Analysis of Asian herbs for the presence of aflatoxins using AFLACARD TOTAL for screening and immunoaffinity columns for quantification

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Summary

A method for the analysis of aflatoxins in a range of Asian herbs was developed using AFLACARD TOTAL, an antibody based card test after clean up with a solid phase column or with an immunoaffinity column. In this study ten types of herb were examined for aflatoxins B₁, B₂, G₁ and G₂, using a number of different extraction methods and clean-up techniques prior to analysis with AFLACARD TOTAL. Results were confirmed using EASI-EXTRACT[®] Aflatoxin immunoaffinity columns in conjunction with HPLC. Extractions using 80 % methanol were effective for simple herbs and required only clean-up with a solid phase syringe column, provided in the test kit to remove pigments. For more complex samples, a 75 % acetonitrile solution was required in conjunction with an immunoaffinity column prior to analysis with AFLACARD TOTAL in order to improve extraction and sample clean up. In all cases, except a few species, the screening results achieved with the card were in agreement with the HPLC results and recoveries were above 70 % for all herbs.

Key words : Aflatoxin, AFLACARD, Herbs, Immunoaffinity Column, HPLC

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Introduction

Aflatoxins are toxic metabolites produced by the moulds *Aspergillus flavus* and *Aspergillus parasiticus* on crops following prolonged exposure to a high humidity or damage through stressful conditions such as drought¹⁻³. They are known carcinogens and can be found in a wide range of food samples including cereals, animal feed, nuts, nut products, dried fruits and spices¹. Aflatoxins have also been found in a wide range of medicinal herbs including peach kernel, zedoary, nelumbo seed and guarana. The use of plants in Asia as sources of food, poison and medicine dates back to prehistoric times, although the first official investigation of medicinal herbs only took place in the 8th century. In the 5th century, Chinese medicine had already been introduced to Japan via Korea. As an increased number of people are now using medicinal herbs as alternative therapies, there is now a focus on the safety of such products as they are regulated in a completely different manner to conventional medicines.

In the EU⁵, there are regulations covering aflatoxin levels in food and feed which are currently set at 4 µg/kg for total aflatoxin and 2 µg/kg for aflatoxin B₁ in groundnuts, nuts, dried fruit and processed products. These levels also apply to cereals intended for human consumption. Levels for spices including those from

the *Capsicum* and *Piper* species are 10 µg/kg for total aflatoxins and 5 µg/kg for aflatoxin B₁. Though Japan has an official level and method for aflatoxin B₁ in all foods including herbs, they are not for total aflatoxin, therefore total aflatoxin levels are being considered for these matrices. As a result there is an urgent requirement for suitable extraction methods and tests for screening and quantification. Since these commodities are often highly coloured, complex matrices and have a range of clinical and biological properties they are often difficult to analyze by standard analytical techniques. Immunoaffinity columns when used in conjunction with HPLC are ideal clean-up tools for concentrating mycotoxins in a variety of difficult commodities and have already been used successfully for analysis of spices, herbs, dried fruit and animal feed with good recoveries and accurate results⁴. For laboratories or companies who do not have access to analytical equipment there is however also the need for simple screening tests to detect aflatoxins in medicinal herbs. Many screening techniques such as ELISA's, fluorometry and lateral flow devices have been found to be unsuitable for analysing aflatoxins in medicinal herbs. This is mainly due to the color, pigments and components in these samples, which can often interfere with or mask test results. This study aims to examine various extraction methods for the analysis of a range of different types of herbs using both screening and quantitative tests.

Materials and methods

Chemicals and equipment All reagents were HPLC grade with acetonitrile and water purchased from Rathburn (Walkerburn) and methanol supplied by Fisher Scientific (Loughborough). Sodium hydroxide and Tween 20 were obtained from Sigma Aldrich Ltd (Dorset) and the TOTAL AFLATOXIN STANDARD SOLUTION and Phosphate buffered saline (PBS) were supplied by R-Biopharm Rhône Ltd (Glasgow). The TOTAL AFLATOXIN STANDARD SOLUTION 1 mL comprises with each 250 ng of aflatoxin B₁, B₂, G₁ or G₂, respectively.

EASI-EXTRACT[®] Aflatoxin immunoaffinity columns, AFLACARD TOTAL and associated reagents including solid phase syringe columns and sample diluent buffers were also supplied by R-Biopharm Rhône Ltd. AFLACARD TOTAL is a qualitative enzyme immunoassay performed on a card. The screening level of AFLACARD TOTAL can be adjusted as required depending on the extraction method and on the volume of sample extract applied to the card.

