# Identification of Individual Mycotoxin Threat Agents from Mycotoxin Mixtures Using Nuclear Magnetic Resonance, Mass Spectroscopy, and Chemometrics

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# Report Documentation Page

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| **16 NOV 2004** | **N/A** | **-** |

| 4. TITLE AND SUBTITLE | 5a. CONTRACT NUMBER |
|---|---|
| **Identification of Individual Mycotoxin Threat Agents from Mycotoxin Mixtures Using Nuclear Magnetic Resonance, Mass Spectroscopy, and Chemometrics** | 5b. GRANT NUMBER |
| | 5c. PROGRAM ELEMENT NUMBER |
| 6. AUTHOR(S) | 5d. PROJECT NUMBER |
| | 5e. TASK NUMBER |
| | 5f. WORK UNIT NUMBER |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U. S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD 21010-5424** | 8. PERFORMING ORGANIZATION REPORT NUMBER |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) | 10. SPONSOR/MONITOR'S ACRONYM(S) |
| | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) |

12. DISTRIBUTION/AVAILABILITY STATEMENT
**Approved for public release, distribution unlimited**

13. SUPPLEMENTARY NOTES
**See also ADM001849, 2004 Scientific Conference on Chemical and Biological Defense Research. Held in Hunt Valley, Maryland on 15-17 November 2004., The original document contains color images.**

14. ABSTRACT

15. SUBJECT TERMS

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | |
| **unclassified** | **unclassified** | **unclassified** | **UU** | **19** | |

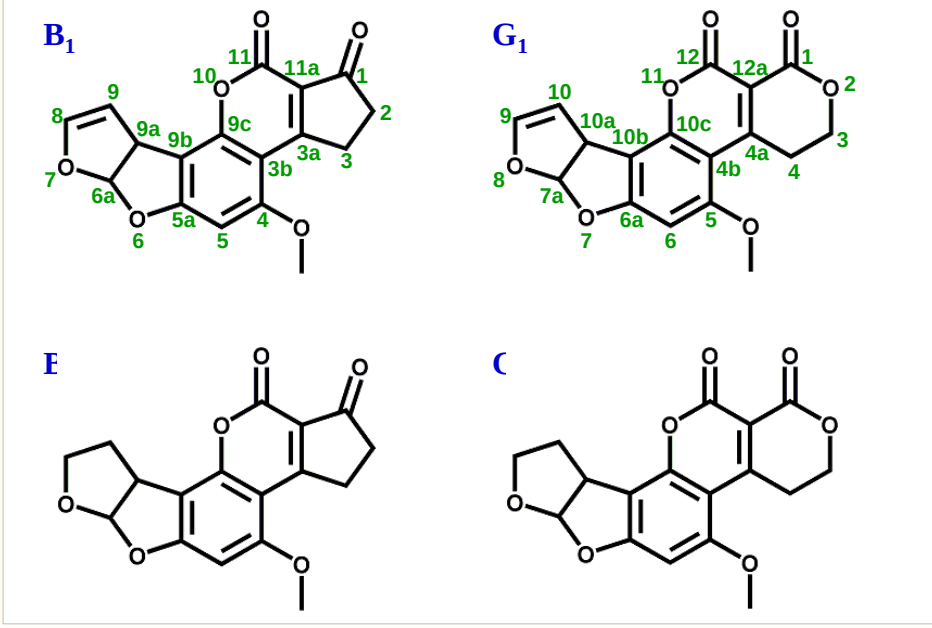
# ABSTRACT

**The U. S. government still claims that the Soviet Union and its allies in Laos and Cambodia used a mixture of mycotoxins to engage in toxin warfare from 1975 to 1984. While the government's case for toxin warfare is suspect [Tucker, J. B. (2001) "The Nonproliferation Review"], mycotoxin mixtures are potent enough to be considered threat agents. Therefore, the need exists for the capability to identify individual toxins from mixtures. We are investigating techniques to accurately and efficiently identify specific mycotoxins from mixtures of mycotoxins using NMR, MS, and chemometrics. We used LC-MS and 2D NMR to identify aflatoxins G1, G2, B1 and B2 in mixtures based on fingerprint spectral regions. Either method is reasonably efficient for mixtures containing few components. However, the higher the structural similarity between toxins and/or the increased number of components in a mixture, the more cumbersome the task becomes. We explored chemometrics as a means to overcome this inherent difficulty by carrying out a preliminary analysis using chemometrics in combination with 1D $^{1}$H NMR. Chemometrics allowed accurate identification of individual aflatoxins G1, G2 and B2 from a mixture of the three without requiring 2D experiments. Also, for samples containing a contaminant (with similar molecular weight to aflatoxins), the analysis flagged these samples as containing an "unknown" component. This work shows that chemometrics combined with NMR and/or MS is promising as a robust solution to the identification of threat toxins from complex mixtures.**

# BACKGROUND

**Mycotoxins are produced naturally by certain fungi species during the spoiling of food stuffs. Aflatoxins in particular are produced mainly by *Aspergillus* molds. They are highly toxic and carcinogenic.**

