EVALUATION OF ELISA SCREENING TEST FOR DETECTING AFLATOXIN IN BIOGENIC DUST SAMPLES

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A RESEARCH PROJECT REPORT

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DISCLAIMER

ABSTRACT

Aflatoxin is a carcinogenic chemical that is sometimes produced when agricultural commodities are infested by the fungi *Aspergillus flavus* and *A. Parasiticus*. Aflatoxin has been found to be present in air samples taken around persons handling materials likely to be contaminated. The purpose of this investigation was to demonstrate the feasibility of using an Enzyme Linked Immunosorbent Assay (ELISA) test kit that was developed to screen for aflatoxin in bulk agricultural commodities, to an air sample. Samples were taken from two environments likely to be contaminated with aflatoxin, a dairy farm feed mixing operation and a peanut bagging operation. The dust collected from these environments was considered to be biogenic, in that it originated primarily from biological materials.

Feed materials were collected from a feed mixing area at a dairy farm in Alabama. The material was mixed and sieved to < 125 μm to isolate the aerosolizable fraction. The dust was extracted using a 4:1 methanol:water solution. The extract was cleaned using solid phase extraction and analyzed using high performance liquid chromatography (HPLC)- tandem mass spectrometry. No quantifiable levels of

aflatoxin could be found in the dairy farm dust. Settled peanut dust was collected using an office vacuum cleaner and sieved to < 125 μm. This material was extracted using 4:1 methanol:water and cleaned using SPE. Analysis was by HPLC with ultraviolet detection. No detectable levels of aflatoxin could be found in the peanut dust. The samples were then spiked with aflatoxin B1 at 2.5, 3.8, and 5.0 ppb by weight of the dust. Ten replicates of the ELISA test were run at each level.

For the dairy farm dust, the ELISA kit detected the aflatoxin in the 5 ppb dairy farm dust extract 100% of the time. At the 2.5 ppb spiking, the ELISA kit detected aflatoxin 2 out of 10 replicates. At the 3.8 ppb spiking, the kit detected aflatoxin in 5 out of 10 replicates.

In peanut shell dust, the ELISA kit detected the aflatoxin at 5 ppb only 10% of the time. At the 2.5 ppb spiking, the ELISA detected aflatoxin 2 out 10 replicates. At the 3.8 ppb spiking, the kit detected aflatoxin 3 out 10 replicates. To see if the low detection rate was due to the dark color of the extract, peanut shell dust extract was spiked with 10 ppb aflatoxin B1 and diluted 2:1. The ELISA kit detected aflatoxin 100% of the time. The manufacturer of the ELISA kit reported a detection rate of 8% and 30% for

a 2.5 ppb and 3.8 ppb spikings of aflatoxin in methanol:water. The manufacturer also reports that the ELISA kit detected 5 ppb spiking of aflatoxin B1 100% of the time. In conclusion, the ELISA test could be utilized on agricultural dusts, with a 2 fold change in threshold for dusts similar to the peanut dust.

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BACKGROUND

Mycotoxins first became an issue in modern times with the outbreak of a disease affecting livestock and poultry in England, known as Turkey X Disease (1). This disease was later shown to be associated with grain infested with the mold *Aspergillus flavus*. This mold was shown to be producing a toxin which was later designated aflatoxin (2). Aflatoxin has been shown to also be produced by *Aspergillus parasiticus* (3). Aflatoxin is toxic in a variety of animal species, with LD 50's ranging from 0.3 mg/kg body weight for the rabbit, to 17.9 mg/kg body weight for the female rat (1). The primary target organ of aflatoxin is the liver. Aflatoxin is considered to be a mutagen, a carcinogen, an immune toxin, and a teratogen (1). A strong correlation was shown between aflatoxin contamination in food sources and liver cancer incidence in several counties in Mozambique (r^2=0.8792, $p < 0.001$)(4), although the results were confounded by potential synergism with hepatitis B virus (HBV). A later study used aflatoxin B1-guanine adducts in urine as a biomarker of exposure and a skin antigen test to assess exposure to HBV. The researchers examined the association between the two potential factors, and found no association between HBV exposure and primary hepatocellular

carcinoma (PHC) (r=0.19) , and moderate correlation (r=0.75) between aflatoxin exposure and liver cancer incidence, although the researchers did not report a level of significance. No synergistic or additive effects on PHC rates were noted from co-exposure to both aflatoxin and HBV (5).

There are five different forms of aflatoxin which are of concern to the public health professional (Figure 1). Aflatoxin B1 is the most prevalent and is also considered the most toxic. Aflatoxins B2, G1 and G2 are less toxic and are produced at concentrations lower than aflatoxin B1. Aflatoxin requires bioactivation to exert its toxic effect, and its toxicity for a given species is the result of a balance between bioactivation and detoxification (6). The metabolic pathways for aflatoxin B1 are illustrated in Figure 2. An important metabolite is aflatoxin M1, which is excreted via lactation. Another main metabolite is aflatoxicol, which serves as a reversible reservoir of the aflatoxin (6). Aflatoxin B1 8-9 epoxide is proposed as the primary toxic metabolite of aflatoxin (6). The 8-9 epoxide will bind to DNA and RNA forming adducts in genetic code, and possibly initiating carcinogenesis (6).

FIGURE 1 Aflatoxin B_1, B_2, G_1, G_2, and M_1

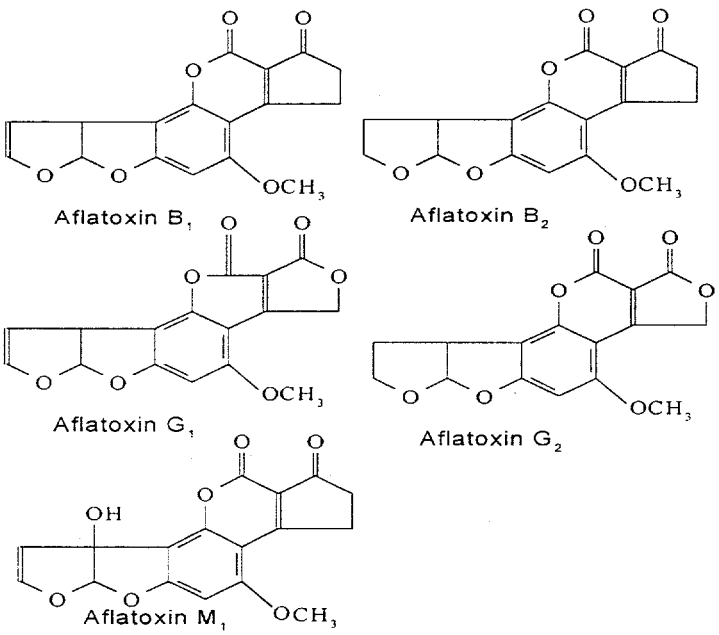
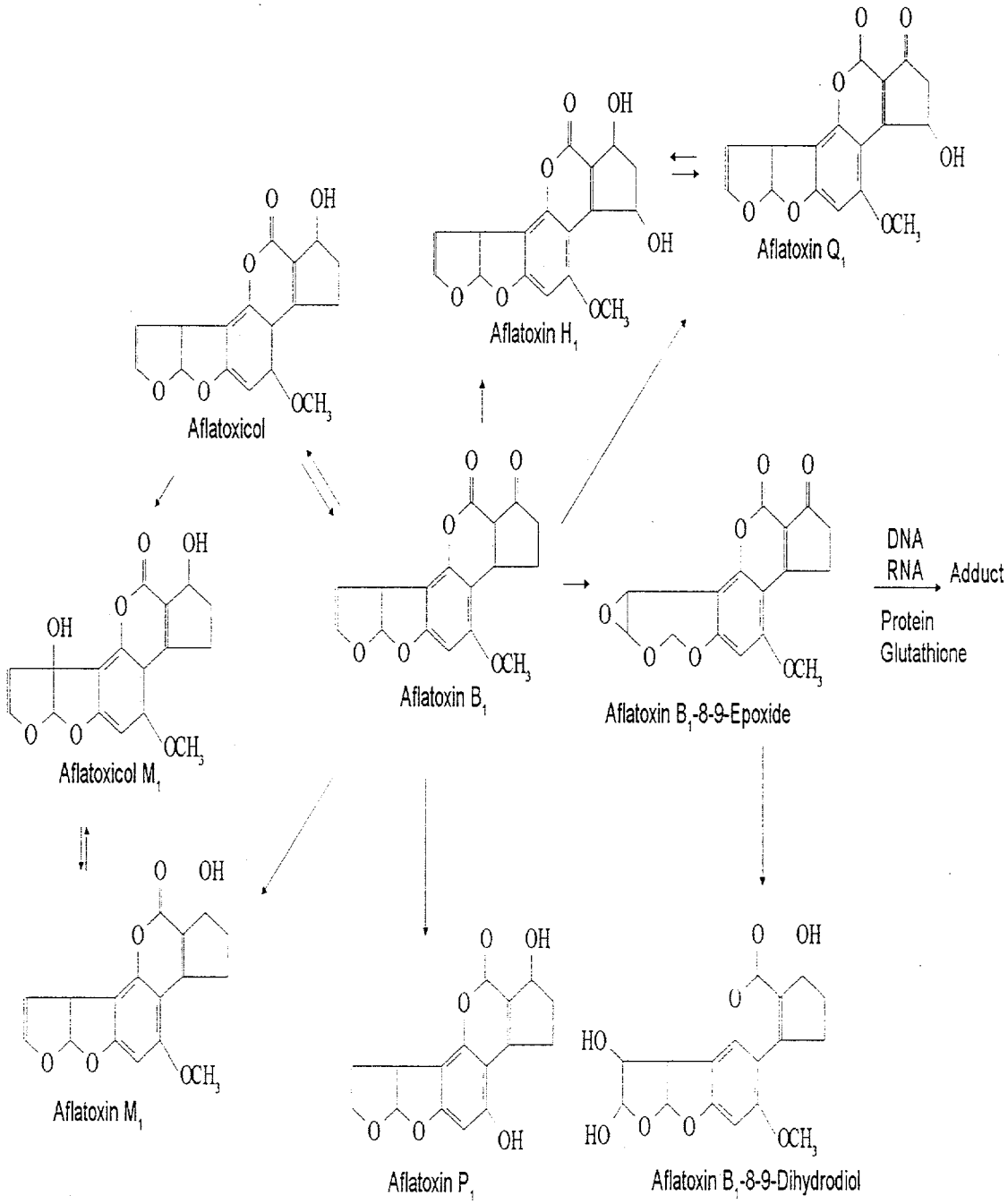


Figure 2 Metabolic Pathways of Aflatoxin B_1



Toxicology of inhaled aflatoxin

Aflatoxin has the potential to be biotransformed in human lung tissue (7). The biotransformed product is capable of long term binding to the DNA and causing permanent lesions in genetic code, which can initiate cell transformation. Ball *et al.* demonstrated different formation rates of DNA adducts in tracheal epithelium of the hamster, rabbit, and rat (8). The rabbit, despite having fewer non-ciliated (NC) epithelium cells (the cells that have the largest amount of p450 in the lung) than the hamster, had a higher rate of adduct formation. The rat, which does not have NC cells in its lung, had the lowest rate of activation. The investigators concluded that the number of NC cells does not predict the rate of adduct formation. In a subsequent study, Ball and Coloumbe followed up the above investigation with an examination of aflatoxin metabolism by NC cells (9). Ball and Coloumbe observed that the hamster and rat NC cells possessed a higher ability to detoxify the activated aflatoxin B1 than the rabbit, hence the higher adduct formation potential in the rabbit.

Coloumbe *et al.* examined two groups of rats given aflatoxin B1 intratracheally, one group was given aflatoxin bound to grain dust, the other crystalline aflatoxin (10).

Aflatoxin B1 was more slowly absorbed when bound to the dust, giving the aflatoxin a longer residence time in the respiratory tract. The pharmacokinetics of aflatoxin showed a two compartment model and a first order absorption rate constant of 0.0083 min^{-1} with elimination rate constant being 0.00016 min^{-1}.

A study performed on rabbits showed cellular immune activity in the lung was suppressed after 2 weeks of sub-lethal dosing of the animals (11). The animals were orally dosed with capsules containing a given dose of aflatoxin B1. The animals were then sacrificed by injection of 2 mL of succinyl choline, and alveolar macrophages and serum were harvested from the animals. Alveolar macrophage activity was measured by exposing the macrophages to *Aspergillus fumigatus* spores for a period of time and by measuring the fraction of macrophages that absorbed spores. The macrophage activity was observed with the sera from aflatoxin dosed and control rabbits. Macrophages from control animals were tested in sera from control and dosed animals. At the lowest dose measured (0.01 mg/kg), macrophage activity was lowest with the control animal's macrophages in serum from aflatoxin dosed animals. At the

highest dosing (0.03 mg/kg), macrophage activity was lowest in the dosed animal macrophages in serum from dosed animals. The rabbit is highly sensitive to aflatoxin, the Lethal Dose 50% (LD50) for the rabbit is only 0.3 mg/kg (7).

Reproductive toxicity has been assessed using the mink (12). The mink study examined birth weight and survivability of kits whose mother was fed aflatoxin contaminated feed. There were three dosing groups of ten animals in the mink study (11). The animals were bred in accordance with standard mink breeding guidelines, allowed to carry their kits to term, and nurse them while being exposed to 0, 5 and 10 ppb aflatoxin B1 in their feed (exposure period 90 days). There was no significant effect on mating success, kit size, or feed consumption. There were effects noted in kit weight at three weeks for the 5 ppb group. The 10 ppb group's kits had reduced weight at birth and at three weeks as well as significantly increased mortality at three weeks. One problem with this study was that feed consumption and animal weight were not reported, so a lowest observable adverse effect level (LOAEL) could not calculated.

Occupational Epidemiology

Olsen *et al.* conducted a retrospective follow up study of 241 feedstock processing facilities in Denmark. Employees

had an approximate exposure to aflatoxin of 170 ng aflatoxin B1 per day from 1964 to 1984 (13). This exposure was approximated from average aflatoxin concentration in processed feeds, types of feeds imported into Denmark, and 131 total dust measurements made by the Danish Labor Inspection Service. When accounting for a ten year latency period, the authors found an increase in risk ($p < 0.05$) for liver cancer (Standardized Proportionate Incidence Ratio, or SPIR of 246%) and biliary cancer (SPIR= 298%). There were also increases in rates of salivary gland tumors (SPIR = 498%) and multiple melanoma (SPIR=238%), but the authors could not find results significant because of study limitations.

Hayes *et al.* concluded in a retrospective cohort study of 71 Danish oil press workers exposed to aflatoxins from 1961 to 1969 (14). The investigators found the workers had a standardized mortality ratio (SMR) of 250% and lung cancer SMR of 253% when compared to an aged adjusted cohort from the Danish population. Aflatoxin exposures were estimated by the authors to be 2.5 μg/exposure week for baggers to 0.04 μg/exposure week for other workers.

Dvoracka investigated pulmonary fibrosis in three dead workers potentially exposed to aflatoxins (15). Two of the workers were farmers and the other was a textile worker. Lung tissue was examined for the presence of aflatoxins. Aflatoxin was found in all three cases, with the textile worker showing the highest concentrations of 54 ng/g, and the two farmers showing 19.9 ng/g and 10 ng/g. Dvoracka concluded from this study that workers exposed to organic dusts are potentially co-exposed to aflatoxin.

Methods of Analysis

Aflatoxins were first purified using thin layer chromatography (TLC) (16) Today, aflatoxins are determined using thin (TLC), and high performance liquid chromatography (HPLC). Methods are detailed in the Association of Official Analytic Chemistry's (AOAC) Official Methods of Analysis (17). The basic steps in aflatoxin analysis are sample preparation, extraction, clean up, final separation, detection and quantification, and confirmation (3). Clean up of the extractant is critical for success using HPLC, particularly if UV detection is used for quantification (18). Methods of detection and quantification range from densitometry and fluorescence under UV light when using TLC,

to UV and fluorescence detection of aflatoxin B1 conjugates with the HPLC method. UV detection has a detection limit of 5 to 10 ng with good sample clean up (18). Fluorescence detection requires conjugation of aflatoxin B1 with trifluoroacetic acid (TFA) to produce a highly florescent molecule. The fluorescence detector can detect below 1 ng of aflatoxin B1 (18).

Modified versions of the TLC and HPLC methods have been developed for analysis of aflatoxin in airborne dust. Selim and Tsuei used a method for 2 grams of dust (19). The method was based on an earlier TLC method published by Shotwell *et al.* (20), except that HPLC was used to quantify the aflatoxin. Selim and Tsuei's extraction procedure used 15 mL water, 150 mL of chloroform, and 15 g of celite, blended for 30 min. The extractant was filtered and then evaporated to near dryness. The residue is suspended using methylene chloride, and was evaporated to near dryness. The residue was then suspended using methanol and the solution cleared of matrix interferences using either a LC-CN solid phase extraction (SPE) column or a silica gel column. Analysis of the column eluent was performed using two dimensional TLC or HPLC analysis with UV detection (19).

Shotwell *et al.* reported a detection limit of 0.5 ng using a TLC method that he developed to detect aflatoxin in as little as 0.5 g corn dust (20).

Selim and Tsiuei explored using super critical fluid extraction (SFE) to improve the extraction efficiency and to reduce the amount handling and toxic solvents used (19). The procedure they developed used carbon dioxide maintained at 2000 psi and 40 °C. with 250 μl of acetonitrile being added after 15 min. to improve the sensitivity and specificity of extraction. The extractant was then analyzed by HPLC with UV detection. The authors reported a 1 ng detection limit with UV detection, and lower detection limits using fluorescence detection. In comparison with the solvent based extraction procedure, the SFE procedure detected aflatoxin B1 in 15/18 samples, while the solvent procedure detected aflatoxin in only 7/18 samples. Aflatoxin B1 concentrations ranged from non-detectable to 120 ng/g in the samples that were extracted using the solvent based system, while SFE produced samples that contained aflatoxin B1 ranging from non-detectable to 983 ng/g. The authors also noted that the procedure for solvent extraction took 2 to 2.5 hours to perform versus 25 to 30 min. using SFE.

Due to the high analytical costs and long turn around time, several screening techniques have been developed by agricultural concerns to detect aflatoxins in agricultural commodities. A simple technique is to crack several corn kernels and examine them under UV light and look for fluorescence (21). Rough guidelines for judging the concentration of aflatoxin by the number of fluorescing kernels have been used, but are not very accurate (3). Another technique involves using a minicolumn. The minicolumn is described in the AOAC's Official Methods of Analysis as a screening tool for aflatoxin above 5 ppb (17). The minicolumn is packed with layers of anhydrous calcium sulfate, silica gel, alumina, florisil and more anhydrous calcium sulfate. The silica and alumina layers serve as chromatographic separation layers for the aflatoxin. The aflatoxin will bind with the florisil and will fluoresce under UV light. This method, however, requires considerable extraction and clean up steps. The sample is extracted with acetone and water, filtered, and is then blended with NaOH and $FeCl_3$. $CuCO_3$ and diatomaceous earth are mixed in, and then the sample is again filtered. The final filtrate is

mixed and extracted using $CHCl_3$, and then added to the minicolumn.

The most recent screening tool uses a competitive binding enzyme linked immunosorbent assay (ELISA), which is commercially available in a kit. The ELISA test uses competitive enzyme binding to detect the presence of the aflatoxin. The enzymes will react with aflatoxin B1 and to a lesser extent, very similar chemical structures such as aflatoxin B2, G1, and G2. (22). The ELISA test is based on binding of an enzyme to free aflatoxin and horse radish peroxidase (HAP). The captured aflatoxin/HAP enzyme substrate conjugate is then exposed to another enzyme that normally would react to produce a blue color when exposed to the non-aflatoxin bound substrate. The presence of aflatoxin is indicated when a blue color does not appear on the port of the test kit, indicating that free aflatoxin, and not the enzyme conjugate, is bound to the antibody. The manufacturer evaluated the ELISA test kit using spiked solutions of aflatoxin B1 equivalent to the concentration of a 0, 1.3 2.5, 3.8, and 5 ppb (ng/g) of aflatoxin in a substrate prior to extraction and analysis. The results of these tests are shown on Table I. Selim and Tsuei state that the ELISA methods are not sufficient for use in air

monitoring because of their poor selectivity, although they do not site information on how specific these methods are (18).

Table I EDITEK 5 ppb ELISA Test Performance

Aflatoxin Level (ppb, w/w)	Accuracy	# Assays
5.0	100%	52
3.8	70%	44
2.5	92%	48
1.3	100%	44
0.0	100%	69

The extraction steps for the ELISA procedure involve taking 50 grams of contaminated material and extracting with 100 mL of 80:20 methanol water. One milliliter is then passed through a 0.45 micron filter and is added to 2 mL of buffer solution provided in the ELISA kit. The one milliliter pre-buffered solution will contain 2.5 ng of aflatoxin B1 if the original 50 grams of substrate was contaminated with 5 ppb (w/w) of aflatoxin B1.

Cross reactivity of ELISA test can be a problem, as the enzymes will react with structures that are similar to Aflatoxin B1. The manufacturer reports cross reactivity for the EDITEK EZ-Screen (tm) card was evaluated for aflatoxins B2, G1, and G2 to be 35%, 100%, and <1% respectively. Another potential problem with this test is the reliance of lack of color change to indicate the presence of the aflatoxin. The manufacturer of the EZ-Screen specifically warns that the mold inhibitor gentian violet can be extracted with methanol:water, yielding a purple solution. This purple color will prevent detection of any color change during the ELISA test. Another problem with the ELISA system is that solvent extraction systems other than methanol:water can interfere with the action of the enzymes in the test kit.

Assuming no interferences, one may estimate how sensitive the EDITEK card would be in detecting aflatoxin in an air sample. By extracting the filter with methanol:water and concentrating the extract to 1 mL, the ELISA would be able to detect 2.5 ng/mL. Therefore by sampling 2 liters of air per minute, an investigator would capable of detecting 2.6 ng/m^3 [f] from an air sample. Assuming an average working breaths 10 m^3 per working day, then a daily intake of 26 ng

could be detected using the ELISA test card. This is one sixth of the Olsen *et al.* exposure cohort that had a 246% SPIR and an estimated occupational intake of 170 ng of aflatoxin a day.

Industrial Hygiene Investigations

Burg *et al.* investigated exposed workers handling corn contaminated with aflatoxins (23). They used high volume samplers and collected airborne dust onto 8 by 10 inch glass fiber filters and also a high volume 4 stage Andersen sampler. Personal samples were also collected, but the investigators failed to collect enough material to analyze using the TLC method they had adapted (21). The researchers investigated two processes in handling of the corn: pouring the corn through a bournier divider and the other was transporting the corn into and out of the storage bin. Average aflatoxin concentration in the aerosolized dust at the bournier divider was 3886 ppb with a range of 2560 to 4560 ppb (w/w) aflatoxin. Bulk samples collected from this corn was analyzed and determined to contain 2250 ppm aflatoxins (w/w). Burg *et al.* explained the higher air concentrations by kernels being shattered and releasing

otherwise unextractable aflatoxin into the environment. Another explanation could be the higher surface area to solvent ratio during extraction, arising from the smaller particle sizes. Samples resulting from the transport of the corn into and out of the storage bin resulted in an average concentration in the aerosolized dust of 138 ppb and a range from non-detectable to 241 ppb aflatoxins (w/w). Accounting for the variable dust concentrations, air concentrations ranged from non detectable to 107 ng/m^3. The farmer handling the transport process was probably exposed to greater than measured concentrations because the investigators did not sample near the farmer when he spent two hours sweeping out the storage bin with no respiratory protection, a process which generated a visible dust.

Sorenson *et al.* examined dust collected at port grain terminals in Minnesota, Wisconsin and Georgia (24). The samples from the northern states consisted of oat, barely, spring wheat, corn, flax, durum wheat, sunflower seeds and rye dust collected during the fall of 1977. The samples from Georgia consisted of corn collected during August of 1978 at a grain dumping station. The samples from the northern states did not contain detectable levels of aflatoxin. The Georgia corn dust contained an average of

130 ppb (w/w) aflatoxin. On a weight per weight basis, the Georgia corn dust had two percent of its particles below 7 μm. The particles on the size range of seven to eleven μm were contaminated with 695 ppm (w/w) aflatoxin B1, and in two samples, particles below 7 μm contained an average of 1185 ppb and 1814 ppb (w/w)aflatoxin B1. The authors concluded that whole dust samples may underestimate the actual aflatoxin intake levels from the respiratory route.

Palgrem *et al.* examined dust settled on surfaces around grain elevators in New Orleans for mycotoxin contamination (25). The investigators extracted the dust using methylene chloride and analyzed the extract by TLC for aflatoxin, ochratoxin (a mycotoxin produced by *Aspergillus ochra*ceus) and zearelanone (a mycotoxin produced by *Fusarium sp.*). The authors further analyzed the dust by plating out strains of mold present in the dust. Ten of the fifteen samples contained zearelanone. Palgrem also isolated strains of the genus *Aspergillus, Penicillin,* and *Fusarium.* None of the samples contained either ochratoxin or aflatoxin. The authors also noted that the species of Fusarium found were not noted as a severely toxigenic species.

Silas *et al.* used high volume Andersen samplers to sample dust in two Georgia corn processing facilities (26). None of the airborne dust samples contained aflatoxin at detectable levels. However, settled dust samples contained aflatoxin from 0.15 to 8 ppb. The average effective diameter of the dust in the corn processing facilities was 2 to 3 μm.

STATEMENT OF PROBLEM

Epidemiology and toxicology studies suggest that aflatoxin may pose a cancer risk to persons exposed to dusts contaminated with aflatoxin. The contamination will occur on particles that are sized such that they will enter the lung and enter the deep air passages, where aflatoxin can be activated by cytochrome p450 containing cells, or be distributed to other parts of the body. The aflatoxin present on the dust will present a chronic risk to the exposed.

Current methods for analyzing aflatoxin require extensive handling and preparation, as well as sophisticated detection equipment to attain the best extraction efficiencies and sensitivity. Therefore, there is a need for a method that is both inexpensive, rapid and sensitive to aflatoxin in the work place. In agriculture, such a method exists in ELISA screening techniques, and might applicable for use in industrial hygiene applications, if the lack of specificity alleged by Selim and Tsiuei is not an over riding factor (19).

OBJECTIVES OF STUDY

The overall objective for this study was to investigate the use of the ELISA screening test to evaluate biogenic dust samples that may contain aflatoxin. To characterize the ELISA test's utility, the ELISA method's response was evaluated by using dust from two environments that are spiked with known amounts of aflatoxin. The null hypothesis for this study is: the ELISA test's accuracy with two biogenic dusts is not different from the accuracy reported by the manufacturer of the ELISA kit when determining aflatoxin in methanol.

MATERIALS AND METHODS

A list of chemical supplies is provided in Appendix I.

Experimental Design

The experimental design is shown in Table II. Bulk dust was collected from two environments that have the potential for aflatoxin formation. A peanut roasting and bagging facility and a dairy farm feed mixing process area were used as collection sites. The dust was sieved to inhalable sizes and extracted using a method detailed below. The extractant's aflatoxin concentration was determined using either high performance liquid chromatography with ultraviolet detection (HPLC-UV) or liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS). To determine the performance of the ELISA test kit, known masses of aflatoxin B1 were spiked to aliqouts of the extractant, and then analyzed using the ELISA procedure detailed below.

Collection of Bulk Dust

Bulk feed materials (old silage, feed meal, cotton seed, cotton seed hulls, and hay) were collected from the dairy farm feed mixing operation. These materials were mixed in proportion to current feed mixing recipe (43%

Silage, 34% feed meal, 8% hay, 8.6% cotton seed, and 6.8% cotton seed hulls) and sieved using an electric sieve to < 125 μm. The final mass of dairy farm dust that was extracted was 46.3 grams. Settled dust was collected from a peanut roasting facility's shake out operation. A Hoover Office Machine Vacuum (model #C2093) was used with a bag that was rated 99.5% efficient to 5 μm (Hoover Bag #4110100A). This material was then sieved using an electric sieve to < 125 μm. Fifty grams of peanut dust was sieved.

TABLE II. Experimental Design

1. Collect and sieve dust to inhalable size
2. Extract entire inhalable dust sample using methanol:water
3. Remove appropriate amount of extract solution to detect minimum of 2.5 ng/mL aflatoxin B1 (LC-MS-MS for dairy farm dust, HPLC-UV for peanut shell dust).
4. Spike two 1-mL aliquots of extract to 1.25 ng/mL aflatoxin B1.
5. Run 10 ELISA tests plus two controls
6. Spike two 1-mL aliquots of extract to 1.9 ng/mL aflatoxin B1.
7. Run 10 ELISA tests plus two controls.
8. Spike two 1-mL aliquots of extract to 2.5 ng/mL aflatoxin B1.
9. Run 10 ELISA tests plus two controls

Extraction and Storage

The sieved dust from the two environments were immediately extracted with 100 mL of 4:1 methanol:water and filtered through a Whatman #3 qualitative filter. Extractant was stored in a silinized bottle, wrapped in aluminum foil, and stored in a darkened refrigerator at approximately 5° C.

Analysis of Dusts

Initially, an HPLC method using an isocratic solvent system was proposed. However, because of extensive interferences in the chromatogram produced by the proposed HPLC method and problems in instrument performance, two different procedures were developed to analyze for aflatoxin. Details of the proposed HPLC method and results of initial experiments are given in Appendix II.

Determination of Aflatoxin B1 in Dairy Farm Dust

Dairy farm extract was cleaned of interferences by using a solid phase extraction (SPE) technique developed by Supelco, inc. (27). One milliliter of extract was added to 4 mL of 0.5% aqueous acetic acid, and passed through a Superclean LC-CN cartridge (Supelco, Inc. Catalogue # 5-7013). The LC-CN cartridge was then washed with 2 mL

hexane, dry packed (air was pulled through the SPE column for approx. 3 min), washed with 3 mL of 25% Tetrohydrofuran (THF) in hexane, and then dry packed for 1 min. Final elution of the aflatoxin was with 2 washings of 2 mL of 1% THF in methylene chloride. This eluent was evaporated to dryness (Meyer N-EVAP Analytic Evaporator, Organomation Associates, Inc.), and the residue dissolved in 200 μl of methanol.

