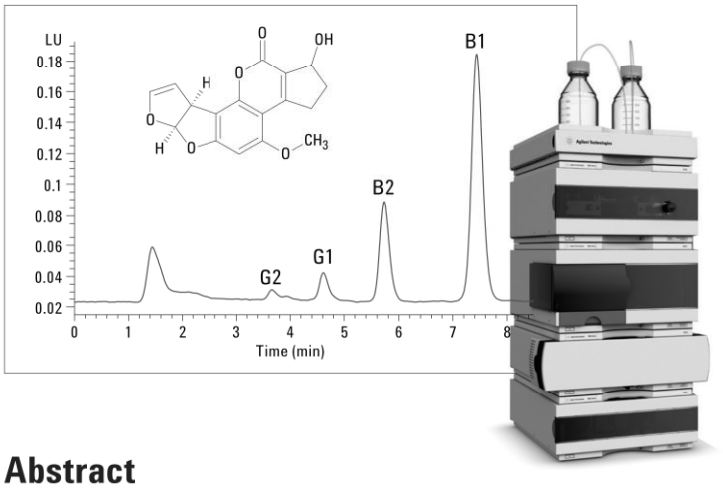
Sensitive fluorescence analysis of aflatoxins using post-column UV derivatization on the Agilent 1260 Infinity LC.

Application Note

Food Testing & Agriculture



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Abstract

The analysis of aflatoxins in food matrices such as grain and nuts is an important task for the food industry to ensure product safety. This Application Note shows the analysis of the four major aflatoxins after sample preparation using an immunoaffinity column, post column UV derivatization, and fluorescence detection. We show that with this method the LOD and LOQ required from the regulatory agencies can be achieved easily. This method offers a less hazardous alternative compared to the well-established, post-column electrochemical derivatization method.

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Introduction

Aflatoxins are toxic and carcinogenic compounds produced as metabolites by many *Aspergillus* species. This fungus invades habitats such as soil, grain, nuts, or decaying vegetation whenever suitable conditions such as high moisture and temperature are met. B1 is the most toxic of at least 14 different types of naturally occurring aflatoxins. Therefore, many countries and regulatory agencies impose strict limits on B1 as well as other common aflatoxins such as B2, G1, and G2 despite exhibiting lower toxicity. The European Commission, for example, has set maximum levels for B1 between 2.0 and 8.0 µg/kg (ppb) and between 4.0 and 15.0 µg/kg (ppb) for the sum of the four above mentioned aflatoxins in crops such as nuts, grains, and dried fruits[1,2]. The U.S. FDA set the action level to 20 µg/kg (ppb) for human food and to 300 µg/kg (ppb) for animal feed[3].

The determination of aflatoxins is usually done by HPLC with fluorescence detection. However, due to the quenching of the fluorescence activity of some aflatoxins in aqueous mobile phases, techniques were developed to derivatize aflatoxins into nonquenchable forms[4]. One common derivatization technique is the post-column electrochemical bromation using a Coring cell[5,6]. While this method enables determination of the aflatoxins at the required sensitivities, the use of hazardous mobile phase (nitric acid, *in-situ* generated bromine) and the required maintenance of the cell are disadvantages of this method.

Other derivatization methods use UV light in the presence of water. Since water is part of the mobile phase this method appears to be ideal for reversed phase HPLC determination. It has been shown that sensitivities comparable to the electrochemical derivatization can be achieved[7].

This Application Note shows the sensitive analysis of the aflatoxins B1, B2, G1, and G2 using the Agilent 1260 Infinity Quaternary LC System with fluorescence detection after photochemical derivatization. It also shows that the LOD and LOQ achieved with this method are well below the limits required by the regulatory agencies.

Experimental

System

The Agilent 1260 Infinity LC System consisted of the following modules:

- Agilent 1260 Infinity Quaternary Pump (G1311B)
- Agilent 1260 Infinity Standard Autosampler (G1329B)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316A)
- Agilent 1260 Infinity Fluorescence Detector (G1312B), equipped with standard flow cell (8 µL)
- Post column UV derivatization module. Acquired from LCTech, Germany (UVE UV Derivatization Module for the Analysis of Aflatoxins). This module was connected to the flow path between the column and the fluorescence detector (FLD).

Column

Agilent Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7 µm (p/n 699975-902)

The immunoaffinity column for sample preparation (RIDA Aflatoxin column) was purchased from Biopharm Co.