**Figure 1. Structures of aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$.**



# PURPOSES

1. **Identify individual mycotoxins out of mixtures solely by NMR or MS techniques.**
2. **Identify individual mycotoxins out of mixtures of mycotoxins by a combination of NMR and MS techniques.**
3. **Identify individual mycotoxins from mycotoxin mixtures by chemometric techniques using NMR and/or MS spectra as input.**

# MATERIALS and METHODS

**NMR Sample Preparation:** Between 9 and 10 mg of individual aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ were dissolved in 1.0 mL of $CDCl_3$ and added to separate NMR sample tubes. The mixture of $B_2$, $G_1$ and $G_2$ aflatoxins was created by combining 0.3 mL aliquots from the 1.0 ml individual aflatoxin solutions. This tube was inverted to mix and the roughly equal weight mixture was transferred to a new NMR sample tube. 1.5 mg of Benzo[a]pyrene in 0.1 ml of $CDCl_3$ was added to the mixture of $B_2$, $G_1$ and $G_2$ aflatoxins described in the above text.

**MS Sample Preparation:** Between 0.1 and 1.0 mg of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ were weighed out and dissolved in 50% $CH_3CN/H_2O$ to produce 1 mg/ml solutions of individual aflatoxins. Equal volumes of these 1 mg/ml individual aflatoxin solutions were combined to produce a sample containing roughly equal weights of $B_1$, $B_2$, $G_1$ and $G_2$ aflatoxins in one sample.

**NMR Experiments:** NMR experiments were carried out on a Varian UNITY INOVA 600 MHz spectrometer at a regulated temperature of 298 K. All pulse sequences used in this study were supplied by the manufacturer. For 2D experiments, 2048 complex points were collected in the directly detected dimension and 512 points were collected in the indirect dimension.

**MS Experiments:** MS experiments were carried out on a Finnigan TSQ-Quantum MS spectrometer. The instrument was set up to select for masses in the range of 50 to 550 mass units. The liquid chromatography (LC) portion of the LC-MS experiment on the aflatoxin mixture used a Thermo $C_{18}$ column from Hypersil-Keystone, aqueous 0.1 M ammonium acetate as mobile phase A and $CH_3CN$ as mobile phase B. The LC gradient went from 0% B to 30% B in 25 minutes followed by a step of 30% B to 100% B in 15 minutes.

**Chemometric Analyses:** Partial Least Squares Regression (PLS) and Principal Component Analysis (PCA) were carried out using The Unscrambler version 8.0 (Camo Process AS, Oslo, Norway, 2003). The data here was not segmented ("binned," "bucketed") because the software does not require it. PLS and PCA were carried out using leverage correction and full cross validation as validation methods, respectively.

**Materials:** Aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ and Benzo[a]pyrene were purchased from Sigma-Aldrich.

# RESULTS

**Figure 2. 1D $^{1}H$ NMR spectrum of aflatoxin $G_2$. From this experiment three proton groups can be assigned unambiguously. $H_{7a}$ is the only proton expected to give a doublet in the spectrum and the integral value of ~1 is as expected for the $H_{7a}$ peak. $H_6$ and the OMe group are the only singlets. The $H_6$ peak integral value is ~1 and the OMe group's peak integral value is ~3. 2D COSY (Fig. 3), HMQC, and HMBC spectra were used to complete the proton assignments.**