LC-MS-MS was used to quantify the amount of aflatoxin B1 present in the dairy farm dust extract. The sample was analyzed using HP 1050 liquid chromatograph with a 100 mm x 2.1 mm Brownlee Aquapore RP 300, 300 Å pore size, C8 column. Detection was with a Perkin Elmer Sciex API III Biomolecular Mass Analyzer using mass reaction monitoring (MRM). A parent ion of M/Z of 313 was used with a daughter of M/Z 241. Solvent A was 10 mM NH_4OAc in water, solvent B was 10 mM NH_4OAc in Methanol. A flow of 0.1 mL/min was used with the following program: using a linear gradient, 0 - 100% B over 12 min. 100% B was maintained for 2 min and then returned to 100% A using a linear gradient over the next 2 min. External standards of 15 ng/mL and 30 ng/mL were used to standardize the response of the LC-MS-MS. A standard curve for the LC-MS-MS method is given in Table III

and Figure 3. A standard of aflatoxin B1, B2, G1, and G2 was used to determine retention times and major ion pairs.

One milliliter of dairy farm dust extract was cleaned using the Supelco SPE procedure and analyzed using LC-MS-MS for aflatoxin B1. Additionally, a ten milliliter sample was concentrated in the N-EVAP and suspended in 1 mL of 4:1 methanol:water. The sample was then cleaned using the Supelco SPE procedure and analyzed using LC-MS-MS.

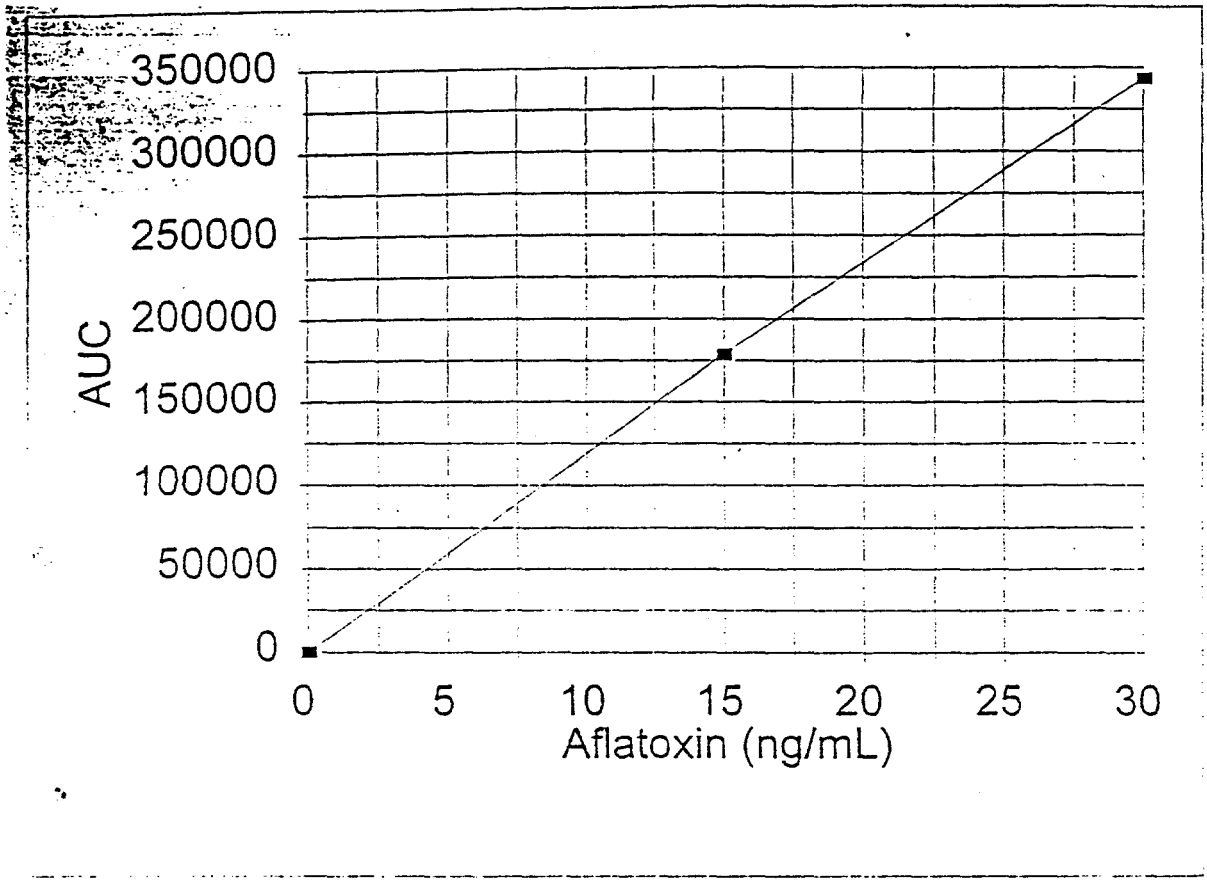
TABLE III Standard Curve Results for LC-MS-MS

Concentration(ng/mL)	Area Under Curve
0	0
25	178,809
50	343,492

Regression Output:

Constant:	2354.333
Std. Err of Y est	5766.915
r^2	0.999
No. Observations	3
Degrees of Freedom	1
X Coefficient	11449.73
Std. Err of Coef.	271.855

Figure 3 LC-MS-MS Standard Curve



Determination of Aflatoxin B1 in Peanut Shell Dust

HPLC-UV detection was used with the peanut dust extract. A Hewlett-Packard HP1050 Liquid Chromatography System was used, with a HP1050 variable wavelength UV detector. A 250 mm x 15 mm Alltech Econosphere C18, 5 μm pore size analytic column was used. A linear gradient system was developed (appendix II). The following solvents were used: A= HPLC grade water, B= HPLC grade methanol, C=HPLC grade acetonitrile. Solvent flow was 2 mL/min with the following program: 80% A, 10% B, 10% C on a linear gradient to 20% A, 40% B, 40% C over 10 min. This system was maintained for 10 min and then returned to 80% A, 10% B, and 10% C over the last 5 min. Retention time for the aflatoxin B1 was 13.8 min. The injection volume was 200 μl. External standards of 100 ng/mL, 75 ng/mL, 50 ng/mL, and 25 ng/mL in methanol were used to quantify the HPLC's response (Table IV and Figure 4).

A 10 mL aliquot of the peanut dust extract was concentrated in the N-EVAP to dryness and suspended in 1 mL of 4:1 methanol:water. This concentrate was then cleaned

using the Supelco SPE procedure and analyzed with the HPLC-UV method described above.

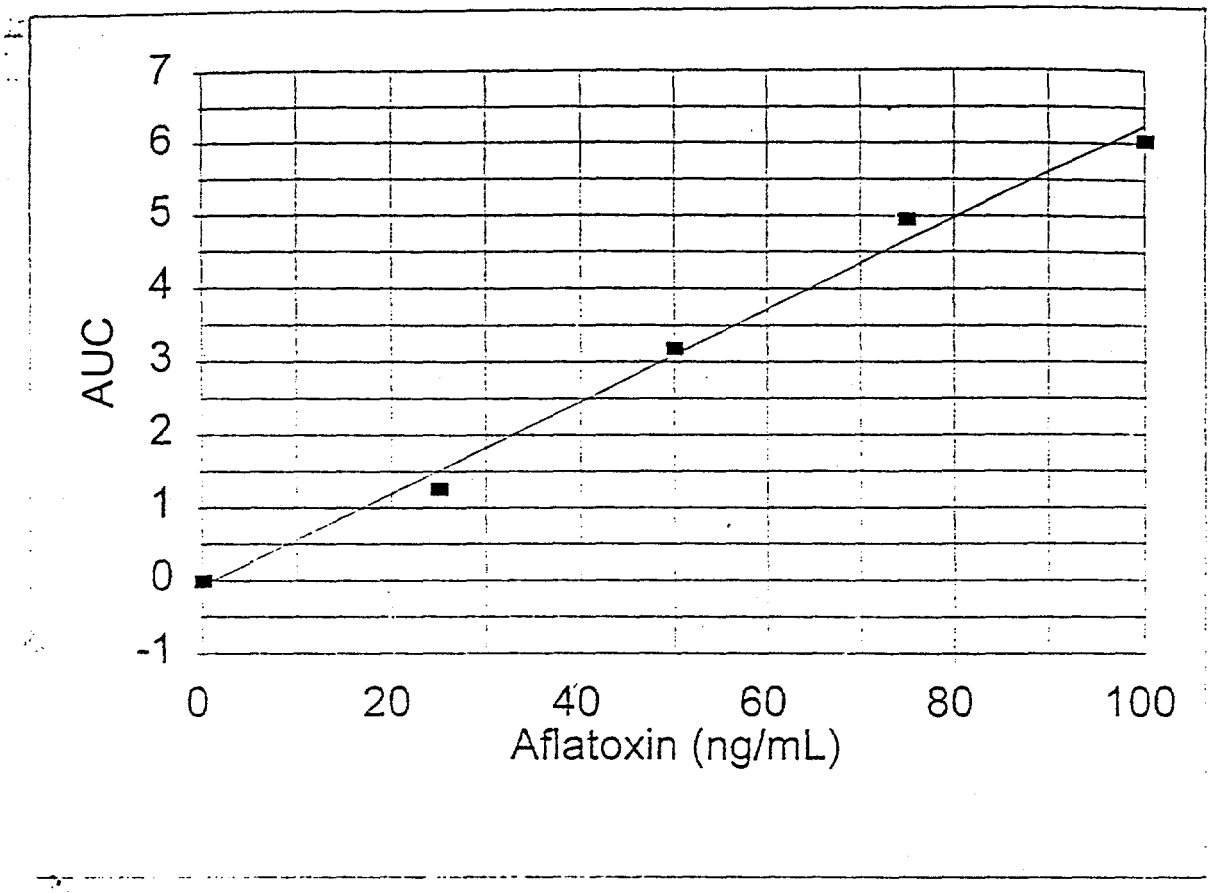
TABLE IV Standard Curve Results for HPLC- UV

Concentration(ng/mL)	Area Under Curve
0	0
25	1.26
50	3.187
75	4.956
100	6.036

Regression Output:

Constant:	-0.065
Std. Err of Y est	0.260303
r^2	0.992
No. Observations	5
Degrees of Freedom	3
X Coefficient	0.063064
Std. Err of Coef.	0.003293

Figure 4 HPLC-UV Standard Curve



Preparation of Spiked Samples

Dairy Farm Extract

The recovery of aflatoxin B1 with our cleaning and analytic procedures was determined by spiking three 1 mL aliquots of dairy farm dust extract spiked with 6 ng of aflatoxin B1 to create three solutions of 6 ng/ mL dairy farm extract. These aliqouts were cleaned by the Supelco SPE procedure and analyzed using LC-MS-MS.

Methanol:water Samples

Ten milliliters and 1 mL of 4:1 methanol:water were spiked with 6 ng of aflatoxin B1 to give solutions of 6 ng/mL and 0.6 ng/mL. The ten milliliter spiked solution was concentrated in the N-EVAP and dissolved in 1 mL of 4:1 methanol:water. Both spiked solutions were processed using the Supelco SPE procedure, and analyzed using LC-MS-MS.

Peanut Shell Dust

A ten milliliter peanut dust aliquot was spiked with 25 ng of aflatoxin B1 to give a 2.5 ng/mL peanut shell extract. This solution was then concentrated in the N-EVAP to dryness, and then suspended in 1 mL of 4:1 methanol:water.

The spiked peanut shell extract sample was then cleaned using the Supelco SPE procedure, and analyzed with HPLC-UV.

ELISA Testing of Dust Extracts

Dust extracts were spiked with aflatoxin B1 and analyzed using the EDITEK 5 ppb aflatoxin B1 test kits. Two 1 mL aliquots of dust extracts were spiked with aflatoxin to produce 1.3, 1.9 or 2.5 ng/mL aflatoxin B1. These solutions were added to the 2 mL buffer solution provided in the test kit. These buffered solutions were then analyzed on five sites on the ELISA test kits, along with the negative control on the sixth site. A total of 2 test kits were used at each spiking level, allowing for 10 individual tests and two negative controls to be performed. Details of the ELISA test procedure are provided in Appendix III.

RESULTS

A list and copies of chromatograms and mass spectra are given in Appendix V.

Percent Recovery

Spiked Dairy Farm Dust Extract Recovery

Percent recovery was determined by comparing the area under the curve (AUC) of the three spiked dairy farm dust extracts to the AUC of a 30 ng/ mL external standard of aflatoxin B1 in methanol. A 30 ng/mL external standard was used because of the five-fold concentration step in the SPE clean up procedure. Recovery for the LC- MS-MS was determined to be 48% with coefficient of variation of 4.4%.

Methanol:water Recovery

Percent recovery was determined by comparing the AUC of the 6 ng/mL and the 0.6 ng/mL (10 mL concentrated to 1 mL, yielding a solution with an expected concentration of 6 ng/mL) with the AUC of a 30 ng/mL external standard. The 6 ng/mL and the 0.6 ng/mL, 4:1 methanol:water solution gave recoveries of 45% and 68% respectively.

Peanut Shell Dust Recovery

The aflatoxin peak on the chromatogram had merged with an adjoining peak. This prevented a direct calculation of recovery based on AUC. To obtain recovery, the AUC of the adjoining peak was determined from an unspiked sample. This unspiked AUC was subtracted from the spiked sample's AUC to obtain the AUC that was due to the presence of aflatoxin B1. This calculated AUC was then compared to an external standard curve shown on Table IV and Figure 4. Percent Recovery was determined to be 66%.

Detection Limits

The lowest level analyzed by LC-MS-MS was used to determine a detection limit. Since the height 0.6 ng/mL was approximately four times the ambient noise, a detection limit was estimated to be 0.15 ng/mL (with a 20 μl injection, 3 picograms) by dividing 0.6 ng/mL by 4. In the peanut shell dust extract, the aflatoxin B1 peak was about the same height as the surrounding peaks. Therefore, the HPLC-UV procedure had a limit of detection of 2.5 ng/mL (with a 200 μl injection, 0.5 ng) in an actual sample.

Natural Background Aflatoxin Levels in Dusts

No aflatoxin B1 was detected in the 1 mL or 10 mL dairy farm dust extracts that were analyzed using LC-MS-MS (<0.15

ng/mL). No aflatoxin B1 was detected in the peanut dust extract sample (<2.5 ng/mL).

ELISA Test Kit Results

ELISA test results are shown on Tables V and VI.

TABLE V. ELISA Results for Dairy Farm Dust

	Spiking Level		
	2.5 ppb	3.8 ppb	5.0 ppb
#Positive	2	5	10
#Negative	8	5	0

TABLE VI. ELISA Test Results for Peanut Dust

	Spiking Level		
	2.5 ppb	3.8 ppb	5.0 ppb
#Positive	2	3	1
#Negative	8	7	9

To compare these results with manufacturer's data, a value for accuracy was calculated. Following the manufacturer's definitions, any negative at the 2.5 and the 3.8 ppb was assumed a "true result", and any positive at the 5.0 ppb level to be a "true result". To compare accuracy

with the ELISA manufacturer's's data, a value of accuracy was calculated at each spiking level. Accuracy for a each spiking level was determined by dividing the number of "true results" by the total number of tests. Table VII compares results with manufacturer's testing of methanol spiked with levels of aflatoxin B1.

Since the results of the ELISA are based on the lack of a color change to a blue-gray, we speculated that the ELISA's poor detection with the peanut dust extract could be a result of the dark color of the extract interfering with the reading of the test results. By diluting the extract five fold, we clarified the extract. We then spiked the diluted sample extract at 12.5 ng/mL and tested it with the ELISA kits. This corresponded with a concentration in the peanut shell dust of 25 ppb. The kit successfully detected the presence of the aflatoxin 5 out of 5 times. To see if we could reduce the amount of dilution necessary, we tested a 5 ng/mL sample extract, diluted 2:1. This corresponded to 10 ppb in the peanut shell dust. The kit successfully detected aflatoxin 5 of 5 times.

TABLE VII. ELISA Test Kit Accuracy

	Spiking Level		
	2.5 ppb	3.8 ppb	5.0 ppb
Dairy Farm Dust	80%	50%	100%
Peanut Dust	80%	70%	10%
Manufacturer's Data	92%	70%	100%

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

The EDITEK 5 ppb aflatoxin kit successfully detected aflatoxin B1 in extracts of two biogenic dusts sieved to inhalable sizes. The ELISA kit was capable of detecting aflatoxin B1 at 2.5 ng/mL in dairy farm dust extract (equivalent of 5 ppb in dust) and 5 ng/mL in peanut shell dust extract(equivalent of 10 ppb in dust). Particularly in the dairy farm dust extract, the ELISA kit detected aflatoxin at levels less than the manufacturer's reported threshold. This may mean that the ELISA kit's threshold was decreased. Another possibility is that the ELISA's response to less than 2.5 ng/mL was enhanced by the presence of the dust itself.

Recovery from the clean up procedure for HPLC analysis was a problem in this study. Recovery could have been affected by the concentration steps during the clean up-procedure. Contrary to this notion, however, was the fact that recovery from a spiked 10 mL methanol:water sample that was concentrated by evaporation to 1 mL, gave better recovery than an original 1 mL spiked methanol:water sample. Since only one sample was analyzed, the higher recovery may have been due to imprecision of the method. A future study

could explore improving the SPE technique, or adopting another technique. Past researchers have used silica gel columns to purify aflatoxin (23,25).

Further investigations exploring the use of the ELISA kit in air sampling should be done. Future studies need to examine recovery from sample media or from the dust itself. If we assume that recovery of aflatoxin from the dust is near 100%, a positive test kit result from a personal air sample would be cause for concern.

In the two biogenic dusts in this study, the ELISA kit exhibited the ability to detect of 2.5 ng and 5 ng of aflatoxin B1 in an actual air sample. This means by sampling at 2.1 L/min for 8 hours, a personal air sample could detect an exposure from 2.5 ng /m^3 to 5.0 ng/m^3 of aflatoxin B1. These values fall well within the range of airborne aflatoxin in the Burg *et al.* study, suggesting that this test would be useful to screen an 8 hour personal air sample. Assuming a worker breathes 10 cubic meters of air during a work shift, an ELISA test could optimally detect an exposure of 25 ng /day or 50 ng/ day, depending on the environment. Assuming an average human body mass of 70 kg, these exposures would result in a inhalation dose of 0.33 ng/kg/day and 0.71 ng/kg/day. To determine if these doses

might be of concern, a slope factor for inhaled aflatoxin was estimated based on data from the Olsen *et al.* epidemiology study, and is presented in Appendix III (14).

From the slope factor estimated in Appendix III, the inhalation virtually safe dose (VSD) representing a 1/1000 excess risk of liver cancer from inhaled aflatoxin was determined to be 1.6 ng/kg/day, and the inhalation VSD representing 1/10,000 risk was determined to be 0.16 ng/kg/day. In a personal air sample, the ELISA test kit appears to provide the potential to screen for an aflatoxin B1 exposure representing between a 1/10,000 and 1/1,000 working lifetime risk of cancer.

These test kits should be further evaluated personal or area sampling to identify the persons who have a significant occupational exposure to aflatoxin B1.

There are several recommendations for future research that can be made from this study. Since the ELISA test kit evaluated requires methanol:water to be used for extraction, a future study should be done to determine the methanol:water extraction efficiency for aflatoxin in biogenic dusts. Future studies should also explore the application of the ELISA test kits to actual air sampling.

Actual air sampling probably should be done using a filter media. This is because aflatoxin is going to be a bound on the dust, and aflatoxin has a very low volatility. No data could be found on percent recovery of aflatoxin from filter media using methanol:water as a solvent, so a future study could focus on determining an optimal sampling filter for aflatoxin.

Once an optimal filter is found, a study could be done to determine the actual sensitivity and specificity of the ELISA test kits by performing air sampling and analyzing the extract by both LC-MS-MS and ELISA. LC-MS-MS is recommended because of its sensitivity (3 pg injected). A cheaper alternative to LC-MS-MS is HPLC-UV florescence detection. This technique offers sensitivity below one nanogram injected, but requires derivitization of the aflatoxin B1 with trifloroacetic acid.

Although Editek kits were used in this study, aflatoxin test kits are available from the Neogen Corporation. A study could compare the performance of Neogen's test kits to EDITEK's test kits.

Lastly, there are ELISA kits for other mycotoxins. Neogen and Editek make mycotoxin kits for ochratoxin, T-2 toxin, fumonisin, and zearelanone. These mycotoxins have

not been as extensively examined in the industrial hygiene literature as aflatoxin. A future study could attempt to detect these mycotoxins using an ELISA kit.

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APPENDIX I CHEMICAL SUPPLIES

Chemical	Supplier	Comments
Methanol	Fischer Scientific	HPLC grade
	JT Baker (LC-MS-MS)	HPLC grade
Water	Fischer Scientific	HPLC grade
	JT Baker (LC-MS-MS)	HPLC grade
Acetonitrile	EM Science	HPLC grade
n-Hexane	Fischer Scientific	
Glacial Acetic Acid	Fischer Scientific	
Tetrahydrofuran	EM Science	
Methylene Chloride	JT Baker	
Dimethyl-dichlorosilane	Sigma Chemical	
Aflatoxin B1 3 ug/mL in 98:2 Benzene:acetonitrile	Supelco, Inc.	
Aflatoxin B1, 5 mg.	Sigma Chemical	>99% pure
Aflatoxin B + G mix 25 µg B1,G1; 7.5 µg B2, G2	Sigma Chemical	>99% pure
Ammonium Acetate	Fischer	

APPENDIX II HPLC METHOD DEVELOPMENT

Aliqouts of the extracts were spiked with aflatoxin B1 and cleaned using the SPE procedure previously described in the determination of aflatoxin in Dairy Farm Dust section in the materials and methods. The eluate from the SPE procedure was dissolved in 100 μl of methanol and 100 μl of 0.5% aqueous acetic acid.

Initial HPLC analysis was to be performed on a Hewlett-Packard HP 1050 liquid chromatograph. However, this instrument was abandoned due to an consistent back pressure build up. Later, this pressure was attributed to a dirty frit, which was immediately replaced.

The HPLC that was used for the isocratic analysis method comprised of a Waters 510 HPLC pump, a Waters U6K Injection loop, a Waters 486 Tunable Absorbance Detector, and a Waters 745 Data Module. The column was a 150 mm x 4.6 mm Alltech C18 column, with a 5 μm pore size. A Alltech C18 guard column was used. An isocratic system was used, consisting of 55% water, 22.5% methanol, and 22.5% acetonitrile. A flow of 2.5 mL/min was used. Retention time for Aflatoxin B1 was 3.7 min. Injection volume was 100 μl.

Post-clean up samples of dairy farm dust extract was spiked with aflatoxin B1 (Supelco, Inc.) at 300 ng/mL, 600 ng/mL, and 1.5 μg / mL. Chromatograms are shown on figures 4, 5 and 6. Figure 7 shows the instrument response to the spiked samples. By method of standard additions, there was no aflatoxin present in the dairy farm dust extract. However, this determination is confounded by a negative y-axis intercept, and a poor r^2 value (0.989).

Aflatoxin B1 eluted at retention time of 3.8 min., unfortunately next to an interfering peak that had a retention time of 3.5 min.. There were also two larger peaks that were present at retention times 1.64 and 4.25 min. The method failed to give adequate baseline separation of the aflatoxin B1 from any of these peaks. It was felt that a new procedure needed to be developed. One method used LC-MS-MS as described in the Analysis of Dairy Farm Dust in the Materials and Methods section.

Another method used the HP1050 liquid chromatograph (after the fret was replaced). A linear gradient system was developed based on the previous isocratic method. The initial program went from 100% water to 20:40:40 water:actonitirile:methanol over 10 min., maintained this

mix for 10 min., and then returned to 100% water over the last 5 min. The flow was at 1.5 mL/min. This system resulted in bubbles being formed in the HPLC during the run. The next modification was making the initial system 80:10:10 water:acetonitrile:methanol and following the same gradients as previously attempted. Retention time was greater than 20 min., so flow was increased to 2 mL/min. This method is described in the Analysis of Peanut Shell Dust in the Materials and Methods section.

Figure 5. 300 ng/mL spiked diary farm dust extract

MN= 0. REM PE= 1. PS= 1.

PW = 6. PT = 12. PH = 0.
TB = 0.

TT(1)=	0.02	TF(1)="PM"	TV(1)= 1.
TT(2)=	0.02	TF(2)="AT"	TV(2)= 0.5
TT(3)=	5.	TF(3)="ER"	TV(3)= 1.

30ng

=30 ng/ml

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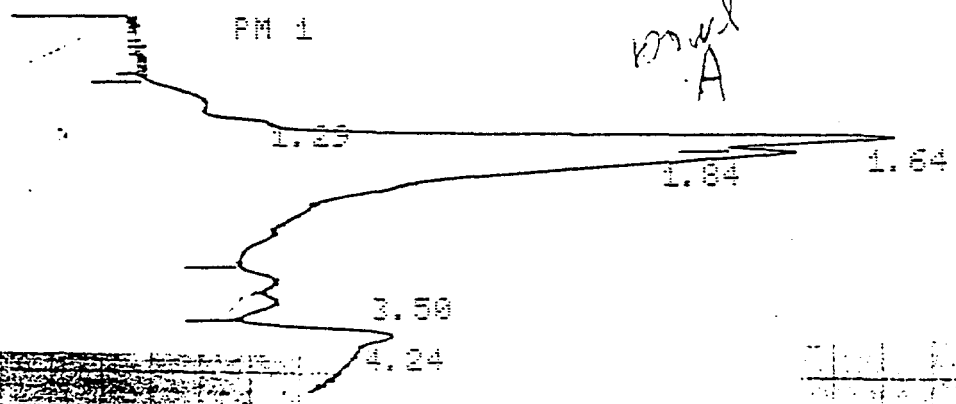

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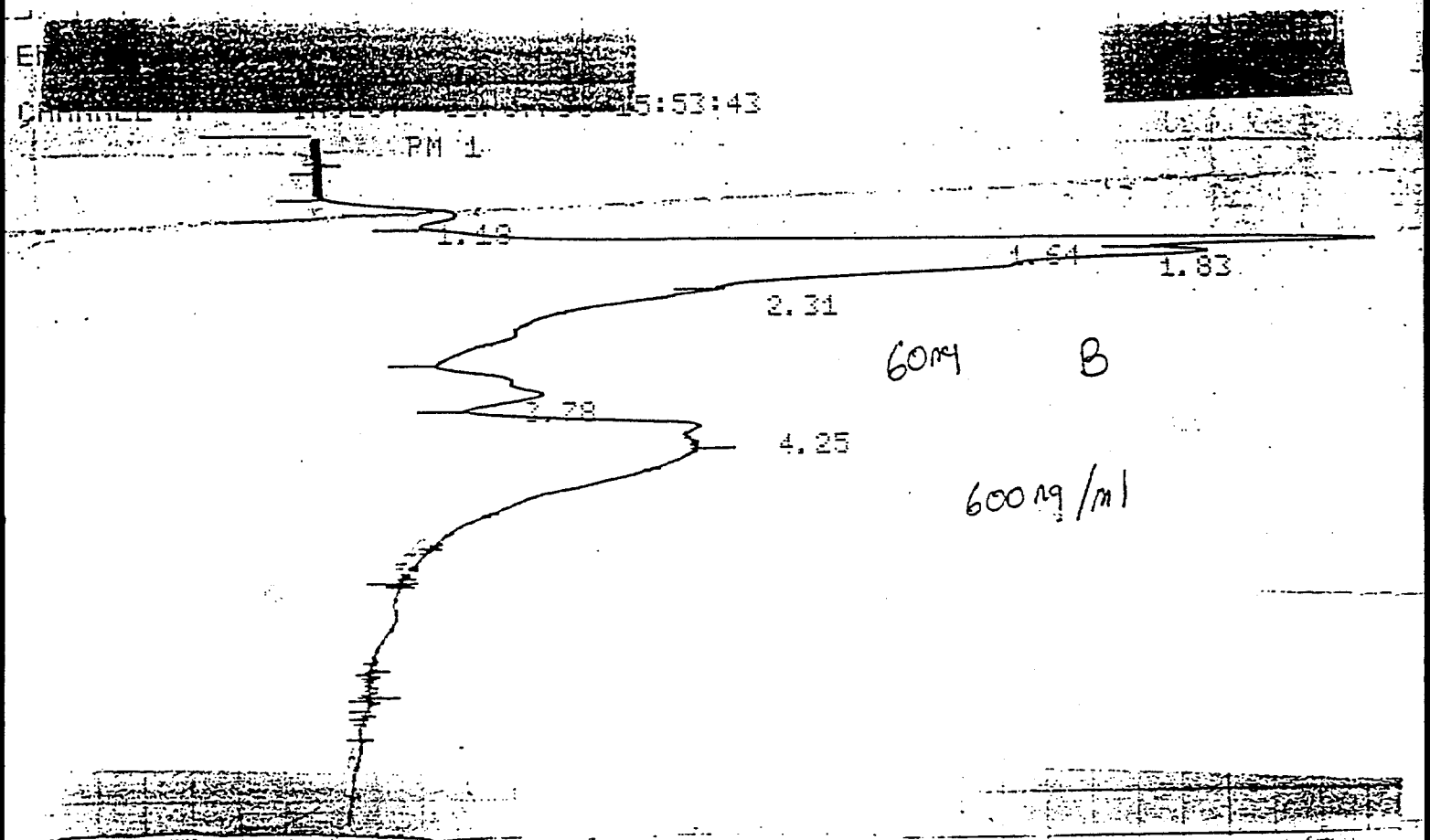
FILE 1. METHOD 0. RUN 1 INDEX 1

PEAK#	AREA%	RT	AREA BC
1	3.04	1.29	701 02
2	31.447	1.64	7252 02
3	51.498	1.84	11876 02
4	4.08	3.5	941 02
5	9.935	4.24	2291 03

