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A NOVEL APPROACH FOR MIXED MYCOTOXIN ANALYSIS

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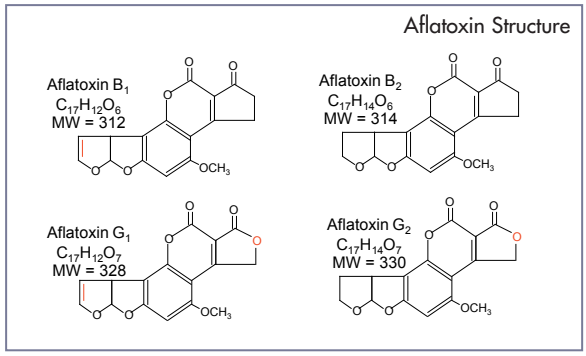
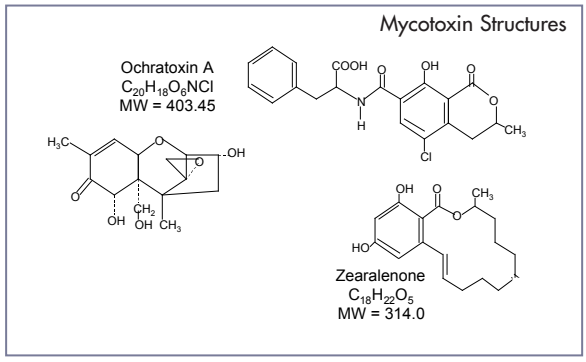
Introduction

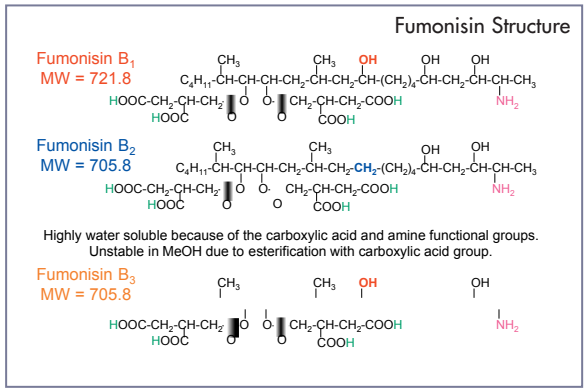
Agricultural products and food stuffs make an excellent substrate for the growth of mold, fungus and other microbiological forms. Aflatoxins are produced by several species of Aspergillis: A. flavus, A. parasiticus and A. nominus mold on peanuts and corn. Aspergillis ochraceus and Penicillium verrucosum molds yield 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; fumonisin B_1 and B_2, B_2 is the most abundant. Fumonisins often occur together with other the mycotoxins.

Because mycotoxins can potentially invade many of the primary raw food ingredients, affecting food safety, the analysis of these toxins in is critical. If not detected, consequences include liver cancer, kidney failure and intestinal distress, amongst their potential teratogenic and reproductive effects. To help avoid these unpleasant consequences, sensitive, validated LC analysis methods have been established. Recently, because of cost and expediency, it has become desirable to integrate several mycotoxins into a single analytical method.

This poster will describe a novel approach for analyzing these toxins using conventional photodiode array, fluorescence and post column derivatization fluorescence in two methods, and the future approach of single multi-analyte toxin method using mass spectrometry.







AOAC Aflatoxin Method: Post Column Derivatization and Fluorescence Detection

AOAC Method 991.31 uses a sample preparation utilizing an affinity SPE column and procedure specific for alfatoxins, manufactured by Vicam. Analysis is achieved using a reverse phase column and an isocratic MeOH/water mobile phase, followed by post column derivatization with saturated Iodine and fluorescence detection.

