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Application Note

Multi-Analyte Mycotoxin Analysis

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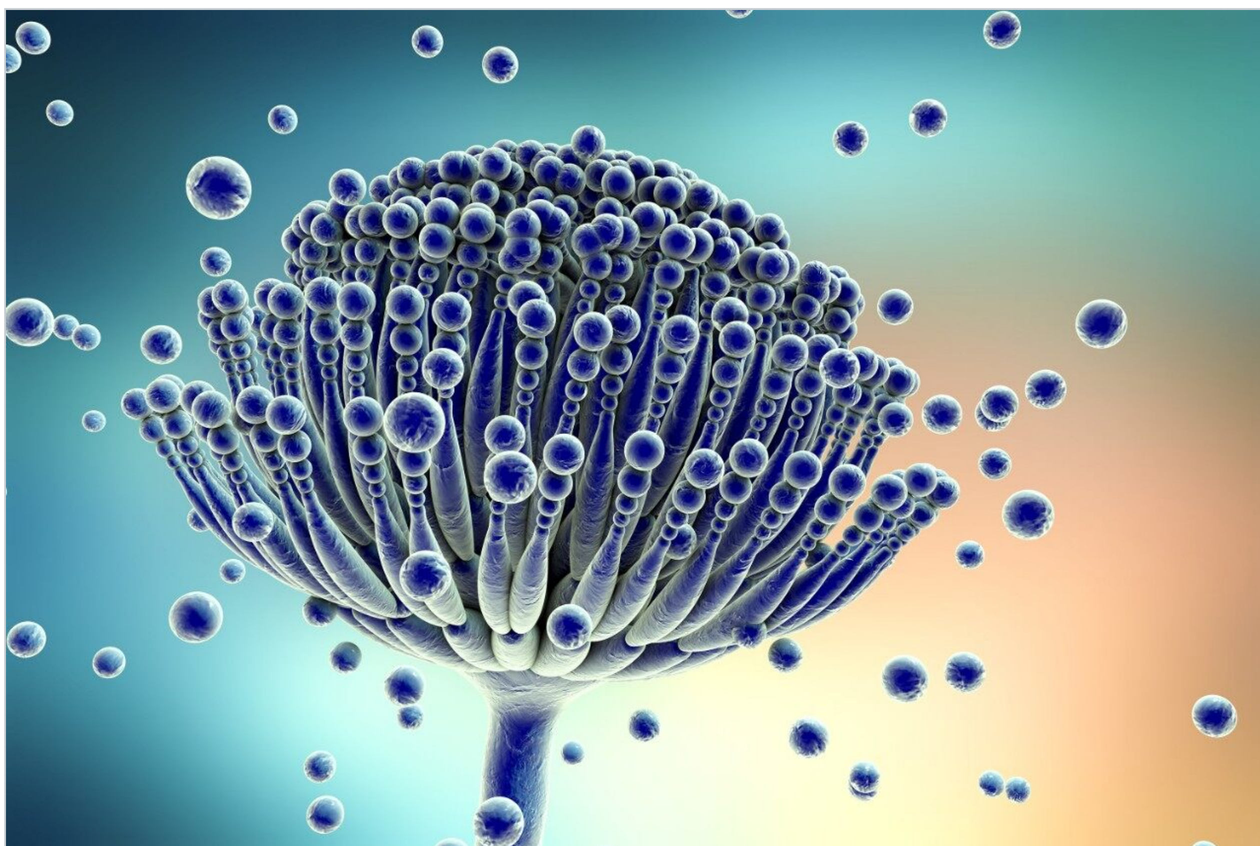

Abstract

This application note demonstrates a multi-analyte, multi-detection method for mycotoxin analysis.

Introduction

Agricultural raw commodity foodstuffs, such as grains, vegetables and fruits, are subject to microbiological contamination during harvesting, storage, and transport. Various molds and fungi are the most prevalent creating toxins that can contaminate foods. The toxins of concern are the 4 alfatoxins, DON (deoxynivalenol), ochratoxin A, zearalenone, and the 3 fumonisins.

