in edible bird nest was conducted using aqueous solutions containing a high concentration of the water-miscible solvent, such as methanol [9]. A mixture solvent containing methanol:water (MeOH:H₂O, 70:30, v/v) was used in this experiment, and it showed a good result in recovery test.

Immonoaffinity column clean-up using in-house AF.2 IAC was also conducted to check the ability of monoclonal antibody coupled with AFB1, B2, G1, and G2. Performance of in-house AF.2 IAC was checked by spiking aflatoxins standard solution in PBS. Spiked PBS was loaded into in-house AF.2 IAC and analyzed by HPLC. Mean ± SD of AFB1, AFB2, AFG1 and AFG2 for recovery test of spiked PBS were 95.0±0.0%, 92.3±3.5%, 96.7±0.6%, and 97.0±0.0%, respectively. The precision of the analysis method was evaluated by calculating intra-day repeatability in terms of relative standard deviation (RSD). Relative standard deviation of AFB1, AFB2, AFG1 and AFG2 for recovery test of spiked PBS were 0.0%, 3.8%, 0.6%, and 0.0%, respectively. These results showed that the performance of in-house AFs.2 IAC was very good and can bind with all aflatoxins.

Table 1. Performance of in-house AF.2 IAC, n=3.

| AFs conc. (ng/mL) | AFB1 (%) | AFB2 (%) | AFG1 (%) | AFG2 (%) |
|------------------|----------|----------|----------|----------|
| Mean ± SD RSD    | Mean ± SD RSD | Mean ± SD RSD | Mean ± SD RSD |
| 0.5              | 95.0 ± 0.0 0.0 | 92.3 ± 3.5 3.8 | 96.7 ± 0.6 0.6 | 97.0 ± 0.0 0.0 |

3.3. Analytical method performance
Performance and validity of our method were measured by conducting recovery test and checking its intra-day precision, linearity, the limit of detection (LOD) and limit of quantification (LOQ). To perform the recovery test, Indonesian edible bird nest (IBN) blank sample was used, which means that the IBN was free from aflatoxin contamination. Chromatograms of IBN blank sample and spiked IBN with aflatoxins standard solution (B₁, B₂, G₁, G₂) are shown in figure 2.

The retention time of AFG2, AFG1, AFB2, and AFB1 for spiked IBN sample with 2 ppb of each aflatoxin B₁, B₂, G₁, G₂ was 5.060 minutes, 6.203 minutes, 7.435 minutes and 9.252 minutes, respectively. During separation by HPLC, AFG2 was the first aflatoxin detected by fluorescence detector and followed by AFG1, AFB2, and AFB1.
Figure 2. HPLC chromatograms. (A) IBN blank sample, (B) Spiked IBN sample with 2 ppb of each aflatoxin B$_1$, B$_2$, G$_1$, G$_2$.

3.3.1. Recovery test. Recovery test of edible bird nest after spiking with aflatoxins and cleaned-up by in-house AF.2 immunoaffinity column was also conducted, and the result is shown in table 2. There were three levels of spiking concentration of aflatoxin standard solution, which were 4 ppb, 8 ppb and 24 ppb for total aflatoxins.

Mean ± SD of AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$ for recovery test of 4 ppb spiked IBN were 96.3±2.9%, 98.5±3.5%, 100.5±3.1%, and 79.8±1.7%, respectively. Mean ± SD of AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$ for recovery test of 8 ppb spiked IBN were 86.5±2.9%, 88.0±2.8%, 82.8±3.0%, and 94.3±1.7%, respectively. Mean ± SD of AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$ for recovery test of 24 ppb spiked IBN were 96.3±6.2%, 87.8±4.6%, 85.3±2.9% and 79.8±3.0%, respectively. The recovery test of our method was higher than the study of aflatoxin M1 from reconstituted powdered milk using in-house IAC-HPLC method which were ranged from 73.6% to 86.3% [10]. Our recovery test was also higher than recovery test of aflatoxins M1, B1, B2, G1, G2 in the bovine liver which were ranged from 45% to 75% [11]. Our results showed that both recovery test of spiked PBS and recovery test of 3-level aflatoxin concentration spiked IBN were good ranged from 79.8±3.0% to 100.5±3.1%, and could be used as a standard method for aflatoxin analysis in the edible bird nest.
Table 2. Performance of analytical method in IBN sample, n=4.

