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Application Note

Quantitative Analysis of Aflatoxin in Edible Nuts by Using High-Performance Liquid Chromatography Coupled with Florescence Detector

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1. Introduction

Aflatoxins are toxic metabolites produced by molds such as Aspergillus flavus and Aspergillus parasiticus. These fungi can contaminate nuts when conditions like high humidity and warm temperatures occur during cultivation, drying, storage, or transport.

Nuts including peanuts, almonds, pistachios, cashews, walnuts, and Brazil nuts are particularly susceptible. Even low levels of contamination can pose serious health risks. Early detection and control of aflatoxin contamination help prevent economic losses and safeguard consumers from long-term toxic effects. Reliable and sensitive detection of B_1, B_2, G_1, and G_2 aflatoxins in nut products is therefore critical, particularly in view of the strict limits set by the European Union (e.g., a maximum of 2 µg/kg for aflatoxin B_1 and 4 µg/kg for total aflatoxins in nuts intended for direct human consumption)[1]. The analysis commonly begins with immunoaffinity cartridges, which selectively capture aflatoxins while removing other matrix components. This step not only concentrates the toxins but also reduces interference, enhancing the accuracy and reproducibility of subsequent measurements.

For detection, post-column photochemical derivatisation is employed to increase the natural fluorescence of aflatoxin B_1 and G_1, improving sensitivity and lowering detection limits. This derivatisation method is rapid, reagent-free, and well-suited for routine monitoring of aflatoxins in complex food samples.

2. Materials and methods

Aflatoxin standards B_1, B_2, G_1, and G_2 were procured from Biopure, Germany.

A total of five commercially available edible nuts were collected from the local market.

Sample clean-up was performed using AflaCLEAN immunoaffinity cartridges from LC Tech, Germany. For chromatographic analysis, an i-Series LC-2060C 3D system coupled with a fluorescence detector RF-20Axs manufactured by Shimadzu, Japan, and a photochemical derivatization chamber from LC Tech, Germany, was employed. The analytical conditions are summarized in Table 1 and the system configuration is illustrated in Fig 1.

2.1. Analytical Conditions

Table 1 Analytical conditions LC

Column	:	Shim-pack™ GIST C18*1 (150 mm*4.6 mm I.D, 5 µm)
Mobile phase	:	Water/Methanol/Acetonitrile = 60 : 30 : 15
Elution mode	:	Isocratic
Flow rate	:	1.2 mL/min
Column temperature	:	35 ˚C
Injection volume	:	10 µL
Florescence Ex.	:	365 nm
Florescence Em.	:	460 nm
Run Time	:	10.0 min

*1 P/N: 227-30017-07

2.2. Sample preparation

For analysis, 20 g of finely crushed nut samples were weighed. To this, 2 g of NaCl, 50 mL of methanol–water mixture (80:20, v/v), and 50 mL of n-hexane were added. The mixture was shaken for 15 minutes to allow extraction of aflatoxins. The resulting crude extract was filtered and then centrifuged at 5000 rpm for 10 minutes to achieve effective separation of the n-hexane and methanolic phases.

From the centrifuged mixture, 10.5 mL of the lower methanolic phase was collected and diluted with 64.5 mL of PBS buffer. A 50 mL portion of this diluted sample was then loaded onto an AflaCLEAN immunoaffinity column. After sample loading, the column was washed with 10 mL of water and then dried by flushing nitrogen through it. 2 ml of methanol was added and allowed to soak for 5 minutes, and then the bound aflatoxins were eluted. The eluate was collected and directly injected into the HPLC system for analysis[2].

A series of calibration standards ranging from 0.05 µg/kg to 0.4 µg/kg were prepared by diluting with methanol. Peanut and cashew nut samples were spiked with known concentrations of aflatoxins: 0.4 µg/kg for G_1 and B_1, and 0.1 µg/kg for G_2 and B_2.

Fig. 1 Shimadzu LC-2060C 3D

3. Result and Discussion

The calibration curve showed excellent linearity, with a correlation coefficient (R^2) of ≥ 0.998 and accuracy within the range of 80–120% indicating a high degree of accuracy and reliability in the measurements.

Peanut and cashew samples were spiked with aflatoxins (0.4 µg/kg for G_1 and B_1, and 0.1 µg/kg for G_2 and B_2), yielding recoveries between 80% and 100%. The regression coefficients and slopes of the compounds obtained from the calibration curves are presented in Table 2.

