Final Report

on

ASSESSMENT OF OCCUPATIONAL EXPOSURE TO AFLATOXIN

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I. INTRODUCTION

The broad objective of this project was to develop a better means to assess the exposures of farmers to aflatoxin B_1 in airborne grain dust. We believe repeated low level exposures during various on-farm grain handling activities represents a significant health risk to farmers and potentially to full-time grain handlers.

The project was initially designed in two phases. The first funded phase (Phase I), involved laboratory method development and optimization for the analysis of aflatoxin B_1 and other toxins (particularly fumonisin B_1) in small airborne grain dust samples. The later portion of Phase I involved pilot-testing the analysis of field samples of bulk corn and airborne dust to establish a correlation between the level of aflatoxin in the dust from these processed bulk corn samples and simultaneously collected air samples. This preliminary data comparing the aflatoxin in bulk and airborne dusts was generated in a small cross-sectional survey of farming operations in Iowa. Surveys of other states in the region of the eastern US are planned to be carried out during the second unfunded phase (phase II) to confirm the applicability of this correlation over a wider range of temperature, humidity and soil conditions, and local storage or grain handling practices.

This method development phase (phase I) was approved for funding on May 1, 1991. However, actual work on the method developed did not start until July 1, 1991, until receiving the award and hiring needed staff. In spite of these expected delays at the start-up time, the acquisition of the commercial SFE equipment facilitated a great deal of progress in the proposed method development activities. Further delays were also caused by the acquisition and installation of the GC,HPLC/MS system due to the need for a lengthy facility renovation.

The objectives of the second phase and new proposed ones, include:

1. To establish a means to estimate a farmer's exposure to airborne aflatoxin (and other mycotoxins) based on its measurement in bulk corn. Implicit in obtaining this goal is to demonstrate a correlation between toxins in bulk grain and in airborne dust on as many on-farm grain handling activities and as wide a geographic area as are feasible. This correlation will greatly extend the utility of future bulk grain surveys for aflatoxin, and it can potentially increase the interest of farmers individually and collectively to the content of aflatoxin or other mycotoxins in their grains because of their repeated and frequent exposure.

2. To identify and quantify other natural toxins such as fumonisin, ochratoxin, and vomitoxin which may be detectable during the course of aflatoxins analysis in grain and grain dust in these same on-farm activities and geographic areas.

3. To assess the change in the aflatoxin and other mycotoxin content of the grain over time as affected by seasonal temperature and humidity, grain

moisture content at storage, and grain handling history. The effect of this history upon the correlation of aflatoxin in prepared bulk and airborne samples will also be examined.

4. To investigate the primary site (foci) of aflatoxin/mycotoxin within the dust. One indicator of site is the proportion of aflatoxins in particles of different diameter. Our approach to this goal is to assess the effect of seasonal temperature, rainfall or humidity, and grain handling history on the distribution of aflatoxin in particles of different size in airborne dust samples. Again the effect of this history upon this size distribution will also be examined.

The long-term objective of this project is to establish the statistical data and exposure model needed to initiate or participate in an epidemiologic assessment of the occupational risk to farmers resulting from their exposure to aflatoxin B_1 and other mycotoxins in grain dust. The availability of the Iowa State Health Registry for both cancer and birth defects at the Department of Preventive Medicine and Environmental Health, The University of Iowa, represents a substantial advantage for the long-term objective of this study.

II. BACKGROUND AND SIGNIFICANCE

Aflatoxins are secondary metabolites of Aspergillus flavus and A. Parasticcuis fungi which are naturally found in soil and flourish late in the grain growing season under conditions of high humidity and temperatures (see Appendix A for chemical structure). Fungal invasion of agricultural crops such as corn has been attributed to hot and humid weather and plant stress caused by poor agronomy or drought and mechanical damage caused by birds or insects[2]. The damaged corn ears become accessible for Aspergillus flavus to grow and subsequently to produce aflatoxin in corn before harvest. Moisture and substrate conditions in the mid-range of seed development provide optimum conditions for fungal development and toxin production[3]. Post-harvest production of aflatoxin in contaminated corn is usually limited under proper storage conditions.

Aflatoxins, particularly Aflatoxin B_1, have been recognized as one of the most potent chemical carcinogens known[4]. Acute toxic effects of aflatoxins have been fully documented for a large number of animal species and humans[5-7]. Aflatoxin B_1 has been recognized to produce cancer in the liver as well as other organs in a broad variety of animal species[8]. Epidemiologic studies in Africa[9,10] and South-East Asia[11] have shown a strong correlation between the incidence of human liver cancer and the level of aflatoxin contamination in the daily diet. A recent epidemiological study in Swaziland, which is dependent on imported grains, showed evidence for the association between the incidence of liver cancer and the estimated levels of aflatoxin in the daily dietary intake[12].

Mycotoxins are commonly attributed as the etiologic agent behind the clinical picture of farmers with sudden acute illness following silo uncapping or other work with moldy silage. The negative immune reaction sharply distinguish these patients from those with farmer's lung disease. All patients subsequently returned to health after their exposure to moldy silage. Emanuel et al referred to the observed lung disease as pulmonary mycotoxicosis attributed to the inhalation of fungal toxins from the work environmental[15]. This disease of fungal origin is now known as organic dust toxic syndrome (ODTS)[16,17].

Although the most publicized route for human and animal exposure to aflatoxins is dietary, evidence has been accumulating to support the possibility that aflatoxins in airborne dust particles may also constitute both potential acute and chronic hazards from exposure via the respiratory route[13,14].

Dvorackova[18] reported a case of pulmonary adenomatosis in a chemical engineer who became acutely ill after he was exposed to high levels of the mold Aspergillus flavus for three month while working on a process to sterilize Brazilian peanut-meal. The condition of the patient became worse and he died three months after the onset of his illness. Analysis of the lung tissue, using the technique of thin layer chromatography (TLC), revealed the presence

of aflatoxin B_1, which provides plausible evidence for an acute occupational risk associated with aflatoxin inhalation.

Three patients, two agricultural workers and one textile worker, died of acute lung disease, clinically diagnosed as bronchopneumonia or influenza[19]. Histological investigation of the lungs after death revealed interstitial fibrosis in all three cases. Radioimmunoassay (RIA) analysis has shown the presence of aflatoxin B_1 in samples of lung tissue from the autopsy of the three patients.

Chronic effects associated with airborne exposure are limited in part by the lack of a method to detect low-level airborne exposure data. Continuing epidemiological studies of about 70 Dutch workers exposed to airborne aflatoxin at a peanut and linseed oil processing plant represents the earliest and most direct evidence for an occupational risk associated with airborne exposure to aflatoxin contaminated grains[20-21]. These workers were exposed from 1961 to 1969 to dust from oil-press residues which were dried and bagged for use as animal feed. Aflatoxin exposures were estimated to be on the order of 5 pg/m^3 with the content of aflatoxin in the airborne dust in the range of 250 to 410 ppb and the time weighed average (TWA) dose of baggers estimated from 0.04 to 2.5 ug per week. In 1973 the authors found rates of cancer generally and liver cancer specifically among exposed workers to be more than three times that in a matched control group, but the number of workers exposed was too small to provide statistical significance[20]. However, in a 1984 follow-up study of this same population[21], the mortality occurring between 1963 and 1980 was significantly elevated for cancer of all types (SMR = 2.5) in comparison to matched controls at $p < .01$; while the SMR for respiratory cancers in this same time period was also 2.5, the small sample size kept the statistical significance to $p < .05$. The other (non-respiratory) tumors identified were at a variety of sites but primarily digestive.

Cancer studies among grain handlers or farmers are few and variable. The incidence of liver cancer (not overall cancer) among 2649 recently studied Swedish grain millers working for the period from 1961 to 1979 was significantly elevated[22]. A recent review of U.S. statewide epidemiologic studies of farmers and rural residents scattered over the past 15 years, found low overall cancer rates[23,24], low rates for the common cancers related to lung, esophageal, and mouth[25], but high rates for various specific sites such as leukemia, Hodgkin's disease, Non-Hodgkin's lymphoma, and stomach (Appendix F). The low rates of respiratory cancer are attributed to low rates of smoking, approximately 17% of farmers smoke compared to 34% of the general population[26]. The high rate but diverse pattern of other cancers is commonly (and perhaps wishfully) attributed to pesticides but is not inconsistent with some findings related to aflatoxin[13,21].

Exposure levels among farmers are poorly documented and no doubt highly variable. In surveys by Donham[27-29] dust levels in swine barns ranged from 2.4 to 16 mg/m^3, were largely of organic origin (ca. 25% protein content typically associated with feed corn), and included 16 A. flavus CFU/mg dry dust (no analyses were made for aflatoxin per se). Shotwell and Burg[30] found 12 to 200 ppb and up to 25 ng/m^3 aflatoxin contamination in corn dust during their pilot surveys. Sorenson et al[31] reported that while the dust may be coarse, aflatoxin was more concentrated on the smaller particles (in the 3-5 μm

diameter characteristic of A. flavus spores). In another small survey focusing on analytical methodology, Zennie[32] found aflatoxin in grain dust from four central Illinois grain elevators to range from zero to 3.5 ppb. Airborne exposures can be implied by multiplying the airborne dust levels by the concentration of aflatoxin on related materials.

Farmers are usually exposed to grain dust through their year-round activities, from harvest and grain storage to animal feeding in confined buildings and bin clean-out operations. The presence of aflatoxin in corn[33] and corn dust[30,31] during relatively normal years, its extrapolation to intermittent exposures of 1 to 20 ng/m^3 on the farm,[14] and the increased risk of Aspergillus flavus infestation during drought conditions[30] suggest that airborne agricultural exposures could be of considerable and growing concern. A dose capable of producing cancer could result from repeated low level toxin concentrations which are usually below the detection limits of current analytical methods. Therefore, a more sensitive and efficient chemical method is needed in order to obtain reliable exposure data for accurate assessment of the occupational risk to farmers exposed to aflatoxins.

Past methods for the analysis of aflatoxins in grain dust[34] are adopted from the official method used for determining aflatoxins in bulk corn samples,[35] by using <1 to 10 g of dust instead of 50 g of corn. These methods are based on extracting aflatoxins from the dust samples with chloroform or methylene chloride, followed by several lengthy concentration and clean-up steps. The concentrated extract is then analyzed by thin layer chromatography (TLC). If no aflatoxin is detected the entire sample concentrate is applied to the thin layer plate. This time consuming sample manipulation usually results in significant analyte losses which consequently limits its overall detection limits. In addition, the final analysis also suffers from the inherent limitations of the TLC method, namely limited sensitivity, poor selectivity, and inaccurate quantitative measurements. The utilization of other more quantitative methods, such as gas liquid chromatography (GLC) or high pressure liquid chromatography (HPLC), require the same laborious liquid extraction, concentration and clean-up procedure.

The need to overcome the complex and inefficient sample clean up procedure has led to the development of enzyme-linked immunosorbent assay (ELISA) for aflatoxin B_1 in the extract of agricultural commodities.[36,37] Although this method is highly sensitive and faster than previous TLC or HPLC methods, it is still prone to interfering substances in the crude sample extract.[38] As a result, the coefficient of variation in some ELISAs is still relatively high.[36] The extract may be purified by column chromatography to eliminate matrix interferences, but this clean-up step prolongs the analysis time and eliminates the main advantage of ELISA, namely its speed.

Aside from the speed and sensitivity of TLC and ELISA, both methods have limited selectivity and are thus susceptible to producing false results due to interferences from complex sample matrices. Therefore, in order to obtain reliable data a second analytical method (e.g. GC/MS or HPLC) must be used for confirmation of the positive results obtained from the TLC or ELISA screening tests.[39]

The growing need for reliable, faster, and more sensitive analytical methods has led to the recent development of the techniques of supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC).[40,41] The separating power of supercritical fluid chromatography bridges the gap between the capabilities of GLC and HPLC and extends beyond the limitations of both techniques. The use of supercritical fluids for effective extraction of organic compounds from their solid matrices has been proven successful during the last few years.[42-44] The advantages of SFE stem from the unique physical properties of supercritical fluids. Above the critical temperature the compressibility of the supercritical fluid is large, and any small changes in pressure result in large changes in density and consequently the solvating power of the fluid.[45] Increasing the pressure, increases the fluid density and its molecular interaction; consequently, the solvating power of the fluid approaches that of the liquid. However, the viscosity and solute diffusivity remain approximately the same as those for the gaseous state, thus allowing more rapid mass transfer of solute than possible with liquids. The solubilizing power of the supercritical fluid can be increased even more by the selective addition of small amounts of polar organic solvents (see preliminary studies of these "modifiers").

The primary goal of this research project is to determine the average yearly exposure of farmers to aflatoxin B_1 in airborne grain dust from repeated low level exposure during various on-farm grain handling activities. This goal created the need to develop and validate a more sensitive and efficient method for the determination of low levels of aflatoxin B_1, as well as other toxins, in grain dust samples. Our proposed analytical approach is based on the use of supercritical fluid extraction followed by on-line HPLC/MS of SFC/MS analysis.
The long-term objective of this project is use this new method to establish statistical field data to assess the occupational risk to farmers resulting from their exposure to aflatoxin B_1 from their year-round grain handling operations.

III. EXPERIMENTAL METHODS

Scope of the Analytical Methods:

Samples of total and airborne dust, bulk corn, ground corn and dislodgable dust were analyzed using supercritical fluid extraction (SFE) followed by off-line HPLC with diode array UV detection. Fluorescent detection was used in line with UV detection to enhance the detection sensitivity for Aflatoxin B_1.

Although the analytical procedure for aflatoxin B_1 was optimized to allow simultaneous identification and quantification of the other aflatoxins (B_2, G_1, & G_2), as shown earlier for method development data (see HPLC chromatograms, Appendix B, Figure B4), the focus of our screening of field samples was limited to aflatoxin B_1 due to its natural prevalence and known carcinogenic potential.

Multi-toxin screening methods are currently available for aflatoxins, ochratoxin, vomitoxin, and zearalenone using TLC and HPLC.[66] The use of the mass spectrometer (MS) as a chromatographic detector for on-line SFE/HPLC/MS or SFE/GC method facilitated the positive identification of all toxins of interest, even if they are not chromatographically resolved. The use of the selected ion monitoring (SIM) capabilities of the MS allows the identification of closely retained compounds and enhances the sensitivity of mass spectrometric detection.

However, it was not possible to analyze all mycotoxins of interest under the same SFE conditions for aflatoxins due to the broad difference in their chemical structures (Appendix A). Fumonisin B_1 is a highly polar compound. It was not possible to isolate fumonisin from corn until very recently because of the lack of an adequate analytical method for its separation and identification. The advent and application of combined HPLC/MS allowed a group of scientists at the Research Institute of Nutritional Diseases in South Africa to separate and characterize that toxin very recently.[67] But since fumonisin is an organic acid, we expected it could be amenable to direct extraction and analysis by the new technique of on-line SFE/SFC/MS, following a procedure similar to current SFC methods for the analysis of free fatty acids.[68,69] Alternatively, we able to develop a new method for the analysis of fumonisin in grain dust samples using off-line SFE and HPLC.[67] Further confirmation was carried out using on-line HPLC/MS.

SFE Instrumentation:

The SFE unit we obtained is the ISCO supercritical fluid extraction module SFX 2-10 (ISCO, Inc., Lincoln, NE) which incorporates a temperature controller, a heating block with two wells for two sample cartridges, high-pressure inlet valves, and fittings for connection to the fluid inlet and outlet. The associated fluid delivery system consists of two ISCO model 260D syringe pumps, and a series D multipump controller/programmer. The dual pump system allows selectable routines for constant addition of modifiers to the

supercritical fluid as well as continuous delivery with altering refill or constant flow/constant pressure operations. A schematic diagram showing the components of the SFE instrumentation is provided in Appendix D, Figure D1.

