1982

New analytical techniques for mycotoxins in complex organic matrices

Merlin K. L. Bicking
Iowa State University

INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure you of complete continuity.

2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame. If copyrighted materials were deleted you will find a target note listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in "sectioning" the material. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.

University
Microfilms
International

300 N. ZEEB RD., ANN ARBOR, MI 48106

Bicking, Merlin K. L.

NEW ANALYTICAL TECHNIQUES FOR MYCOTOXINS IN COMPLEX ORGANIC MATRICES

Iowa State University PH.D. 1982

New analytical techniques for mycotoxins in complex organic matrices

by

Merlin K. L. Bicking

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Chemistry
Major: Chemistry (Organic - Analytical)

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1982

TABLE OF CONTENTS

INTRODUCTION

The Ames Solid Waste Recovery System (ASWRS) accepts trash and garbage and processes it into Refuse Derived Fuel (RDF), which is suitable for combustion. This processing generates much airborne material of unknown danger to the public health. Studies of airborne bacteria levels in the processing plant were found to be orders of magnitude above ambient locations outside the plant. Fungal levels were also found to be higher than ambient levels. This work was undertaken to evaluate the chemical hazard posed by the high levels of fungi.

Mycotoxins are defined as toxic compounds produced by fungal contaminants. The toxicity syndromes resulting from ingestion of such contaminants have been termed mycotoxicoses. Although many toxic fungal metabolites are known, relatively few are known to have caused natural outbreaks of myctoxicoses. The principal fungal species involved in reported mycotoxicoses are Aspergillus, Penicillium, and Fusarium [1]. These organisms are considered "imperfect fungi" because they only reproduce asexually [2].

Mycotoxins were first identified as the cause of the mysterious "Turkey-X" disease in England. A peanut meal contaminated with Aspergillus flavus contained a toxic component [3]. The toxic compounds subsequently isolated were

called, appropriately, aflatoxins. Many other mycotoxins have since been isolated and identified.

Table I presents a summary of data obtained at the ASWRS during the period July - September, 1978. These fungal levels are two to three orders of magnitude above those at locations outside the plant. Such data are indicative of a possible mycotoxin contamination problem. Thus, the present study was undertaken. Initially, existing analytical techniques were found to be inadequate to identify and quantify the mycotoxins which were present.

This dissertation describes new analytical techniques developed to analyze RDF for mycotoxins. In addition, the techniques were found to be applicable to a variety of other substrates where mycotoxin contamination is of interest. The chapters in Part I chronicle the development of each individual step in the analytical scheme, along with pertinent background information and a discussion of the results. Part II describes the isolation and identification of the spore pigment in the aflatoxin species Aspergillus flavus.

Table I

Occurrence of fungi at the Ames Solid Waste Recovery System.
July - September 1978

	CFU/m^3[a]
Aspergillus flavus	22000
A. fumigatus	53000
A. niger	13000
A. ochraceus	360
A. terreus	600
A. niveus	240
Penicillium spp[b]	4800
Fusarium spp[b]	1200

[a]Colony Forming Units per m^3.

[b]Unidentified species.

PART I. DEVELOPMENT OF AN ANALYTICAL METHOD FOR MYCOTOXINS

INTRODUCTION

The structures of the mycotoxins pertinent to this study are given in Figure 1. In addition, the fungal species known to produce each mycotoxin are listed [4].

Aflatoxins

The names of the four highly carcinogenic aflatoxins, B1, B2, G1, and G2, are derived from the color fluorescence under long-wave ultraviolet light. The "Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC)" lists separate methods for analyzing peanuts, cocoa beans, soybeans, eggs, corn, cottonseed products, green coffee, and pistachio nuts [5]. These methods are subjected to rigorous collaborative study and are reviewed each year.

Extraction has been done in chloroform, methanol-water, or acetone-water, depending on the substrate. Originally, detection was achieved by visual estimation of the fluorescent intensity on Thin Layer Chromatography (TLC) plates. However, this method rarely has produced better than 20-30% accuracy. A "minicolumn" packed with Florisil has been developed to provide a quick screening method [6]. More recently, High Performance Liquid Chromatography (HPLC)

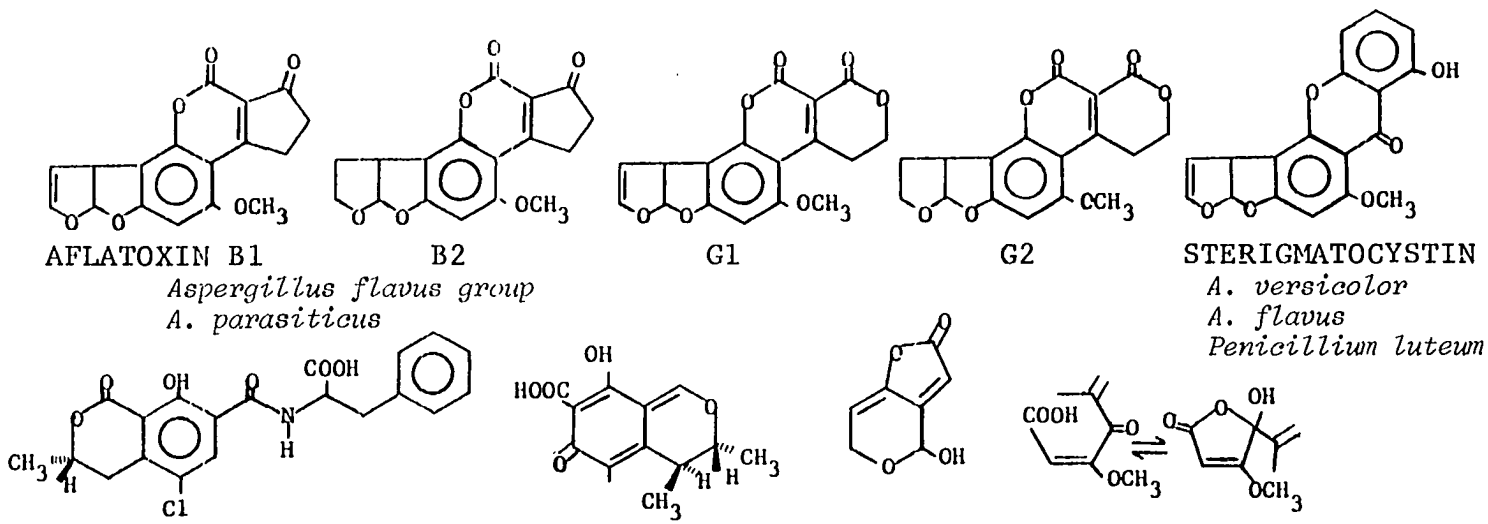


Figure 1. Structures of the nine mycotoxins of interest and the fungal species known to produce them

methods have been described. Detection limits of 5 ng with ultraviolet detection [7] and sub-ng levels with a novel silica gel-packed fluorescence flow cell [8] are reported. However, the fluorescence quantum efficiencies of aflatoxins are low in the solvent systems often used in normal phase HPLC [9] (Table II). Thus, reverse phase HPLC can provide pg detection limits [10]. This requires that B1 and G1 be converted to the more fluorescent B2a and G2a, respectively. These derivatives are hemiacetals formed by the acid-catalyzed hydration of the double bond in the terminal furan ring. Mass spectral data have also been used for detection and confirmation [11,12].