Software

Agilent OpenLAB CDS ChemStation Edition for LC & LC/MS Systems, Rev. C.01.03 [37]

Solvents and samples

Solvents

Methanol (HPLC Grade) was purchased from Dikma. Ultra-pure water was prepared using a MilliQ system from Millipore Co.

Standards

The aflatoxin standard (99%+ containing B1: 1.055 µg/mL, B2: 0.327 µg/mL, G1: 1.082 µg/mL and G2: 0.309 µg/mL in toluene/acetonitrile (98:2)) was purchased from Supelco.

Samples

Corn powder was purchased at a local supermarket. For the spiked corn powder, the standard (concentration level 4 in Table 1) was added before sample preparation. The certified content corn powder (containing 9.00 ppb B1 and 0.6 ppb B2) was purchase from Trilogy Co. Sesame paste containing 4.57 ppb aflatoxin B1 and the other aflatoxins in an unknown amount was received from a customer. The *Semen Ziziphi Spinosae* powder, a TCM sample in the Chinese Pharmacopeia that must be tested for aflatoxins, was purchased at a local pharmacy.

Sample and standard preparation

For preparation of standards, 100-µL standard solution was transferred into a 10-mL vial and blown dry with nitrogen at room temperature. The compounds were redissolved in 10 mL methanol/water (50:50, v:v) and diluted to the concentrations shown in Table 1.

For sample preparation, 5 g of the sample (see Table 2) and 0.5 g sodium chloride was weighed into a 50-mL centrifugal tube and 25 mL of HPLC grade methanol/water (70:30, v:v) was added. After high speed homogenization for 1 minute and centrifugation at 5,000 rpm for 5 minutes, exactly 5 mL of the supernatant was added to 15 mL of water. The entire sample (20 mL) was passed through the immunoaffinity column.

For cleanup with the immunoaffinity column the following protocol was used:

1. Rinse the column with 2 mL distilled water.
2. Pass sample extract solution slowly and continuously through the column (1 drop/second), use positive pressure with syringe, discard the eluent.
3. Rinse the column with 10 mL distilled water, discard the eluent.
4. Air purge the column for about 10 seconds to remove remaining eluent.
5. Elute with 0.5 mL methanol (100 %) with a low flow (1 drop/second) rate through the column to ensure complete elution of the aflatoxins.
6. Collect all remaining eluent by purging air through the column.
7. Add 0.5 mL distilled water to the eluent and mix with a vortex mixer.
8. Filter sample through a 0.22 µm syringe filter.

Level conc. in ppb	1	2	3	4	5	6
B1	0.1055	0.2638	0.5275	1.055	5.275	10.55
B2	0.0327	0.0818	0.1635	0.327	1.635	3.25
G1	0.1082	0.2705	0.541	1.082	5.41	10.82
G2	0.0309	0.0772	0.1545	0.309	1.545	3.09

Table 1
Concentration of standard levels.

	Corn powder	Corn powder (spiked)	Corn powder (certified content)[3]	Sesame paste (customer sample)	*Semen Ziziphi Spinosae* powder
Weight(g)	5.03	5.00	4.99	4.96	4.98

Table 2
Samples.

Chromatographic conditions

Column:	Agilent Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7 µm (p/n 699975-902)
Mobile phase:	Water/Methanol 65:35 (v:v), isocratic
Stop time:	8.5 min
Flow:	1.2 mL/min
Injection volume:	30 µL
Column temperature:	30 °C
FLD:	Ex.: 365 nm, Em.: 440 nm, Data rate: 1.16 Hz, Gain: 10
	Standard flow cell (8 µL)

Results and discussion

Using the method described above, a blank injection (red) and an injection of the aflatoxin standard (level 4, blue) was done. Figure 1 shows the baseline separation of four aflatoxins in 8.5 minutes. The relatively broad peaks are caused by the volume of the reaction coil inside the UV derivatization module required to ensure long enough exposure of the compounds to the UV light.

Area and retention time precision

To determine area and retention time precision, six consecutive injections of the standard (level 4) were done. The retention time precision was below 0.1% RSD for all four aflatoxins and the area precision was below 2% RSD (Table 3).

