

Discrimination of aflatoxin contamination level in nutmeg by fluorescence fingerprint measurement

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1 **Discrimination of aflatoxin contamination level in nutmeg by**
2 **fluorescence fingerprint measurement**

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9 **Keywords:** mycotoxin; excitation-emission matrix; spice; partial least squares regression

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16

17 **Abstract**

18 A fluorescence fingerprint (FF), also known as an excitation–emission matrix, was used to
19 develop a new method of classifying nutmeg contaminated with aflatoxins. The experimental
20 samples were collected from nutmeg with a wide range of fungal contamination levels. After
21 grinding the samples, FF measurement and high–performance liquid chromatography (HPLC)
22 analysis were carried out. The total concentration of aflatoxins ($AFB_1 + AFB_2 + AFG_1 + AFG_2$;
23 total AF concentration) in the samples was determined using HPLC, which varied from 0.0 to
24 1781.8 ppb. The FFs of the same samples were measured with a fluorescence
25 spectrophotometer. Although the FF patterns changed with increasing total AF concentration,
26 the trend was unclear at lower concentrations. Therefore, models for predicting or classifying AF
27 contamination in nutmeg from FF patterns were developed using partial least–squares
28 regression (PLSR). The total AF concentrations predicted by the PLSR model showed a
29 positive correlation to the actual concentration with a coefficient of determination of 0.69.
30 Moreover, the variable importance in projection plot indicated that fluorescence from AFs as
31 well as from kojic acid derivatives was important for the prediction of total AF concentration.
32 Finally, it was indicated that samples with a total AF concentration of 10 ppb or higher could be
33 reliably discriminated by setting the threshold to 2.2 ppb in the partial least–squares
34 discriminant analysis (PLSDA). **While no false negative was observed in the discrimination, the**
35 **false positive rate was 13.3%.** Future studies on nutmeg samples with different origins are
36 necessary to confirm feasibility of FF as a rapid and simple method of predicting aflatoxin
37 contamination of nutmeg.

38

39

40 1. Introduction

41 Aflatoxins (AFs ; aflatoxins B1, B2, G1, and G2) are cardinal toxic metabolites in human
42 food such as rice, wheat, fruits, nuts, and spices in the world (Patterson & Jones, 1977)(Zöllner
43 & Mayer-Helm, 2006)(Do & Choi, 2007) . They are known as a strong carcinogen, an
44 immunosuppressive substance, and a mutagen. Therefore, there are regulations for AFs
45 concentration in many organizations and countries; the Japanese acceptable guideline for AFs in
46 food and feed is 10.0 ppb (Fujita, Hidaka, Tkehito, Mizuki, & Sugiyama, 2016) , the US FDA
47 guideline is 20.0 ppb (NGFA U.S, 2011), the Codex and Australian guideline is 15.0 ppb (Bash,
48 2015)(Authority, n.d.) and the **European Union** total aflatoxin limit is 4.0 ppb (The Commission
49 of the European Communities, 2010).

50 AFs are produced with other secondary metabolites such as kojic acid derivatives by
51 *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. The fluorescence emitted by colonies of these
52 fungi, which is due to kojic acid derivatives, is called bright greenish–yellow fluorescence
53 (BGYF)(Steiner, Rieker, & Battaglia, 1988). It is a presumptive indicator of AF presence but
54 not used as an inspection method because there are *Aspergillus* spp. that produce kojic acid
55 derivatives but not AFs (Steiner et al., 1988).

56 For the reason mentioned above, several chemical methods of detecting AFs in food have
57 been reported (Do & Choi, 2007). Thin–layer chromatography (TLC) and high–performance
58 liquid chromatography (HPLC) are methods of detecting AFs using the autofluorescence of AFs.
59 HPLC with mass spectrometry has high sensitivity and quantitativity (Zöllner & Mayer-Helm,
60 2006)(Lei Bao and Chengzhu Liang et al., 2013)(Tanaka et al., 2002)(Abbas, Shier, Horn, &
61 Weaver, 2004). Recently, the use of ELISA–based AFs screening kits has been developed as a
62 simple and rapid method of detecting AFs (Yu, Gribas, Vdovenko, & Sakharov, 2013).
63 However, these methods require a skilled operator, considerable time, toxic solvents and
64 expensive disposable columns such as immunoaffinity and multifunctional columns. These

65 make the aflatoxin test difficult to perform in the production field. Thus, the number of reports
66 on aflatoxin-contaminated spices (McKee, 1995)(Romagnoli, Menna, Gruppioni, & Bergamini,
67 2007) tended to increase and the regulation of import spices has become stricter over the years
68 (Commission, 2016).