A Waring blender with 1 litre glass jar was used to blend the samples for the extraction of the aflatoxins. Whatman No.113 filter papers were used with 7.5 cm funnels for filtration. The HPLC system was purchased from Dionex (UK). The HPLC equipment consists of a system interface module, an HPLC pump, an injection valve, a precolumn, an analytical column, a Kobra Cell and a scanning fluorescence detector. The Kobra Cell was supplied by R-Biopharm Rhône Ltd.

Materials In order to carry out this study, 10 samples of Asian medicinal herbs were sent to R-Biopharm Rhône Ltd in Glasgow including peach kernel (*Persicae semen*: tōnin), apricot kernel (*Armeniaca semen*: kyōnin), zedoary (*Zedoariae rhizoma*: gajutsu), nelumbo seed (*Nelumbis semen*: renniku), guarana (*Guarana semen*), turmeric (*Curcumae rhizoma*: ukon), nutmeg (*Myristicae semen*: nikuzuku), citrus unshiu peel (*Aurantii nobilis pericarpium*: chinpi), bitter orange peel (*Aurantii pericarpium*: tōhi) and immature orange peel (*Aurantii fructus immaturus*: kijitsu).

Preparation of standards/Spiking for recovery To check the performance of the extraction methods, the

samples were tested first using EASI-EXTRACT[®] Aflatoxin to ensure that they did not contain any aflatoxins, prior to spiking with aflatoxins. The samples were then spiked with 10 µg/kg or 20 µg/kg of total aflatoxin and retested using the chosen extraction procedure with AFLACARD TOTAL and EASI-EXTRACT[®] Aflatoxin.

A 4 point standard calibration curve for use with the EASI-EXTRACT[®] Aflatoxin column methods based on the assumption of 1 g equivalent being passed through the immunoaffinity column was prepared. The standard was prepared by taking 80 µL of 1,000 ng/mL TOTAL AFLATOXIN STANDARD SOLUTION up to 2 mL methanol: water (1 : 1 v/v) (= 40 µg/L or 80 ng). Take 1 mL of 40 µg/L up to 2 mL methanol: water (1 : 1 v/v) (= 20 µg/L or 40 ng). Then, make 10 µg/L or 20 ng, and 5 µg/L or 10 ng standard solutions by stepwise dilution with methanol: water (1 : 1 v/v). The diluted standard solution of total aflatoxin was prepared freshly on the day of use with the 1,000 ng/mL total aflatoxin standard returned to the refrigerator at 4 °C where it was stable until the expiry date printed on the vial.

The following method was used for spiking samples at 10 µg/kg total aflatoxin concentration:

The sample was thoroughly blended and mixed prior to spiking. 50 g of the sample material was taken and placed in a suitable vessel. Then 0.5 mL of total AFLATOXIN STANDARD SOLUTION (1,000 ng/mL) was added to the sample resulting in a 10 ng/g spiked sample, it was then covered and left for 2-3 h at room temperature. The spiked sample can be stored at 4 °C in the dark overnight. Before analysis, the sample was mixed thoroughly and the assay performed using the EASI-EXTRACT[®] Aflatoxin immunoaffinity column as per the method. Finally 100 µL of the final sample was injected onto the HPLC and the aflatoxin concentration recovered from the sample was calculated.

Preparation of samples

For peach kernel, apricot kernel, zedoary and nelumbo seed, 50 g of each material was weighed and extracted with 100 mL of methanol : water (8 : 2 v/v). The sample was blended at high speed for 2 min before being filtered. The pH of the filtrate was adjusted to 7.4 using 0.5 mol/L sodium hydroxide.

For guarana, turmeric and nutmeg, 25 g of each material was weighed and extracted with 100 mL of acetonitrile: water (75 : 25 v/v). The sample was blended and the pH was adjusted as the same manner described above.

For citrus unshiu, bitter orange peel and immature orange peel, 25 g of sample was weighed and extracted with 150 mL of acetonitrile:water (75 : 25 v/v). The sample was blended and the pH was adjusted as the same manner described above.

Analysis using AFLACARD TOTAL and EASI-EXTRACT[®] Aflatoxin immunoaffinity columns

Peach Kernel, Apricot Kernel, Zedoary and Nelumbo Seed

For screening at 4 µg/kg level using AFLACARD TOTAL, 1 mL of filtrate was added to 1 mL of 80 % methanol: water (v/v) and then 1 mL of this solution was diluted with 3 mL of sample diluent buffer which was provided in the AFLACARD TOTAL kit. The solution was gently mixed and passed through a solid phase syringe column also provided in the kit with 500 µL of resulting solution being added to the test port on the card and the assay performed as per the kit instructions.

