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Development and Validation of HPTLC-MS Method for the Detection of Aflatoxins in Dry Fruits

KEYWORDS: Aflatoxins; Contamination; Validation; HPTLC-MS

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ABSTRACT *A simple HPTLC method of quantification of aflatoxins has been developed using Silica gel 60 F254s HPTLC plates, optimized solvent system of chloroform: acetone (8:2; v/v) in a twin trough chamber saturated for 5 min. Validation was carried out by testing its specificity, linearity (2-12 ng), accuracy (95.952–98.720% and 88.987-90.678%), precision (CV-0.819% and 0.968 %), limits of detection (32.610pg and 16.279pg) and quantification (98.818pg and 49.331pg) for aflatoxin B1 and B2, respectively. The presence of aflatoxins content was also confirmed using TLC-MS interface.*

Introduction

Among the aflatoxins, B_1 and B_2 are known as the most toxic members of the mycotoxin family formed principally by Aspergillus flavus and Aspergillus parasiticus (Paterson and Lima 2010a). Aflatoxins are of major concern to public health, due to their hepatotoxins, immunotoxic and carcinogenic effects (Creppy 2002). These fungi being heat resistant are not destroyed during processing, spoiling 5–10% of agricultural products worldwide (Topal 1993). Food products susceptible to aflatoxin risk include spices, nuts, seeds, dried fruits and grains. The European Union has posed strict limitations on total aflatoxins (2-4 ng/g) in nuts and spices (Commission Regulation 2010). Highly sophisticated methods such as HPLC (Quinto et al. 2009), Electrokinetics (Gilbert et al 2003) and LC-MS (Blesaa et al. 2003) are available for the purpose.

However, for the laboratories not having expensive infrastructure, for which HPTLC, is proved to be a boon. It is therefore important to develop a rapid and routine analysis of aflatoxins in foods stuffs like dry fruits.

Materials and Method

Chemicals and Reagents

Aluminium baked Silica gel 60 F_{254s} HPTLC plates (E. Merck, Darmstadt, Germany), standard aflatoxin B_1 and B_2 (Sigma Aldrich Pvt. Ltd., USA), acetone and chloroform (HPLC grade) were used.

Instrumentation

HPTLC system (Camag, Muttanz, Switzerland) consists of a sample applicator Linomat V with 100 µL syringe (Hamilton, Bonaduz, Switzerland), connected to a nitrogen cylinder; a TLC Scanner 3, connected to a PC running win-CATS software (version 4.4.1), a TLC Visualizer and twin trough chambers.

HPTLC-MS analysis (Camag TLC-MS Interface) was carried out with acetonitrile as eluting agent at a flow rate of 1 L/min as an attachment. The contents were automatically transferred to single-quadrupole mass spectrometer and mass spectra was recorded with capillary voltage +8 to -8 kV, nebulizer gas pressure 20 psi at temperature 320°C, fragmentator voltage 100V, gain 1, threshold 15 and step size 0.25.

Standard and Sample preparation

Standard solution of aflatoxin B_1 and B_2 were prepared in acetonitrile (1 µg/mL). Sample of dry fruits (almond, maize corn, cashew nuts and ground nuts) were purchased from the local market and analyzed in three replicates for aflatoxin contamination. The extraction of aflatoxin from samples was carried out by dissolving them in acetonitrile (1 L) and sonicated for 120 minutes. The process was repeated three times to ensure the maximum extraction of aflatoxins from samples. The solution was filtered using Whatman filter paper no. 42 (0.50 µm) and concentrated up to 20mL.

Chromatographic analysis

Each HPTLC plate (20X10 cm^2; 0.2 mm thick) contains 12 tracks of samples and standards under conditions: band width 8 mm, 12.3 mm apart from each other, 10mm from lower edge and 15 mm from left and right edge of the plate sprayed using Linomat V sample applicator with 100 µl syringe at temperature 22±2°C and relative humidity 65±5%, application volume of standard aflatoxins 2-12µl in the concentration range of 2-12ng. Loaded plates were developed to a distance of 80 mm using optimized mobile phase chloroform: acetone (8:2; v/v) in camag twin-trough plate development chamber. The developed plate was dried using warm air and scanned at 366nm. UV scanner was set for maximum light optimization with the following settings: slit dimension: 6.00mm×0.30mm (micro), scanning speed: 100 nm/s and data resolution: 1 nm/step. Regression analysis and statistical data were automatically generated by the win-CATS software. Densitometric measurements, spectra recording and data processing were carried out using HPTLC Scanner 3 Camag system.

Further confirmation of the target species in the sample was carried out using TLC-MS interface. It extracts circular zones in the form of bands from the developed HPTLC plate using acetonitrile as solvent. The eluted material is directly transferred into the detector of mass spectrometer.

Method Validation

For linearity, standard aflatoxins were applied on HPTLC plate with increasing concentration (2-12ng). Each sample (20µl) was loaded on the same plate and quantified. The calibration curve was plotted between peak areas versus concentrations with the help of win-CATS software. For accuracy, pre-analyzed samples were spiked with known amount of standard aflatoxins and percentage recovery was measured. For intraday precision, the experiment was

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carried out with six replicates while for inter day precision, the same experimental protocol was repeated for five consecutive days and results are expressed in terms of coefficient of variability. The limit of detection and quantification were determined in terms of signal to noise ratio using progressively lower concentration loaded over the plate resolving the target compounds into a single peak.

RESULTS AND DISCUSSION

Method development

HPTLC method was optimized for resolution based on the simulation for chemical composition of mobile phase, nature of stationary phase, time of saturation. Standardized conditions for HPTLC analysis were as follows, optimized mobile phase: chloroform: acetone (8:2; v/v); stationary phase: silica gel 60 F_{254s}; time of saturation: 5 min. Densitometric scanning at 366 nm resulted into a chromatogram consisting of two peaks of aflatoxin B_1 and B_2 at R_f value of 0.73 and 0.69, respectively. Visualization was carried out at white light and images were recorded.