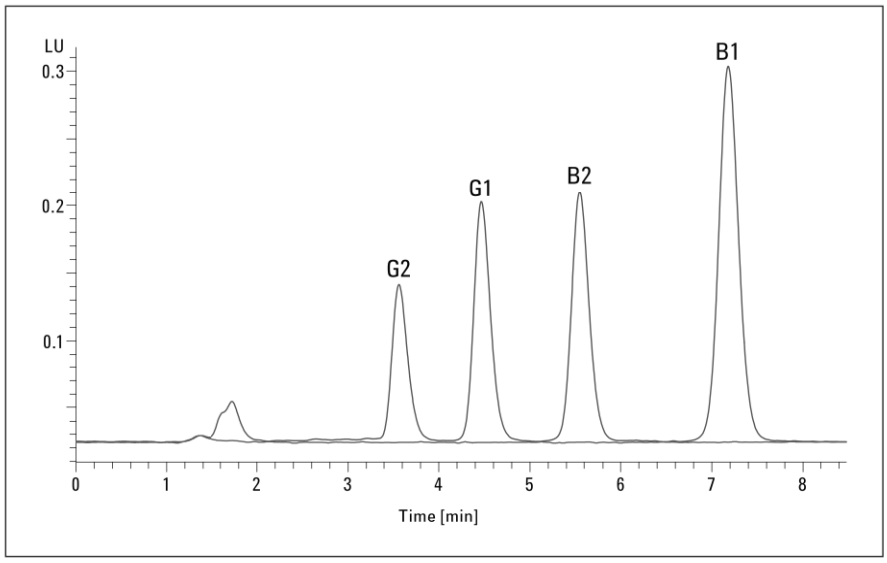

Figure 1
Blank injection (red) and analysis of standards (blue).

RSD	B1	B2	G1	G2
Rt (%)	0.09	0.07	0.06	0.07
Area (%)	0	0	1.8	0

Table 3
Retention time and area precision (n = 6).

Limit of detection and limit of quantification

To determine LOD, S/N = 3 and LOQ, S/N = 10 the standards (level 1 and level 2) were injected (Figures 2A and 2B). Table 4 summarizes the LOD and LOQ results. The values are well below the required limits from the European Commission and the U.S. FDA.

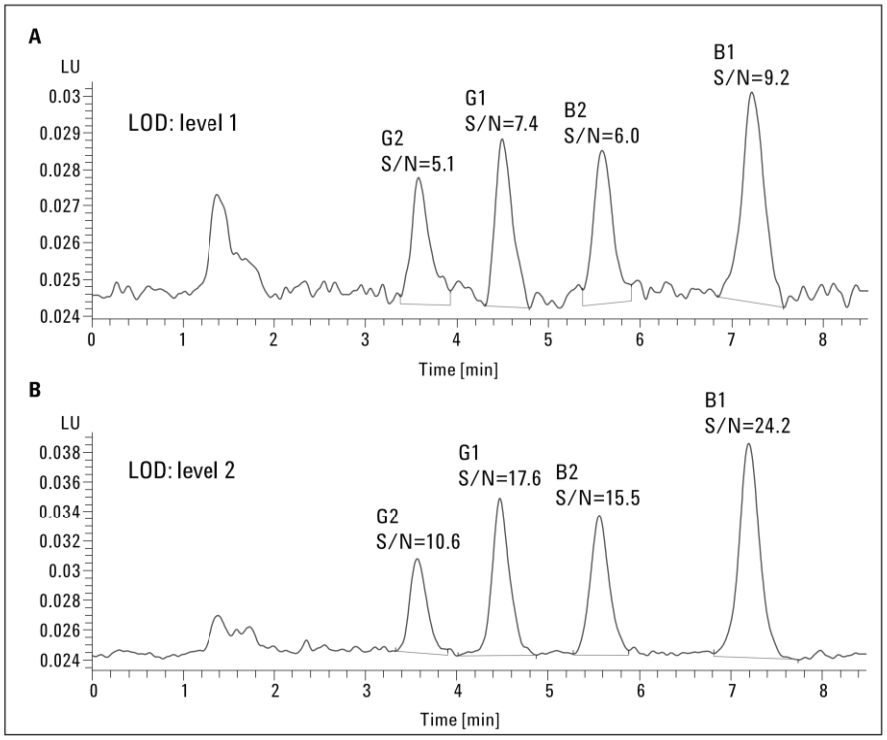

Figure 2
Determination of LOD and LOQ.