23061

Figure 6. 600 ng/mL spiking of dairy farm dust extract



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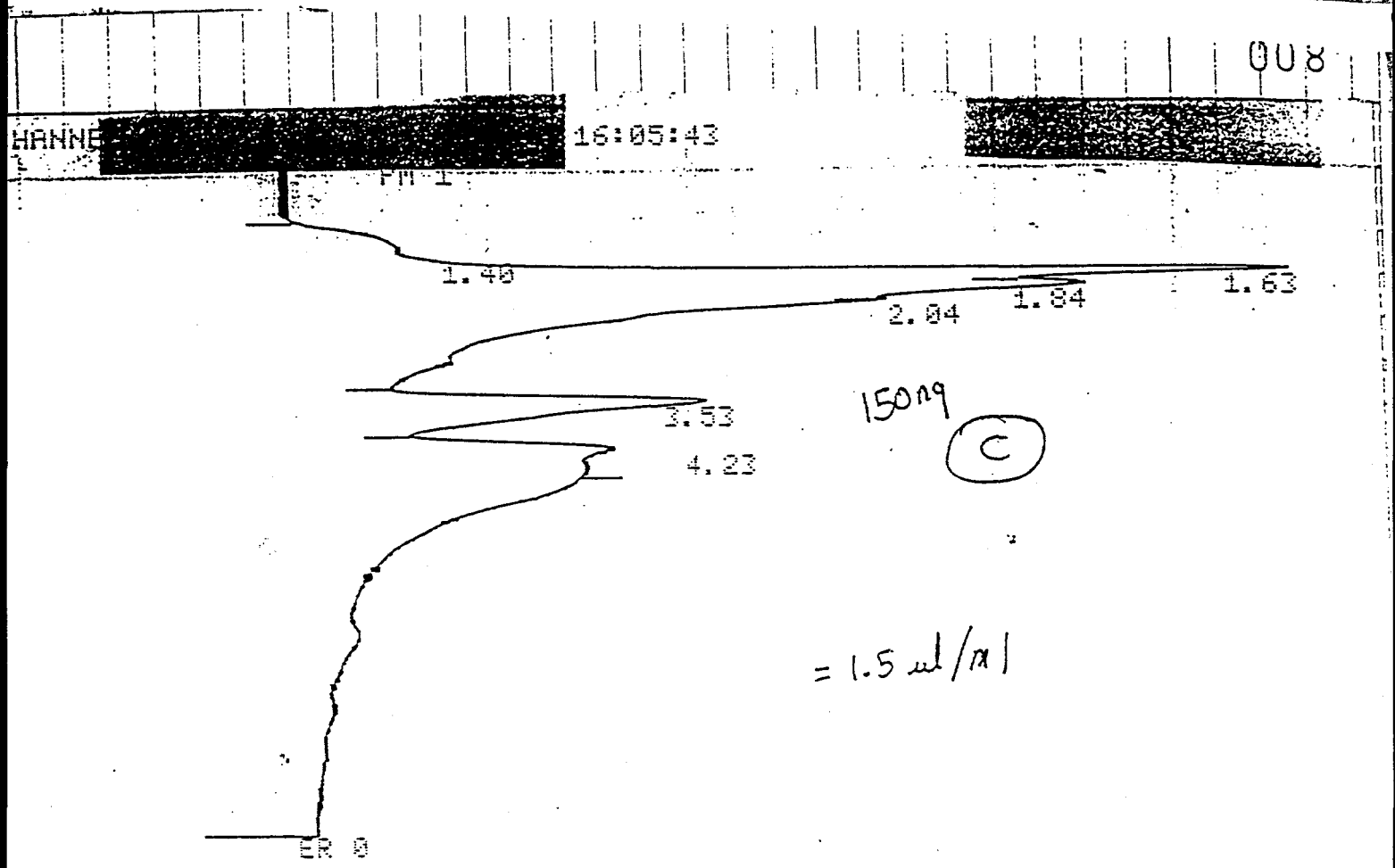
FILE 1. METHOD 0. RUN 1 INDEX 1

PEAK#	AREA%	RT	AREA	BC
1	4.727	1.18	1594	02
2	28.119	1.64	9483	02
3	40.787	1.83	13755	02
4	17.575	2.31	5927	02
5	4.267	3.78	1439	02
6	4.525	4.25	1526	03
TOTAL	100.		33724	

Figure 7. 1.5 μg/mL spiking of dairy farm dust extract

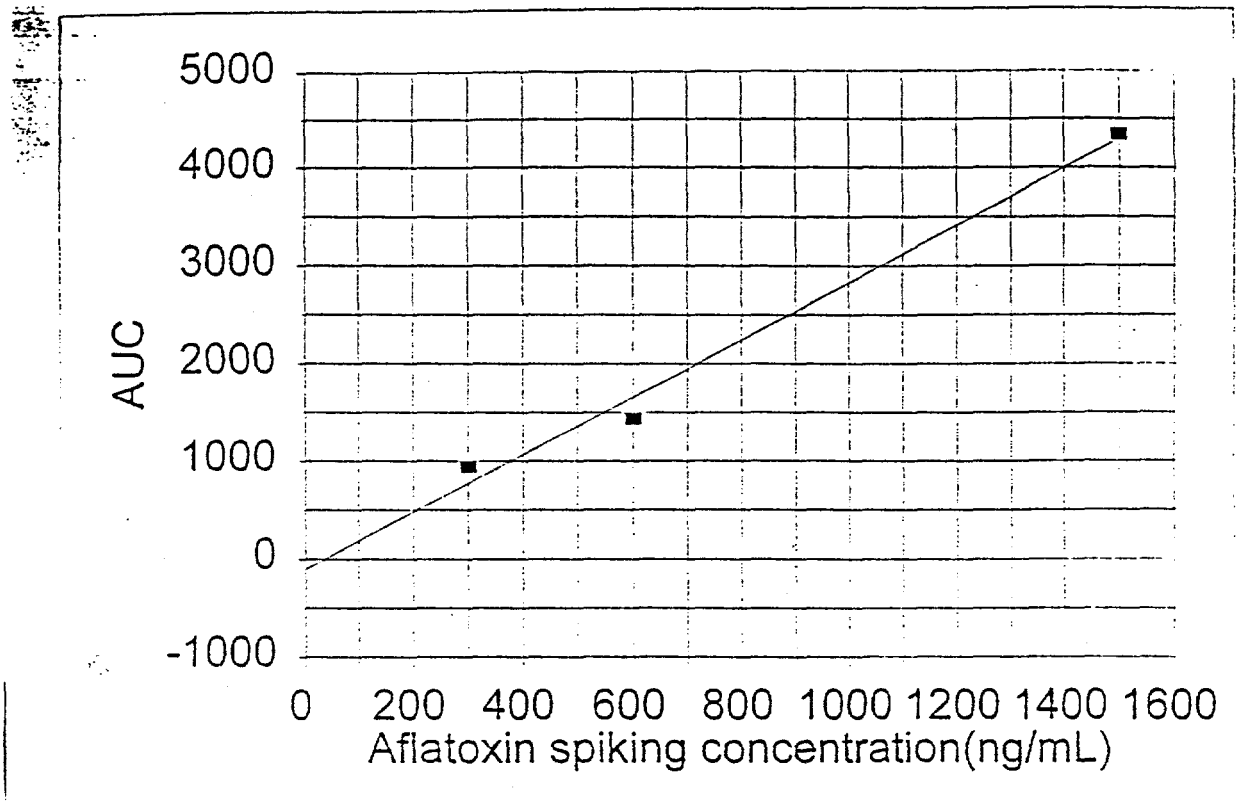


ZY 03/07/96 16:05:43 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 2 INDEX 2

PEAK#	AREA%	RT	AREA BC
1	4.489	1.4	1539 02
2	26.703	1.63	9154 02
3	22.718	1.84	7788 02
4	28.115	2.04	9638 02
5	12.675	3.53	4345 02
6	5.3	4.23	1817 03
TOTAL	100.		34281

Figure 8. Method of Standard Additions for Dairy Farm Dust



0		-100.077
300	941	778.0767
600	1439	1656.23
1500	4345	4290.692

Regression Output:

Constant	-100.077
Std Err of Y Est	276.916
R Squared	0.988656
No. of Observations	3
Degrees of Freedom	1
X Coefficient(s)	2.927179
Std Err of Coef.	0.313546

EZ-SCREEN: QUIK-CARD TEST PROCEDURE ON A 6-SITE CARD

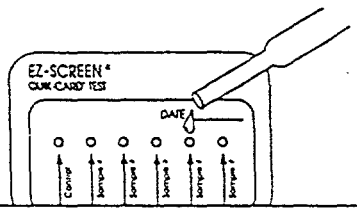
Preparation of the Reagents:

- Remove foil pouch from the refrigerator and allow all reagents in the pouch to reach ambient temperature before opening.
- Prepare the Negative Control (green-capped tube) by removing the plastic shrink seal from around the dropper cap.
- Prepare the Enzyme (red-capped tube) by squeezing the plastic dropper tube to break the inner glass ampule. Tilt the vial back and forth for approximately 10 seconds to rehydrate and mix the contents. Do not shake vigorously or the contents will foam. Remove the plastic shrink seal from around the dropper cap.
- Prepare the Substrate (blue-capped tube) by squeezing the plastic dropper tube to break the inner glass ampule. Shake the vial vigorously for approximately 10 seconds to rehydrate and mix the contents. Remove the plastic shrink seal from around the dropper cap.

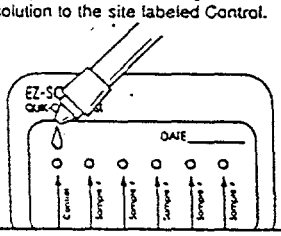
NOTE:

- When preparing the diluted sample extract, use the dilution buffer provided in the kit.
- When applying the sample or the test reagents to the QUIK-CARD*, hold the pipette tip or reagent tube tip above the test sites and allow the drops to fall freely.
- After the addition of the sample or the test reagents as shown below, allow the drops to absorb into the test sites before proceeding to the next step.

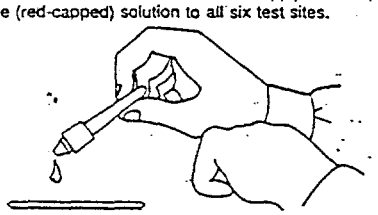
*STEP 1. Using the pipette provided, apply one drop of the prepared Sample Extract to the sites labeled Sample.



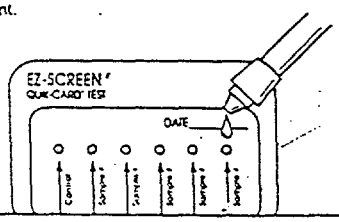
STEP 2. Apply one drop of the Negative Control (green-capped) solution to the site labeled Control.



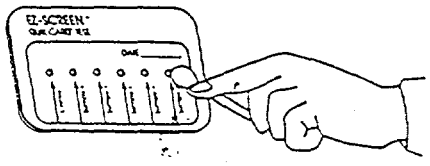
STEP 3. DISCARD THE FIRST DROP. Apply one drop of Enzyme (red-capped) solution to all six test sites.



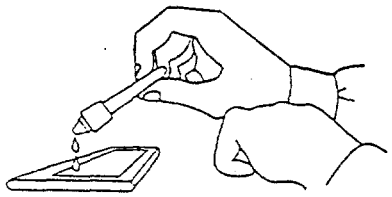
STEP 4. Apply one drop of Negative Control solution to all six test sites. In this step, the Negative Control is used as a Wash Reagent.



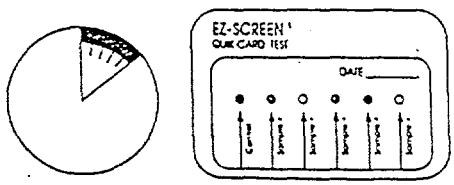
STEP 5. Carefully remove excess fluid from around the *outside* of all test sites with a cotton-tipped applicator or tissue. Do not touch the ports directly.



STEP 6. DISCARD THE FIRST DROP. Apply two drops of the Substrate (blue-capped) solution to all six test sites.



STEP 7. Interpret results in 5 minutes.



*See package insert for more detailed information.

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EZ-SCREEN®: QUIK-CARD® TEST

GENERAL PRINCIPLES

The EZ-SCREEN: QUIK-CARD system is a sequential competitive enzyme immunoassay in which a sample containing the analyte of interest competes with analyte coupled to an enzyme for binding to antibody. The system consists of 4 key components. The first is a solid support which serves as the location on which all of the test reactions occur. In the case of the System, the solid support is the QUIK-CARD. The card comes in two port or six port configurations that provide for either one or five tests plus a control. The QUIK-CARD consists of a rigid plastic frame containing a piece of glass fiber filter covered by a single adhesive label with either two or six small holes that serve as the sites for test reactions to take place.

The second test component is capture antibody. During manufacture rabbit antibody capable of reacting with the analyte of interest is added to the reaction sites of the card where it becomes trapped in the filter. The amount of antibody added to the reaction site determines the sensitivity of the test.

The third test component is enzyme conjugate. This material contains the analyte to be detected (e.g. aflatoxin) that has been chemically coupled to the enzyme horseradish peroxidase. The enzyme conjugate will compete with the sample for antibody binding sites at the reaction port.

The fourth test component is the enzyme substrate which will react with the enzyme conjugate in the absence of aflatoxin to produce a blue color.

ILLUSTRATION 1
NEGATIVE TEST

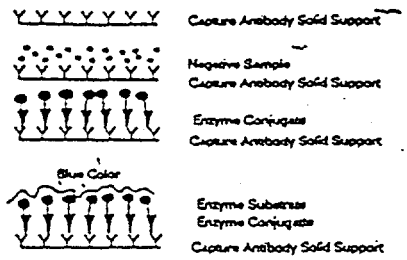


Illustration 1 shows the sequence of events that take place when a test sample does not contain the analyte of interest.

ILLUSTRATION 2
POSITIVE TEST

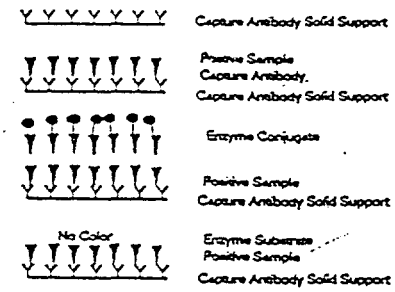


Illustration 2 shows the sequence of events that take place when a test sample contains the analyte of interest at levels equal to or exceeding the stated sensitivity of the assay.

ILLUSTRATION 3
NEGATIVE TEST

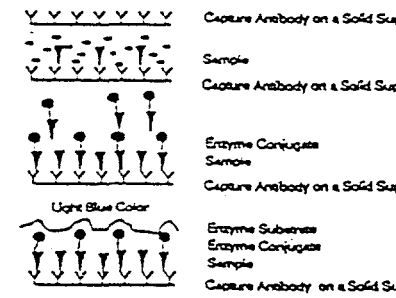
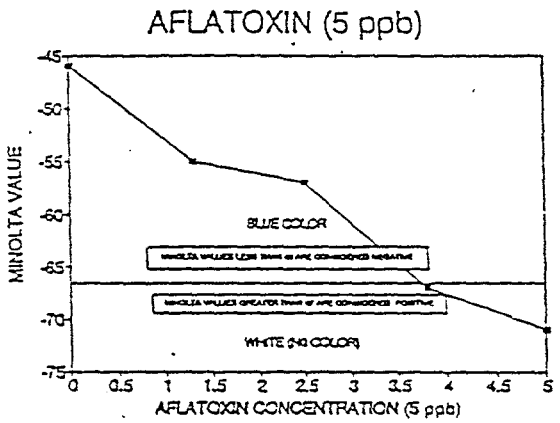


Illustration 3 shows the sequence of events that takes place when a test sample contains the analyte of interest but at a level which is less than the stated sensitivity of the assay (i.e. a level which is below the significant or regulatory level).

These illustrations show that a range of color intensities may be seen in the test. The colors range from dark, which is obtained with a negative sample because it contains no analyte to react with antibody, to white, which is obtained with a positive sample because it contains enough analyte to react with all of the antibody. The EZ-SCREEN: QUIK-CARD test is a qualitative screening procedure, so intermediate color changes in the range from dark to white may not be used to precisely measure the presence of a specific level of analyte in the sample. However, they may be used to evaluate the performance of the EZ-SCREEN: QUIK-CARD assay.

During product quality control evaluation, the presence of color is monitored using an instrument that measures the reflectance of light from a "Spot" or "Point". This instrument called the Minolta Chromometer gives readings known as Minolta values or reflectance meter values. This is illustrated for the EZ-SCREEN:Aflatoxin 5 ppb test.



A standard curve is generated at each phase of the product quality control. The y-axis is divided into Minolta values with 45 being dark and 67 being white. The reflectance meter values provide an objective measure of the degree of color development obtained at each concentration. Values of 67 or greater are evaluated as an "absence of visually detectable color", or POSITIVE while values of 66 or less are evaluated as the "presence of visually detectable color", or NEGATIVE.

Samples containing aflatoxin at a level of 5 ppb or greater will always give a positive result in the EZ-SCREEN:Aflatoxin 5 ppb test while samples containing 2.5 ppb or less will always be negative. The frequency of positive findings in the equivocal range will decrease as the contamination level falls from 5 ppb to 2.5 ppb.

The EZ-SCREEN:AFLATOXIN 5 ppb test is manufactured in accordance with FDA Good Manufacturing Practices (GMP) which requires that specifications and controls be established and that the finished product meet these specifications.

In addition to the general GMP requirements EDITEK employs in-process controls and statistically-based sampling to ensure consistent product quality and conformance to specifications.

Manufactured by EDITEK, Inc., Burlington, N.C.

APPENDIX IV SLOPE FACTOR FOR INHALED AFLATOXIN

Olsen *et al.* failed to report a cohort size, so cohort size had to be inferred from the expected number of cases of liver cancer over the fourteen year period of study. Olsen reported the expected number of liver cancers as 2.8, and a national rate of 10 liver cancers/ 100,000 over the fourteen year period of study. The cohort size was determined to be 28,000 persons (eq. 1).

Equation 1:

$$28{,}000 = \frac{2.8}{\frac{10}{100{,}000}}$$

where 28,000= cohort size
2.8= number of expected cases
10/10,000 = national cancer rate over 14 years

To determine excess liver cancer rate the background rate of liver cancer (2.8/10,000) was subtracted from the observed liver cancer rate (7/10,000)(eq. 2).

Equation 2:

$$\frac{4.2}{10{,}000} = \frac{7}{10{,}000} - \frac{2.8}{10{,}000}$$

where 4.2/10,000 = excess liver cancer rate over 14 yrs.
7/10,000 = observed liver cancer rate over 14 yrs.

representing 1/10,000 risk was determined to be 0.16 ng/kg/day (eq. 4).

Equation 4:

$$1.62 ng/kg/day = \frac{1 ng/kg/day}{0.000617} * \frac{1}{1{,}000}$$

where 1.62 ng/kg/day = daily dose representing 1 in 1000 working lifetime risk of liver cancer

The estimation of the occupational exposure limit is quite precarious. The main problem with the Olsen *et al.* study is the crude approach of estimating the exposure in the cohort by calculating the exposure from dust concentrations around the feed handling operations and then assuming the dust aflatoxin concentration to be equal to the feed concentration. The exposure assessment goes even further to assume homogeneous exposure across all employees in the cohort. Over estimation of exposure will effect the model by underestimating the dose-response slope, ultimately resulting in a less protective risk assessment. The epidemiological data is controlled for age, but not anything

else. Among many variables not modeled, stratified or matched on is smoking or alcohol consumption.

To compare the relative potency of inhaled versus ingested aflatoxin, the inhaled VSD of 1/100,000 for lifetime risk of liver cancer was compared VSD's of 1/100,000 for lifetime risk of liver cancer from risk assessments that evaluated ingested aflatoxin risk. Based on the slope factor calculated from the Olsen *et al.* data, the inhaled VSD for a 1/100,000 risk over 50 years is 0.016 ng/kg/day. In a risk assessment of oral intake of aflatoxin, Bruce gives an acceptable dose for a 1/100,000 risk of liver cancer of 50 years as 0.12 ng/kg/day (28). The dose recommended in another risk assessment was 0.14 ng/kg/day based on human data, 0.15 ng/kg/day based on animal using a NOAEL/5000 method, and 0.023 ng/kg/day based on animal using a Toxic Dose 50% /50000 method (29). Wu-Williams *et al.* calculated an acceptable daily oral intake of aflatoxin for a 1/100,000 lifetime risk of liver cancer as 0.22 mg/kg/day using a multiplicative model of aflatoxin intake and hepatitis B virus status, respectively (30). Therefore, based on Olsen *et al.* study, inhaled aflatoxin appears to be 10 times more potent than ingested aflatoxin.

This apparent increase may be a result of other co-contaminants, such as pesticides, that may be present in the dust. Another possible explanation is that the slope factor calculated from the Olsen et al. data failed to take into account dietary intake of aflatoxin, resulting in an underestimation of total dose.

APPENDIX V HPLC AND LC-MS-MS RESULTS

List of Chromatograms

LC-MS-MS

Chromatogram Number

1....Mass spectra of Aflatoxin B1, G1, B2, G2
2....Daughter Spectra of Aflatoxin B1
3....Daughter Spectra of Aflatoxin G1
4....Daughter Spectra of Aflatoxin B2
5....Daughter Spectra of Aflatoxin G2

6....MRM of 15 ng/mL Aflatoxin B1
7....MRM of 30 ng/mL Aflatoxin B1
8....MRM of diary dust extract
9....MRM of dairy dust extract (313→241,321→257)
10...MRM of dairy dust extract (313→241,321→257)
11...MRM of aflatoxin B1 spiked solution

12...MRM of solution of Aflatoxin B1, G1, B2, G2
13...MRM Elution order of Aflatoxin B1, G1, B2, G2
14...MRM Elution order of Aflatoxin B1, G1, B2, G2
15...MRM of for aflatoxin B1, G1, B2, G2 in dairy farm extract
16...MRM of for aflatoxin B1, G1, B2, G2 in dairy farm extract

17a,b,c...MRM's of 6 ng/mL spiked dairy farm dust extract

18...MRM of dairy farm dust extract
19...MRM of 15 ng/mL Aflatoxin B1
20...MRM of 30 ng/mL Aflatoxin B1

21...MRM of 1 mL of dairy farm dust extract
22...MRM of concentrated 10 mL dairy farm dust extract

23...MRM of 1 mL of methanol:water spiked with 6 ng aflatoxin B1
24...MRM of 10 mL of methanol:water spiked with 6 ng aflatoxin B1
25...MRM of 7.5 ng/mL aflatoxin B1
26...MRM of solvent (blank)

HPLC-UV

27...Solvent Blank
28...Solvent Blank (13.4 min → 15.4 min)
29...25 ng/mL standard
30...50 ng/mL standard
31...75 ng/mL standard
32...100 ng/mL standard

33...100 ng/mL standard
34...200 ng/mL standard
35...75 ng/mL standard

36...Concentrated 10 mL of peanut shell dust extract

37...Concentrated 10 mL of peanut shell dust extract (12 → 15 min)

38...Conc. 10 mL of peanut shell dust extract+25 ng aflatoxin B1

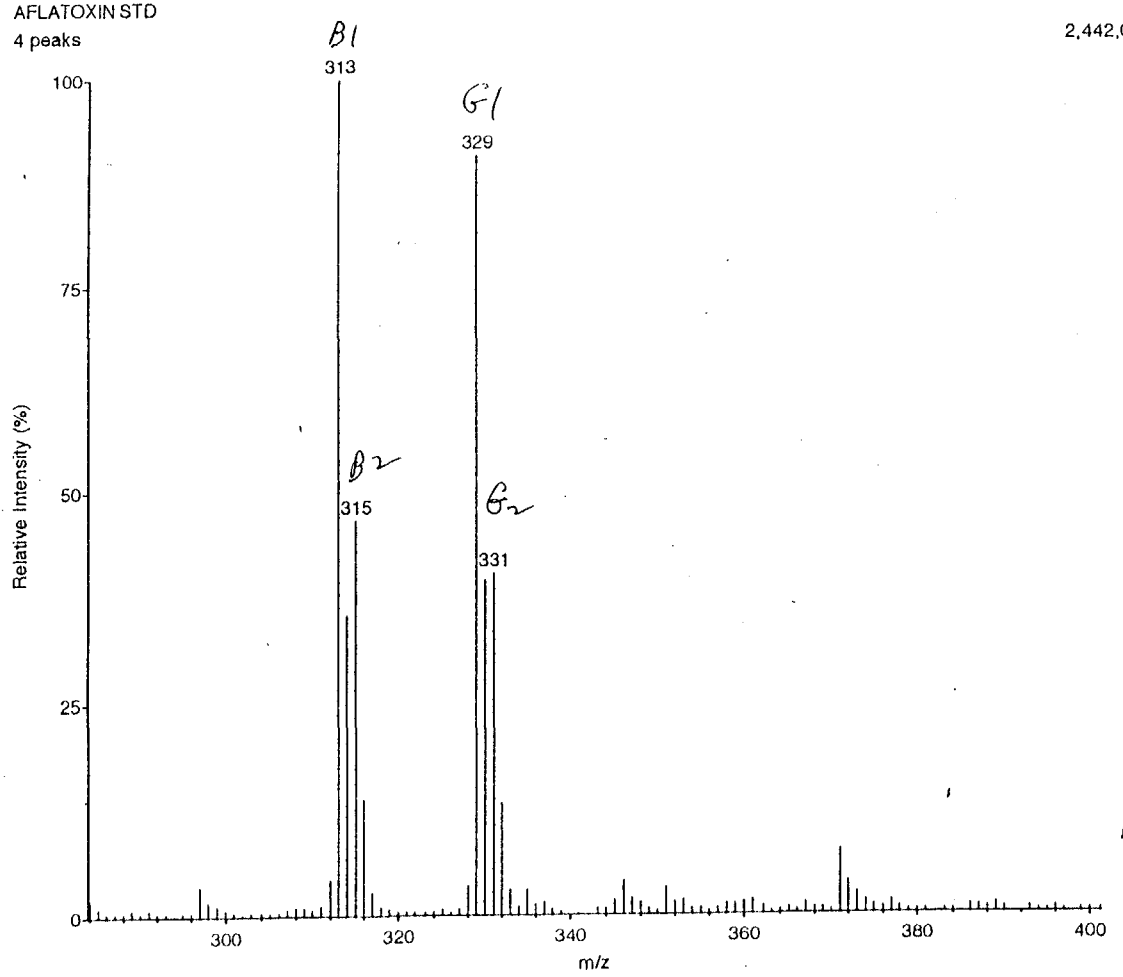
39...Conc. 10 mL of peanut shell dust extract+25 ng aflatoxin B1 (12 → 15 min)

DI	200
ISV	5500
IN	650
OR	70
R0	30
M1	1000
RE1	119.4
DM1	0.190
R1	28.1
L7	0
R2	0
M3	1000
RE3	120.3
DM3	0
RX	-20
R3	-100
L9	-250
FP	-250
MU	-4300
CC	10
CG	Off
DI μA	5.9
CGT	4.0
IS v	5622.9
UV	4342.9

Aflatoxin std/Scans 59-65

+Profile Q1SCAN
Scans 59-65 minus 16-49 Time=1.27 min
Aflatoxin std - 3/20/96 - 8:36 AM
AFLATOXIN STD
4 peaks

2,442,017



Chromatogram 1

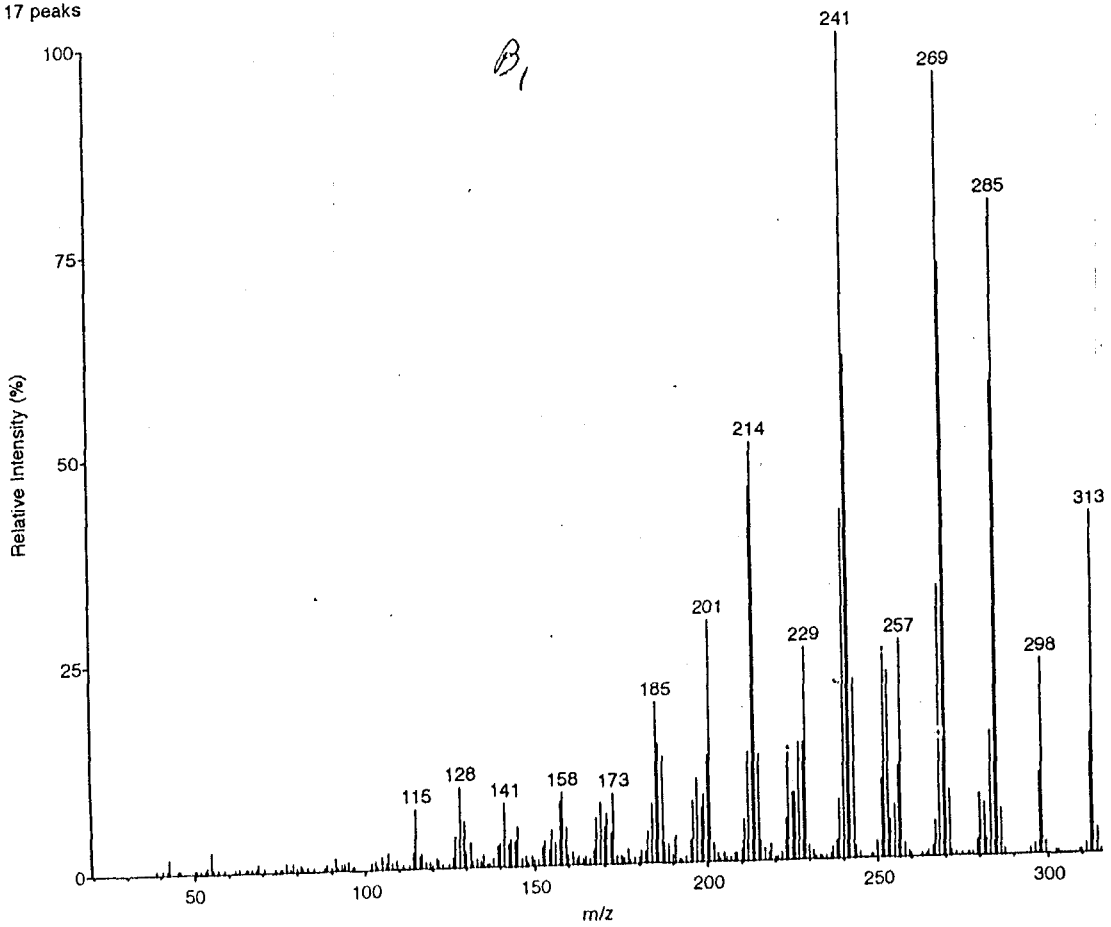
Aflatoxin std MSMS-313/Scans 45-51

+Profile DAUGHTER, Parent = 313
Scans 45-51 minus 25-39 Time=1.27 min
Aflatoxin std MSMS- - 3/20/96 - 8:40 AM
AFLATOXIN STD
17 peaks