Figure 1 HPTLC plate image of aflatoxin contamination in maize corn and cashew nuts at 366nm after development with optimized mobile phase (where S1- almond, S2 – maize corn, S3 – cashew nuts, S4 – ground nuts and standard in 2-12µl concentration)

After optimization, test samples were loaded on the same plate and quantified. UV spectra (200-800 nm) were recorded and matched with that of standard aflatoxins.

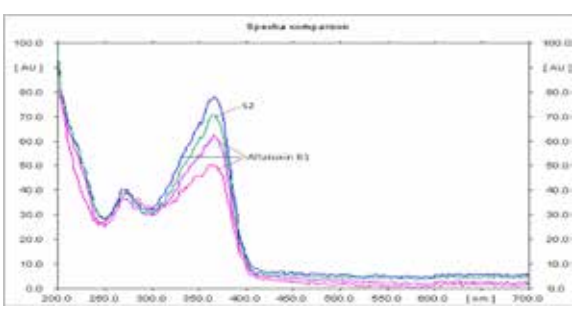

Figure 2 (a) represents UV Spectra for aflatoxin B_1 in three concentration (4,6,8µl) and contaminated sample of maize corn (S2)

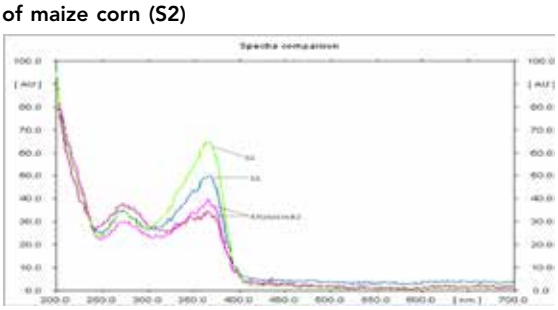

Figure 2 (b) represents UV Spectra for aflatoxin B2 in three concentrations (4,6,8µl) and contaminated sample of maize corn (S2) and cashew nuts (S3)

The characteristic molecular ion peak of aflatoxin B_1 and B_2 at m/e 335 and 337 were recorded in HPTLC-MS spectra confirming aflatoxin contamination in test samples.

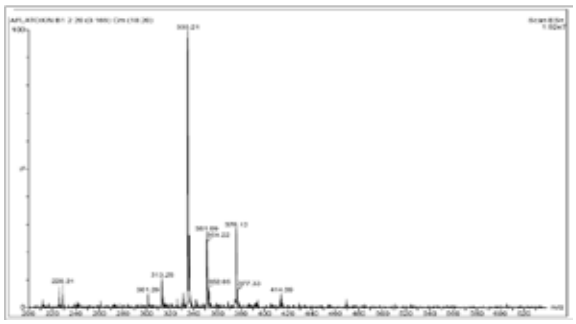

Figure 3 (a) HPTLC-MS Spectra of contaminated maize corn depicting the presence of peak at m/e 335 of aflatoxin B_1

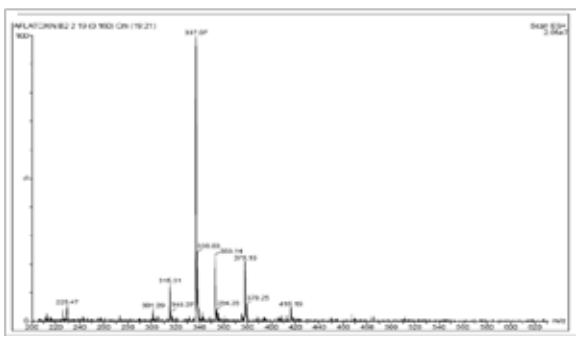

Figure 3 (b) HPTLC-MS Spectra of contaminated maize corn depicting the presence of peak at m/e 337 of aflatoxin B_2

Validation

The validation was carried out using ICH guidelines (1999) with the following parameters. Specificity: HPTLC method was found to be specific as there is no interference of the standard aflatoxins with that of others. Linearity: A linear regression of the data points for standard aflatoxin B_1 and B_2 resulted in a calibration curve with the equation Y=248.974 + 177.985x [regression coefficient (r^2) = 0.99878, standard deviation = 2.46%] and Y=5.980 + 118.859x [regression coefficient (r^2) = 0.99789, standard deviation = 2.95%] for aflatoxin B_1 and B_2, respectively. Samples were analyzed for the presence of aflatoxin content and quantified. Out of the twenty samples studied, mean aflatoxin content of B_1 and B_2 (ng/g± SD) are as follows: maize corn: 25.894±2.34 and Not Found and cashew nuts: 18.976 ± 1.45 and 23.456 ± 1.98. No contamination was observed in any samples of almond and ground nuts. Accuracy: Pre analyzed samples were spiked with known amount of standard solution of aflatoxins in increasing order of concentration and amount recovered after development of the plate was determined and found to be 87.52-93.58%. Precision: The percent coefficient of variability values were 0.819 and 0.968 and for inter day precision 0.741 and 0.290 for aflatoxin B_1 and B_2, respectively. Limit of detections were observed as 32.610 and 16.279pg whereas limit of quantification were 98.818 and 49.331 pg for aflatoxins B_1 and B_2, respectively.

Conclusion

The present communication reports a simple HPTLC method for rapid analysis of contamination of dry fruits by aflatoxin B_1 and B_2. Out of the four dry fruits, sample of maize corn and cashew nuts were found contaminated with aflatoxin B_1 and B_2. Using TLC-MS interface as an attachment confirmation of aflatoxin contamination has been

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performed.

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