	B1	B2	G1	G2
LOD (calculated by S/N in ppb)	0.034	0.016	0.043	0.018
LOQ (calculated by S/N in ppb)	0.109	0.053	0.156	0.073

Table 4
LOD and LOQ (ppb).

Linearity

Five concentrations of the standard (level 2–6) were injected (Figure 3). The linearity (correlation factor) for all four aflatoxins was less than 0.99, the results are summarized in Table 5.

Analysis of samples

Recovery

To investigate the recovery of the sample preparation step and the analytical method, standard and spiked samples were passed through the immunoaffinity column and subsequently analyzed. The fact that the recovery for both standards and samples gave similar results shows that the matrix does not have an influence on the recovery (Table 6). The overall recovery was somewhat lower than reported in the instructions of the immonaffinity column (70–110%).

Figure 4 shows the analysis of corn powder (red) and corn powder spiked with standard (level 4). The corn powder was spiked with the standard prior to sample preparation with the immunoaffinity column as described above. While the unspiked corn powder does not contain any aflatoxins, the spiked corn powder shows good separation of the aflatoxins from any matrix peaks.

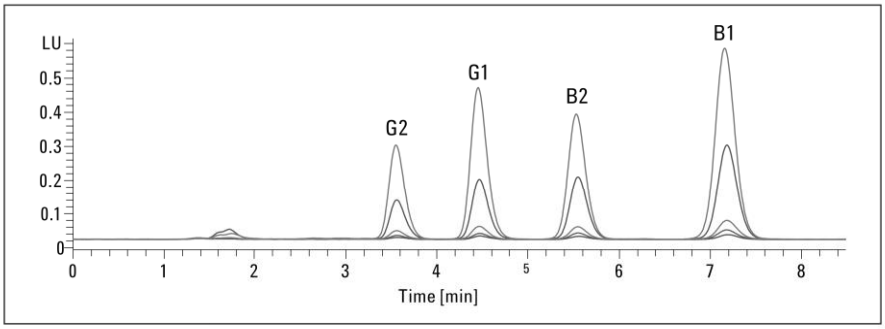

Figure 3
Overlay of five concentrations (standard level 2–6).

	B1	B2	G1	G2
Linearity	0.99996	0.99999	0.99444	0.99649

Table 5
Linearity results.

	B1	B2	G1	G2
Standard	58.0%	69.2%	60.7%	62.1%
Spiked sample	52.8%	74.7%	63.6%	64.6%

Table 6
Recoveries for standard and spiked samples.

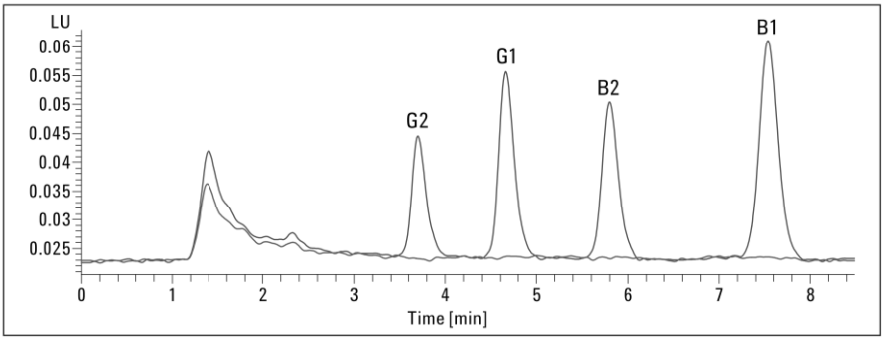

Figure 4
Corn powder (red) and spiked corn powder (blue).

Figure 5 shows an overlay of the spiked corn powder (red) and the certified content corn powder (blue) containing only the aflatoxins B1 (9.00 ppb) and B2 (0.6 ppb).

Figure 6 shows the analysis of the sesame paste containing the aflatoxins B1, B2, G1, and G2. Again, the relevant aflatoxin peaks are well separated from any matrix peaks.

Semen Ziziphi Spinosae is a Traditional Chinese Medicine for tranquilizing, allaying excitement, relieving pain (analgesia) and lowering blood pressure. The analysis (blue) and the overlay with the aflatoxin standards (red) is shown in Figure 7. The analyzed sample does not contain any aflatoxins.

Table 7 summarizes the quantitative results for the different samples.

These results show good agreement of the know aflatoxin concentrations in the samples and the determined recovery values from Table 6.

