Detection of Aflatoxins B₁, B₂, G₁ and G₂ in Nutmeg Extract Using Fluorescence Fingerprint

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A rapid method for predicting total aflatoxin (aflatoxins B₁, B₂, G₁, and G₂) in nutmeg extract was developed using fluorescence fingerprint (FF) and partial least squares (PLS) regression. FF is also known as excitation-emission matrix, which is a series of fluorescence spectra acquired by scanning an excitation wavelength. Nutmeg extract was artificially spiked with an aflatoxin reagent. The FF of spiked nutmeg extract was measured with a fluorescence spectrometer. The FF data was preprocessed to remove signals not related to fluorescence. After preprocessing, 1428 out of 5041 fluorescence intensities remained. They were set as explanatory variables for PLS regression. Then, total aflatoxin concentration was set as the response variable. Eleven out of 21 samples were used as the calibration dataset; the remaining ten were used as the validation dataset. Three latent variables were used to develop the ideal PLS model by cross validation. R² was 0.993 and SEC was 0.2 μg/L for the calibration dataset. Significant correlations were observed between the actual and predicted values for the validation dataset, with R² of 0.773 and SEP of 1.0 μg/L. The PLS regression coefficient, which shows the degree of contribution of each wavelength to the model, indicated that the prediction was mainly based on the fluorescence of aflatoxin itself.

Keywords: Aspergillus, excitation-emission matrix, fluorescence, mycotoxin, PLS regression

Introduction

Aflatoxins are toxic metabolites primarily produced by Aspergillus flavus, A. parasiticus and A. nomius in foodstuffs and feeds. These fungi are widely distributed in nature and frequently contaminate human foodstuffs such as crops, beans, fruits, nuts, and spices. Since aflatoxins are strongly carcinogenic in humans and domestic animals, it is important to be able to reliably analyze foodstuffs for their presence (Patterson and Jones, 1977; Zöllner and Mayer-Helm, 2006; Do and Choi, 2007).

For the above reason, several methods for detecting aflatoxins in agricultural commodities have been reported: thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) with fluorescence detection, HPLC with mass spectrometry, and the use of rapid and simple enzyme-linked immunosorbent assay (ELISA)-based aflatoxin screening kits, which are commercially available for all major types of aflatoxin (AOAC, 2006; Tanaka et al., 2002; Do and Choi, 2007; Zöllner and Mayer-Helm, 2006; Abbas et al., 2004). However, these methods generally require considerable time for sample preparation, and use expensive disposable columns for clean-up, such as the multifunctional column (MFC) and the immunoaffinity column (IAC). In addition, sophisticated handling skills, and expensive equipment and reagents are needed. In particular, aflatoxin analysis of aflatoxins in spices is complex because of the interference of highly colored materials that are coextracted with aflatoxins. For the above method, an elaborate clean-up process, using the above columns, is necessary before

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analysis of aflatoxins in spices (Akiyama et al., 2001; Stroka et al., 2000). The supply of processed spices in Japan is largely dependent on imports from tropical countries. There are several reports (McKee, 1995; Romagnoli et al., 2007) on aflatoxin-contaminated spices (nutmeg) worldwide, and global warming may further increase the number of contamination reports. These circumstances necessitate that nutmeg importers in Japan spend considerable time on food safety checks. Thus, new methods for the rapid, easy, and accurate detection of aflatoxins in nutmeg are anticipated.

Aflatoxins have generally been detected using their fluorescence property (Carnaghan et al., 1963; Pons, 1976). Aflatoxin B group toxins are named for their blue fluorescence (425 nm), and aflatoxin G group toxins for their green fluorescence (450 nm) under UV irradiation (Cole and Cox, 1981). Aflatoxins exhibit strong fluorescence under UV conditions (Cole and Cox, 1981; Panalaks and Scott, 1977). Thus, conventional fluorescence analysis is very useful for aflatoxin determination; however, complicated pre-cleaning for sample preparation, considerable time, and excellent skills are still required. McClure and Farsaie (1980) reported an optical direct detection method for aflatoxin-contaminated pistachio nuts. However, this method was not sufficiently precise for quantitative analysis and is still based on conventional fluorescence methods.