69 Therefore, we developed a rapid method of predicting the total AF concentration in nutmeg
70 extract using fluorescence fingerprint (FF) (Fujita et al., 2013). The FF method has higher
71 detection sensitivity than conventional fluorescence measurements because it uses
72 comprehensive spectral data consisting of excitation \times emission wavelength \times fluorescence
73 intensity. Aflatoxin B group toxins are named for their blue fluorescence (425 nm) and aflatoxin
74 G group toxins for their green fluorescence (450 nm) under UV irradiation (Cole & Cox, 1981),
75 and these autofluorescence were thought to play an important role for the prediction of AF
76 concentration using FF. The method does not require complex preprocessing or expensive
77 columns. However, it entails the extraction of components using organic solvents such as
78 acetonitrile.

79 The objectives of this study were prediction of total AF concentration in nutmeg powder, and
80 discrimination between samples could be accepted and discarded under Japanese guideline, 10
81 ppb, based on FF measurement without the solvent extraction procedure.

82

83 **2. Materials and Methods**

84 *2.1. Samples*

85 Ninety-one samples of roughly ground nutmegs were imported from Indonesia in 2014 and
86 2015. They included nutmegs potentially contaminated with AFs in the production site, which
87 are usually discarded by the sample provider's quality inspection. Samples were passed through
88 a 1 mm sieve for each lot and used as samples for HPLC analysis and FF measurement.

89

90 *2.2. Sample preparation for FF measurement*

91 Samples were ground to fine powder with a multi beads shocker (MB1001FC(S), Yasui Kikai,
92 Osaka, Japan). First, 1.0 g of sample was put into a polycarbonate tube with a metal corn. Then,
93 they were frozen at -80°C for over 12 h to prevent oil effusion. Finally, the frozen
94 polycarbonate tubes sample and a metal corn were set into a multi beads shocker and
95 immediately ground. The grinding speed and duration were set to 1500 rpm and 45 sec,
96 respectively.

97

98 *2.3. FF measurement*

99 FF was measured with a fluorescence spectrometer (F-7000, Hitachi High-Technologies
100 Corporation, Tokyo, Japan). About 300 mg of fine nutmeg powder was put into a dedicated cell
101 for the measurement of the FF of powder samples. The measurement wavelength range was set
102 to 200–900 nm with 10 nm intervals for both excitation and emission. The photomultiplier
103 voltage was set to 500 V. The wavelength scanning speed was set to 30,000 nm/min with a
104 response time of 0.002 sec. The slit width for both excitation and emission was 10 nm. These
105 conditions were decided in a preliminary experiment. The measurement was conducted using
106 the front-face method (Sádecká & Tóthová, 2007). Three replicates were obtained for each
107 sample.

108

109 *2.4. Preprocessing of FF data*

110 Preprocessing of FF data was conducted using R v3.3.1 software with the EEM package
111 v1.1.1 (Trivittayasil, V., 2016). The FF data, which were measured in the excitation
112 wavelength range of 250–700 nm and the emission wavelength range of 260–720 nm, were
113 used for the following data analyses to cut off unnecessary excitation and emission wavelength
114 ranges, whose noises could negatively affect the data analyses. Then the scattering rays and

115 two regions that are not related to fluorescence emission, namely, the regions where the
116 emission wavelength is shorter than excitation light ($EM \leq EX$) and the region above the
117 second-order scattering ($EM \geq 2*EX$), were deleted. Then, the median of the fluorescence
118 intensities of three replicates at each wavelength condition was calculated to construct
119 representative FF data of each sample. Finally, the data was unfolded into 2-dimensional data
120 (Guimet, Ferré, Boqué, & Rius, 2004) whose rows represent samples and columns represent
121 EX/ EM wavelength combinations for the following data analyses.

122

123 *2.5 HPLC analysis*

124 Twenty-five grams of sample was extracted with 250 mL of a solution of acetonitrile, water and
125 methanol (6:4:1). The mixture was centrifuged (2500 rpm, 5 min) and the supernatant was used
126 as an extract. Five milliliters of the extract was adjusted to 50 mL with 2% Tween-20 aqueous
127 solution and filtered with glass fiber filter paper (ADVANTEC GA-55, 15 cm, Toyo Roshi
128 Kaisha, Ltd.). An immunoaffinity column (AFLAKING, Horiba Seisakusho Co., Ltd.) was
129 loaded with 20 mL of the filtrate, washed with 10 mL of PBS containing 0.01% Tween, and
130 further washed with 10 mL of water. After removal of moisture in the column, AFs was eluted
131 with 3 mL of acetonitrile. The eluate was dried and solidified in a nitrogen stream and dissolved
132 in 1 mL of a mixed solution of acetonitrile and water (9: 1) to prepare a test solution. Ten
133 microliters of the test solution were injected into a high-performance liquid chromatograph
134 (LC-20AD, Shimadzu Corporation), and the concentrations of AF B1, B2, G1 and G2 in the
135 sample were determined from the calibration curves.