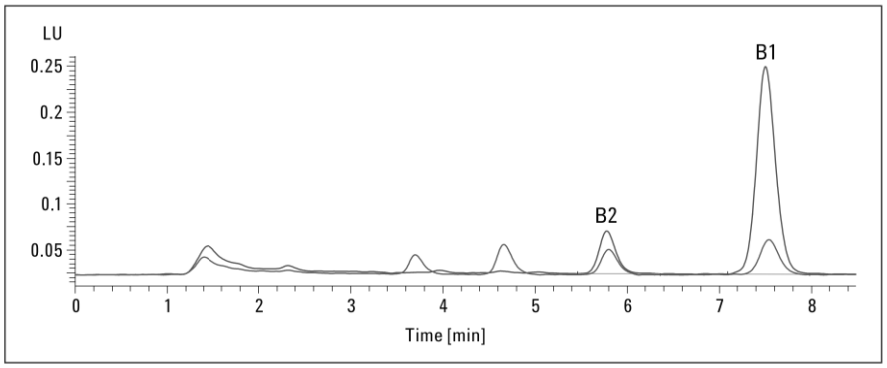

Figure 5
Spiked corn powder (red) and certified corn powder (blue).

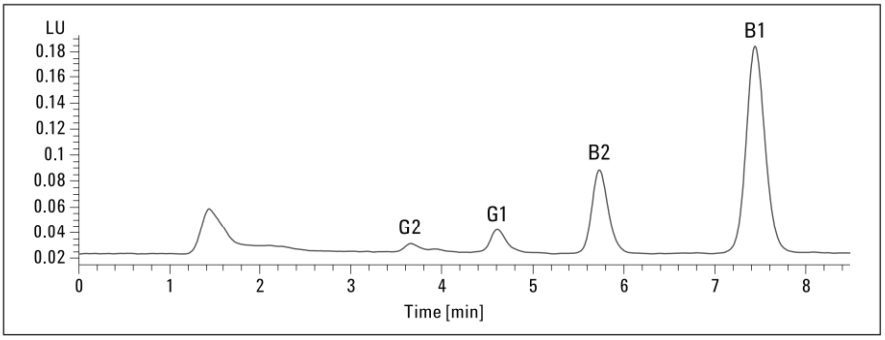

Figure 6
Analysis of certified content sesame paste.

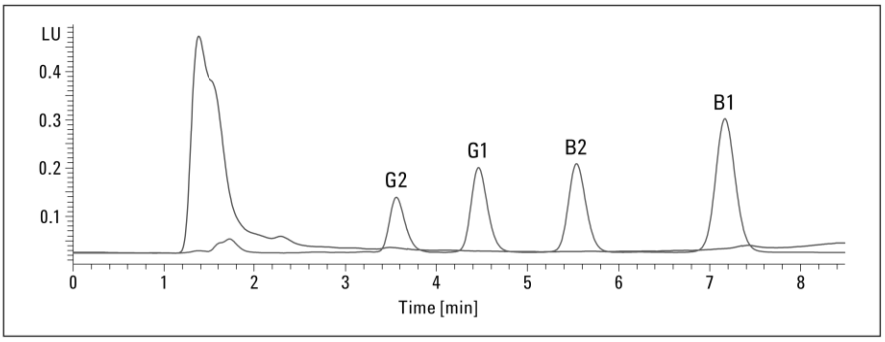

Figure 7
Analysis of *Semen Ziziphi Spinosae* (blue) and aflatoxin standards (red).

Content (ppb)	Corn powder	Corn powder (spiked)	Corn powder (certified content)	Sesame paste (customer sample)	*Semen Ziziphil Spinosae* powder
B1	N.D.	0.680	4.036	2.839	N.D.
B2	N.D.	0.277	0.381	0.538	N.D.
G1	N.D.	0.987	N.D.	0.693	N.D.
G2	N.D.	0.271	N.D.	0.100	N.D.

Table 7
Quantitative results for the analyzed samples.

Conclusion

This Application Note shows the analysis of the aflatoxins B1, B2, G1, and G2 with fluorescence detection after post-column derivatization using a simple UV device. The achieved LOD's and LOQ's allow the quantitative measurement of the compounds well below the required limits of the regulatory agencies. Compared to the well-established electrochemical post-column derivatization method, the advantages are the less hazardous mobile phase and the lower required maintenance of the derivatization device.

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