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Rapid Detection of *Aspergillus flavus* Contamination in Peanut with Novel Delayed Luminescence Spectra

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ABSTRACT

Employing a luminescence spectrometer the usefulness of light-induced delayed luminescence (DL) for the detection of aflatoxinB1 (AfB1) contamination in whole peanut was studied. Peanut was artificially contaminated with *Aspergillus flavus* (7.5×10^5conidia/mL) and incubated for 0–72 h. The DL and fluorescence spectra of contaminated peanut were obtained by a luminescence spectrometer. The correlation between the spectra and contamination levels was established. The DL and fluorescence intensity has a negative correlation with the AfB1 concentration. Our results suggested that the DL technique might be useful for the rapid and noninvasive evaluation of AfB1 contamination levels in whole peanut.

INTRODUCTION

All organisms can emit weak light spontaneously at very low rates; this phenomenon is called luminescence. When illuminated with a pulse of light, reemission occurs at a much higher rate and decays hyperbolically within seconds to minutes. This is called delayed luminescence (DL), and its lifetime extends from 10^{-7} to greater than 10 s. It has been found in many biological systems that a close connection exists between the status of living organisms and their DL (1–3).

Aflatoxins are a group of toxic secondary metabolites of fungi and most commonly produced by certain strains of *Aspergillus flavus* and all strains of *A. parasiticus*. A variety of crops including corn, cotton seed, peanut, wheat, copra, peas, millet, spices and dry fruits may be contaminated by aflatoxins, particularly aflatoxinB1 (AfB1). Among animals, poultry livestock are particularly endangered by acute aflatoxicosis. Prolonged exposure to subacute levels of aflatoxins is detrimental to human health. The aflatoxin contamination of agricultural commodities is an established concern at both national and international levels. The stringent regulations governing aflatoxin levels in food and feed with an enforcement of regulatory limits has become mandate globally. Chromatographic methods (4–7) using liquid chromatography (LC) in conjunction with fluorescence detection (FLD) (8) or mass spectrometry detection (MS) (9) are the most commonly employed to measure AfB1 at the concentrations present in food commodities. Niedwetzki and Geschwill (10) developed an LC-based automatic workstation for determining aflatoxins in food.

However, to date, DL has not been used for analysis of peanut quality and possible contaminations. This report presents novel DL spectra methods for determining *A. flavus* contamination in peanut. The novel methods were tested in peanut samples artificially contaminated with various concentrations of *A. flavus* conidia suspension. The sensitivity and specificity of newly developed methods were compared with the commonly used fluorescence detection method as a tool for routine analysis of AfB1 contamination in peanut. It is the first time that the detection of AfB1 concentration, therefore, the levels of contamination, with DL spectra has been reported.

MATERIALS AND METHODS

Reagents and peanut

All chemicals were AR grade, they include: methanol–water (55:45 v/v), petroleum ether, $NH_4H_2PO_4$–$HgCl_2$ solution (mixture of 8.69×10^{-2} mol/L $NH_4H_2PO_4$ and 5×10^{-4} mol/L $HgCl_2$) and ethanol. Peanut samples were bought from a local market. *A. flavus* As312890 was kindly provided by Dr. Xuanqiang Liang (Crops Research Institute, Guangdong Academy of Agricultural Sciences). AfB1 were purchased from the Sigma Chemical Co. A standard AfB1 solution was prepared by dissolving AfB1 into the $NH_4H_2PO_4$–$HgCl_2$ solution.

Peanut sample preparation

Peanut sample (20 g) was first disinfected with 70% ethanol for 1 min and washed 3 times with 5 mL of autoclaved distilled water. Washed peanut sample was then inoculated with 1 mL of a homogeneous suspension of *A. flavus* (7.5×10^5 conidia/mL) and stirred with a sterile scoop for 1 min before pushing out the suspension. Spiked peanut sample was transferred to a sterile glass vessel and incubated at 25°C in darkness for 0, 12, 24, 36, 48 and 72 h, respectively, in an artificial climate chamber (LRH-250-GS; Guangdong Medical Appliances Plant, Guangdong, China). AfB1 extract analysis was performed as described previously (11, 12). Briefly, peanut samples were dried at 115°C for 1 h, and then skived thoroughly. 2 g peanut was mixed with 10 mL of methanol–water (55:45 v/v) and 3 mL petroleum ether. After vigorously shaking for 1 min, the sample was allowed to settle for 30 min before separating the top layer of cloudy solution. The cloudy solution was discarded, and the rest of the leftover solution was filtered through a filter paper (Whatman No. 4). The volume of filtration was recorded. The extraction procedure took approximately 50 min to complete. The filtration of 200 μL (0–36 h incubation) or 20 μL (48–72 h incubation) was then diluted in 3 mL aqueous solution containing $NH_4H_2PO_4$ (8.69×10^{-2} mol/L) and $HgCl_2$ (5×10^{-4} mol/L). The diluted extracts (3.02–3.2 ml) were used for fluorescence spectra analysis.