Aflatoxins are produced by several species of Aspergillis: *A. flavus. A. parasiticus, and A. nominus* mold on peanuts and corn. *Aspergillis ochraceus* and *Penicillium verrucosum* molds yields ochratoxin in peanuts, corn and other grain staples. *Fusarium graminearum* and *F. culmorum* molds produce a heat stable vomitoxin, also called DON (deoxynivalenol) in cereal grains such as wheat, barley, oats, rice, and corn. Different species of fusariums, especially *F. culmorum and F. crookwellense* produce zearalenone. Several species of mold produce 15 closely related compounds of fumonisins, fumonisins B1, B2 being the most abundant. Fumonisins often occur together with other the mycotoxins.

Because these mycotoxins can potential invade many of the primary raw food ingredients, and affecting food safety, the analysis of these toxins in the raw food stuff is critical. The consequences are liver cancer, kidney failure, and intestinal distress, amongst their potential teratogenic and reproductive effects. This is why sensitive, validated LC methods have been established for their analysis.

This is a worldwide issue with varying degrees of acceptance. The acceptable concentrations for mycotoxins are country dependant, as shown in Table 1.

	US (in ppb, ng/g)	World
Total Aflatoxins	20	0 - 20
Ochratoxin A	4	5 - 200
Zearalenone	—	60 -1000
Vomitoxin (DON)	1000	500 - 750
Total Fumonisins	2000 - 4000	2 -1000

Table 1. Different countries have differing mycotoxin action limits.

Currently, there are numerous methods for mycotoxin analysis, several for the four aflatoxins, another for the fumonisins, a third for ochratoxin A, and a fourth for zearalenone. Refer to Figure 1 for mycotoxin structures. Recently, for cost and expediency, it has become desirable to integrate several mycotoxins into a single analytical method.

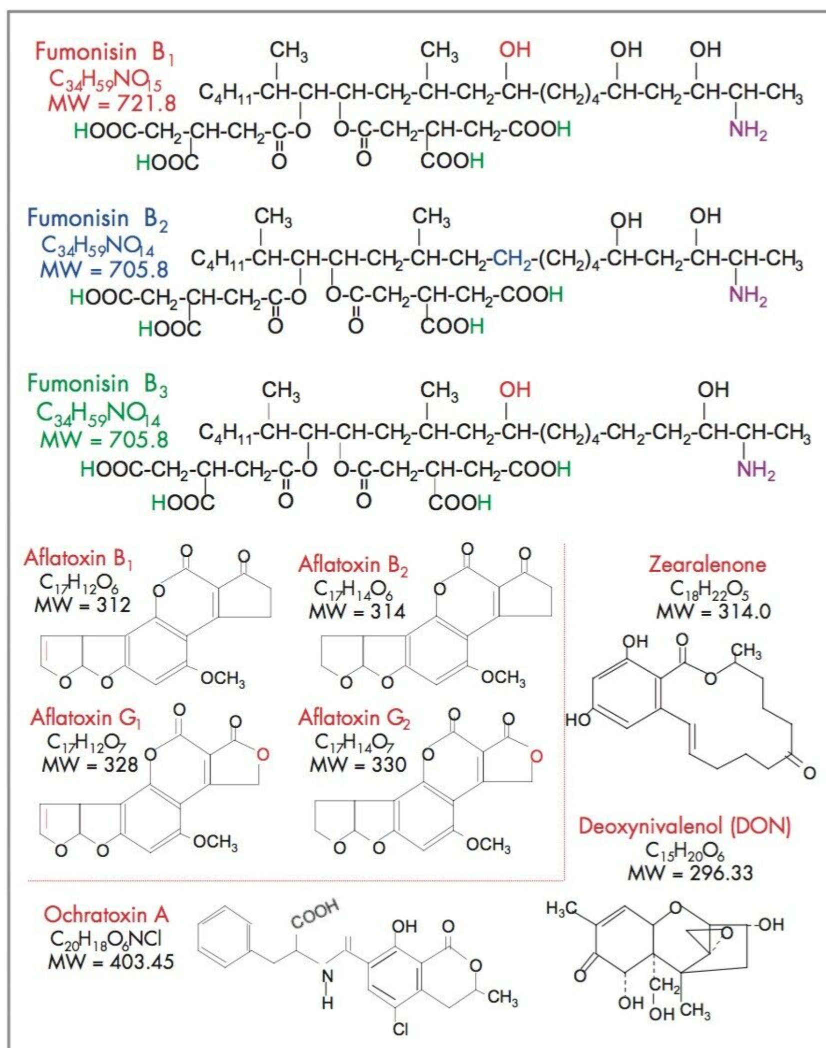

Figure 1. Mycotoxin structures.

