Aflatoxin Analysis by HPLC with Fluorescence Detection using Post-Column Derivatization and Pre-column Immunoaffinity Cleanup

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Abstract

HPLC with fluorescence detection and reversed phase separation has been the cornerstone of aflatoxin analysis for many years. To achieve the lowest detection limits, derivatization of aflatoxins B1 and G1 is required to improve their fluorescence yield. In recent years, post-column bromination reactions have replaced older derivatization methods like pre-column TFA (trifluoroacetic acid) or post-column Iodine. Additionally, sample preparation has been improved due to the use of immunoaffinity column (IAC) cleanup methods.
We have comparison tested the various methods including those above which may offer higher sensitivity, faster analysis time, simplified sample preparation, and good configuration flexibility.

Introduction

In the recent developments for aflatoxin analysis, two bromination approaches have been thoroughly tested: generation of bromine via post-column addition of pyridinium hydrobromide perbromide (PBPB) or electrochemical generation of bromine from potassium bromide acidified with nitric acid. Both are cited in official AOAC and other methods(1) and will be positioned here in comparison with earlier methods.

Perhaps most remarkable is the development of SPE devices impregnated with aflatoxin-specific antibodies that promise to deliver cleaner samples than conventional SPE (solid phase extraction) methods. A wide variety of IAC columns exist, for aflatoxins and other mycotoxins, though most are offline manual procedures. Converting the IAC cleanup to an online method is highly desirable, though problematic due to the limited robustness, both chemically and physically, of most IAC materials. Our work here is the beginning of a larger project to improve sensitivity and workflow for labs involved in aflatoxin and, in other ongoing work, mycotoxins as a whole.

Experimental

Agilent 1200 series Rapid Resolution LC, consisting of:
G1379B micro vacuum degasser
G1312B binary pump SL
G1311A quaternary pump (reagent delivery)
G1367C high performance autosampler SL
G1316B thermo. column compartment SL
G1321A FLD Fluorescence Detector
ChemStation 32-bit version B.03.02

Experimental

Chromatographic Conditions:

Optimized aflatoxin separation on:
Zorbax SB-Aq 4.6x150mm 5um column (pn 883975-914). 40C, 1ml/min,
40% 4/6 ACN/MeOH, 60% water.
FLD signal (ex 360nm, em 455nm) shown with and without 0.3ml/min post-column addition of bromination reagent.

1. PBPB 50mg/L solution in mobile phase. Solution delivered post-column with Agilent pump G1311A, 0.2 or 0.3ml/min. Delay coil for reaction consisted of ~40cmx 0.5mm i.d. PTFE at ambient temperature.

2. HNO_3 and KBr added postcolumn (typically this is added precolumn however postcolumn is somewhat more flexible for our evaluation) in amount and flow rate equivalent to simple mobile phase addition.

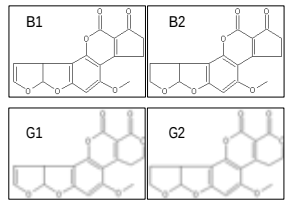

Figure 1. Aflatoxins B1 (upper left) and B2 (upper right) and G1 and G2 lower left and right respectively. Unsaturation at left in B1 and G1 suppresses fluorescence sensitivity. Derivatization reactions are all designed to remove the double bond.

Sample Preparation

Immunoaffinity columns.

—R-RBiopharm Rhône Ltd. (Glasgow, UK).

The basic procedure uses 6/4 MeOH/water to grind the nuts, then filtration, dilution with PBS (phosphate buffered saline) and loading onto a prepared IAC SPE column. Elution with Methanol and a rinse/dilution with water completes the procedure.(1)(2)

---Horiba (Kyoto, Japan)

The basic procedure uses 9/1 ACN/water to grind the nuts, then filtration, dilution with PBS (phosphate buffered saline) and loading onto a prepared IAC SPE column. Elution with ACN completes the procedure.(3)