192,606

B1

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4100
CC	1
CG	Ar
DI μA	4.5
CGT	312.6
IS v	5627.7
UV	4338.06



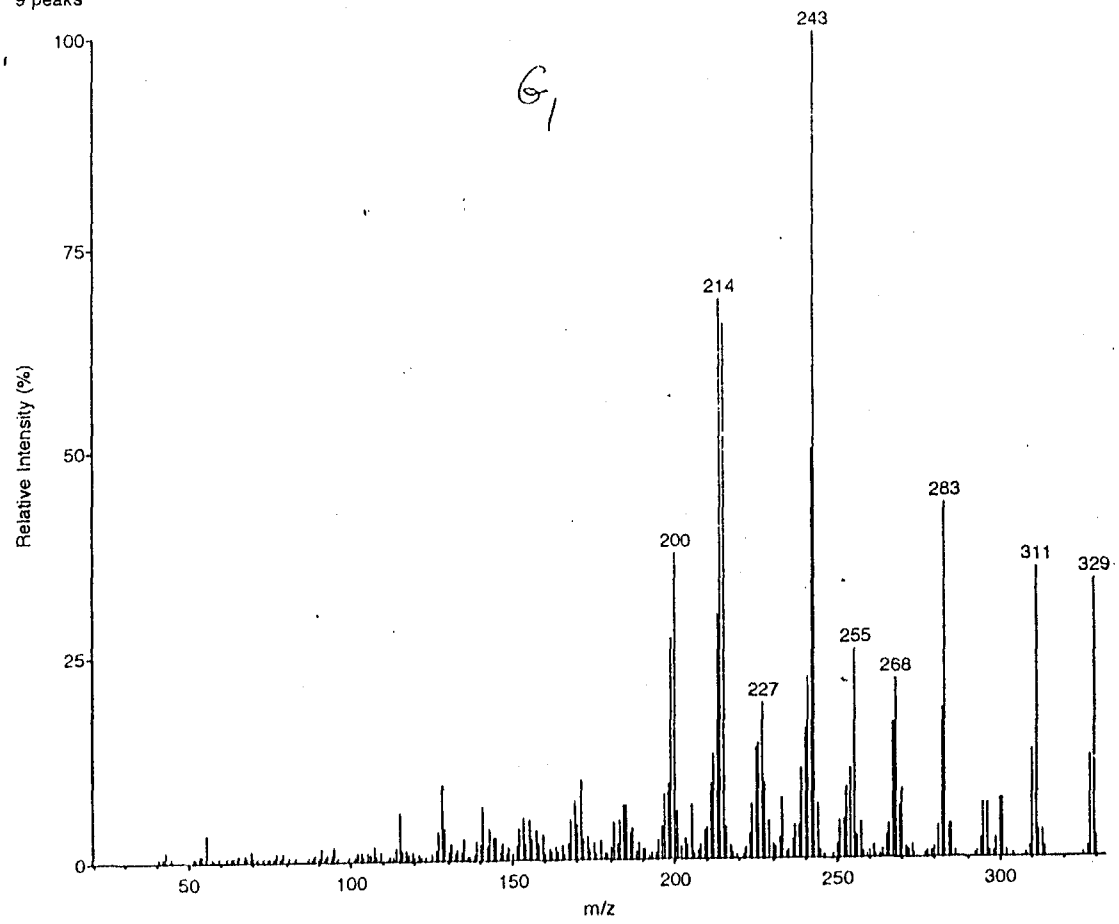
Chromatogram 2

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
.7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
.9	-250
FP	0
MU	-4100
CC	1
CG	Ar
DI μA	4.5
CGT	312.6
S v	5627.7
JV	4211.04

Aflatoxin std MSMS-329/Scans 45-49

+Profile DAUGHTER, Parent = 329
Scans 45-49 minus 15-40 Time=1.24 min
Aflatoxin std MSMS- - 3/20/96 - 8:45 AM
AFLATOXIN STD
9 peaks

214,620



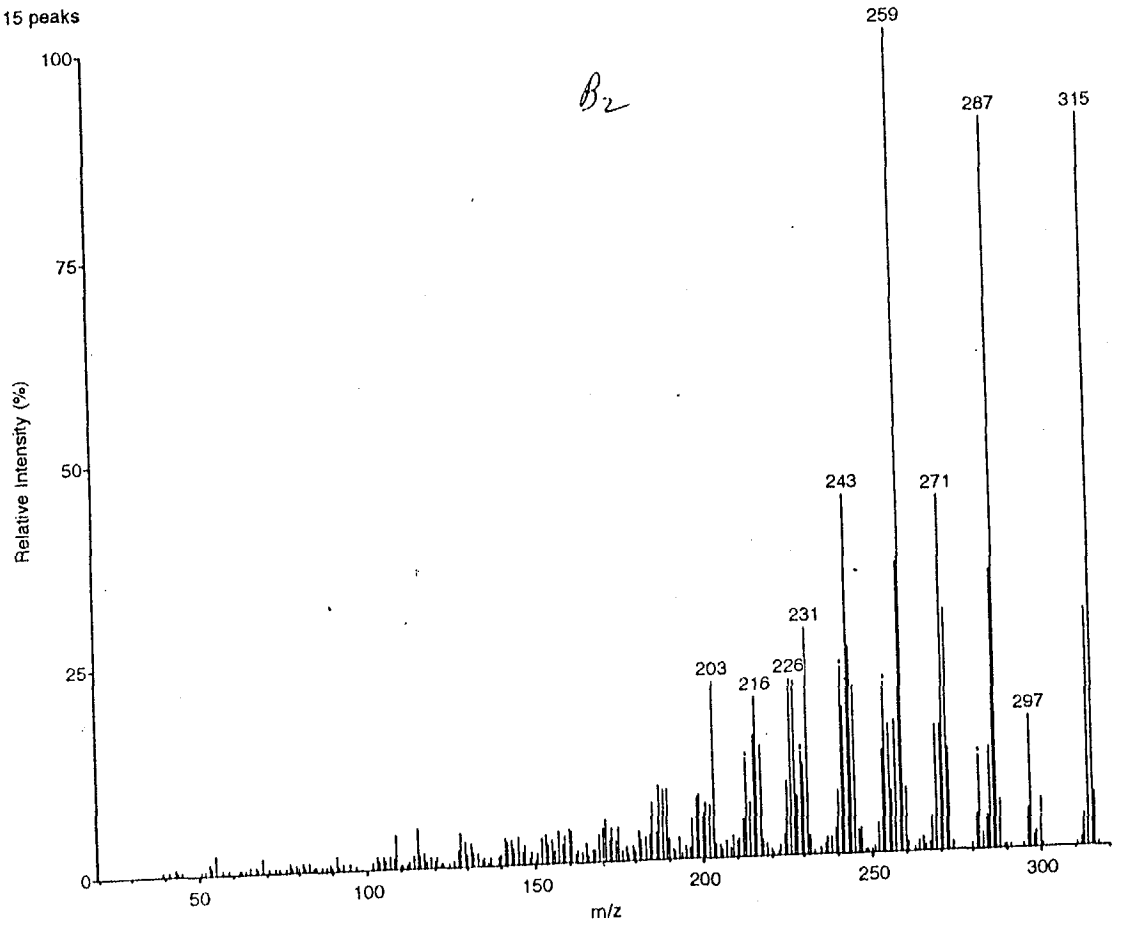
Chromatogram 3

DI	200
ISV	5500
IN	650
OR	80
R0	30
VI1	1000
RE1	115.0
DM1	0.160
R1	27
_7	5
R2	3
VI3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
_9	-250
FP	0
VU	-4100
DC	1
DG	Ar
DI μA	5.4
DGT	312.6
S v	5583.8
JV	4289.2

Aflatoxin std MSMS-315/Scans 50-59

+Profile DAUGHTER, Parent = 315
Scans 50-59 minus 19-42 Time=1.37 min
Aflatoxin std MSMS- - 3/20/96 - 8:50 AM
AFLATOXIN STD
15 peaks

110,508

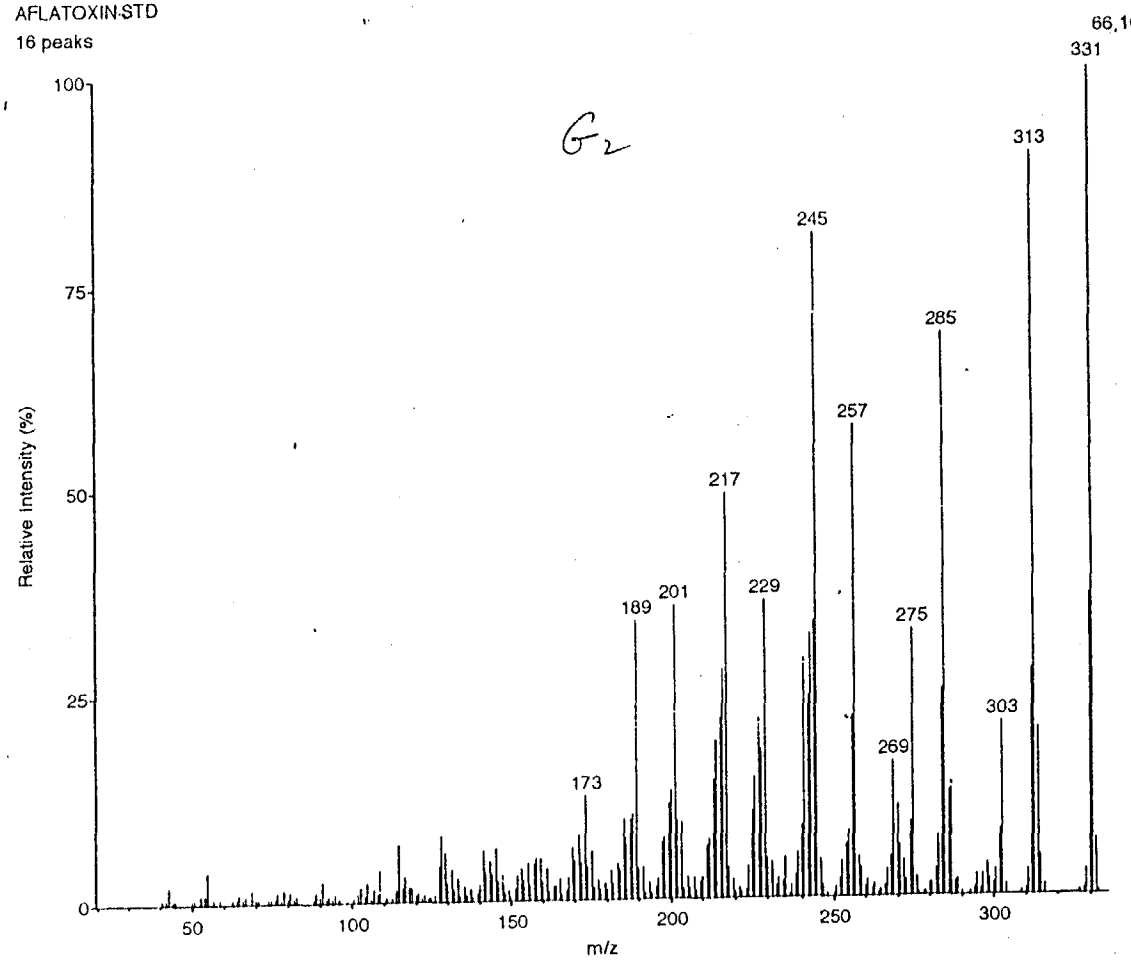


Chromatogram 4

)I	200
SV	5500
N	650
)R	80
?0	30
/1	1000
?E1	115.0
)M1	0.160
?1	27
.7	5
?2	3
/3	1000
?E3	120.3
)M3	0.160
?X	-10
?3	-10
.9	-250
?P	0
/U	-4100
)C	1
)G	Ar
)i μA	6.5
)GT	312.6
? v	5618.0
/V	4181.7

Aflatoxin std MSMS-331/Scans 45-57

+Profile DAUGHTER, Parent = 331
Scans 45-57 minus 27-39 Time=1.34 min
Aflatoxin std MSMS- - 3/20/96 - 8:54 AM
AFLATOXIN-STD
16 peaks



Chromatogram 5

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4100
CC	1
CG	Ar
Di μA	3.05
CGT	313.9
IS v	5613.09
UV	4299.0

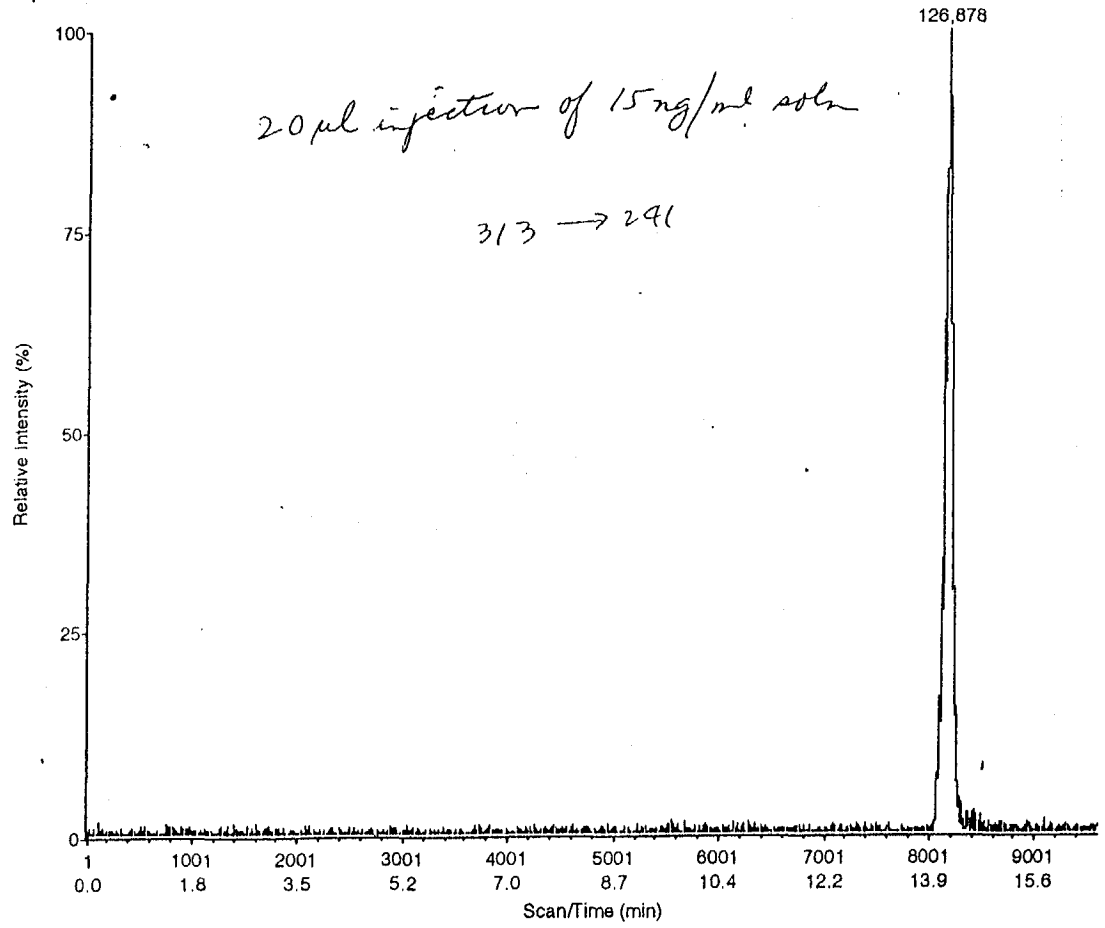
Aflatoxin B1-LCMS-MRM-2/Chromatogram

TIC of all masses

Aflatoxin B1-LCMS-M - 3/11/96 - 8:49 AM
Aflatoxin B1, 15 ng/ml
1 peak

2040

126,878



Chromatogram 6

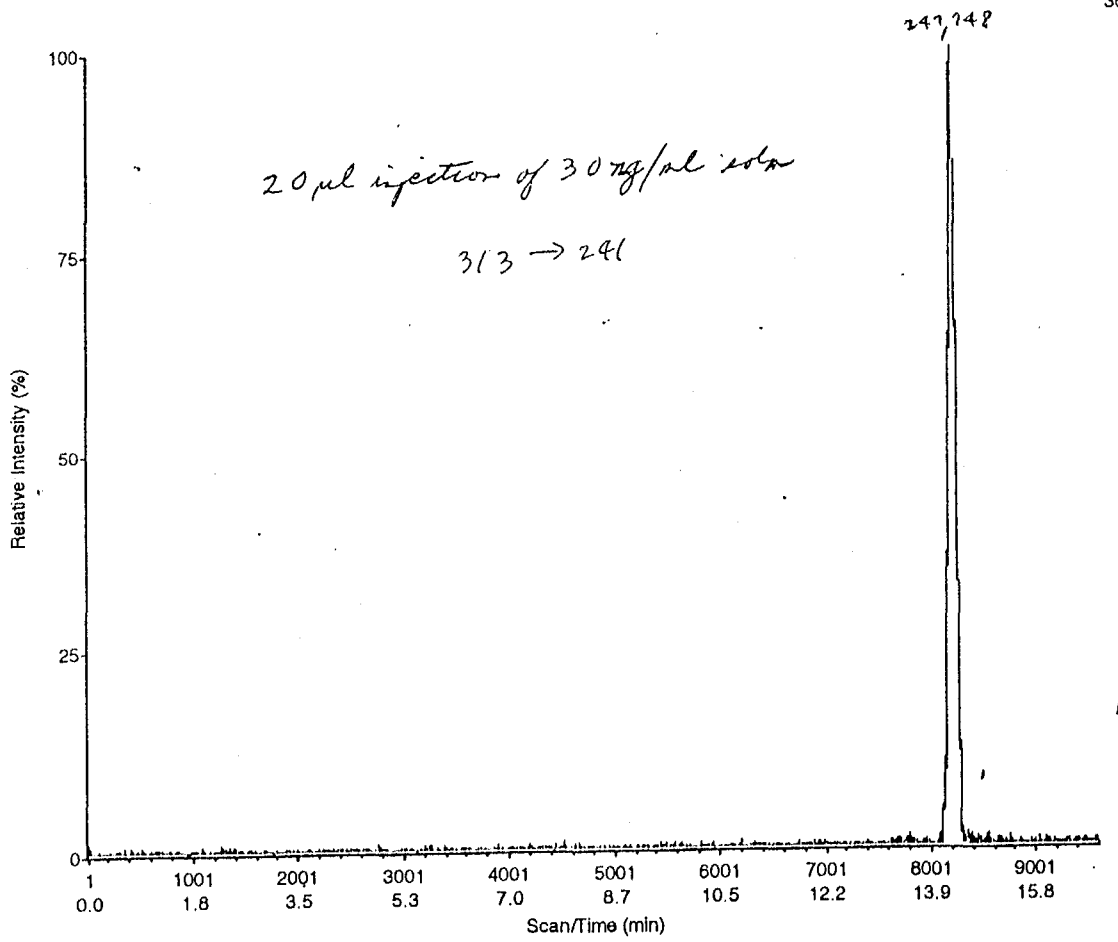
DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4100
CC	1
CG	Ar
DI μA	2.6
CGT	315.2
IS v	5613.09
UV	4386.9

Aflatoxin B1-LCMS-MRM/Chromatogram

TIC of all masses

Aflatoxin B1-LCMS-M - 3/11/96 - 8:20 AM
Aflatoxin B1, 30 ng/ml

3696



Chromatogram 7

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4100
CC	1
CG	Ar
DiμA	3.05
CGT	316.6
IS v	5622.9
UV	4196.4

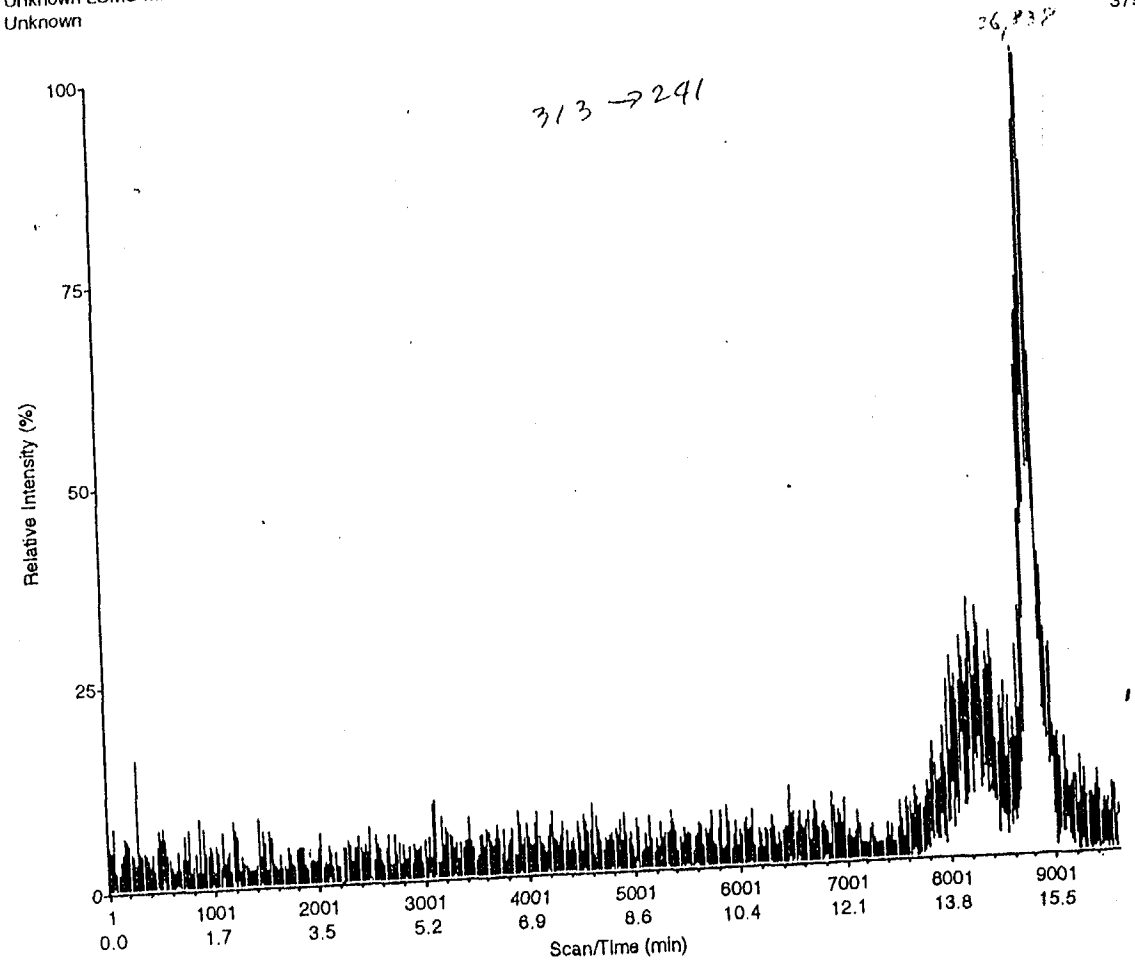
Unknown-LCMS-MRM/Chromatogram

TIC of all masses

Unknown-LCMS-MRM - 3/11/96 - 9:20 AM
Unknown

379

313 → 241



Chromatogram 8

Chromatogram 9

Unknown-LCMS-MRM-2/Chromatogram

TIC of all masses

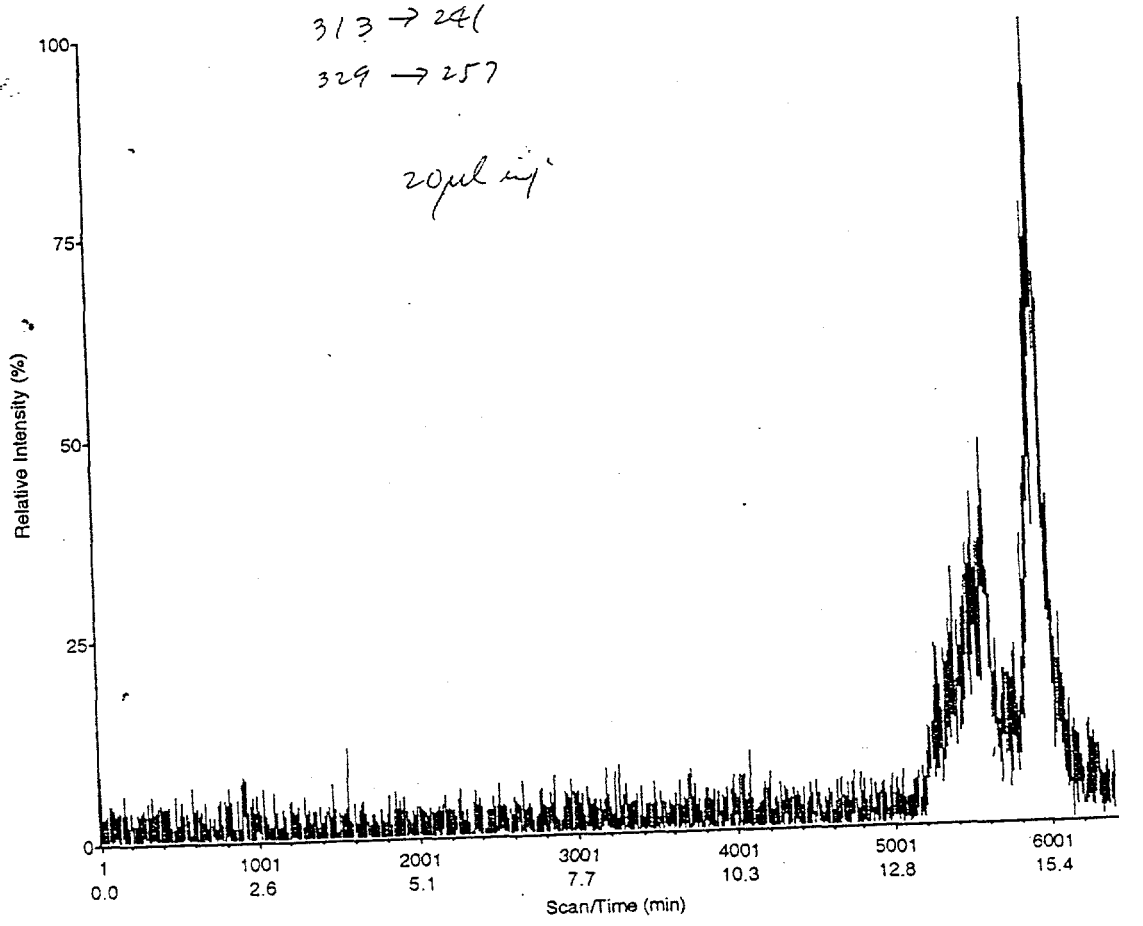
Unknown-LCMS-MRM-2 - 3/11/96 - 10:21 AM
Unknown

489

313 → 241
329 → 257

20µl inj

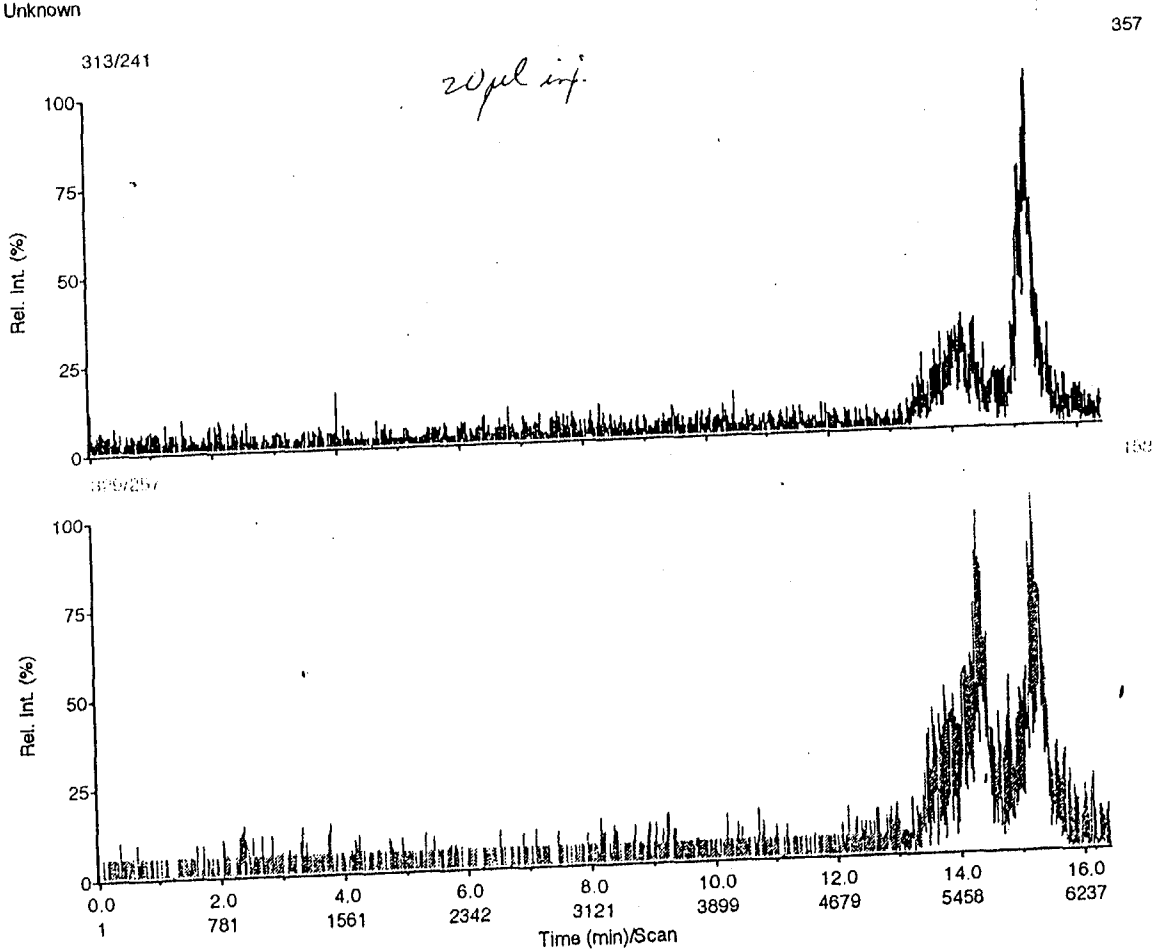
200
5500
650
80
30
1000
115.0
0.160
27
5
3
1000
120.3
0.160
-10
-10
-250
0
-4100
1
Ar
3.2
312.6
5598.4
4294.09