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Abbreviations: AfB1, aflatoxinB1; AR, analytical reagent; DL, delayed luminescence; DW, dry weight; FLD, fluorescence detection; LC, liquid chromatography; LS, luminescence spectrometer; MS, mass spectrometry.

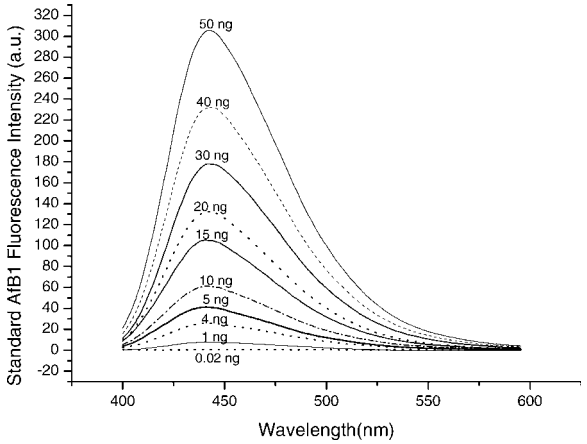


Figure 1. Fluorescence spectra of the standard AfB1 solution. Fluorescence spectra of pure AfB1 (0.02–50 ng/mL) was determined using the luminescence spectrometer under the fluorescence mode with excitation slit of 10 nm, emission slit 10 nm, scan speed of 200 nm/min.

Determination of fluorescence spectra of the extracts

The fluorescence spectra of the diluted extracts and control (extracts of unspiked peanut) were measured using a luminescence spectrometer (LS-55, Perkin Elmer, Boston, MA). The LS has the capacity of measuring both DL and fluorescence of both solid and aqueous specimens; the extracts were measured in a microcell (5/5 mm; PerkinElmer). Fluorescence spectra were obtained at the excitation wavelength of 365 nm under the fluorescence mode with excitation slit of 10 nm, emission slit 10 nm and scan speed of 200 nm/min. The excitation wavelength of 365 nm was chosen for fluorescence spectra generation because it is known that AfB1 can be excited at this wavelength.

Determination of DL and fluorescence spectra of whole peanut

DL and fluorescence spectra of contaminated peanut cotyledon (3 g) were directly measured using the LS, cotyledon inside faced to spectrometer. The DL spectra and fluorescence spectra of no peanut were measured as the control. The DL spectra and fluorescence of the same peanut sample were measured with a conventional quartz cell (10 mm). DL spectra was obtained at the excitation wavelength of 365 nm under the phosphorescence mode with a delay of 500 ms, a cycle of 520 ms, gate of 1ms and scan speed of 200 nm/min. Fluorescence spectra were obtained at the excitation wavelength of 365 nm under the fluorescence mode with excitation slit of 2.5 nm, emission slit 2.5 nm and scan speed of 200 nm/min.

The AfB1 standard curve was obtained by measuring the fluorescence spectra of pure AfB1 (0–50 ng/mL). The AfB1 concentration of the extracts of the spiked peanut was determined using the same LS under the fluorescence mode with excitation slit of 10 nm, emission slit 10 nm and scan speed of 200 nm/min. Fluorescence spectra of the AfB1-extracting media were measured as the control.

