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HPTLC-fluorescent densitometry for screening aflatoxin B_1 in millet and buckwheat

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ABSTRACT

Given severe health-hazardous effects of aflatoxin B1 (AFB1) widely occurring in cereal grains and animal feeds, it is highly urgent to develop analytical methods for its rapid screening. In this work, we proposed a simple and high-throughput method for the determination of AFB1 in millet and buckwheat samples using high performance thin layer chromatography (HPTLC) linked to fluorescence densitometry. The first step was to optimize the solid-liquid extraction for the crude clean-up of the samples. The QuEChERS (Quick, Easy, Cheap, Effective, Robust and Safe) extraction strategy was used and different solvent systems for their extraction efficiency of AFB1 from the samples were evaluated. Then, trichloromethane:ethyl acetate (7:3, *V/V*) was used as the mobile phase to realize the separation of the targeted compound from background noises on silica gel plates. Quantification was readily performed with densitometry in fluorescence mode. In order to fix the optimal excitation wavelength, spectra scanning ranging 250−400 nm was carried out, revealing that 364 nm light gave the highest signal. With the optimized optical system, high sensitivity to AFB1 was achieved, with a limit of detection (LOD) at 3 μg/kg. Apart from that, good linearity (0.999) was obtained within the range of 1−80 ng/band of AFB1. To assess the analysis accuracy, 2 levels of AFB1 (50 and 100 μg/kg) were spiked into real grain samples. The obtained results showed that the recovery rates were within the range of 81.6%−114.0%. The proof-of-concept results of this work evidenced that HPTLC is a promising analytical tool for the screening of mycotoxin in difficult samples.

1. Introduction

Miscellaneous cereals are a group of highly valuable food for human beings[1-4]. However, they are susceptible to mycotoxin contamination during growth, storage and transportation, which affects product yield and quality and poses food safety risks[5-8]. Aflatoxins (AFs), the secondary metabolites of fungi, are the most potent carcinogens ever discovered and have been classified as Class I carcinogens by the World Health Organization (WHO)[9]. Even at low levels of exposure, aflatoxin B1 (AFB1) may lead to liver damage and thus induce life-threatening cancers[10-12]. According to cancer risk assessment conducted in Europe and the United States, 0.1% of cancers per 100 000 subjects are induced by aflatoxins, with AFB1 being the most toxic and prevalent. A detailed survey of scientific databases found a scarcity of studies on AFB1 in miscellaneous grain crops, with China, a large producer of miscellaneous grains, playing a crucial role in ensuring the quality and safety of miscellaneous grain products and the high-quality development of the agricultural industry[13-17]. Therefore, there is a need to control AFB1 in miscellaneous grains.

Against this background, there is a growing interest in HPTLC as an advanced planar chromatography that serves as a powerful and simple

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screening platform[18-19]. The integration of advanced detectors such as fluorescence densitometry (FLD)[20], mass spectrometry (MS)[21-22], biosensors[23], and laser spectroscopy[24], with HPTLC, achieves separation of complex matrices and paves the way for new frontiers in HPTLC applications. More specifically, this method gives back a precise quantitative response to a large number of samples (high throughput analysis of over 70 samples per run) in a short period of time (60 min), facilitating the straightforward and reliable identification of positive outcomes[25].

The objective of this work was to establish a simple and reliable HPTLC fluorescence density assay for rapid and highly sensitive screening of AFB1 in miscellaneous cereals samples.

2. Experimental

2.1 Chemicals and materials

AFB1 standard (≥ 98% purity) was purchased from Sigma-Aldrich (Shanghai, China). Acetonitrile, methanol, trichloromethane, ethyl acetate with chromatographic purity was provided by Sigma-Aldrich (Shanghai, China). Other chemicals and solvents of analytical grade were from Sinopharm Group (Shanghai, China). Ultra-pure water (> 18 MΩ) was prepared by a Millipore Synergy system (Schwalbach, Germany). HPTLC silica gel plates G60 F_{254} (analytical grade, 0.20 mm layer thickness, 10 cm × 20 cm) were from by Merck (Darmstadt, Germany). Prior to using, all plates were pre-washed with methanol. Six blank miscellaneous cereals samples including three millet samples and 3 buckwheat samples were purchased in the farmers' markets.