| Analyte | Spiking level (ng g⁻¹) | Mean recovery (%) | Intra-day repeatability² | LODb (ng g⁻¹) | LOQc (ng g⁻¹) |
|---------|------------------------|------------------|--------------------------|---------------|--------------|
| AFB₁    | 1                      | 96.3 ± 2.9       | 3.0                      | 0.26          | 0.79         |
|         | 2                      | 86.5 ± 2.9       | 3.3                      |               |              |
|         | 6                      | 96.3 ± 6.2       | 6.4                      |               |              |
| AFB₂    | 1                      | 98.5 ± 3.5       | 3.6                      | 0.04          | 0.13         |
|         | 2                      | 88.0 ± 2.8       | 3.2                      |               |              |
|         | 6                      | 87.8 ± 4.6       | 5.2                      |               |              |
| AFG₁    | 1                      | 100.5 ± 3.1      | 3.1                      | 0.26          | 0.81         |
|         | 2                      | 82.8 ± 3.0       | 3.6                      |               |              |
|         | 6                      | 85.3 ± 2.9       | 3.4                      |               |              |
| AFG₂    | 1                      | 94.3 ± 1.7       | 1.8                      | 0.03          | 0.09         |
|         | 2                      | 79.8 ± 1.7       | 2.1                      |               |              |
|         | 6                      | 79.8 ± 3.0       | 3.7                      |               |              |

³Intra-day repeatability was estimated by analysis of four replicate samples at three concentration levels on the same day.

bLOD, limit of detection
cLOQ, limit of quantification

3.3.2. Intra-day precision/repeatability. Repeatability expresses the precision under the same operating condition over a short interval of time [12]. Intra-day repeatability was estimated by analysis of four replicate spiked IBN samples at 3 level aflatoxins concentration on the same day. Spiking concentration levels were 4 ng/g, 8 ng/g and 24 ng/g for total AFB₁, AFB₂, AFG₁, and AFG₂. The relative standard deviation of AFB₁, AFB₂, AFG₁, and AFG₂ for recovery test of 4 ppb spiked IBN were 3.0%, 3.6%, 3.1%, and 1.8%, respectively. The relative standard deviation of AFB₁, AFB₂, AFG₁, and AFG₂ for recovery test of 8 ppb spiked IBN were 3.3%, 3.2%, 3.6%, and 2.1%, respectively. The relative standard deviation of AFB₁, AFB₂, AFG₁, and AFG₂ for recovery test of 24 ppb spiked IBN were 6.4%, 5.2%, 3.4%, and 3.7%, respectively. Our repeatability in terms of RSD was better compared to aflatoxin M₁ in cheese and cheddar which are ranged from 7% to 10% [13].

3.3.3. Linearity. Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample [12]. The linearity test was constructed by diluting AFs standard solution in MeOH:H₂O (50:50, v/v) into three levels of concentration (0.65 ng/mL, 0.3125 ng/mL, and 0.0625 ng/mL). The linearity of calibration curve was calculated using an equation, y = Ax + B. Calibration curve was constructed from the area value of HPLC analysis. Calibration curve of aflatoxins standard solution is shown in figure 3. The calibration curve was linear in the range of 0.0625 ng/mL to 0.625 ng/mL. Coefficient of linear correlation of AFB₁, AFB₂, AFG₁, and AFG₂ were 0.99997, 1, 0.99999 and 0.99998, respectively. The coefficient of linear correlation of this study was higher than the coefficient of linear regression from the analysis of aflatoxin contamination in chilli [14].
3.3.4. Limit of detection (LOD) and limit of quantification (LOQ). Limit of detection of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value, and limit of quantification is the lowest amount analyte in a sample which can be quantitatively determined with suitable precision and accuracy [12]. Limit of detection and limit of quantification were determined based on the standard deviation of the response (y-intercept) divided by the slope. Limit of detection was defined as 3 times SD y-intercept/slope, while LOQ was defined as 10 times SD y-intercept/slope. Limit of detection of AFB₁, AFB₂, AFG₁ and AFG₂ were 0.018 ng/mL, 0.003 ng/mL, 0.018 ng/mL and 0.002 ng/mL, respectively. Each LOD values was corresponding to 0.26 ng/g, 0.04 ng/g, 0.26 ng/g, 0.03 ng/g for AFB₁, AFB₂, AFG₁, AFG₂, respectively. Limit of quantification of AFB₁, AFB₂, AFG₁ and AFG₂ were 0.055 ng/mL, 0.009 ng/mL, 0.056 ng/mL and 0.006 ng/mL, respectively. Each LOQ values was corresponding to 0.79 ng/g, 0.13 ng/g, 0.81 ng/g, 0.09 ng/g for AFB₁, AFB₂, AFG₁, AFG₂, respectively. Both LOD and LOQ values are shown in table 2. Based on validation parameters such as recovery test, intra-day repeatability, linearity, LOD, and LOQ which had been tested, our method can be used for validated method for aflatoxin analysis in edible bird nest.

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