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A HPLC Method to Assay Mycotoxins - Fumonisin, Ochratoxin and Zearalenone

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A HPLC METHOD TO ASSAY MYCOTOXINS - FUMONISIN, OCHRATOXIN AND ZEARALENONE

by

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A HPLC METHOD TO ASSAY MYCOTOXINS - FUMONISIN, OCHRATOXIN AND ZEARALENONE

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A HPLC method to quantitate the mycotoxins Fumonisin B1(FB1), Ochratoxin A (OA), and Zearalenone (ZON) was developed and validated. Mycotoxins are secondary metabolites of fungi like Fusarium, Asperigillus and Penicillium that contaminate crops. They have a wide array of chemical structures which facilitate their analytical detection.

The analytical method consists of reverse phase chromatography and fluorescence detection. Sample cleanup was performed using SAX and C18 cartridges prior to analysis on the HPLC.

The method developed for FB1 is unique in that it is more sensitive, accurate, and reproducible than the existing methods. Automation is one of the salient features that improves sensitivity of the method and overcomes the limitations due to the instability of the FB1-OPA derivative. Simultaneous analysis of OA and ZON was made possible by varying the HPLC conditions. Extraction optimization and statistical validation studies were completed for both the methods.

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Nalini Sadagopan

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CHAPTER I

INTRODUCTION

Origin

Mycotoxins are toxic secondary metabolites of molds that contaminate agricultural products like corn, peanuts, wheat, barley, and oats (Scott et al., 1985). They are produced by fungi like Fusarium, Aspergillus and Penicillium before harvest and even during storage. It has been shown that foods and feeds are susceptible to fungal growth at some stage during production, processing, transportation and storage (Frisvad and Samson, 1992). The occurrence of mycotoxins in food is determined mainly by biological and environmental factors. The weather during growth (temperature, humidity, moisture, air) and harvest (moisture, temperature, rapidity of drying, rewetting, relative humidity) has an effect on the presence or absence of mycotoxins (Abramson and Miller, 1989). The presence of mycotoxins in foods and feeds is potentially hazardous to animal and human health. Some of the diseases caused by these mycotoxins are listed in Table 1.

They can be present as contaminants in crops all over the world (Trucksess,1995). Many countries have enacted legislation that limits the amount of certain mycotoxins permissible in foods and feed (Trucksess, 1995). Sensitive, specific, accurate and precise

methods of analysis are needed for enforcement of mycotoxin regulations, to determine potential human and animal exposure, and for research purposes (Trucksess, 1995).

Table 1

Diseases Caused by Mycotoxins in Animals

Mycotoxin	Diseases
Fumonisin	Leukoencephalomalacia (horses) (Cawood et al., 1991), pulmonary edema (pigs) (Harrison et al., 1990), hepatotoxicity (rats) (Gelderblom et al., 1991)
Ochratoxin	Nephrotoxicity (rats) (Kuiper-Goodman et al., 1989)
Zearalenone	Reproductive problems and estrogenic effects (swine) (Chang et al., 1979)

Chemical Structure and Properties

Mycotoxins have a wide array of chemical structures. Fumonisin B1 (FB1) is mainly produced by *Fusarium moniliforme* and *Fusarium proliferatum*. FB1, as shown in Figure 1 is the diester of 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxy cosane and propane 1,2,3-tricarboxylic acid, in which the C14 and C13 hydroxyl groups are esterified with one of the terminal carboxy groups of the tricarboxylic acid (Benzuidenhout et al., 1988). It also has a primary amine moiety available for derivatization. Fumonisins B2 and B3 are other types of fumonisin that are known to occur in crops. They differ from fumonisin B1 in the hydroxylic groups. In FB2 the hydroxyl group on C10 is absent and in FB3 the hydroxyl group on C5 is absent.

Hydrolyzed fumonisins have been known to be present and are suspected to be equally hazardous as the unhydrolyzed fumonisins themselves.

Ochratoxin A (OA) and B produced by contamination of *Penicillium verucosum* and *Aspergillus ochraceus,* are derivatives of isocoumarin linked to L-phenylalanine as depicted in Figure 2. The IUPAC name of OA is (R)-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)carbon- yl -L-phenylalanine (Seidel et al., 1993). Ochratoxin B has the same structure as ochratoxin A with the chlorine atom replaced by hydrogen.

Zearalenone (ZON) is a (6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid μ-lactone as shown in Figure 3. The fungi *Fusarium sacchari* var. *subglutinans* and *Fusarium graminearum* (Ichinoe et al., 1983) produce zearalenone via the diastereomeric precursors α and β zearalenol.

Toxicology of the Mycotoxins

The fumonisins are structurally similar to sphingosine which is a constituent of different sphingolipids. FB1 and FB2 have been found to inhibit sphingolipid biosynthesis at the level of sphingosine N-acyl-transferase in rat primary hepatocytes and pig kidney cells in culture (Norred et al.,1992, Wang et al., 1991). Sphingolipids are highly bioactive components of cell membranes and their metabolic disruption could affect cell growth, differentiation, and behavior (Merrill, 1991). FB1 and FB2 are the first known naturally occurring specific inhibitors of sphingosine biosynthesis (Wang et al., 1991).

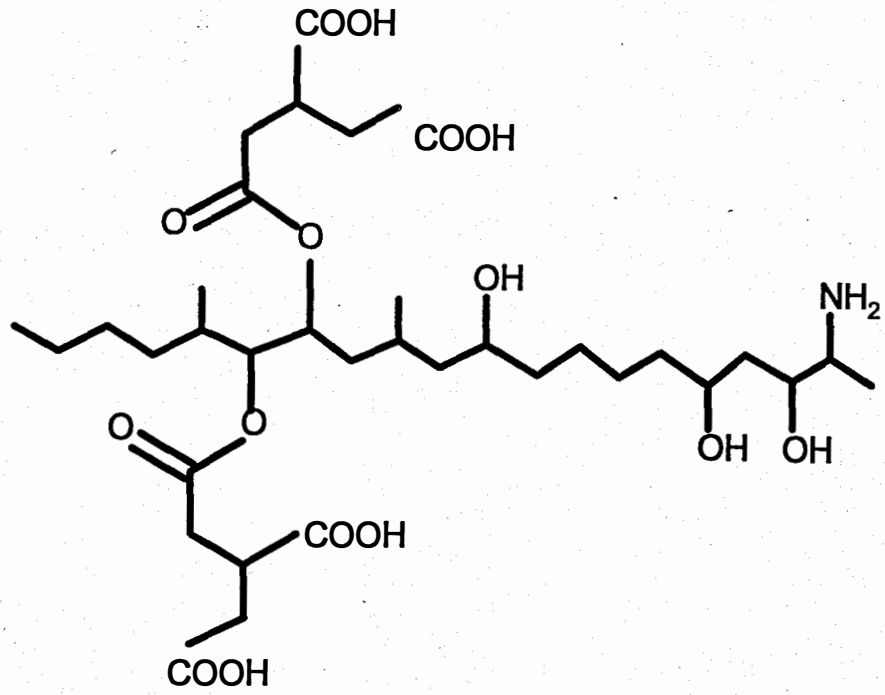

Figure 1. Structure of FB1 .

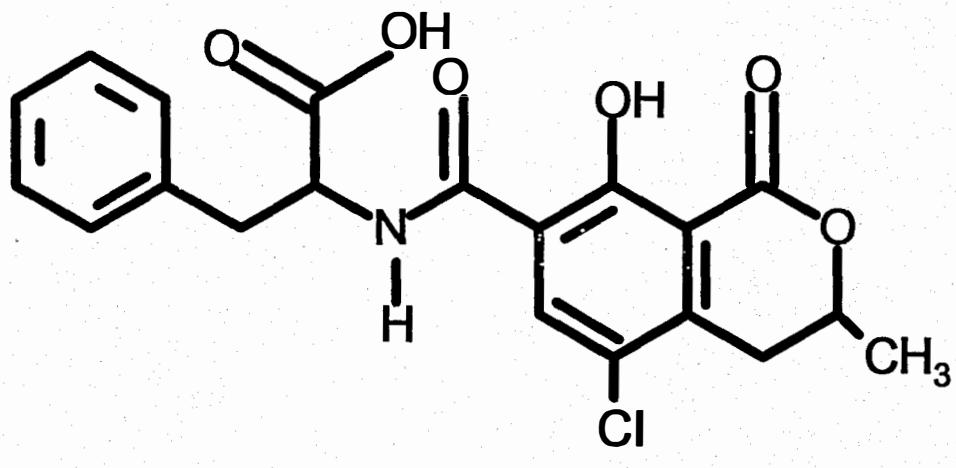

Figure 2. Structure of OA .

OA inhibits tRNA- synthetase accompanied by a reduced protein synthesis in many different microorganisms and hepatoma cells (Roschenthaler et al., 1984). Also a major biochemical effect of OA is the inhibition of mitochondrial respiration (Wei et al., 1985). It also has an effect on hepatic microsomal calcium sequestration in rats which may contribute to its toxicity in liver (Khan et al., 1989).

ZON produces hyper estrogenic effects, which affects fertility (Schiefer, 1985). It also has anabolic properties which result in weight gain. (Becci et al., 1982).

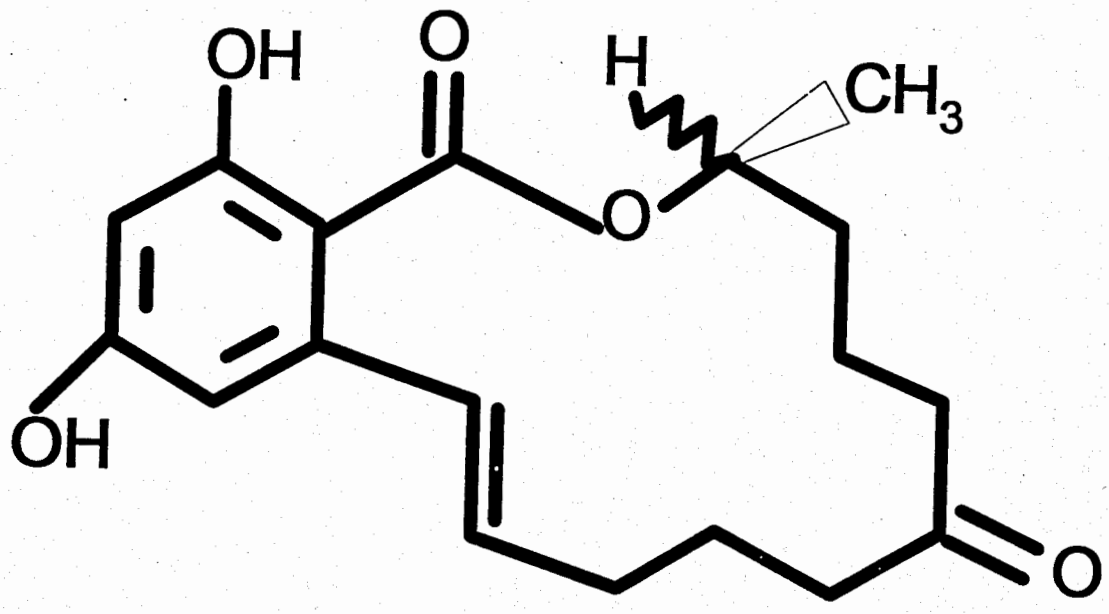

Figure 3. Structure of ZON.