2.2 Preparation of standard solutions

An amount of (0.10 ± 0.01) mg reference standard of AFB1 was added into a 10 mL volumetric flask. The flask was filled with 10 mL methanol, resulting in 0.01 mg/mL standard stock solution, and stored at 4 °C in a refrigerator at a constant temperature protected from light. Before analysis, the working solutions for establishing calibration curves were prepared by further diluting stock solutions with methanol to 0.010−0.001 mg/mL.

2.3 Sample extraction and spiking

Extraction of the AFB1 from samples was based on the protocol described by Ouakhssase et al.[26], and followed the QuEChERS strategy, with slight modifications. For extracting analytes from miscellaneous cereals samples, acetonitrile, methanol, and acetonitrile-methanol (40:60, *V/V*) were tested as extraction solvents. Briefly, 10 g of millet and buckwheat samples (crushed and sieved through 20 mesh sieve) were mixed with 10 mL of extraction solvent; containing 1 g NaCl and 4 g $MgSO_4$ in each extraction solvent for maximum recovery. If necessary, 0.1 mL stock solution of AFB1 (0.1 mg/mL) was spiked into the extraction mixture of the blank samples, resulting in artificial contamination at 1 mg/kg. Following a treatment of 30 °C ultrasonic bath for 30 min, the suspension was separated by centrifugation at 4 000 r/min for 10 min. Then, 2 mL of supernatant was pipetted into a 10 mL centrifuge tube and blown dry with a nitrogen blower in a water bath. Finally, the residue was miscible with 1 mL of methanol and passed through a 0.45 μm cellulose filter membrane to remove solid particles, to avoid needle blockage. The resulting clarified solution was used directly for HPTLC analysis.

2.4 HPTLC steps

Carried by a 0.5 MPa nitrogen gas stream, 1−8 μL of AFB1 working solution and 10 μL of sample extract were accurately applied onto HPTLC silica gel plates surface by Limonat 5 semiautomatic sampler (CAMAG Switzerland) equipped with a 100-μL syringe, at the delivery rate of 150 nL/s and 0.2 μL pre-dosage. Application parameters settings: the band array was initially 15 mm from the left edge and 8 mm from the bottom, bandwidth 6 mm with automatically calculated interval from each other. For establishing the calibration curve for real samples, 1, 5 μL working solution (0.001 mg/mL) and 1, 2, 4, 8 μL of working solution (0.01 mg/mL) were applied, resulting in gradient concentrations at 1, 5, 10, 20, 40, 80 ng/band, respectively.

After application, HPTLC silica gel plates were developed in an ADC-2 (CAMAG, Muttenz, Switzerland) to realize reproducible separation. First, 10 mL of the mobile phase was poured into each of the two side tanks. Developing parameter settings: 30 s drying time, 3 min humidity control with saturated $MgCl_2$, 10 min tank saturation, 10 min plate pre-conditions, 80 mm migration distance, and 5 min post-chromatographic drying. The fully automated pre-saturation and pre-condition effectively avoids the problem of deformation of the unfolding front and ensures the normality and reproducibility of the method. Then, the HPTLC silica gel plates were dried at 80 °C for 5 min by a TLC Plate Heater (CAMAG, Muttenz, Switzerland) to evaporate residues of organic solvents, which was of crucial importance for obtaining a clean back-ground.

2.5 Plate documentation and densitometry

For plate documentation, the ProViDoc-System DD70 (Biostep GmbH, Germany) consisting of a 366 nm illumination with a Canon EOS700D digital camera was used. In order to quantitation, the plates were densitometric ally evaluated with a TLC Scanner 3 (CAMAG, Switzerland) in fluorescence mode. To determine the optimum excitation wavelength, the excitation spectra of AFB1 standards and spiked samples on the HPTLC plates were recorded with in the range of 250−400 nm, using Hg lamp and K400 optical filter. Quantitative scanning was performed with parameter settings: fluorescent mode, mercury lamp, excitation wavelength 364 nm, K400 optical filter, slit dimension 3 mm × 0.3 mm, scanning speed 20 mm/s, and data resolution 25 mm/step. The operation of the instrument and the collection and analysis of the quantitative data were performed using Win CATS software (version 1.4.7).