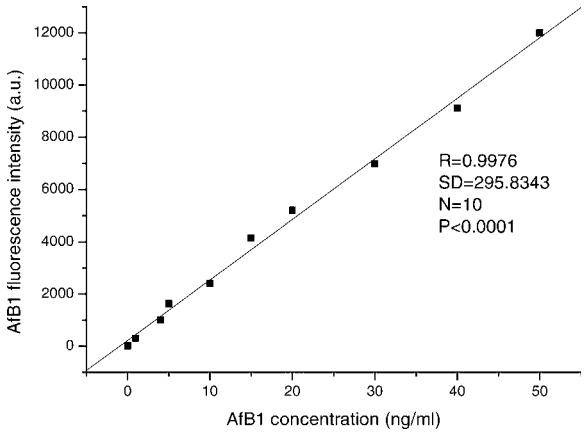
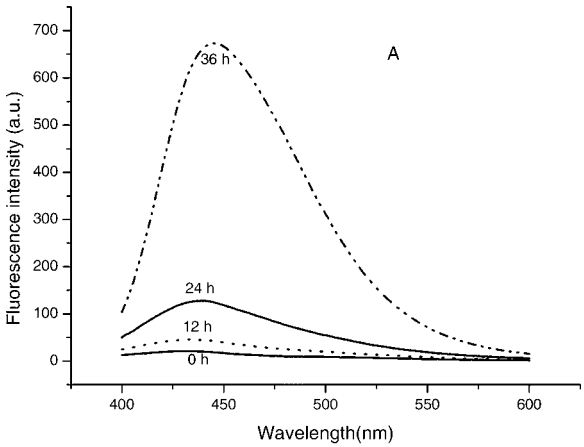


Figure 2. AfB1 standard curves.



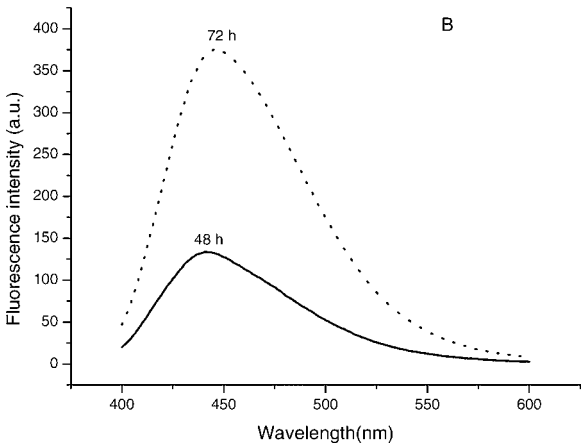


Figure 3. The fluorescence spectra of AfB1 extracts. A: The extracts of spiked peanut incubated for 0–36 h. Filtration of 200 μL; B: The extracts of spiked peanut incubated for 48–72 h. Filtration of 20 μL.

All measurements were conducted in complete darkness in three replicates at 20°C and 65% of the relative humidity. The results of measurements presented in the text were the average DL or fluorescence intensity of the sample of three replicates that took out the control. The data acquisition of DL and fluorescence intensity was obtained at the wavelength between 400 and 600 nm The AfB1 concentration was determined from the peak (440 nm) intensity (peak wavelength ± 10 nm, *i.e.* 440–460 nm) and normalized to ng/g dry weight (ng/g dw) after subtracting the blank control of the extracting media. The intensity of DL and fluorescence with the whole peanut was determined from the total intensity of 440–460 nm.

RESULTS

Determination of the AfB1 concentration

Peanut was artificially contaminated with *A. flavus* conidia (7.5×10^5 conidia/mL) and incubated for 0–72 h. AfB1 was extracted and its concentration was determined with the fluorescence spectra using an LS. Figure 1 shows the fluorescence spectra (400–600 nm) of standard AfB1 solutions (0.02–50 ng/mL). The total fluorescence intensity was obtained between 430 and 450 nm, and its value was used to make the standard curve of AfB1 concentration. The AfB1 concentration had a positive correlation to the

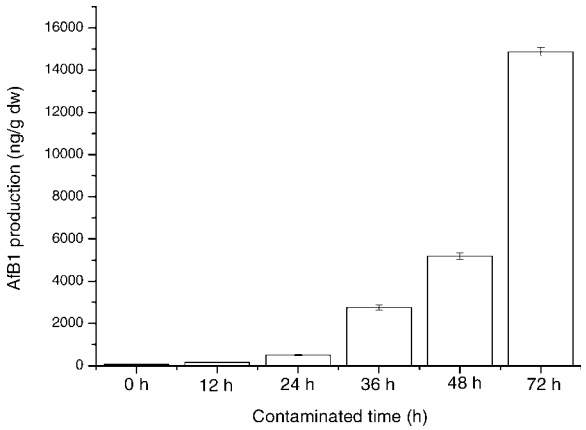


Figure 4. Relationship between the incubation time and AfB1 concentration. The AfB1 concentration of each time points was calculated from Eq. 1 and expressed in ng AfB1 per gram dry weight. Note the sharp increase between 24 and 36 h and between 48 and 72 h.

