Determination of Four Aflatoxins in Hazelnuts by Immunoaffinity-SPE with HPLC-FLD Detection without Photo Derivatization

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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 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]. 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.

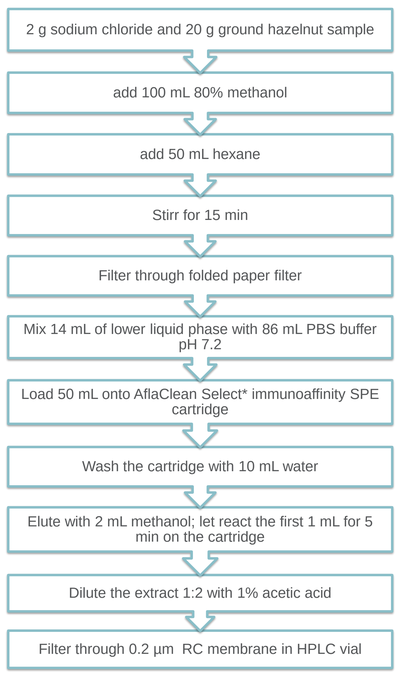
Table 1. Calibration Levels and added concentrations in [µg/kg] to a purified sample extract.

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

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.



* purchased from a reputable vendor

Instrumentation and LC conditions

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)

Column	Thermo Scientific™ Acclaim™ C18 column, 100 x 3 mm, 3 µm
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
Injection volume	20 µL
Column temp.	30° C (forced air mode, fan speed 5)
FLD Excitation	365 nm
FLD Emission	450 nm
Sensitivity	8
Lamp mode	HighPower

Data Analysis

The data acquisition and processing was done with Thermo Scientific™ Chromeleon™ 7.2.8 Chromatography Data System (CDS).

RESULTS

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.

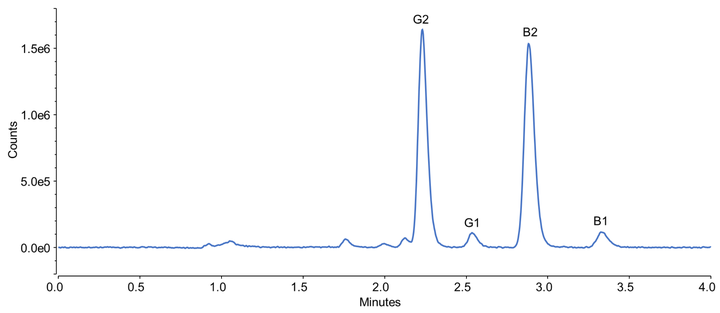


Figure 1. FLD chromatogram of a standard solution of the four aflatoxins: G2, G1, B2 and B1 at concentrations of 0.9 µg/kg for G2 and B2 and 2.9 µg/kg for G1 and B1

Calibration results

For 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 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 (R2) 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).

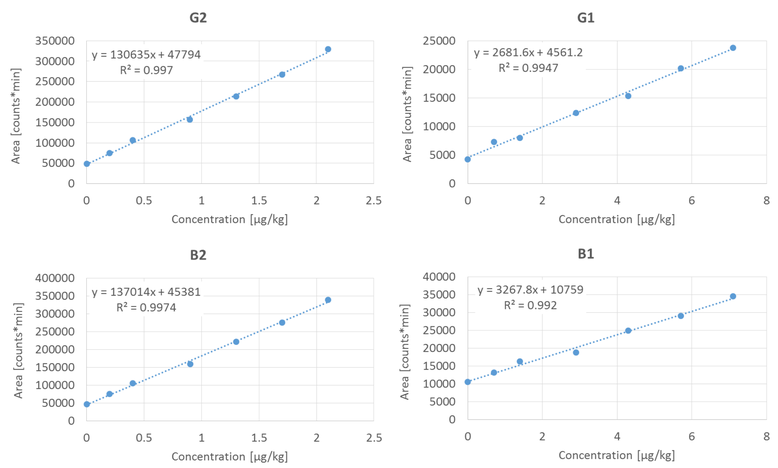


Figure 2. Calibration curves for all four aflatoxin analytes

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 and LOD, LOQ results.

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

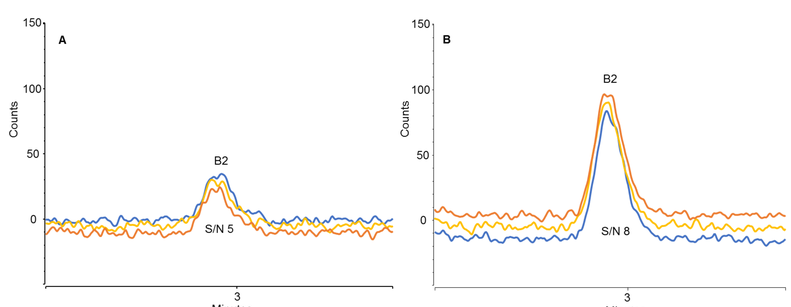


Figure 3. A) Determination of LOD with S/N of approximately 3 of aflatoxin B2 (triplicate injection); B) determination of LOQ with S/N of approximately 10 of aflatoxin B2 (triplicate injection)

Quantitation results

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 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. 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 the removal of impurities which come from the standard solutions.

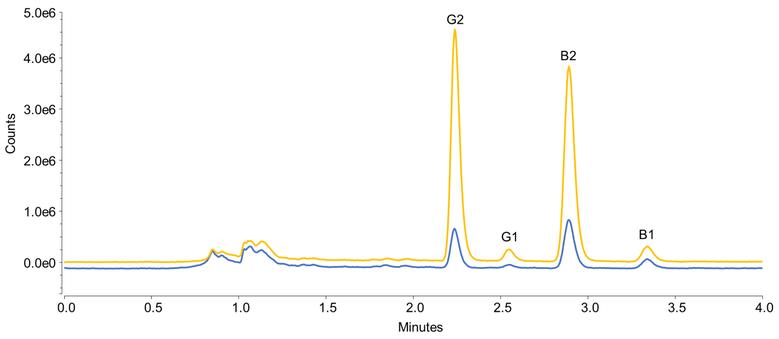


Figure 4. Overlay of spiked (recovery) sample (orange) and non-spiked (blue) hazelnut sample

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

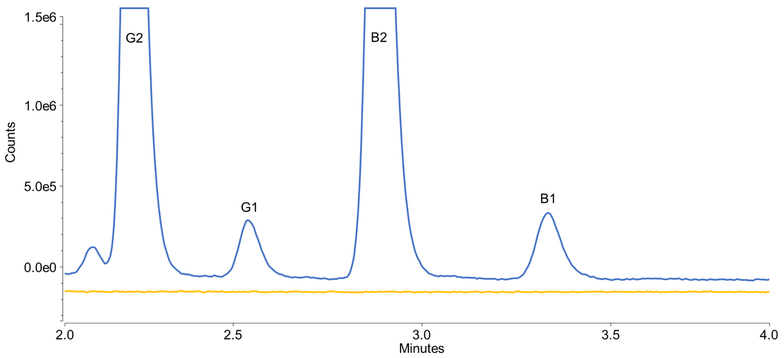


Figure 5. Zoomed overlaid chromatograms of processed sample spiked with the highest calibration concentration (blue) and consecutive blank injection (orange).

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 their 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 found amount is 3.4 µg/kg, which is below the maximum limit. The sum of all four compounds should not exceed 10 µg/kg, but was found to be 6.3 µg/kg.

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)..

TRADEMARKS/LICENSING

PO72789-EN 0718S