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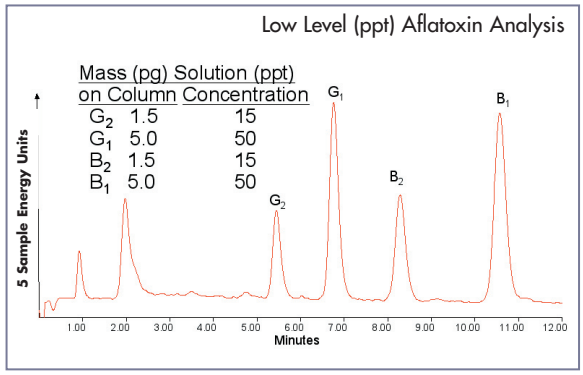
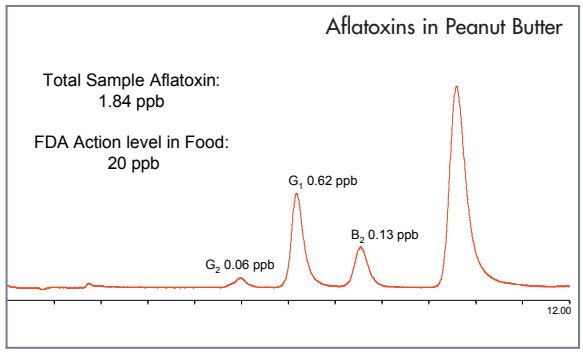
System:	Waters Alliance® System for Carbamate Analysis
Column:	Waters Symmetry® C18, 5 µm, 3.9 x 150 mm
Col Temp:	30° C
Mobile Phase:	40% MeOH/60% DI
Flow Rate:	1 mL/min
Inj Vol:	100 µL
Reagent Manager:	Aqueous Saturated Iodine (I_2) @ 0.5 mL/min @80° C
Detection:	Waters 2475 Multi-λ Fluorescence Detector Ex 365 nm, Em 455 nm





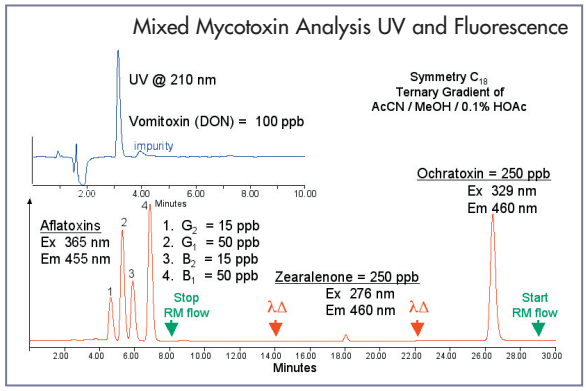
Multi-Analyte Mycotoxin Analysis

The Action Limits for these toxins are country dependant and are approximated:

	US	World
Total Aflatoxins	20 ppb	0-20 ppb
Ochratoxin	4 ppb	5-200 ppb
Zearalenone	---	60-1000 ppb
Vomitoxin (DON)	1000 ppb	500-750 ppb
Total Fumonisins	2000-4000 ppb	2-1000 ppb

A current trend in LC analysis is multi-analyte methods. With changes in mobile phase composition, it is possible to detect the four aflatoxins, ochratoxin, zearalenone and vomitoxin in a single analysis.

Based on work done at Vicam and from Dr. Roswitha Gobel, IVPT, Bernau, Germany, the following chromatogram can be obtained.



This method uses the Waters Alliance System for Carbamate Analysis, integrating post-column derivatization for aflatoxins, fluorescence detection for derivatized aflatoxin and natural fluorescence for zearalenone and ochratoxin. A Waters 2996 Photodiode Array (PDA) Detector was placed in series before the post-column hardware for the simultaneous UV detection and confirmation of vomitoxin (DON).

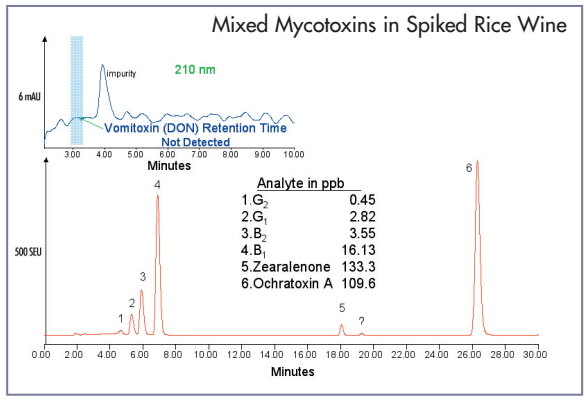
This approach requires that the post column I_2 aflatoxin reagent flow be stopped and flushed from the tubing before zearalenone elutes; otherwise, the natural zearalenone and ochatoxin fluorescence is quenched. This is accomplished using the system control capabilities of the Waters Alliance System and Empower™ Software. Reagent Manager (RM) flow is stopped at 8 minutes, with sufficient time allocated to flush the tubing prior to zearalenone elution. The 2475 Fluorescence Detector was timed and programmed to change excitation and emission wavelengths (Δλ) for analyte response optimization.

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.