Experimental

Aflatoxin Analysis

Aflatoxin analysis has been an HPLC method for decades with numerous methods published. Of the many derivitization methods for aflatoxins found in the literature, three tend to stand out: post column iodine addition, which is the basis of the official AOAC method, electrochemically generated bromine using the Kobra Cell, and photochemical UV (Phred). Response comparison of the three methods is shown in Figure 2 with detection limits given in Table 2.

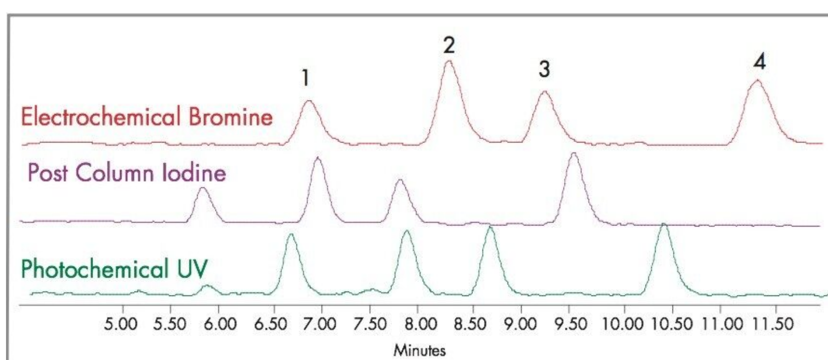

Figure 2. Response comparison 1: 12 ppt G2, 2: 40 ppt G1, 3: 12 ppt B2, 4: 40 ppt B1.

Analyte/Method	Iodine	Bromine	Photochemical
G_2	1.26	1.26	0.94
G_1	4.08	2.20	1.57
B_2	1.88	0.94	0.63
B_1	2.51	3.46	4.08

Table 2. Detection limits calculated as 3.14 times standard derivatization of peak area for 7 replicates.

While each derivitization method for aflatoxins has its advantages, incorporation into a mixed mycotoxin scheme presents complications.

Fumonisins require a post column addition of o-phthaldehyde—2-mercaptoethanol for derivitization. This reagent interferes with Iodine addition for aflatoxins. On the other hand, the KBr-HNO_3 required for electrochemical bromine generation introduce UV active species into the eluent which can diminish the

response of DON at 220 nm. This leaves photochemical UV as the suitable alternative for aflatoxin derivitization in a mixed mycotoxin analysis.

Other Mycotoxins

DON(deoxynivalenol), does not have fluorescence activity and hence the only means of detection is low wavelength UV, 220 nm. At this wavelength, many other organics will also be detected and requires sample preparation for optimal results. Use of photodiode array detection (PDA) will provide a UV spectra which can be compared against a spectral library for identification, and PDA peak purity calculations aids is assessing peak homogeneity.

Alfatoxins G2 and B2, ochratoxin-A and zearalenone are naturally fluorescent, whereas the alfatoxins G1 and B1 require pre-column derivatization as described earlier, and the fumonisins require an o-phthaldehyde post-column derivatization to make these analytes fluorescent. The fumonisins have a primary amine functionality that can be derivatized as shown in Figure 3.