Lasers as excitation sources provide at least two orders of magnitude more photons that conventional sources. Thus, using a pulsed N_2 laser (337.1 nm) it is possible to take advantage of the absorption characteristics from aflatoxins and measure their fluorescence directly on a TLC plate [13]. However, this is the only reported application of laser light to TLC analysis of aflatoxins. Furthermore, the supporting equipment is crude and unsuitable for quantitative work. A quantitative detection of 0.2 ng aflatoxin B1 is reported. Solution detection limits of 7.8×10^{-12}M (2.5 ppt) have also been reported using a nitrogen laser as the excitation source [14]. Sub-pg detection limits have

Table II

Emission Maxima and Fluorescence Quantum Efficiencies (ϕ) of Aflatoxins in different solvents

	B1		B2		G1		G2	
	$100 \cdot \phi$	λ_{em} (nm)	$100 \cdot \phi$	λ_{em} (nm)	$100 \cdot \phi$	λ_{em} (nm)	$100 \cdot \phi$	λ_{em} (nm)
H_2O	1.3	450	70	450	1.8	475	67	475
C_6H_6	0.36	415	0.03	410	10	430	17	430
$CHCl_3$	0.29	415	0.30	410	30	430	36	430
CH_3OH	2.3	440	15	435	3.8	460	59	460

been reported with a novel laser fluorescence detector coupled with HPLC [15]. This unique detector design requires no optics and provides the lowest detection limits reported to date.

Sterigmatocystin

Sterigmatocystin is also a known carcinogen and one of its fungal sources, A. versicolor, is known to putrify animal and vegetable products in soil. Official methods for barley and wheat involve extraction with CH_3CN-4%KCl, followed by silica gel column chromatography. After TLC development, quantitation can be accomplished by observing directly a brick-red fluorescence or producing the more fluorescent yellow $AlCl_3$ derivative [5].

Other published methods use florisil for sample preparation [16] or HPLC and Exclusion Chromatography (EC) [17] to achieve 50 and 25 µg/kg detection limits, respectively. Recent work with TLC has shown 5 µg/kg detection limits [18].

At present, data documenting the natural occurrence of sterigmatocystin are rare [19]. It is questionable whether or not there is a significant health risk, but sterigmatocystin is presumed to be a biosynthetic precursor of aflatoxins [20] and therefore may also be present with the aflatoxins.

Patulin

Originally isolated as an antibiotic, patulin has since been shown to be mutagenic [5]. However, its toxicity to humans is unknown. It is common in apples and apple products and is extracted with ethylacetate. This extract is prepared with silica gel and analyzed by two-dimensional TLC using a fluorescent 3-methyl-2-benzothiazoline hydrazone hydrochloride derivative [5].

Two recent reports claim detection limits in fruit juices by TLC and HPLC of 20 and 5 ppb, respectively [21,22].

Ochratoxin A

Ochratoxin A causes kidney and liver damage. Samples are extracted from barley or green coffee with $CHCl_3$/0.1M H_3PO_4. This compound, a carboxylic acid, is separated from its esters and other matrix components on a column of $NaHCO_3$-diatomaceous earth. Analysis is by TLC, observing a blue-green fluorescence [5]. Solvent-related spectral data have been published [23], along with HPLC methods [24].

Citrinin

Citrinin is found on rice, barley, and dried fish. There are no official analytical methods, but new techniques are being developed. Recent reviews [19,25] have stressed that quantitative extraction from natural substrates is the major analytical problem. Analysis is complicated by the fact that citrinin "streaks" with almost every TLC developing

solvent used. Published work reports TLC detection limits of 0.5 μg/g [26] and 0.3 ng by HPLC [27].

Penicillic Acid

Penicillic acid exists in two tautomeric forms. There are no official analytical methods. A fluorodensitometric assay on TLC plates using an NH_3 derivative has been published [28]. Also, 25 ng of the tetramethyl silyl derivative may be detected by Gas Chromatography (GC) [29]. Currently, two-dimensional TLC with a p-anisaldehyde spray reagent is recommended [19].

Multiple Mycotoxin Methods

Many multiple mycotoxin detection methods have been reported. Three recently published methods are representative [30-32]. All employ TLC for some form of quantitation. Table III lists the detection limits for these three methods and a very recent one [33]. This latter work reports significantly lower detection limits using High Performance Thin Layer Chromatography (HPTLC) and a novel multiple development technique. However, the equipment is somewhat specialized and only results for standard mixtures are evaluated. The method has not been applied to real samples.

Finally, Table III lists results from a method using Sephadex LH-20, which is separation by EC [34].

Table III

Detection limits for 5 multiple mycotoxin methods

	Detection Limits				
Reference	[30]	[31]	[32]	[33]	[34]
Mycotoxin	(μg/kg)	(ng)	(μg/kg)	(ng)	(μg/kg)
Aflatoxins	10	10	4-5	.005	5
Sterigmatocystin	40-60	1000	140	0.2	10
Patulin	--	100	750-800	0.2	50
Ochratoxin A	40-60	10	140	.005	10
Citrinin	80-200	100	600-750	.010	--
Penicillic Acid	--	1000	3400-3650	2.0	--

EXTRACTION OF THE SAMPLE

Introduction

Mycotoxins are usually extracted from mold-contaminated samples using official methods approved by the Association of Official Analytical Chemists (AOAC) [5]. A variety of organic solvents have been used, depending on the particular matrix being extracted. The extractions require either a Soxhlet extractor or wrist action shaker. After extraction, the matrix is removed by filtration and the solvent is evaporated. The residue is used for the next step in the analytical scheme.

Collection of the Sample

Initially, dust samples were taken from the floor immediately above the processing area of the solid waste processing plant. It was assumed that this material had settled out from the air. This assumption seemed reasonable since the area in the processing area was visibly "dusty", and all surfaces (handrails, walkways, etc.) were coated with a thick layer of dust.

Later samples were obtained directly from the air using a high volume air sampler. Particulates were trapped on an 8 x 10 inch quartz fiber filter. In a typical experiment, the air sampler was placed on a walkway immediately above the

processing area. Material was collected on quartz fiber filters. The sampler operated at approximately 1400 L min^{-1} for 8 hours and filters were changed every 2 hours. The gray material could be easily removed from the filter, giving 4.7 g for the sample collected (3/5/82). The sample appeared similar to the blank, a sample of dust found in the apartment of a typical midwestern graduate student.

Extraction of the Sample

Initial samples were placed in a 43 x 123 mm cellulose thimble and extracted 6 hours with chloroform ($CHCl_3$) in a Soxhlet apparatus (ca. 6 cycles/hour). A small amount of colored material was still being removed after 6 hours, but this did not represent a significant amount of material. The $CHCl_3$ appeared dark brown or black after filtering and dilution (1 g sample/10 mL). The extracted material represented less than 10% by weight of the original sample.

Unfortunately, this extract fluoresced blue, as do the aflatoxins. The levels of this background fluorescence remained high through the remaining steps in the analytical method, preventing analysis of aflatoxins.

Other common procedures [5] for extracting aflatoxins include $CHCl_3/H_2O$ (100+10), methanol/H_2O (55+45), and acetone/H_2O (85+15). The $CHCl_3/H_2O$ mixture gave a brown extract, much like that described above for $CHCl_3$. The methanol mixture gave a red-orange solution, while the acetone

mixture was green. All solutions fluoresced blue, but the levels were considerably less for methanol and acetone.

Background levels were acceptable for either methanol or acetone mixtures, but interfering peaks prevented analysis of all four aflatoxins. It was found that methanol, with no H_2O added, provided good extraction efficiency with few interferences.