Decontamination

Although the ideal defense against mycotoxins is prevention, its presence is sometimes unavoidable. There are a few methods of decontamination for these mycotoxins. Physical methods involve color separating damaged kernels, heating to

destroy the thermally unstable mycotoxin, milling and irradiation with UV light. Chemical methods involve ammoniation which reduces OA (Madsen et al., 1983) and fumonisin. Liquid treatments with acids, alkali, bleach, have also been studied (Doyle et al., 1982). Treatment of ZON contamination with formaldehyde has proven effective (Bennet et al., 1981). Biological methods using enzymes and microorganisms can degrade mycotoxins (Doyle et al., 1982).

Methods of Analysis

Understanding the safety of mold contaminated grains in animal feeds and food products requires strong analytical support. Also quantitative analyses of mycotoxins is of critical importance for research and regulatory work.

Currently there are many different methods of analysis, as listed in Table 2, for these mycotoxins. They are limited due to their sensitivity, ease of analysis and cost. Currently there is no regulation for FB1, OA or ZON in the Unites States on food. However there are a few European countries which have levied regulations on these mycotoxins. Some of the regulated levels are in the lower part per billion levels. Thus a sensitive method for quantitation should be able to measure these mycotoxins in the lower ppb levels. Methods available for OA and ZON analysis in food and feed are primarily ELISA and HPLC (Seidel et al., 1993; Merino et al., 1993). The detection limit with ELISA kits manufactured by Neogen Corporation, East Lansing, MI, for OA is 2.5 ppb and ZON is 125 ppb. Most of the various HPLC methods developed so far use chloroform and methylene chloride (Hald et al., 1993; Merino et al., 1993)

as their extraction solvent and mobile phase. The AOAC General Referee reports for the Committee on Natural Toxins recommend: development of a simple screening method for Fumonisins; development of an efficient method of analysis for ZON and to study the AOAC Official Methods for mycotoxins and find alternative solvents to replace benzene, chloroform and methylene chloride (Trucksess 1995).

Table 2

Available Methods of Analysis for FB1

Method	Advantages	Disadvantages
Thin Layer Chromatography	Ease of testing	Detection limit < 100 ppm (Sydenham et al., 1990)
HPLC - UV	-	Detection limit 10 ppm (Sydenham et al., 1990)
Enzyme Linked Immuno Sorpent Assay (ELISA)	Rapid	Detection limit 0.5 ppm (Pestka et al., 1993)
GC-MS	Specific	Detection limit 0.1 ppm; expensive instrumentation; (Plattner et al., 1994)
HPLC-Fluorescence	Sensitive	Detection limit 10 ppb; inefficient clean up procedures (Stack & Eppley, 1992; Hopmans & Murphy, 1993; Rice & Ross, 1994)

Based on the above mentioned requirements the goal of this research was to develop a HPLC method which would be able to quantitate the mycotoxins FB1, OA, and ZON in lower ppb levels.

CHAPTER II

METHODOLOGY

HPLC System for FB1

The FB1 molecule has a long hydrocarbon backbone and thus a reverse phase column is suitable for its analysis. The HPLC system for FB1 consists of a Waters model 510 reciprocating pump, a Waters model 717 autosampler, a μ bondapak C18 column (3.9 x 150 mm, particle size 10 μ), a Waters model 470 fluorescence detector. The mobile phase used was a 50:50:1, acetonitrile:water:acetic acid (Stack and Eppley, 1992) with a pH of 3.35. The pump flow rate was maintained at 1 ml/min.

FB1 standards were purchased from Sigma Chemical Company, St.Louis, Missouri. They were labelled to be 98% pure which was confirmed by an outside laboratory. The FB1 was obtained from cultures of Fusarium moniliforme. A stock standard solution of 100 ppm was made up by dissolving 1 mg in 10 ml of 1:1 acetonitrile:water. Standards of different concentrations were made up from the 100 ppm stock by dilution with the same solvent. FB1 is neither uv absorbing or fluorescent, hence it is necessary to derivatize FB1 with a fluorigenic reagent such as o-pthaldialdehyde (OPA) (Shepard et al., 1990). The primary amino group reacts with the OPA in the presence of 2-mercaptoethanol(MCE) in a borate buffer to form a 2-alkyl-1-isoindole derivative. The derivative is fluorescent at an excitation wavelength 335

nm and emission wavelength of 440 nm. The addition reaction between the FB1, OPA and MCE is shown in Figure 4. The OPA derivative is unstable and it decomposes after 30 minutes to form a nonfluorescent 2,3 dihydro compound via an intramolecular rearrangement (Blau and Halkett, 1993).

The derivatization of FB1 with the OPA reagent is performed by the autosampler. The OPA reagent is prepared by dissolving 40 mg of crystalline OPA (Sigma Chemical Company, St.Louis, Missouri) in 1 mL HPLC grade methanol (Stack and Eppeley, 1992). It is then diluted with 5 mL of 0.1 M borax solution. Then 50 μL of 2-mercaptoethanol (Sigma Chemical Company, St.Louis, Missouri) is added. The OPA solution is placed in a common vial and 50 μL of the samples are placed in successive vials. The auto sampler is set in the auto transfer mode. In this particular mode it is programmed to transfer a 100 μL of OPA from the common vial into a sample vial. Then it is programmed to mix the OPA and the FB1 solution by drawing a 100 μL of the total mixture and redelivering the drawn solution into the original sample vial. Maximum peak area was obtained when the autosampler was programmed for 5 mixes. Then a 10 μL aliquot of the FB1-OPA derivative is injected onto the column which starts the program on the data collection station. Once the derivative is injected into the column the separation takes place and the peak corresponding to the FB1-OPA derivative appears at the retention time 5.45 min. There are other peaks due to the OPA reagent before 4 min and after 8 min.

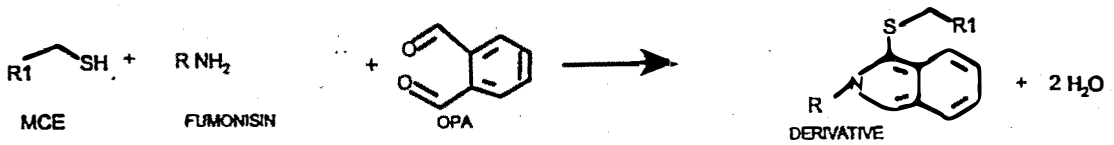

Figure 4. Reaction of FB1 With OPA .

HPLC System for OA and ZON

OA and ZON are naturally fluorescent. OA has an excitation wavelength of 333 nm and an emission wavelength of 460 nm, while ZON has an excitation wavelength of 274 nm and an emission wavelength of 440 nm.

OA and ZON were chromatographed on the C18 column (Waters μBondapak, 150mm x 3.5mm, 10 μ in particle size) as used in the above FB1 method. The same HPLC instrumentation as that of FB1 method was used except for the detector. A Waters model 474 fluorescent detector with greater sensitivity was used. The mobile phase used was 40:60 acetonitrile:water with a flow rate of 1 mL/min. The detector was time programmed for wavelength monitoring. The retention time of OA is 5.5 min and that of ZON is 14.3 min. Thus the detector was programmed to monitor at the wavelengths of OA from time 0 to 10 min and then switch to the wavelengths of ZON from 10 min till 16 min.

Extraction of the Mycotoxins From the Sample Matrix

The extraction of FB1, OA and ZON from a sample matrix can be done using the extraction solvent 1:1 acetonitrile : water. A 25 g portion of the sample is weighed into a disposable container. Then 100 mL of the extraction solvent is added and the mixture is shaken for one hour. A 2.00 mL aliquot of the extract is sufficient for the preconcentration of FB1 and another 2.00 mL for both OA and ZON.

Solid Phase Extraction

For FB1

The 2 mL aliquot is diluted with 8 mL of 3:1 methanol:water. If the pH is not in the range of 7-9, it is adjusted with 1 N NaOH. A strong anion exchange (SAX) cartridge (purchased from Romer Laboratories, Missouri) is preconditioned with 5 mL of methanol and then 5 mL of 3:1 methanol:water. The extract is then applied to the cartridge carefully maintaining the flow rate to be less than 1 mL/min. The cartridge is washed with 8 mL of 3:1 methanol:water followed by 2 mL methanol. The FB1 is then eluted off the cartridge using 2 mL of 95:5 methanol:acetic acid.

For OA and ZON

The sample preparation for OA and ZON is done on a C18 (Waters Sep-pak cartridge). The extract (2 mL) is diluted with 8 mL of water. The pH of the solution as measured by a pH meter should be 7 + 1. If not, adjust with 1 N NaOH. The

C18 cartridge is preconditioned with 3 column volumes (8 mL) of acetonitrile and then with 3 column volumes of water. The extract is applied to the cartridge and the flow rate should be less than 1 ml/min. The cartridge is then washed with 2 ml of 1:4 acetonitrile:water to remove loosely bound hydrophilic impurities. Then the OA and ZON are eluted with 2.5 mL of 95:5 acetonitrile:acetic acid.

CHAPTER III

FUMONISIN B1

Method Development

The method for FB1 was originally designed based on the method of Stack and Eppeley, 1992. The sensitivity using this method was 10 ppb. Also SAX extraction using cartridges from Varian were performed. The final eluate (14 mL) in this sample preparation had to be evaporated to reconcentrate the FB1. This evaporation period was lengthy and also the recovery was only 30-60% and inconsistent.