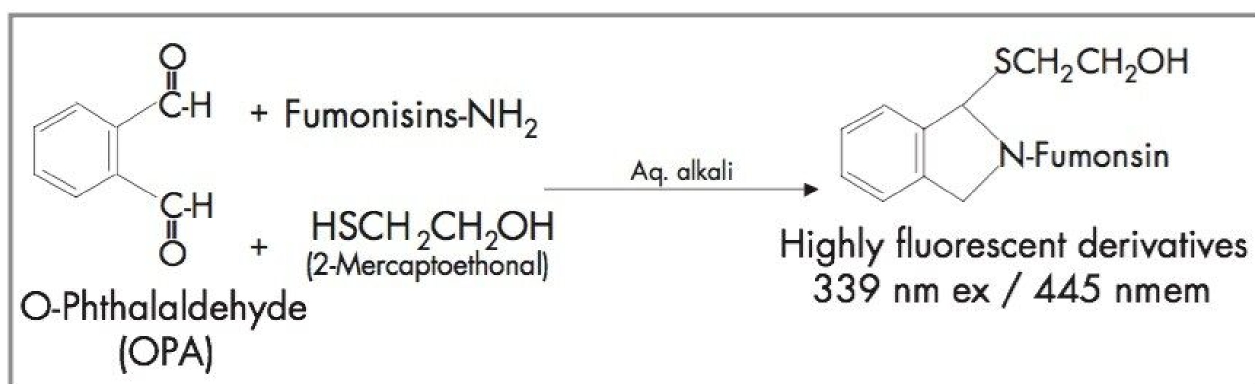

Figure 3. Post-column derivatization chemistry for any primary amine.

Results and Discussion

Through use of UV detection at 220 nm for DON, a PHRED unit (Photochemical Reactor for Enhanced Detection) for aflatoxins, and post column addition pump for fumonisins, the analysis for DON, the four aflatoxins G2, G1, B2, B1, three fumonisins B1, B2, and B3, zearalenone and ochratoxin-A has been condensed into a single 30 minute analysis, Figure 4.

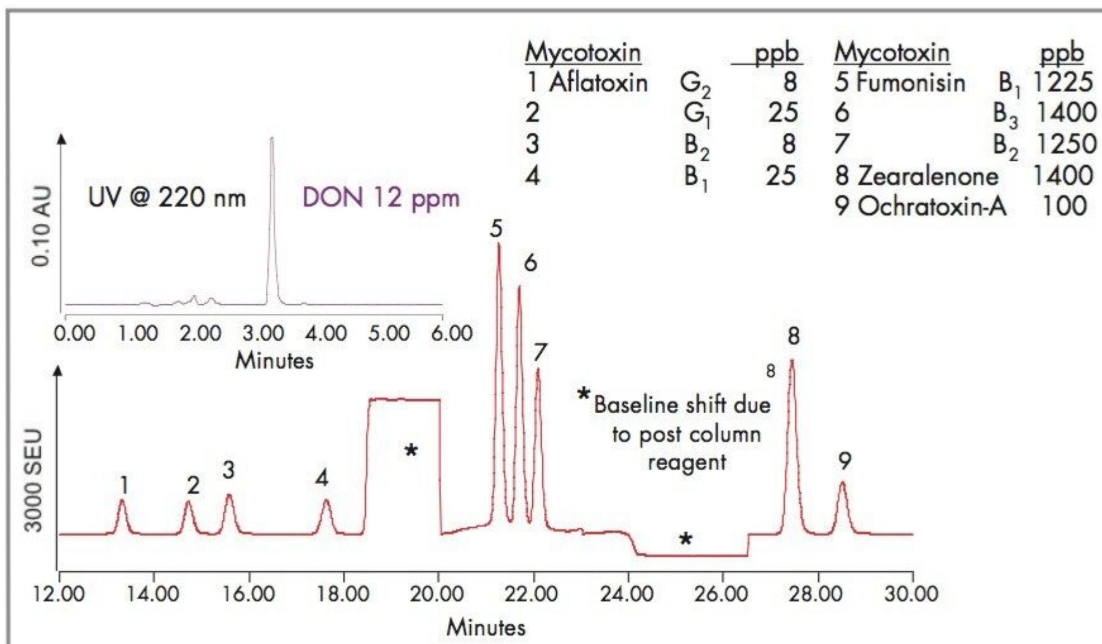

Figure 4. Chromatogram of multi-analyte mycotoxins.

This multi-analyte method uses a Waters Alliance HPLC System with a Post Column Reaction Module, integrating fluorescence detection for UV derivitized aflatoxins, post column derivitization for fumonisins, and natural fluorescence for zearalenone and ochratoxin-A. A Waters 2996 Photodiode Array Detector was placed in series before the post-column hardware for the simultaneous UV detection and confirmation of vomitoxin (DON).