The visual representation of chromatograms of standards and samples is shown in Fig. 2-5.

The presence of four types of aflatoxins—G_2, G_1, B_2, and B_1 was analyzed in various nut samples, including pine nuts, almonds, cashews, peanuts, and walnuts. Aflatoxins G_2 and G_1 were found to be below the limit of detection (LOD) in all nut samples. However, measurable levels of aflatoxin B_2 and B_1 were detected in certain samples.

Pine nuts showed the highest contamination, with aflatoxin B_2 at 0.066 µg/kg and aflatoxin B_1 at 0.346 µg/kg. Peanuts also contained detectable levels of aflatoxins, with 0.015 µg/kg of B_2 and 0.057 µg/kg of B_1 .

In contrast, almonds, cashews, and walnuts did not show detectable levels of any aflatoxins. The result summary is displayed in the Table 3.

All detected aflatoxin levels were well below the maximum limits set by the European Union for nuts intended for human consumption, which aim to protect consumer health by restricting B_1 and total aflatoxin concentrations.

The limits of detection (LOD) and quantification (LOQ) for aflatoxins G_2 and B_2 were 0.008 µg/kg and 0.002 µg/kg, respectively, while for G_1 and B_1 they were 0.020 µg/kg and 0.006 µg/kg, respectively. Additionally, the signal-to-noise ratio for all analytes was greater than 10, indicating good sensitivity and reliable detection.

Table 2. Coefficient of determination, slope of each calibration curve

Compound	Standard concentration Range (µg/kg)	Linearity (R^2)	Slope
G2	0.05-0.25	0.9980	1.068e+004
G1	0.2-0.1	0.9981	4.6e+003
B2	0.05-0.25	0.9981	1.045e+004
B1	0.2-1	0.9976	5.5e+003

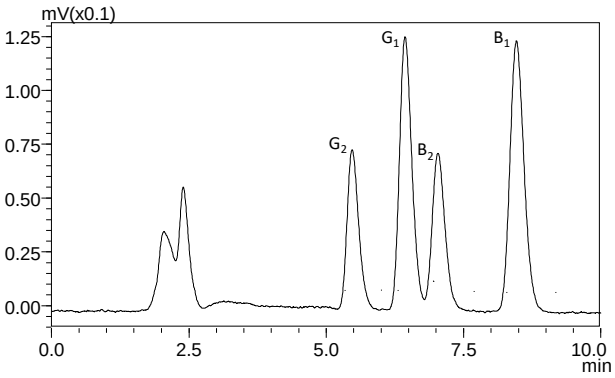

Fig.2 Chromatogram of Aflatoxin standard (0.4 µg/kg - G_1 and B_1, and 0.1 µg/kg - G_2 and B_2)

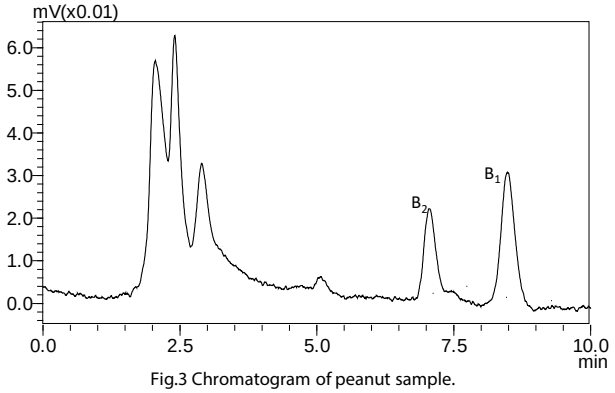

Fig.3 Chromatogram of peanut sample.

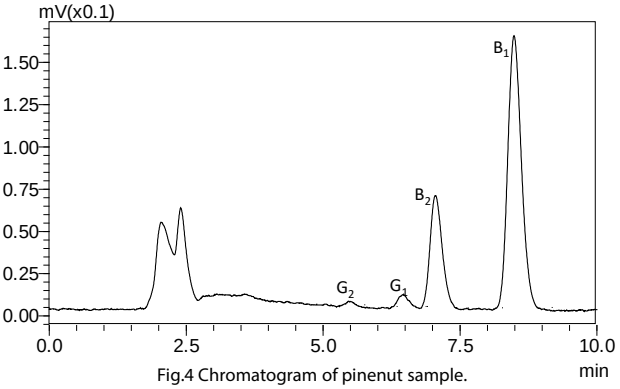

Fig.4 Chromatogram of pinenut sample.