On the other hand, fluorescence fingerprint (FF), also known as excitation-emission matrix (EEM), is a series of fluorescence emission spectra acquired at consecutive excitation wavelengths (Tsuta et al., 2007; Fujita et al., 2010). The pattern of an FF diagram is unique for each constituent, like a fingerprint, making it possible to identify a particular substance in a mixture (Andersen and Mortensen, 2008; Booksh et al., 1996; Shimoyama and Noda, 1992; Tsuta et al., 2007). The FF method is highly sensitive compared with typical fluorescence measurements because the FF method uses all spectral data, consisting of excitation × emission × fluorescence intensities. The capacity to obtain large amounts of information is one of the major advantages of the FF method over the conventional fluorescence method.

FF measurement has several merits in practical and commercial applications, since it requires no complex pre-processing and is nondestructive (Andersen and Mortensen 2008; Yin et al., 2009; Shibata et al., 2011; Fujita et al., 2012; Kokawa et al., 2011). Fujita et al. (2012) successfully predicted the concentrations of two Fusarium mycotoxins in fungus-contaminated wheat flour using FF, with high accuracy and nondestructively. Therefore, a simple method for aflatoxin detection by FF measurement analysis was presumed possible. The objectives of this study were 1) to investigate the original aflatoxin FF pattern at a wide range of wavelengths, and 2) to develop a method for predicting the aflatoxin concentration of a crude nutmeg extract using FF.

**Materials and Methods**

**Sample preparation** Figure 1 shows the flowchart of sample preparation. The reagents for aflatoxins B₁, B₂, G₁, and G₂ used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (Wako Pure Chemical Industries, Osaka, Japan) and ultrapure water from a Milli-Q system (Millipore Biocel A10, Nihon Millipore, Tokyo, Japan) were used. Aflatoxin solutions (0, 15, 60, 150, and 600 μg/L) were prepared in 90% acetonitrile solution for

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Fig. 1. Sample preparation.
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Nutmeg extract was prepared from uncontaminated nutmeg powder. The extraction method was based on standard analysis methods of the Japan Food Hygiene Association (2005). Fifty grams of ground nutmeg was placed in 400 mL of 90% acetonitrile solution and mixed using a shaker for five min. Then, solid substances were eliminated by paper filtration. Finally, the nutmeg extract was artificially spiked with aflatoxin at 5% volume of the extract. The aflatoxin concentration of the nutmeg extract was prepared to be 0, 2.5, 5, 10, 20, 30, and 60 µg/L. The spiked nutmeg extract was diluted to one-tenth with 90% acetonitrile solution to prevent pigment effects.

Twenty-one samples with seven aflatoxin concentrations were prepared from the above spiked nutmeg extract and divided into two groups: 11 were used as the calibration dataset to develop a calibration model, and the remaining 10 were used as the validation dataset to validate the calibration model.

Fluorescence fingerprint measurement FF was measured with a fluorescence spectrometer (F-7000, Hitachi High-Technologies Corporation, Tokyo, Japan). An optional photomultiplier (R928, Hamamatsu Photonics K.K., Shizuoka, Japan) was used as a light detector that extends the upper wavelength range to 900 nm. The measurement range for the excitation and emission wavelengths was 200 – 900 nm, with 10 nm wavelength increments. The slit widths on the excitation and emission sides were fixed at 10 nm. The photomultiplier voltage was 400 V, and the wavelength scanning speed was set to 30,000 nm/min with a response time of 0.002 sec.

The artificially spiked nutmeg extract in each aflatoxin concentration was diluted to one-tenth with 90% acetonitrile solution to prevent pigment effects. On the other hand, the aflatoxin solutions were measured without dilution because they were already transparent. Aliquots (300 µL) of samples were pipetted into 1.0 mL microcells (FM20-SQ-3, GL Sciences Inc., Tokyo, Japan). The diluted solution was referred to as the spiked nutmeg extract. FF was acquired three times for each sample solution. The FF contour map was created using Matlab 2008 (The MathWorks, MA, Japan).

Preprocessing of FF data FF data were preprocessed for statistical analysis on the basis of previous studies (Fujita et al., 2010; Shibata et al., 2011). Fluorescence is an emission with a longer wavelength than excitation (Karoui and Blecker 2011). All data whose emission wavelength was shorter than the excitation wavelength were therefore removed. The FF data include scattered light and second-, third- and fourth-order lights (Lakowicz, 2006). These lights were generated by light scattering from the surface of a diffraction grating and were not fluorescence. They appeared at the same excitation wavelength or several times higher on the emission axis. They were removed as high-order lights (scattered light, ±30 nm from the excitation wavelength; second-order light, ±30 nm from the excitation wavelength; third-order light, ±40 nm from the excitation wavelength; and fourth-order light, ±40 nm from the excitation wavelength).