The H_2O is apparently present to "release" the aflatoxins from the matrix. This is similar to deactivation of chromatographic absorbents with H_2O. A $CHCl_3/H_2O$ mixture was found to be superior to either $CHCl_3$ or methanol for extracting corn or peanut meal, but methanol was chosen for RDF samples.

PRECIPITATION OF INTERFERING MATERIALS IN THE SAMPLE EXTRACT

Precipitation of interfering components in a particular matrix extract has been used often [5]. However, in such cases the matrix and its components were well-defined, allowing specific chemical reactions to be used. Virtually nothing is known about the chemical composition of dust generated in an RDF processing facility. Nuclear Magnetic Resonance (NMR) spectra of RDF extracts show only aliphatic hydrogens. The fluorescing species are present at low levels, and are confirmed by the UV spectrum, showing steadily increasing absorbance at shorter wavelengths. Only very general comments can be made about the composition of the extracts.

Sample extracts were injected onto a Styragel column as the next step in the analytical scheme. However, with either chloroform ($CHCl_3$) or methanol (CH_3OH) extracts, some material precipitated near the inlet to the column. This colored material could not be eluted using the normal mobile phase 30% acetonitrile/chloroform. This could not be explained by the solvent effects proposed for that system (discussed in next chapter). The column was not overloaded under these conditions.

The problem could be explained in terms of simple solubility. The extract, in $CHCl_3$, was injected into a system

consisting of 30% $CH_3CN/CHCl_3$. Thorough mixing of sample and mobile phase would occur when the sample began to pass through the packed column. Any compounds not soluble in 30% $CH_3CN/CHCl_3$ would, by necessity, precipitate onto the surface of the stationary phase. For the $CHCl_3$ extract, this would be the less-polar components, since 30% $CH_3CN/CHCl_3$ is a polar mixture. For the CH_3OH extract, polar components would precipitate from the less polar mobile phase.

To eliminate this problem, all extracts were filtered and the solvent removed using a rotary evaporator. The residue was dissolved in 30% $CH_3CN/CHCl_3$ and the insoluble residue removed by filtration. For the $CHCl_3$ extract, a considerable amount of brown material would not dissolve. A red-brown oily residue remained from the CH_3OH extract, and exhibited a bright green fluorescence.

It is unlikely that any aflatoxins are lost in this step. First, sufficient time was allowed for the mobile phase to extract the aflatoxins from the residue before filtering. Second, the aflatoxins are known to be readily soluble in 30% $CH_3CN/CHCl_3$ because standards chromatograph readily in this mobile phase (i.e., chromatographic retention is a measure of solubility in this system).

The effect of this precipitation step is illustrated in Figure 2, which shows the UV detector response due to material eluting from the Styragel column. Figure 2a was

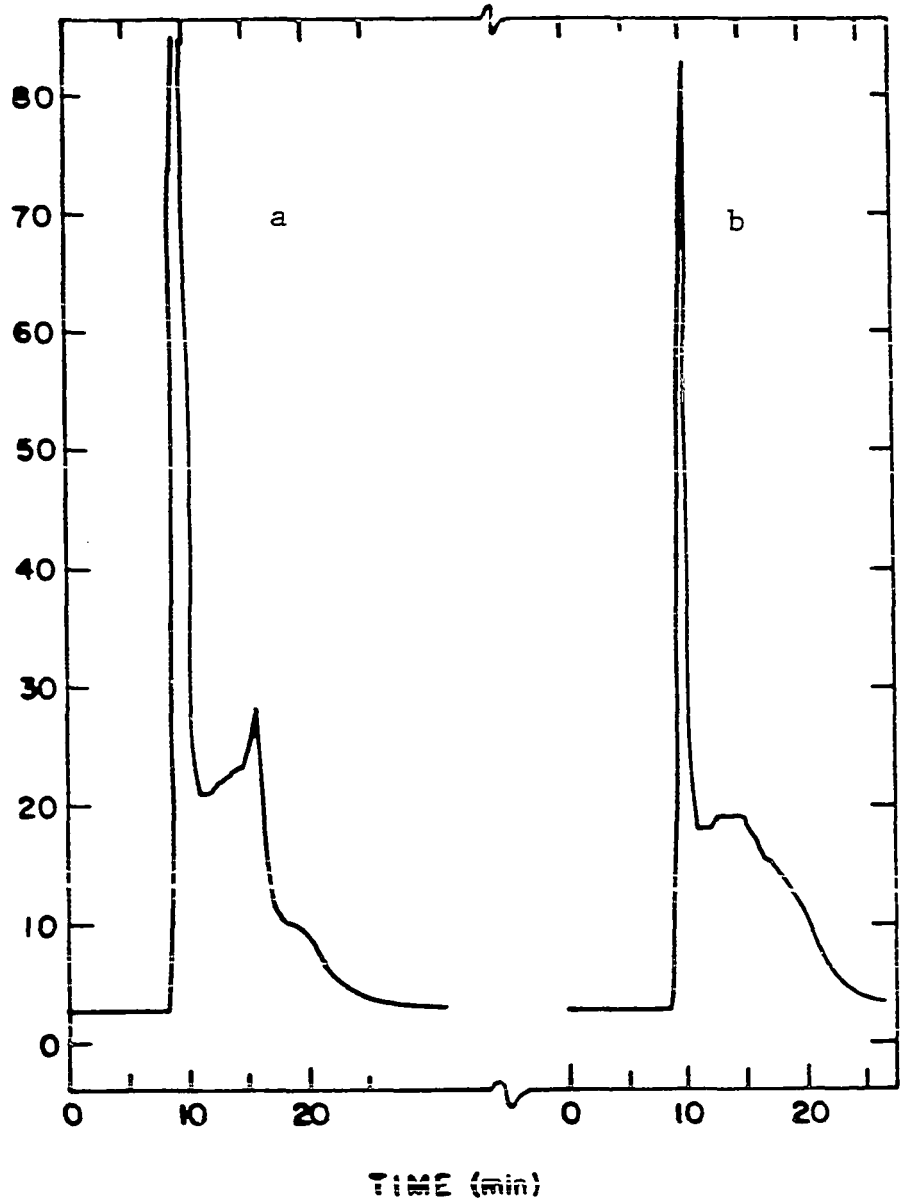


Figure 2. UV detector response of an RDF extract eluting from 1.27x20.4 cm Styragel column: (a) extract dissolved in $CHCl_3$, (b) extract dissolved in 30% $CH_3CN/CHCl_3$

produced by injection of the $CHCl_3$ extract directly onto the column. Figure 2b was produced by injection of the 30% $CH_3CN/CHCl_3$ soluble material. In this system, non-polar components elute later. Note that, after precipitation, some late eluting (i.e., non-polar) material has been removed. Note also that the amount of material eluting at 10 minutes has decreased. This is not due to dilution of the sample, but indicates that some components have been removed. However, the amount of material eluting with the more polar aflatoxins (11-14.5 min. fraction) shows less change.

In summary, a simplification of the sample matrix and extended column life can result by dissolving the sample in the mobile phase before injection.

EXCLUSION CHROMATOGRAPHY ON STYRAGEL

Introduction

Exclusion Chromatography (EC), also known as Gel Permeation Chromatography, has been a well established technique for the analysis of molecules based on their molecular size. Several mechanisms have been proposed to explain the separation process, including restricted diffusion, steric exclusion, and thermodynamic properties only [35]. Steric exclusion has been accepted generally as the most descriptive. The "gels" used as chromatographic stationary phases have a semi-solid consistency formed by the interaction of the gel forming compound with a solvating medium [36].