Choice of Clean-up Cartridge

The C18 cartridge clean up methods also did not give reproducible results. When standard spikes were used to test the effectiveness of these cartridges, there was some flow through of FB1 observed during sample application and washing. Another problem during the analysis of FB1 analyte is the presence of coelutants. Extracts of noncontaminated corn samples when analyzed underivatized did not have any peaks at the retention time of FB1. When the same extract is derivatized with OPA a peak was observed at the retention time of FB1. This means that an effective cleanup step is necessary to prevent coelution. Since the HPLC column is a C18, using a column which is orthogonal for sample preparation would minimize the coelution. Thus a SAX

cartridge was chosen. Also on trying the different cleanup cartridges the SAX cartridges from Romer Labs seemed to retain the FB1 more efficiently with minor changes in flow rate of the applications or washings.

Elimination of Evaporation

The evaporation step was eliminated by increasing the strength of the eluting solvent from 1% acetic acid to 5% acetic acid. The evaporation step was time consuming, and it was suspected that FB1 adheres to the glass vessels used during evaporation. Hence the glass vessels had to be silanized. Using a stronger eluent resulted in the final volume of the eluate being 2.5 mL rather than 14 mL. Thus this effectively reduces the time for analysis and increases the recovery by eliminating another step.

Choice of Derivatives and Automation

Although the OPA derivative of FB1 is more sensitive than other derivatives like flourescamine and naphthalene dicarboxaldehyde (NDA), it is unstable. The derivative of fluorescamine gives two peaks and NDA uses NaCN in the derivatization process. Since the fluorescence of OPA derivative decays in solution after 30 min it was necessary to derivatize each sample immediately before being analyzed on the HPLC. Thus constant presence of the analyst is necessary, so that the next sample can be analyzed. To eliminate this limitation, automation of the derivatization process was introduced. The autosampler used was able to perform the auto transfer process which allowed it to transfer OPA from a common vial into the sample vial just before

chromatography and mix it with the sample. Thus 30 samples could be analyzed continuously before manual supervision is necessary.

The autosampler works pneumatically and it produces efficient mixing. The precision is high since the derivatization by the auto sampler is repeatable in the same way and the duration is the same for every sample. Sensitivity of the method improved because of the increased fluorescence of the derivative produced by the autosampler as compared to the manual derivative process for the same concentration of FB1.

The amount of OPA required to derivatize a 50 µL of the sample manually is 200 µL whereas using the autosampler the amount of OPA was reduced to 100 µL. This reduces the cost of the analysis to some extent. Thus the automation part of the method development has been very helpful in improving the analysis.

Experimental Design and Results

Extraction Efficiency

Extraction efficiency can be defined as the ability of the solvent to bring into solution all of the analyte of interest in a specific period of time. Since FB1 is most stable in acetonitrile : water (1:1) (Visconti et al, 1992), this extraction solvent was chosen. A corn matrix which contained no FB1 (also tested negative by ELISA) was used as a blank. The FB1 standard (250 µl of 100 ppm) was spiked into the 25 g of blank corn in 100 ml of the extraction solvent so that the final eluate would contain 0.25 ppm of FB1. The mixture was shaken at a speed of 360 rotations/min. Aliquots (2.00

mL) of the mixture were taken at each time interval (5-120 min). They were subjected to the SAX extraction and the eluate chromatographed after derivatization. The peak at 5.5 min was identified as FB1 and the peak area was obtained for quantitaion. A plot of time in (minutes) against peak area (thousands) was made to determine the time at which the extraction is maximum. Also the peak area obtained at this time was compared with the area of a standard 0.25 ppm standard run on the HPLC on the same day.

The data for this study is shown in Figure 5. The peak area increases at every time interval until it reaches 60 min and remains virtually the same there after. This shows that the maximum extraction is achieved only at 60 min. Comparing the peak area (732453) of a 0.25 ppm standard with the peak area at 60 min, gives us the result that the extraction efficiency at 60 min. is 96.5%:

Extraction Efficiency = (706538/732453) x 100 = 96.5 %

Thus 60 min is chosen to be the optimum time for maximum extraction of FB1 from a sample matrix.

Optimization of the Auto Sampler

Transfer of the manual method to the autosampler required method development. The peak area at a particular concentration varied with the number of mixes the autosampler performed (Figure 6). Thus a study of the peak area of a standard solution

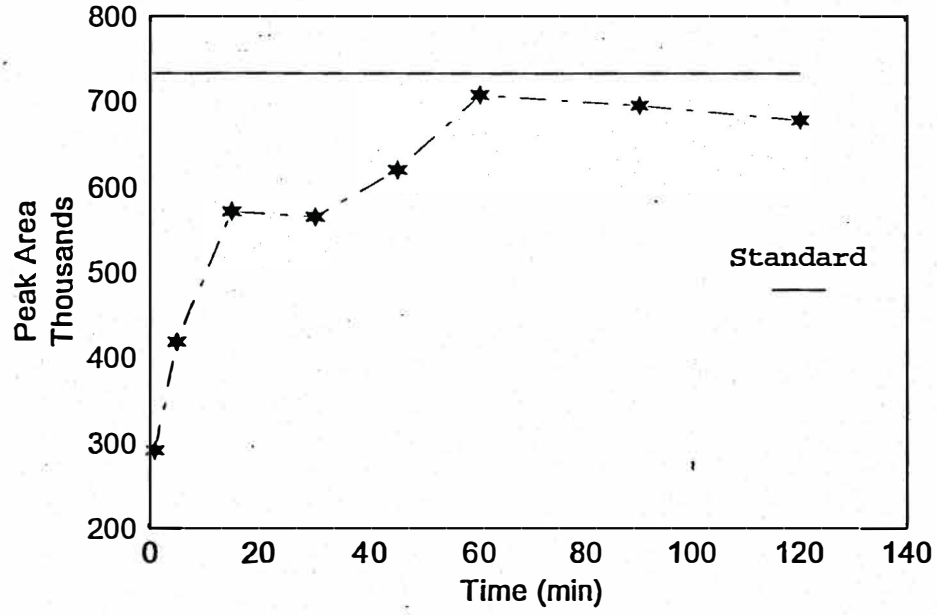

Figure 5. Extraction Efficiency for FB1 Analysis.

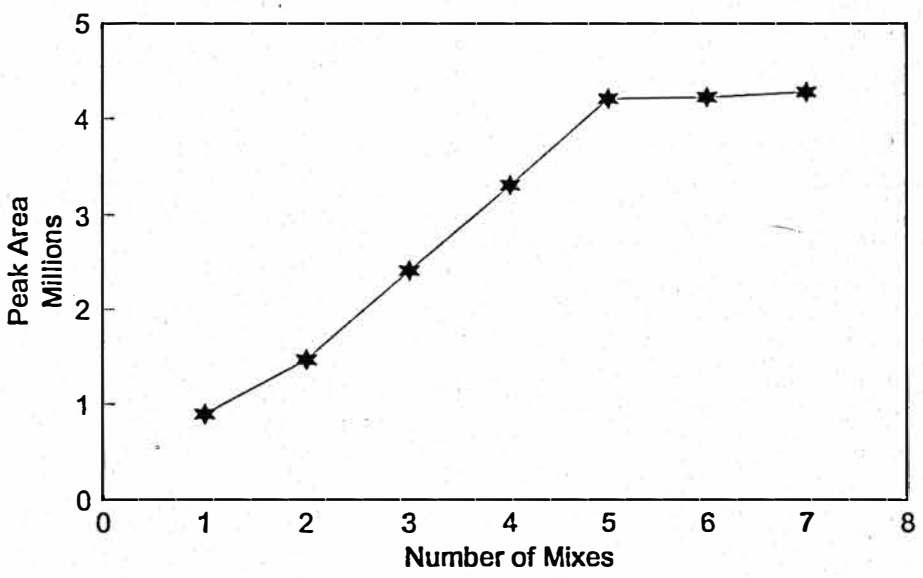

Figure 6. Optimization of the Autosampler for FB1 Analysis.

(1.5 ppm) of FB1 with the different number of mixes (1-7) performed by the autosampler was studied.

The results for this study is shown as plot of the number of mixes against the peak area in Figure 6. The number of mixes at which maximum peak area was obtained, 5 mixes, was determined to be the optimum.

Resolution Map and Ruggedness

A commercially available software program called "DRYLAB" by Waters was used to estimate the ruggedness of the system. This program requires 2 different analyses at two different organic percentages in the mobile phase. Thus two different analyses at 50% and 60% acetonitrile were performed and the retention times of the fumonisin peak were obtained. An input of the chromatographic conditions such as column type, length, temperature, solvent strength and the two retention times gives a map called the resolution map, which plots resolution against % organic. DRYLAB defines that the less steep the curve in the resolution map the more rugged the system. Thus the resolution map in Figure 7, shows that this system is rugged for slight changes in mobile phase. Ideally "DRYLAB" is used to obtain information on modifications that would yield a separation between two closely appearing peaks. During the process of multimethod development this information of FB1 was obtained from the program.

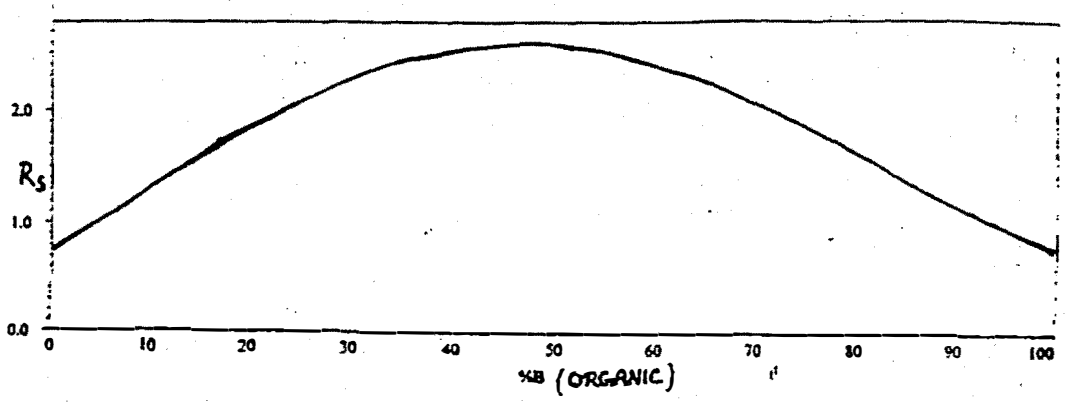

Figure 7. Ruggedness of the FB1 system - Resolution Map.