The low intensity of the Xe lamp and the low sensitivity of the photomultiplier at a short excitation wavelength and a long emission wavelength, respectively, resulted in some noise in the data. Therefore, data at an excitation wavelength < 220 nm or at an emission wavelength > 800 nm were removed (Shibata et al., 2011).

Chemometrics Quantification models of aflatoxin concentration for the aflatoxin solutions or contaminated nutmeg extract were developed using JMP 8.0 (SAS Institute, Tokyo, Japan). PLS regression with leave-one-out cross validation was applied to the analysis of the FF data of the calibration samples, as well as of the true aflatoxin concentrations, to develop a calibration model. The performance of PLS models depends on the number of latent variables (LVs) used. The optimum number of LVs was determined by minimizing the root-mean-square error of the prediction of cross validation. The calibration model was applied to the analysis of the validation dataset to evaluate the accuracy of the model. The fitting of the calibration model to the validation datasets was finally evaluated using the coefficient of determination ($R^2$), standard error of calibration (SEC), and standard error of prediction (SEP).

Results and Discussion

FFs of aflatoxin solutions Figure 2 shows the FF contour maps of all concentrations of aflatoxin solutions. The oblique lines with strong-emission light, which run from an excitation wavelength of 200 nm and emission wavelengths of 200, 400, and 600 nm, are the scattered light and second-, third- and fourth-order lights, respectively (Lakowicz, 2006). Three specific peaks were observed visually, as well as highly significant characteristic profile patterns at emission wavelengths of approximately 420 to 450 nm, as shown in Figure 2.

Aflatoxins exhibit a strong fluorescence characteristic under UV conditions (Cole and Cox, 1981; Panalaks and Scott, 1977). The fluorescence intensities and number of peaks of each aflatoxin differ. Cole and Cox reported that aflatoxin B$_1$ peaks at excitation wavelengths of 223, 265, and 362 nm; aflatoxin B$_2$ also has three peaks at excitation wavelengths of 222, 265, and 363 nm; aflatoxin G$_1$ peaks at excitation wavelengths of 243, 257, 264, and 362 nm; and G$_2$ peaks at excitation wavelengths of 214, 265, and 363 nm. The fluo-
Fig. 2. Fluorescence fingerprints of aflatoxin reagent.
The ovals show the fluorescence at approximately Ex356 nm/Em450 nm in each FF. The two arrows show the fluorescence at approximately Ex220 nm/Em420 nm and Ex250 nm/Em420 nm in each FF.

Fig. 5. Fluorescence fingerprints of contaminated nutmeg extract.

Fig. 4. Distribution of PLS regression coefficients of aflatoxin solution.
PLS: Partial least squares.

Fig. 7. Distribution of PLS regression coefficients of contaminated nutmeg extract.
PLS: Partial least squares.
Fluorescence emission wavelengths also differ between aflatoxins in the B group (Em425 nm) and G group (450 nm). Although the fluorescence features are not the same, Figure 2 shows that the three peaks for the aflatoxin solutions observed in this study are in agreement with the typical profile pattern of wavelength locations for excitation (Ex220, 260, and 360 nm) and emission (Em420 and 450 nm). Therefore, these peaks were confirmed using the FFs of all the aflatoxin solutions. It was thus shown that FFs could be used to detect the presence of aflatoxins in simple acetonitrile solutions.

**Aflatoxin quantitation in solution using PLS regression**

The original FF consists of 5041 intensities recorded under the excitation and emission wavelengths conditions. Some of the wavelength conditions were removed by preprocessing. After preprocessing, 1428 fluorescence intensities remained, and this total was set as the independent variable for PLS regression.

Figure 3 shows the relationship between the actual and predicted aflatoxin concentrations in the acetonitrile solutions of the dataset. The optimum number of LVs in the PLS regression was three. R² was 0.999 and SEC was 7.36 μg/L. This shows an excellent correlation between the actual and predicted aflatoxin concentrations in the standard acetonitrile solutions. This indicates that signals for aflatoxins were present in the FF data and that the aflatoxin concentration in solutions can be predicted using PLS regression.

