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APPLICATION NOTE 72686

Determination of underivatized aflatoxins B2, B1, G2, and G1 in ground hazelnuts by immunoaffinity solid-phase extraction with HPLC-FLD detection

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Keywords

Aflatoxins, immunoaffinity solid-phase extraction, FLD, Vanquish Fluorescence Detector F

Goal

Determination of four aflatoxins in ground hazelnut by immunoaffinity solid-phase extraction sample purification and subsequent HPLC measurements with fluorescence detection

Application benefits

- Rapid chromatographic method for the determination of four aflatoxins in hazelnuts by fluorescence detection without derivatization
- Clear advantage of selective purification and enrichment of the aflatoxins on immunoaffinity solid-phase extraction cartridges for improved limit of detection and quantification

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 application 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]

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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. Since aflatoxins B1 and G1 fluoresce less intensely than G2 and B2, it may be necessary to use derivatization reagents or mass spectrometry to enable trace level quantitation.

Another common approach to improve the specificity, and therefore the limit of quantitation (LOQ), is to use immunoaffinity solid-phase extraction (SPE) to selectively purify and enrich the aflatoxins[2] prior to the HPLC analysis. With this sample purification method, low LOQ values can be achieved even in highly complex and fatty matrices.

This application note describes the determination and quantification of four aflatoxins in ground hazelnuts by using AflaCLEAN™ Select immunoaffinity SPE for clean-up followed by HPLC-FLD analysis without derivatization. The Thermo Scientific™ Vanquish™ Flex Fluorescence Detector (FLD) provides sufficient trace level detection performance to determine aflatoxins in this matrix far below the limits defined by the European Commission.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Chemical™ Optima™ Methanol LC/MS grade (P/N 10767665)
- Fisher Chemical™ Optima™ Acetonitrile LC/MS grade (P/N 10001334)
- Fisher Scientific™ Hexane, HPLC grade (P/N 10703611)
- Fisher Scientific™ PBS buffer (phosphate buffered saline), pH 7.2 (P/N 11530546)
- Fisher Scientific™ Acetic acid LC/MS grade (P/N 10860701)
- Fisher Scientific™ Sodium chloride, purris. (p.a.) (P/N 15626770)
- Aflatoxin mixture (purchased from a reputable vendor)

Equipment

- Magnet stirrer, Fisher Scientific
- Folded filter paper, Grade 597½, Ø 185 mm, Fisher Scientific (P/N 10433141)
- Syringe filter, regenerated cellulose (RC), Ø 15 mm, 0.2 µm, Fisher Scientific (P/N 10712712)
- AflaCLEAN Select immunoaffinity SPE columns, 3 mL (purchased from a reputable vendor)
- Conical tubes (15 mL), Fisher Scientific (P/N 11307211)
- Vials (amber, 2 mL), Fisher Scientific (P/N 15508760)
- Septa (silicone/PTFE), Fisher Scientific (P/N 11548180)

Preparation of standards

Quantification was done by standard addition calibration. To prepare the calibration standards, the stock solution containing all four aflatoxins, with a concentration of 857 µg/kg for G2 and B2 and of 2856 µg/kg for G1 and B1 in methanol (stock solution 1), was diluted 1:100 using 1% acetic acid (stock solution 2). Further dilutions of stock solution 2 were made to add defined concentrations to a purified sample extract after immunoaffinity SPE (see next section). Table 1 gives an overview of the calibration levels and corresponding concentrations.