Unknown-LCMS-MRM-2/Extract

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4100
CC	1
CG	Ar
Di μA	3.2
CGT	312.6
IS v	5598.4
UV	4294.09

Unknown-LCMS-MRM-2 - 3/11/96 - 10:21 AM
Unknown



Chromatogram 10

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4100
CC	1
CG	Ar
DI μA	4.2
CGT	312.6
IS v	5608.2
UV	4352.7

Spiked sample-LCMS-MRM/Chromatogram

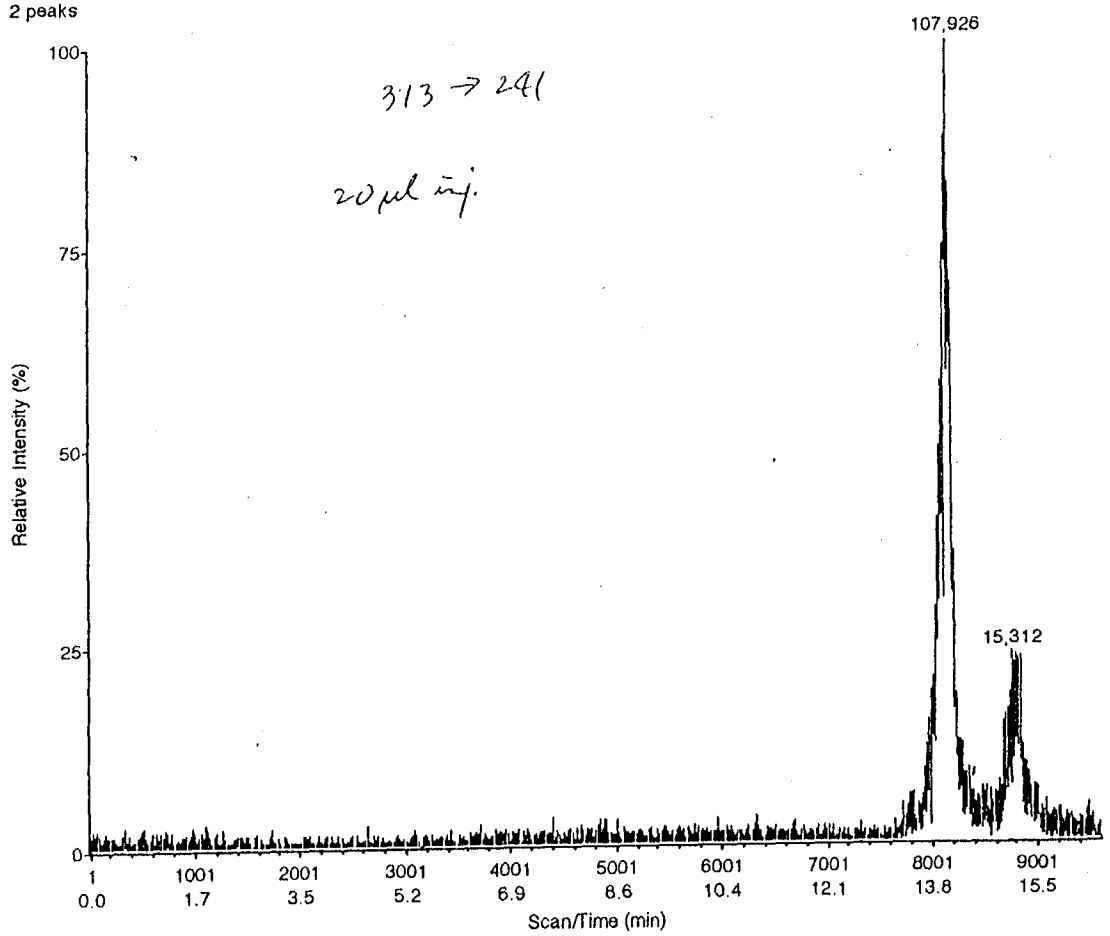
TIC of all masses

Spiked sample-LCMS- - 3/11/96 - 9:50 AM
Spiked sample
2 peaks

1090

313 → 241

20 μl inj.



Chromatogram 11

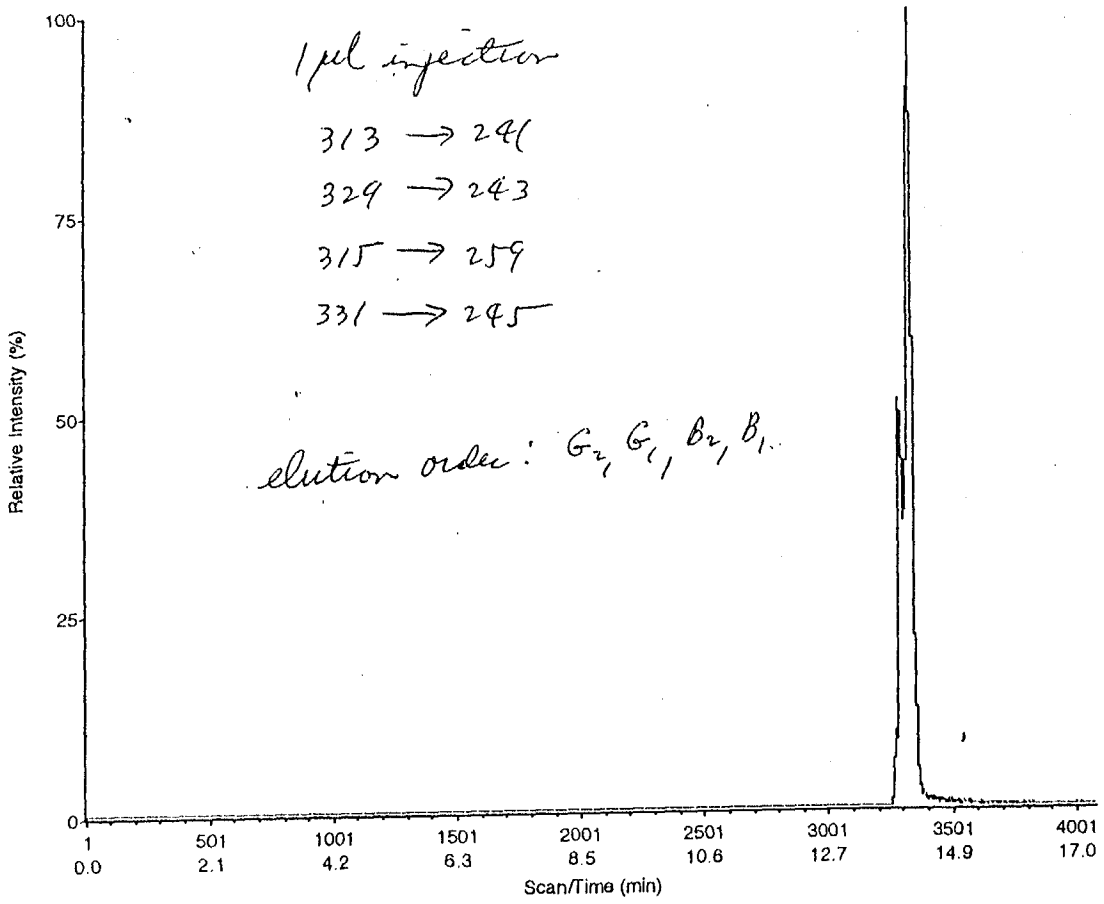
DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
DI μA	4.4
CGT	307.3
IS v	5598.4
UV	4372.3

Aflatoxin std LCMS-MRM/Chromatogram

TIC of all masses

Aflatoxin std LCMS- - 3/20/96 - 11:44 AM
Aflatoxin std

25,254

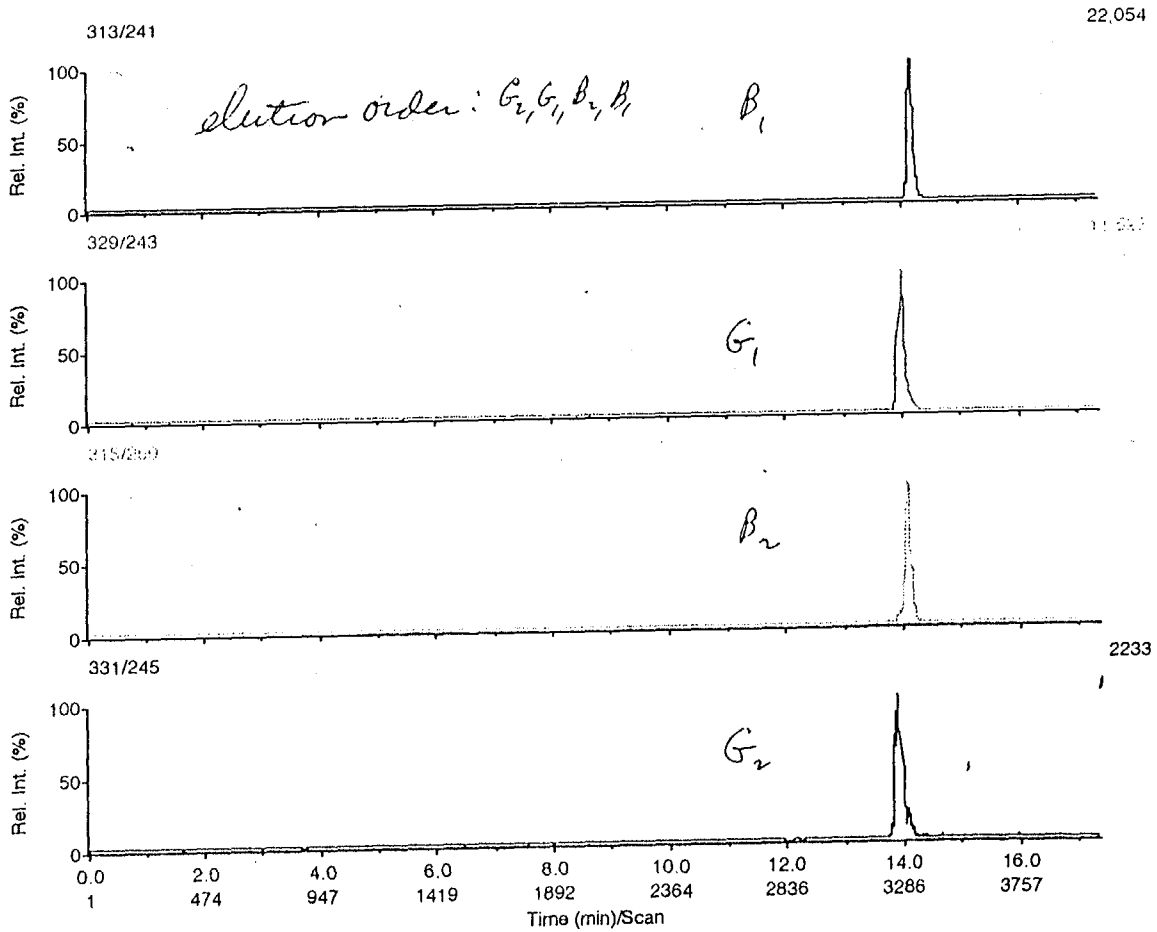


Chromatogram 12

Aflatoxin std LCMS-MRM/Extract

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
DiμA	4.4
CGT	307.3
IS v	5598.4
UV	4372.3

Aflatoxin std LCMS- - 3/20/96 - 11:44 AM
Aflatoxin std



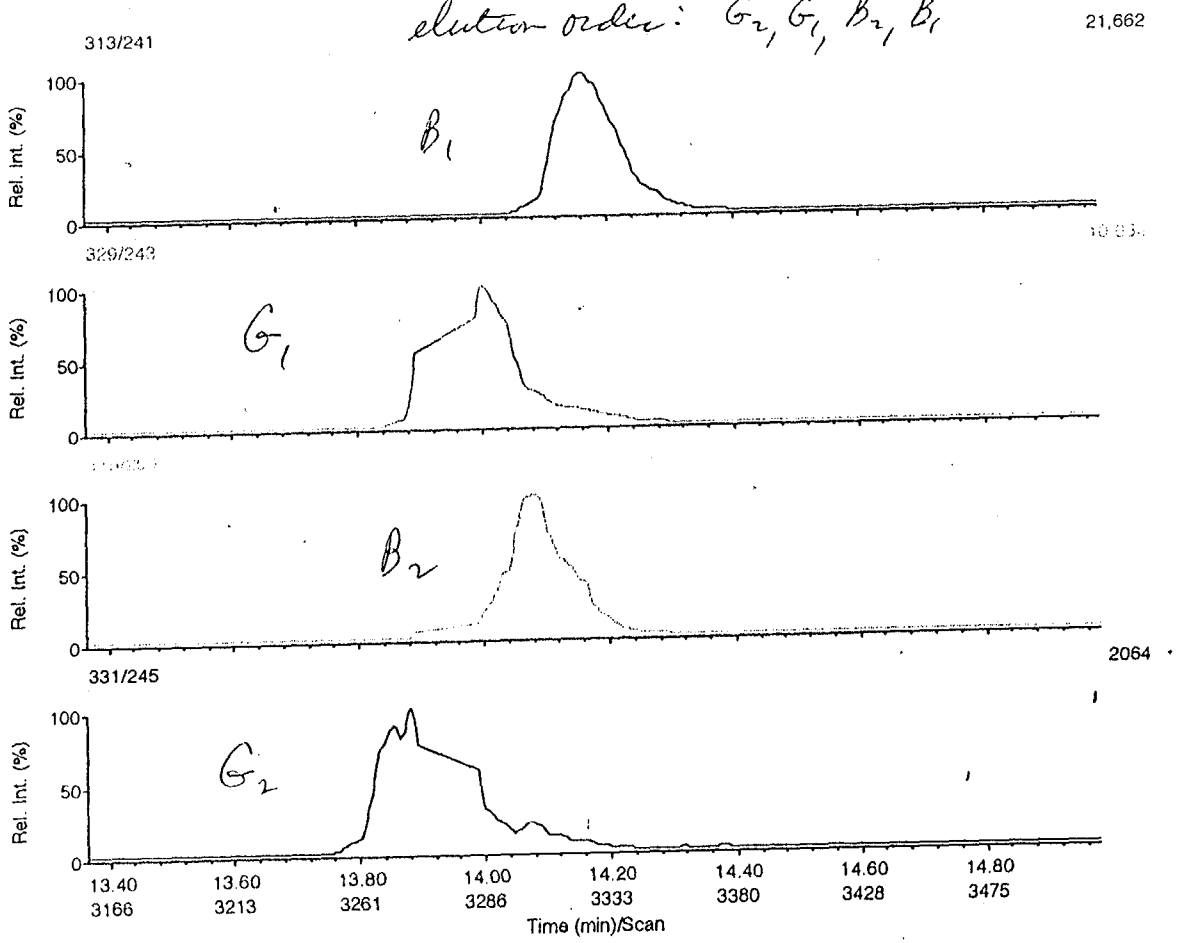
Chromatogram 13

Aflatoxin std LCMS-MRM/Extract

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
DI μA	4.4
CGT	307.3
IS v	5598.4
UV	4372.3

Aflatoxin std LCMS- - 3/20/96 - 11:44 AM
Aflatoxin std

elution order: G_2, G_1, B_2, B_1



Chromatogram 14

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
DI μA	3.4
CGT	308.6
IS v	5642.4
UV	4240.4

Unknown LCMS-MRM-2/Chromatogram

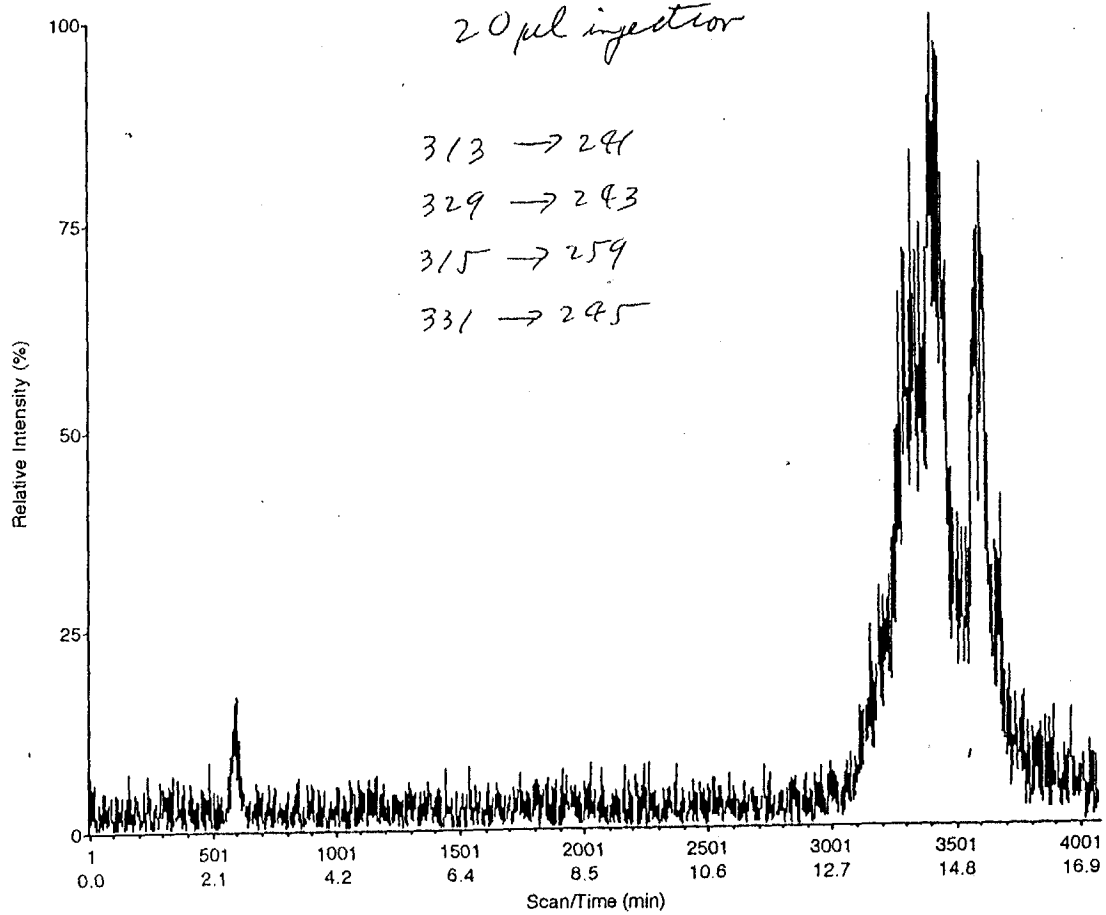
TIC of all masses

Unknown LCMS-MRM-2 - 3/20/96 - 12:39 PM
Unknown

608

20 μl injection

313 → 291
329 → 293
315 → 259
331 → 295

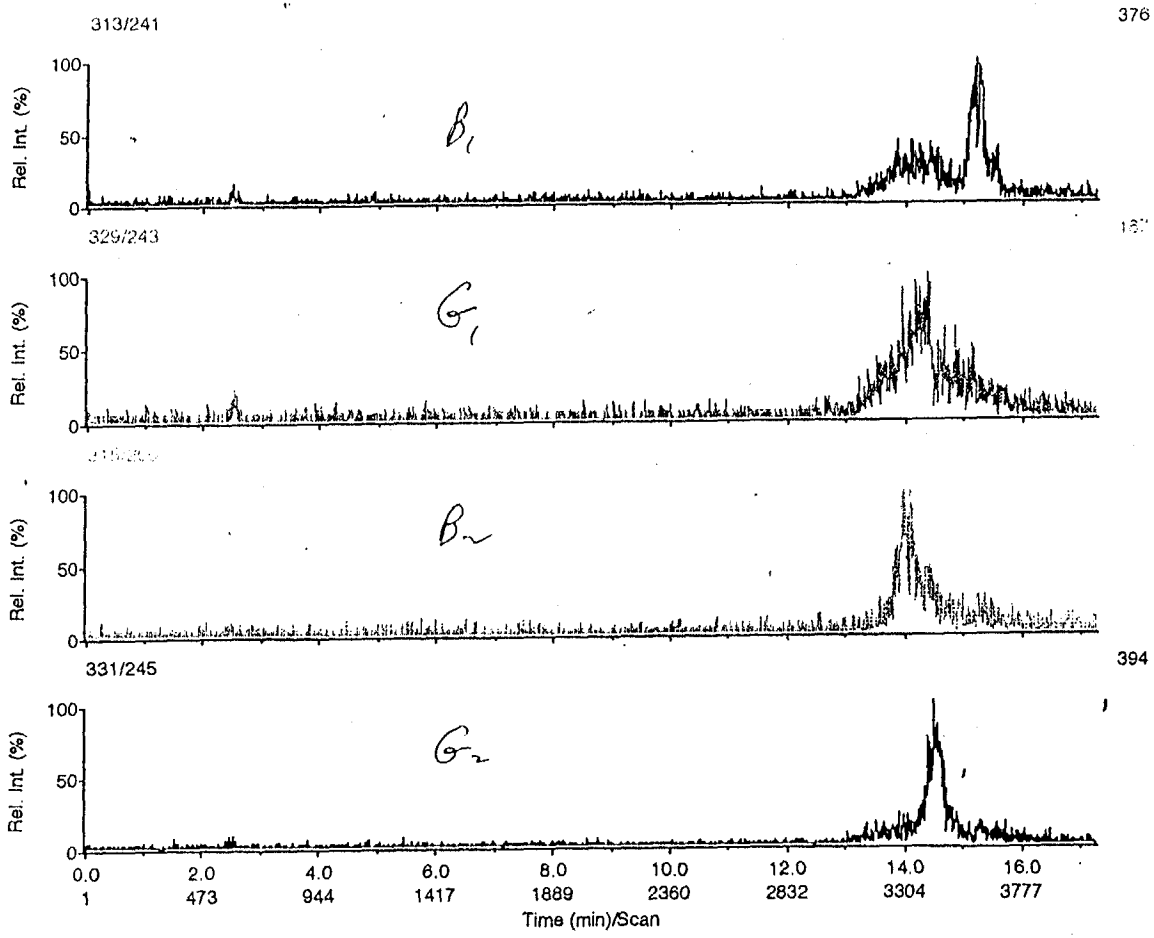


Chromatogram 15

Unknown LCMS-MRM-2/Extract

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
DI μA	3.4
CGT	308.6
IS v	5642.4
UV	4240.4

Unknown LCMS-MRM-2 - 3/20/96 - 12:39 PM
Unknown



Chromatogram 16

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MJ	-4300
CC	1
CG	Ar
DiμA	3.4
CGT	311.3
IS v	5618.0
UV	4372.3

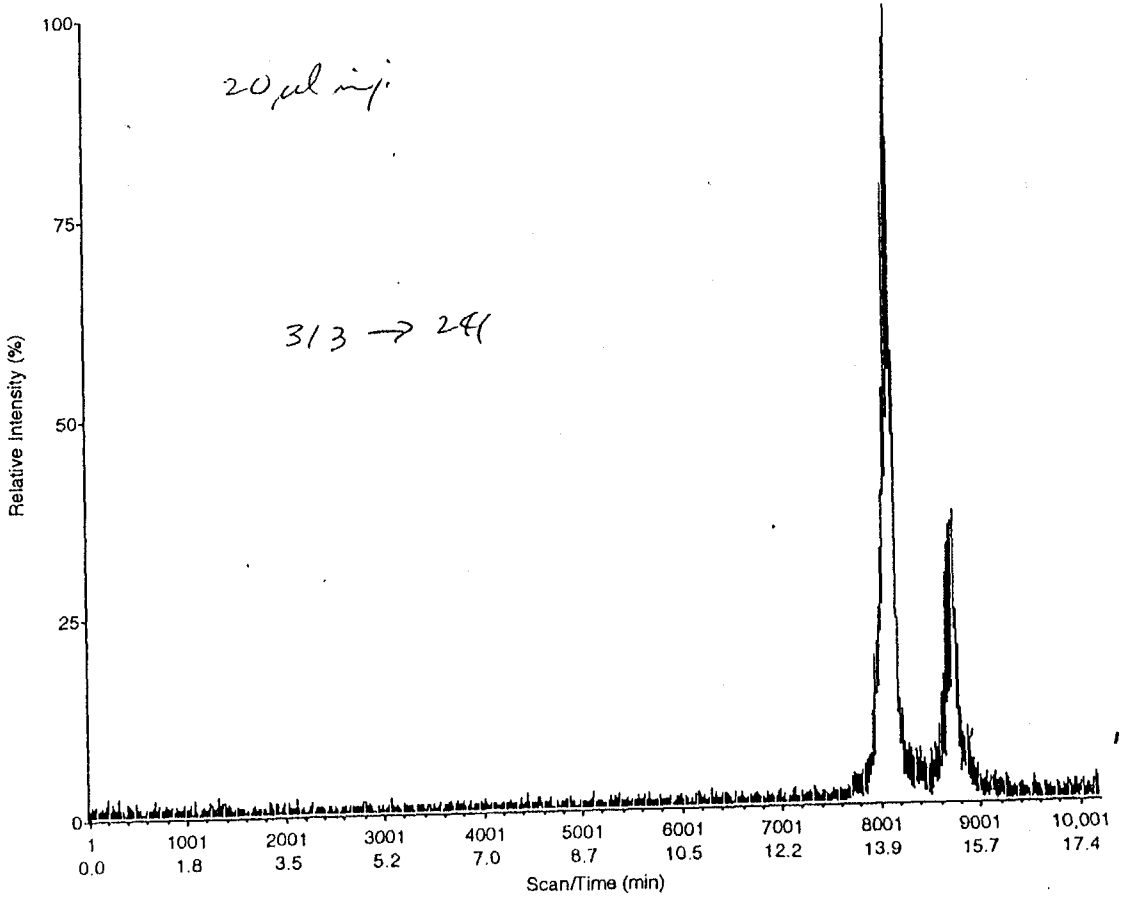
Spiked sample A LCMS-MRM/Chromatogram

TIC of all masses

Spiked sample A LCM - 3/20/96 - 10:02 AM
Spiked sample A

160639

1660



Chromatogram 17

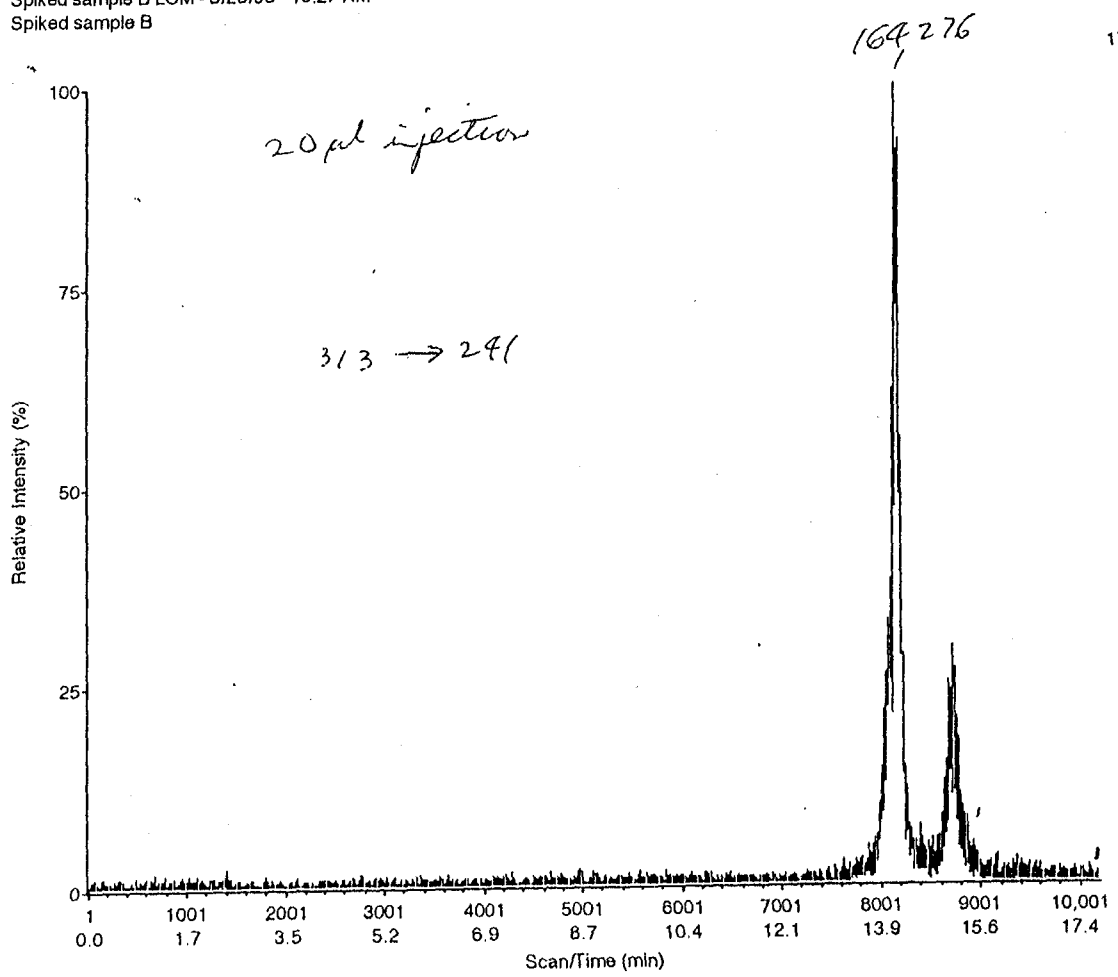
DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
DiμA	3.2
CGT	311.3
IS v	5618.0
UV	4313.6