SFE/HPLC/MS Instrumentation:

Figure 2 shows a schematic diagram for the on-line SFE/HPLC/MS system. The primary component of this system is the Hewlett-Packard MS Engine (Hewlett-Packard, Avondale, PA). The MS Engine is a multi-dimensional GC/MS system with capabilities for thermospray or particle beam HPLC/MS interface. The mass spectrometer of the MS Engine has a mass range up to 2000 amu, and can be programmed for automatic switching from electron impact (EI) to chemical ionization (CI) modes, which allows flexibility and convenience during method development and routine sample analysis. The GC oven of the MS Engine can be used for normal capillary gas chromatography or supercritical fluid chromatography (SFC) by the addition of the ISCO SFC-500 Microflow pump for supercritical-fluid delivery (not shown in Figure 1), as described above under SFC instrumentation (Appendix D, Figure D2).

For on-line SFE/HPLC, the outlet effluent from the SFX 2-10 extraction module is connected to the HPLC column, passing through a CDS 335 cryogenic trap (Chemical Data Systems, Oxford, PA) to concentrate the extracted analytes at the head of the column. The cryogenic trap is programmed for cooling and subsequent thermal desorption through the CDS 335 Cryfocusing controller unit. A zero-volume valve may be connected between the cryogenic trap and the GC column to allow venting of the CO_2 during the extraction and concentration process.

For off-line SFE, a capillary restrictor (fused silica tubing 15-30 cm long x 15-50 μm ID) is connected to the fluid outlet of the SFX 2-10 extraction module, and the free end of the capillary is inserted into a small vial containing suitable solvent for collecting the extract (e.g. methanol).

The SFE module (ISCO SFX 2-10) and two syringe pumps (ISCO 260D, only one is shown in Figure 2) were the only equipment requested in the budget of this proposal. All other instrumentation, except for the GC/MS system were available at the Analytical Toxicology Laboratory (ATL), where all the proposed work was performed. Due to needed space renovation, the HPLC,GC/MS was not completely installed until the very end of the proposed work period for this project.

The experimental procedures for the analytical method development and optimization are described in detail in the Appendices.

IV. RESULTS OF THIS STUDY

Detection of Aflatoxin in Grain Dust:

Recent droughts of 1988 and 1989 have created favorable conditions for substantial growth of Aspergillus flavus on the corn crop in the Midwest, where approximately one-third of the world's corn is produced. Analysis of bulk corn samples revealed unacceptable levels of aflatoxins in one third of the official samples in Iowa in 1989, which caused many grain elevators to close their doors for receiving any corn.[46] Many farmers were forced to keep their corn crop for animal consumption, and others were discouraged to harvest their poor and unmarketable crop. The situation generated tremendous concern regarding the possible health risk to farmers associated with handling aflatoxin contaminated grain.

Four local farms were selected for collection of airborne samples during the 1991 harvest. Personal air sampling pumps were used to collect dust samples on fiber glass filters at two locations, inside and outside the cab of the harvesting tractor. In one of the four farms, extra air samples were collected during the unloading of the grain and inside a hog confinement building, where some of the harvested grains were used for animal feeding.

Collected dust samples were analyzed for aflatoxin B_1 using a modification of the thin layer chromatography (TLC) procedure described in the literature.[34,47] Table I (Appendix B) summarizes the results of these analysis for all the samples collected. As shown in Table I (Appendix B), aflatoxin B_1 was detected in the airborne dust samples in two of the four farms selected even using the TLC method. In farm number 2, aflatoxin was detected in the dust sample collected from inside the tractor cab but was not detected in the outside sample, primarily because of strong crosswinds.

In farm number 4, aflatoxin B_1 was not detected in the field dust samples but was found in the dust samples collected during corn unloading and inside the hog confinement building where the same corn was used for animal feeding. Aflatoxin B_1 concentrations of 66.6 ng/m^3 and 92.6 ng/m^3 are comparable to more recent farm level measurements of Burg and Shotwell,[48] and are significantly higher than the Netherlands epidemiologic study (roughly five pg/m^3) which showed 2.5 times the risk of cancer among 60 to 70 peanut and flax seed processors.

There are no literature data currently available on aflatoxin exposure levels inside animal confinement facilities (farms 4-6). This type of exposure is highly important in determining the total yearly exposure because the animal feeding operation is a year-round activity. In addition, grains which are unmarketable due to their contamination with aflatoxin producing fungi, are usually used for on-farm animal feeding. Improper storage of such contaminated grain can result in fungal spread and increased Aflatoxin production. To investigate this possibility, we collected and analyzed airborne dust samples (approx. 4-5 m^3 of air) along with bulk feed samples and settled dust from two animal confinement buildings during the summer of 1989. The aflatoxin B_1 TLC results from these samples are shown in Table II. These

preliminary data (Tables I and II, Appendix B) demonstrate that even in less drought-stressed corn, aflatoxin B_1 is present at detectable levels throughout various on-farm grain handling operations. Therefore, year-round grain handling in animal confinement buildings may contribute the largest portion of the overall farmer's exposure to aflatoxin contaminated dust. More data are needed in order to determine the contribution of all possible exposure sources to the total dose of farmer's exposure to aflatoxins from their yearly farming activities.

Our experience with this limited number of samples has brought to our attention several factors to consider in future sampling. The effects of temperature, humidity, and wind velocity and direction are crucial in determining the amount of dust sampled. Multiple sampling of the same farm under different weather conditions should be used. The content of aflatoxins in the dust should be compared with the level of aflatoxins in bulk corn from the same sampling location.[14] Moreover, the use of personal air sampling pumps is important to determine the actual dose delivered to the exposed worker. Analysis of the small dust samples collected by the personal air sampler (which collects ≤ 1 m^3/day) will necessitate the use of a more sensitive analytical method such as the one developed herein.

Supercritical Fluid Extraction of Aflatoxins from Grain Dust:

We were able to successfully apply supercritical fluid extraction (SFE) with carbon dioxide to the separation of aflatoxins from grain dust and spiked fiber glass filters. The initial SFE instrumentation used (Appendix B, Figure B1) consisted of an SFC-grade liquid carbon dioxide tank, a computer controlled syringe pump for delivering liquid CO_2, and an extraction vessel (empty HPLC column) placed in a GC heating oven. The inlet of the extraction vessel was connected to the syringe pump using 1/16" stainless steel tubing, and the outlet was connected to a capillary restrictor which consists of a piece of fused silica tubing of approximately 15" long x 50 μm ID. The free end of the capillary tubing was immersed inside a small vial containing methanol for collecting the extracted material.

The SFE procedure consists of packing the solid sample into the extraction vessel followed by pumping liquid CO_2 into it while maintaining an extraction temperature above the critical temperature of CO_2 (T_c = 31.3°C). The pressure is also maintained above the critical pressure of CO_2 (P_c = 72.9 atm) through proper selection of the restrictor dimensions and CO_2 pumping rate. With this simplified instrumentation, we were able to carry out fast and efficient extraction of aflatoxin B_1 from spiked dust samples and fiber glass filters. Table III in Appendix B lists the percentage recovery for aflatoxin B_1 from fiber glass filters using both the liquid/liquid extraction and SFE. Table IV lists the percentages recovery of aflatoxin B_1 from settled hog dust samples using classical liquid/liquid extraction and supercritical fluid extraction (SFE). As shown in Tables III & IV in Appendix B, the recovery and reproducibility of the SFE method are much superior to the classical liquid extraction method. The increased precision when analyzing settled dust (Table IV, Appendix B) reflects the added capability of SFE in relation to the liquid extraction method to deal with background interferences.

The efficiency and reliability of the SFE method was further validated by its application to the extraction of aflatoxin B_1 from naturally contaminated corn samples. Extracts from both liquid extraction and SFE experiments were analyzed directly by HPLC and TLC without any column cleanup. Table V in Appendix B shows a comparison between the concentration of aflatoxin measured after classical liquid extraction and the concentrations obtained using SFE with CO_2 only and SFE with CO_2 in the presence of varying amounts of organic modifiers (methanol and acetonitrile).

From the data listed in Table V (Appendix B), it is evident that the use of supercritical CO_2 without any additive modifiers can achieve the same extraction efficiency as classical liquid/liquid extraction. The addition of increasing quantities of methanol to supercritical CO_2 can progressively increase the concentration of detected aflatoxin B_1 (Appendix B, Figures B2 and B3). However, the addition of methanol $\geq$ 5% results in the extraction of greater quantities of interfering components which precludes reliable quantification of the aflatoxin B_1 peak (see Appendix B, Figure B3). As a result, an unquantified peak for aflatoxin B_2 can be seen in the HPLC chromatogram of the CO_2-modifier B extracts. For this reason we have tried other modifiers in search of an efficient and selective SFE system for aflatoxin B_1. The use of supercritical CO_2 plus 5% acetonitrile has resulted in a much cleaner extraction and the highest detectable concentration of aflatoxin B_1 (see Appendix B, Figure B4). The efficiency and cleanliness of the extract obtained with supercritical CO_2 and acetonitrile was confirmed by thin layer chromatographic analysis. SFE with acetonitrile did not show any significant matrix interferences with Aflatoxin B_1, or even with the other aflatoxins (B_2, G_1 and G_2). These observations were all confirmed by TLC analyses of all SFE extracts.

Supercritical fluid extraction of aflatoxin was initially re-evaluated or just evaluated using the new ISCO SFX 2-10 (ISCO, Inc., Lincoln, NE) purchased on this grant, was initially evaluated for SFE of aflatoxin by comparing its performance with the classical liquid extraction method described in Appendix C.

Since spiked samples may not always represent the extractability of naturally contaminated matrices, a naturally contaminated dust sample was used to compare the efficiencies of both extraction techniques.

The amount of aflatoxin B_1 recovered using the liquid extraction and the SFE method are listed in Table II for a set of 18 grain dust samples. With liquid extraction, aflatoxin was detected in less than half (7/18) of the samples, while the use of SFE resulted in the detection of aflatoxin in most of the samples (14/18). Although the SFE conditions used were not optimized, the improved efficiency of SFE is visible. In addition to the improved detection capability of the SFE method, tremendous saving of time and material was realized, relative to the liquid extraction method. While liquid extraction requires a minimum of 2 - 2.5 hrs per sample, SFE required only 25-30 minutes, and resulted in a cleaner extract that saved cleanup time and materials.

A systematic approach was later used to study the effect of various SFE parameters on the efficiency of extracting aflatoxin from naturally contaminated grain dust samples. The results of the SFE method optimization

for aflatoxin are shown in the publication in Appendix F, which appeared in the April issue of the American Industrial Hygiene Journal.

A similar optimization procedure was followed for the optimum extraction conditions for fumonisin B_1. The optimization procedure and the results obtained are shown in Appendix G. Method optimization and validation were carried out using naturally contaminated dust samples.

On-Line SFE/HPLC Analysis:

On-line SFE/HPLC analysis for Aflatoxin was carried out using the ISCO SFX-10 on line with Hewlett-Packard 1090 Series II/L HPLC (Hewlett-Packard Co., Palo Alto, CA). The on-line SFE extraction procedure involves placing the sample into the extraction vessel (2.5 mL) which is inserted into the extraction chamber of the SFX-10 module. The sample is then extracted using 2 mL of supercritical carbon dioxide with 5% acetonitrile-methanol (2:1) at 1200 psi and 45°C. The SFE extract is passed through a concentration cartridge containing C-18 packing material (37-50μm). Following the SFE extraction, valve switching allows the C-18 cartridge to be connected on-line with the analytical HPLC column, where chromatographic separation takes place. The analytical column was an ODS-Hypersil reversed phase column, 100 x 2.1 mm. , 5 μm particle size. The mobile phase was methanol-acetonitrile-water (35:35:30). Dual detection systems were used for these on-line experiments; UV (diode array) at a wavelength of 265 nm and Fluorescent detection at an excitation wavelength of 366 nm and emission of 425 nm. The latter detection system was more useful for lower concentration range.

Particle beam HPLC/MS detection was found to be less sensitive than both the diode array-UV and fluorescent detection and was not then used during the optimization of the on-line SFE/HPLC procedure for low concentration airborne dust samples. For this reason we intend to use fluorescent detection for future on-line SFE/HPLC analysis and Thermospray/MS interface for on-line confirmation of the analytical results by mass spectrometry.

The advantages of the SFE Method:

1. SFE provides increased sensitivity and tunable selectivity for mycotoxins. These features are attributed to the following characteristics of SFE:

    - Compatibility of the SFE effluent with chromatographic mobile phases allows cryogenic trapping and concentrating the analyte at the head of the analytical column (GC, SFC), a feature particularly useful for the analysis of small and low-mass samples such as airborne dust samples.

    - No analyte losses due to adsorption on glassware or due to solvent partitioning while being extraction.

- No liquid interferences from solvents, reagents, and labware commonly used for sample preparation during classical liquid extraction.

- Capability for selective extraction as a function of fluid solvation power and/or the addition of fluid modifiers to isolate the toxins of interest from various matrix interferences.

2. SFE produces better quantitative results than liquid extraction techniques. Our analysis of aflatoxins in corn, settled dust, and fiber glass filters provided more quantitative recovery and better reproducibilty with SFE than with classical liquid-liquid extraction.

3. SFE represents a tremendous savings in the cost and the time of the analysis due to the elimination of the time consuming and laborious liquid extraction, concentration, and source clean up processes Additional cost savings are gained from the elimination of the use and disposal of organic solvents and column chromatography materials used for the classical liquid-liquid sample extraction and clean up.

4. SFE eliminates the health hazard associated with the exposure of laboratory personnel to harmful organic solvents during the classical liquid-liquid extraction process. This advantage is particularly important with increasing occupational health regulations related to hazardous chemicals in laboratories (OSHA, 29 CFR 1910.1450).

With the above listed advantages make this technique of on-line SFE/HPLC or SFE/SFC highly suitable for the separation and quantitative determination of aflatoxins in airborne grain dust samples.

Difficulties Encountered with the SFE Method Development:

Several difficulties were encountered during the on-line SFE/HPLC procedure related to instrumentation and personnel training. Delays in the acquisition and installation of the GC/HPLC/MS system contributed significantly to the delays in addressing some of the other difficulties or optimization steps.

1. Require tremendous personnel training and personnel skills for proper use and understanding of the SFE process.

2. Instrumental limitations due to frequent to system leaks and frequent clogging of capillary lines and filters due to the incompatibility of some materials used (e.g. switching valve seat and trapping cartridge seals). These problems are eliminated through use of more compatible hardware materials, proper system operation, frequent maintenance, and use of quality control samples.

3. Filters and trapping materials (e.g. C_{18}) which were unstable under the SFE conditions or overloaded with sample extract, leading to breakthrough and inconsistent recovery. In addition the extraction efficiency was found to vary with the type of air sampling filter used. PVC filters were found to provide the highest recovery (84%) of

aflatoxin spikes at a concentration of 10 ng per sample, whereas the lowest recovery was obtained with fiber glass filters. This filter evaluation process requires further verification and evaluation, particularly with naturally contaminated dust samples.

Further Method Refinements:

1. Optimization of the on-line SFE procedure for Aflatoxin and fumonisin through testing and selection of the most compatible filter and trapping materials or using cryogenic trapping on-line with SFE. The SFE conditions for both aflatoxin B_1 and fumonisin B_1 were significantly different such that their combined extraction with not possible during the course of this project. Combined extraction required programming the Ph of the supercritical fluid modifier and the HPLC mobile phase, which was not possible with available instrumentation. On-line derivatization, under the SFE conditions, may provide an alternative approach to overcome this difficulty.