However, many examples of "anomalous retentions" are known that do not follow a size exclusion pattern. For example, Sephadex, a dextran-based gel, has been studied extensively.

Effects of temperature [37] on retention, pH [38], resonance energy [39], and hydrophobic character [40,41] have been reported. A wide variety of functional groups has been examined for adsorption, partitioning, and other effects [42-44].

Non-exclusion effects have been reported with porous poly(styrene)-divinyl benzene (PS-DVB) gels. In some cases, only weak interactions are proposed [45-47] while other work

proposes more specific mechanisms, including adsorption [48-50], partitioning [51], and solvent-solute hydrogen-bonding [52-54]. An empirical approach to correct for changes in molar volume due to the presence of various functional groups has also been employed [55]. Other parameters have been considered in attempts to predict elution orders [56-59]. It seems likely, therefore, that a single calibration curve (elution vs. some size parameter) is not possible for a wide variety of functional groups [60]. A survey of non-exclusion effects is given in two recent reviews on exclusion chromatography [61,62].

The Hildebrand solubility parameter, δ, is a measure of solvent "polarity". It has been used to explain solvent-gel and solvent-solute interactions [51,63,64]. However, a clear, concise explanation for the role of the solvent did not evolve. This work considers the change in retention of a series of mycotoxins and test solutes as a function of the total solubility parameter. From these data, a more unified interpretation of solvent-related non-exclusion effects is proposed. A better understanding of anomalous retentions in exclusion chromatography has allowed more meaningful decisions to be made regarding the separation process. This has resulted in improved separations in real samples.

The Role of the Solvent

With few exceptions, most workers have ignored a fundamental interaction between solvent and polymer molecules. Dissolution of a polymer is governed by equation 1.

$$\Delta G = \Delta H - T\Delta S \qquad (1)$$

Since dissolution occurs with a large increase in entropy, ΔH determines the sign of ΔG. The heat of mixing can be shown to be dependent on $(\delta_1 - \delta_2)^2$, where δ_1, and δ_2, are the solubility parameters for solvent and polymer. Thus, to assure solution, $(\delta_1 - \delta_2)^2$ should be as small as possible (preferably zero). That is, two substances will be most miscible when their δ values are nearly equal. Similarly, any cross-linked polymer will be most soluble in a solvent with the same δ value, and will show the greatest degree of swelling in that solvent [65]. Under these conditions, a cross-linked polymer swells to form a kind of stationary phase "sponge" of solvent and gel.

Recently, it has been proposed that the separation mechanism in exclusion chromatography is a function of the relative polarity of the solvent. By varying the solvent polarity, one chould change the elution behavior from a reverse-phase to a normal-phase system. Solubility parameters were used to indicate solvent "polarity", relative to that of the stationary gel phase.

When $\delta_{solvent} = \delta_{gel}$, the heat of mixing is nil, and "solution" is assured on the basis of entropy considerations mentioned earlier. Such a solution resembles a liquid-liquid partition chromatographic system where the mobile phase and stationary phase (solvent in swollen gel) are identical. Separations should be primarily size exclusion, since there are no preferential interactions between the two phases.

If the δ-value (polarity) of the mobile phase is changed by addition of a modifier, the stationary phase should retain the original solvent, based on the thermodynamic considerations mentioned above. Now, a true liquid-liquid partition system exists between the original gel stationary phase sponge and a modified mobile phase. A solute molecule's retention would be further influenced by its distribution ratio between the two phases. The nature of the modifier would determine the type of partition; i.e., normal-phase when $\delta_{mobile} < \delta_{stationary}$, and reverse-phase when $\delta_{mobile} > \delta_{stationary}$.

When the base solvent does not have a δ-value similar to that of the gel, the strength of the solvent-gel interactions is low and swelling occurs to a smaller extent. Hence, there is no stationary phase sponge, but more likely a layer of sorbed solvent molecules on the gel. The mechanism now involves sorption rather than partition, a

distinction that is often overlooked. The preceding discussion is in agreement with that in references [66] and [67].

Choice of Stationary Phase and Solvent Systems

References [66] and [67] use PS-DVB gels as a stationary phase. Styragel, a similar material, was used for this study. It was designed for the analysis of low molecular weight materials (M.W. <500). This was contrary to the large pore gels used in other studies, but was considered necessary here. The analysis of low molecular weight solutes requires such gels, and solvent effects on them should be observed directly.

For PS-DVB copolymers, $\delta = 9.1$ [65]. A review of various solvents [68] indicates three with similar δ-values that should function well for exclusion chromatography, viz. chloroform ($CHCl_3$) (δ=9.3), benzene (δ=9.2), and tetrahydrofuran (THF) (δ=9.1). Coincidentally, these solvents are commonly used as EC solvents, but usually from empirical considerations (i.e., they work). Toluene also has an acceptable δ-value (8.9), but was not studied here.

Tetrahydrofuran has been shown to be very reactive chromatographically toward hydrogen-bonding molecules [53], which is not desirable for this study. It was also not compatible with the chromatographic system used, and consequently was not studied as a base solvent.

Other work on solvent effects used alcohols as modifiers [66,67]. However, solubility parameter theory breaks down for strongly hydrogen-bonding solvents [69]. Thus, alcohols should be avoided as solvents.

Obviously, the total solubility parameter value is only a general indicator of the intermolecular interactions occurring in solution. More specific interactions have been identified, assigned solubility parameter values, and tabulated for a variety of solvents [70,71]. The five specific solubility parameter values are disperion, δ_d, orientation, δ_o, induction, δ_{in}, proton-donor, δ_a, and proton-acceptor, δ_b. An expanded treatment of the solubility parameter has been reported which applies these specific interactions to chromatography [68].

Since the purpose of this study is to investigate the role of solvent polarity, particular attention must be paid to the above interactions. It is well-established that a mobile phase elution series based solely on δ is inappropriate [70,71]. A binary solvent system, however, will provide a gradual change in solvent interactions. In addition, it is desirable to measure only one "solvent" variable at a time, with all others kept constant, or nearly so.

Thus, in order to measure a retention change as a function of the total solubility parameter, the other specific solubility parameters (δ_d, δ_{in}, δ_o, δ_a, δ_b) must not

change drastically as δ changes. This can be accomplished by a judicious choice of solvent modifiers. Thus, the solvent binary systems chosen are: chloroform/1-chloropropane (ClPr), chloroform/acetonitrile (CH_3CN), benzene (C_6H_6)/ cyclohexane (C_6H_{12}), benzene/acetonitrile. Table IV lists the differences in δ values for these solvents. Some compromises have been made to accommodate UV absorbance measurements. The problem is most severe for the polar modifier, but the difference in δ-values is also greater. Note that the minus signs are important to the relevant equations, which will be discussed later.

Nine mycotoxins and a series of test solutes were chosen to provide a wide variety of functional groups and suitable absorption at 254 nm. The test solutes chosen were phenol, benzene, toluene, pyridine, 2-butanone (methyl ethyl ketone), nitrobenzene, and acetone.

The Chromatographic System

The mobile phase was originally delivered by a peristaltic pump. However, the irreproducibility of the flow rate necessitated substituting a constant volume piston pump. A low pressure pulse dampener was used to reduce the flow variations caused by a single-piston pump.