Spiked sample B LCMS-MRM/Chromatogram

TIC of all masses

Spiked sample B LCM - 3/20/96 - 10:27 AM
Spiked sample B

1793



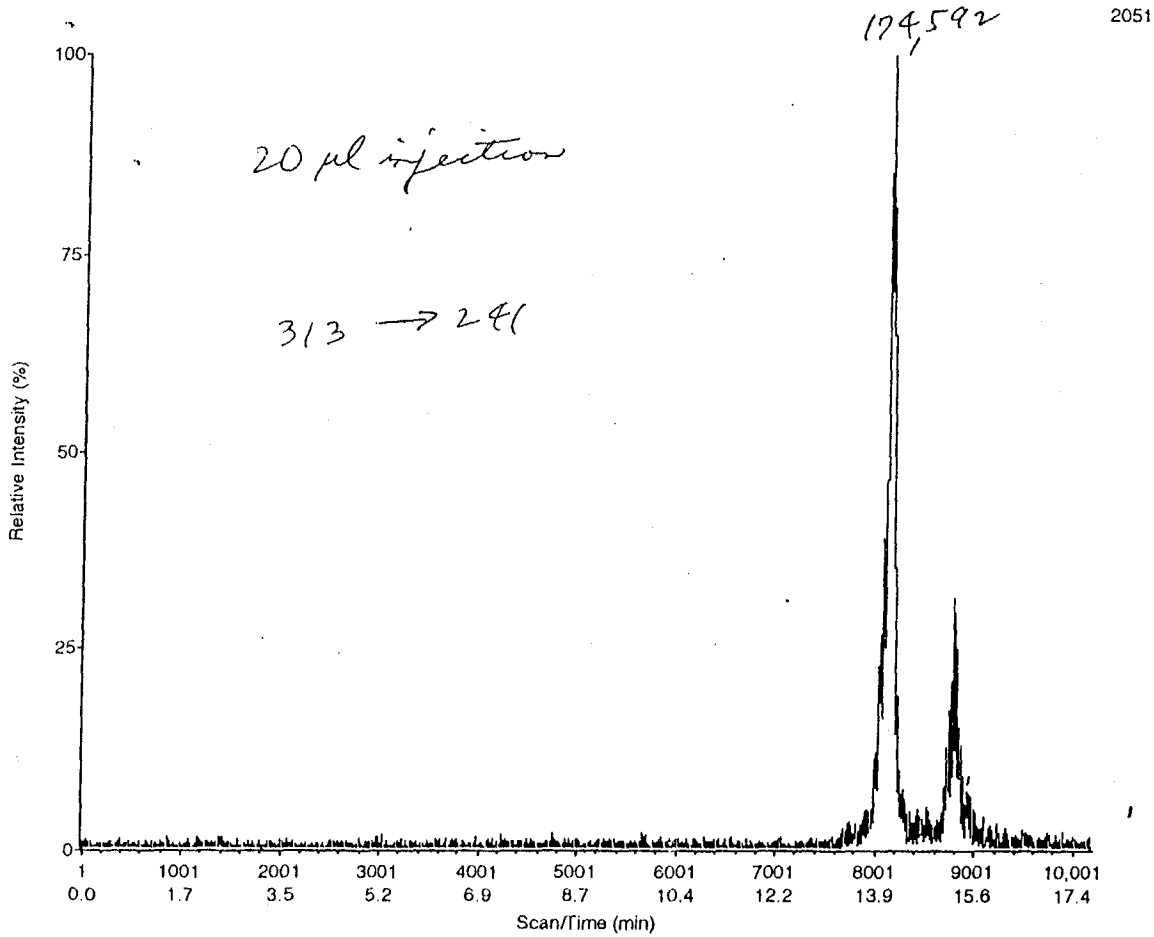
Chromatogram 17

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
Di μA	3.05
CGT	308.6
IS v	5588.7
UV	4328.3

Spiked sample C LCMS-MRM/Chromatogram

TIC of all masses

Spiked sample C LCM - 3/20/96 - 10:53 AM
Spiked sample C



Chromatogram 17

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
DiμA	3.5
CGT	310.0
IS v	5593.6
UV	4313.6

TIC of all masses

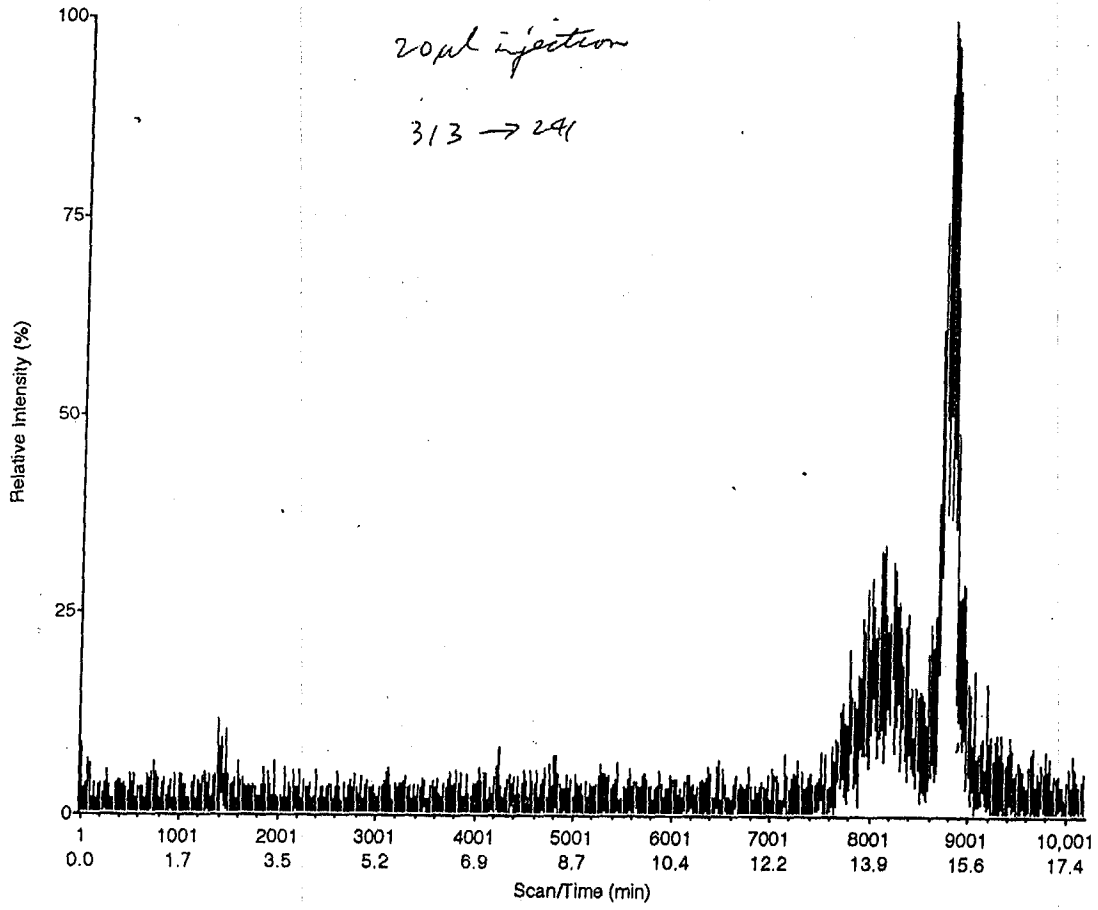
Unknown LCMS-MRM - 3/20/96 - 11:18 AM
Unknown

Unknown LCMS-MRM/Chromatogram

457

20μl injection

313 → 241



Chromatogram 18

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
Di μA	2.7
CGT	312.6
IS v	5627.7
UV	4352.7

Aflatoxin B1 LCMS-MRM-2/Chromatogram

TIC of all masses

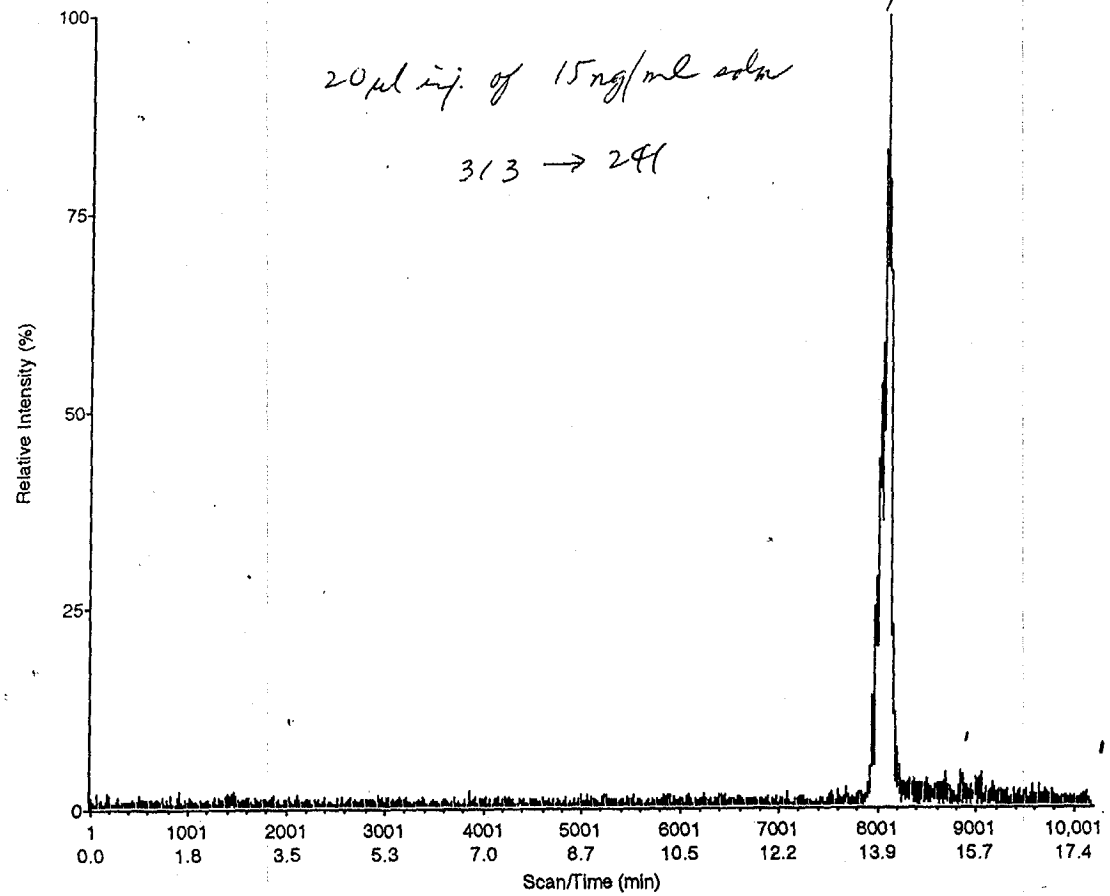
Aflatoxin B1 LCMS-M - 3/20/96 - 9:37 AM
AFLATOXIN B1,15 ng/ml

1889

178,809

20 µl inj. of 15 ng/ml soln

313 → 241



Chromatogram 19

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
Di μA	3.5
CGT	310.0
IS v	5637.5
UV	4225.7

Aflatoxin B1 LCMS-MRM/Chromatogram

TIC of all masses

Aflatoxin B1 LCMS-M - 3/20/96 - 9:11 AM
AFLATOXIN B1,30 ng/ml

19 178,809

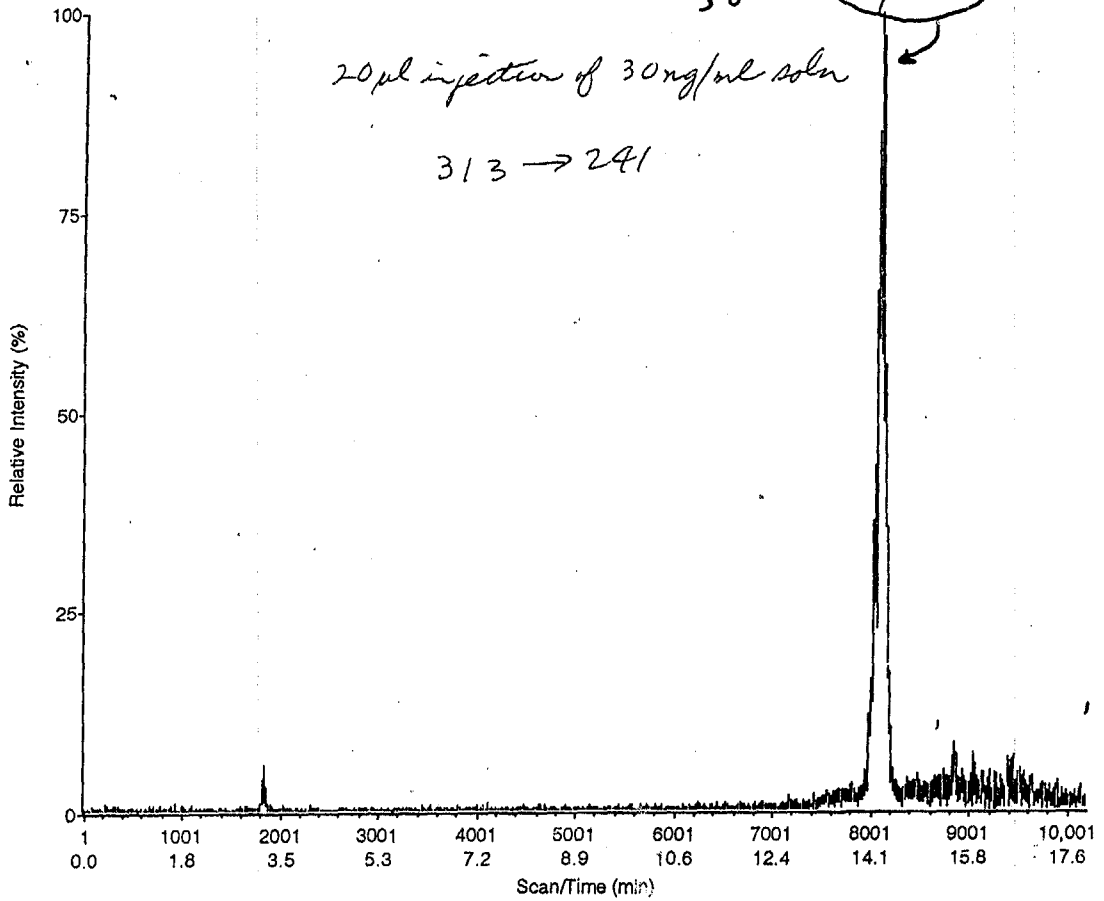
343492

30

3710

20μl injection of 30ng/ml soln

313 → 241



Chromatogram 20

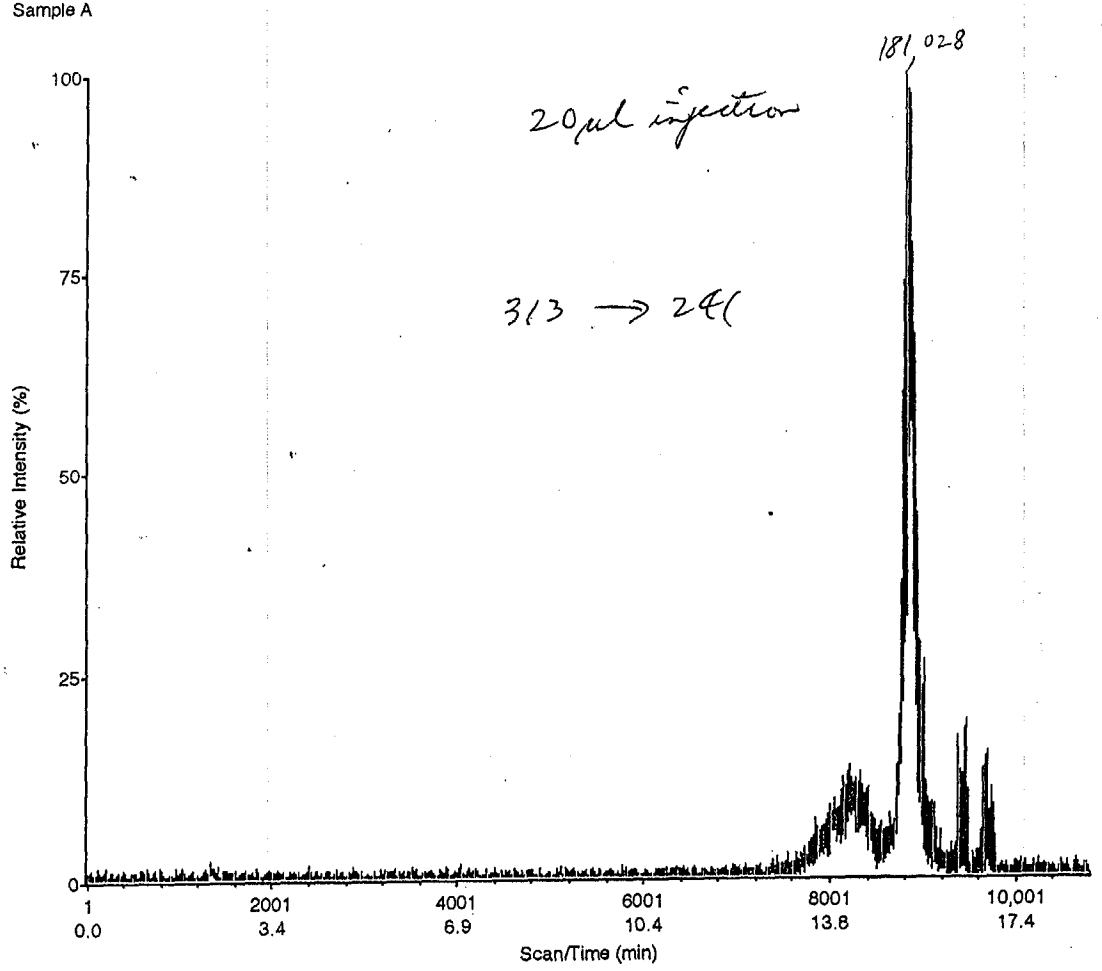
DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MJ	-4300
CC	1
CG	Ar
Di μA	3.9
CGT	308.6
IS v	5588.7
UV	4396.7

Sample A LCMS-MRM/Chromatogram

TIC of all masses

Sample A LCMS-MRM - 3/25/96 - 9:27 AM
Sample A

1813



Chromatogram 21

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
Di μA	4.2
CGT	310.0
IS v	5613.09
UV	4406.4

Sample B LCMS-MRM/Chromatogram

TIC of all masses

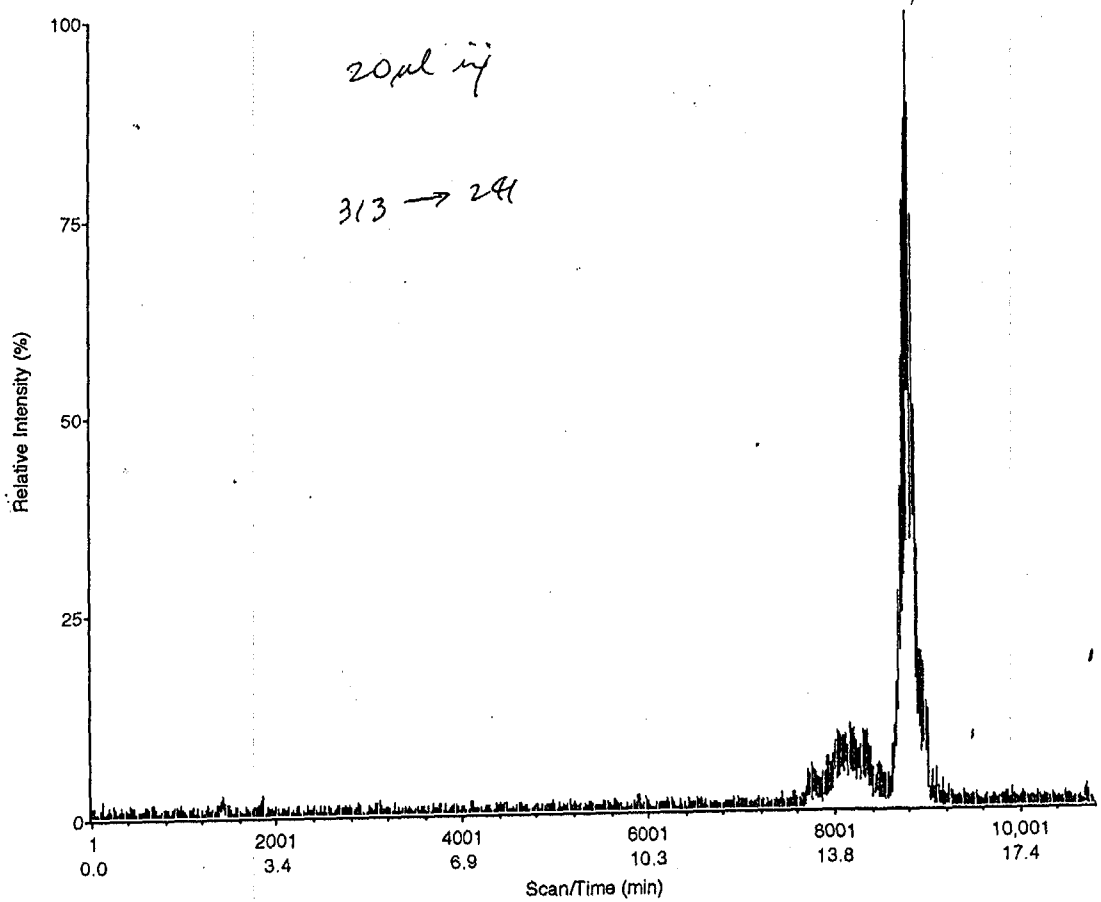
Sample B LCMS-MRM - 3/25/96 - 9:54 AM
Sample B

1724

170,566

20μl inj

313 → 241



Chromatogram 22

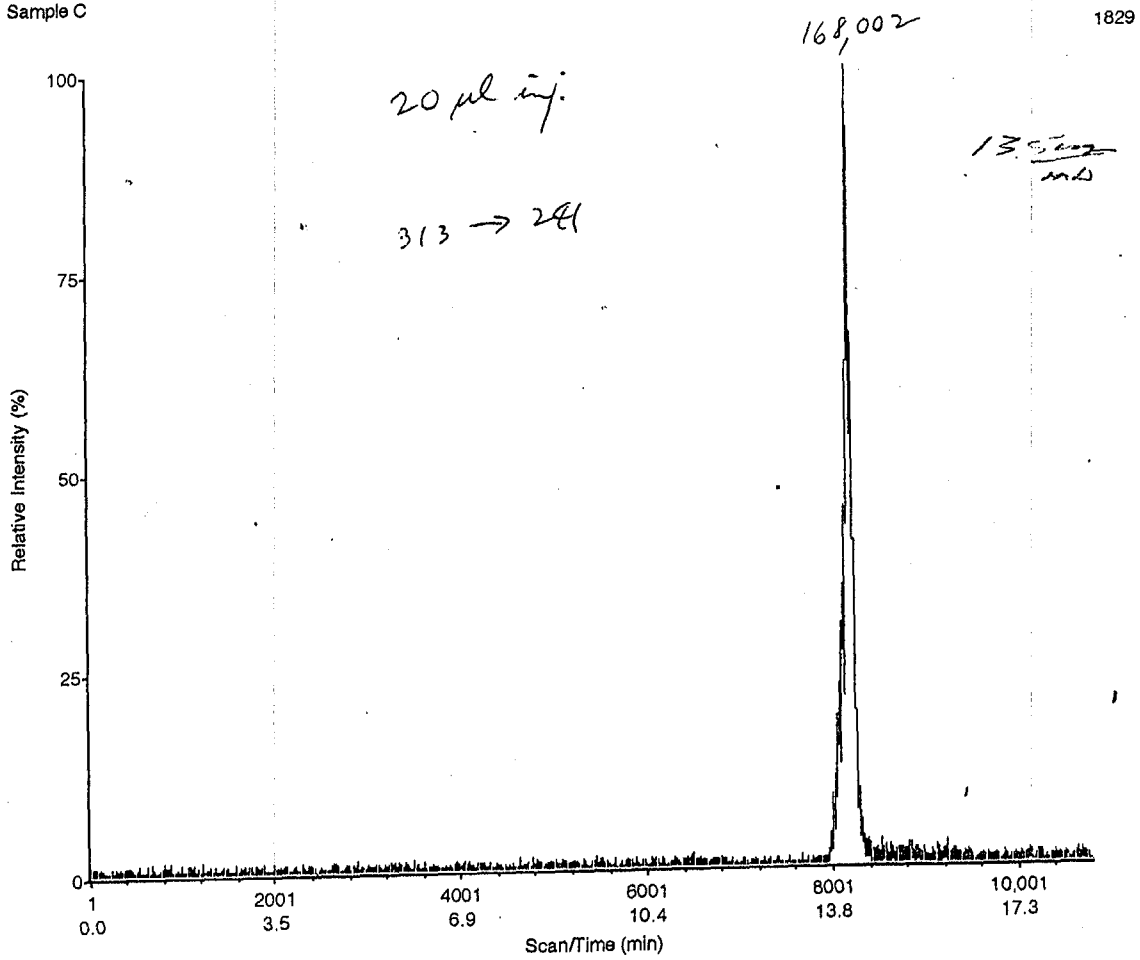
DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
DI μA	4.3
CGT	310.0
IS v	5608.2
UV	4386.9

Sample C LCMS-MRM/Chromatogram

TIC of all masses

Sample C LCMS-MRM - 3/25/96 - 10:20 AM
Sample C

1829



Chromatogram 23

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
DI μA	3.7
CGT	308.6
IS v	5588.7
UV	4386.9

Sample D LCMS-MRM/Chromatogram

TIC of all masses

Sample D LCMS-MRM - 3/25/96 - 10:46 AM
Sample D

Blank Solvent

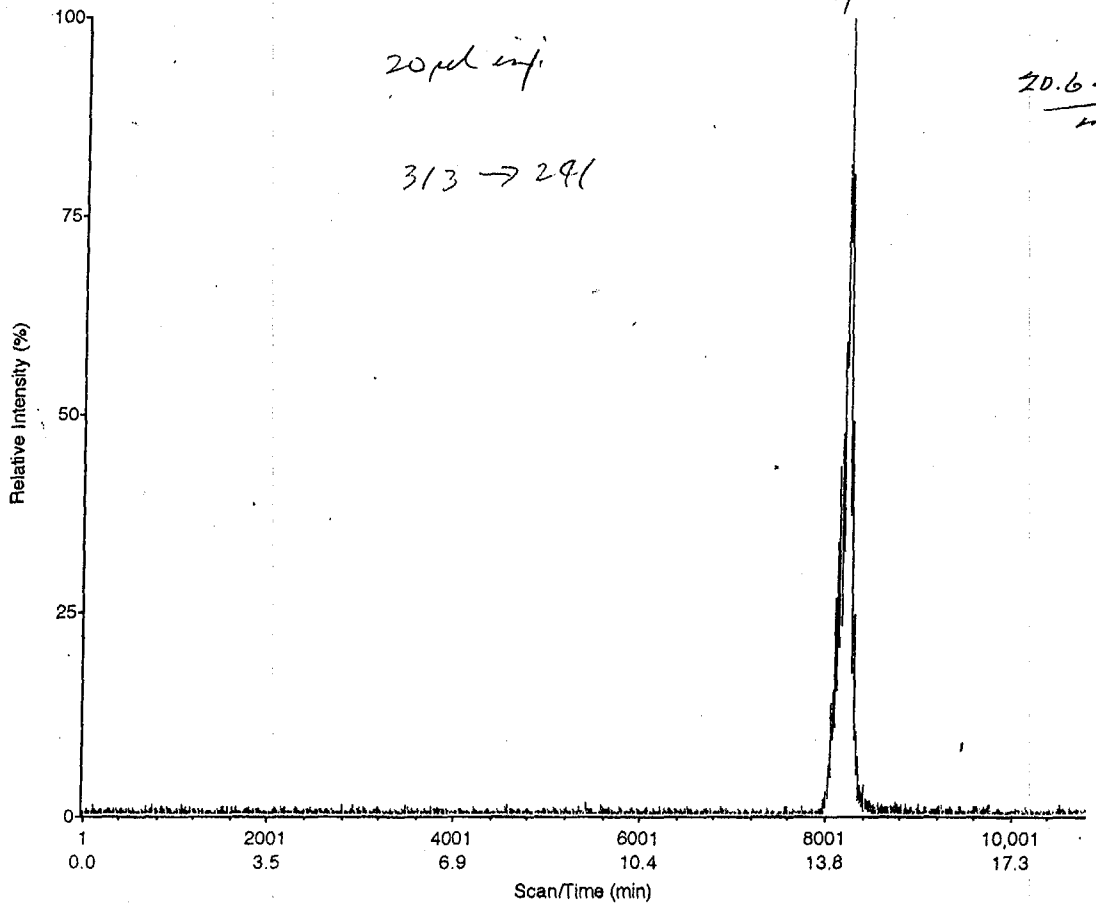
255,605

2611

20μl inj.