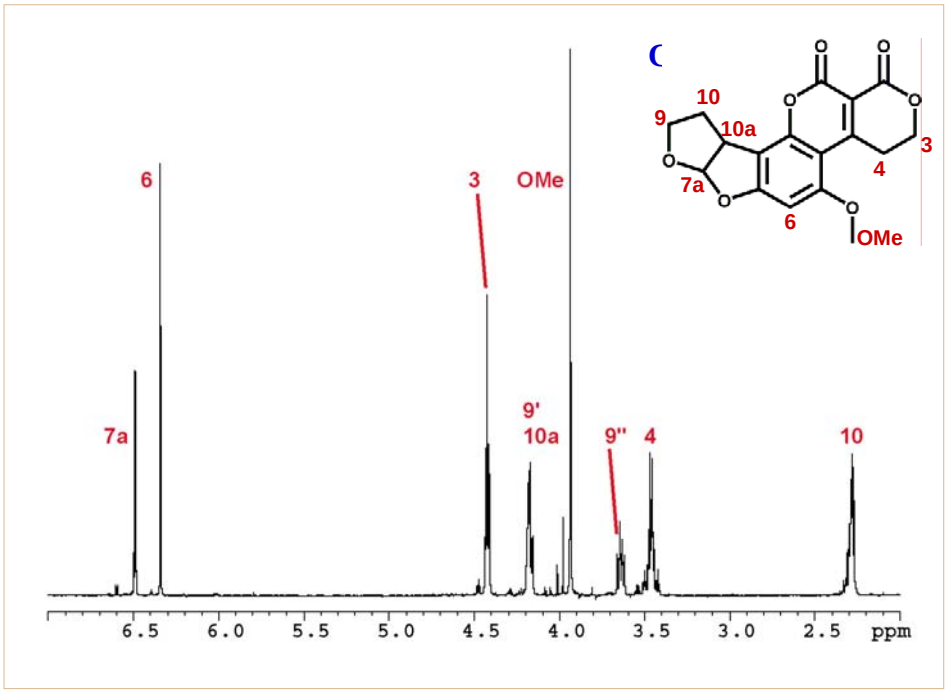


**Figure 3. 2D COSY NMR spectrum of aflatoxin $G_2$. $H_{10a}$ can be identified by its connection to $H_{7a}$, which was previously identified in the 1D $^1$H NMR spectrum. Other $^1$H assignments were carried out in a similar fashion.**

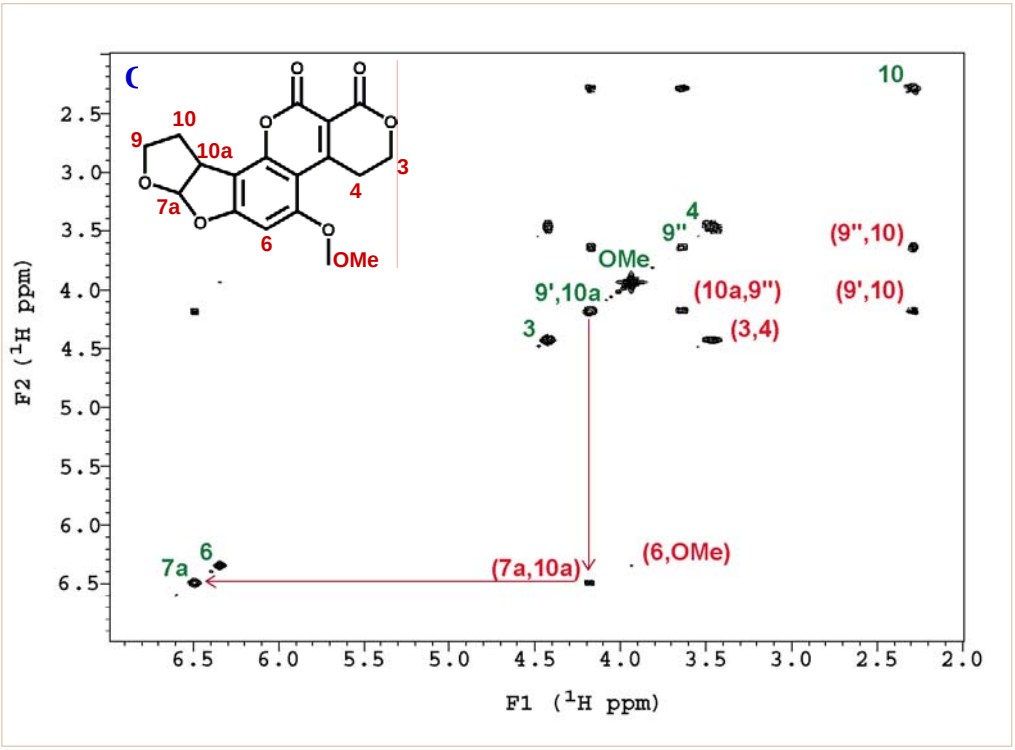


**Figure 4. 1D $^{13}C$ NMR spectrum of aflatoxin $G_2$. The number of peaks matches the expected 17 carbons. Assignment of the carbon chemical shifts (labeled here) required the HMQC (Figure 5) and HMBC (Figure 6) spectra.**