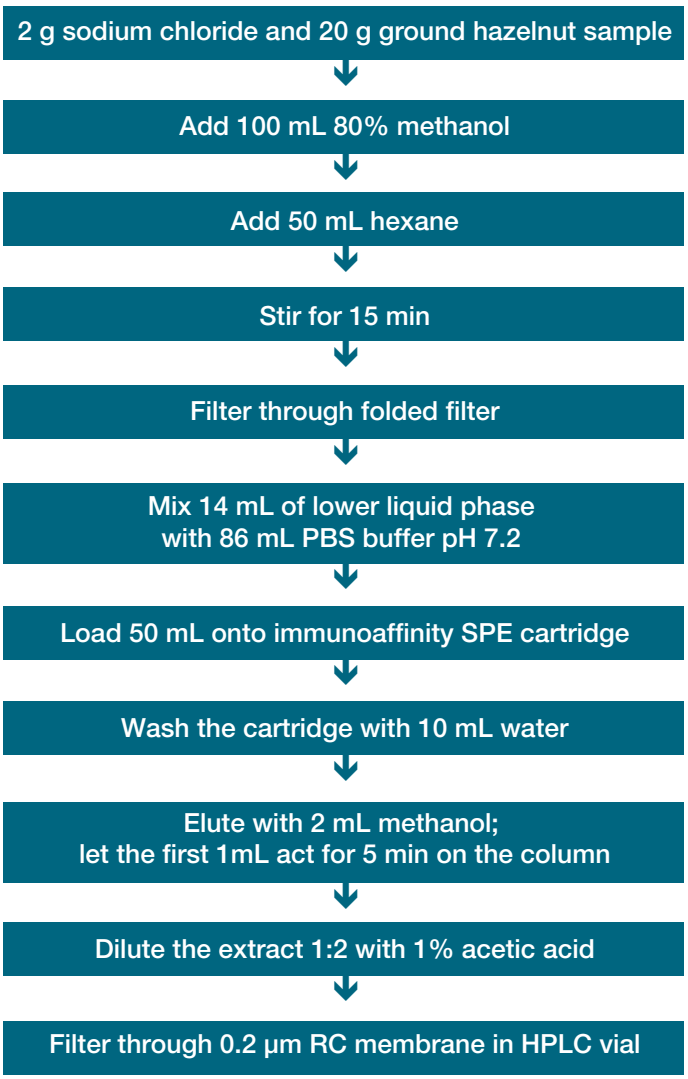
Table 1. Calibration levels and added concentration in µg/kg to a purified sample extract

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

The recovery experiment was carried out by adding 113 µL of the aflatoxin stock solution 1 to 20 g of the ground hazelnut sample prior to sample preparation. The resulting concentration of the recovery sample was 1.7 µg/kg for G2 and B2 and 5.65 µg/kg for G1 and B1 when 100% recovery is assumed.

Preparation of samples

Spiked and non-spiked samples were prepared in triplicate.



Instrumentation

Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:

- System Base Vanquish Flex (P/N VF-S01-A-01)
- Quaternary Pump F (P/N VF-P20-A-01)
- Split Sampler FT (P/N VF-A10-A-01)
- Column Compartment (P/N VH-C10-A-01)
- Fluorescence Detector F (P/N VF-D51-A-01)
  - Standard Bio Flow cell, 8 µL, 20 bar (P/N 6079.4230)

LC conditions

Column:	Thermo Scientific™ Acclaim™ C18, 100 × 3 mm, 3 µm (P/N 076186)
Mobile phase:	A: Water B: Methanol C: Acetonitrile
Flow rate:	0.5 mL/min
Isocratic mobile phase condition:	50% A, 30% B, 20% C
Isocratic run time:	4 min
Mixer volume:	350 + 50 µL
Column temp.:	30 °C (forced-air mode, fan speed 5)
Active pre-heater temperature:	30 °C
Autosampler temperature:	4 °C
FLD Excitation wavelength:	365 nm
FLD Emission wavelength:	450 nm
Sensitivity:	8
Lamp mode:	HighPower
UV data collection rate:	10 Hz
UV response time:	0.5 s
Injection volume:	20 µL
Needle wash:	90/10 water/methanol (v/v)

Data processing and software

The data acquisition and processing was done with Thermo Scientific™ Chromeleon™ 7.2.7 Chromatography Data System (CDS) software.