Samples were injected with a Teflon® loop injector. The volume of the "100 μL loop" was calibrated by filling

Table IV. Differences in Solubility Parameter Values for Binary Solvent Systems

k/j	$(\delta^2)^j-(\delta^2)^k$	$\delta^j - \delta^k$				
		δ_d	δ_o	δ_{in}	δ_a	δ_b
$CHCl_3/PrCl$	-15.9	0.8	0.1	-0.1	6.5	-0.2
$CHCl_3/CH_3CN$	59.9	-1.6	5.2	2.3	-6.5	3.3
C_6H_6/C_6H_{12}	-17.4	1.0	0	0	0	0.6
C_6H_6/CH_3CN	61.8	-2.7	8.2	2.8	0	3.2

it with an I_2 solution of known absorbance and pumping the solution from the loop into a volumetric flask. After dilution, the absorbance of the solution was measured. From the dilution factor obtained, the volume of the loop could be calculated. The "100 µL loop" was found actually to inject 190 µL. The 500 µL loop injected 630 µL.

The chromatographic columns were constructed of borosilicate glass, 12.7 mm inner diameter. Each column contained a movable bed support at each end for adjusting the bed height of the stationary phase. This allowed a change in column length, without repacking, whenever the stationary phase changed in volume.

The ultraviolet absorption detector contained a 2-mm, 8 µL semipreparative flow cell and could be operated at either 254, 280, 340, or 365 nm. Wherever possible, standard Cheminert® (1/4-28 NF) fittings were used. Detector responce was monitored with a strip chart recorder.

Figure 3 presents data for the effective plate height (HEEP) vs. the linear velocity (µ). Linear velocity and HEEP are described by equations 2, 3, and 4,

$$\mu = L/\ t_M \tag{2}$$

$$HEEP = L/N \tag{3}$$

$$N = 5.545\left(\frac{t_R'}{w_h}\right)^2 \tag{4}$$

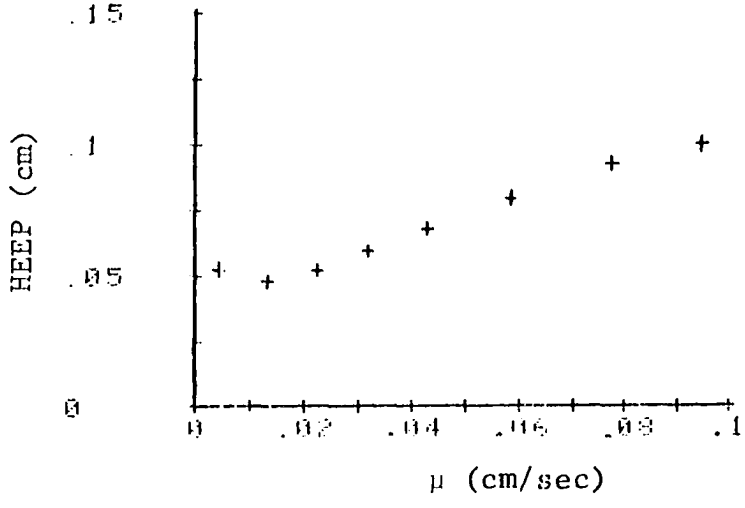


Figure 3. Height Equivalent of an Effective Plate (HEEP) vs. linear velocity, μ, for a Styragel column

where L is the length of the column, N the effective plate height, t_M the mobile phase hold-up time, t_R' the adjusted retention time for toluene, and w_h the peak width at half-height. The hold-up time of the mobile phase was measured with a poly(styrene) standard of molecular weight 36,000. For purposes of convenience and reproducibility, the linear velocity chosen was, 0.037 cm s^{-1}, which corresponds to a flow rate of 1.00 mL $min.^{-1}$.

Figure 4 presents data for HEEP vs. the injected volume of carbon tetrachloride. The figure demonstrates the possible dangers of overloading the column. All injected solutions of test solutes and mycotoxins were of sufficiently low concentration to avoid such problems.

The data for Figures 3 and 4 were collected on a 20 cm long column. The mobile phase was 30% (v/v) $CH_3CN/CHCl_3$. Figure 5 gives an example of the chromatograms, recorded at a flow rate of 1.00 mL $min.^{-1}$. In general, asymmetry factors for all measurements were less than 1·15.

Results and Discussion

Table V gives the bed height as a function of solvent composition. As expected, when the difference between the δ value of the solvent and the gel became greater (increasing modifier), the gel tended to shrink. As the gel shrank, solvent "voids" appeared near the column inlet. Such large

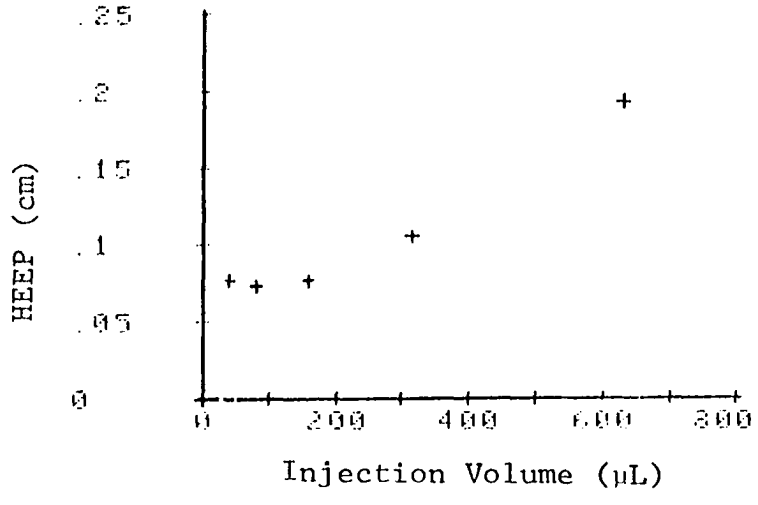


Figure 4. Height Equivalent of an Effective Plate (HEEP) vs. injected volume (µL) of CCl_4

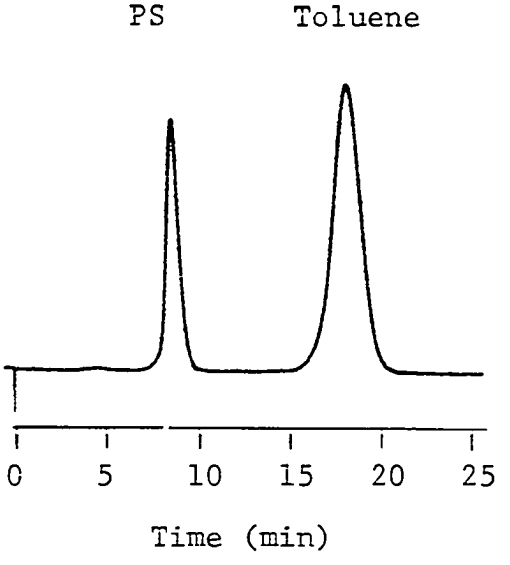


Figure 5. Chromatogram of poly(styrene) and toluene on Styragel. Conditions: column, 1.27x20.4 cm; flow rate, 1.00 mL min^{-1}; mobile phase: 30% $CH_3CN/CHCl_3$