For screening at 10 µg/kg level, 1 mL of filtrate was added to 4 mL of 80 % methanol: water (v/v). Then the dilution, the column filtration and the application on the card were carried out with the same manner

described above.

For the quantification and confirmation using EASI-EXTRACT[®] Aflatoxin with HPLC, 2 mL of filtered extract was diluted with 8 mL of PBS and passed through the immunoaffinity column according to the EASI-EXTRACT[®] Aflatoxin instructions for use and 100 µL of diluted eluate was injected onto the HPLC.

Guarana, Turmeric and Nutmeg

For screening at 4 µg/kg level, 1 mL of filtrate was added to 29 mL of 10 % Tween 20 and water. 30 mL of solution was passed through the EASI-EXTRACT[®] Aflatoxin column at a flow rate of 3 mL per minute. The column was washed with 20 mL of water. The toxin was eluted from the column using 1.5 mL of 100 % methanol and collected in a suitable vial. 0.7 mL of the solution was added to 3 mL of the sample diluent buffer provided in the AFLACARD TOTAL kit. This was mixed gently and 2×500 µL of solution were applied to the test port on the card and the assay performed as per the kit instructions.

For quantification and confirmation, 1 mL of filtrate was then added to 29 mL of 10 % Tween 20 and water. 30 mL of solution was passed through the EASI-EXTRACT[®] Aflatoxin immunoaffinity column at a flow rate of 3 mL per minute according to the EASI-EXTRACT[®] Aflatoxin instructions for use and 100 µL of diluted eluate was injected onto the HPLC.

Citrus Unshiu, Bitter Orange Peel and Immature Orange Peel

For screening at 4 µg/kg level, the sample dilution and the immunoaffinity column chromatography were carried out in the same manner as the guarana, turmeric and nutmeg. The eluate was diluted with the sample diluent buffer as described above and was mixed gently, and then 2×750 µL of solution were applied to the test port on the card and the assay performed as per the kit instructions.

Quantification and confirmation were carried out in the same manner as the guarana, turmeric and nutmeg.

Results and discussion

For peach kernel, apricot kernel, zedoary and nelumbo seed, all of the cards showed reasonable results, as shown in Table 1. Significant natural contamination was recognized in peach kernel and zedoary samples. In these samples, the recovery was corrected with subtraction of the contamination. Zedoary and nelumbo seed gave low recovery <60 %. It is possible that the lower recoveries obtained with these samples were caused by an uneven distribution of toxins.

For guarana, turmeric and nutmeg, the samples required a more rigorous extraction with 75 % acetonitrile and more thorough clean up using an immunoaffinity column prior to application onto the AFLACARD TOTAL. The weight of sample was also reduced from 50 g to 25 g in this protocol to enable better extraction. All of the card results were consistent with results obtained using EASI-EXTRACT Aflatoxin immunoaffinity columns with HPLC, as shown in Table 2.

For citrus unshiu peel, bitter orange peel and immature orange peel the samples required a more rigorous extraction with 75 % acetonitrile and the sample required greater clean up using an immunoaffinity column prior to application onto the AFLACARD. The weight of sample was also reduced from 50 g to 25 g and a greater volume of extraction solvent was used for the extraction of these samples, as they were more

Table 1. Recovery after clean-up with EASI-EXTRACT Aflatoxin and results on the AFLACARD TOTAL (I)

Sample	Spiked	HPLC analysis after using EASI-EXTRACT Aflatoxin	Recovery with using EASI- EXTRACT Aflatoxin	AFLACARD TOTAL Result at a 4 µg/kg or 10 µg/kg screening level
Peach kernel	No	3.87 µg/kg	N/A	Negative (<10 µg/kg)
	20 µg/kg	18.45 µg/kg	72.9 %	Positive (≥10 µg/kg)
Apricot kernel	No	<LOQ	N/A	Negative (<4 µg/kg)
	10 µg/kg	7.95 µg/kg	79.5 %	Positive (≥4 µg/kg)
Zedoary	No	6.24 µg/kg	N/A	Negative (<10 µg/kg)
	20 µg/kg	16.7 µg/kg	52.4 %	Positive (≥10 µg/kg)
Nelumbo seed	No	<LOQ	N/A	Negative (<4 µg/kg)
	10 µg/kg	5.70 µg/kg	57.0 %	Positive (≥4 µg/kg)

Aflatoxins were extracted with 100 mL of 80 % methanol. The samples for the AFLACARD were not cleaned up with the EASI-EXTRACT Aflatoxin column.

N/A: not available. LOQ: Limit of quantification, 0.3 µg/kg.