136

137 *2.6. Prediction of total AF concentration using FF*

138 The total AF concentration of nutmeg powder has been predicted by partial least squares
139 regression (PLSR) analysis. The preprocessed FF data and the total AF concentration measured

140 by chemical analysis served as explanatory and objective variables, respectively. The unfolded
141 FF data matrix contains a total of 91 samples with 2162 variables (46 excitation wavelengths ×
142 47 emission wavelengths). The data were sorted according to total AF concentration in
143 descending order. Then every third sample from the top was chosen as validation and the rest
144 was served as calibration. Thus, 61 samples were used as the calibration dataset, and 30 samples
145 were used as the validation dataset. Logarithmic transformation and mean centering as
146 preprocessing methods were applied to the FF data matrix and total AF concentration. Cross-
147 validation was performed within the calibration group to determine the suitable number of latent
148 variables. PLS regression was conducted using R v3.3.1 software with pls package v2.5-0
149 (Mevik & Wehrens, 2007).

150

151 *2.7. Discrimination of total AF concentration by FF*

152 FF data has been discriminated based on the total AF concentration by partial least- squares
153 discriminant analysis (PLSDA), which was carried out with caret v6.0-71 packages (Kuhn,
154 2008) using the same data as PLSR.

155

156 **3. Results and Discussion**

157 *3.1. FF measurement*

158 Figure 1 shows examples of the FF contour maps. The intensity of FF changed with increasing
159 AF concentration. There was no visible difference in FF pattern between 0.0 and 13.4 ppb,
160 whereas there was a definite difference in FF pattern between 0.0 and 153.9 ppb or 1781.8 ppb.
161 Especially, fluorescence intensity around excitation / emission wavelength range of 400-550/
162 450-600 nm was higher in the FF contour map of 1781.8 ppb than others.

163

164 *3.2. Prediction of total aflatoxin concentration*

165 Figure 2 shows a histogram of aflatoxin concentrations of calibration and validation data sets.
166 The number of samples below and above 10 ppb were 37 and 24 for calibration, and 18 and 12
167 for validation, respectively. Figure 3 shows the correlation between the measured and predicted
168 aflatoxin concentrations of nutmeg by PLSR. The number of latent variables (LVs) was
169 determined to be two by cross-validation. The coefficient of determination for calibration (R^2C)
170 was 0.6 and the root-mean-squared error of calibration (RMSEC) was 0.59 log (ppb), which is
171 equivalent to 3.9 ppb. The log notation is omitted hereafter. The coefficient of determination for
172 prediction (R^2P) was 0.69 and the root-mean-squared error of prediction (RMSEP) was 3.1 ppb.
173 These results indicated difficulty in the quantitative prediction of total AF concentration,
174 because there were large errors for both calibration and prediction relative to 10 ppb, which is
175 the regulation value of total aflatoxin concentration in Japan.

176

177 3.3. Discrimination of total AF concentration by FF

178 In Fig. 3, the vertical and horizontal dashed lines both indicate 10 ppb for the measured and
179 predicted concentrations, respectively. Samples located above the horizontal line could be
180 considered as contaminated ones. The total AF concentration of those located on the right side
181 of the vertical line but under the horizontal one was under estimated to 10 ppb or less, although
182 the actual measured concentration is 10 ppb or more. In this manner, the results of PLSR
183 analysis could be used for pseudo discriminant analysis of AF contamination in the nutmeg
184 samples. Another discriminant analysis was carried out by PLS-DA. When the threshold of
185 predicted concentration was 10.0 ppb, there was a 10% false negative error in **calibration and**
186 **validation datasets** results. Since the false negative error should be 0% in the production field to
187 prevent contaminated samples deemed as fine ones, the threshold of predicted concentration
188 was reduced from 10.0 to 1 ppb stepwisely to reduce the false negative error. In the case of
189 PLSR, there was no false negative error when the threshold of predicted concentration was set

190 at 4.0 ppb, as shown by the horizontal long dashed–dotted line in Fig. 3. This indicates that the
191 samples with the predicted total AF concentration of 4.0 ppb or less were within the regulation
192 value. Also, the proportion of false positive error under such a condition was 17.6% (Fig. 4).