2. Optimize the detection for the on-line SFE process. The sensitivity for the particle beam (PB) HPLC/MS interface does not meet the needed lower detection limit for the on-line process. Enhanced sensitivity for the on-line process can be achieved as follows:

   a. Use of thermospray (TS) HPLC/MS interface.

   b. Use of fluorescent detection, either separately or on line with the TS/MS interface.

   c. Using on-line chemical derivatization followed by negative ion chemical ionization (NCI) technique to enhance MS sensitivity. Pentafluoro-benzyl-hydroxylamine was used as a derivatizing agent to enhance the sensitivity for aflatoxin with NCI/MS. Further work is needed to optimize this method for on-line SFE/HPLC/MS.

V. SIGNIFICANT FINDINGS

1. An efficient, more sensitive, and highly specific analytical method for the determination of low levels of aflatoxin B_1 in airborne grain dust was developed and optimized (Appendix F). Supercritical fluid extraction was used to extract aflatoxins from small amounts of grain dust samples. A detection limit of 1 ng of aflatoxin B_1 per sample are obtained using off-line SFE and HPLC analysis. Lower detection limits are obtained using on-line SFE-HPLC with fluorescent detection.

2. A new method for the analysis of fumonisin B_1 in grain dust, was developed and optimized using the off-line supercritical fluid extraction and HPLC analysis (Appendix G). The detection limits of this method are 50 and 5 ppb using UV and fluorescence detection respectively.

3. The distribution of aflatoxin B_1 in aerosolized grain dust was studied during manual bin clean-out and grain handling operations (Appendix H). Significant correlation was found between the concentration of aflatoxin B_1 and the particle size of the airborne dust. The highest average concentration of aflatoxin B_1 was found on dust particles ≤ 1 μm in diameter (160 ppm). The average concentration of aflatoxin B_1 on dust particles ≥ 7 μm was 6.5 ppm.

   Under the sampling conditions used in this study, the average pulmonary exposure dose is estimated to be 0.22 μg aflatoxin / hour.

REFERENCES

1. Stephen, J. and R.A. Pietrowski: Bacterial Toxins: American Society for
Microbiology, 1986.
2. Stoloff, L.: *Aflatoxin - An Overview. In Mycotoxins in Human and Animal Health.* Edited by J.V. Rodricks, C.W. Hesseltine, and M.A. Mehlman, Pathotox Publishers, Inc., Park Forest South, IL. pp. 7-28 (1977).
3. Lillehoj, E.B., J.H. Wall, and E.J. Bowers: Preharvest Aflatoxin Contamination: Effect of Moisture and Substrate Variation in Developing Cottonseed and Corn Kernels. *Appl. and Envir. Microbiol.* 53:584-86 (1987).
4. International Agency for Research, Cancer Working Group: *IARC Monographs, Cancer Res.* 40:1 (1980).
5. World Health Organization: *Environmental Health Criteria II. Mycotoxins.* Geneva, Switzerland: WHO (1979).
6. International Agency for Research on Cancer (IARC): *IARC Monographs Eval.*
Carcinog. Risk Chem. Hum. Vol. 20. Lyon, France: IARC, October, 1979.
7. Smith, J.E. and M.O. Moss: *Mycotoxins.* New York, NY: John Wiley and Sons, p. 7, 1985.
8. Sinnhuber, R.O., J.D. Hendricks, J.H. Wales, and G.B. Putnam: Neoplasms in
Rainbow Trout, A Sensitive Animal Model for Environmental Carcinogenesis. *Ann. N.Y. Acad. Sci.* 298:398-402 (1977).
9. Peers, F.G. and C.A. Linsell: Dietary Aflatoxins and Liver Cancer - A Population Based Study in Kenya, *Br. J. Cancer* 27:473 (1973).
10. Van Rensburg, S.J., J.J. Van der Watt, I.F.H. Purchase, L. Pereira Coutinho, and R. Markham: Primary Liver Cancer and Aflatoxin a High Cancer Area. *S. Afr. Med. J.* 48:2508a (1974).
11. Shank, R.C., N. Bhamarapravati, J.E. Gordon, and G.N. Wogan: Dietary Aflatoxins and Human Liver Cancer IV. Incidence of Primary Liver Cancer in Two Municipal Populations in Thailand. *Fd. Cosmet. Toxicol.* 10:170- (1972).
12. Peers, F.G., X. Bosch, J. Kaldor, C.A. Linsell, and M. Pluumen: Aflatoxin
Exposure, Hepatitus B Virus Infection and Liver Cancer in Swaziland. *Int. J. Cancer* 39:545-53 (1987).
13. Baxter, C.S., H.E. Wey, and W.R. Burg: A Prospective Analysis of the Potential Risk Associated with Inhalation of Aflatoxin-Contaminated Grain Dust. *Food Cosmet. Toxicol.* 19:765-69 (1981).
14. Popendorf, W., K.J. Donham, D.N. Easton, and J. Silk: A Synopsis of Agricultural Respiratory Hazards. *Am. Ind. Hyg. Assoc. J.* 3:154-161 (1985).
15. Emanuel, W.A., B.S. Wenzel, and B.R. Lawton: Pulmonary Mycotoxicosis. *Chest*
67:293-97 (1975).
16. Donham, K.J.: Hazardous Agents in Agricultural Dusts and Methods of Evaluation. *Am. J. Ind. Med.* 10:205-20 (1986).
17. Rylander, R.: Lung Diseases Caused by Organic Dust in the Farm Environment. *Am. J. Ind. Med.* 10:221-27 (1986).

18. **Dvorackova, I.:** Aflatoxin Inhalation and Alveolar Cell Carcinoma. *Br. Med. J.* 1:691-93 (1976).
19. **Dvorackova, I. and V. Pichova:** Pulmonary Interstitial Fibrosis with Evidence of Aflatoxin B_1 in Lung Tissue. *J. Toxicol. Envir. Hlth.* 18:153-57 (1986).
20. **Van Nieuwenhuize, J.P., R.F.M. Herber, A. Debruim, I.P.B. Meyer, and W.C. Duba:** Aflatoxins--Epidemiological Study on the Carcinogenicity of Prolonged Exposure to Low Levels Among Workers of a Plant. *Tijdschr. Soc. Geneesk.* 51:754-59 (1973).
21. **Hayes, R.B., J.P. Van Nieuwenhuize, J.W. Raatgever, and F.J.W. Ten Kate:** Aflatoxin Exposures in the Industrial Setting: an Epidemiological Study of Mortality. *Fd. Chem. Toxic.* 22(1):39-43 (1984).
22. **Alavanja, M.C., H. Malker, and R.B. Hayes:** Occupational Cancer Risk Associated with the Storage and Bulk Handling of Agricultural Foodstuff. *J. Tox. & Environ. Health* 22:247-54 (1987).
23. **Blair, A. and D.W. White:** A death certificate study of leukemia among farmers from Wisconsin. *J. of the National Cancer Inst.* 66:1027-1030 (1981).
24. **Blair, A., H. Malker, K.P. Cantor, L. Burmeister, and K. Wiklund:** Cancer Among Farmers: A Review. *Scand. J. Work Envir. and Hlth.* 11:397-407 (1985).
25. **Burmeister, L.F., G.D. Everett, S. Van Lier, and P. Isacson:** Selected cancer
mortality in farm practices in Iowa. *Am. J. Epidem.* 118:72-77 (1983).
26. **Pomrehn, P.R., R.B. Wallace, and L.F. Burmeister:** Ischemic heart disease mortality in Iowa farmers. *J. Am. Med. Assoc.* 248:1073-76 (1982).
27. **Donham, K.J., M. Rubino, T.D. Thedell, and J. Kammermeyer:** Potential Health
Hazards to Agricultural Workers in Swine Confinement Buildings. *J. of Occupational Med.* 19(6):383-87 (1977).
28. **Donham, K.J. and W. Popendorf:** Ambient Levels of Selected Gases Inside Swine
Confinement Buildings. *Am. Ind. Hyg. Assoc. J.* 46:658-61 (1985).
29. **Donham, K.J., L.J. Scallon, W. Popendorf, M.W. Treuhaft, and R.C. Roberts:**
Characterization of Dusts Collected from Swine Confinement Buildings. *Am. Ind. Hyg. Assoc. J.* 47(7):404-10 (1986).
30. **Shotwell, O. and W. Burg:** Aflatoxin in Corn: Potential Hazard to Agricultural Workers." *Ann. Am. Conf. Gov. Ind. Hyg.* 2:69-86 (1982).
31. **Sorensen, W.G., J.P. Simpson, M.J. Peach, T.D. Thedell, and S.A. Olenchock:**
Aflatoxin in Respirable Corn Dust Particles. *J. Toxicol. Envir. Hlth.* 7:669-72 (1981).
32. **Zennie, T.M.:** Identification of Aflatoxin B1 in Grain Elevator Dust in Central Illinois. *J. Tox. Envir. Hlth.* 13:589-93 (1984).
33. **Schmitt, S.G. and C.R. Hurburgh,** "Measurement and Distribution of Aflatoxin in 1983 Iowa Corn." ASAE paper No. 85-3533, American Society Agricultural Engineers, St. Joseph, MI (1985).
34. **Shotwell, O.L., W.R. Burg, and T. Diller:** Thin Layer Chromatographic Determination of Aflatoxin in Corn Dust. *J. Assoc. Off. Anal. Chem.* 64:1060-63 (1981).

35. **Association of Official Analytical Chemists (AOAC): Official Methods of Analysis, 13th Edn.** Edited by S. Williams. Arlington, VA, Secs 26.049, 26.026-26.031, 1980.
36. **Fun, S.C., T.S.L. Fan, G. Zhang, Y. Xu, S. Faust, and P.L. McMahon:** Improved Enzyme-Linked Immunosorbent Assay for Aflatoxin B_1 in Agricultural Commodities. *J. Assoc. Off. Anal. Chem.* 70:854-57 (1987).
37. **Park, D.L., B.M. Miller, L.P. Hart, G. Yang, J. McVey, S.W. Page, J. Pestka, and L. Brown:** Enzyme-Linked Immunosorbent Assy for Screening Aflatoxin B_1 in Cottonseed Products and Mixed Feed - Collaborative Study. *J. Assoc. Off. Anal. Chem.* 72:326-32 (1989).
38. **Rauch, P., L. Fukal, P.B. Brezina, and J. Kas:** Interferences in Radioimmunoassay of Aflatoxins in Food and Fodder Samples of Plant Origin. *J. Assoc. Off. Anal. Chem.* 71:491-93 (1988).
39. **Dashek, M.V., T. Eadie, G.C. Llewellyn, S.A. Olenchock, and G.H. Wirtz:** Thin Layer Chromatographic Analysis of Aflatoxins Within Grain Dusts. *J. Am. Oil Chem. Soc.* 60:563-66 (1983).
40. **Charpentier, B.A. and M.R. Sevenants, eds.:** *Supercritical Fluid Extraction and Chromatography - Techniques and Applications.* ACS Symposium Series 366: American Chemical Society, Washington, DC, 1988.
41. **Johnston, K.P. and J.M.L. Penninger, eds.:** *Supercritical Fluid Science and Technology*. ACS Symposium Series 406, American Chemical Society, Washington, DC 1989.
42. **Hawthorne, S.B. and D.J. Miller:** Directly Coupled Supercritical Fluid Extraction-Gas Chromatographic Analysis of Polycyclic Aromatic Hydrocarbons and Polychlorinated Biphenyls From Environmental Solids. *J. Chromatogr.* 403:63-76 (1987).
43. **McNally, M.L.P. and J.R. Wheeler:** Increasing Extraction Effiency in Supercritical Fluid Extraction From Complex Matrices - Predicting Extraction Effiency In Supercritical Fluid Extraction Using Supercritical Fluid Chromatographic Retention. *J. Chromatogr.* 447:53-63 (1988).
44. **Selim, M.I., A.J. Weinrich, and W.J. Popendorf:** "Occupational Exposure to Aflatoxins in Agricultural Workers." Paper presented at the American Industrial Hygiene Conference, Orlando, Florida, May, 1990.
45. **McHugh, M.A. and V.J. Krukonis:** *Supercritical Fluid Extraction - Principles and Practice*. Boston, MA: Butterworths (1986).
46. **Wall Street Journal:** Fungus in Corn Crop, A Potent Carcinogen Invades Food Supplies. Des Moines. Vol. LXX No. 91, February 23, 1989.
47. **Stahr, H.M.:** *Analysis of Submicro Amounts of Aflatoxins by Thin Layer Chromatography*. In Advances in Thin Layer Chromatography, edited by J.C. Touchstone. New York: John Wiley and Sons, pp. 176-93 (1982).
48. **Burg, R.W. and O.L. Shotwell:** Aflatoxin Levels in Airborne Dust Generated from Contaminated Corn During Harvest and at an Elevator in 1980. *J. Assoc. Off. Anal. Chem.* 67:309-12 (1984).
49. **Look, D.:** *Aflatoxin-poisoned Corn in E. Iowa Called World's Worst Case.* Des Moines Register, p. 1A (Apr. 12 1990).

50. Remington, R.D. and Schork, M.A.: Statistics with Applications to the Biological and Health Sciences, 2nd ed. Prentice-Hall, Englewood Cliffs, New Jersey, 1985.
51. Kraemer, H.C. and Thiemann, S.: How Many Subject? Statistical Power Analysis in Research. Sage Publications, Beverly Hills, California, 1987.
52. Iwata, Y., J.B. Knaak, R.C. Spear, and R.J. Foster: Procedure for the Determination of Dislodgable Pesticide Residues on Foliage. *Bull. Envir. Contam. Toxicol.* 18(6):649-55 (1977).
53. Popendorf, W.J. and J.T. Leffingwell: Procedures for the Determination of
Dislodgable Dust on Foliage as Related to Worker Re-entry Hazards. *Bull. Envir. Contam. Toxicol.* 18(6):787-88 (1977).
54. Westlake, W.E., F.A. Gunther, and G.E. Carman: Worker Environment Research:
Dioxathion (Delnav^{R}) Residues on and in Orange Fruits and Leaves, in Dislodgable particulate matter, and in the Soil beneath Sprayed Trees. *Arch. Envir. Contam. Toxicol.* 1(1):60-83 (1973).
55. Spear, R.C., W.J. Popendorf, J.T. Leffingwell, T.H. Milby, J.E. Davies, and W.P. Spencer: Fieldworkers' Response to Weathered Residues of Parathion. *J. Occup. Med.* 19(6):406-10 (1977).
56. Popendorf, W.J. and J.T. Leffingwell: Natural Variations in the Decay and
Oxidation of Parathion Foliar Residues. *J. Ag. and Food Chem.* 26(2):437-41 (1978).