3. Results and discussion

3.1 Optimization of chromatographic conditions

To achieve separation of interfering matrices in the samples, the mobile phases for the separation were optimized, with the Rf value of the target on a thin layer plate and the separation from the matrix were used as the basis for the selection. Guided by the natural blue

fluorescence of AFB1 (366 nm UV conditions), the types and ratios of solvents (trichloromethane, acetone, ethyl acetate, and methanol) were continuously adjusted. After comparison, the best separation results were achieved with a mobile phase consisting of a mixture of trichloromethane:ethyl acetate (7:3, *V/V*). As shown in Fig. 1, it is straightforward to notice that the AFB1 blue band was clearly visible in the window at 0.3 Rf, whereas the millet and buckwheat interfering substrates migrated in the range of 0.5−0.9 Rf. With the advantage of the image acquisition of the HPTLC plate, the visual reading can be used to make a "positive/negative" judgement based on whether the Rf of the sample is the same as that of the AFB1 standard or not. Additionally, it allows to make a preliminary estimation of the amount of AFB1 (the sensitivity of the visual detection is as low as 1 ng/spot). Leveraging the image acquisition of the HPTLC plate facilitates visual interpretation of the sample, allowing for a "positive/negative" judgement based on whether the Rf value of the sample is the same as that of the AFB1 standard. Furthermore, it enables a preliminary estimation of the amount of AFB1 (with the sensitivity of the visual detection being as low as 1 ng/spot), thereby making it possible to make a simple and definite judgement of the attainment of the desired concentration. This method demonstrated excellent efficiency and convenience in screening a large number of miscellaneous cereals samples for AFB1.

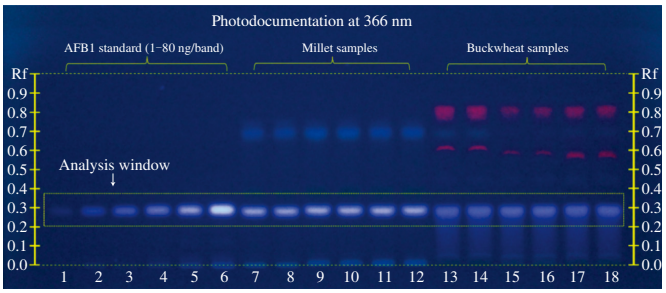

Fig. 1 Separation of AFB1 standard and 6 extracts of miscellaneous cereal samples (40 ng/band AFB1 were added in every sample). Track assignment: 1−6 AFB1 standard at 1, 5, 10, 20, 40, 80 ng/band; 7−8 millet 1; 9−10 millet 2; 11−12 millet 3; 13−14 buckwheat 1; 15−16 buckwheat 2; 17−18 buckwheat 3.

3.2 *Optimization of fluorescent densitometry*

To further assess the accuracy of the primary screening results, the color developed results were scanned and measured using a thin layer chromatography scanner to provide more accurate quantitative results. At the same time, in order to exclude the potential influence of the sample matrix effect on the optical density scanning, the fluorescence mode was selected for scanning and quantification of the separation results. As shown in Fig. 2a, the spectral profiles of the analytes under different conditions exhibited a high degree of similarity, verifying the purity between bands with the same Rf value, and the optimum excitation wavelength of AFB1 appeared at 364 nm. Therefore, a 364 nm mercury lamp was used as the excitation light source and equipped with a K400 filter, and optical density scans were obtained. As shown in Fig. 2b, the signal peaks of the analyte spots are clear even in the presence of interfering matrices in the test samples, while the good signal-to-noise ratio further validates the applicability of the optical parameters. The FLD quantitative detection is also very efficient, from scanning the chromatographic plate to the presentation of integral quantitative results in 3−5 min.