313 → 291

20.6 ng/mL



Chromatogram 24

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
Di μA	3.9
CGT	327.1
IS v	5593.6
UV	4372.3

Sample F LCMS-MRM/Chromatogram

TIC of all masses

Sample F LCMS-MRM - 3/25/96 - 9:01 AM
Sample F, 7.5 ng/ml B1

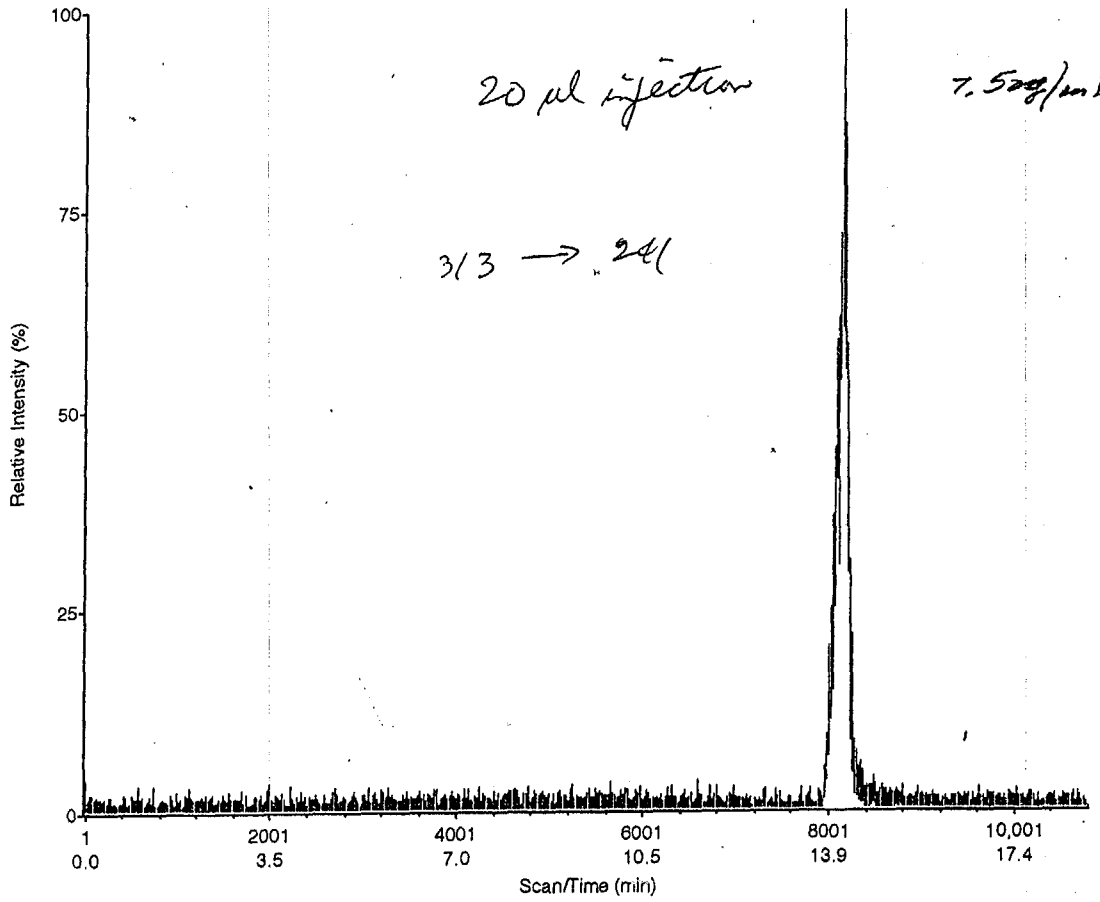
1066

93,261

20 μl injection

7.5 ng/mL

313 → 241



Chromatogram 25

DI	200
ISV	5500
IN	650
OR	80
R0	30
M1	1000
RE1	115.0
DM1	0.160
R1	27
L7	5
R2	3
M3	1000
RE3	120.3
DM3	0.160
RX	-10
R3	-10
L9	-250
FP	0
MU	-4300
CC	1
CG	Ar
Di μA	3.4
CGT	308.6
IS v	5598.4
UV	4426.0

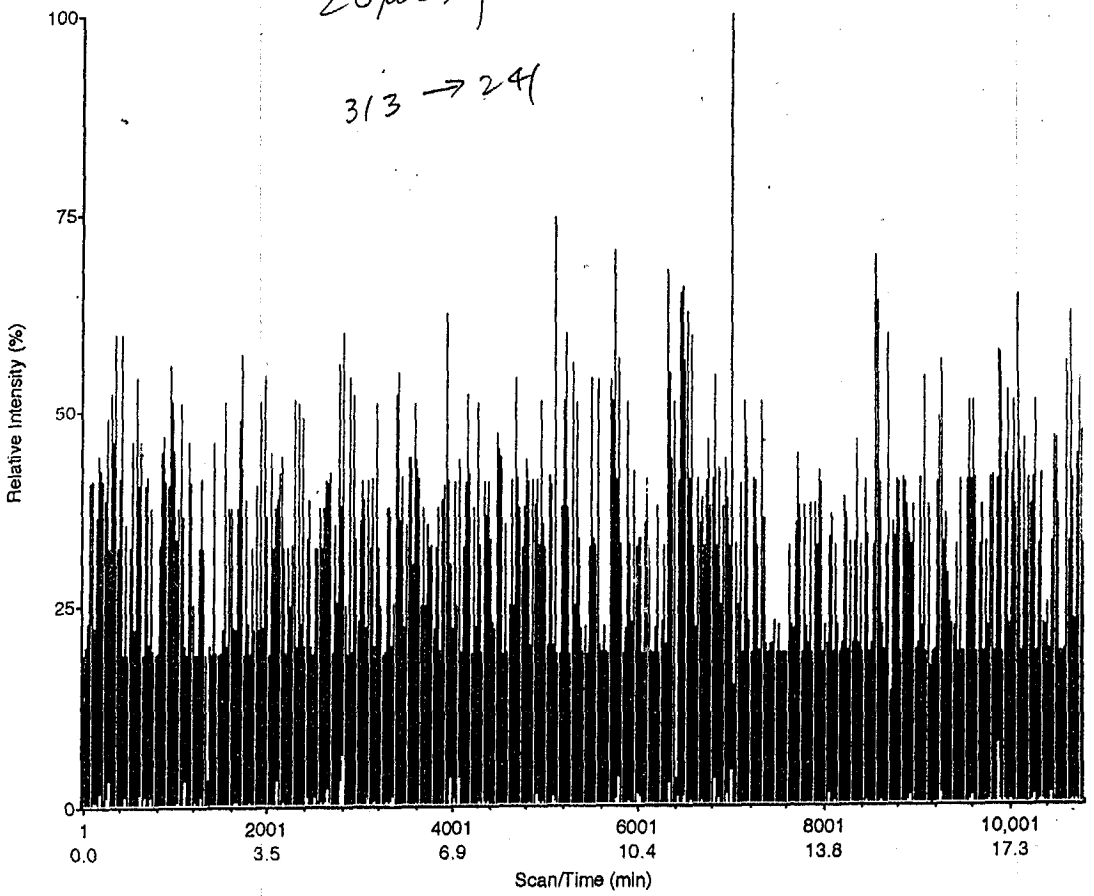
Sample E LCMS-MRM/Chromatogram

TIC of all masses

Sample E LCMS-MRM - 3/25/96 - 11:12 AM
Sample E blank

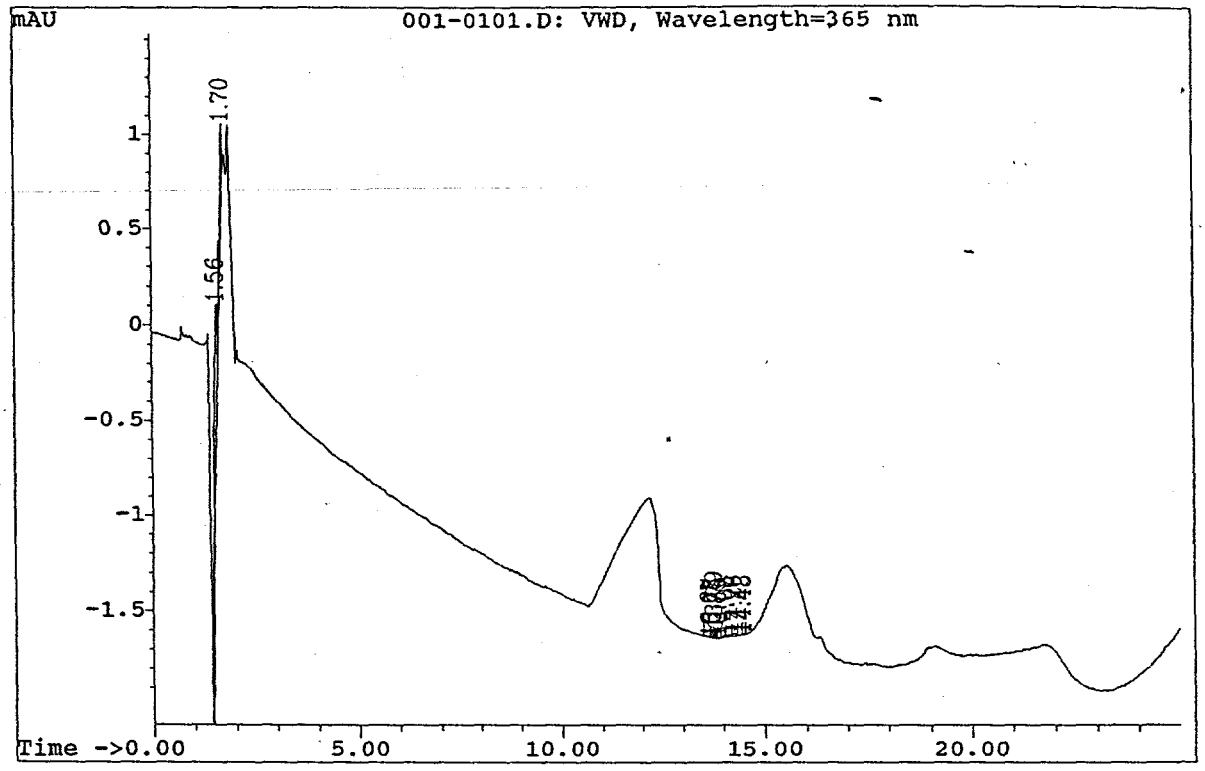
20μl inj.

313 → 241



Chromatogram 26

Chromatogram 27



Area Percent Report

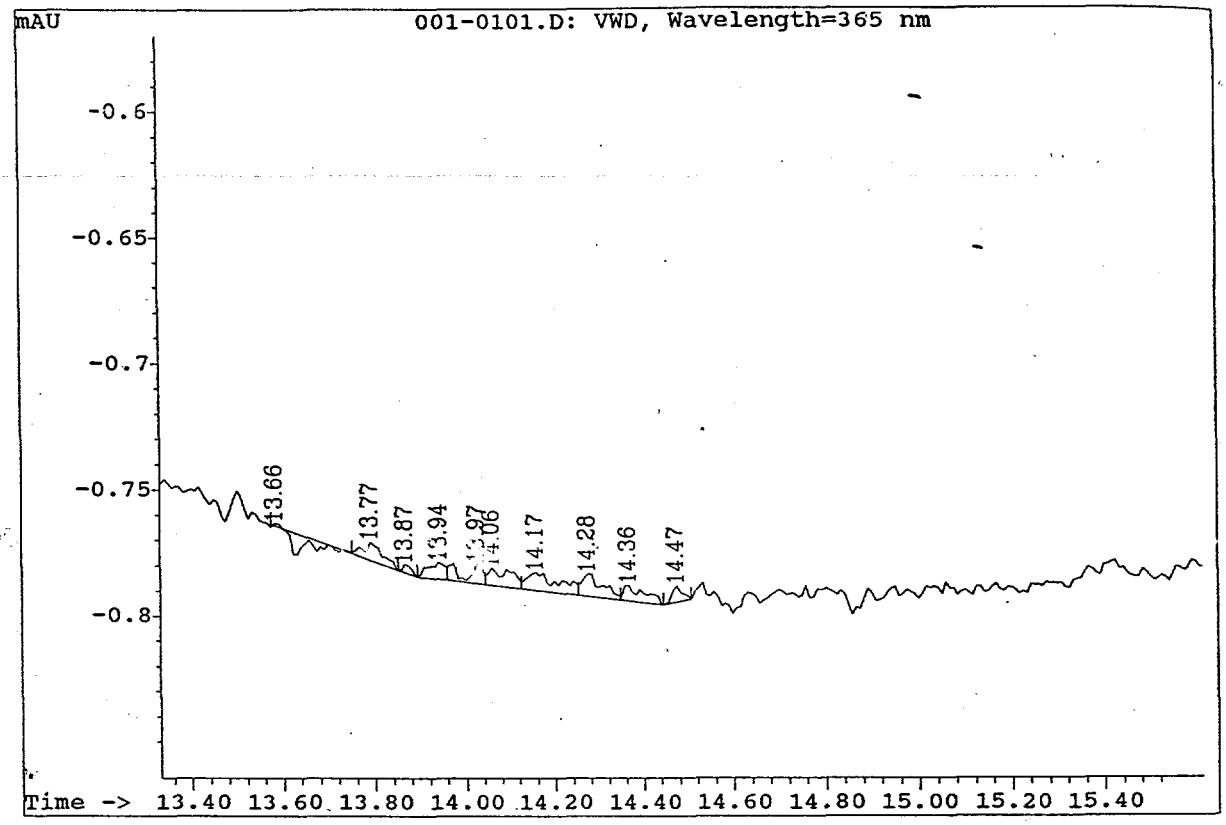
Data File Name : C:\HPCHEM\1\DATA\AP30\001-0101.D
Operator :
Acquired on : Tue Apr 30 12:42:06 1996
Sample Name : blank
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

Instrument : VWD
Vial Number : 1
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	1.562	1.333	0.586	BV	0.043	33.3231
2	1.704	2.302	0.463	VB	0.075	57.5292
3	13.568	0.012	0.005	BV	0.044	0.2930
4	13.667	0.002	0.002	VV	0.018	0.0531
5	13.717	0.009	0.006	PV	0.028	0.2350
6	13.748	0.012	0.007	VV	0.027	0.2926
7	13.793	0.061	0.046	PV	0.022	1.5244
8	13.847	0.005	0.003	VV	0.022	0.1144
9	13.894	0.010	0.005	PV	0.033	0.2518
10	14.024	0.053	0.014	VV	0.065	1.3267
11	14.083	0.081	0.013	VV	0.101	2.0198
12	14.260	0.088	0.016	VV	0.074	2.1867
13	14.422	0.024	0.005	PV	0.066	0.6022
14	14.485	0.010	0.005	PB	0.035	0.2480

Chromatogram 28



Area Percent Report

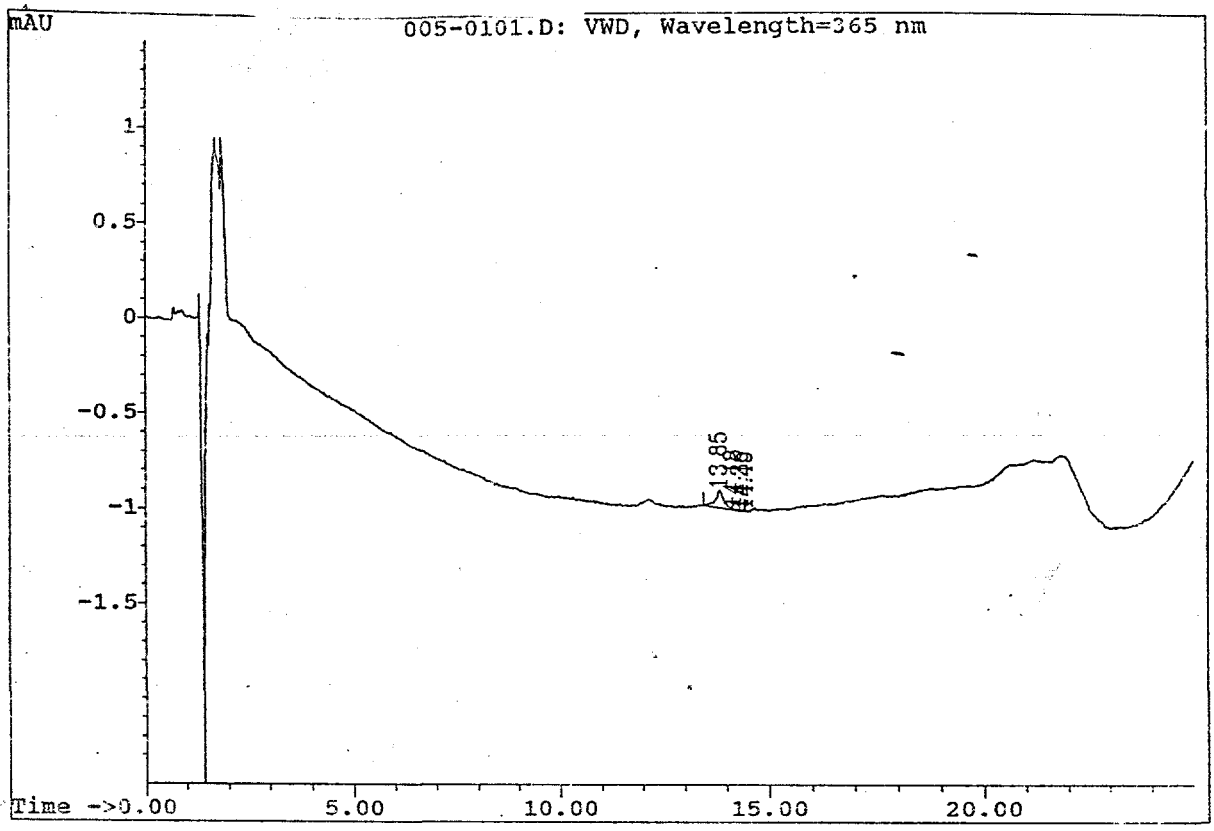
Data File Name : C:\HPCHEM\1\DATA\AP26\001-0101.D
Operator :
Acquired on : Fri Apr 26 12:19:17 1996
Sample Name : blank
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

Instrument : VWD
Vial Number : 1
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	13.658	-0.017	-0.001	BV	0.336	-10.3340
2	13.767	0.022	0.004	VV	0.095	12.8617
3	13.869	0.004	0.004	PV	0.020	2.6427
4	13.943	0.017	0.007	PV	0.042	10.1241
5	13.972	0.021	0.008	VV	0.046	12.5145
6	14.058	0.025	0.007	VV	0.060	14.7807
7	14.173	0.036	0.008	VV	0.080	21.4720
8	14.278	0.029	0.009	VV	0.044	17.2585
9	14.363	0.021	0.007	VV	0.054	12.6436
10	14.475	0.010	0.007	PB	0.026	6.0363

Total area = 0.2


mAU
005-0101.D: VWD, Wavelength=365 nm
1
0.5
0
-0.5
-1
-1.5
13.85
Time ->0.00
5.00
10.00
15.00
20.00

Area Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP26\005-0101.D
Operator :
Acquired on : Fri Apr 26 14:21:57 1996
Sample Name : 25 ng/ml
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

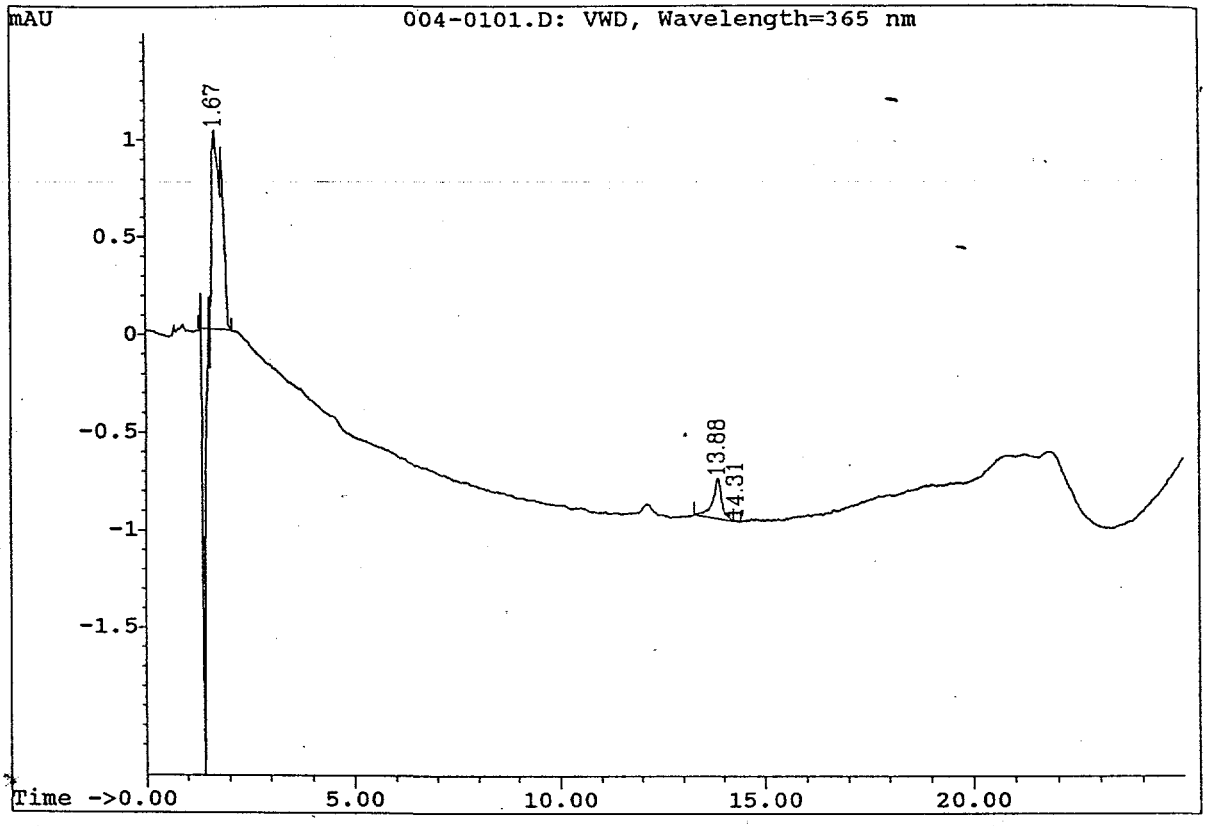
Instrument : VWD
Vial Number : 5
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	13.850	1.262	0.090	BV	0.176	92.0659
2	14.184	0.072	0.008	VV	0.107	5.2392
3	14.357	0.027	0.006	PV	0.070	1.9548
4	14.449	0.010	0.006	VV	0.030	0.7401

Total area = 1

Chromatogram 30



Area Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP26\004-0101.D
Operator :
Acquired on : Fri Apr 26 13:51:17 1996
Sample Name : 50 ng/ml
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

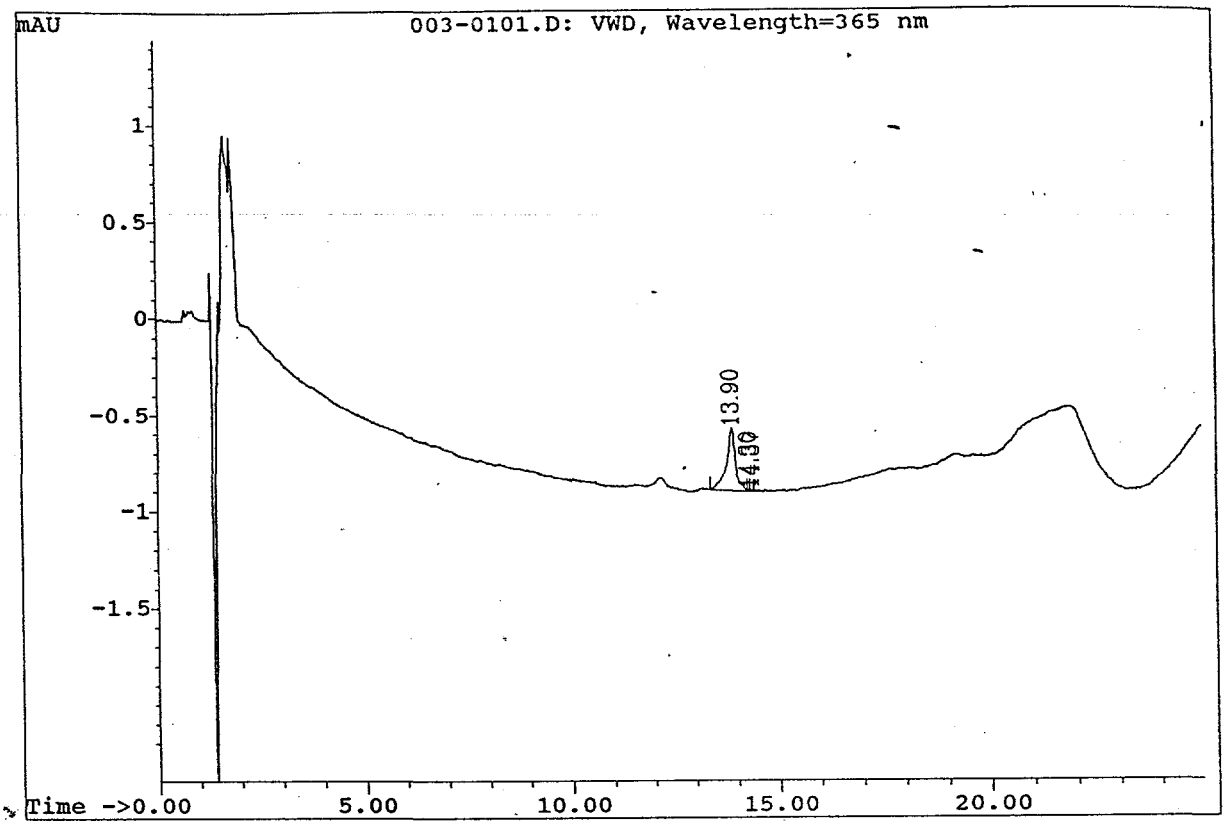
Instrument : VWD
Vial Number : 4
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	1.672	6.156	1.022	BB	0.162	65.6528
2	13.876	3.187	0.208	BV	0.219	33.9945
3	14.311	0.033	0.005	PV	0.122	0.3527

Total area = 9

Chromatogram 31



Area Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP26\003-0101.D
Operator :
Acquired on : Fri Apr 26 13:20:36 1996
Sample Name : 75 ng/ml
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

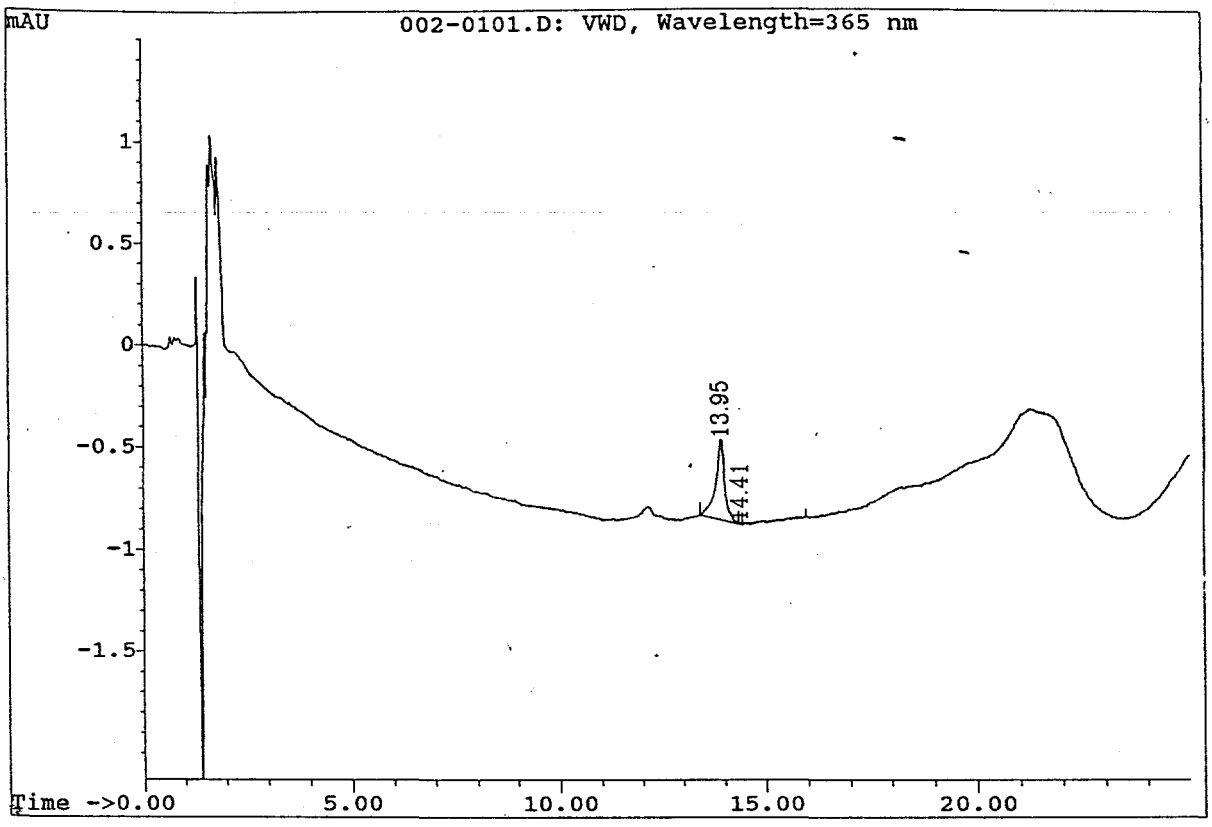
Instrument : VWD
Vial Number : 3
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	13.904	4.956	0.327	BV	0.206	99.0529
2	14.300	0.020	0.008	VV	0.043	0.4091
3	14.371	0.027	0.008	VV	0.050	0.5380

Total area = 5

Chromatogram 32



Area Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP26\002-0101.D
Operator :
Acquired on : Fri Apr 26 12:49:57 1996
Sample Name : 100 ng/ml
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

Instrument : VWD
Vial Number : 2
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