fluorescence intensity (Fig. 2). The correlation between the fluorescence intensity and the AfB1 concentration can be expressed as:

$$\text{AfB1 concentration} = (\text{fluorescence intensity} - 220)/232(\text{ng/mL}) \quad (1)$$

The fluorescence intensity of the extracts from artificially contaminated peanut were measured after 0–72 h incubation, and the AfB1 concentrations were determined according to the above equation and dilution factor. Figure 3 shows the fluorescence spectra of the extracts of different incubation time, *i.e.* the correlation of the fluorescence intensity and the contamination levels. The AfB1 concentrations were plotted against the incubation time (Fig. 4). There was no significant increase in AfB1 concentration between 0–24 h. However, between 24 and 36 h there was a sharp increase and the AfB1 concentration was greater than 2 μg/mL (equivalent to dry weight) at 36 h. Between 36 and 48 h there was a steady increase in AfB1 concentration. Between 48 and 72 h there was another sharp increase, and the AfB1 concentration was greater than 15 μg/mL (equivalent to dry weight) at 72 h.

Detection of DL and fluorescence spectra in the whole peanut

This study used artificially spiked peanut as a model to test the feasibility of DL spectra for the rapid and specific detecting contamination levels of AfB1 in peanut. Peanut was contaminated with *A. flavus* conidia (7.5×10^5 conidia/mL) and incubated for 0–72 h. The DL and fluorescence spectra in the peanut (a whole peanut was divided two cotyledons, cotyledon inside faced to spectrometer) were measured by an LS, and the AfB1 concentration was determined based on the method described in the previous section.

Figure 5 shows peanut appearance and coloration after being treated with *A. flavus*. Peanut color changed from original color to yellowish at 36 h, indicating the onset of the massive production of AfB1 (see Fig. 4). Yellow color became darkened when the incubation time increased at 72 h, implying the massive production of AfB1 again (see Fig. 4). DL and fluorescence spectra (400–600 nm) of the contaminated peanut (3 g) were directly detected. The excitation wavelength (365 nm) was widely used for AfB1 fluorescence spectra. Therefore, the excitation wavelength of

Figure 5. Appearance and coloration of peanut contaminated with *A. flavus*. 1, 2, 3, 4, 5 and 6 stand for 0, 12, 24, 36, 48 and 72 h after inoculating *A. flavus*. Peanut samples were spiked with the same amount of *A. flavus* (7.5×10^5 conidia /20 g peanut) and incubated at 25°C for 0–72 h. Coloration became visible at 36 h. Peanut color changed from original color to yellowish. Yellow color became darkened when the incubation time increased.

365 nm was chosen in this study for both DL and fluorescence spectra generation. The following experimental parameters for DL spectra measurement would give the best detection signals and avoid signal saturation: excitation wavelength of 365 nm, excitation slit of 2.5 nm, emission slit 2.5 nm under the phosphorescence mode with a delay of 500 ms, a cycle of 520 ms, gate of 1 ms and scan speed of 200 nm/min.

Figure 6 shows the DL spectra of peanut samples obtained at different incubation times. The peak intensity appeared between 440 and 460 nm There was a negative relationship between incubation time (*i.e.* contamination level) and DL intensity. The total DL intensity was plotted against the incubation time. The longer the incubation time or the higher the contamination level, the weaker the DL intensity (Fig. 7).