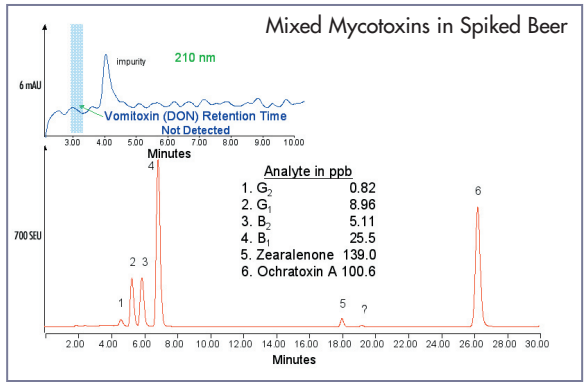
The Alcohol section of the U.S. Treasury Department of ATTTB in Walnut Creek, CA monitors alcoholic beverages for mycotoxins and desires a multi-toxin method to maximize throughput and minimize costs. Dr. Darsa Siantar is working with chemists at Vicam and Waters to develop the appropriate sample preparation and analysis method.

Dr. Siantar prepared two samples, rice wine and beer, using the following protocol:

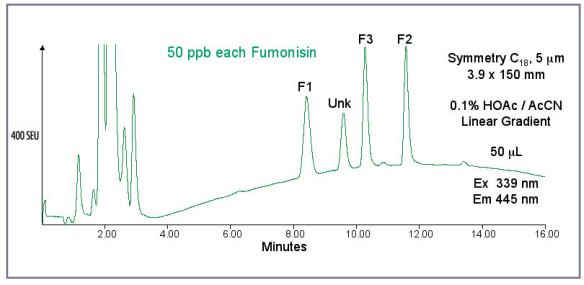
- Pre-treat the rice wine and beer to remove the alcohol
- Spike the samples with known amounts of mycotoxins
- Pass through a Vicam mixed bed immunoaffinity column
- Wash with DI water and air dry
- Elute mycotoxins with 100% MeOH
- Take to dryness under N_2
- Reconstitute in 50% AcCN/water



After Vicam mixed mode Immunoaffinity column



After Vicam mixed mode Immunoaffinity column



This approach works well for these mycotoxins, but not for the fumonisin mycotoxins. They have a primary amine group that can be derivatized post-column with o-OPA reagent (o-Phthaldehyde) to form a fluorescent analyte. Employ a reverse-phase gradient to resolve the three fumonisins, and use fluorescence detection for specificity and sensitivity.

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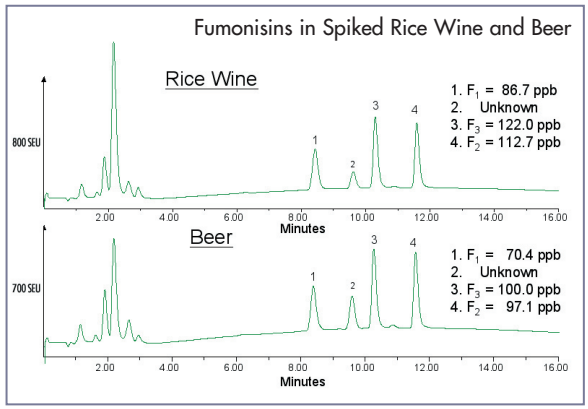

After Vicam Mixed mode Immunoaffinity column

The same spiked rice wine and beer sample were analyzed for fumonisins. The Carbamate Analysis System has two Reagent Manager capabilities, the aflatoxin RM is off and the OPA RM is on. The new fumonisin gradient method, saved in a Waters Empower Software project using the AcCN and 0.1% acetic acid and fluorescence wavelength changes, are entered into the Waters Alliance System. The system is equilibrated and samples are run. (This scenario can be pre-programmed using Empower software.)

Summary

This presentation demonstrates the feasibility of doing a multi-toxin analysis for the four aflatoxins, ochratoxin, zearalenone, vomitoxin (DON) and the three fumonisins. Although this currently requires two methods, both using similar mobile phases and post-column derivatization techniques, the Alliance System for Carbamate Analysis can be set-up to run both methods on the same column.

After sample preparation with the Vicam mixed bed immunoaffinity column, samples can be analyzed using the first method, re-programmed, equilibrated and the entire batch re-analyzed using the second method. Having a PDA detector in series with any detection technique provides additional detector selectivity, such as shown with vomitoxin (DON) analysis at 210 nm.

Single quadrupole, positive electrospray scanning Mass Spectrometry was also used to analyze the same standards and samples. The preliminary data, not shown, suggests that a single, multi-toxin method for all the toxins is achievable using a reverse phase column and binary of 0.1% acetic acid and acetonitrile gradient.

As with all food and beverages samples, sample preparation is critical to overall performance of any LC method. Development of mixed mode affinity columns specific for the toxins in conjunction with LC/MS or LC/MS/MS demonstrates the future trend in multi-analyte methods.

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