# A Unified Method for Mixed Mycotoxin Analysis Using UV and Fluorescence Detection

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## Introduction:

Agricultural products and foodstuffs 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 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, fumonisin $B_1$, $B_2$ being the most abundant. Fumonisins often occur together with the other mycotoxins.

Because mycotoxins can potential invade many of the primary raw food ingredients, affecting food safety, the analysis of these toxins in raw food stuffs is critical. If not detected, consequences include liver cancer, kidney failure, intestinal distress, teratogenic and reproductive effects.
The range limits for these toxins are jurisdiction dependant:

| | 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 | 2-4 ppm | 2- 1000 ppb |

To help avoid these unpleasant consequences, sensitive, validated LC methods have been established for their analysis. Recently, for cost and expediency, it is desirable to integrate several mycotoxins into a single analytical method. This poster will describe a novel unified approach to analyze six families of mycotoxins in a single analysis using UV for DON, PHRED for the aflatoxins, OPA derivitization of the fumonisins and natural fluorescence for ochratoxin A and zearalenone

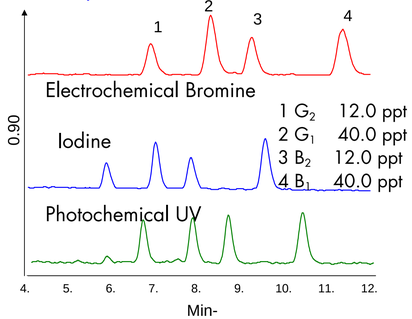
## Derivatization of Aflatoxins

Of the many derivitization methods for aflatoxins found in the literature, three tend to stand out; iodine which is the basis of the official AOAC method, electrochemically generated bromine ( e.g. Kobra Cell), and photochemical UV. Analysis has been

System: Waters Alliance® System for Carbamate Analysis
Column: Waters Symmetry® $C_{18}$, 3.5 mm, 4.6 x 150 mm
Col Temp: 30°C
Mobile Phase: 0.1 % $H_3PO_4$ / Methanol / Acetonitrile ternary gradient
Flow Rate: 1.0 mL / min
Inj Vol: 50 µL
Detection: Waters 2475 Multi-l Fluorescence Detector Ex- 365 nm, Em- 455 nm

1 Reagent Manager: Aqueous Saturated Iodine ($I_2$) @ 0.5 mL / min @ 80° C
2 Kobra Cell: ( Rhone Diagnostics Ltd ) Electrochemically generated bromine @ 100 µa
Eluent for Kobra Cell is 60% water, 25% methanol, and 15 % acetonitrile to which

## Comparison of Aflatoxin Derivitization Methods Limit of Detection*




1 $G_2$ 12.0 ppt
2 $G_1$ 40.0 ppt
3 $B_2$ 12.0 ppt
4 $B_1$ 40.0 ppt

MDL(ppt)* = Std $Dev_{PA}$ X 3.14

| Method | $I_2$ | $Br_2$ | UV |
|---|---|---|---|
| $G_2$ | 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 |

*MDL calculated using EPA protocols

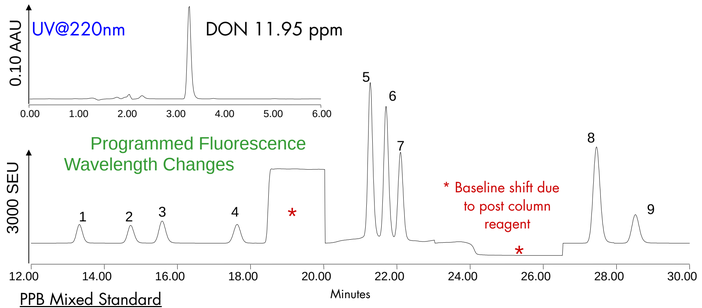
While each derivitization method for aflatoxins has its advantages, incorporation into a mixed mycotoxin scheme presents complications. Fumonisins require a post column addition of o-phthdialdehyde -2-mercaptoethanol for derivitization. This reagent inteferes with Iodine addition for aflatoxins. On the other hand, the KBr / $HNO_3$ required for electrochemical bromine generation introduce UV active species into the mobile phase which can diminish the response for DON at 220 nm. This leaves photochemical UV as a suitable alternative for aflatoxin derivitization for a mixed mycotoxin analysis.