Table 2. Recovery after clean-up with EASI-EXTRACT Aflatoxin and results on the AFLACARD TOTAL (II)

Sample	Spiked	HPLC analysis after using EASI-EXTRACT Aflatoxin	Recovery with using EASI- EXTRACT Aflatoxin	AFLACARD TOTAL Result at a 4 µg/kg screening level
Guarana	No	<LOQ	N/A	Negative
	10 µg/kg	8.52 µg/kg	85.2 %	Positive
Turmeric	No	<LOQ	N/A	Negative
	20 µg/kg	15.2 µg/kg	76.0 %	Positive
Nutmeg	No	<LOQ	N/A	Negative
	20 µg/kg	19.56 µg/kg	97.8 %	Positive

Aflatoxins were extracted with 100 mL of 75 % acetonitrile. The samples for the AFLACARD were cleaned up with the EASI-EXTRACT Aflatoxin column.

LOQ: Limit of quantification, 0.3 µg/kg.

N/A: not available.

Positive: above 4 µg/kg level,

Negative: below 4 µg/kg level.

absorbent than the previous matrices analysed. Cleaning up with the immunoaffinity column allowed recoveries of >70 % for most samples and the cards showed reasonable results, as shown in Table 3.

Conclusions

Specific analytical methods were found to be suitable for the analysis of aflatoxin in a range of medicinal herbs. For peach kernel, apricot kernel, zedoary and nelumbo seed an 80 % methanol extraction solvent was found to be the best and easiest extraction method requiring only a standard clean-up with a solid phase syringe column, provided in the kit to remove pigments. For more difficult samples such as guarana, turmeric,

Table 3. Recovery after clean-up with EASI-EXTRACT Aflatoxin and results on the AFLACARD TOTAL (III)

Sample	Spiked	HPLC analysis after using EASI-EXTRACT Aflatoxin	Recovery with using EASI- EXTRACT Aflatoxin	AFLACARD TOTAL Result at a 4 µg/kg screening level
Citrus unshiu peel	No	<LOQ	N/A	Negative
	20 µg/kg	17.46 µg/kg	87.3 %	Positive
Bitter orange peel	No	<LOQ	N/A	Negative
	20 µg/kg	23.04 µg/kg	115 %	Positive
Immature orange peel	No	<LOQ	N/A	Negative
	20 µg/kg	16.42 µg/kg	81.9 %	Positive

Aflatoxins were extracted with 150 mL of 75 % acetonitrile. The samples for the AFLACARD were cleaned up with the EASI-EXTRACT Aflatoxin column.

N/A: not available.

LOQ: Limit of quantification, 0.3 µg/kg.

Positive: above 4 µg/kg level,

Negative: below 4 µg/kg level.

nutmeg, citrus unshiu peel, bitter orange peel and immature orange peel, extraction with 75 % acetonitrile was required to improve extraction and increase recoveries. For these matrices the use of an EASI-EXTRACT® Aflatoxin immunoaffinity column was also included prior to screening with AFLACARD TOTAL to provide better clean-up and concentration of the aflatoxin from the sample for detection. In this study AFLACARD TOTAL enabled screening of medicinal herbs for aflatoxins at levels of 4 µg/kg and/or 10 µg/kg, while EASI-EXTRACT® Aflatoxin allowed recoveries of >70 % for most samples with the exception of zedoary and nelumbo seed, which gave recoveries of <60 %.

In conclusion, AFLACARD TOTAL and EASI-EXTRACT® Aflatoxin immunoaffinity columns with HPLC were found to be suitable for screening and quantification of aflatoxins respectively in medicinal herbs.

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生薬中のアフラトキシンの市販カード式 ELISA キットおよびイムノアフィニティーカラムによるスクリーニング法

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生薬、香辛料中を汚染するアフラトキシンの簡便なスクリーニングと定量のための前処理法の確立を目的にして、市販カード式 ELISA キット (アフラカード・トータル), 固相カラム, イムノアフィニティーカラム (イージーエクストラクト・アフラトキシンの) の使用法を検討した。

トウニン, キョウニン, ガジュツ, レンニクでは, 80 % メタノールによる抽出, キットに備えつけの固相カラムによる精製によって, カードで妥当な定性反応が示された。ガラナ, ウコン, ニクズク, チンピ, トウヒ, キジツでは, 75 % アセトニトリルによる抽出, イムノアフィニティーカラムによる精製によって, 70 % 以上の高い回収率が得られ, カードで妥当な定性反応が示された。

キーワード : アフラトキシン, アフラカード, 生薬, イムノアフィニティーカラム, HPLC