193 Figure 5 shows the changes in the ratio of false negatives to false positives based on results of
194 PLSDA. The false negative rate reached 0% at 2.2 ppb, and the false positive rate was 13.3%,
195 which was lower than that in the case of PLSR analysis.

196

197 *3.4. Variable importance in projection (VIP) value derived from the PLS model*

198 Figure 6 shows the VIP value derived from the PLSR model. Wavelengths with higher VIP
199 values contribute more to the PLS model (Wold, Sjöström, & Eriksson, 2001). There were some
200 peaks at excitation wavelengths of 250, 320, 390, 460, 520 nm, and at emission wavelengths of
201 420, 420, 490, 720, 640 nm, respectively. According to previous studies, the fluorescence peaks
202 of AFs are located at excitation wavelengths of 220, 260, 360 nm, and at emission wavelengths
203 of 420, 420, 450, respectively (Fujita et al., 2013). The fluorescence peak of kojic acid
204 derivatives is located at an excitation wavelength of 395 nm and a fluorescence wavelength of
205 495 nm (Hruska et al., 2014). Therefore, the peak observed at Ex390/Em490 nm, indicated by
206 arrow A, probably reflected the fluorescence of kojic acid derivatives and the peak observed at
207 Ex250/Em420 nm, indicated by arrow B, reflected that of AFs. On the other hand, it was
208 difficult to observe the peak at Ex360/Em450 nm, because it seemed to overlap with the peak of
209 the kojic acid derivatives (arrow A). **Components corresponding to the peak at Ex320/Em410**
210 **nm could not be identified in this study.** Thus, this model included not only information on
211 typical fluorescence peaks of AFs located at Ex250/Em420 nm but also those of kojic acid
212 derivatives located at Ex390/Em490 nm. On the other hand, the VIP values at Ex250/Em420
213 nm and Ex390/Em490 nm were 1.5 and 3.3, respectively. This suggests that the model largely
214 reflects the behavior of kojic acid derivatives in nutmeg rather than AFs.

215

216 **4. Conclusion**

217 In this study, we could develop a PLS model using FF measurement without organic solvent
218 extraction and expensive immunoaffinity columns. This method would be more suitable for
219 production field inspection than ELISA-based AFs screening kits, because it does not require a
220 skilled operator with scientific knowledge. The PLS model seemed to reflect the behavior of
221 AFs and kojic acid derivatives in contaminated nutmeg. It was difficult to quantify the total AF
222 concentration, because BGYF derived from kojic acid derivatives had a significant effect on the
223 PLS model. However, with 13.3% false positive rate, we could completely classify AF-
224 contaminated nutmeg samples by setting the classification threshold to 2.2 ppb, which indicated
225 the potential of FF as a rapid and simple method of predicting aflatoxin contamination of
226 nutmeg. The threshold for the discrimination of AF contamination was optimized using the
227 samples prepared for this study. Therefore, further studies on samples of different lots and
228 production areas are necessary to confirm feasibility of FF. Furthermore, in practical
229 applications, the discrimination threshold and corresponding acceptable false positive and false
230 negative rates should be determined on the basis of the number of samples for examination,
231 accuracy, and cost required at the inspection site.

232

233 **Acknowledgements**

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235 Fisheries of Japan (Research project for improving food safety and animal health). We thank
236 Vox trading Co. Ltd. for providing us with uncontaminated samples as well as with potentially
237 contaminated sample of nutmeg, which are usually discarded on the production site during
238 inspection.