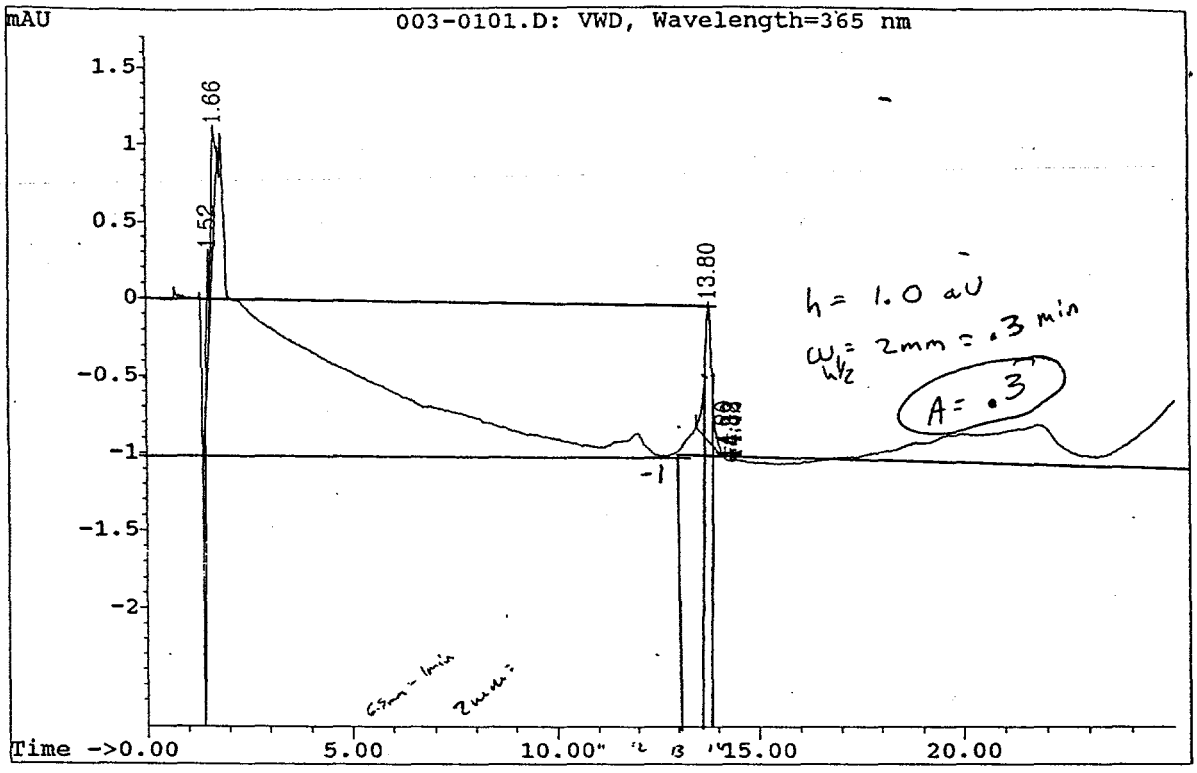
Pk#	Ret Time	Area	Height	Type	Width	Area %
1	13.948	6.036	0.392	BV	0.205	99.3719
2	14.409	0.038	0.010	VB	0.055	0.6281

Total area = 6

001-0101.D: VWD, Wavelength=365 nm
C:\HPCHEM\1\DATA\AP30S\001-0101.D\VWD1A.CH Blank

Peak#	Ret Time	Type	Width	Area	Start Time	End Time
1	1.379	BV	0.082	3451	0.783	1.504
2	13.571	BV	0.182	7.08	13.417	13.733
3	13.948	VV	0.232	76.09	13.733	14.111
4	14.197	VV	0.168	55.93	14.111	14.419
5	14.962	VV	0.384	358.50	14.419	15.717
6	18.523	BV	0.693	54.31	17.392	18.675

Chromatogram 33



Area Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP30\003-0101.D
Operator :
Acquired on : Tue Apr 30 13:43:27 1996
Sample Name : 100 ng/ml
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

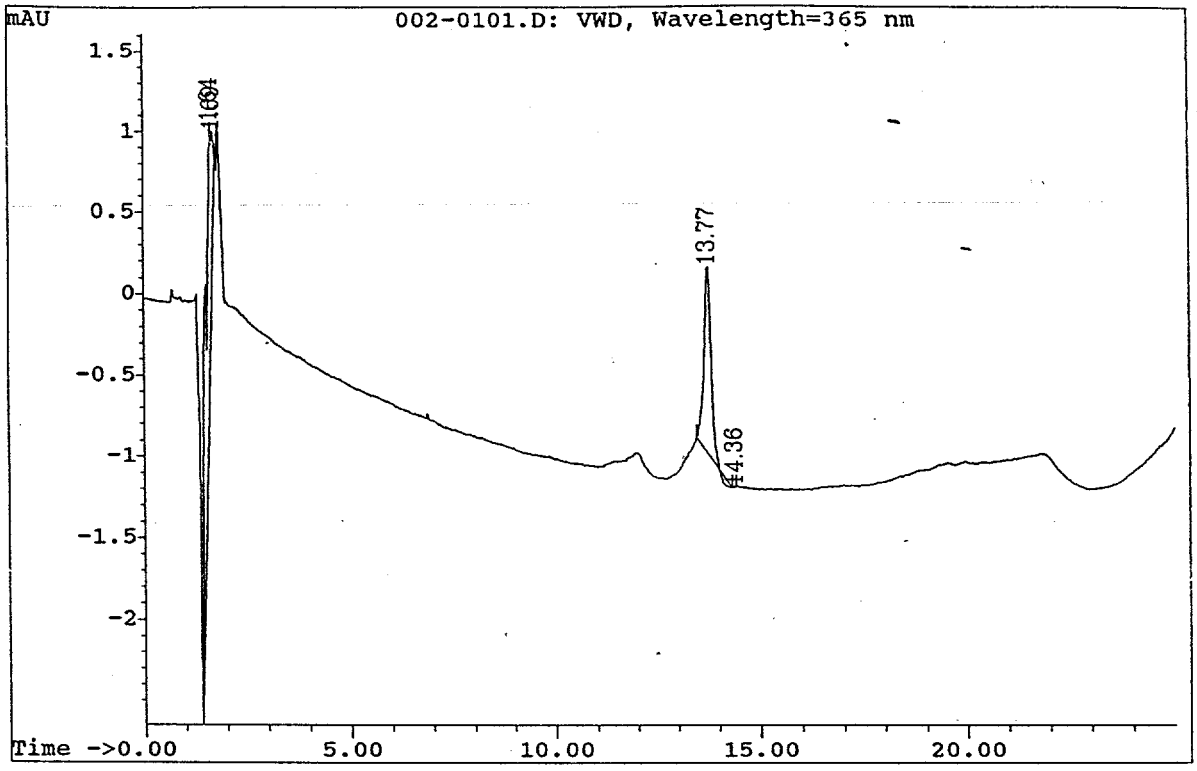
Instrument : VWD
Vial Number : 3
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	1.524	-3.173	0.710	BV	0.000	-23.2663
2	1.661	5.376	0.764	PB	0.106	39.4229
3	13.799	11.417	0.907	BV	0.184	83.7241
4	14.199	0.000	0.005	PV	0.001	0.0014
5	14.333	0.004	0.003	PV	0.022	0.0324
6	14.384	0.006	0.006	PV	0.024	0.0472
7	14.424	0.005	0.003	VV	0.032	0.0384

Total area = 14

Chromatogram 34



Area Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP30\002-0101.D
Operator :
Acquired on : Tue Apr 30 13:12:47 1996
Sample Name : 200 ng/ml
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

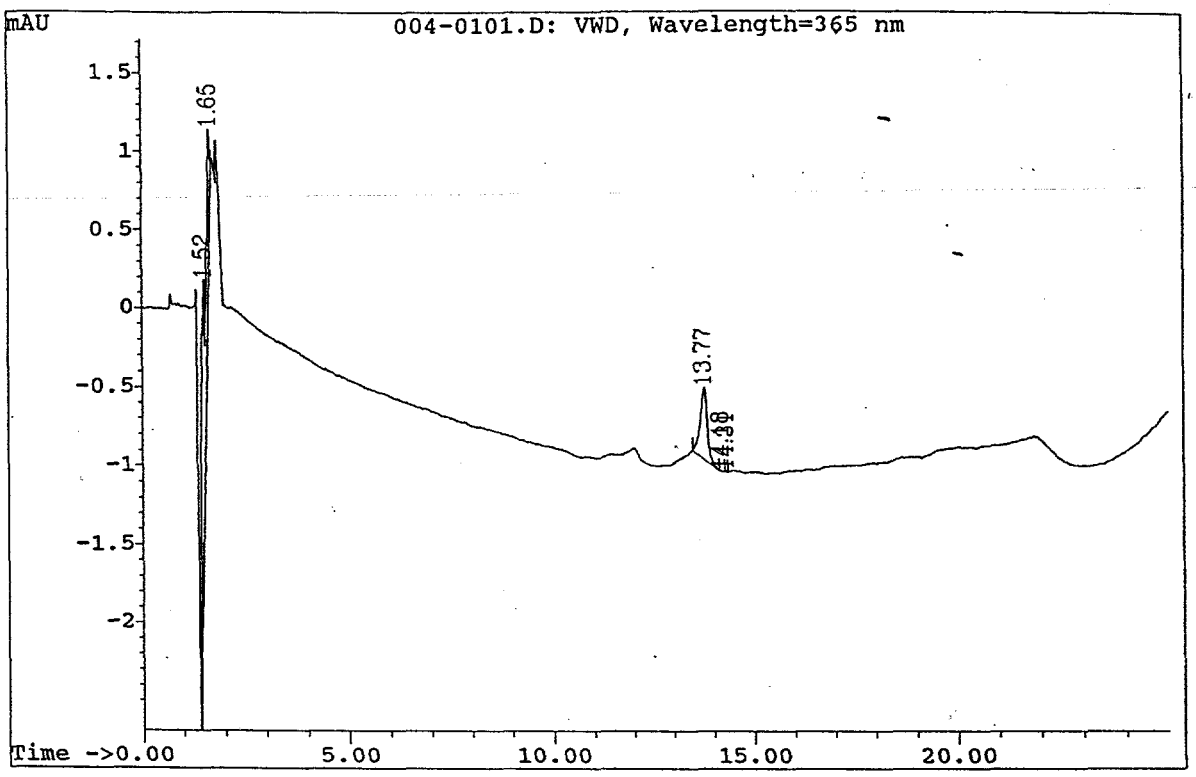
Instrument : VWD
Vial Number : 2
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	1.643	17.466	1.315	BV	0.163	51.8051
2	1.689	3.025	0.811	VB	0.062	8.9728
3	13.767	13.211	1.156	BV	0.171	39.1862
4	14.360	0.012	0.005	PV	0.038	0.0360

Total area = 34

Chromatogram 35



Area Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP30\004-0101.D
Operator :
Acquired on : Tue Apr 30 14:14:07 1996
Sample Name : 75 ng/ml
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

Instrument : VWD
Vial Number : 4
Injection Number: 1
Sequence Line : 1

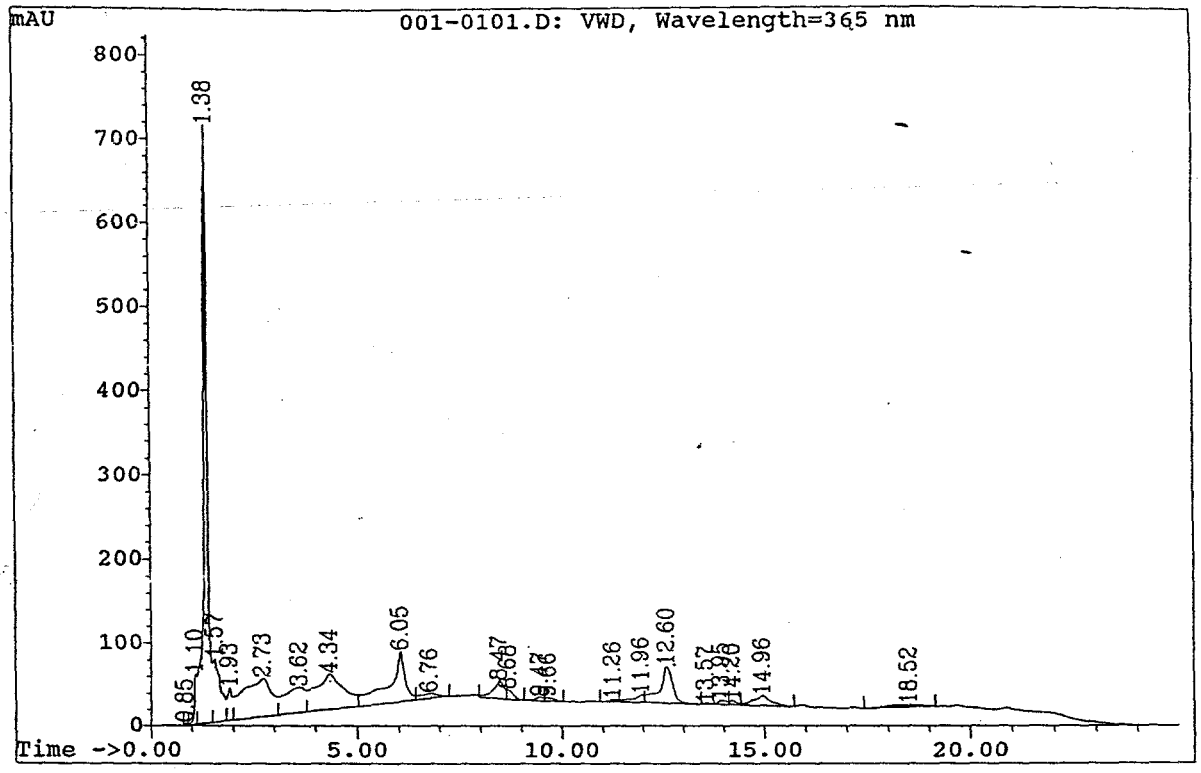
VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	1.516	9.119	1.470	PV	0.082	44.4063
2	1.653	5.791	0.785	VB	0.097	28.2004
3	13.770	5.595	0.458	BV	0.164	27.2461
4	14.185	0.013	0.006	PV	0.039	0.0626
5	14.306	0.017	0.007	PV	0.039	0.0845

Total area = 21

Initial 80% H_2O / 10% Acetonitrile / 10% Methanol — 10 min to 20% H_2O / 40% Acetonitrile / 40% Methanol — for 5 min

Chromatogram 36



Area Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP30S\001-0101.D
Operator :
Acquired on : Tue Apr 30 14:47:17 1996
Sample Name : 10 mg concentra
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

Instrument : VWD
Vial Number : 1
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	0.851	13.588	2.955	PV	0.067	0.0827
2	1.099	250.807	60.756	VV	0.061	1.5263
3	1.380	4744.242	719.045	VV	0.098	28.8708
4	1.572	1025.363	77.450	VV	0.163	6.2398
5	1.927	318.164	39.741	VV	0.105	1.9362
6	2.732	2324.218	46.783	VV	0.628	14.1439
7	3.620	1138.745	31.614	VV	0.520	6.9298
8	4.338	2095.403	42.966	VV	0.622	12.7515
9	6.051	1743.884	60.554	VV	0.369	10.6123
10	6.762	191.123	6.374	VV	0.398	1.1631
11	8.466	407.231	20.588	VV	0.276	2.4782
12	8.680	125.509	11.255	VV	0.186	0.7638
13	9.467	63.384	4.842	PV	0.187	0.3857
14	9.664	63.091	4.459	VB	0.201	0.3839

Height Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP30S\001-0101.D
Operator :
Acquired on : Tue Apr 30 14:47:17 1996
Sample Name : 10 mg concentra
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

Instrument : VWD
Vial Number : 1
Injection Number: 1
Sequence Line : 1

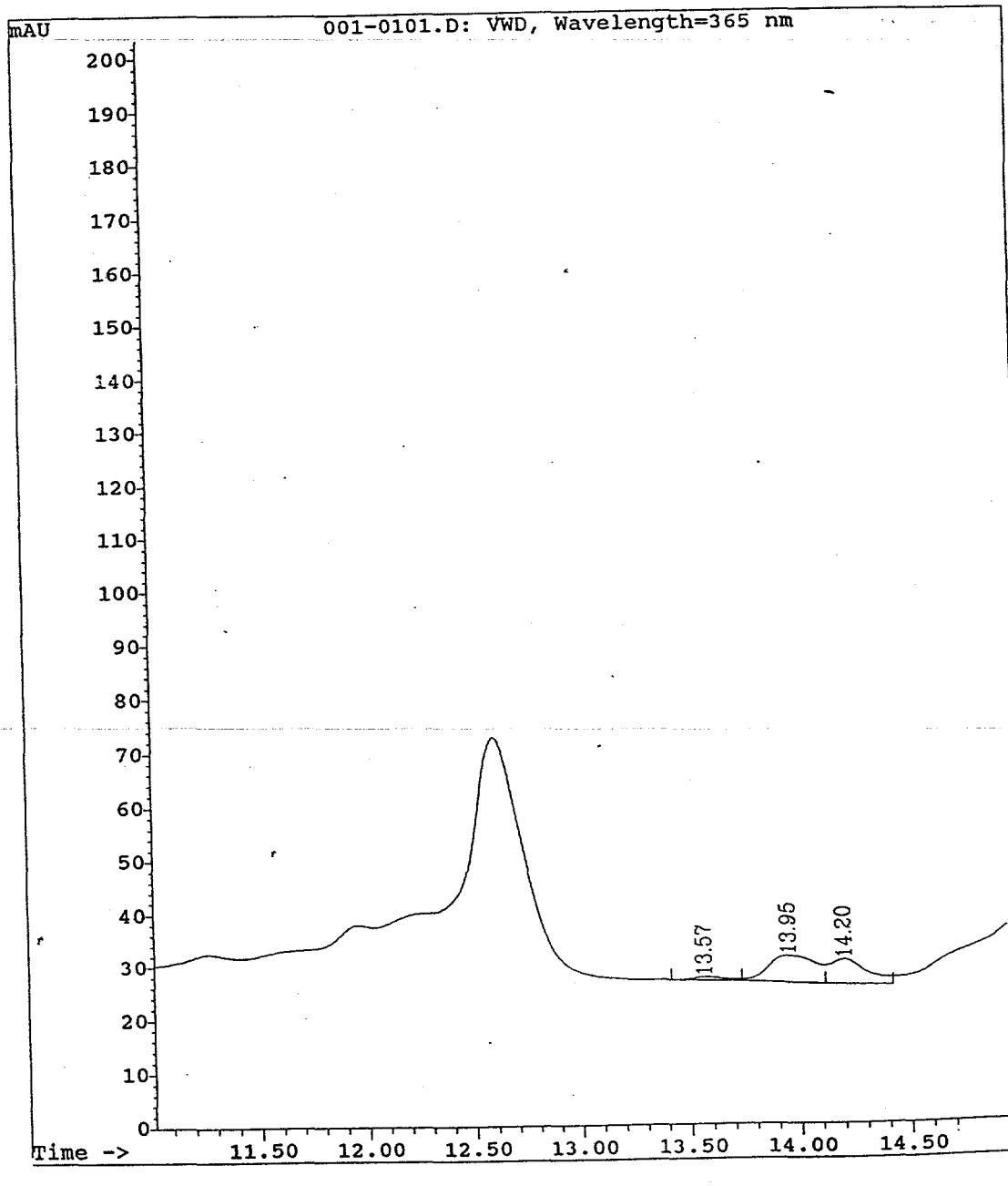
VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Height %
1	0.851	13.588	2.955	PV	0.067	0.2467
2	1.099	250.807	60.756	VV	0.061	5.0731
3	1.380	4744.242	719.045	VV	0.098	60.0406
4	1.572	1025.363	77.450	VV	0.163	6.4671
5	1.927	318.164	39.741	VV	0.105	3.3184
6	2.732	2324.218	46.783	VV	0.628	3.9064
7	3.620	1138.745	31.614	VV	0.520	2.6398
8	4.338	2095.403	42.966	VV	0.622	3.5877
9	6.051	1743.884	60.554	VV	0.369	5.0563
10	6.762	191.123	6.374	VV	0.398	0.5322
11	8.466	407.231	20.588	VV	0.276	1.7191
12	8.680	125.509	11.255	VV	0.186	0.9398
13	9.467	63.384	4.842	PV	0.187	0.4043
14	9.664	63.091	4.459	VB	0.201	0.3723
15	11.263	34.597	2.396	BV	0.203	0.2001
16	11.957	155.916	7.897	VV	0.264	0.6594
17	12.600	808.913	42.596	VH	0.276	3.5568
18	13.571	-30.243	-2.735	PHA	0.184	-0.2284
19	13.950	49.974	4.040	BH	0.203	0.3373
20	14.962	297.750	11.249	BV	0.350	0.9393
21	18.523	168.017	2.773	BB	0.832	0.2315

Total height = 1198

Chromatogram 37

File: C:\HPCHEM\1\DATA\AP30S\001-0101.D
Operator:
Date Acquired: Tue Apr 30 14:47:17 1996
Method File Name: AFLTX.M
Sample Name: 10 mg concentra
Misc Info:
Bottle Number: 1



Height Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP30S\002-0101.D
Operator :
Acquired on : Tue Apr 30 15:17:57 1996
Sample Name : 10 mg+ 25ng aflt
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

Instrument : VWD
Vial Number : 2
Injection Number: 1
Sequence Line : 1

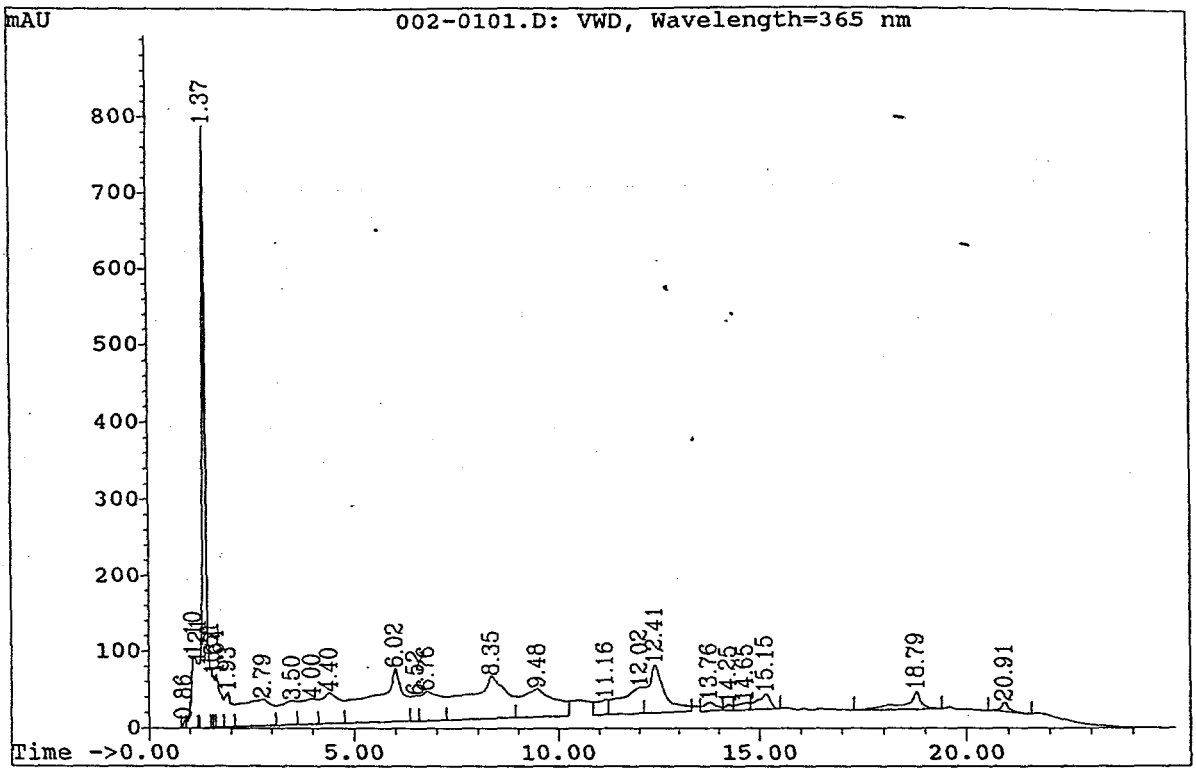
VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Height %
1	0.857	44.428	10.536	PV	0.062	0.5872
2	1.103	932.360	92.863	VV	0.145	5.1753
3	1.206	185.737	83.464	VV	0.037	4.6515
4	1.373	4471.122	794.847	VV	0.076	44.2972
5	1.513	257.044	78.350	VV	0.055	4.3665
6	1.574	210.746	67.263	VV	0.052	3.7486
7	1.643	587.263	68.523	VV	0.111	3.8188
8	1.926	577.533	44.604	VV	0.162	2.4858
9	2.789	1818.100	33.601	VV	0.679	1.8726
10	3.505	883.351	30.482	VV	0.388	1.6987
11	4.003	956.874	32.864	VV	0.397	1.8315
12	4.404	1261.181	39.354	VV	0.419	2.1932
13	6.016	3478.687	69.406	VV	0.620	3.8680
14	6.520	441.157	32.385	VV	0.227	1.8048
15	6.762	1309.860	37.522	VV	0.452	2.0911
16	8.348	3598.635	55.202	VV	0.812	3.0764
17	9.480	2099.312	35.711	VV	0.722	1.9902
18	11.160	389.883	18.424	VV	0.282	1.0268
19	12.024	1313.203	33.046	VV	0.662	1.8417
20	12.409	1801.819	62.150	VV	0.395	3.4637
21	13.764	255.299	11.303	VV	0.315	0.6299
22	14.655	185.623	8.717	VV	0.355	0.4858
23	15.154	421.273	19.159	VV	0.298	1.0677
24	18.793	607.684	22.363	BV	0.353	1.2463
25	20.914	154.879	12.210	BB	0.183	0.6804

Total height = 1794

25ng. + 10µl Peanut extract concentrated → 1ml

Chromatogram 38



Area Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP30S\002-0101.D
Operator :
Acquired on : Tue Apr 30 15:17:57 1996
Sample Name : 10 mg+ 25ng aflt
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

Instrument : VWD
Vial Number : 2
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	0.857	44.428	10.536	PV	0.062	0.1568
2	1.103	932.360	92.863	VV	0.145	3.2907
3	1.206	185.737	83.464	VV	0.037	0.6555
4	1.373	4471.122	794.847	VV	0.076	15.7806
5	1.513	257.044	78.350	VV	0.055	0.9072
6	1.574	210.746	67.263	VV	0.052	0.7438
7	1.643	587.263	68.523	VV	0.111	2.0727
8	1.926	577.533	44.604	VV	0.162	2.0384
9	2.789	1818.100	33.601	VV	0.679	6.4169
10	3.505	883.351	30.482	VV	0.388	3.1177
11	4.003	956.874	32.864	VV	0.397	3.3772
12	4.404	1261.181	39.354	VV	0.419	4.4513
13	6.016	3478.687	69.406	VV	0.620	12.2778
14	6.520	441.157	32.385	VV	0.227	1.5570

Height Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP30S\002-0101.D
Operator :
Acquired on : Tue Apr 30 15:17:57 1996
Sample Name : 10 mg+ 25ng aflt
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

Instrument : VWD
Vial Number : 2
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Height %
1	0.857	44.192	10.497	PV	0.062	0.6031
2	1.103	929.926	92.690	VV	0.144	5.3255
3	1.206	185.225	83.236	VV	0.037	4.7823
4	1.373	4465.958	794.528	VV	0.076	45.6496
5	1.513	255.636	77.956	VV	0.055	4.4789
6	1.574	209.314	66.836	VV	0.052	3.8400
7	1.643	581.206	68.058	VV	0.110	3.9103
8	1.926	567.010	43.986	VV	0.161	2.5272
9	2.789	1758.810	32.517	VV	0.679	1.8683
10	3.505	839.423	29.010	VV	0.387	1.6668
11	4.003	905.274	31.123	VV	0.397	1.7882
12	4.404	1186.391	37.396	VV	0.416	2.1486
13	6.016	3233.418	66.577	VV	0.602	3.8252
14	6.520	398.518	29.283	VV	0.227	1.6825
15	6.762	1178.576	34.289	VV	0.446	1.9701
16	8.348	3195.580	51.112	VV	0.781	2.9366
17	9.480	1722.715	31.009	VV	0.684	1.7816
18	11.160	265.035	12.814	VV	0.277	0.7362
19	12.024	998.022	26.969	VV	0.617	1.5495
20	12.409	1336.936	55.864	VV	0.336	3.2097
21	13.764	73.230	5.602	BV	0.204	0.3219
22	14.655	121.817	6.369	VV	0.319	0.3659
23	15.154	376.455	18.197	VV	0.283	1.0455
24	18.793	607.684	22.363	BV	0.353	1.2849
25	20.914	154.879	12.210	BB	0.183	0.7015

Total height = 1740

Height Percent Report

Data File Name : C:\HPCHEM\1\DATA\AP30S\002-0101.D
Operator :
Acquired on : Tue Apr 30 15:17:57 1996
Sample Name : 10 mg+ 25ng aflt
Run Time Bar Code:
Instrument Method: AFLTX.M
Analysis Method : AFLTX.M

Instrument : VWD
Vial Number : 2
Injection Number: 1
Sequence Line : 1

VWD, Wavelength=365 nm

Pk#	Ret Time	Area	Height	Type	Width	Height %
1	0.857	44.192	10.497	PV	0.062	0.6037
2	1.103	929.926	92.690	VV	0.144	5.3313
3	1.206	185.225	83.236	VV	0.037	4.7875
4	1.373	4465.958	794.528	VV	0.076	45.6988
5	1.513	255.636	77.956	VV	0.055	4.4838
6	1.574	209.314	66.836	VV	0.052	3.8442
7	1.643	581.206	68.058	VV	0.110	3.9145
8	1.926	567.010	43.986	VV	0.161	2.5300
9	2.789	1758.810	32.517	VV	0.679	1.8703
10	3.505	839.423	29.010	VV	0.387	1.6686
11	4.003	905.274	31.123	VV	0.397	1.7901
12	4.404	1186.391	37.396	VV	0.416	2.1509
13	6.016	3233.418	66.577	VV	0.602	3.8293
14	6.520	398.518	29.283	VV	0.227	1.6843
15	6.762	1178.576	34.289	VV	0.446	1.9722
16	8.348	3195.580	51.112	VV	0.781	2.9398
17	9.480	1722.715	31.009	VV	0.684	1.7836
18	11.160	265.035	12.814	VV	0.277	0.7370
19	12.024	998.022	26.969	VV	0.617	1.5512
20	12.409	1336.936	55.864	VV	0.336	3.2131
21	13.764	49.594	5.149	BH	0.164	0.2962
22	14.655	94.440	5.361	VV	0.294	0.3084
23	15.154	357.225	17.784	VV	0.275	1.0229
24	18.793	607.684	22.363	BV	0.353	1.2863
25	20.914	154.879	12.210	BB	0.183	0.7023

Total height = 1739

Chromatogram 39

File: C:\HPCHEM\1\DATA\AP30S\002-0101.D
Operator:
Date Acquired: Tue Apr 30 15:17:57 1996
Method File Name: AFLTX.M
Sample Name: 10 mg+ 25ng aflt
Misc Info:
Bottle Number: 2