57. Popendorf, W.J., R.C. Spear, J.T. Leffingwell, Y. Yager, and E. Kahn: Harvester Exposures to Zolone^{R} (Phosalone) Residues in Peach Orchards. *J. Occup. Med.* 21(3):189-94 (1979).
58. Nigg, H.N., J.H. Stamper, and R.M. Queen: "The Development and use of a Universal Model to Predict Tree Crop Harvester Pesticide Exposure." *Am. Ind. Hyg. Assoc. J.* 45(3):182-86 (1984).
59. Zweig, G., J.T. Leffingwell, and W. Popendorf: The Relationship between Dermal Pesticide Exposure by Fruit Harvesters and Dislodgable Residues. *J. Envir. Sci. Hlth.* B20(1):27-59 (1985).
60. Popendorf, W.J^I^U^^xploring Citrus Harvesters' Exposure to Pesticide Contaminated Foliar Dust. *Am. Ind. Hyg. Assoc. J.* 41(9):652-59 (1980).
61. Popendorf, W., A. Pryor, and H.R. Wenk: Mineral Dust in Manual Harvest Operations. In Proceedings of the ACGIH Agricultural Respiratory Hazards Symposium, *Ann. Am. Conf. Gov. Ind. Hyg.* 2:101-15 (1982).
62. Woodget, B.W. and Cooper: Samples and Standards: John Wiley and Sons, pp. 63-70, 1987.
63. Wright, Bob W., S.R. Frye, D.G. McMinn, and R.D. Smith: On-Line Supercritical Fluid Extraction-Capillary Gas Chromatography. *J. Anal. Chem.* 59:640 (1987).
64. Trucksess, M.W., W.C. Brumley, and S. Nesheim: Rapid Quantitation and Confirmation of Aflatoxins in Corn and Peanut Butter, Using a Disposable Silica Gel Column, Thin Layer Chromatography, and Gas Chromatography/ Mass Spectrometry. *J. Assoc. Off. Anal. Chem.* 67:973-75 (1984).
65. Goto, T., M. Matsui, and T. Kitsuwa: Determination of Aflatoxins by Capillary Column Gas Chromatography. *J. Chromatogr.* 447:410-14 (1988).
66. Kamimura, H., M. Hishijima, K. Yasuda, K. Saito, A. Ibe, T. Nagayama, H.

Ushiyama, and Y. Naoi: Simultanous Detection of Several Fusarium Mycotoxins in Cereals, Grains, and Foodstuffs. *J. Assoc. Off Anal. Chem.* 64:1067-73 (1981).

67. **Sydenham, Eric W., W.C.A. Gelderblom, P.G. Thiel, and W.F.O. Marasas:** Evidence for the Natural Occurrence of Fumonisin B_1, a Mycotoxin Produced by Fusarium Moniliforme, in Corn. *J. Agr. Food Chem.* 38:288 (1990).
68. **Markides, K.E., S.M. Fields, and M.L. Lee:** Capillary Supercritical Fluid Chromatography of Labile Carboxylic Acids. *J. Chromatogr. Sci.* 24:254 (1986).
69. **David, P.A. and M. Novotny:** Separation of α-Keto Acids by Capillary Supercritical Fluid Chromatography. *J. Chromatogr.* 452:623-29 (1988).
70. **Castegnaro, M., M. Friesen, J. Michelon, E.A. Waler:** *American Industrial Hygiene Association*, 42:398-401, 1981.
71. **Selim, M.I. and J.R. Strubinger:** Partition of Supercritical n-Pentane into SE-54 and SE-30 Stationary Phases in Capillary Supercritical Fluid Chromatography. *Fresenius Z. Anal. Chem.* 330:246-49 (1988).
72. **Selim, M.I. and J.R. Strubinger:** Validation of Recent Theory for the retention Mechanism in Supercritical Fluid Chromatography. *J. Chromatogr. Sci.* (Under publication).
73. **Donham, K.J., J.A. Merchant, W. Popendorf, and L. Burmeister:** Preventing Respiratory Disease in Swine Confinement Workers: Intervention through Applied Epidemiology, Education, and Consultation. *Am. J. Ind. Med.* In press.

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Table I

Concentration of Aflatoxin B1 in Airborne Dust
Using TLC on Samples Collected
During Harvest and Handling of the 1988 Corn Crop in Iowa

Farm	Concentration of aflatoxin B_1 in airborne dust (ng/m^3)		
	Harvest	unloading	feeding
Farm #1	ND	ND	NA
Farm #2	66.6(i)	ND	NA
Farm #3	ND	ND	NA
Farm #4	ND	92.1	8.1

i = inside tractor cab
NA = Not available, samples were not collected
ND = not detected

Table II

Concentration of aflatoxin B_1 in Airborne Dust,
Animal Feed and Settled Dust in Animal Confinement Buildings
Summer 1989

Farm	Concentration of Aflatoxin B_1		
	Feed ng/g	Settled Dust ng/g	Airborne Dust ng/m^3
Farm #5	ND	125 (n=2)	6.3 (n=2)
Farm #6	27.7 (n=3)	227 (n=3)	5.5 (n=2)

ND = Not detected
TLC Detection Limits were ≈ 2 ng

TABLE III

Recovery of Aflatoxin B_1 from Fiber Glass Filters

Extraction Method	Spiking Level (μg/filter)	Mean % Recovery	Standard Deviation	Coeff.of Variance
Liquid/Liquid	0.2	79.47 (n=3)	16.67	20.99%
SFE with CO_2	0.1	91.60 (n=3)	8.47	9.24%

TABLE IV

Recovery of Aflatoxin B_1 from Settled Hog Dust

Extraction Method	Spiking Level (ppm)	Mean % Recovery	Standard Deviation	Coeff.of Variance
Liquid/Liquid	25	60.65 (n=3)	6.83	11.27%
SFE with CO_2	18	75.67 (n=3)	0.58	0.76%

Table V

Extraction of Aflatoxin B_1 from Naturally Contaminated

Extraction Method	% Modifier	Concentration Aflatoxin B_1 (ppb)
Classical Liquid / Liquid	NA	25.0
SFE with CO_2	0	25.6
SFE with CO_2 + Methanol	0.6	29.8
SFE with CO_2 + Methanol	1.3	33.1
SFE with CO_2 + Methanol	3.1	94.8
SFE with CO_2 + Methanol	5.0	NQ
SFE with CO_2 + Acetonitrile	5.0	98.7

NA= Not applicable
NQ= Not quantified due to background interfernces.

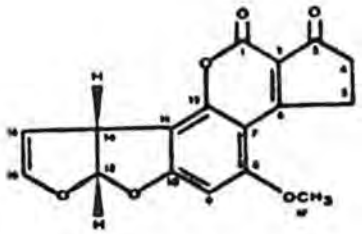
Table VI

Concentration of Aflatoxin B_1 in Airborne Dust
During Bin Clean-Out Operation

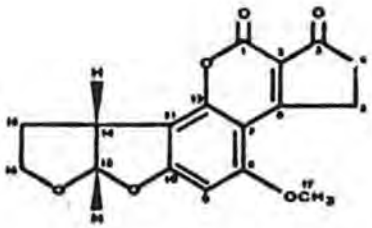
FARM NUMBER	Aflatoxin Conc. in Dust (ppm)	Aflatoxin Conc. in Bulk Corn (ppm)	Aflatoxin Conc.in Dust/ Conc. in Corn
1	5.960	0.016	373.5
2	21.360	0.309	69.1
3	0.590	0.015	393.3
4	3.350	0.035	95.7
5	6.510	0.082	79.4
6	ND	0.010	0.0
7	1.460	0.040	36.5
8	7.280	0.207	35.2
9	7.100	0.120	59.2
10	60.120	0.002	300060.0
11	65.430	ND	0
12	1.550	ND	0
13	ND	ND	0
14	2.450	0.041	59.8

Appendix A

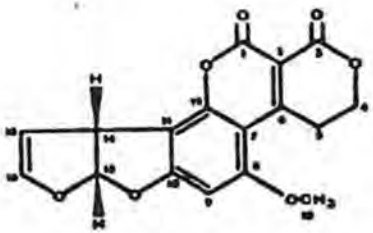
Chemical Structure of Aflatoxins and Other Mycotoxins of Interest to this Project



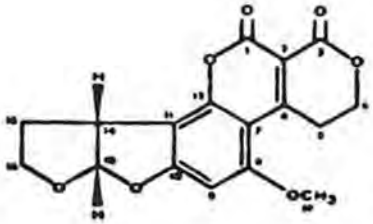
Aflatoxin B_1



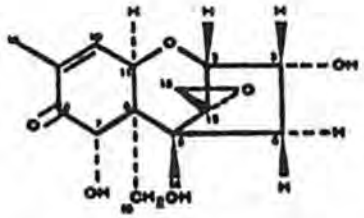
Aflatoxin B_2



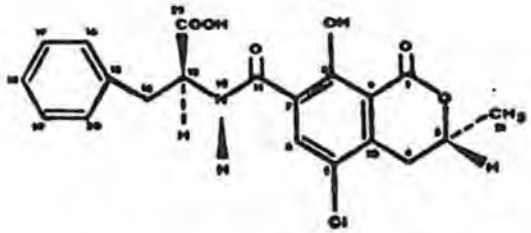
Aflatoxin G_1



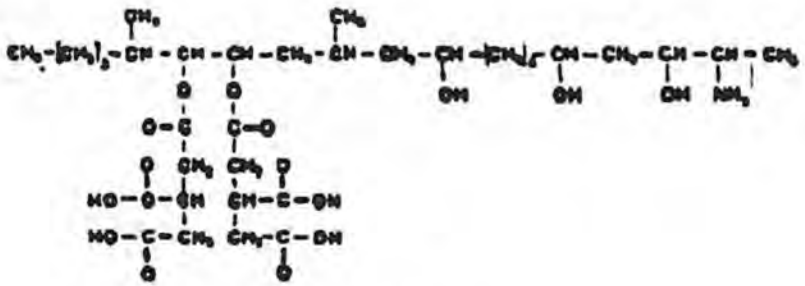
Aflatoxin G_2



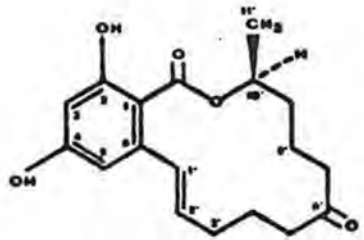
Deoxynivalenol (Rd toxin) (Vomitoxin) (3α, 7α,15-Trihydroxy-12,13-epoxytrichothec-9-en-8-one)



Ochratoxin A (7-Carboxy-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocoumarin-7-L-β-phenylalanine)



Fumonisin B_1



(*S*)-Zearalenone (F-2)([6-(10-Hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone])

Appendix B

Figure B1:	Schematic Diagram for the Off-line SFE System used in the Preliminary Work.
Figure B2-B4:	Examples of the Off-Line HPLC Analysis of Aflatoxins in Naturally Contaminated Corn - Effect of Modifiers on the Efficiency and Selectivity of SFE.
Figure B5:	Conditions for the SFE and HPLC used in the Preliminary Work.
Tables I-V:	Preliminary Data on SFE Method Development and Analysis of Field Samples for Aflatoxins.

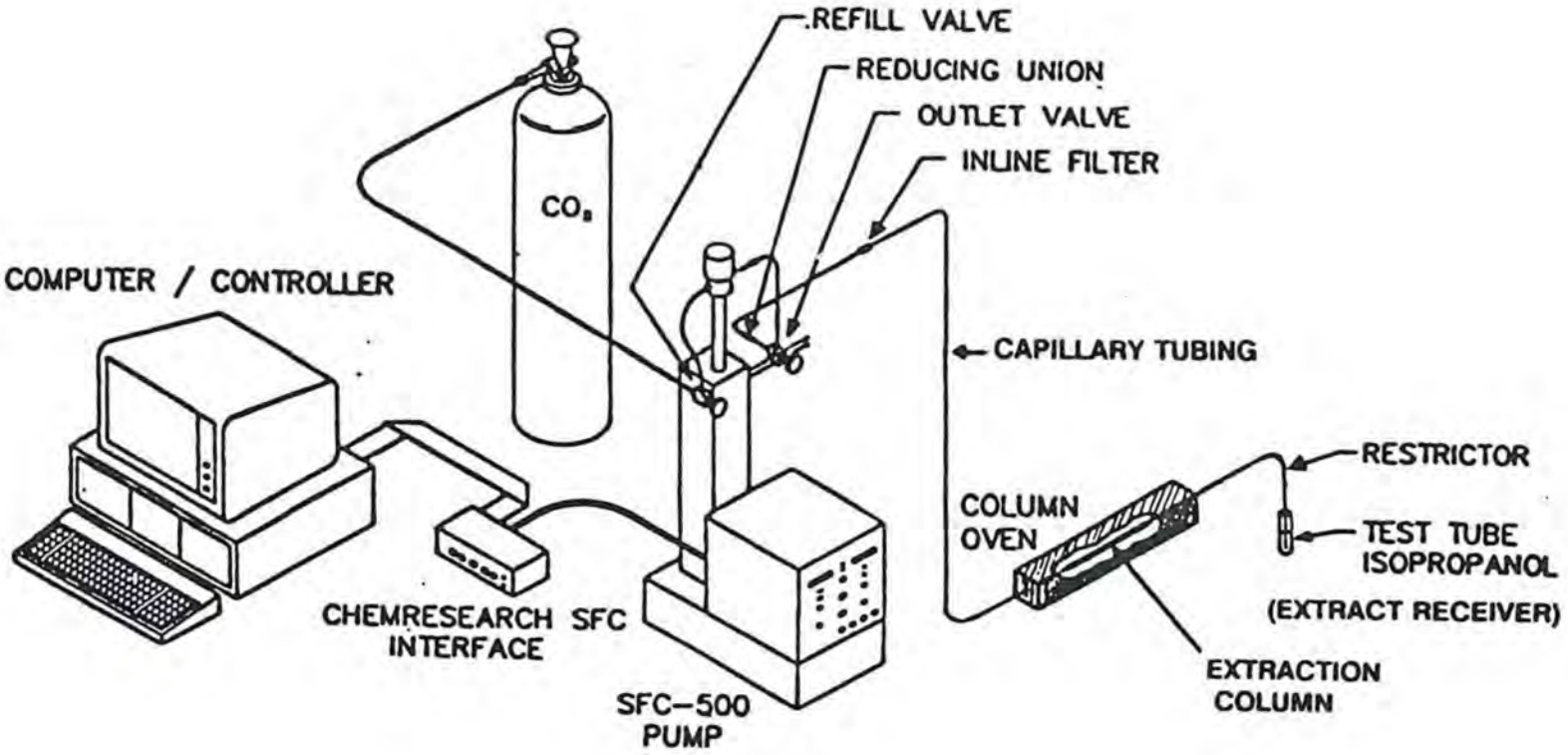


Figure B1: Schematic Diagram for the Preliminary off-Line Supercritical Fluid Extraction (SFE) System.