## The Unified Method For Mixed Mycotoxin Analysis

Through use of UV detection for DON, a PHRED unit, Photochemical Reactor for Enhanced Detection for aflatoxins, and a programmable post column reagent pump for OPA -2-mercaptoethanol addition for fumonisins, we have been able to consolidate DON, the four aflatoxins $G_2$, $G_1$, $B_2$, $B_1$, three fumonisins $B_1$, $B_2$, and $B_3$, zearalenone, and ochratoxin-A into a single analysis.

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 and terminated after the elution of aflatoxin $B_1$ but before the elution of zearalenone respectively. This is accomplished using the system control capabilities of the Waters Alliance HPLC System and Empower™ Software. Reagent Manager flow is initiated at 17.5 minutes and stopped at 22.5 minutes. The 2475 Fluorescence Detector was timed programmed to change excitation and emission wavelengths for analyte response optimization.




PPB Mixed Standard

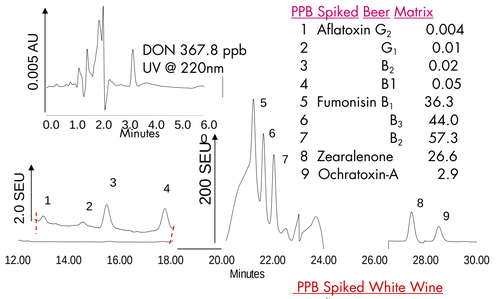
| | | |
|---|---|---|
| 1 Aflatoxin | $G_2$ | 7.7 |
| 2 | $G_1$ | 26.9 |
| 3 | $B_2$ | 8.4 |
| 4 | $B_1$ | 24.9 |
| 5 Fumonisin | $B_1$ | 1225 |
| 6 | $B_3$ | 1400 |
| 7 | $B_2$ | 250 |
| 8 Zearalenone | | 1381 |
| 9 Ochratoxin A | | 101.8 |

## MDL for Mixed Mycotoxin Method ( ppb )

| | | | |
|---|---|---|---|
| Don | 5.76 | $FB_1$ | 2.0 |
| $AG_2$ | 0.03 | $FB_2$ | 2.0 |
| $AG_1$ | 0.04 | $FB_3$ | 2.0 |
| $AB_2$ | 0.02 | Zearalenone | 0.56 |
| $AB_1$ | 0.04 | Ochratoxin-A | 0.03 |

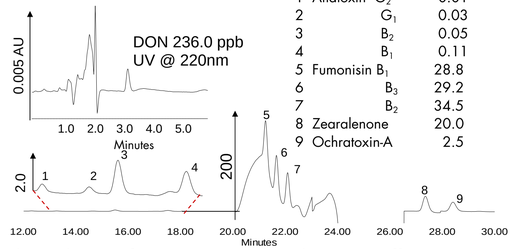
## Mycotoxins in Beer and Wine Matrix

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 desire 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 mixed mycotoxin immunoaffinity cartridge sample prep for this analysis method. The immunoaffinity column is a mixed bed resin for DON, aflatoxin, fumonisin, zearalenone, ochratoxin- A ,( DAFZO ) under development by VICAM.




PPB Spiked Beer Matrix

| | | |
|---|---|---|
| 1 | Aflatoxin $G_2$ | 0.004 |
| 2 | $G_1$ | 0.01 |
| 3 | $B_2$ | 0.02 |
| 4 | B1 | 0.05 |
| 5 | Fumonisin $B_1$ | 36.3 |
| 6 | $B_3$ | 44.0 |
| 7 | $B_2$ | 57.3 |
| 8 | Zearalenone | 26.6 |
| 9 | Ochratoxin-A | 2.9 |




PPB Spiked White Wine

| | | |
|---|---|---|
| 1 | Aflatoxin $G_2$ | 0.01 |
| 2 | $G_1$ | 0.03 |
| 3 | $B_2$ | 0.05 |
| 4 | $B_1$ | 0.11 |
| 5 | Fumonisin $B_1$ | 28.8 |
| 6 | $B_3$ | 29.2 |
| 7 | $B_2$ | 34.5 |
| 8 | Zearalenone | 20.0 |
| 9 | Ochratoxin-A | 2.5 |

Through use of Vicam's Mixed Bed Immunoaffinty Column, a variety of sample matrices can be prepared to provide reliable results at low limits of detection.