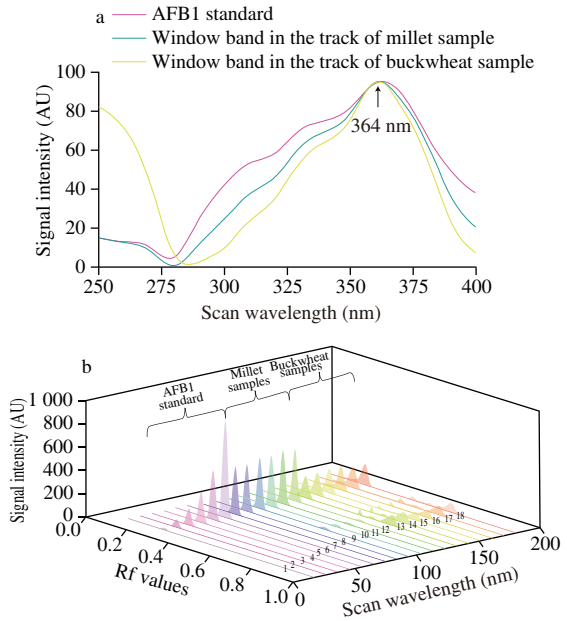

Fig. 2 (a) Full spectra of a AFB1 band deposited on silica gel plate, from 250−400 nm. (b) Optical density diagram of the separated samples with the optimal excitation wavelength at 364 nm. Track assignment: 1−6 AFB1 standard at 1, 5, 10, 20, 40, 80 ng/band; 7−8 millet 1; 9−10 millet 2; 11−12 millet 3; 13−14 buckwheat 1; 15−16 buckwheat 2; 17−18 buckwheat 3.

3.3 *Evaluation of quantitative capacity*

To validate the quantitative ability of the optimized FLD assay, the limit of detection (LOD) and limit of quantification (LOQ) were determined in the linear range of AFB1 detectability (1−80 ng/band). As shown in Table 1, the results obtained by HPTLC-FLD exhibited good linearity and had a high linearity coefficient (R^2 = 0.999). On this basis, the LOD and LOQ were determined to be 0.3 ng/band and 1 ng/band, respectively, with a confidence level of 95% according to the German method DIN 32645[27]. This means that the LOD and LOQ of AFB1 in the millet and buckwheat samples were 3 and 10 μg/kg, respectively, and the sample extracts were spotted in 10 μL volumes with a spot concentration of 10 g/mL. The detection limit of AFB1 in grains (5 μg/kg) was met. Based on this result, it was clear that the HPTLC fluorescence-density method showed high quantitative performance in the rapid screening of AFB1. If the content of the target component in the sample is too low, the sample volume can be increased so that the concentration reaches the concentration within the linear range of determination, which can further improve the detectability of the results.

Table 1
Quantitative performances of the established HPTLC-densitometry analysis

Analytical performances	AFB1
Linear range (ng/band)	1−80
Regression	y = 1 068.171x + 222.833
Correlation coefficient (R^2)	0.999
LOD[a] (ng/band)	0.3
LOD[a] (μg/kg)	3
LOQ[a] (ng/band)	1
LOQ[a] (μg/kg)	10

Note: [a]Mean value of 3 replicates.

3.4 Optimization of sample extraction method

The accuracy of the assay can be greatly improved by optimizing the extraction method. In the present study, the QuEChERS method was used for the extraction to satisfy the new extraction concepts of rapidity, precision and environmental protection as much as possible. Comparisons were made using different solvent systems by adding 1 mL of 10 μg/mL standard AFB1 to the sample. The extraction efficiency was determined by finding the recovery of AFB1 in the extract. As shown in Fig. 3, it is clear that pure acetonitrile is the best choice for the extraction of the target compounds from the 2 miscellaneous grain samples, which is comparable to the method of Miró-Abella et al.[28] and Yogendrarajah et al.[29].