Results

Standard Linearity

Standard solutions were prepared, using a 100 ppm stock solution, ranging from 2.5 ppb to 10 ppm, and their chromatograms were obtained (Figure 8). Three replicates of each concentration were analyzed in a random order using the developed method. Mean, standard deviation, coefficient of variation for each concentration were calculated and are listed in Table 3. Using the least-squares linear regression model ($y = mx + b$), a plot of the average result for each solution versus the concentration was obtained. This result is shown in Figure 9, and the $r^2 = 0.9948$.

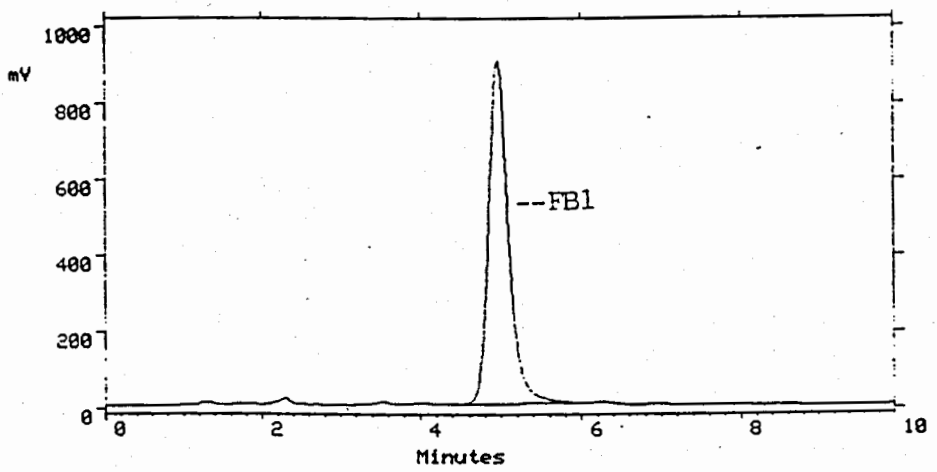

Figure 8. Chromatogram of a FB1 Standard.

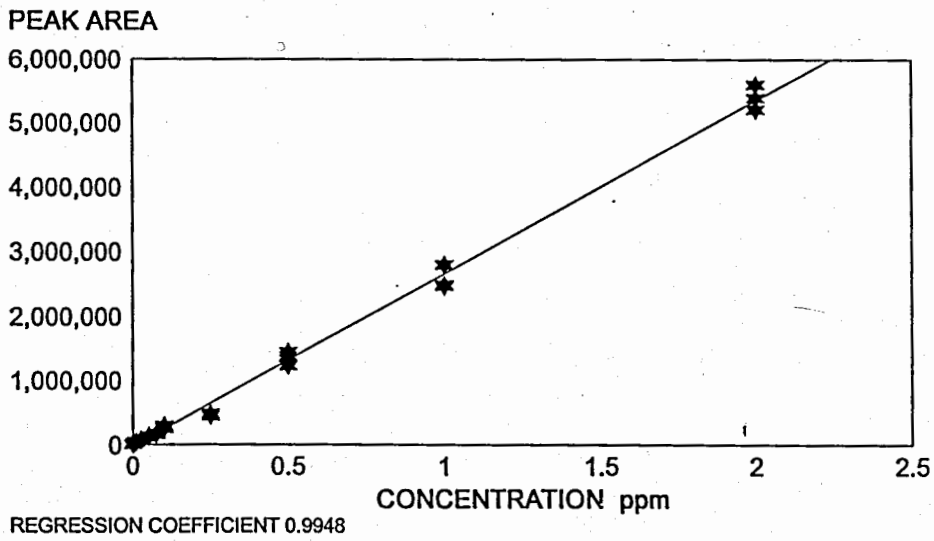

Figure 9. Linearity Plot for FB1.

Residuals

The residuals were also calculated. Residual is defined as a difference between actual value and predicted value. The residuals graph uses the x-axis as concentration and y-axis as the residual value. This allows for comparison of the differences in residuals over concentration.

The residual plot for FB1 is shown in Figure 10. The deviation from the predicted value is minimal from 0 - 0.1 ppm, while it increases with increasing concentration. Even though concentrations of 2.5 ppb - 10 ppm were studied for linearity, there were more deviations at higher concentrations. Those above 2 ppm did not seem to be in the linear range. Hence it would be necessary to dilute samples

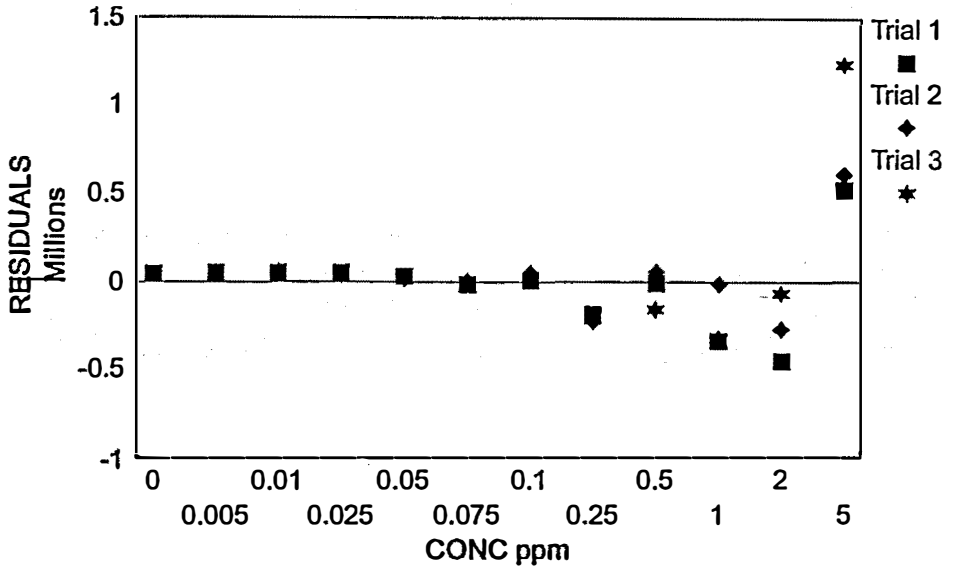

Figure 10. Residuals Plot for FB1.

with higher concentrations of FB1 into the linear range for an accurate quantitaion.

Table 3

Linearity Data for FB1

Concentration (ppm)	Mean Peak Area	Standard Deviation	Co-efficient of Variation
0	1811	134	7.42
0.005	2294	2155	9.40
0.01	39473	4636	11.75
0.025	71964	5937	6.25
0.05	126532	9276	7.33
0.075	166066	10154	6.11
0.1	274116	21613	7.88
0.25	462062	21161	4.58
0.5	1348599	109305	8.10
1	2587587	183929	7.11
2	5402636	194712	3.50

Limit of Reliable Measurement

This is the minimum level of analyte which a method can quantitate at the 95% confidence level. Seven(n) replicates of the analyte solutions were prepared at a concentration of 5 ppb. The data was analyzed to determine mean, standard deviation(s), and coefficient of variation, and the results are listed in Table 4. The experimental "t" statistic was calculated which compares the mean (x) and the theoretical mean(u).

$$t=(x-u)\sqrt{n}/s$$

Table 4

LRM Data for FB1

Data for LRM	
Theoretical Mean	0.005
Standard Deviation	0.0012
Coefficient of Variation	22.53
Number of replicates	7
Mean	0.00562
Experimental T-value	1.299
Student T-value	2.447

The Student's - t critical value was obtained from the t-distribution table for a 95% confidence interval and for 7 measurements. Since the experimental "t" was less than the Student's - t value, the 5 ppb level was statistically approved to be the limit of reliable measurement. Even though the linearity study included the 2.5 ppb level, the signal at this concentration was not reproducible and hence 5 ppb was chosen to be the LRM and the lowest point in the curve.

Accuracy

Accuracy was determined by spiking standard solutions of FB1 into a FB1 free corn matrix. Standard solutions were spiked into the 25 g corn matrix in a 100 mL of acetonitrile : water (1:1). The spike levels were such that the concentration in the final eluate was 0.07 ppm, 0.6 ppm and 1.0 ppm. The spiked samples were analyzed. The accuracy, as listed in Table 5, was determined by comparing the concentration equivalent for the observed peak area against the expected theoretical concentration calculated from the amount spiked.

$$\text{Accuracy} = (\text{Conc Obtained} / \text{Conc Expected}) \times 100$$

The % recovery at the 3 levels of concentrations are higher than 90. In some cases the recovery is greater than a 100% and possible explanations can be due to derivatization, the instability in the signal from the detector, and matrix interferences not being eliminated completely. The chromatogram of the blank corn matrix which

has no peak at the retention time of FB1 (5.5 min) is shown in Figure 11. Thus the reproducibility of the method was tested.

Table 5

Accuracy Data for FB1

Amount Spiked (ppm)	Recovery(%)
0.07	114
0.60	90
1.0	110

Precision

Three different corn matrices were used to study the precision. The samples were prepared as mentioned in the methodology. Standards (1.25 ppm) were analyzed along with samples every day. Triplicates of each sample (Figure 12) were analyzed each day for three days over a weeks time. Concentration (μg/g) in the samples were calculated from the peak area. Statistical analysis of the data and its resulting mean, standard deviation, variance, coefficient of variation for all three matrices for all three days combined is listed in Table 6.