239

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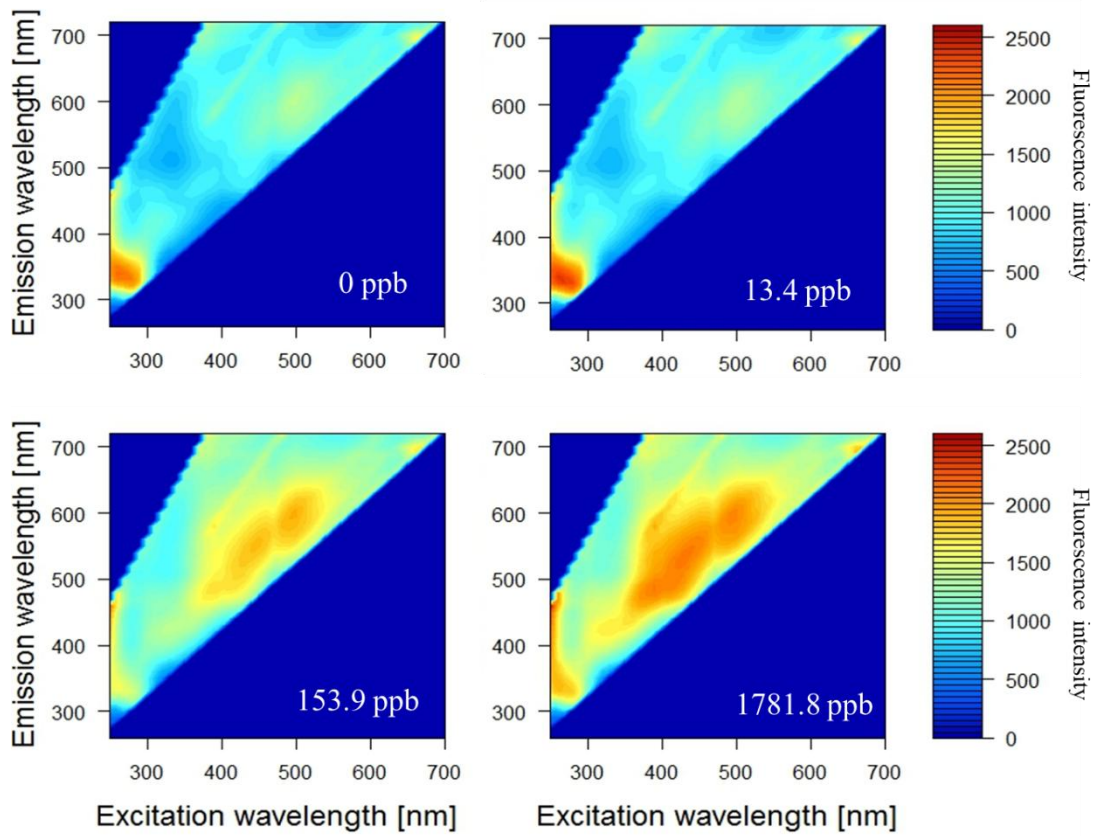
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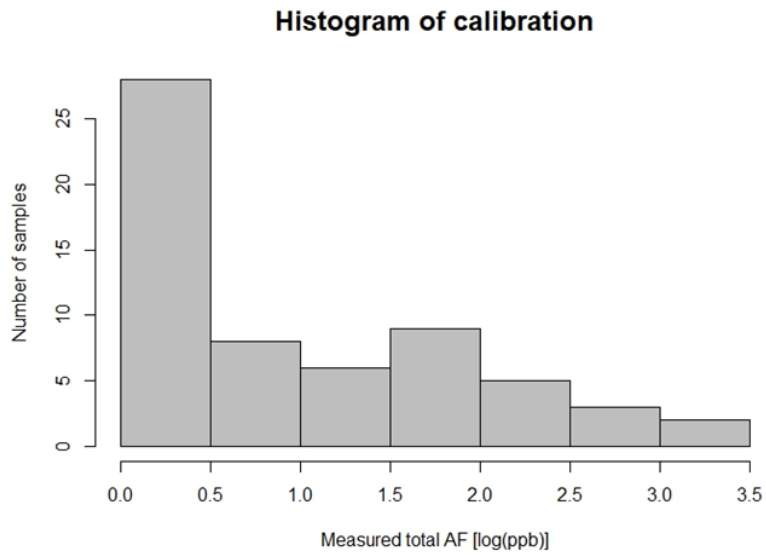
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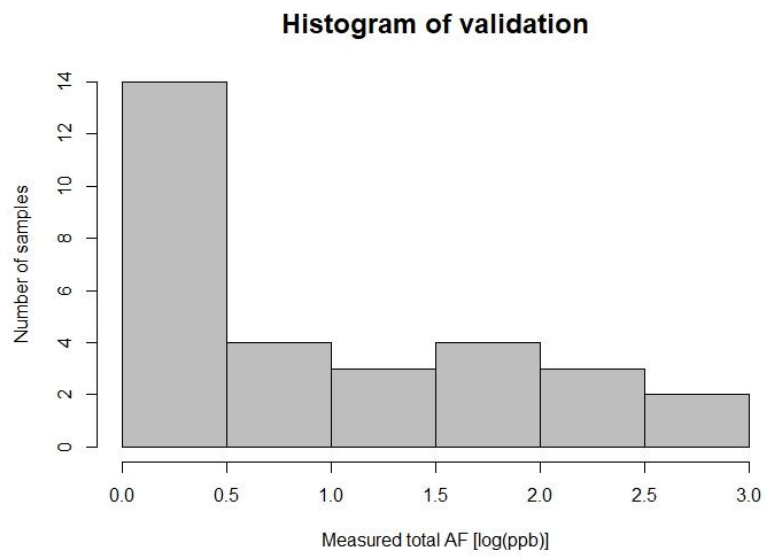
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Fig. 1. Examples of the FF contour maps.