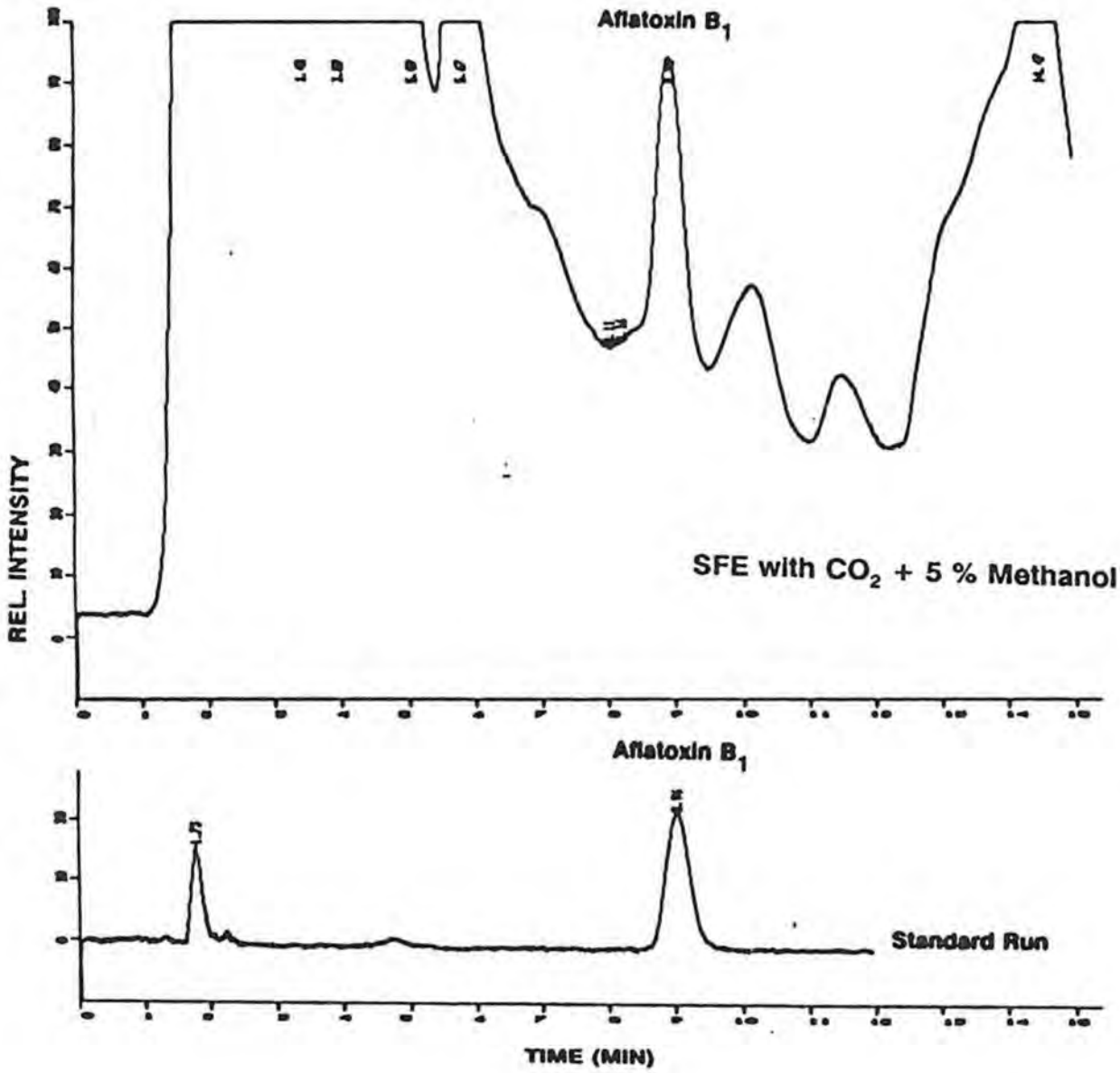


Figure B3: Effect of Increasing Methanol on the SFE of Aflatoxin

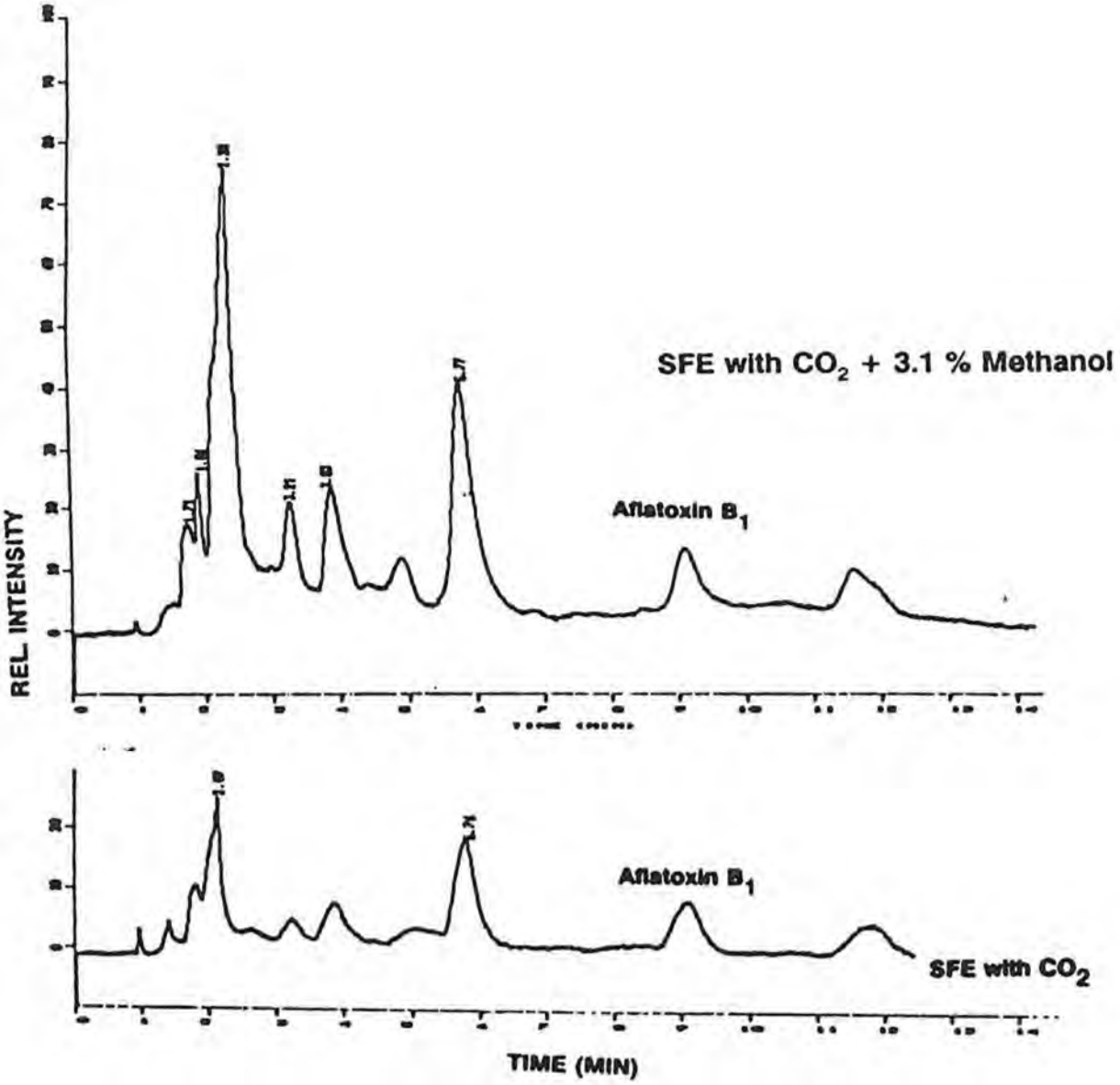


Figure B2: Effect of Methanol on the SFE of Aflatoxin

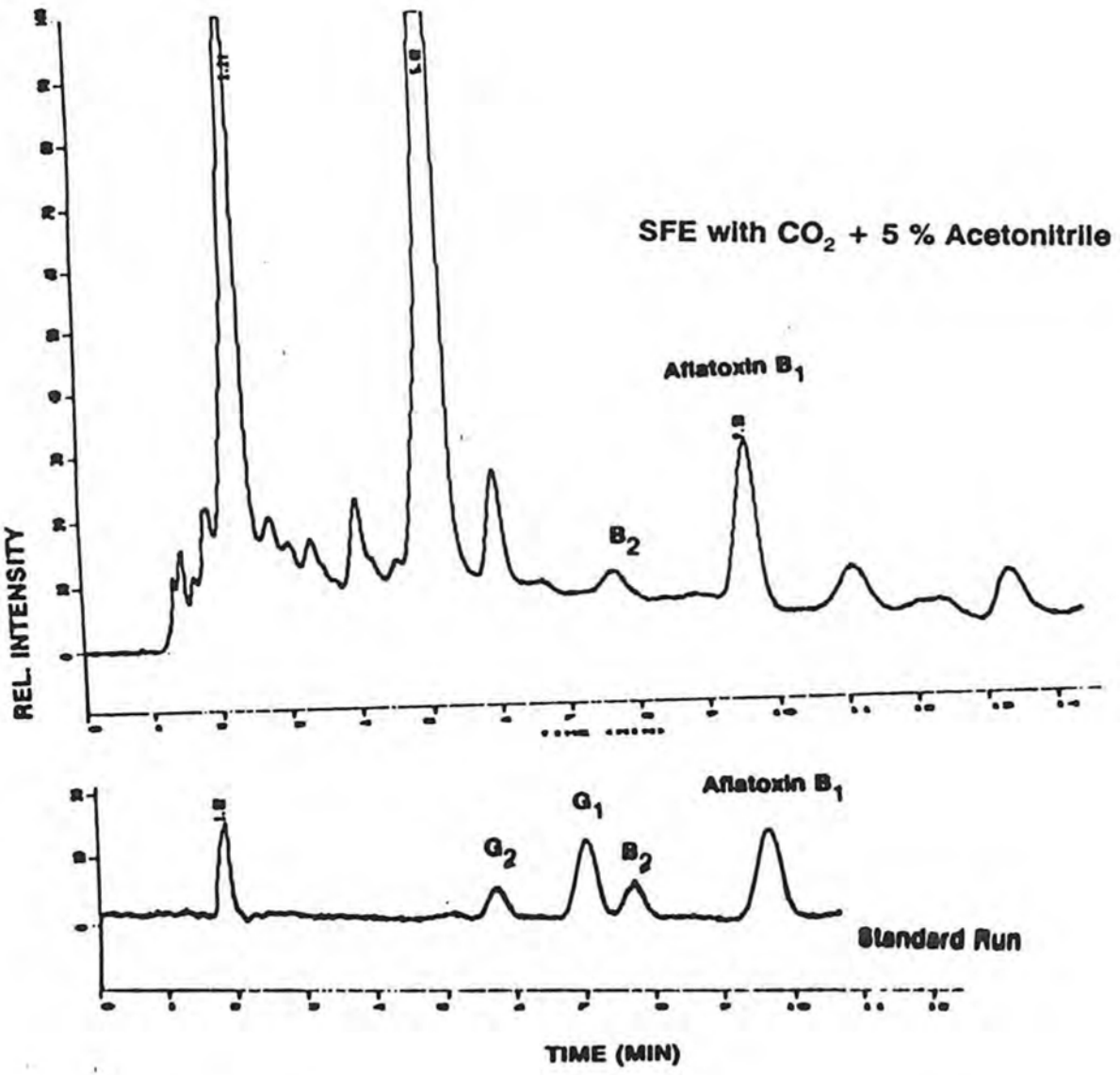


Figure B4: Effect Acetonitrile on the SFE of Aflatoxin

Conditions for the Supecritical Fluid Extraction (SFE)

and

High Pressure Liquid Chromatography (HPLC) Analysis

Used in the Preliminary Study

SFE Conditions:

Static extraction with supercritical carbon dioxide

for 10 minutes followed by dynamic extraction

under the following conditions:

Temperature	**: 40 °C**
Pressure	**: 4,000 psi**
CO_2 Volume	**: 5 x thimble volume (void volume of the extraction cartridge)**

Total extraction time is approximately 20 minutes.

HPLC conditions :