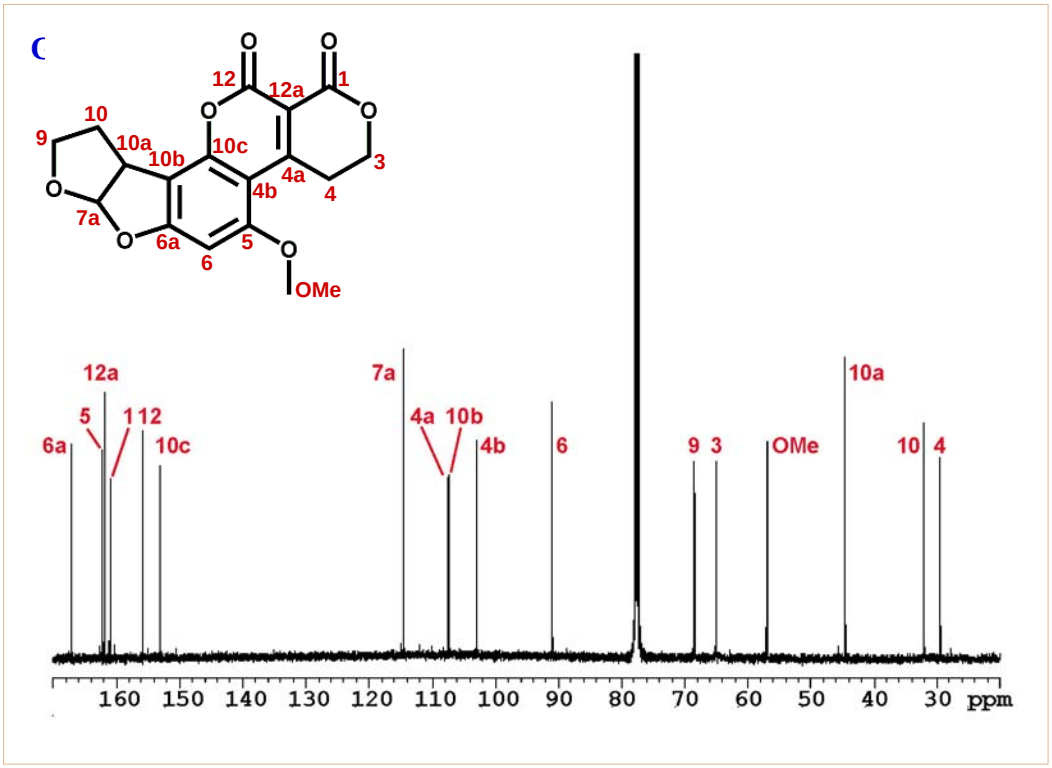


**Figure 5. 2D $^{1}H$-$^{13}C$ HMQC spectrum of aflatoxin $G_2$. The chemical shift assignments for carbons bonded to protons are labeled. Carbons that lack directly attached protons are assigned from the HMBC spectrum (Figure 6).**

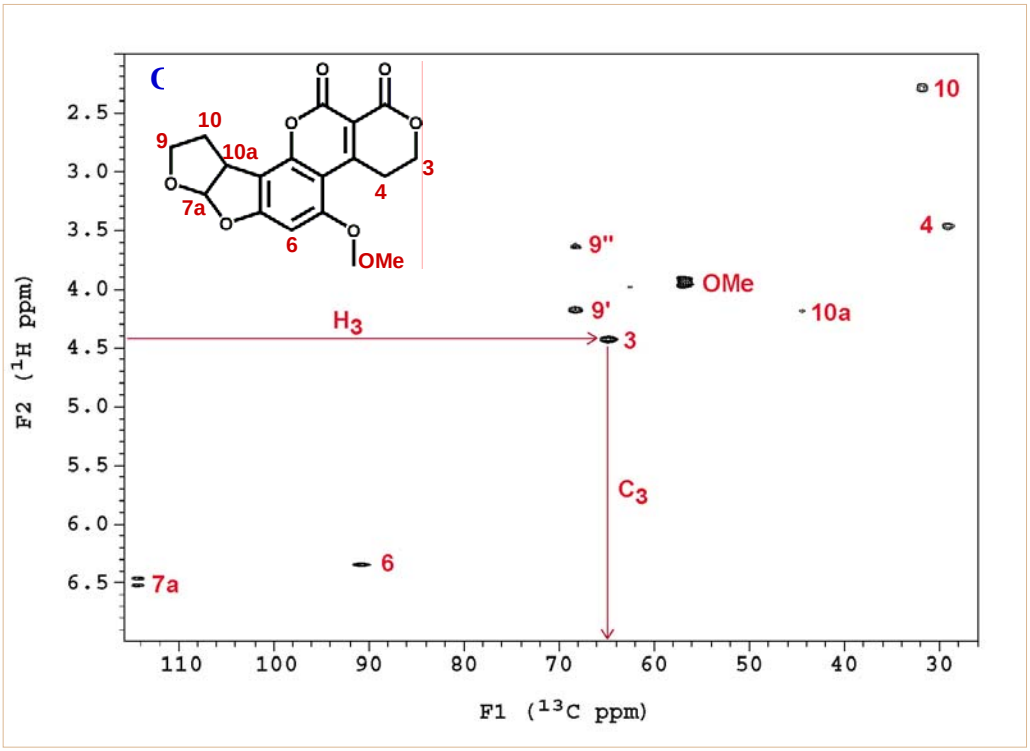


**Figure 6. A portion of the 2D $^{1}H$-$^{13}C$ HMBC spectrum of aflatoxin $G_2$. The HMBC allowed assignment of chemical shifts to those carbons that lack directly attached protons. In the portion of the HMBC shown here, proton 6 connects to carbons 6a and 5 (2 bonds away), carbons 4b and 10b (3 bonds away), and carbon 10c (4 bonds away).**