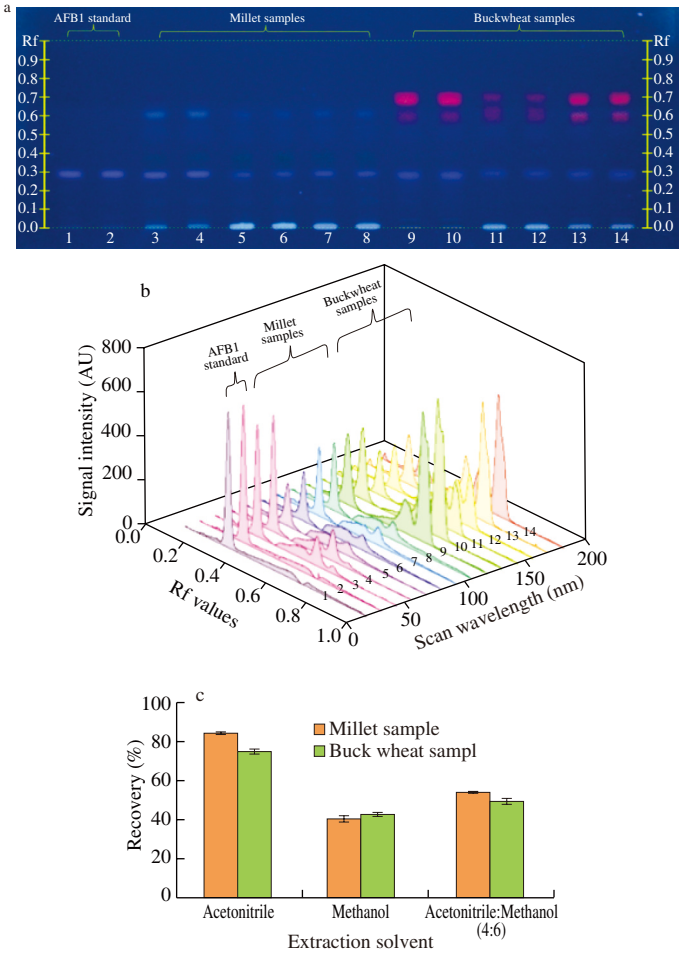

Fig. 3 (a) HPTLC separation results of AFB1 standard and AFB1 from millet and buckwheat samples extracted with three extraction solvents (30 ng/band AFB1 were added in every sample). (b) Optical density diagram of the separated samples. Track assignment: 1−2 AFB1 standard at 30 ng/band; 3−4 acetonitrile extract in millet; 5−6 methanol extract in millet; 7−8 acetonitrile-methanol (40:60, *V/V*) extract in millet; 9−10 acetonitrile extract in buckwheat; 11−12 methanol extract in buckwheat; 13−14 acetonitrile-methanol (40:60, *V/V*) extract in buckwheat. (c) effect of different extraction solvents on AFB1 recovery in miscellaneous cereal samples.

3.5 Method validation

To further evaluate the accuracy and precision of the HPTLC fluorescence density method, spiked recoveries were determined for each of the 6 miscellaneous grain samples, including millet and buckwheat samples from 3 different origins. The spiked amounts of AFB1 in the samples were 5 and 10 ng, respectively, and the spot volume of the samples was calculated as 10 μL of concentration at 10 g/mL, which was converted to 50 and 100 μg/kg, respectively. The results were shown in Table 2 and the calculated recoveries were in the range of 81.6%−114.0%. It showed that the spiked recovery of the HPTLC densitometric method was good and the quantitative accuracy of the method was high. At the same time, the samples were determined reproducibly, and each of the 6 samples was analyzed in parallel 3 times. The relative standard deviation (RSD) of the chromatographic peak area of AFB1 in the grain samples was less than 4.7%, indicating that the reproducibility of the instrumental detection was good. The specific chromatographic results are shown in Fig. 4. In addition, the developed HPTLC fluorescence density method was analyzed in terms of efficiency and cost. The HPTLC fluorescence density method allows the simultaneous determination of up to 17 sample (in this paper) orbitals on a 10 cm × 20 cm thin-layer chromatographic plate, with a total time from dispensing-expanding-imaging to integration and quantification of approximately 1 h. The HPTLC fluorescence density method can be used for the simultaneous determination of up to 17 sample orbitals on a 10 cm × 20 cm thin-layer chromatographic plate. Although liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is currently the most commonly used technique for the detection of mycotoxins, it is necessary to measure each of the 17 samples individually for a total time of about 8−9 h[30-31]. The comparison further demonstrated that the method is more simple, efficient and cost-effective, showing the advantages of the HPTLC fluorescence density method in the screening of a large number of samples.

Table 2
Assessment on method accuracy.