The above approach requires the post column addition of OPA be initiated after the elution of aflatoxin B1 and terminated before the elution of zearalenone. This is accomplished using the system control capabilities of the Waters Alliance HPLC System and Empower Software. Reagent Manager flow is initiated at 18.5 minutes and stopped at 24.5 minutes. The 2475 Fluorescence Detector was timed programmed to change excitation and emission wavelengths for analyte response optimization.

This multi-analyte, multi-detection method shows excellent linearity within the typical calibration range for these analytes, shown in Figure 5. The detection limits, Table 3, are well below the analytes action limit suggesting that this approach is excellent for analyte screening.

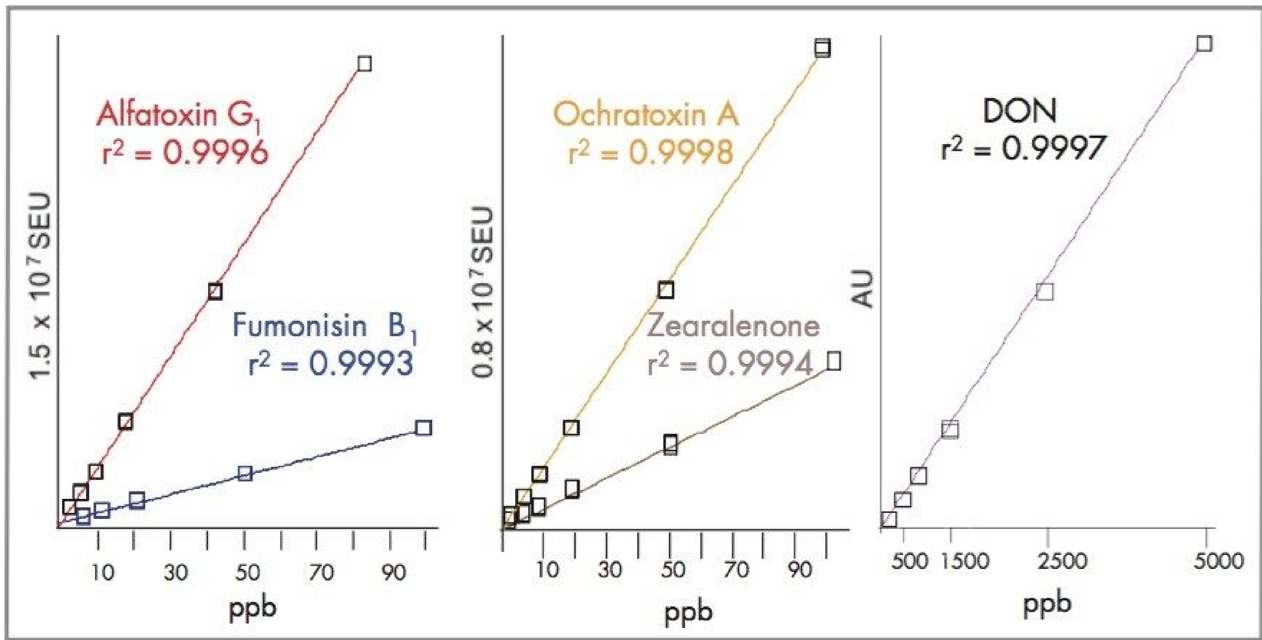

Figure 5. Multi-analyte, multi-detection linearity.

Method Detection Limits (ppb) with 50 µL Injection			
DON	5.76	Fumonisin B_1	2.0
Aflatoxin G_2	0.003	Fumonisin B_2	2.0
Aflatoxin G_1	0.004	Fumonisin B_3	2.0
Aflatoxin B_2	0.002	Zearalenone	0.56
Aflatoxin B_1	0.004	Ochratoxin- A	0.03

MDL calculated per EPA 40 CFR Ch 1 Pt 136 Appendix B

Table 3. Multi-analyte, multi-detection detection limits.

Sample Preparation

Sample preparation is strongly recommended when doing mycotoxin analysis. Vicam, a leader in immunoaffinity chemistries, markets several sample preparation kits, the AflaTest for aflatoxins, FumoniTest

for fumonisins, ZearalaTest, OchraTest and DON test HPLC. As a result of the trend to perform multi-analyte analysis, Vicam is developing a new test cartridge containing monoclonal antibodies for all the mycotoxins (DAFZO), and should be commercially available in early 2005.

