Immunoaffinity Solid-Phase Extraction with HPLC-FLD Detection for the Determination of Aflatoxins B2, B1, G2, AND G1 in Ground Hazelnut

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ABSTRACT

- Determination of aflatoxins, G2, G1, B2 and B1 in food ingredients meeting the maximums levels set by the European Commission
- Immunoaffinity solid-phase extraction for sample pre-treatment and FLD detection without photo derivatization
- Trace level detection of all four aflatoxins in ground hazelnut down to 1 µg/kg for B1 and G1 and 0.1 µg/kg for B2 and G2

INTRODUCTION

Mycotoxins are naturally occurring fungal toxins that were first found in fungus aspergillus flavus. Most of them are very stable and are not destroyed during processing or cooking procedures. One common group are the aflatoxins, of which 20 naturally occurring forms are known. Aflatoxin B1 is considered to be the most toxic to human health, but in addition the aflatoxins, B2, G2, G1, and the milk-derived derivatives M1 and M2 also have high importance. The B and G aflatoxins occur in various foods, such as nuts, grains, and spices, while the M derivatives are found in dairy products. The focus of this work is the determination of the toxins B2, B1, G2, and G1 in ground hazelnuts. The European Commission has set various maximum levels of aflatoxins in several foods under consideration of their consumption and use [1]. The maximum level for aflatoxin B1 ranges from 2 to 12 µg/kg for foods used for direct consumption or as an ingredient, with the exception of baby food products, which allow for a maximum level of 0.10 µg/kg. The limit sum of all four aflatoxins varies between 4 and 15 µg/kg. Therefore, a sensitive and accurate analytical method is required to monitor the low levels in various foods. For reliable identification and quantification, high-performance liquid chromatography (HPLC) coupled with fluorescence detection (FLD) is one of the most common techniques.

MATERIALS AND METHODS

Standards and Calibration

Quantification was performed by standard addition calibration to correct matrix influences, due to the absence of a hazelnut sample that was free of any aflatoxin content.

Table 1 gives an overview on the calibration levels and corresponding concentrations.

Calibration Level	Toxins G2, B2 Concentration [µg/kg]	Toxins G1, B1 Concentration [µg/kg]
1	0.2	0.7
2	0.4	1.4
3	0.9	2.9
4	1.3	4.3
5	1.7	5.7
6	2.1	7.1

Table 1. Calibration Levels and added concentrations in [µg/kg] to a purified sample extract

Sample preparation

Spiked and non-spiked samples were prepared in triplicate.

The recovery experiment was carried out by adding aflatoxin solution to the ground hazelnut sample prior to sample preparation.

2 g sodium chloride and 20 g ground hazelnut sample
↓
add 100 mL 80% methanol
↓
add 50 mL hexane
↓
Stirr for 15 min
↓
Filter through folded paper filter
↓
Mix 14 mL of lower liquid phase with 86 mL PBS buffer pH 7.2
↓
Load 50 mL onto AflaClean Select* immunoaffinity SPE cartridge
↓
Wash the cartridge with 10 mL water
↓
Elute with 2 mL methanol; let react the first 1 mL for 5 min on the cartridge
↓
Dilute the extract 1:2 with 1% acetic acid
↓
Filter through 0.2 µm RC membrane in HPLC vial

* purchased from a reputable vendor

Data Analysis

Data acquisition and processing was done by Thermo Scientific™ Chromeleon™ Chromatography Data System software 7.2.8.

INSTRUMENTATION AND METHOD

Thermo Scientific™ Vanquish™ Flex UHPLC system:

- System Base Vanquish Flex
- Quaternary Pump F
- Split Sampler FT
- Column Compartement
- Fluorescence Detector F with Standard Bio Flow Cell (8 µL, 20 bar)

Table 2. Chromatographic condition

Parameter	value
Column	Thermo Scientific™ Acclaim™ C18, (100 x 3 mm; 3 µm)
Mobile Phase	A: water B: methanol C: Acetonitrile
Flow rate	0.5 mL/min
Isocratic mobile phase composition	50% A, 30% B, 20% C
Run time	4 min
Column Temperature	30 °C (forced air mode, fan speed 5)
FLD	Excitation wavelength = 365 nm Emission wavelength = 450 nm Sensitivity = 8 Lamp mode = HighPower
Injection volume	20 µL

RESULTS

Separation of aflatoxins

Figure 1 shows the separation of a standard aflatoxin solution on the Acclaim 120 C18 column with excitation at 365 nm and emission at 450 nm. No immunoaffinity SPE clean-up was used prior to injection. As can be seen, there are some peaks eluting before the first target aflatoxin G2. These unknown peaks are impurities in the standard solution and were not observed in the blank. All aflatoxin analytes are baseline separated within 4 min.