Sample	Spiked level (μg/kg)	Recoveries (%) [a]	Repeatability (%RSD) [a]
Millet 1	50	114.0 ± 0.4	0.3
	100	97.0 ± 0.1	0.1
Millet 2	50	113.7 ± 0.5	0.4
	100	96.1 ± 0.1	0.1
Millet 3	50	113.9 ± 1.2	1.0
	100	98.4 ± 1.2	1.2
Buckwheat 1	50	87.2 ± 1.7	1.9
	100	83.7 ± 3.9	4.7
Buckwheat 2	50	86.6 ± 0.5	4.6
	100	81.6 ± 2.4	3.0
Buckwheat 3	50	87.4 ± 3.7	4.2
	100	82.6 ± 3.3	4.0

Note: [a] RSD (%) values for mean results corresponding to each analyte with 3 replicates.

In conclusion, in this study, sample solutions extracted and cleanly prepared by the QuEChERS method were subjected to a fully automated unfolding separation on a thin-layer chromatographic plate. Subsequently, quantitative analysis was performed using a visual imaging device and an FLD. The specificity of the HPTLC method was determined post quantification by comparing the purity of the AFB1 peaks in the sample extracts with the spectra of the reference standard, AFB1. Finally, the applicability and efficacy of the method were validated using 2 representative samples of mixed grains, millet, and buckwheat.

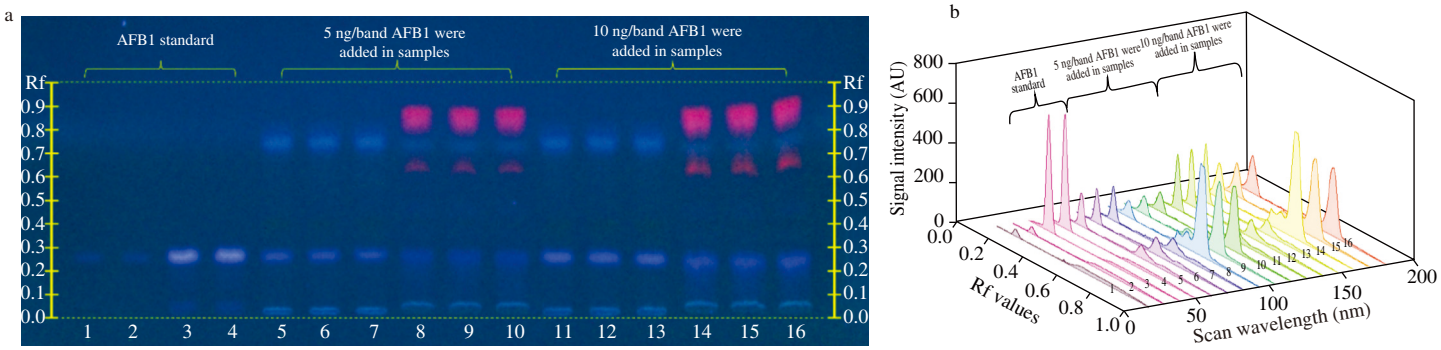

Fig. 4 (a) Image of the separation results of AFB1 standard and millet and buckwheat samples spiked with 5 and 10 ng standards, respectively. (b) Optical density diagram of the separated samples. Track assignment was the same to (a). Track assignment: 1−2 AFB1 standard at 1 ng/band; 3−4 AFB1 standard at 20 ng/band; 5−7 millet samples spiked with 5 ng/band AFB1; 8−10 buckwheat samples spiked with 5 ng/band AFB1; 11−13 millet samples spiked with 10 ng/band AFB1; 14−16 buckwheat samples spiked with 10 ng/band AFB1.

4. Conclusion

In this work, a novel analytical method combing of HPTLC and FLD was developed for the screening of AFB1 in miscellaneous grain samples (millet and buckwheat), reaching an ideal balance between simplicity and robustness. The first step was to optimize the solid-liquid extraction for the crude cleanup of the samples. Trichloromethane:ethyl acetate (7:3, *V/V*) was used as the mobile phase, and the results separated the target compounds, obtaining the limit of detection of 3 μg/kg and a good linearity (R^2 = 0.999) in the range of 1−80 ng/band. Validation with real samples showed that the method has high accuracy (recoveries 81.6%−114.0%). This study demonstrated that HPTLC is a detection and analytical platform with multiple advantages, including simplicity, high throughput and sensitivity, thus holding promise for the future of food analysis.

Competing interests

Authors declares that he has no conflict of interest.

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