Column: **C_{18}, 25 cm x 4.6 mm (5 μm packing).**

M. Phase: **Methanol-Acetone-water (22.5:22.5:55)**

Flow rate: **1.5 mL/min.**

Detection: **UV (365 nm, 0.02 AUFS).**

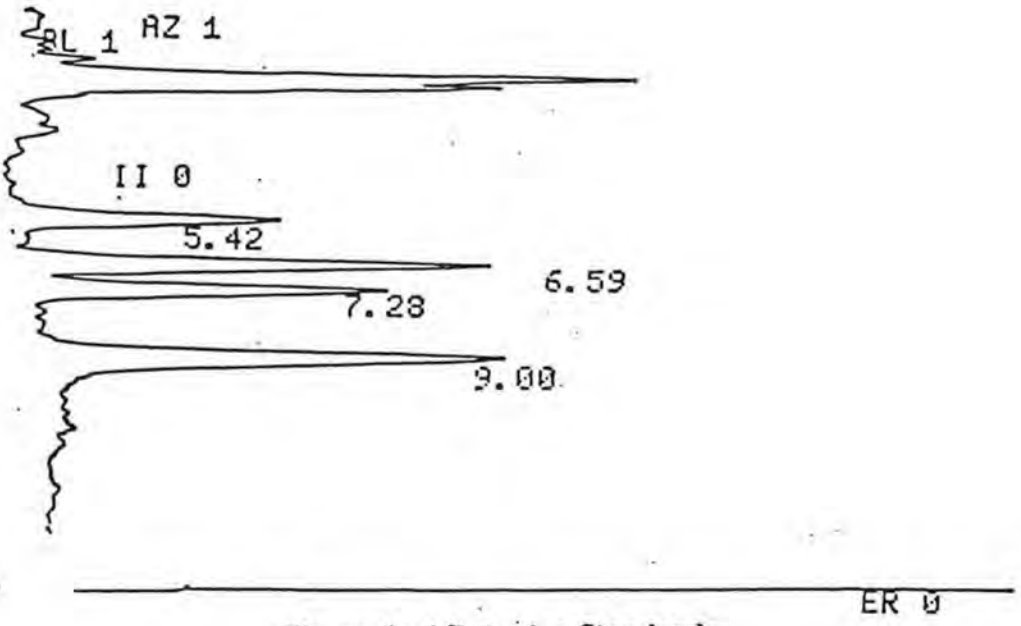


Figure A: Aflatoxins Standards

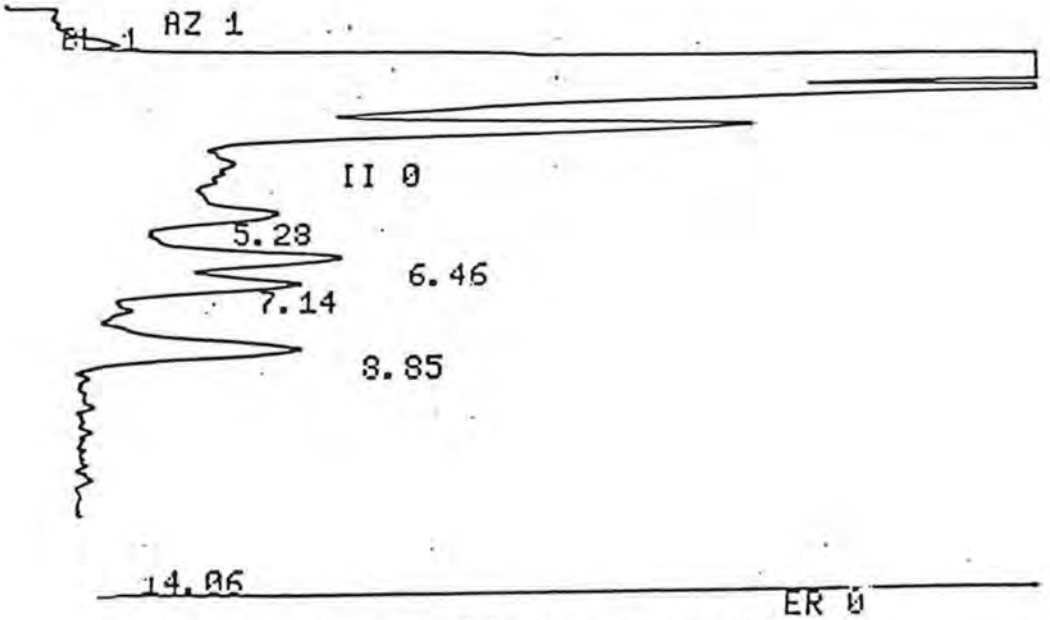


Figure B: Grain Dust Sample Matrix
Spiked with Aflatoxins Standards

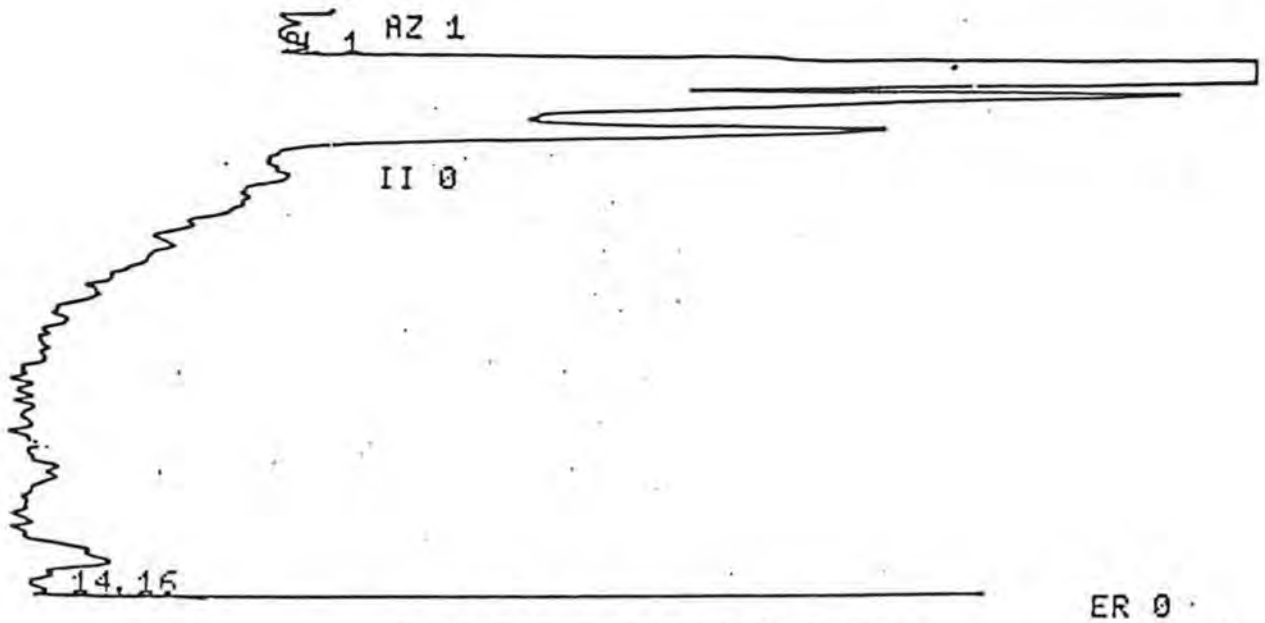


Figure C: Grain Dust Sample without Aflatoxins Contamination

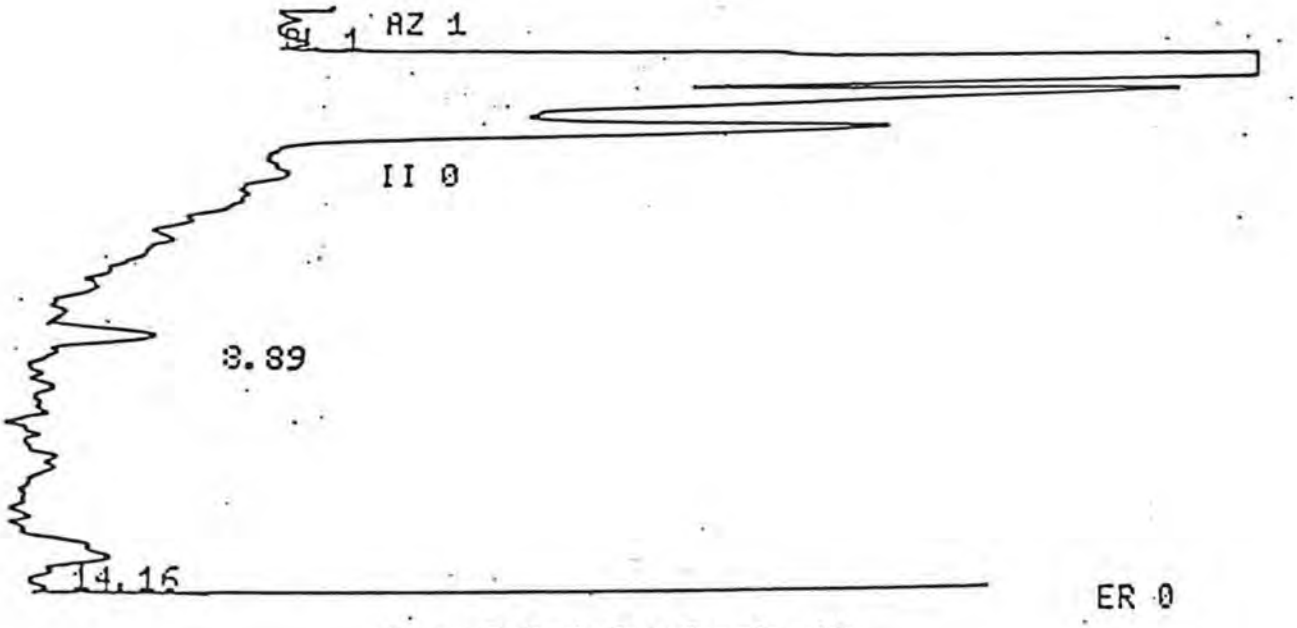


Figure D: Grain Dust Sample with Aflatoxin B_1 Contamination

Appendix C

- Reference Analytical Methods:

  a. Classical Liquid Extraction and Cleanup Methods for Aflatoxins from Corn and Grain Dust.
  b. TLC and HPLC Methods for Aflatoxins.
  c. Classical Liquid Extraction of Fumonisins.
  d. TLC and HPLC Method for Fumonisins

- Optimization of SFE for Aflatoxins and Fumonisins.

- Multimycotoxin Screening

- Quality Control and Quality Assurance Plan.

- Safety Precautions.

Appendix C

Reference Analytical Methods

Extraction of Aflatoxins from Corn and Grain Dust - Classical Methods:

The following methods were used in our preliminary work for comparison with the proposed supercritical fluid extraction (SFE) method. These methods are based on the literature citations listed below. The analyte (aflatoxins) is solvent extracted from its solid matrix, followed by solvent exchange through liquid/liquid extraction, extract defatting, extract concentration, and extract clean-up.[1,2] The clean extract is then analyzed by thin layer chromatography (TLC)[1-4], or high pressure liquid chromatography (HPLC)[5,6], gas chromatography (GC)[7], or gas chromatography/mass spectrometry (GC/MS)[8].

A. Aflatoxins From Contaminated Corn:

1. Extract 50 g of corn using 200 ml of acetonitrile-4% aqueous potassium chloride by blending in a Waring blender 5-10 minutes or shaking on a wrist-action shaker for 30 minutes.
2. Filter the extract through Whatman #1 filter paper.
3. Transfer 50 ml of the extract to a 250 ml separatory funnel and dilute with an equal volume of 10% NaCl solution.
4. Defat by extraction twice with 30 ml iso-octane, hexane or petroleum ether. Discard the defating extract (iso-octane, hexane or petroleum ether layer).
4. Extract aflatoxins from the defated extract with 3x30ml chloroform.
5. Evaporate the chloroform extract to dryness on a steam bath under nitrogen.

B. Aflatoxins from Grain Dust:

1. Dust samples collected on glass fiber filters are extracted with 150 Ml chloroform, 15 mL water, and 15 g Celite.
2. Wash the residue thoroughly with chloroform and combine the washings with the filtered chloroform extract.
3. Evaporate the combined chloroform on a steam bath under nitrogen.
4. Use 2-3 mL methylene chloride to dissolve the residue and transfer it quantitatively to a preconditioned silica sep-pack column for cleanup.
5. Follow the washing and elution procedure for the silica columns.
6. Evaporate the final eluate on a steam bath under nitrogen.
7. Dissolve the residue in 200 μL of the HPLC mobile phase for HPLC analysis, or 200 μL of benzene-acetonitrile (98+2) for TLC analysis.

C. Solid Phase Extraction (SFE) - Extract Clean-up:

All three types of commercial SFE columns, silica gel[9], C_{18}[10], & cyano[11], can be used for cleanup of aflatoxin extracts from corn and corn dust. We have evaluated the columns using standard spikes and naturally contaminated corn

samples. Cleaner extract and greater recoveries (approximately 100%) were obtained with cyano columns, and Supelclean LC-CN (Supelco, Inc., Supelco Park, Bellefonte, PA).

Elution or Clean-up Step	Column Type		
	Silica Gel	C_{18}	Cyano-
Col. Conditioning	3 ml hexane followed by 3 mL chloroform	Water followed by 80% methanol/20% water	2mL of .5% acetic acid in water
Sample Loading	methylene chloride	water or methanol or combination	1mL extract (in 20% water/80% methanol) + 2mL of .5% acetic acid in water
Col. Wash	3 mL hexane 3 mL anhydrous ethyl ether 3 mL chloroform	Col volume distilled water followed by 1-mL hexane. Air dry column under vacuum for 5 minutes	.5mL of THF:0.5% acetic acid (2:8), followed by 2mL hexane. Pass N_2 gas for 2 min to dry the packing. Pass 3mL. Wash w/ THF:hexane (1:3) then dry with N_2 for 1 minute.
Elution	6 mL chloroform-acetone (9+1)	Elute w/ (3) 500µl aliquots methylene chloride. Constitute to 2-ml. For HPLC evaporate to dryness under nitrogen and reconstitute with mobile phase.	2mL CH_2Cl_2:THF (99:1). Collect eluent in a silanized vial that is rinsed with methanol just before use. Take eluent to dryness and redissolve in appropriate TLC spotting solvent.

Note: In the loading and elution steps allow solvent to drip freely without vacuum. If vacuum is needed, do not exceed flow rate of 3 mL/min.

D. Thin Layer Chromatographic Analysis (TLC) :

One-Dimensional TLC:

1. Dissolve final extract residue in 200 μL benzene-acetonitrile (98+2).
2. Spot 10 - 20 μL of the extract to the TLC plate, Whatman K6 Silica Gel (Whatman International Ltd., Maidstone, England), 250 μm thickness.
3. Spot 5, 10 & 20 μL of a reference standard of the following concentrations:
   0.5 μg/mL aflatoxin B_1.
   0.1 μg/mL aflatoxin B_2.
   0.5 μg/mL aflatoxin G_1.
   0.1 μg/mL aflatoxin G_2.
4. Develop the TLC plate in chloroform-acetone-water (91+9+1) in an unlined tank.

Two-Dimensional TLC:

1. Develop first direction as in one-dimensional TLC above.
2. Develop the second direction in toluene-ethyl acetate-formic acid (60+30+10) in unlined tank.

Confirmation of the Identity of Aflatoxins from TLC Analysis:

A. View Under Long Wavelength UV:
   B_1, B_2: produce bluish fluorescent spots
   G_1, G_2: produce greenish fluorescent spots

B. Formation of Adducts with Trifluoroacetic acid:

1. Spray the 2-dimensional plate with 10% trifluoroacetic acid solution in acetone.
2. Heat in an oven for 110 °C for 5 minutes.
3. Observe under long wave UV light.
4. Compare the color and the R_f value of the unknown with those of the standard.

C. HPLC Analysis of Aflatoxins :

1. For further confirmation of the identity of aflatoxins, analyze the extract with HPLC using the following HPLC conditions.
   Column: C_{18}, 25 cm x 4.6 mm (5 μm packing).
   M. Phase: Methanol-Acetone-Water (22.5:22.5:55)
   Flow rate: 1.5 ml/min.
   Detection: UV (365 nm, 0.02 AUFS).
2. Compare the retention times of the unknown peaks in the extract with the retention times of aflatoxin standards.
3. Run a multilevel calibration (minimum of three concentrations bracketing the unknown concentration), and use the calibration curve to calculate the concentration of aflatoxin in the sample.

HPLC with Post-Column Derivatization :

This method allows the determination of aflatoxins B_1, B_2, G_1, G_2 down to 1 ppb levels. Aflatoxins are separated on the HPLC column and derivatized on-line with ß-cyclodextrin followed by fluorescence detection[6]. This highly sensitive method will be used as an alternative for the GC/MS method, since all the HPLC-post column derivatization instrumentation are available at our Analytical Toxicology Laboratory.

Optimization of the Supercritical Fluid Extraction Procedure for Aflatoxins:

Corn samples spiked with known concentrations of aflatoxins will be used to study the optimum laboratory conditions for the extraction and removal of aflatoxins from corn, using supercritical carbon dioxide. Figure 1 shows a schematic diagram for the laboratory apparatus which will be used in this study. Figure D1 (Appendix D) shows a schematic diagram for the proposed (requested) SFE instrumentation, which will be combined with the GC/MS as shown in Figure D3 (Appendix D) for on-line SFE/GC/MS analysis of aflatoxins in corn and dust samples.

The efficiency and selectivity of the SFE procedure (described on page 51-52) will be optimized using spiked corn and dust samples. Actual method performance will then be validated using naturally contaminated samples.

Preparation of Aflatoxin Spikes:

Representative corn and dust samples[12] will be analyzed for their aflatoxin content using the liquid extraction and HPLC analysis as described above, then used for preparation of spikes for studying the recovery and selectivity of the SFE method for aflatoxins. The corn samples are ground in a clean grinder, sieved to a consistent mesh size of 1 to 2 mm. The meal is stored in a desiccator and used for the preparation of the spikes for the SFE work.

Two methods will be used for preparing aflatoxin spikes, homogeneous mixing of the spike with the meal matrix, or direct application of the spiking solution to the meal in the extraction vessel. Both methods will be evaluated for their accuracy and reproducibility, the more reliable method will be used in the rest of the study.

Direct addition of the toxins to the meal is a fast and convenient spiking procedure, but does not provide realistic representation of the naturally contaminated corn samples. In addition, minimum volume of standard solutions must be used in order to avoid the effect of the solvent on the SFE process.

Homogeneous meal spikes are prepared by adding known volume of a standard aflatoxin solution to a known weight of the meal in a round bottom flask. Sufficient amount of methanol is then added to cover the surface of the meal. The round bottom flask is mounted on a rotary evaporator and the solvent is evaporated under vacuum and gentle heating in a water bath. The flask is rotated slowly, during solvent evaporation, in order to allow even distribution of

aflatoxins in the meal matrix. After complete evaporation of the solvent, the spiked meal is transferred from the round bottom flask to a porcelain dish and placed in a vacuum oven to complete dryness. The dry spiked-meal is stored in a desiccator and used in the SFE optimization experiments.

Optimization of the SFE Procedure for Aflatoxin B_1:

In order to optimize the SFE conditions, several experimental parameters will be considered. These include[13,14]:

1. temperature of the extraction column.
2. density of supercritical carbon dioxide.
3. extraction time - fluid/matrix contact time.
4. effect of fluid modifiers, selective modifiers which have determined in our preliminary work and referred to as modifiers A and B.
5. superficial velocity
6. physical condition of corn matrix before extraction, e.g., moisture content and mesh size of ground samples.

Multimycotoxin Screen:

The following reference methods will be used for simultaneous analysis of aflatoxins, zearalenone, vomitoxin, and ochratoxin in corn and corn dust samples[9].

These methods will be used during the later part of phase II.

Materials:

High speed blender, filter paper (Whatman #4), separatory funnel, beakers.

All chemicals used were purchased from Fisher. The solvents used were pesticide grade.

Mycotoxin Standards:

Aflatoxin Standard mixture (Applied Sciences Laboratories)
Ochratoxin A (Aldrich Chemical Company)
Desoxyinvalenol (Vomitoxin) (Myco Lab. Co.)
All other standards were obtained from Sigma Chemical Company.