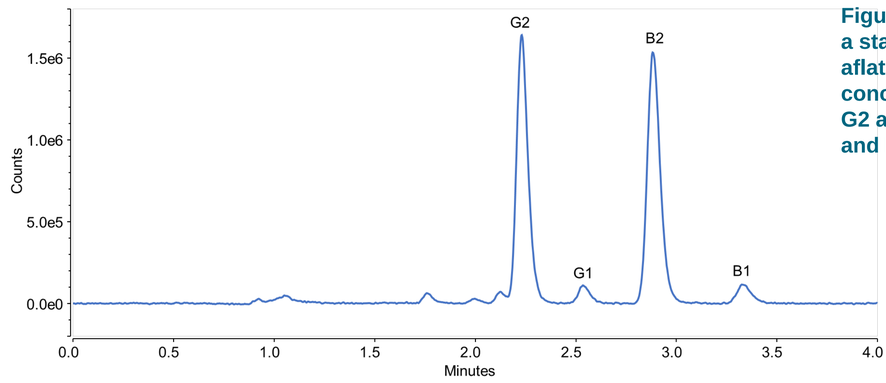


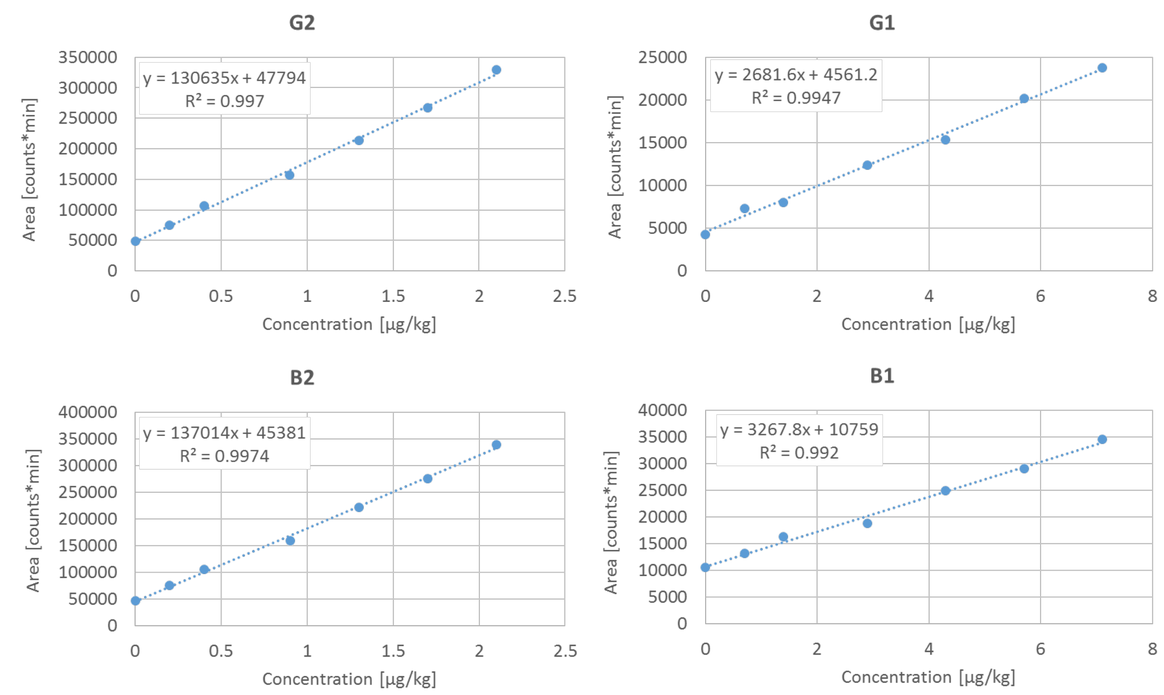
Figure 1. FLD chromatogram of a standard solution of the four aflatoxins: G2, G1, B2 and B1 at concentrations of 0.9 µg/kg for G2 and B2 and 2.9 µg/kg for G1 and B1