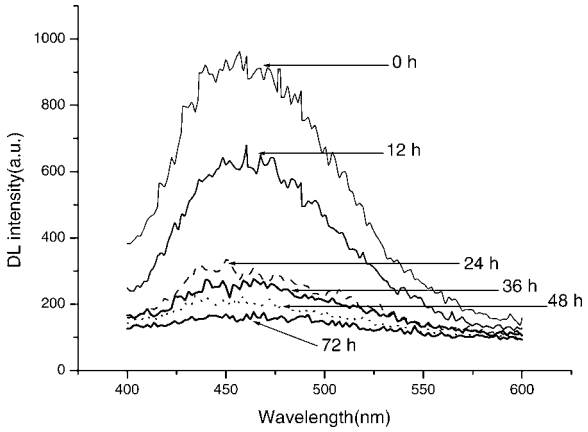


Figure 6. DL emission spectra of contaminated peanut. DL spectra were obtained at the excitation wavelength of 365 nm under the phosphorescence mode with a delay of 500 ms, a cycle of 520 ms, gate of 1ms, scan speed of 200 nm/min. The longer the incubation time, the weaker the DL intensity.

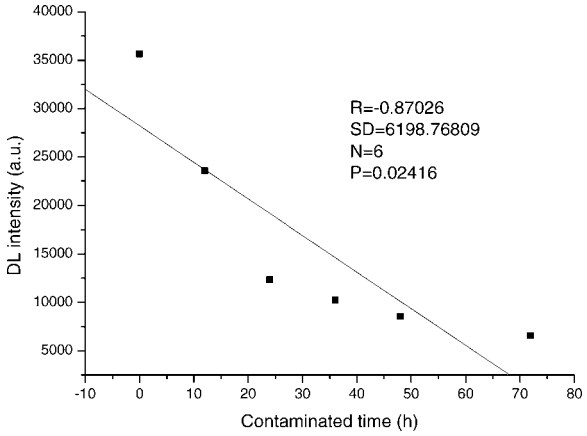


Figure 7. Relationship between the contamination level and the DL intensity. The DL intensity was the total intensity obtained 440–460 nm There was a negative correlation between the contamination level and the DL intensity.

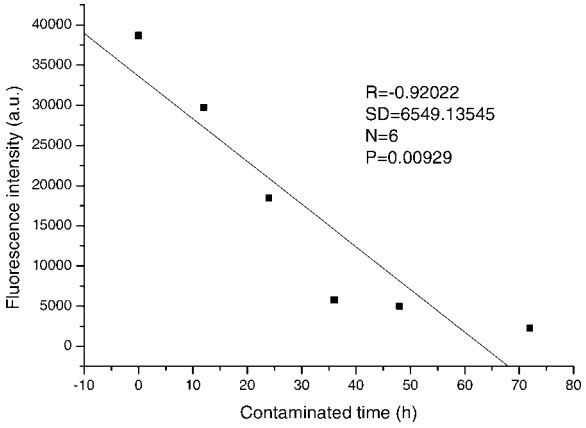


Figure 9. Relationship between the contamination level and the fluorescence intensity. The fluorescence intensity was the total intensity obtained 440–460 n*M*. There was a negative correlation between the contamination level and the fluorescence intensity.

The fluorescence spectra of the whole peanut sample was obtained at the excitation wavelength of 365 nm under the fluorescence mode with excitation slit of 2.5 nm, emission slit 2.5 nm and scan speed of 200 nm/min. Fluorescence emission spectra of contaminated peanut are shown in Fig. 8. The results showed that the profile of the fluorescence emission spectra was similar to that of the DL spectra. The emission peak was also around 450 nm (see Fig. 6). The total intensity of the fluorescence between 440 and 460 nm showed a negative correlation with the AfB1 contamination levels (Fig. 9).

To establish the relationship between DL intensity and AfB1 concentration, the DL intensity of peanut sample was plotted against the AfB1 concentrations determined by the linear regression (Fig. 4). The logarithm of the intensity of DL (Fig. 10) at the total 440–460 nm values had negative correlation to the logarithm of AfB1 concentration (Fig. 10). Similarly, the logarithm of the fluorescence intensity of the contaminated whole peanut had a negative correlation to the logarithm of the AfB1 concentration (Fig. 11), which was correlated well with the relationship between DL intensity and AfB1 concentration.