Results and discussion

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

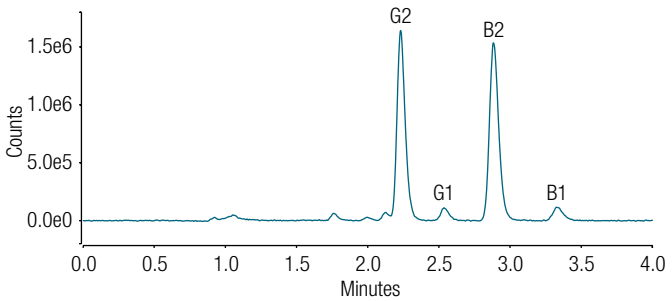


Figure 1. FLD chromatogram of a standard solution of the four aflatoxins: G2, G1, B2, and B1 at concentrations of 0.9 µg/kg for G2 and B2 and 2.9 µg/kg for G1 and B1

For quantification, 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 contained all analytes, was set to zero, 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% (Table 2).

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the sample extract diluted to a S/N ratio approximately of 3 for LOD and a S/N ratio of 10 for LOQ of each analyte and injected in triplicate. One example chromatogram of LOD and LOQ determination of aflatoxin B2 in the diluted sample extract is given in Figure 3. Table 2 shows a full summary of calibration, linearity, LOD, and LOQ results.