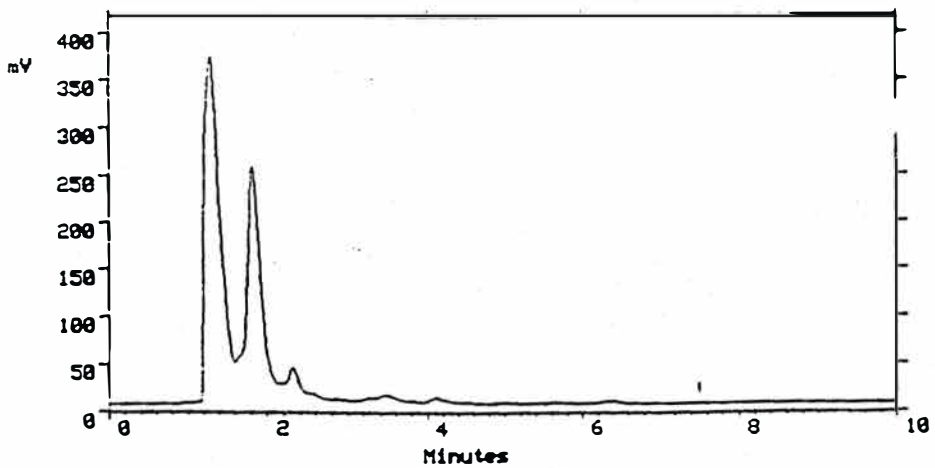

Figure 11. Chromatogram of a FB1 Free Corn Sample.

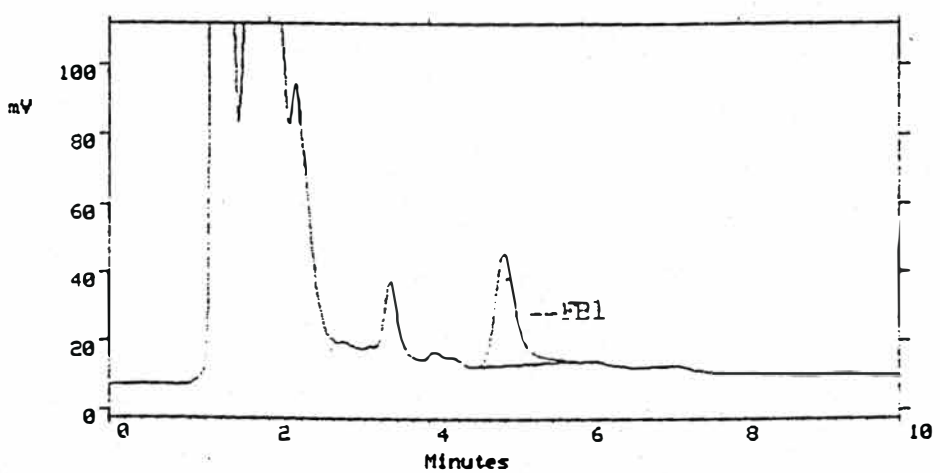

Figure 12. Chromatogram of a FB1 Containing Corn Sample.

Table 6

Statistical Analysis of Precision Data for FB1

Matrix	Mean (μg/g)	Standard Deviation	Variance	Coefficient of Variation (%)	Minimum Difference to Detect (μg/g)
1	0.459	0.0569	0.00324	12.4	0.1767
2	0.124	0.0219	0.00048	17.6	0.0635
3	0.368	0.0268	0.00072	7.3	0.0840

The daily averages for each matrix were not significantly different for all three matrices. The calculated F_{max} and tabulated F_{max} are listed in Table 7. Since the calculated F_{max} is lesser than the tabulated F_{max} the daily variances for all the 3 matrices were not significantly different. Of the percent of total variance, the between - day variances were lower than the with-in day variance, which means that the assay variability parameters (pH of the samples, pipet volumes, voltage fluctuations, etc) in a day should be studied carefully and maintained to be minimal. This would prevent variances within a day from being high. The minimum difference to detect at the level of FB1 present in the matrix for duplicate assays was calculated using the total assay variability and are listed in the table.

Table 7

Comparison of Variances for FB1

Matrix	Calculated F_{max} [Tabulated $F_{max}(\alpha = 0.05)$ = 202.000]	With-in Day Variances (%)	Between Day Variances (%)
1	9.308	83	17
2	31.000	94	6
3	7.000	100	0

Thus statistically the method was validated to be linear between 5 ppb - 2 ppm, limit of reliable measurement being 5 ppb. The experimental range being 2.5 ppb - 10ppm, and the linear range was chosen based on the statistical analysis of the data and residual study. The LRM 5 ppb can also be called the detection limit of the method. The method is over 90% accurate between 0.07 - 1 ppm, and precise at a 95% confidence level. The studies were done using corn matrices. Hence this is a reliable method for identification and quantitation of FB1 in corn type matrices.

CHAPTER IV

OCHRATOXIN AND ZEARALENONE

Attempt for a Multi-Method

Once the method for FB1 was established, an attempt to analyze OA and ZON was made. Standards of OA and ZON when analyzed on the same system as that for FB1 eluted at 5.6 min and 6.0 min respectively. Thus a mixture of OA and ZON gave an unresolved chromatogram (Figure 13). Even though both OA and ZON fluoresce at either set of wavelengths there is a compromise in the sensitivity. Thus conditions had to be modified to obtain a complete separation of OA and ZON such that time programmed wavelength switching is possible where each of them could be detected at their own optimum wavelengths.

Variations on the Physical Factors Affecting Resolution

Resolution is given by the equation

$$Rs = 1/4\,(\alpha - 1)\,\alpha\sqrt{N}\,(k/k+1)$$

where α=selectivity, N=efficiency and k=capacity.

Increasing the efficiency of the column which is a physical factor should increase resolution (Figure 14).

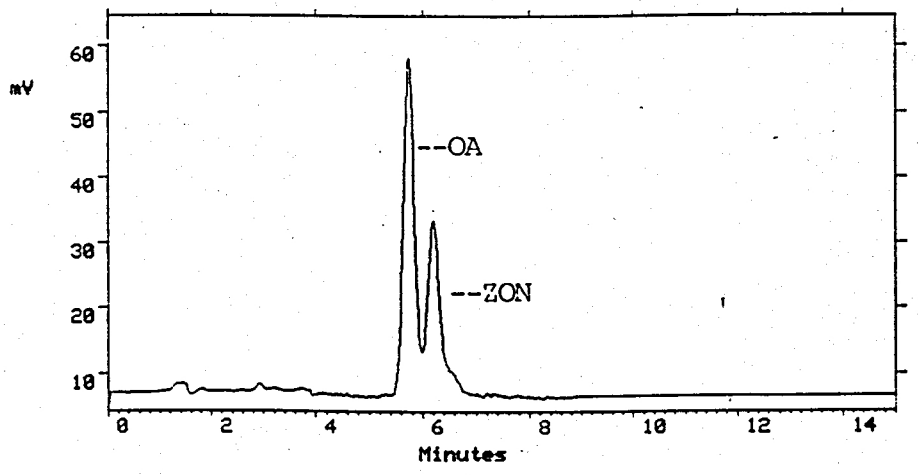

Figure 13. Chromatogram With Unresolved Peaks for OA and ZON, R_s = 0.73, 15 cm Column; 50% Organic Mobile Phase.

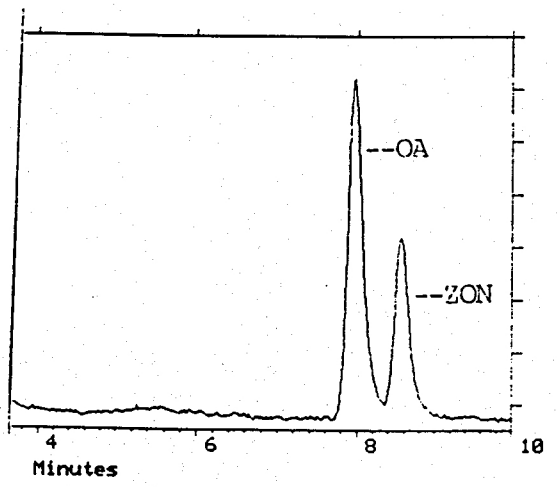

Figure 14. Chromatogram With Unresolved Peaks for OA and ZON , R_s= 1.0, 30 cm Column; 50% Organic Mobile Phase.

$$N=L/\sigma$$

where L is the length of the column and σ is a mathematical factor. Also the size of the particle and packing of the column are other physical factors effecting resolution. Hence a 30 cm Novapak column with 4 μ particle size was chosen. Resolution was calculated using the equation

$$Rs=2(t2-t1)/(w2-w1)$$

where t_2 is the retention time and w_2 is the width of the base of ZON peak and t_1 is the retention time and w_1 is the width of the base of OA peak.

Using the set of conditions used for FB1, the resolution was 0.73. Resolution calculated after using the new column was 1.0 (Figure 14). This was not sufficient to perform a time programmed wavelength switching, which would require a resolution of more than 2.0.

Chemical Factors Affecting Resolution

The factors affecting resolution chemically are the selectivity α and the capacity k. They can be improved by altering the organic composition of the mobile phase. DRYLAB predicts maximum resolution using 40% acetonitrile. This can be seen from the resolution map (Figure 15). The resolution obtained when a 40% acetonitrile was used in the mobile phase (Figure 16) was greater than 2. The OA peak was at 8.5 min and the ZON peak at 17 min. This resolution satisfied the criteria required for

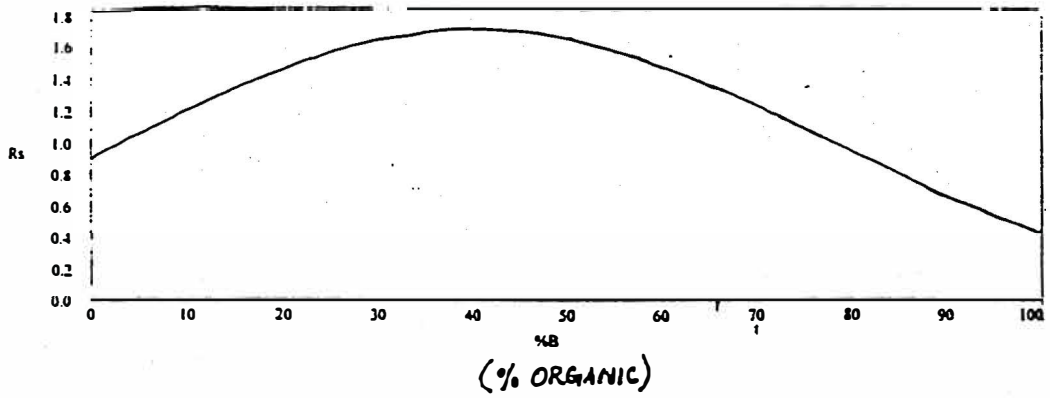

Figure 15. Resolution Map for OA and ZON.