To understand the structure of the prediction model, the PLS regression coefficients in a contour map of the regression model developed are shown in Figure 4. The PLS regression coefficient is the degree of contribution of each wavelength to the predicted result (Fujita et al., 2012; Saranwong et al., 2010). Thus, the higher the absolute value of the regression coefficient, the higher the contribution of each wavelength to the prediction. In this experiment, the area of the higher regression coefficient includes a typical peak pattern of aflatoxin solutions (Figures 2 and 4). Aflatoxin fluorescence exhibits a wide range of emission at UV wavelengths from 200 to 400 nm (Rasch et al., 2010). Yao et al. (2010) reported that the aflatoxin concentration of individual corn kernels could be estimated using emission wavelengths from 400 to 600 nm at an excitation wavelength of 365 nm. This suggests that the prediction model proposed here is based on information of the aflatoxin itself. Subsequently, the aflatoxin concentration of spiked nutmeg extract was predicted using FF and PLS regression.

**FF of aflatoxin in spiked nutmeg extract**

Figure 5 shows...
the FF of nutmeg extract spiked with three aflatoxin concentrations.

A typical peak and pattern similar to that in Figure 2 can be observed at approximately Ex370/Em450 nm in the spiked nutmeg extracts. However, the fringe of the peak at Ex250 nm is hardly discernible. Similarly, the remarkable difference is that the scatter light and its higher-order lights between Ex250 and 350 nm were drastically weakened. These phenomena indicate that the nutmeg pigment caused the UV absorption between Ex250 and 350 nm, which reduced fluorescence intensity. In such a case, dilution of the sample can effectively decrease UV absorption, thereby increasing fluorescence signal intensity. This is the reason we diluted the spiked nutmeg extract to one-tenth in the final stage of sample preparation. Although such differences in color scale cannot be observed by the naked eye (Figure 5), the digitized data provides quantifiable fluorescence information. As a result, it is worth applying PLS regression to the analysis of the data in Figure 5.

Prediction of aflatoxin concentration in spiked nutmeg extract

The same preprocessing as that used to obtain the FFs of the aflatoxin solutions was carried out. 1428 fluorescence intensities were acquired from the FF data of diluted nutmeg extract. PLS regression was applied to the analysis of 11 samples as the calibration dataset. The remaining 10 samples were used as the validation dataset. Figure 6 shows the correlation between the actual and predicted aflatoxin concentrations of the spiked nutmeg extract. Three LVs were used to develop the best PLS mode by cross validation. R² was 0.993 and SEC was 0.2 μg/L in the calibration dataset. Thus, the calibration model is considered to be good. To evaluate its robustness for prediction, the model was applied to the analysis of the validation dataset. A good fit was observed between the actual and predicted values of the validation dataset, with R² of 0.773 and SEP of 1.0 μg/L. Although the nutmeg extract prepared for calibration and validation datasets is identical, the SEP for validation in Figure 6 shows five times as much as that for calibration. It is thought that the main reason for this variance is the dispersion or non-uniformity of the nutmeg extract. Though the SEP of the diluted nutmeg extract was 1.0 μg/L, the actual SEP of the original nutmeg extract was ten times greater, 10 μg/L. Compared with the SEC of the aflatoxin solution (7.36 μg/L) in Figure 3, the SEP of 10 μg/L seems to be quite reasonable.

Figure 7 shows the PLS regression coefficient map for identifying which wavelengths most effectively contribute to prediction. Although the map indicated that important information is dispersed widely across all wavelength conditions, the regression coefficient from Ex 250 – 400 nm/Em 400 – 800 nm was relatively high. This range of wavelengths is also similar to the range of the aflatoxin solution in this study (Figure 4). Hence, the predicted attribute seems to be based on information of the aflatoxin itself. It is clear that FFs provide sufficient information on aflatoxins. To predict aflatoxin quantity, we used not only peak signals but also all other fluorescence signals. In addition, PLS regression was applied to mining for objective information, e.g., aflatoxin quantity. In spite of the simple process of extraction from nutmeg, and the inclusion of pigments and other constituents, the results indicate that rapid and quantitative aflatoxin detection by FF measurement is possible.

We found that UV absorption due to the nutmeg pigment prevents the detection of fluorescence signals related to aflatoxin in this study. To achieve a more precise prediction, it is necessary to eliminate the effect of UV absorption. As the next step, the FF of nutmeg powder and the accumulation of seasonal data are expected to be useful in facilitating the practical detection of aflatoxins.

References


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