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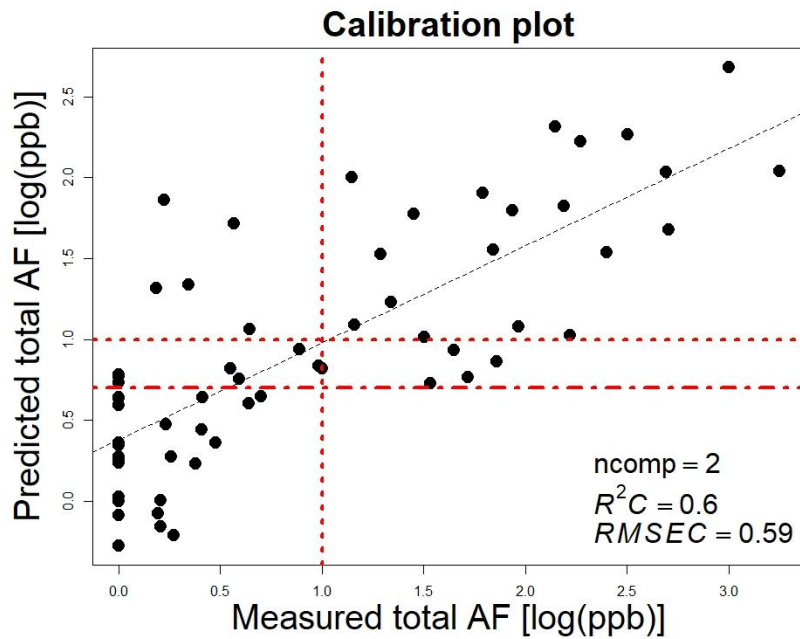


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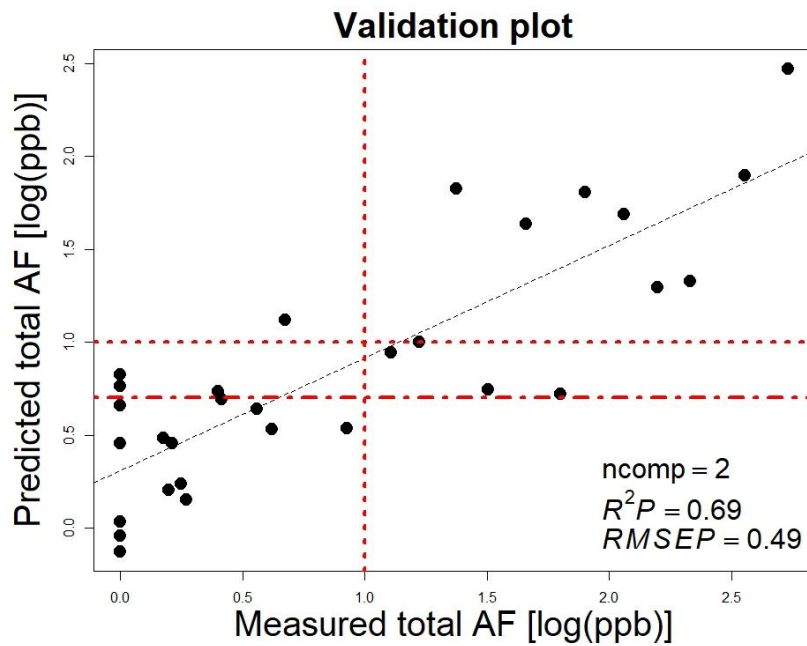


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Fig. 2. Histograms of total AF concentration in the calibration and validation data sets.