Beer, spirits, and wine use various cereal grains and fruits as the substrate for the fermentation process. These agricultural raw materials are also the prime substrate for mold and bacterial growth and mycotoxin contamination. The Alcohol section of the Bureau of Alcohol and Tobacco Tax and Trade Bureau in Walnut Creek CA monitors alcoholic beverages for mycotoxins, and desires a multi-toxin method to maximize throughput and minimize costs. Dr. Darsa Siantar is collaborating with the chemists at Vicam and Waters to develop the appropriate sample prep for this analysis method. Figures 6 and 7 are representative chromatograms of beer and white wine respectively.

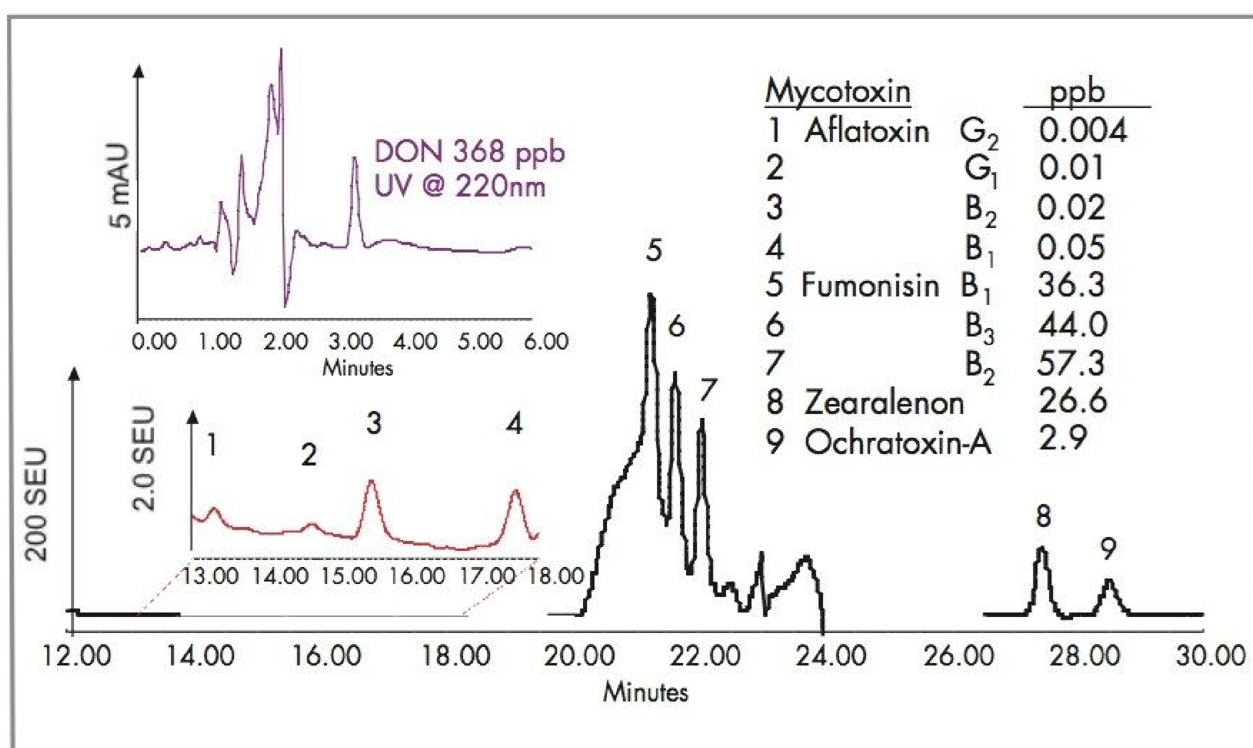

Figure 6. Chromatogram of multi-mycotoxins in spiked beer after Vicam DAFZO sample prep.