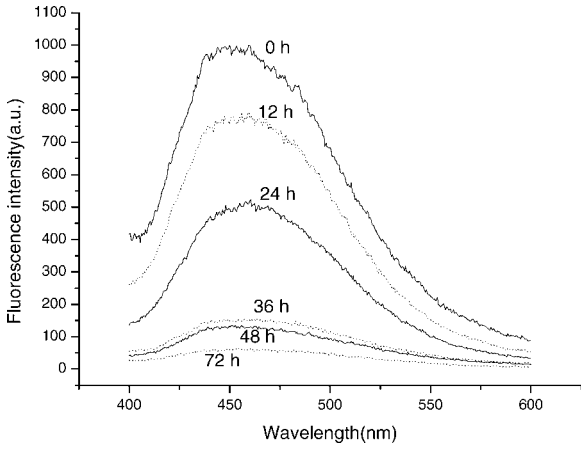


Figure 8. Fluorescence emission spectra of contaminated peanut. Fluorescence spectra was obtained at the excitation wavelength of 365 nm under the fluorescence mode with excitation slit of 2.5 nm, emission slit 2.5 nm and scan speed of 200 nm/min. The longer the incubation time, the weaker the fluorescence intensity.

DISCUSSION

It is known that *A. flavus*–contaminated food can produce AfB1. It is always a challenge to determine low concentrations of AfB1 in complex mixtures. Many of the analytical methods currently used for the determination of AfB1 employ either solvent extraction, which involves toxic solvents, or immunoaffinity columns and multifunctional columns, which are very expensive (14–16). There is a need to develop a sensitive and simple technique to detect *A. flavus* contamination in foods. The connection between the biological state and physical characteristics of DL has been demonstrated in many biological systems (1–3). This study established an optical method for a rapid detection of *A. flavus* contamination in whole peanut with DL spectra, without using toxic solvents and extracting progress. The DL method could detect the different levels of *A. flavus* contamination in whole peanut.

Our preliminary data showed that a clear negative correlation existed between the degrees of the contamination and DL intensity, *i.e.* the longer the incubation time or the higher contamination level

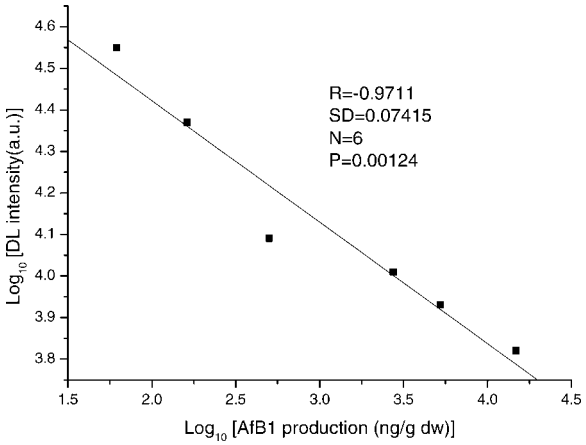


Figure 10. Relationship between the AfB1 concentration and DL intensity of the whole peanut.

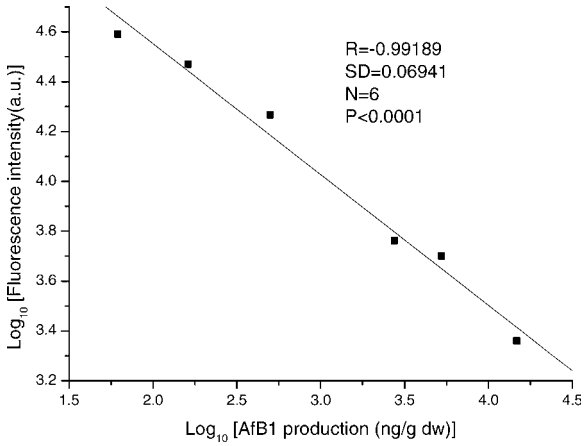


Figure 11. Relationship between the AfB1 concentration and fluorescence intensity of the whole peanut.

the weaker DL intensity (see Fig. 7). This might attribute to the strong absorbance of the *A. flavus* to the emission luminescence around 450 nm. This phenomenon suggests that using 450 nm as an excitation wavelength and adjusting the excitation light emission intensity we might observe the emission spectra of *A. flavus* and link it to the contamination level in peanut.

In this study, we also used a common and easy fluorescence method to extract and detect the AfB1 production in peanut (12). Fluorescence detection is a common method used for the determination of AfB1 in contaminated foods (13). We used mixtures of methanol–water for aflatoxins extraction in this study. We compared the intensity of DL and fluorescence of the same peanut specimen. Results demonstrated that the total values 440–460 nm had a negative correlation. Therefore, measuring the DL of whole peanut can indirectly detect the AfB1 production and *A. flavus* contamination. The DL of whole peanut might be a potential technique for a fast, quantitative and non-toxic method for the determination of peanut quality.