Reagents:

1. Extraction solvent - Acetonitrile/KCl (4%; aqueous) 9:1
2. Iso-Octane
3. Ferric chloride gel (100 ml dist. H_2O, 80 ml 0.2 N NaOH, 10 ml ferric chloride (10% in H_2O)), 3 gms of cupric carbonate and a 100 ml beaker full of Infusorial earth. All mixed well.
4. Chloroform
5. Sodium sulfate

6. Spotting solvent - Benzene:Acetonitrile (98:2)
7. TLC plates - Baker Silica 250F
8. Developing solvents:
   A. Toluene-Ethyl acetate-Formic Acid (30:15:5)
   B. Toluene-Ethyl acetate-Acetone (30:20:10)
9. p-Anisaldehyde spray:
   Mellanol-glacial acetic acid-conc.- H_2SO_4- p-Anisaldehyde (85:10:5:0.5)

Liquid Extraction:

1. Extract 50 gm corn sample in a blender with 200 mL of extraction solvent (1) for 2 minutes.
2. Filter the mixture through Whatman #1 filter paper and transfer the extract to a separatory funnel.
3. Defat the filtrate with 50 mL of Iso-Octane (2).
4. Allow phases to separate and drain the lower (Acetonitrile/aq.KCl) phase into a beaker containing the Ferric chloride gel (3).
5. Discard the upper Iso-Octane phase.
6. Mix the gel and acetonitrile phase thoroughly, then filter.
7. Transfer the filtrate to a separatory funnel and extract with 100 mL Chloroform (4).
8. Filter the chloroform extract through a layer of anhydrous sodium sulfate into a beaker.
9. Evaporate the chloroform extract to dryness.

TLC (Thin Layer Chromatography) Analysis:

1. Redissolve the sample extract in 500 μL of spotting solvent (6).
2. Spot 50 μL on a thin layer plate (7) along with the appropriate Mycotoxin Standards.
3. Develop the plate first in solvent system (8a) to about 2/3 of the plate.
4. Air dry the Plate and submit to the second solvent system (8b).
5. Develop the plate to 1 to 2 inches of the top.
6. Allow solvents to evaporate and examine under UV light for the presence of **aflatoxins, zearalenone and ochratoxin.**
7. Spray the plate with p-Anisaldehyde spray (9) for the detection of **Vomitoxin** (and also T-2 toxin). After spraying, the plate is heated in a oven for 10 minutes at 120°C.

Summary of the TLC Method for Fumonisin:

Reagents:

1. 1 % potassium chloride
2. Developing solution: 3:2 methanol: 1 % KCL
3. 0.1 M sodium borate
4. fluorescamine (100 mg in 100 ml of acetonitrile)
5. 0.01 M boric acid: acetonitrile (6:4)

Procedure:

1. Evaporate extracted samples to 0.5 ml.
2. Spot 20 μl of sample.
3. Place in development solution.
4. Spray with sodium borate.
5. Immediately spray with fluorescamine.
6. Wait for 1 minute, then spray with acetonitrile/boric acid.
7. Observe under longwave UV light. (Fumonisin appear as bright greenish fluorescent band)

Derivatization:[15-19]

The derivatization method uses fluorescamine to form flurophoric derivative.

Reagents:

1. 0.1 M Borate Buffer, pH 8.01
2. Fumonisin Stock Solution (213.52 μg/ml in methanol)
3. dry acetone (redistilled acetone to remove any amine contamination)
4. Fluorescamine solution (28 mg/ 100 ml dry acetone)

Procedure:

1. Place 250 μl of the fumonisin stock solution in a small test tube.
2. Evaporate to dryness under N_2.
3. Add 250 μl of 0.1 M borate buffer to the test tube and vortex well.
4. Add 100 μl of fluorescamine to the mixture and vortex it vigorously for a period of 30 minutes.

The fluorophoric derivative formed was found to be stable for at least four hours at room temperature and 24 hours if maintained in subzero temperatures.

<u>HPLC Method for Fumonisin</u>:

Reagents:

1. HPLC grade methanol
2. 0.05 M potassium chloride (pH 7.5 and 8.5)

Conditions:

1. Pump: ISCO model 2350
2. Column: Supelco LC-18, 5 μm particle size (15 cm x 4.6 mm)
3. Eluant: methanol: potassium chloride (1:9)
4. flow rate: 1.0 ml/min.
5. Retention time: 3.25 minutes
6. Detector: ISCO V_4
7. Wavelength: 390 nm

Liquid-liquid Extraction for Fumonisin:[20]

Reagents:

1. Ethyl Acetate
2. Methanol:Water (3:1)

Procedure:

1. Measure 1 gram of sample.
2. Extract sample with 2 ml of ethyl acetate. (do twice)
3. Reextract with 1 ml methanol:water. (do this three times)
4. Evaporate to dryness.
5. Reconstitute in 1 ml of methanol:water.
6. Filter through a .45 μm filter and then a .2 μm filter.

Quality Control/Quality Assurance and Safety Precautions:

Method sensitivity, accuracy, detection limit, and linear quantification range will be established using pure standards and matrix spikes. Bulk corn and settled dust with undetectable levels of aflatoxins will be used for the preparation of the matrix spikes. Method performance will be validated using the parallel field samples, which will be purposely collected during the method development phase.

All laboratory equipment (e.g. analytical balances, pH meters, etc.) and analytical instrumentation (e.g. GC, SFC, HPLC, MS, etc.) will be routinely calibrated, and their performance will be recorded consistent with good laboratory practices (GLPs). Raw data will be maintained in duplicate-copy notebooks, and reviewed routinely by the PI. Preparation of standards, laboratory calibrations and QC-charts will be maintained for routine check of reliability of the standards and the accuracy and precision of analytical methods. Detailed standard operating procedure, and quality control protocol will be documented by the end of phase I, before application of the selected methods to actual field samples.

Safety Precautions:

Good laboratory practices and extreme caution will be followed during the course of this work to avoid exposure of laboratory or field personnel to mycotoxins. All sample handling will be carried out in an efficient hood. Preparation and handling of stock standards and dilutions will be performed in a glove box. Disposable rubber gloves and dust respirators will be worn during sample handling. Organic vapor respirators with activated carbon will be worn during handling of concentrated stock standards. A 5% by volume sod hypochlorite, containing 5% by volume acetone, and pH adjusted to 7.8 - 8.0 by HCl, will be used to decontaminate lab ware from aflatoxins. Safety data sheets for the mycotoxins used and in-house safety and sample handling protocol will be made available to all personnel involved.

References:

1. Shotwel, O., W.R. Burg, and T. Diller: Thin Layer Chromatographic Determination of Aflatoxin in Corn Dust. *J. Assoc. Off. Anal. Chem.* 64:1060-1063 (1981).

2. Association of Official Analytical Chemists (AOAC): Official Methods of Analysis, 13th Edn. Edited by S. Williams. Arlington, VA, Secs 26.049, 26.026-26.031, 1980.

3. Dashek, M.V., T. Eadie, G.C. Llewellyn, S.A. Olenchock, and G.H. Wirtz: Thin Layer Chromatographic Analysis of Aflatoxins Within Grain Dusts. *J. Am. Oil Chem. Soc.* 60:563-566 (1983).

4. Stahr., H.M.: *Analysis of Submicro Amounts of Aflatoxins by Thin Layer Chromatography*. In Advances in Thin Layer Chromatography, edited by J.C. Touchstone. New York: John Wiley and Sons, pp. 176-193 (1982).

5. Scott, P.M.: Liquid Chromatography in the Trace Analysis of Mycotoxins. In Trace Analysis. Vol. 1. Academic Press, Inc., 1981. pp. 193-266.

6. Francis, O.J., G.P. Kirschenheuter, G.M. Ware, A.S. Carman, and S.S. Kuan: ß-cyclodextrin Post Column Fluorescence Enhancement of Aflatoxins for Reverse-Phase Liquid Chromatographic Determination in Corn. *J. Assoc. Off. Anal. Chem.* 71:725-728 (1988).

7. Goto, T., M. Matsui, and T. Kitsuwa: Determination of Aflatoxins by Capillary Column Gas Chromatography. *J. Chromatogr.* 447:410-414 (1988).

8. Trucksess, M.W., W.C. Brumley, and S. Nesheim: Rapid Quantitation and Confirmation of Aflatoxins in Corn and Peanut Butter, Using a Disposable Silica Gel Column, Thin Layer Chromatography, and Gas Chromatography/ Mass Spectrometry. *J. Assoc. Off. Anal. Chem.* 67:973-975 (1984).

9. Ehrlich, K.C. and L.S. Lee: Mycotoxins in Dust: Method for the Analysis of Aflatoxins, Ochratoxins A, Zearalenone, Vomatoxin, and Secalonic Acid D. *J. Assoc. Off. Anal. Chem.* 67:963-67 (1984).

10. Supelco, Ic.: Rapidly Extract Aflatoxins from Corn Meal by Solid Phase Extraction - HPLC Bulletin 847. Bellfonte, PA: Supelco, Inc. 1986.

11. Baker, J.T.: Solid Phase Extraction for Sample Preparation. Phillipsburg, NJ: J.T. Baker, Inc. 1988.

12. Woodget, B.W. and D. Cooper: Samples and Standards. New York: John Wiley & Sons. 1987.

13. McHugh, M.A., and V.J. Krukonis: *Supercritical Fluid Extraction - Principles and Practice*. Boston, MA: Butterworths (1986).

14. McNally, M.L.P. and J.R. Wheeler: Increasing Extraction Effiency in Supercritical Fluid Extraction From Complex Matrices - Predicting Extraction Efficiency In Supercritical Fluid Extraction Using Supercritical Fluid Chromatographic Retention. *J. Chromatogr.* 447:53-63 (1988).

15. Dalby, Archives of Biochemistry and Biophysics, 63, 390 and Leimgruber, W.

16. Stein, S., Bohlen, P., Stone, J., Dairman, W., and Udenfriend, S. (1973) *Archives of Biochemistry and Biophysics* 155, 202.

17. Samejima, K. (1974) *J. of Chromatography* 96, 250.

18. Felix, A.M. and Jimenez, M.H. (1974) *J. of Chromatography* 89, 361.

19. Bohlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) *Archives of Biochemistry and Biophysics* 155, 213.

20. Gelderblom, W.C.A., Jaskiewicz, K., Marasas, W.F.O., Thiel, P.G., Horak, R., Kriek, N.P.J. (1988) *Applied and Environmental Microbiology* 54, 1806.

Appendix D

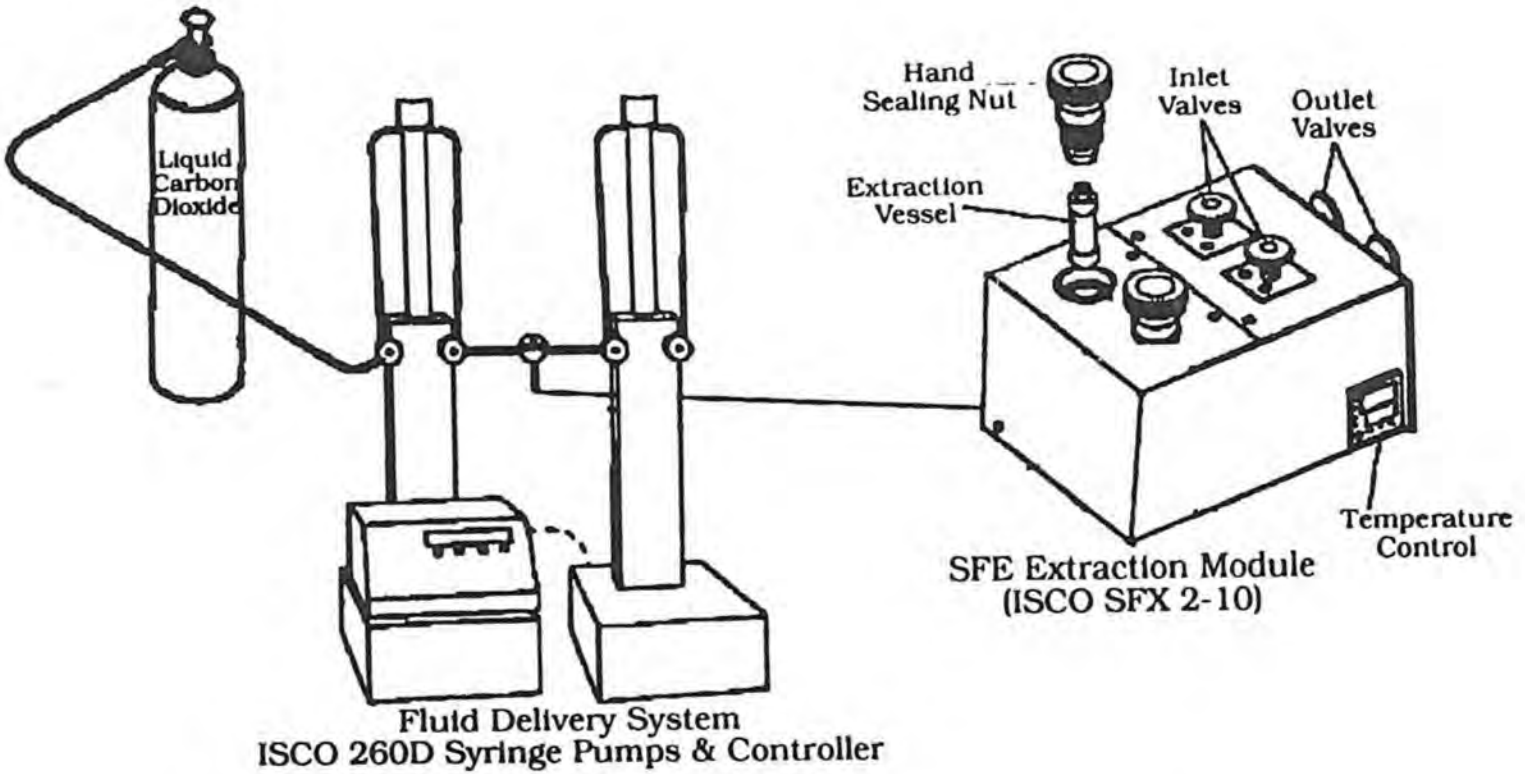


Figure D1: Schematic Diagram for the Proposed SFE Instrumentation.