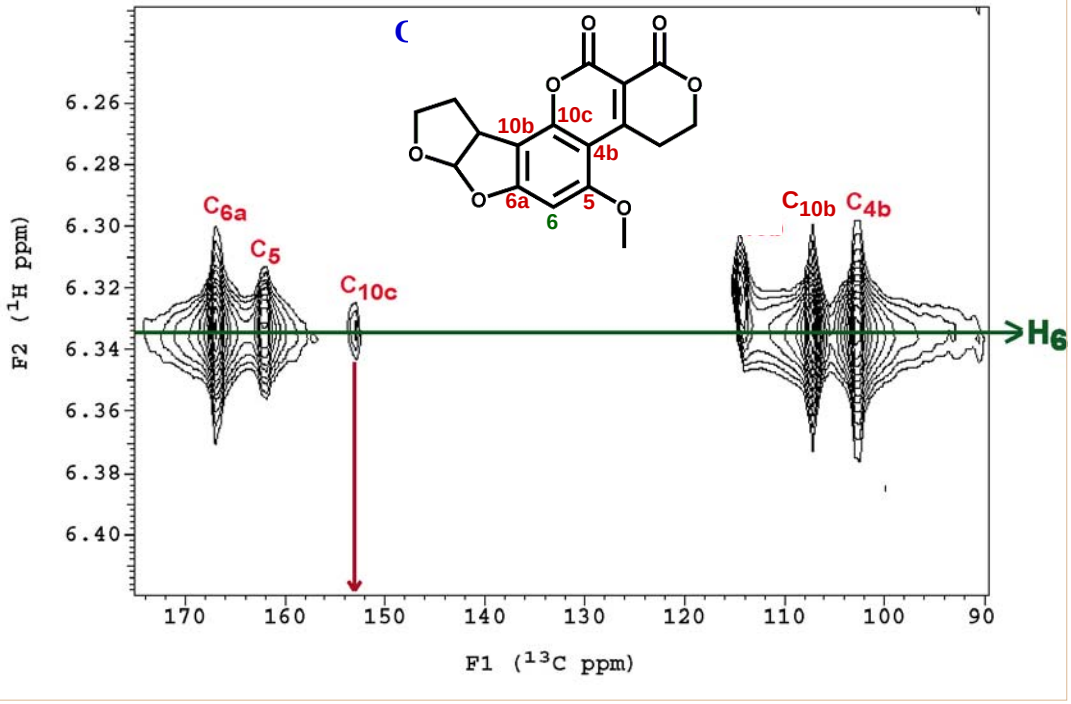


**Figure 7. 1D $^{1}H$ spectrum of a mixture of aflatoxins $B_2$, $G_1$, and $G_2$. Using the $^{1}H$ chemical shift table above and this spectrum, type 1 ($B_1$ + $G_1$), type 2 ($B_2$ + $G_2$) and B and G type aflatoxins can be distinguished. The spectrum is lacking peaks that will unambiguously identify the individual toxins if the content of the mixture was unknown before doing the experiment.**

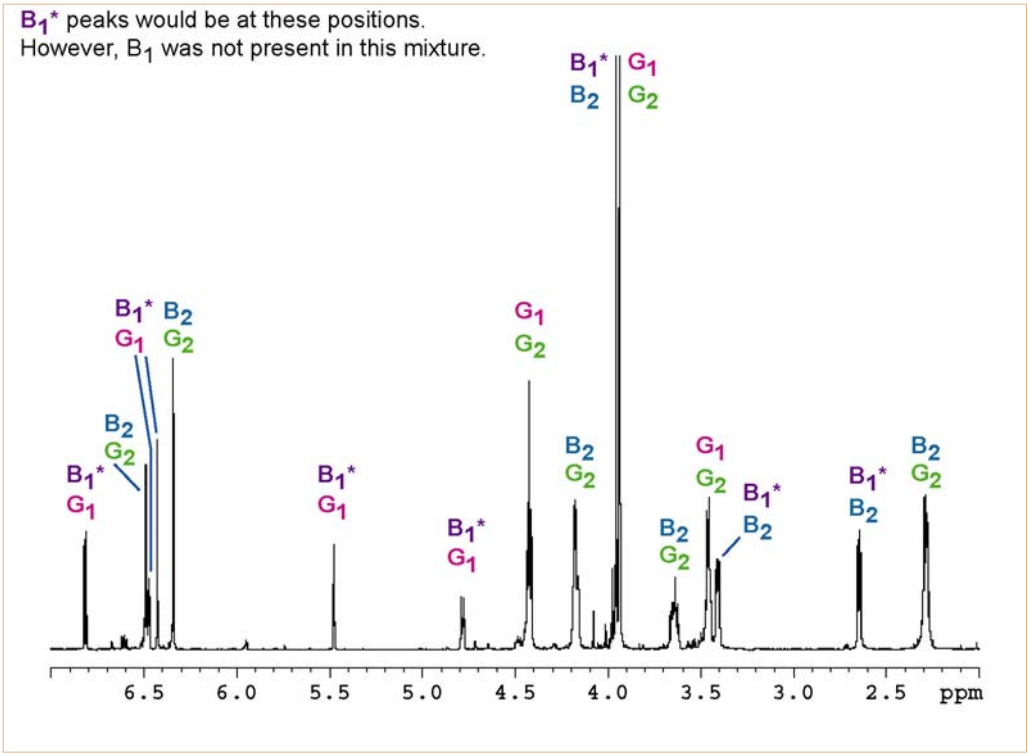


**Figure 8. 1D $^{13}C$ spectrum of a mixture of aflatoxins $B_2$, $G_1$, and $G_2$. Using the $^{13}C$ chemical shift table above and this spectrum, type 1 ($B_1$ + $G_1$), type 2 ($B_2$ + $G_2$) and B and G type aflatoxins can be identified. A few representative type assignments are shown. Individual aflatoxins $B_2$, $G_1$, and $G_2$ can be identified by their 5a/6a carbon chemical shift positions. The fact that aflatoxin $B_1$ is missing from this mixture is evidenced by the absence of its $C_{5a}$ peak.**