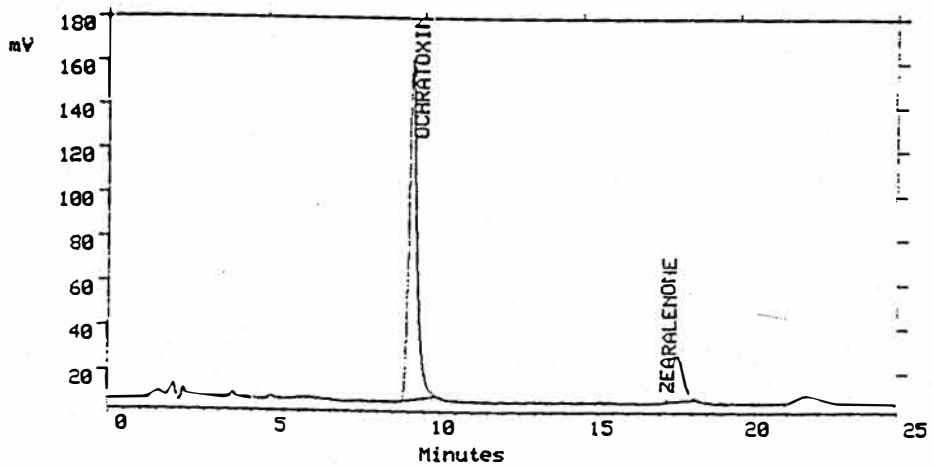

Figure 16. Chromatogram With Resolved Peaks for OA and ZON, 30cm Column, 40% Organic Mobile Phase.

simultaneous determination. The α was calculated to be 2.4 after the separation was achieved. This shows that the capacity of the column to separate the 2 analytes with such a mobile phase composition is reliable.

Since the change in mobile phase produced such a drastic change in the separation of OA and ZON on the 30 cm column the same mobile phase was tried on the 15 cm column. A resolution was achieved even on the 15 cm column (Figure 17), where the retention time for OA was 5.3 min and ZON was 14.3 min. The impedance for the analysis of all the three mycotoxins under study is that the retention times of OA and FB1 are the same.

Thus using the same system and by just switching the mobile phases the analysis of FB1 and OA and ZON can be performed in two different runs by this newly developed method.

Extraction Efficiency

A similar type of extraction study as for FB1 was performed for OA and ZON using the same extraction solvent. A standard spike (125 µL of 100 ppm) of both OA and ZON were added into a 25 g of the blank corn matrix in 100 mL of the extraction solvent such that a 0.1 ppm concentration should be obtained in the final eluate. The aliquots at different intervals of time (5 min - 60 min) were tested for OA and ZON. The results for this study is shown in Figure 18.

The peak area did not vary much with time, but the maximum (99.5%) extraction was at 30 min for zearalenone and hence 30 min of shaking was considered to be the

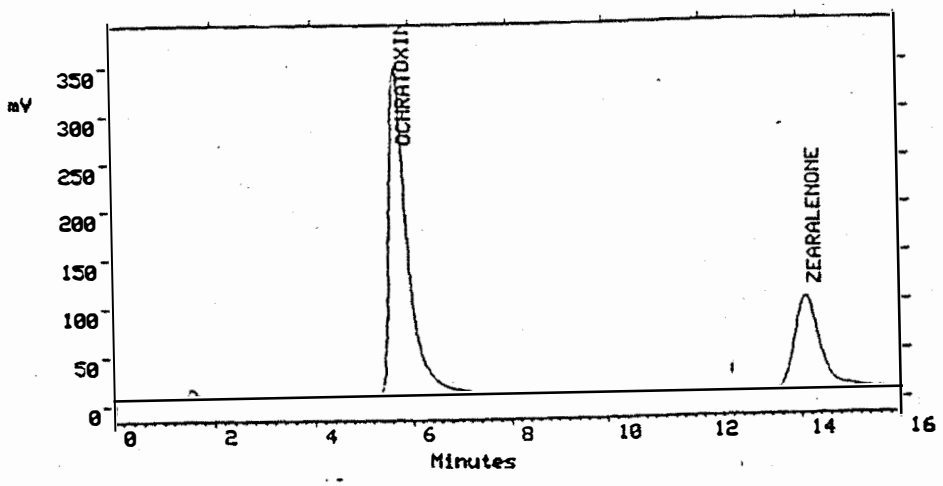

Figure 17. Chromatogram With Resolved Peaks for OA and ZON, 15 cm Column, 40% Organic Mobile Phase.

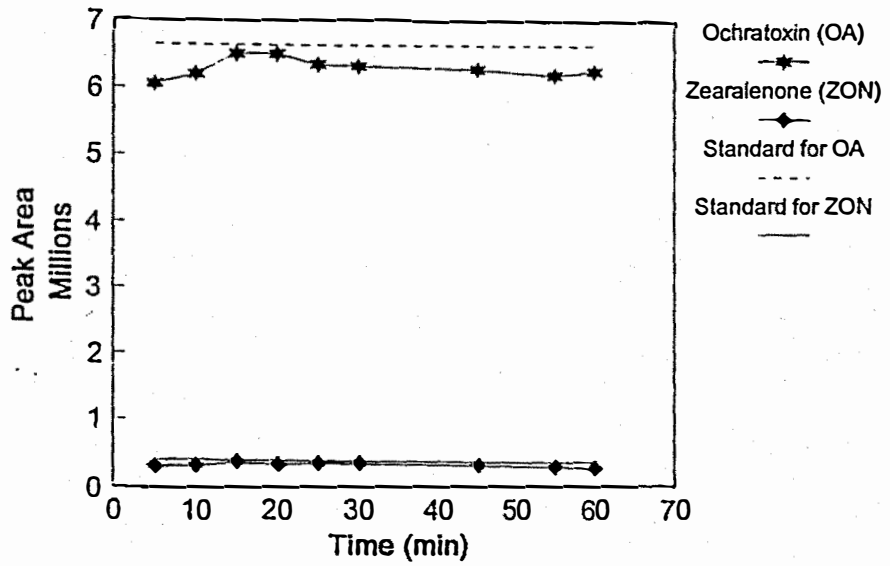

Figure 18. Extraction Efficiency for OA and ZON.

optimum period for extraction. Even though the Figure 18 shows 15 min to be optimum for extraction, 30 min was chosen because the study was conducted on a spiked blank matrix, but it would require a longer period for naturally contaminated matrix. When a matrix contains all the three mycotoxins shaking for 60 min would be preferred, since FB1 would be extracted completely only at 60 min. The peak areas for OA and ZON are not significantly changed at 60 min as seen from the graph. But when quantitating OA and ZON 30 min would be the optimum.

Choice of Solid Phase Extraction

The C18 (Waters Sep pak) cartridge was chosen for the solid phase extraction so that OA and ZON can be selectively extracted from the matrix. Using a SAX would require pH of the sample solution to be different than FB1, for OA and ZON to be absorbed by the cartridge and thus two different extraction procedures would be needed. Since a C18 clean up would require a neutral pH despite the analyte of interest, it would not pose a problem for simultaneous extraction. The extraction method was optimized based on the required steps when using a C18 cartridge. The cartridge should be washed with at least 10 mL of acetonitrile and 10 mL of water to thoroughly condition. The washing solution was chosen to be 1:5 acetonitrile: water, because higher proportions of acetonitrile resulted in loss of OA. The eluting solvent acetonitrile (2.5 mL) was sufficient to elute just OA. This does not elute ZON, thus a stronger eluent was the option. A 5% acetic acid in acetonitrile was sufficient to elute both OA and ZON with just 2.5 mL. Thus the solid phase extraction was designed and optimized.

Results

Standard Linearity

Standard solutions ranging from 1.25 ppb to 0.1 ppm for OA and 50 ppb to 5ppm for ZON were prepared using a 100 ppm stock. Three replicates of each concentration were run in a random order using the developed method. The results and their mean, standard deviation, coefficient of variation for each concentration are listed in Tables 8 and 9.

Table 8

Linearity Data for OA

Concentration (ppm)	Mean Peak Area	Standard Deviation	Co-efficient of Variation
0	0.00	0.00	none
0.00125	82771	5674	6.86
0.0025	191365	4771	2.49
0.005	436065	30848	7.07
0.01	922547	24807	2.69
0.02	2190512	54762	2.50
0.1	10102691	56297	0.5

Table 9

Linearity Data for ZON

Concentration (ppm)	Mean Peak Area	Standard Deviation	Co-efficient of Variation
0	0.00	0.00	none
0.025	87853	4843	5.51
0.05	201826	9659	4.79
0.125	609535	20485	3.36
0.5	1970377	116153	5.90
1.0	3202699	124134	3.88
2.5	8408261	292954	3.48
5.0	18426781	406933	2.26

Using the least-squares linear regression model ($y = mx + b$), a plot of the average result for each solution versus the theoretical concentration was obtained (Figures 19 and 20). The CVs and standard deviations for each concentration were sufficiently low. The data were analyzed and the range 2.5 ppb - 0.1 ppm for OA and 25 ppb - 1 ppm for ZON was statistically found to be linear.

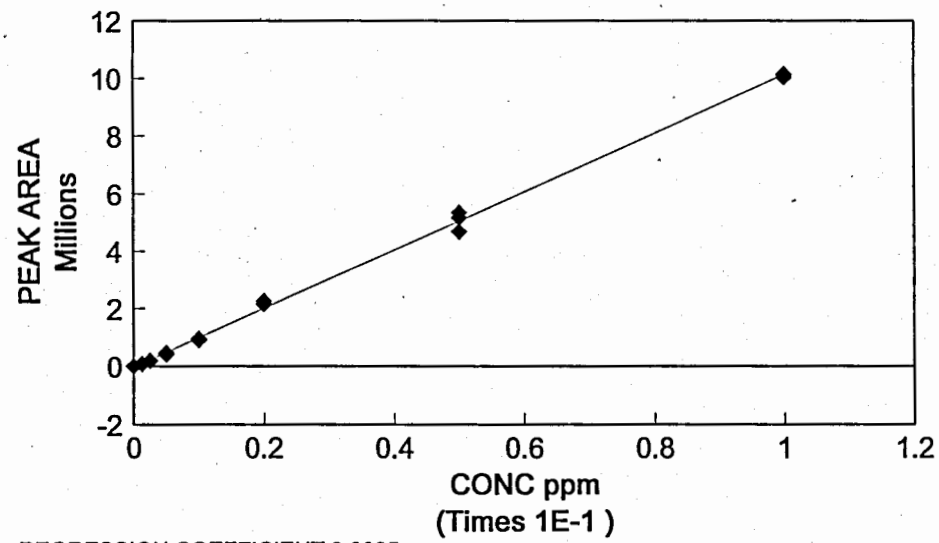

Figure 19. Linearity Plot for OA.