We will require further research to perform the following experiments: (1) to treat noncontaminated peanuts with different concentrations of standard AfB1; or (2) using one concentration of standard AfB1 to treat peanuts with different contamination levels of *A. flavus*. The excitation wavelength will be 365 nm, and the changes of DL and fluorescence spectra will be compared to samples with no addition-pure AfB1. Judging the strong absorbance of the *A. flavus* to the emission luminescence around 450 nm is related to AfB1 itself.

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REFERENCES

1. Ho, M. W., F. Musumeci, A. Scordino and A. Triglia (1998) Influence of cations in extracellular liquid on delayed luminescence of Acetabularia acetabulum. *J. Photochem. Photobiol. B: Biol.* **45**, 60–66.
2. Scordino, A., A. Triglia, F. Musumeci, F. Grasso and Z. Rajfur (1996) Influence of the presence of atrazine in water on the in-vivo delayed luminescence of Acetabularia acetabulum. *J. Photochem. Photobiol. B: Biol.* **32**, 11–17.
3. Chen, W. L., R. Van Wijk and D. Xing (2005) Effects of isoflurane on measurements of delayed lumininescence in *Acetabularia acetabulum*. *Luminescence* **20**, 16–19.
4. Otta, K. H., E. Papp and B. Bagocsi (2000) Determination of aflatoxins in food by overpressured-layer chromatography. *J. Chromatogr. A* **882**, 11–16.
5. Richard, J. L., G. A. Bennett, P. F. Ross and P. E. Nelson (1993) Analysis of naturally occurring mycotoxins in feedstuffs and food. *J. Anim. Sci.* **71**, 2563–2574.
6. Issaq, H. J. (1997) Capillary electrophoresis of natural products. *Electrophoresis* **18**, 2438–2452.
7. Stroka, J. and E. Anklam (2000) Development of a simplified densitometer for the determination of aflatoxins by thin-layer chromatography. *J. Chromatogr. A* **904**, 263–268.
8. Jaimez, J., C. A. Fente, B. I. Vazquez, C. M. Franco, A. Cepeda, G. Mahuzier and P. Prognon (2000) Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis. *J. Chromatogr. A* **882**, 1–10.
9. Ventura, M., A. Gomez, I. Anaya, J. Diaz, F. Broto, M. Agut and L. Comellas (2004) Determination of aflatoxins B1, G1, B2 and G2 in medicinal herbs by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **1048**, 25–29.
10. Niedwetzki, G. and G. L. Geschwill (1994) Determination of aflatoxins in food by use of an automatic work station. *J. Chromatogr. A* **661**, 175–180.
11. Blesa, J., J. M. Soriano, J. C. Molto, R. Marin and J. Manes (2003) Determination of aflatoxins in peanuts by matrix solid-phase dispersion and liquid chromatography. *J. Chromatogr. A* **1011**, 49–54.
12. Stroka, J., E. Anklam, U. Jorissen and J. Gilbert (2000) Immunoaffinity column cleanup with liquid chromatography using post-column bromination for determination of aflatoxins in peanut butter, pistachio paste, fig paste, and paprika powder: collaborative study. *J. AOAC Int.* **83**, 320–340.
13. Xiao, D. R., S. Y. Wang and H. L. Zhan (1999) Rapid identifying method for resistance to aflatoxin production in peanut. *Chinese Journal of Oil Crop Sciences* **21**, 72–76.
14. Akiyama, H., Y. Goda, T. Tanaka and M. Toyoda (2001) Determination of aflatoxins B1, B2, G1 and G2 in spices using a multifunctional column clean-up. *J. Chromatogr. A* **932**, 153–157.
15. Kussak, A., B. Andersson and K. Andersson (1993) Automated sample clean-up with solid-phase extraction for the determination of aflatoxins in urine by liquid chromatography. *J. Chromatogr.* **616**, 235–241.
16. Kussak, A., B. Andersson and K. Andersson (1995) Immunoaffinity column clean-up for the high-performance liquid chromatographic determination of aflatoxins B1, B2, G1, G2, M1 and Q1 in urine. *J. Chromatogr. B Biomed. Appl.* **672**, 253–259.