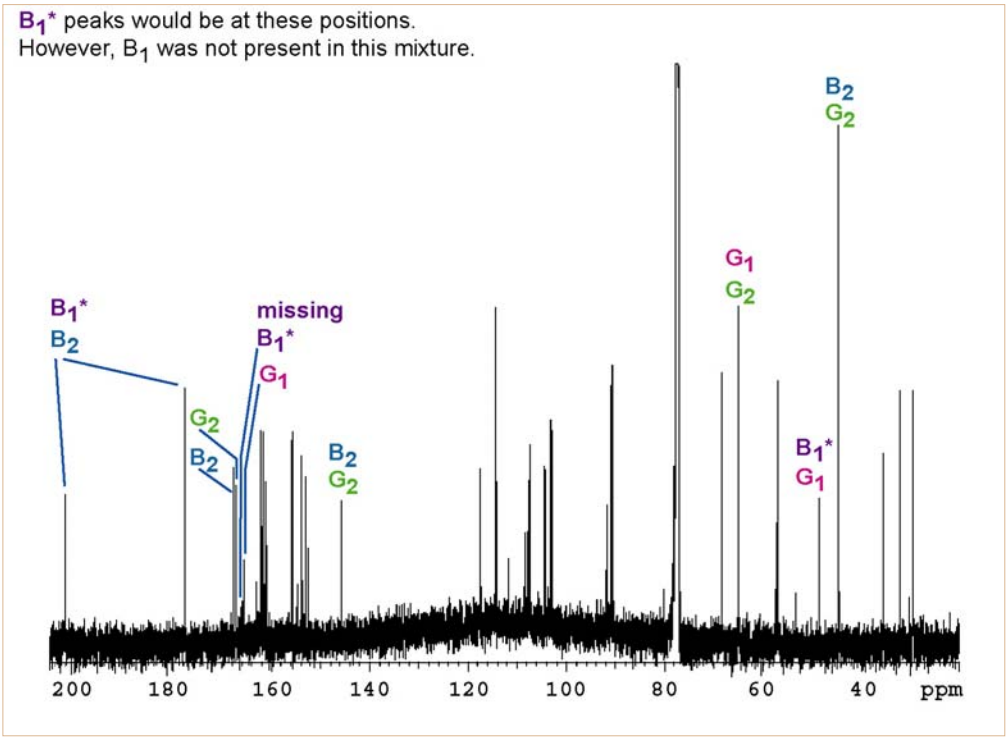


**Figure 9. A portion of the 2D $^{1}H$-$^{13}C$ HMBC spectrum of a mixture of aflatoxins $B_2$, $G_1$, and $G_2$. 2D NMR peaks for the interaction between the 5/6 protons and 5a/6a carbons are present for aflatoxins $B_2$, $G_1$, and $G_2$ and absent for B1.**