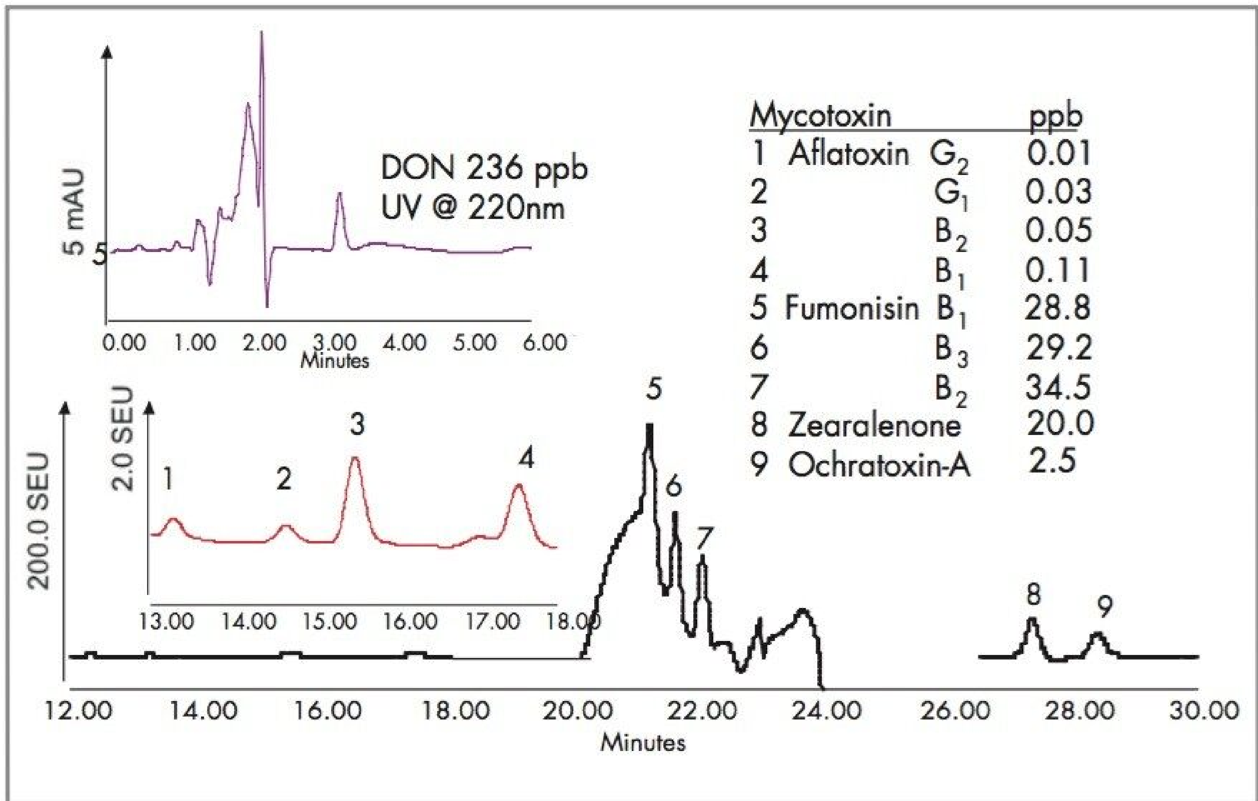

Figure 7. Chromatogram of multi-mycotoxins in spiked white wine after Vicam DAFZO sample prep.

Conclusion

DON, aflatoxins, fumonisins, zearalenone, and ochratoxin can now be analyzed in a single analysis. This method is not limited to these analytes, but can also be used to detect aflatoxins M1 and M2, or the metabolites of DON. This method was accomplished using the Waters Carbamate Analyzer which has been optimized for post column derivatization using OPA. Add to this a UV detector, preferably a photodiode array detector because of its spectral analysis capability, and several applications can be run utilizing the same HPLC System. With the system control of gradients, the on/off of the post column reagent pumps, simultaneous detection from two detectors, fluorometer auto zero with all changes in excitation and emission wavelength, and processing of all the data, the Waters Empower Software is the focal point of all HPLC work.

The key to this multi-analyte, multi-detector method is the sample preparation using the high selectivity

of Vicam's immuno-affinity cartridges. Without sample prep, numerous other matrix components will be detected by UV possibly compromising accuracy for DON, or other primary amines reacting with OPA and co-eluting with the fumonisins.

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