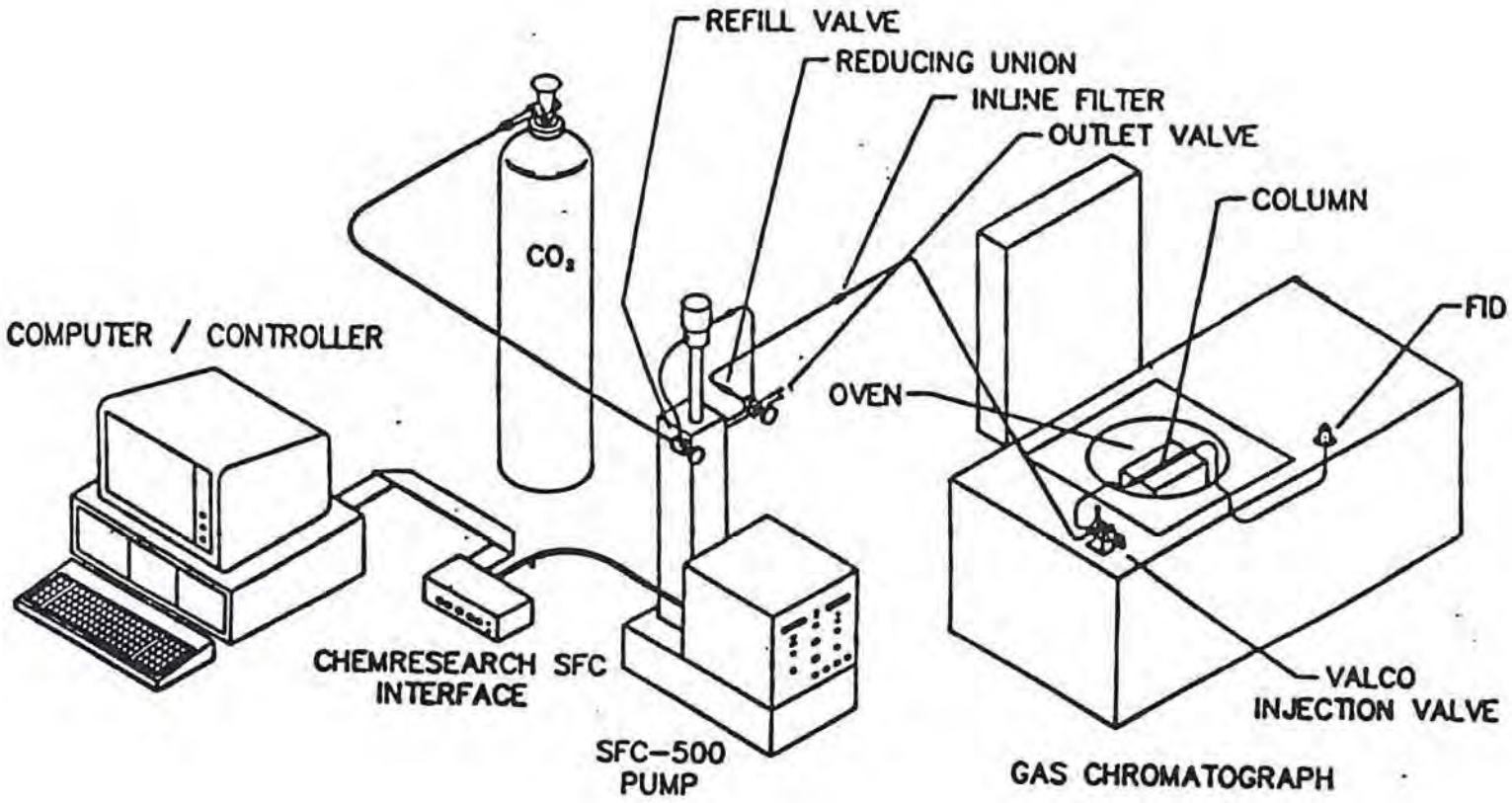


Figure D2: Basic Components of Available Supercritical Fluid Chromatography (SFC) System.

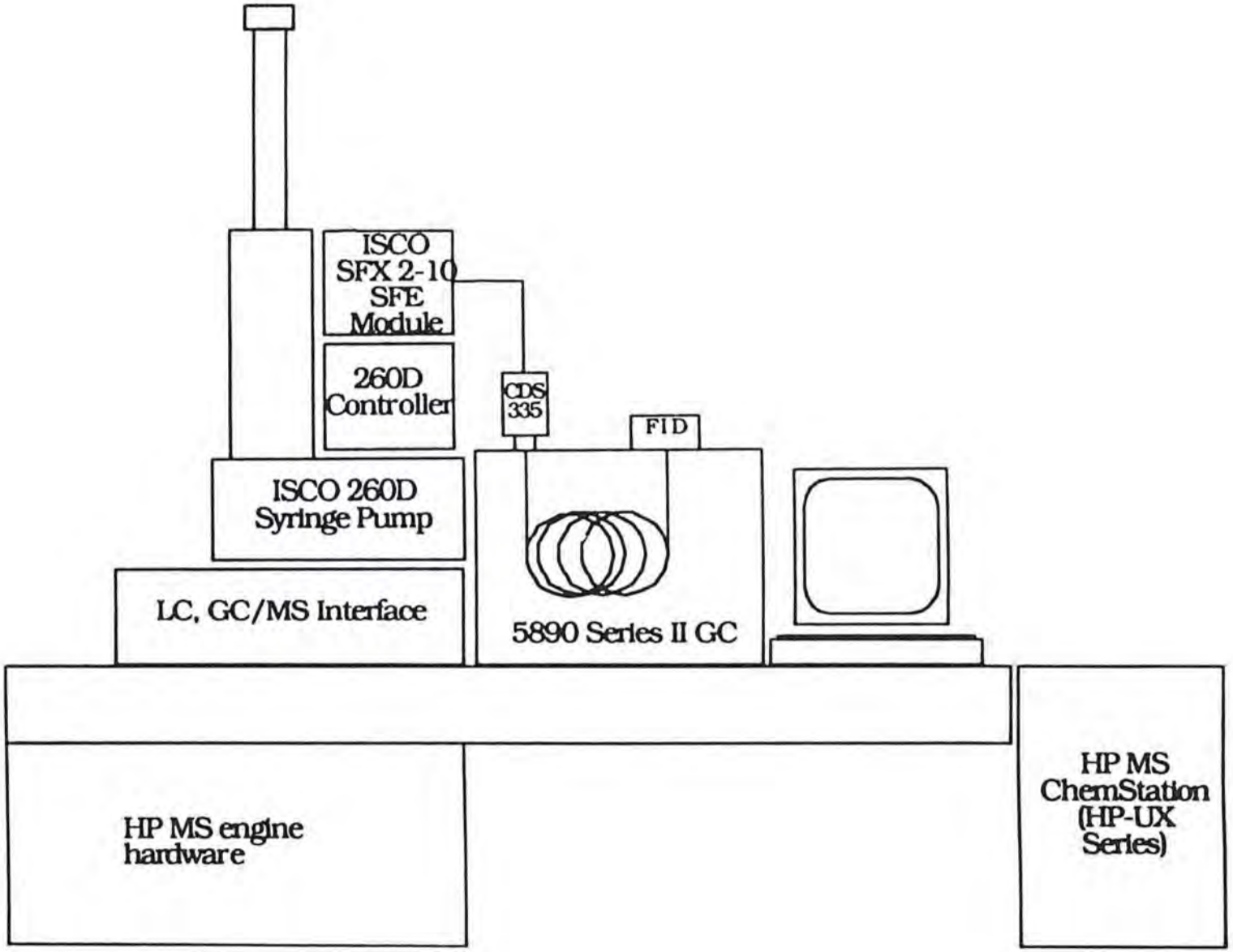
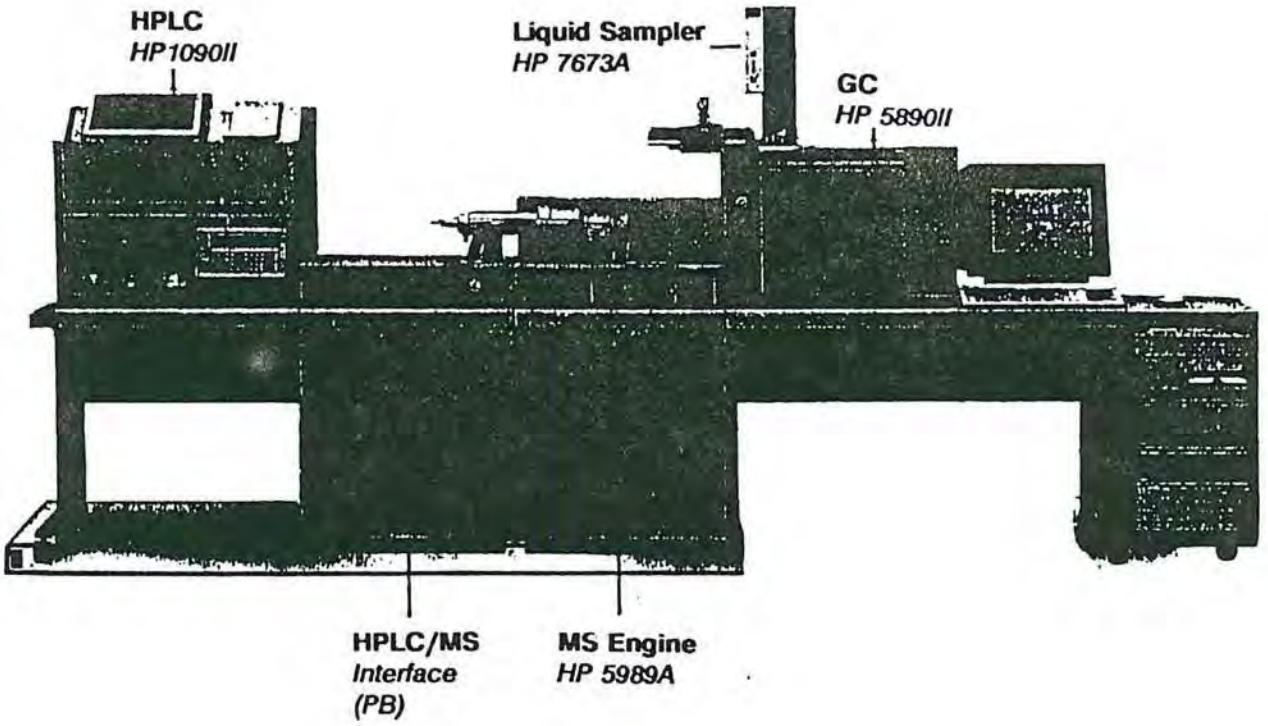


Figure D3: Schematic Diagram for the SFE/GC,SFC,LC/MS System

h-0

FIGURE 4: Newly Installed GC, HPLC/MS



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Appendix E

DEVELOPMENT AND OPTIMIZATION OF A SUPERCRITICAL FLUID EXTRACTION METHOD FOR THE ANALYSIS OF AFLATOXIN B_1

Am. Ind. Hyg. Assoc. J. 54(4):135-141 (1993)

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Appendix F

SUPERCRITICAL FLUID EXTRACTION OF FUMONISIN B_1 FROM GRAIN DUST

Submitted for publication, Am. Ind. Hyg. Assoc. J. (1993)

Mustafa I. Selim
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Appendix G

DISTRIBUTION OF AFLATOXIN B_1 IN AEROSOLIZED GRAIN DUST

An M.S. Thesis by:
Alex Martin Juchems

(Data deing prepared for publication)

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Centers for Disease Control

Memorandum

Date OCT 29 1993

From S. Price Connor, Ph.D., Research Grants Program Officer SPC
Grants Program Office, OD, NIOSH

Subject Final Report Submitted for Entry into NTIS for Grant 1 R01 OH02857-01

To William D. Bennett
Information, Acquisition and Data System Section (IADSS), TIB, DSDTT, NIOSH

The attached Final Report has been received from the principal investigator on the subject NIOSH grant. Please determine if this document should be forwarded to the National Technical Information Service (NTIS) for entry into the NTIS data base.

Attachments
Grant Summary
Final Report

Check E-mail
Masters Thesis

Assessment of Occupational Exposure to Aflatoxin

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108 AMRF - Oakdale Campus
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Other Occupational Needs
1 R01 OH02857-01
05/01/91 – 10/31/92
$192,394 ($192,394 Cum)

Importance to Occupational Safety and Health

Available epidemiologic studies from around the world provide evidence for association between the incidence of lung and other cancers and past exposure to aflatoxins in contaminated grain dust. In the United States, limited exposure data are currently available to assess the potential risk of lung cancer among farmers and agricultural workers due to inhalation of aflatoxin contaminated grain dust.

Past analysis of grain dust samples from the midwest and southeast corn growing belt has demonstrated the presence of aflatoxins in high volume samples of airborne dust. Recent drought conditions have led to flourishing fungal growth and consequent production of some alarming levels of aflatoxins in the corn crop. Our preliminary study indicates once detected, aflatoxin B_1 in airborne dust samples collected during harvesting, continues throughout grain unloading and animal feeding. The possibility for repeated hot and humid seasons creates a growing need to define the possible role of aflatoxins in the etiology of lung and other cancers.

Objectives

The long-term goal of this proposal is to determine the average yearly exposure of farmers to aflatoxin B_1 in airborne grain dust from repeated low level exposures during various on-farm grain handling activities. This goal creates the need for an efficient, more sensitive, and highly specific analytical method for the determination of low levels of aflatoxins in airborne grain dust samples. Therefore, the specific objectives of this proposal are:

1. To develop and validate a one-step extraction and analysis technique for the separation and quantitative determination of aflatoxin B_1 in airborne grain dust samples.
2. To collect and analyze statistically acceptable dust samples in a cross-sectional survey representative of regional on-farm agricultural grain handling operations.
3. To determine the proportion of aflatoxins in respirable dust particles and the effect of high and low seasonal temperature, rainfall or humidity, and grain handling on the aflatoxin content in bulk corn and dust samples.
4. To identify and quantify other natural toxins such as ochratoxin, zearalenone, vomitoxin, and fumonisin, which may be detectable during the course of aflatoxins analysis in grain and grain dust.

Methodology

The analytical methods are based on the application of supercritical fluid extraction of grain dust samples, followed by on-line analysis by SFC or HPLC/MS. Airborne dust samples are directly extracted with supercritical carbon dioxide using an SFE apparatus, in the presence of small amounts of organic solvents as fluid modifiers, followed by off-line analysis of the extract. Off-line

and on-line supercritical fluid extraction methods have been developed for analysis of aflatoxin, and other mycotoxins in grain dust samples. Funding of the above proposed research plan was limited to the laboratory method development only. Anticipated future field sampling will include on-farm grain handling operations in Iowa and three other states with high potential for aflatoxin contamination in grain (e.g. GA, OH, and OK). The proposed field study is pending revision and resubmission.

Significant Findings

Since the beginning of the currently funded method development phase on July 1, 1991, the SFE procedure for aflatoxin has been optimized, leading to a lower detection limit of 1 ng per sample. A new SFE method was also developed and optimized for the detection of fumonisins in grain dust. Both the aflatoxin and fumonisin methods were first developed as off-line extractions with SFE followed by HPLC analysis. SFE analysis of aflatoxin B_1 has been optimized on-line with HPLC/MS. However, greater sensitivities were obtained with on-line SFE/HPLC with fluorescence detection. Further improvement in mass spectrometric detection for aflatoxin B_1 requires derivatization followed by negative chemical ionization, which has been attempted but requires further development. A multi-toxin screening method has been developed for on-line analysis of the SFE extracts using HPLC/MS. These methods are being evaluated using field samples from the 1992 harvest season.

Publications

Selim MI, Tsuei MH: Development and Optimization of a Supercritical Fluid Extraction Method for the Analysis of Aflatoxin B_1 in Grain Dust. Am Ind Hyg Assoc 54(4):135-141, 1993

Selim MI, Tsuei MH: Occupational Exposure to Aflatoxins in Grain Dust. Am Ind Hyg Assoc, in press, 1991

Selim MI, El-Sharkawy S, Padanilam B: Analysis of Fumonisins in Corn Dust. J Toxicol & Environ Health, in press, 1991

Unpublished Articles

Final Report, Mustafa I. Selim, Assessment of Occupational Exposure to Aflatoxin, 04/22/93

Selim MI, El-Sharkawy, Popendorf WJ: Supercritical Fluid Extraction of Fumonisin B_1 From Grain Dust. Am Ind Hyg Assoc submitted 1993

Juchems AM: Distribution of Aflatoxin B_1 in Aerosolized Grain Dust. In preparation, 1993

Tsuei MH: Determination of Aflatoxins in Grain Dust Using Supercritical Fluid Extraction, Thesis, expected graduation December 1991