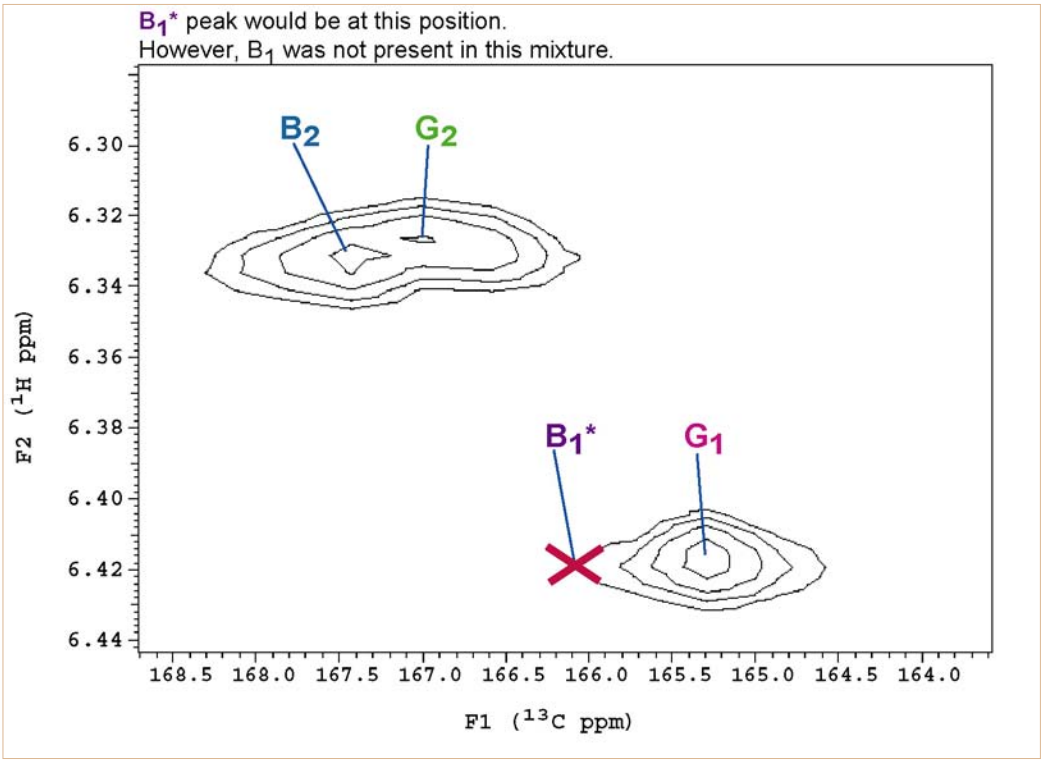
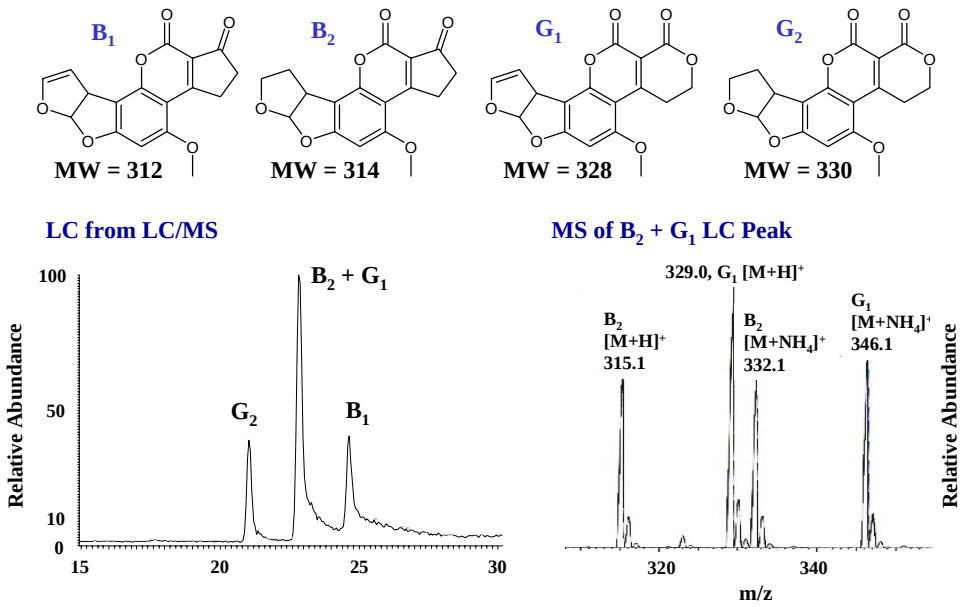


**Figure 10. LC-MS spectrum of a mixture of aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$. All of the individual aflatoxins in this mixture were identified by LC-MS**




**B2 and G1 coelute on LC, but can easily be separated by MS due to 14 g MW difference.**

## Preliminary Chemometrics

**Identification, quantitation of aflatoxins in a mixture:** PLS employed spectral data (X variables) and toxin concentrations (mM, Y variables) to prepare a calibration model based on five well-characterized samples on hand. Four contained single toxins. The fifth was a mixture of $B_2$, $G_1$ and $G_2$ (Table III). The model was calculated with 4 principal components (PCs) using leverage correction. Aflatoxin concentrations in a 6$^{th}$ sample were predicted. Even with this minimally-defined model, the concentrations for $B_2$, $G_1$ and $G_2$ are predicted within 2 mM (Table IV). The predicted value is negative for B1 (not present in sample).

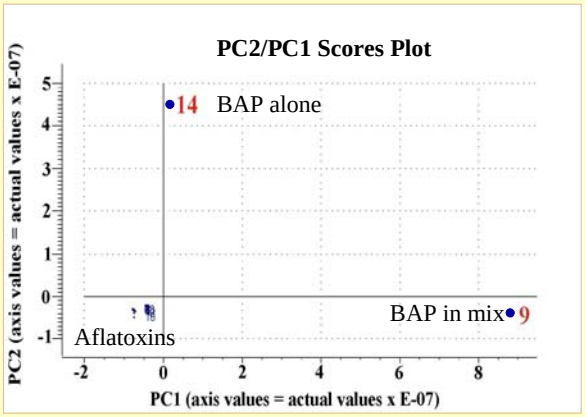
**Table III. Calibration Model Samples**

| Sample | Toxin Conc. (mM) | | | |
|---|---|---|---|---|
| # | $B_1$ | $B_2$ | $G_1$ | $G_2$ |
| 1 | 39.6 | 0 | 0 | 0 |
| 2 | 0 | 31.2 | 0 | 0 |
| 3 | 0 | 0 | 29.2 | 0 |
| 4 | 0 | 0 | 0 | 27.2 |
| 5 | 0 | 10.4 | 9.8 | 9.1 |