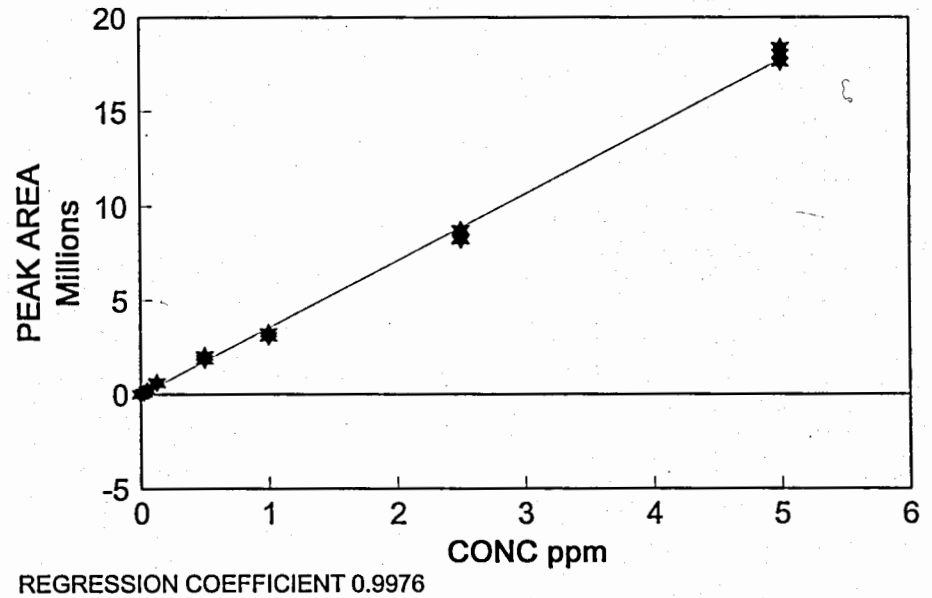

Figure 20. Linearity Plot for ZON.

Residuals

The residuals were also calculated. The residual plot for OA and ZON are shown in Figures 21 and 22. The deviation from the predicted value is minimal from 0 - 0.1 ppm for OA and 0 - 5.0 ppm for ZON, while it increases with increasing concentration. Even though higher concentrations of OA and ZON were studied for linearity, since there were more deviations at higher concentrations, they were not fit to be in the linear range. Hence it would be necessary to dilute samples with higher concentrations of OA and ZON into the linear range for an accurate quantitaion.

Limit of Reliable Measurement

This is the minimum level of analyte which a method can determine with a 95% confidence interval. Seven (n) replicates of the analyte solutions were prepared at a concentration of 1.0 ppb for OA and 30 ppb for ZON. The data were analyzed to determine mean, standard deviation(s), and coefficient of variation (Tables 10 and 11). The experimental "t" statistic was calculated which compares the mean of the data(x) and the theoretical mean(u). The Student's - t critical value was obtained from the t-distribution table for a 95% confidence interval and for 7 measurements. Since the experimental t was less than the Student's t value, the 0.001 ppm for OA and 0.03 ppm for ZON were statistically approved to be the limit of reliable measurement. Even though the linearity study included the 25 ppb level for ZON, the experimental t-value was higher than the Student t-value and was rejected from being the LRM.

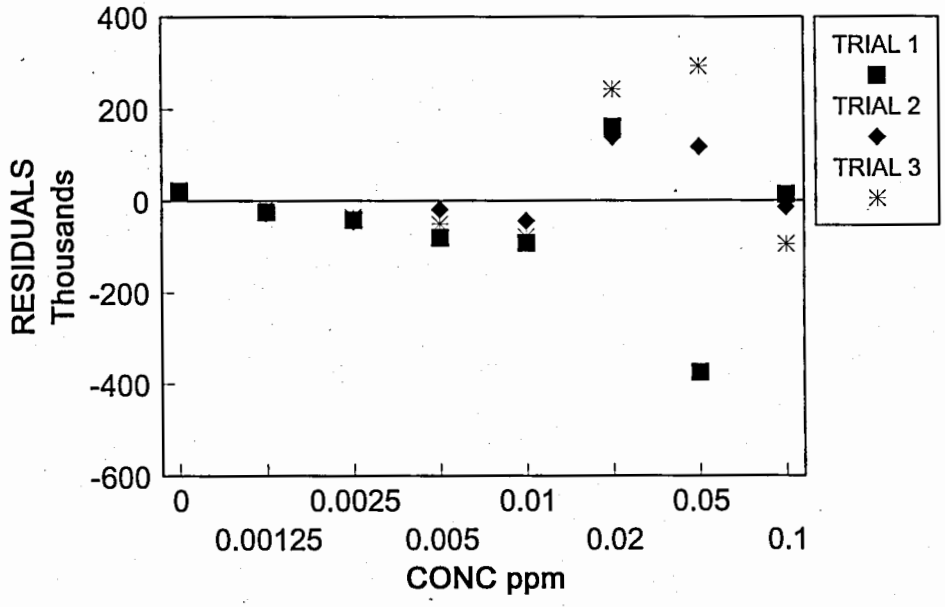

Figure 21. Residuals Plot for OA.

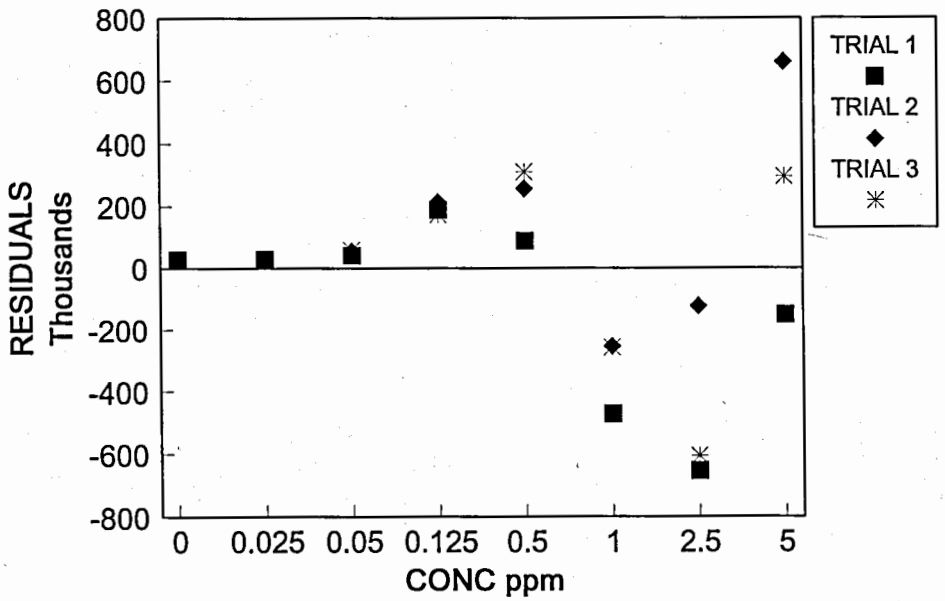

Figure 22. Residuals for ZON.

Table 10

LRM Data for OA

Data for LRM	
Mean	0.00109
Standard Deviation	0.0001
Coefficient of Variation	9.95
Number of replicates	7
Theoretical Mean	0.001
Experimental t-value	2.343
Student t-value	2.447

Accuracy

Accuracy was determined by spiking standard solutions of OA and ZON into blank corn matrix (Tables 12 and 13). The corn matrix was analyzed and no peak was observed at the retention times of OA and ZON (Figure 23). Standard solutions were spiked into the 25 g corn matrix in a 100 mL of acetonitrile : water (1:1). The spike levels were such that the concentration of OA in the final eluate was 0.0016 ppm, 0.008 ppm, and 0.08 ppm and of ZON in the final eluate was 0.04 ppm, 0.2 ppm, and 4 ppm.

Table 11

LRM Data for ZON

Data for LRM	
Mean	0.0332
Standard Deviation	0.004
Coefficient of Variation	12.23
Number of replicates	7
Theoretical Mean	0.03
Experimental t-value	2.135
Student t-value	2.447

The spiked samples were analyzed and the accuracy was determined by comparing the concentration equivalent for the observed peak area against the expected theoretical concentration calculated from the amount spiked. The % recovery at the 3 levels of concentrations are higher than 90. Recoveries greater than 100 % are not observed here as in FB1. It may be because OA and ZON are less complicated to analyze and no derivatization is required. Thus the recovery of the method was tested.

Table 12

Accuracy Data for OA

Amount Spiked (ppm)	Recovery(%)
0.0016	97
0.008	98
0.08	96

Table 13

Accuracy Data for ZON

Amount Spiked (ppm)	Recovery(%)
0.04	92
0.2	97
4	99

Precision

Matrices which had OA and ZON were not found and hence spiking standards into blank corn matrix was followed to do a precision study (Figure 24). A blank corn matrix the same as the one used for accuracy was utilized. Three different levels of

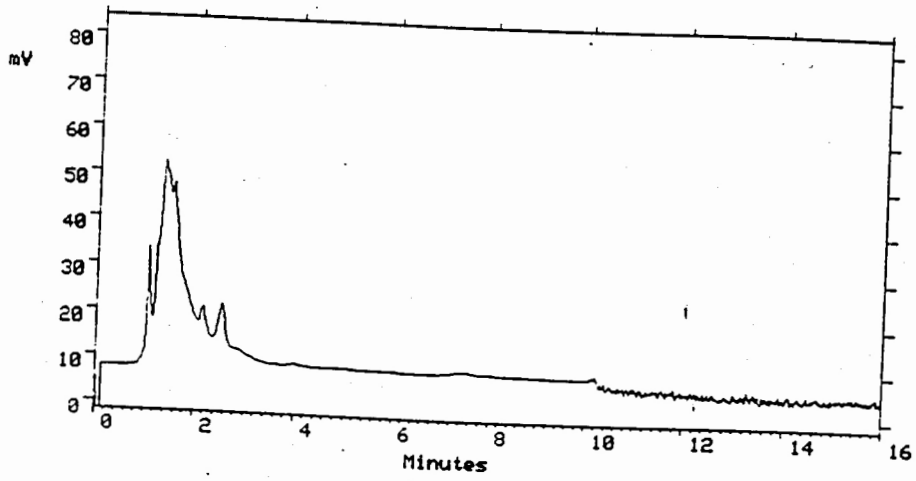

Figure 23. Chromatogram of a OA and ZON Free Corn Sample.