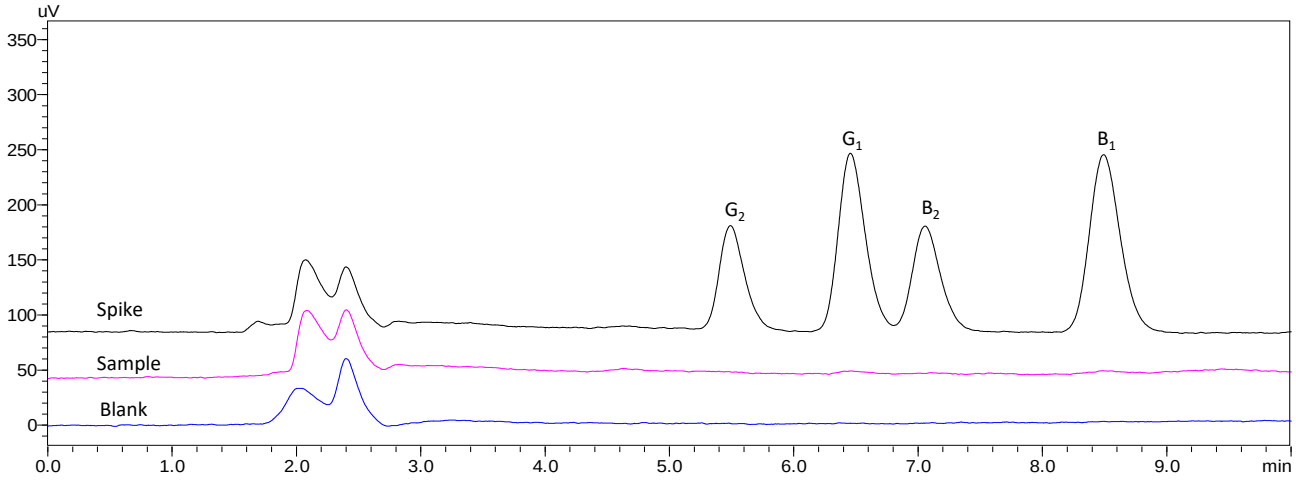

Fig. 5 Comparison chromatograms between Cashew sample and Spike.

Table 3 : Summary Table

	Aflatoxin G_2 in µg/kg / Percent recovery	Aflatoxin G_1 in µg/kg / Percent recovery	Aflatoxin B_2 in µg/kg / Percent recovery	Aflatoxin B_1 in µg/kg / Percent recovery
Pine nuts	< LOQ	< LOQ	0.066	0.346
Almonds	< LOQ	< LOQ	< LOQ	< LOQ
Cashews	< LOQ	< LOQ	< LOQ	< LOQ
Peanuts	< LOQ	< LOQ	0.015	0.057
Walnuts	< LOQ	< LOQ	< LOQ	< LOQ
Cashew Spike	0.086 (86%)	0.347 (87%)	0.090 (90%)	0.385 (96%)
Peanut Spike	0.081 (81%)	0.328 (82%)	0.105 (90%)	0.345 (86%)

*Note : LOQ-Limit of Quantification

4. Conclusion

The developed HPLC–fluorescence method with post-column photochemical derivatization and AflaCLEAN immunoaffinity column clean-up enabled accurate and sensitive detection of aflatoxins (B_1, B_2, G_1, G_2) in edible nuts. The method showed excellent linearity ($R^2 \geq 0.998$), good recoveries (80–100%), and very low detection limits.

All analyzed nut samples were within the permissible EU limits, confirming their safety. The use of immunoaffinity columns significantly reduced matrix interference and improved precision, making this method reliable for routine monitoring of aflatoxins in food safety testing.

5. References

1. European Commission. (2006). Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (as amended, including Commission Regulation (EU) No. 165/2010).
2. LCTech GmbH. (2023). Application Note AN0038 Aflatoxin analysis of almond and sesame products.

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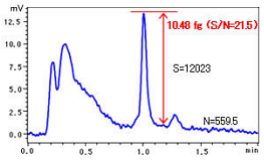

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