**Table IV. Sample 6 Conc. Prediction**

| Toxin | Predicted Conc. (mM) | Expt'al Conc. (mM) |
|---|---|---|
| B1 | -4 | 0 |
| B2 | 13 | 15 |
| G1 | 7 | 6 |
| G2 | 12 | 13 |

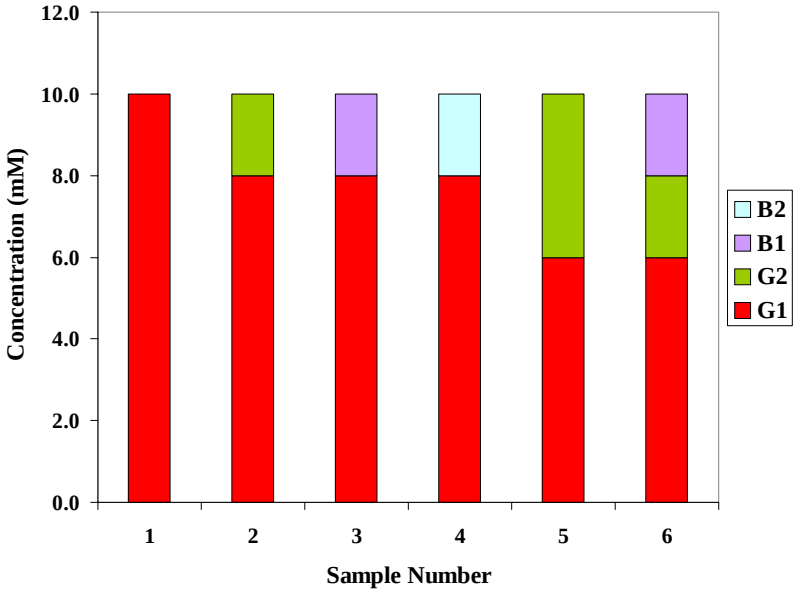
**Figure 11. Scores Plot from PCA involving 14 Samples. PCA readily distinguishes samples containing a possible background compound, Benzo-[a]pyrene (BAP), from those that do not, even when a sample contains both aflatoxins and BAP. Of the 14 samples, 10 contained 1-3 aflatoxins, one contained BAP alone, one contained 3 aflatoxins ($B_2$, $G_1$, & $G_2$) plus BAP, and two contained only $CDCl_3$. PCA was carried out on all spectral regions *other than* toxin peak regions with a weighting of 1.0 for each chemical shift intensity.**



## Training Set Model

**A training set was modeled as a mixture design using The Unscrambler software. The model is a simplex-lattice design built according to the D-optimal principle that includes 4 mixture components ($G_1$, $G_2$, $B_1$ & $B_2$), with each component ranging from 0 to 10 mM. The model was calculated to take interactions between the component spectra into account. The resulting design calls for 59 samples, with one replicate for each of the various mixtures and 3 replicates of the center sample. Experiments will be carried out using a modification of this design with some of the zero concentration points replaced by detection limit concentration values.**

**Figure 12. Aflatoxin concentrations for the first six samples of the chemometric training set model.**



# CONCLUSIONS

1. **NMR and MS can be employed to identify individual toxins out of a mixture of toxins having similar structures.**
2. **Type 1 aflatoxins (vinyl protons) vs. type 2 aflatoxins (no vinyl protons) and B vs. G type aflatoxins may be distinguished by 1D $^{1}H$ or $^{13}C$ NMR .**
3. **Individual $B_2$, $G_1$ and $G_2$ aflatoxins in a mixture and the absence of $B_1$ in the mixture studied here may be distinguished by either 1D $^{13}C$ NMR or 2D $^{1}H$-$^{13}C$ HMBC.**
4. **LC/MS is able to distinguish the individual $B_1$, $B_2$, $G_1$ and $G_2$ aflatoxins from a mixture of all four.**
5. **Preliminary results indicate that chemometrics can be employed with $^{1}H$ 1D data to: a) identify and quantify aflatoxins in mixtures, b) readily flag samples that contain components other-than or in-addition-to aflatoxins for further analysis.**

## FUTURE STUDIES

1. **Prepare a complete training set and apply chemometrics to $^{1}H$ and $^{13}C$ 1D NMR spectra of mycotoxin mixtures for more accurate and efficient identification and quantification of individual toxins.**
2. **Use NMR and MS to investigate additional mycotoxins including trichothecenes, saxitoxins, and brevetoxins individually and in mixtures.**

# REFERENCE and ACKNOWLEDGEMENT

1. **Tucker, J. B. (2001)** ***The Nonproliferation Review.***

**Dr. Carol S. Brevett for discussions regarding PCA and The Unscrambler.**