Calibration results

For quantitation, the ground hazelnut samples and recovery samples were prepared in triplicate. Figure 2 shows the calibration curves of the standard addition calibration method for all four aflatoxins. The original sample, which already contained all analytes, was set to zero amount, which results in a negative x-axis intercept. In this way, the calculated amount of the analytes corresponds to the absolute amount of the negative x-intercept.

Linearity (R^2) was found to be 0.9920-0.9974 for all four aflatoxins and the percentage of relative standard deviation of the retention times (% RSD RT) were all below < 0.2. Limits of detection are in the range of 0.075-1.056 µg/kg and limits of quantification of 0.185-1.329 µg/kg, respectively.

Figure 2. Calibration curves for all four aflatoxin analytes



Quantitation results

As can be seen in Figure 3, the immunoaffinity SPE clean-up results in pure extracts. Some matrix peaks can be observed in the first two minutes of the chromatogram, but there is no interference in the target analyte region from 2 to 4 minutes. Neither the non-spiked sample extract nor the spiked (recovery) extract, where the standard solution was added before the sample preparation, show a poorly resolved peak in front of the toxin G2 as it was observed for the standard solution in Figure 1. This leads to the assumption that a clear advantage of immunoaffinity purification is the removal of impurities which come from the standard solutions.

Figure 3. Overlay of spiked (recovery) sample (yellow) and non-spiked (blue) hazelnut sample

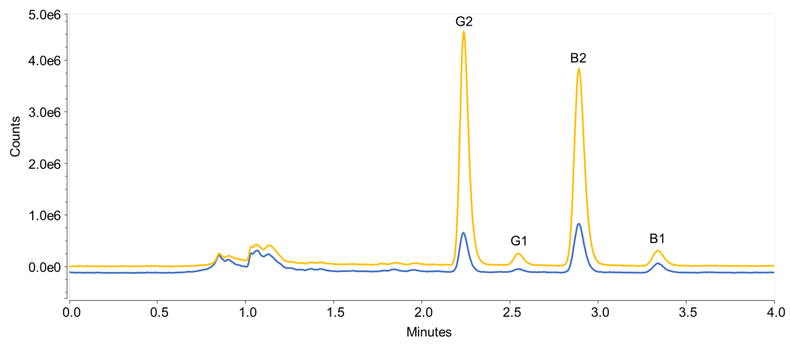


Table 3. Recovery and calculated sample amount of ground hazelnut (averaged from three preparations)

Compound	Recovery [%]	Amount [µg/kg]
G2	100	0.4
G1	72	2.2
B2	100	0.3
B1	95	3.4

CONCLUSIONS

- The combination of immunoaffinity SPE purification and enrichment with FLD detection without derivatization offers a sensitive analytical method for the quantification of the aflatoxins G2, G1, B2 and B1 in ground hazelnuts.
- The Vanquish Fluorescence Detector F provides sufficient trace level detection performance down to 1 µg/kg for aflatoxins B1 and G1 and 0.1 µg/kg for B2 and G2, enabling aflatoxin analysis in ground hazelnuts far below the tolerance levels defined by the European Commission.
- Good selectivity, linearity and recovery for reliable quantitative results were observed with the applied method.
- The method run time of less than 4 min enables a relatively high sample throughput.

REFERENCES

1. COMMISSION REGULATION (EC) No 1881/2006 of 19 December 2006: setting maximum levels for certain contaminants in foodstuffs, ANNEX section 2: mycotoxins (M5)..
2. Thermo Fisher Application note 72686: Determination of underivatized aflatoxins B2, B1, G2, and G1 in ground hazelnut by immunoaffinity solid-phase extraction with HPLC-FLD detection.

TRADEMARKS/LICENSING