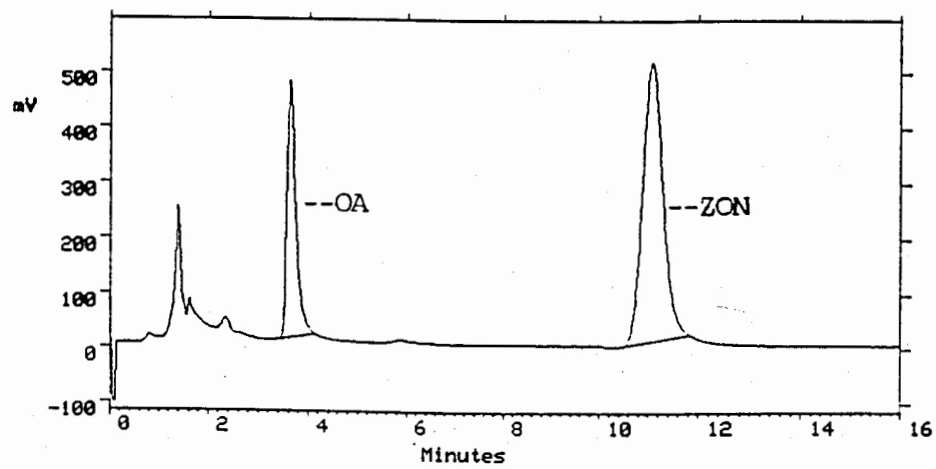

Figure 24. Chromatogram of a OA and ZON Spiked Corn Sample.

spiking was done, at 0, 50 and 100% of the linear range. The samples were prepared and analyzed as mentioned in the methodology section of the report. Standards at all the three levels were analyzed along with samples everyday. Triplicates of each sample were analyzed each day for three days spread over a weeks time. Concentrations (μg/g) of OA and ZON were calculated from the peak area measurements of the samples.

Table 14

Statistical Analysis of Precision Data for OA

Matrix	Mean (μg/g)	Standard Deviation	Variance	Coefficient of Variation (%)	Minimum Difference to Detect (μg/g)
1	0.350	0.0561	0.00315	16.0	0.160
2	0.026	0.0043	0.00002	16.4	0.017
3	0.008	0.0006	0.00000	7.5	0.0034

The mean, standard deviation, variance, coefficient of variation for all three matrices for all three days combined are listed in Table 14 and Table 15. The coefficient of variations were in the acceptable range for the three days over 3 days in both OA and ZON.

The daily averages and daily variances for each matrix were calculated and the results showed that the daily averages were different at lower and mid levels of the linear range for OA and were different only at the mid level of the linear range for ZON. The calculated F_{max} and tabulated F_{max} are listed in Table 16 and Table 17.

Table 15

Statistical Analysis of Precision Data for ZON

Matrix	Mean (µg/g)	Standard Deviation	Variance	Coefficient of Variation (%)	Minimum Difference to Detect (µg/g)
1	20.278	2.0873	4.35694	10.3	5.924
2	2.089	0.3444	0.11861	16.5	1.536
3	0.219	0.0145	0.00021	6.6	0.050

Table 16

Comparison of Variances for OA

Matrix	Calculated F_{max} [Tabulated $F_{max}(\alpha = 0.05)$ =202.000]	With-in Day Variance (%)	Between Day Variance (%)
1	13.000	100	0
2	4.333	32	68
3	21.000	12	88

Since the calculated F_{max} are lesser than tabulated F_{max}, the daily variances for all the matrices were not significantly different for both OA and ZON. Of the % of total variance components, the between day variance were very low for the highest level

of the linear range in both OA and ZON, while they were acceptable in the case of the other two levels of the linear range.

Table 17

Comparison of Variances for ZON

Matrix	Calculated F_{max} [Tabulated $F_{max}(\alpha = 0.05)$ = 202.000]	With-in Day Variance (%)	Between Day Variance (%)
1	28.986	100	0
2	19.000	16	84
3	0	62	38

These variations might have been due to the fact that the precision studies were not performed using naturally contaminated matrices, and spiking into an empty matrix could not exactly simulate such samples. The variations in the daily averages could be minimized with careful analysis. It is difficult to pinpoint just one aspect of the assay which might lead to the variability because there are so many parameters that play a main role in obtaining the final result, and every step of the assay should be followed with care.

Thus the simultaneous assay for the determination of OA and ZON was successfully developed. This method could be used to determine both the toxins in one extraction from the contaminated corn matrix. The linear range, LRM, accuracy, and

precision for both toxins using this method were studied simultaneously and the results were statistically analyzed and validated.

CHAPTER V

CONCLUSION

The extraction efficiency study, efficient SAX clean up, and method automation for fumonisin are the salient features. Separation for Ochratoxin and ZON on a single system with simple mobile phase, in a single run and short analysis time was achieved. The simultaneous extraction of OA and ZON was developed. This thesis also presents the validation of these methods according to the AOAC method validation procedures, except for the collaborative study.

Our objective of developing a simple and cost effective assay was accomplished. Instead of using different sets of instruments for each toxin, a single HPLC design is used to quantitate 3 different mycotoxins. Also the method is reliable enough to avoid any further confirmations as required for the ELISA method.

The validation procedures for these methods were done using corn as the matrix. Even though corn is one of the major crops in which these mycotoxins occur, there are other crops like wheat, barley, oats in which OA and ZON may occur too. Hence the extraction efficiency and reproducibilities for these methods need to be tested using these matrices. Even though it was possible to analyze OA and ZON simultaneously, FB1 couldn't be analyzed at the same time. Further extensive method development procedures might solve this limitation. But this might require total alterations of the conditions like changing the column length and nature of the column.

The existing procedures for FB1 using C18 clean up must be modified to improve the recovery and reproducibility. Use of Sep pak vacuum cartridges instead of Sep pak classic cartridges might reduce the time required and the cumbersome nature of SPE. If the C18 procedure is made to fit the extraction needs for FB1, then all the 3 toxins FB1, OA and ZON can be simultaneously extracted and analyzed using the method.

Another possibility would be to compare the results of the ELISA techniques and the HPLC techniques for samples. The ELISA tests claim to be rapid, and hence employ extraction procedures varying from 3-5 minutes in a methanol solvent which is less stronger than acetonitrile in its extractability. Thus there may not be a complete extraction of the analytes of interest in these studies. A comparison study of the two methods would reveal the discrepancies in the existing procedures. A collaborative study of the developed methods might be a good way of testing the reliability of these methods.

Appendix A

Raw Data From the Method Validation Studies for the Mycotoxins

Data From Linearity Study

Table 1:

For FB1

Concentration (ppm)	Peak Area
0	1957
	1692
	1785
0.005	22289
	25349
	21189
0.01	36016
	37665
	44741
0.025	77405
	72857
	65632
0.075	156524
	176744
	164991
0.1	251032
	293873
	277443
0.25	485866
	445338
	455042
0.5	1376729
	1441148
	1228022

Table 1 - Continued

1.0	2473333
	2799742
	2489627
2.0	5211908
	5394898
	5601103
3.0	9040036
	9121165
	9748235
5.0	13795642
	14425249
	13506066

Table 2:

For OA

Concentration (ppm)	Peak Area
0	0
	0
	0
0.00125	80976
	78212
	89126
0.0025	190559
	187048
	196489
0.005	405463
	467155
	435578
0.01	902194
	950181
	915268
0.02	2170748
	2148376
	2252413
0.05	4681133
	5174016
	5348963
0.1	10147950
	10120473
	10039650

Table 3:

For ZON

Concentration (ppm)	Peak Area
0	0
	0
	0
0.025	90492
	90805
	82263
0.05	191000
	204914
	209564
0.125	606488
	631374
	590744
0.5	1839789
	2009183
	2062161
1	3059380
	3276358
	3272361
2.5	8216050
	8745435
	8263300
5	17614330
	18426781
	18062113

LRM Data

For FB1:

Table 4

Replicates	Concentration (ppm)
1	0.00606
2	0.00717
3	0.00566
4	0.00378
5	0.00433
6	0.00536
7	0.00701

For OA:

Table 5

Replicates	Concentration (ppm)
1	0.001013
2	0.000986
3	0.001093
4	0.001223
5	0.001266
6	0.001086
7	0.00101

For ZON

Table 6:

Replicates	Concentration (ppm)
1	0.041
2	0.031
3	0.037
4	0.031
5	0.031
6	0.031
7	0.031

Precision Data

For FB1:

Table 7

Day	Matrix 1	Matrix 2	Matrix 3
1	0.50	0.16	0.35
	0.41	0.15	0.36
	0.38	0.10	0.38
2	0.37	0.12	0.36
	0.48	0.13	0.41
	0.48	0.13	0.36
3	0.51	0.10	0.34
	0.48	0.10	0.41
	0.52	0.13	0.34

For OA:

Table 8

Day	Matrix 1	Matrix 2	Matrix 3
1	0.31	0.025	0.0071
	0.29	0.030	0.0076
	0.35	0.032	0.0072
2	0.46	0.029	0.0075
	0.39	0.026	0.0076
	0.28	0.029	0.0072
3	0.36	0.020	0.0086
	0.33	0.020	0.0082
	0.38	0.024	0.0087

For ZON:

Table 9

Day	Matrix 1	Matrix 2	Matrix 3
1	19.3	1.8	0.20
	28.5	2.3	0.20
	19.4	2.1	0.22
2	22.9	2.4	0.22
	22.9	2.5	0.22
	18.3	2.5	0.22
3	22.3	1.7	0.25
	17.3	1.6	0.22
	21.3	1.7	0.22

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