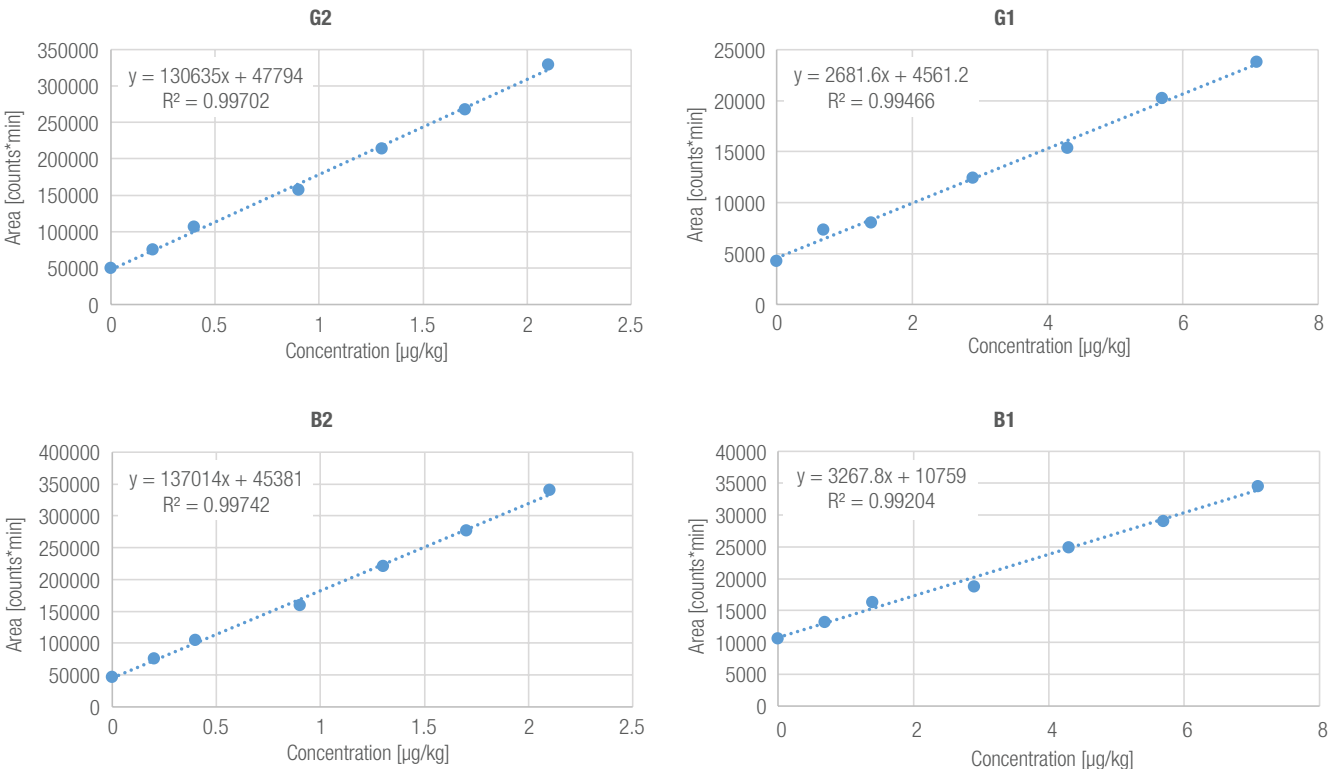


Figure 2. Calibration curves for all four aflatoxin analytes

Table 2. Data of % RSD RT (n=13), calibration range, linearity, LOD, and LOQ with standard deviation (S.D.) (n=3)

Compound Name	% RSD RT	Calibration Range [µg/kg]	R^2	LOD [µg/kg] ± S.D.	LOQ [µg/kg] ± S.D.
G2	0.09	0.2–2.1	0.9970	0.075 ± 0.008	0.185 ± 0.017
G1	0.17	0.7–7.1	0.9947	0.931 ± 0.076	1.329 ± 0.066
B2	0.09	0.2–2.1	0.9974	0.104 ± 0.013	0.206 ± 0.017
B1	0.15	0.7–7.1	0.9920	1.056 ± 0.154	1.122 ± 0.061

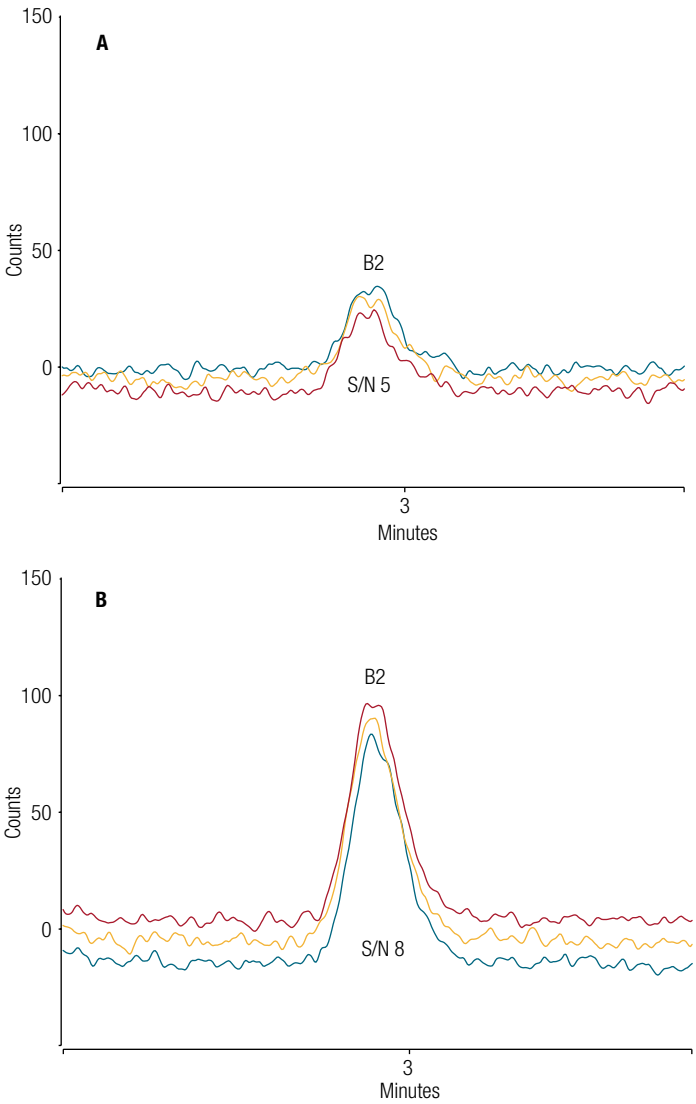


Figure 3. A) Determination of LOD with S/N of approximately 3 of aflatoxin B2 (triplicate injection); B) determination of LOQ with S/N of approximately 10 of aflatoxin B2 (triplicate injection)