Results and Discussion

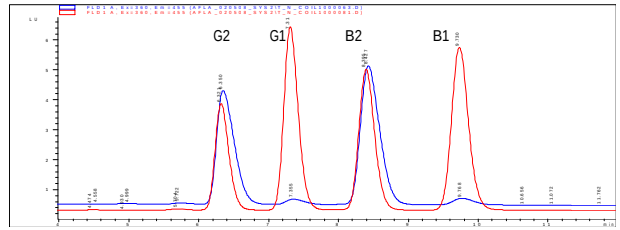

Figure 2. Optimized aflatoxin separation on Zorbax SB-Aq (see Experimental for details). The FLD signal (ex 360nm, em 455nm) is shown with post-column 0.3ml/min water vs. 0.3ml/min PBPB reagent (50mg/L). There is virtually no difference for B2 and G2, as expected. Solutions were delivered with Agilent pump G1311A, 0.2 or 0.3 ml/min. Delay coil for reaction consisted of ~400mm x 0.5mm i.d. PTFE at ambient temperature.

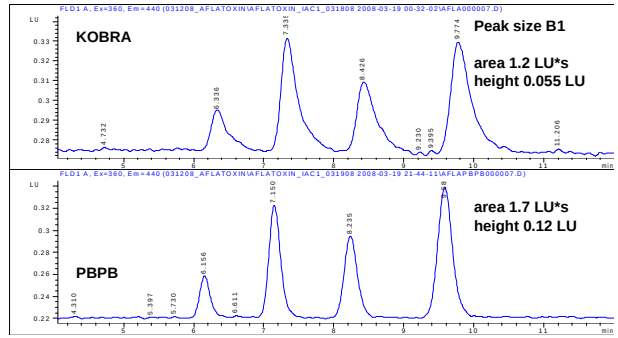

Figure 3. HNO3/KBr reagent and KOBRA cell, 100uA current as recommended, vs. PBPB 50mg/L solution. Though comparable signal was observed, in our hands the peak symmetry and absolute response of PBPB was superior. Eventually, we were forced to abandon the preferred KOBRA cell and complete studies with PBPB. The tailing behavior has not been detailed elsewhere, and it requires further investigation.

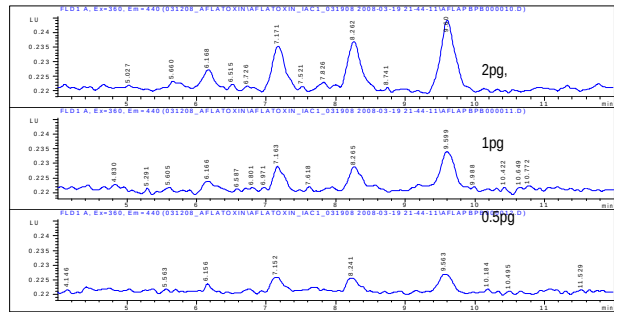

Figure 4. Injection of 2,1 and 0.5pg B1,G1. B2,G2 0.6,0.3,0.15pg each (mass on column) FL 360ex, 440em, default 2 second filter. 0.5pg is the nominal LOD under these conditions. Taking the sample preps into consideration, and a 20ul injection volume, this would be equivalent to a LOD sample concentration of approximately 0.05 ug/kg.

Results and Discussion

RBiopharm IAC Prep'd Samples	g2	g1	b2	b1	B1 recov %	Total recov%
low std NO IAC prep (5pg B1)	1.6	5.7	1.5	5.4	107.8%	108.7%
v low std, NO IAC prep (0.5pg B1)	0.1	0.6	0.1	0.3	69.8%	80.9%
Rb solvent only LOW 100ul spike 75g a	6.2	20.1	9.4	31.3	78.2%	64.4%
Rb solvent only LOW 100ul spike 75g b	5.1	17.1	9.7	32.9	82.2%	62.3%
Rb solvent only HIGH 500ul spike 75g a	22.2	74.9	43.2	154.3	77.2%	56.7%
Rb solvent only HIGH 500ul spike 75g a	20.3	69.8	38.6	138.0	69.0%	51.3%
Rb nut blank 52g no spike 3-18-08 1 a	0.1	0.1	0.0	0.3	0.0%	0.0%
Rb nut blank 52g no spike 3-18-08 1 b	0.1	0.1	0.0	0.1	0.0%	0.0%
Rb nut 46.7g LOW spike 3-18-08 a	3.5	11.2	7.2	23.8	59.5%	44.0%
Rb nut 46.7g LOW spike 3-18-08 b	4.2	14.2	7.5	24.2	60.5%	48.2%
Rb nut 44.4g LOW spike 3-18-08 a	4.4	14.3	7.7	24.8	62.0%	49.3%
Rb nut 44.4g LOW spike 3-18-08 b	3.6	11.5	7.1	23.2	58.0%	43.6%
Rb nut 46.4g High spike 3-18-08 a	19.3	64.9	35.6	120.4	60.2%	46.2%
Rb nut 46.4g High spike 3-18-08 b	16.6	58.6	32.9	114.5	57.2%	42.8%
Rb nut 47.1g High spike 3-18-08 a	17.1	60.0	33.3	115.1	57.6%	43.4%
Rb nut 47.1g High spike 3-18-08 b	23.1	83.3	36.7	127.8	63.9%	52.1%
				Avg.	**65.5%**	**50.3%**