Table V. Bed Height (cm) as a Function of Solvent Composition

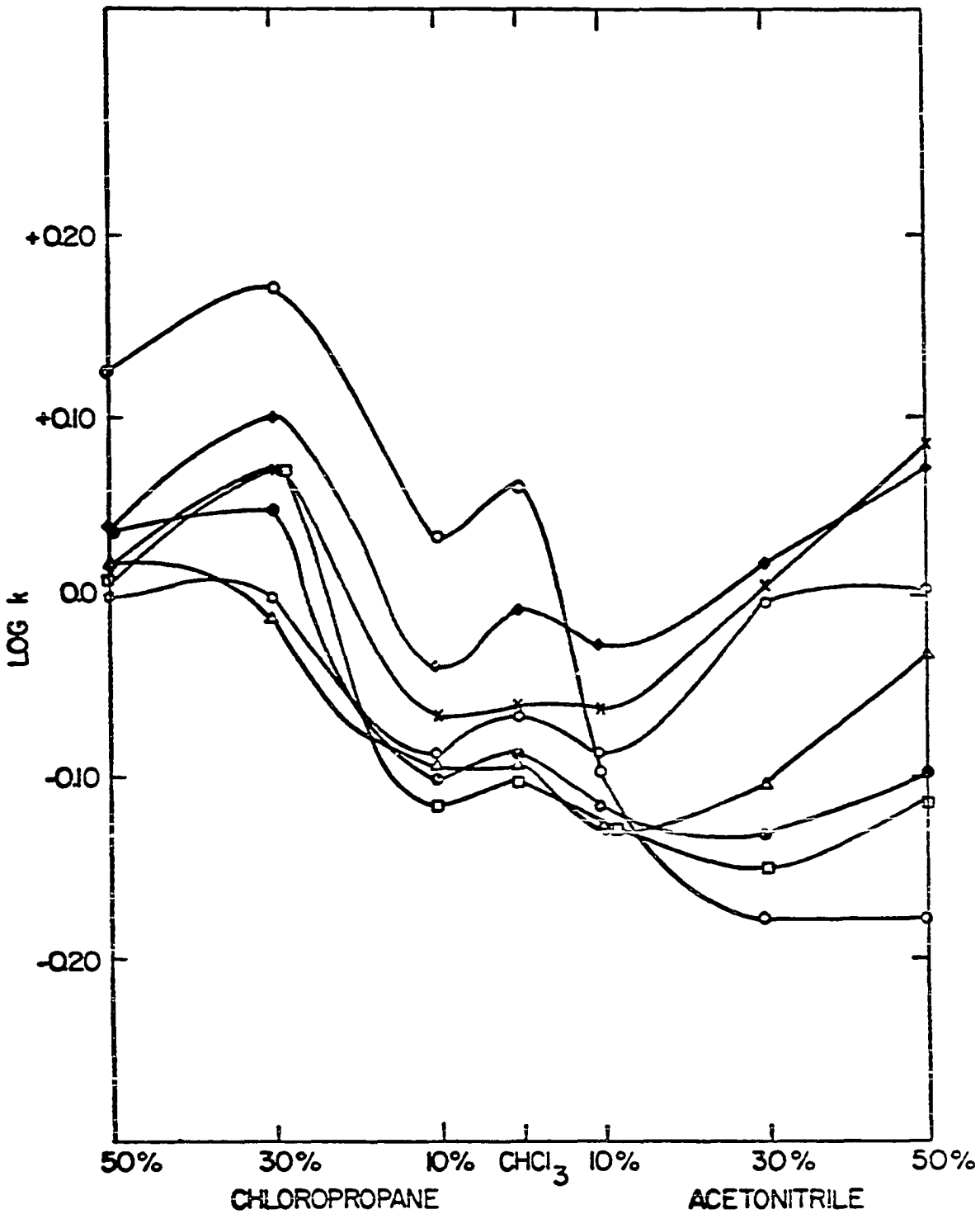
	% Modifier						
	nonpolar				polar		
Base	50	30	10	0	10	30	50
Chloroform	21.8	21.8	23.0	23.0	23.0	20.4	20.3
Benzene	22.0	22.0	22.4	22.4	22.4	22.4	20.8

pockets of solvent within the column decreased column efficiency considerably and increased the void volume. Shoulders on peaks often appeared. Column efficiency could be regained, however, by shortening the bed height with the movable bed supports. This procedure allowed the mobile phase to be changed without repacking each time, as has been suggested elsewhere [67].

Figures 6 and 7 present the data for the chloroform- and benzene-based systems, respectively. The benzene-based system offers no advantage for the separation of the mycotoxins and is of little interest for routine procedures because of the possible health problems associated with the use of benzene. In addition, the column efficiency was considerably less and the peaks too broad to be useful. Hence, the benzene-based system will not be discussed further.

In Figure 6, the solvent systems to the right of the base solvent represent a reverse-phase separation, while those to the left represent normal phase separation. In general, the solutes follow the trends expected from partition theory, with more polar compounds eluting faster (lower k) than non-polar in reverse-phase, and vice versa. However, it is clear that there is no simple explanation and there are exceptions.

Figure 6. Experimental log k vs. % modifier, chloroform-based mobile phase: (○) phenol, (◆) benzene, (x) toluene, (◎) pyridine, (●) 2-butanone, (△) nitrobenzene, (□) acetone