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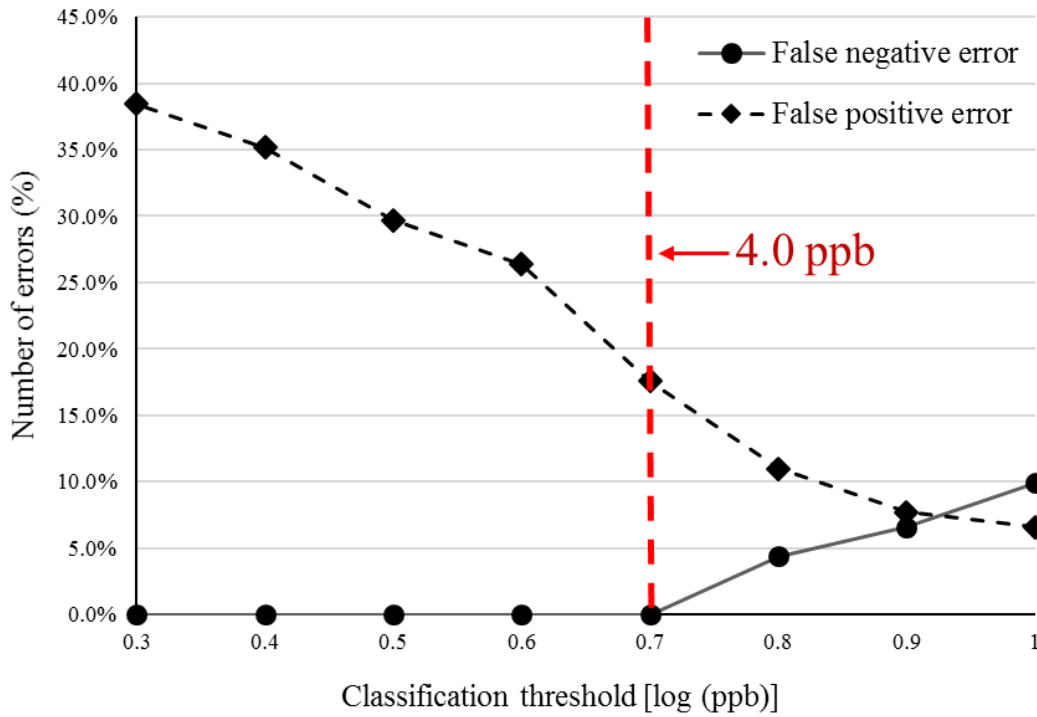


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343 Fig. 3. Correlation between the measured and predicted AF concentrations of the
 344 nutmeg powder for the calibration (top) and validation (bottom) datasets. The horizontal
 345 and vertical long dashed lines refer to 10.0 ppb, and the horizontal long dashed-dotted
 346 line refers to 4.0 ppb.

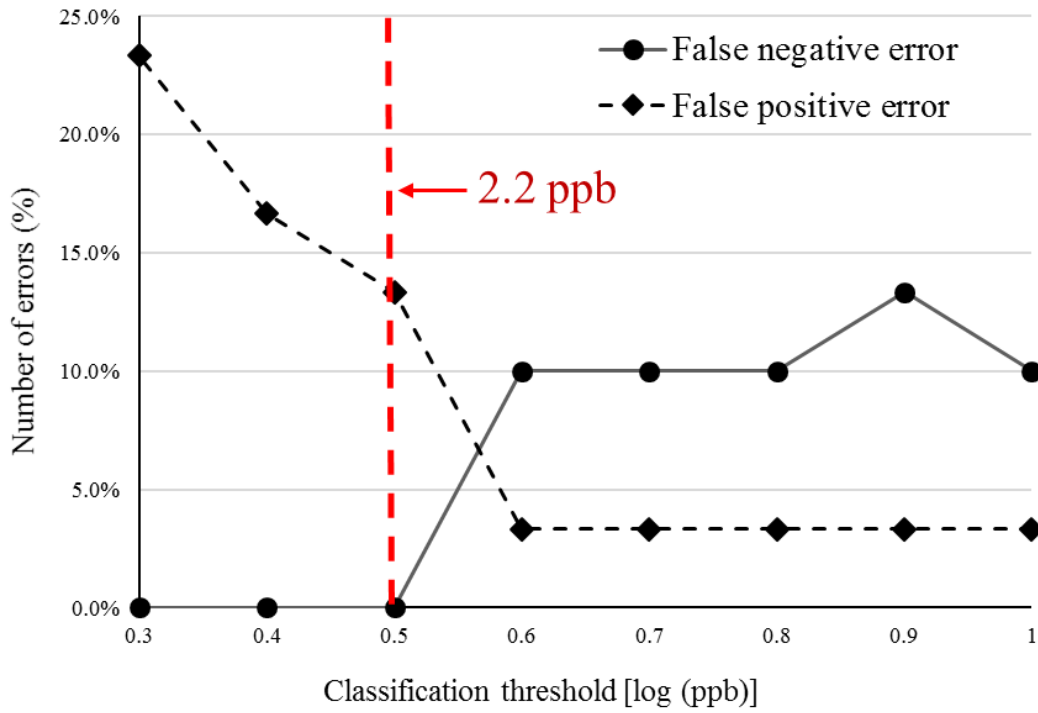
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Fig. 4. Changes in the ratio of false negatives and false positives for the classification of AF contamination on PLSR against the classification threshold.