Horiba IAC Prep'd Samples	g2	g1	b2	b1	B1 recov %	Total recov%
Horiba solv only LOW 100ul spike 75g a	3.1	14.0	4.7	18.7	62.5%	52.1%
Horiba solv only LOW 100ul spike 75g b	8.5	22.3	6.6	20.9	69.6%	74.6%
Horiba solv only HIGH 500ul spike 75g a	42.7	108.3	33.0	100.4	67.0%	72.9%
Horiba solv only HIGH 500ul spike 75g b	45.3	113.5	34.6	104.9	70.0%	76.5%
Horiba nut bl 48.1g no spike 3-18-08 a	0.1	0.1	-	-	0.0%	0.0%
Horiba nut bl 48.1g no spike 3-18-08 b	0.0	0.1	-	0.0	0.0%	0.0%
Horiba nut 43.8g LOW spike 3-18-08 1 a	9.1	23.4	7.2	21.7	72.4%	78.7%
Horiba nut 43.8g LOW spike 3-18-08 1 b	9.4	23.2	7.3	21.6	72.0%	79.0%
Horiba nut 44.5g LOW spike 3-18-08 2 a	9.2	23.9	7.1	21.9	72.9%	79.5%
Horiba nut 44.5g LOW spike 3-18-08 2 b	9.4	24.6	7.2	21.8	72.6%	80.7%
Horiba nut 51.6g High spike 3-18-08 1 a	49.3	120.4	37.4	111.6	74.4%	81.7%
Horiba nut 51.6g High spike 3-18-08 1 b	48.6	124.5	37.7	113.2	75.5%	83.1%
Horiba nut 55.7g High spike 3-18-08 2 a	45.3	116.2	36.1	108.9	72.6%	78.6%
Horiba nut 55.7g High spike 3-18-08 2 b	46.4	117.7	36.2	109.2	72.8%	79.4%
				Avg.	**71.2%**	**76.4%**
low std NO IAC prep (20pg B1)	6.3	19.6	6.3	19.7	98.7%	99.8%
low std NO IAC prep (5pg B1)	1.6	5.1	1.6	4.9	97.8%	101.6%

Tables 1 and 2. Summarizing the recovery and repeatability of the two IAC columns and procedures, we might conclude that the RBiopharm extraction solvent could be higher in Methanol. This method is the mfr's for hazelnuts, and cashew nuts might have slightly higher oil content which would necessitate addition of hexane to the extraction procedure. This has bee reported elsewhere to facilitate defatting the sample and improving aflatoxin recovery. The recovery generally reported is 85%, with fairly low <10% repeatability. We achieved the repeatability but have fallen short on the first evaluation of the procedure.

Conclusion

The Agilent 1200 Rapid Resolution LC system equipped with fluorescence detection provided a rapid separation with good resolution and sensitivity for the aflatoxins. Immunoaffinity cleanup greatly simplified the separation, though more study is required to optimize the recovery for different matrices.
Future work includes expansion of the number of matrices, additional development of IAC for online use and refinement of overall method sensitivity.

References:
1. SENYUVA & GILBERT: JOURNAL OF AOAC INTERNATIONAL VOL. 88, NO. 2, 2005
2. Private communications, RBiopharm,
3. Private communications, Horiba
4. Mycotoxin Protocols, ed. M.W. Trucksess, A.E. Pohland, Humana Press 2001