+0.20
+0.10
0.0
-0.10
-0.20
LOG k
50%
30%
10%
CHCl3
10%
30%
50%
CHLOROPROPANE
ACETONITRILE

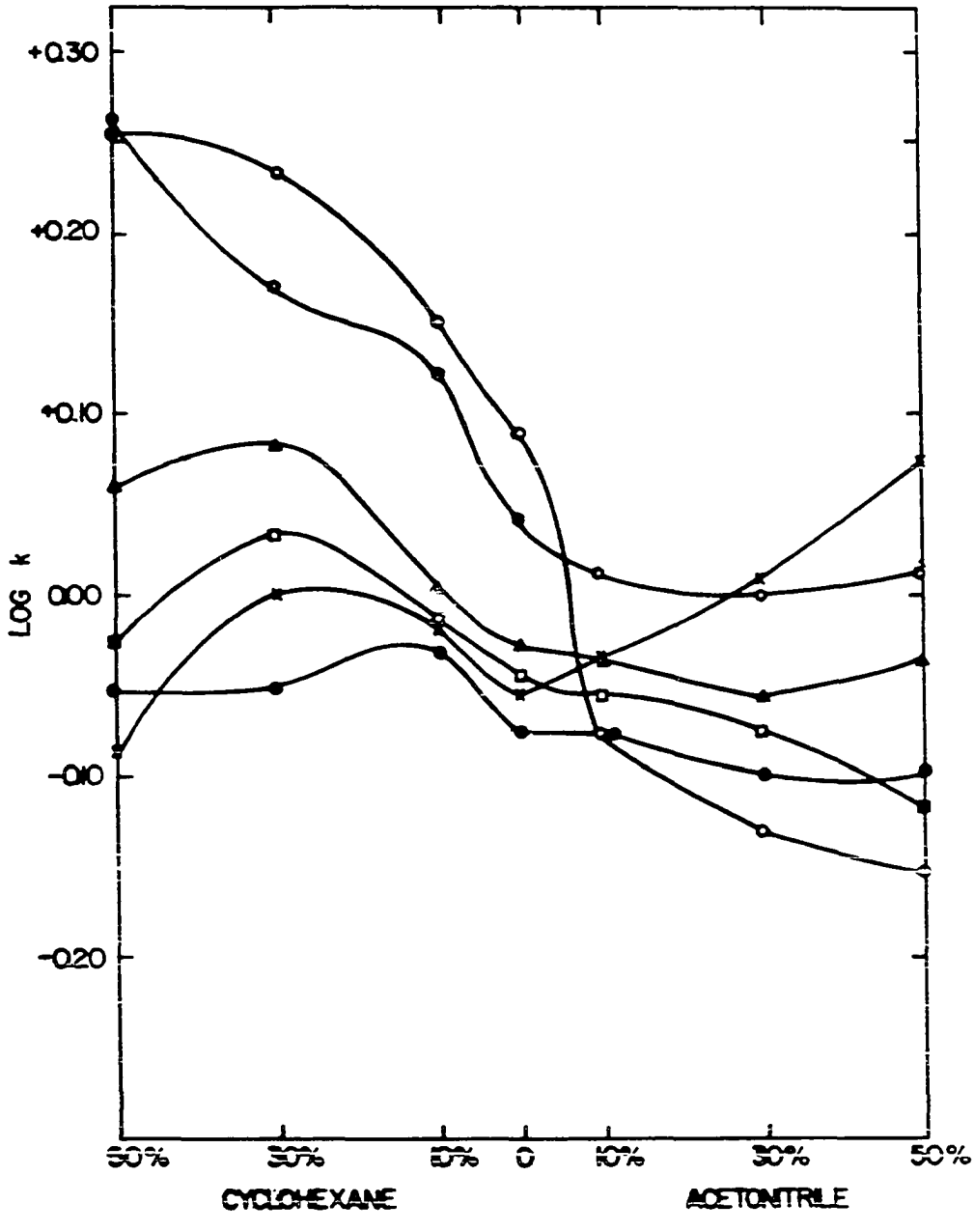


Figure 7. Experimental log k vs. % modifier, benzene-based mobile phase: (○) phenol, (x) toluene, (⊙) pyridine, (●) 2-butanone, (△) nitrobenzene, (□) acetone

Of particular interest is the tendency for the retention, using the base solvent as mobile phase, to increase relative to the adjacent data points. This is inconsistent with the data of Mori [66] where data points are given only for mobile phases containing 0%, 50%, and 100% modifier. The lowest retention was reported at 0% modifier. This indicates that the stationary phase exclusion limit is much greater than in this study and differences in molecular size cannot be distinguished.

The assumption by Mori [66] is that the lowest retention indicates that no preferential interactions are occurring (i.e., exclusion chromatography only). Without any intermediate data, however, this does not constitute proof that a completely different mechanism is operative. In the present study, the increased retention at 0% modifier could be the result of an exclusion process. Perhaps the absence of other solvent effects allows greater permeation through the pores and greater retention when no modifier is present. If one ignores the 0% data, there is a smooth relationship throughout the range of polarity. This is indicative of a liquid-liquid partitioning system, and further evidence for a different mechanism when no modifier is present.

If the separation mechanism is indeed partitioning, it should follow trends predicted by liquid-liquid partition theory. Equation 5 states the retention relationship on the

$$\log K = \frac{\bar{V}}{2.3RT}\left[(\delta_R - \delta_M)^2 - (\delta_S - \delta_R)^2\right] \qquad (5)$$

basis of solubility parameter theory [36], where K is the distribution constant, V the molar volume and δ_R, δ_M, δ_S represent solubility parameter values for solute, mobile phase, and stationary phase, respectively. Since,

$$K = k \cdot \frac{V_M}{V_S} \qquad (6)$$

Equation 5 can be rearranged to give,

$$\log k = \frac{\bar{V}}{2.3RT}\left[(\delta_R - \delta_M)^2 - (\delta_S - \delta_R)^2\right] + C \qquad (7)$$

where C is a term containing several constants. This equation is similar to that given in reference [51].

Figure 8 is a plot of calculated values from Equation 7 using data from reference [70]. Here the stationary phase is assumed to be the chloroform layer distributed interstitially among the strands of the polymer. The mobile phase parameters are calculated from the volume fraction of each component in the binary solvent system. Equation 7 predicts a complete reversal of retention order, which is, of course, not observed.

As mentioned earlier, specific interactions limit the applicability of solubility parameter theory. As expanded treatment using specific interactions [68] is given in Equation 8,

$$\begin{aligned}\log k = [(\delta^k)^2 - (\delta^j)^2 &- 2\delta_d^i(\delta_d^k - \delta_d^j) - 2\delta_{in}^j(\delta_b^k - \delta_d^j) \\ &- 2\delta_d^i(\delta_{in}^k - \delta_{in}^j) - 2\delta_o^i(\delta_o^k - \delta_o^j) \\ &- 2\delta_a^i(\delta_b^k - \delta_b^j) - 2\delta_b^i(\delta_a^k - \delta_a^j)] + C \qquad (8)\end{aligned}$$

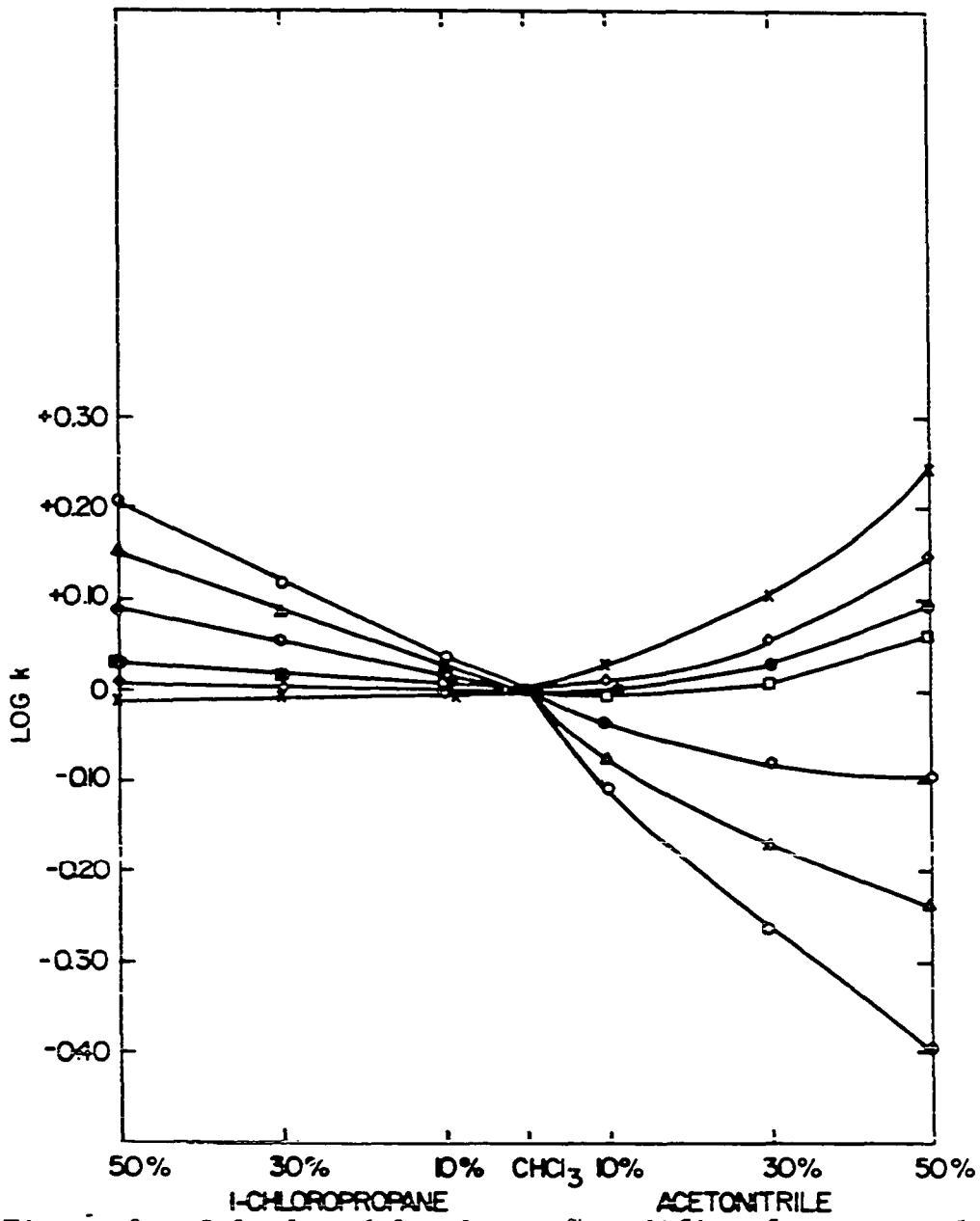


Figure 8. Calculated log k vs. % modifier for test solutes from solubility parameter theory, chloroform-based mobile phase: (○) phenol, (◇) benzene, (x) toluene, (◎) pyridine, (●) 2-butanone, (△) nitrobenzene, (□) acetone