As can be seen in Figure 4, 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 min. Neither the non-spiked sample extract nor the spiked (recovery) extract, where the standard solution was added before the sample preparation, show a peak in front of the toxin G2 as it was observed for the standard solution in Figure 1. In Figure 5, which illustrates a blank injection and one calibration point, an impurity can be detected, as the standard was added after the clean-up procedure to the extract. This leads to the assumption that a clear advantage of immunoaffinity purification is that it eliminates impurities which comes from the standard solution.

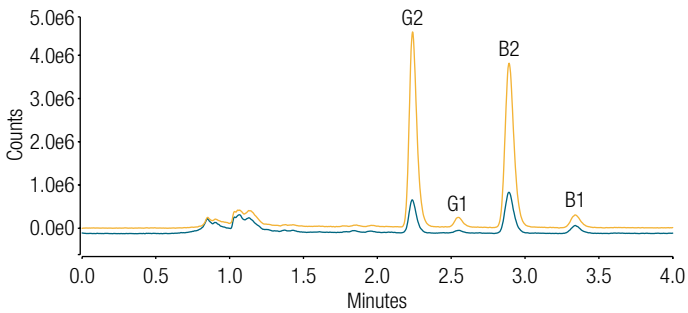


Figure 4. Overlay of spiked (recovery) sample (orange) and non-spiked (blue) hazelnut sample

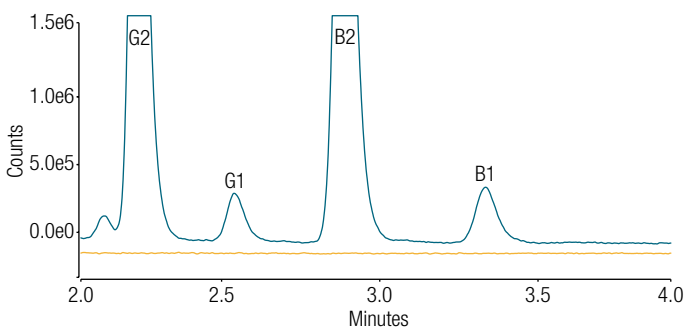


Figure 5. Zoomed overlaid chromatograms of processed sample spiked with the highest calibration concentration (blue) and consecutive blank injection (orange)

In addition, it can be shown in Figure 5 that no carry-over in the blank injection was observed, even after the injection of the processed sample spiked with the highest calibration concentration with 2.1 µg/kg for G2 and B2 and 7.1 µg/kg for G1 and B1.

Quantitative results with recovery rates of each compound and calculated sample amounts (corrected by recovery rate) are summarized in Table 3.

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

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

The recovery rates are excellent for G2, B2, and B1 but inferior for G1. The vendor of the immunoaffinity SPE cartridges reports a minimum recovery of 90% for G1 in the data sheet. However, cartridge stability is limited, and the shelf life is reported to be only a few months under proper storage conditions. The cartridges used in this study were close to their expiration date, which could have caused the lower recovery rate for G1.

The tolerated aflatoxin levels in ground hazelnuts, valid for both direct consumption and ingredient use, is defined by the European Commission Regulation to be 5 µg/kg for B1 and 10 µg/kg for the sum of B1, B2, G1, and G2. All four aflatoxins were detected in the sample. For B1, the amount found was 3.4 µg/kg, which is below the maximum limit. The sum of all four compounds should not exceed 10 µg/kg and was calculated to be 6.3 µg/kg.

Conclusion

- 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 allows a 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. Karsten, M.; Swart, R.; Mcleod, F.; Murphy, B.; Henderson, S.; Richter, B. Fast end Effective Determination of Aflatoxins in Grains for Food Using Accelerated Solvent Extraction followed by HPLC (HPLC 2008 presentation), Chromatography-Foods-Beverages-Contaminants-Applications-Notebook-71476, page 32–33.

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