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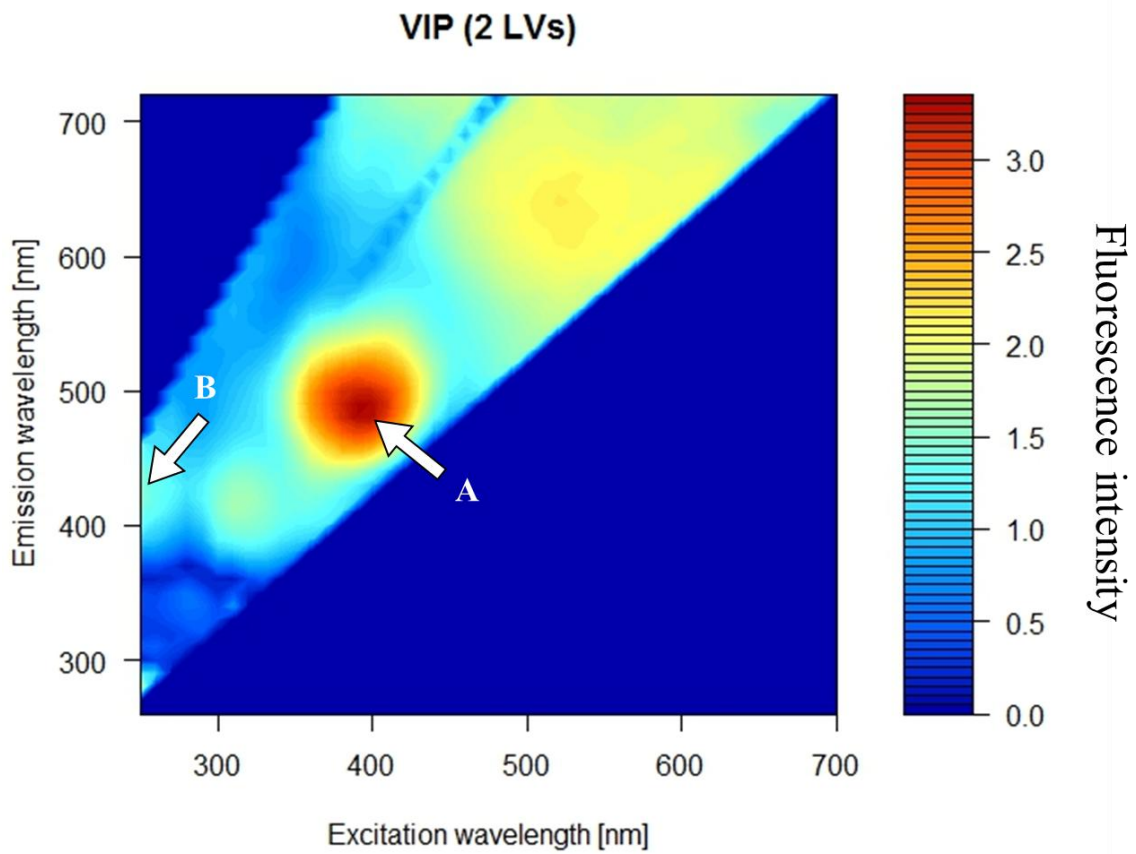
Fig. 5. Changes in the ratio of false negatives and false positives for the classification of AF contamination on PLSDA against the classification threshold.

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Fig. 6. Variable importance in projection (VIP) on the prediction of total AF concentrations. The arrow A indicates the peak at Ex390/Em490 nm, probably reflected the fluorescence of kojic acid derivatives and the arrow B indicates the peak at Ex250/Em420 nm reflected that of AFs

Highlights

- Fluorescence fingerprints (FF) of nutmeg powder samples were measured.
- PLS models to predict aflatoxins (AF) in samples from FF patterns were developed.
- Samples with AF of 10 ppb or higher could be discriminated without false negative.
- PLS models reflected the behavior of AFs and kojic acid derivatives in samples.