where i represents the solute, and j and k represent stationary and mobile phases, respectively.

Figure 9 is a plot of calculated values using Equation 8. This indicates the importance of specific interactions in the chromatographic process. The retention order has changed in several instances. Unfortunately, it still does not match the experimental data.

It is reasonable at this point to assume that the gel is interacting with the solutes. Treatment of the gel phase as a binary mixture has some precedence [51]. The appropriate expression in this case is

$$\delta^*_{stat} = f \cdot \delta^*_{gel} + (1-f) \cdot \delta^*_{CHCl_3}. \qquad (9)$$

where δ^* represents all the specific solubility parameters and f is the fractional contribution from the aromatic rings of the gel. Benzene is used here since the specific solubility parameters for poly(styrene) are not known.

Empirically, it was found that a good approximation of the experimental retention order is obtained when f = 0.25. The order on the polar side is correct, while the non-polar side reflects some inconsistencies. However, the general trends are the same.

Two glaring exceptions involve phenol and pyridine. Phenol elutes later and pyridine earlier than expected. Coincidentally, these two have the highest values for the

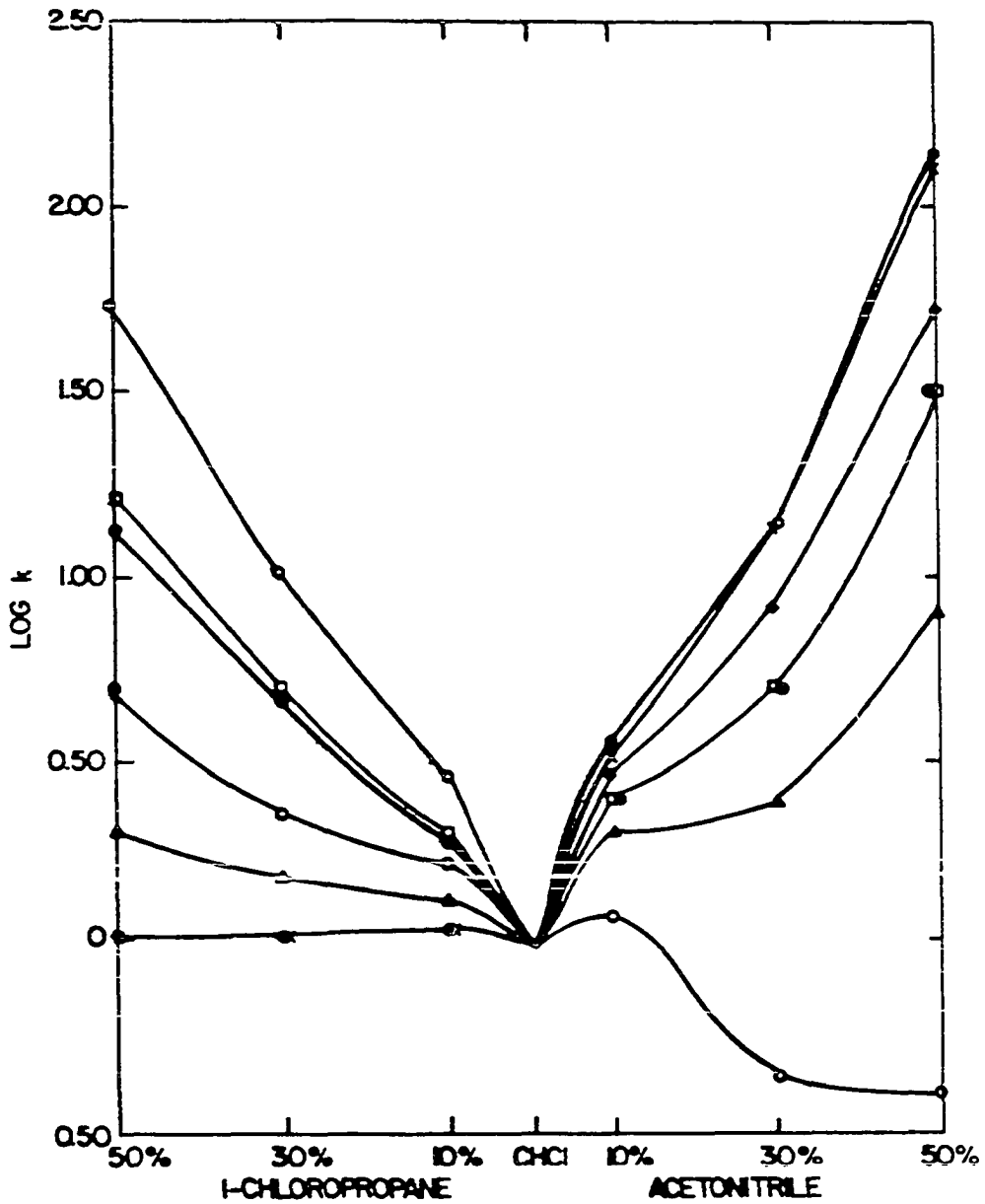


Figure 9. Calculated log k vs. % modifier for test solutes from expanded solubility parameter theory, chloroform-based mobile phase: (○) phenol, (◆) benzene, (x) toluene, (○) pyridine, (●) 2-butanone, (△) nitrogenzene, (□) acetone

hydrogen-bonding parameters δ_a, δ_b. As more approximations were made in deriving these values [71], it is possible the deviation is due to hydrogen-bonding effects.

Note, also, that all calculations predict a general decrease in retention for $CHCl_3$ as the mobile phase. This adds further weight to the contention that, when predicted, size exclusion effects are occurring "predominantly".

Another inconsistency illustrated in Figure 6 is at the extremes of polarity, i.e. 50% modifier. Solutes show a leveling off or actual decrease in retention. All calculations predict an increase. For the partition system, as the modifier concentration is increased, a point will be reached where the thermodynamic and concentration effects no longer allow a distinct $CHCl_3$/gel phase to exist. At this point the liquid-liquid system has broken down and a liquid-solid sorption mechanism has taken its place. This change in mechanism is contrary to that proposed by Mori [66].

Figure 10 illustrates this change in mechanism. The mycotoxin citrinin shows pronounced tailing in normal adsorption chromatography. Figures 10a-c indicate very little tailing for this partitioning system. However, citrinin could not be eluted from the column with 50% CH_3CN. The mobile phase was changed to 30% CH_3CN and citrinin injected again, Figure 10d. The retention time has changed and tailing is observed. Even after changing the mobile phase

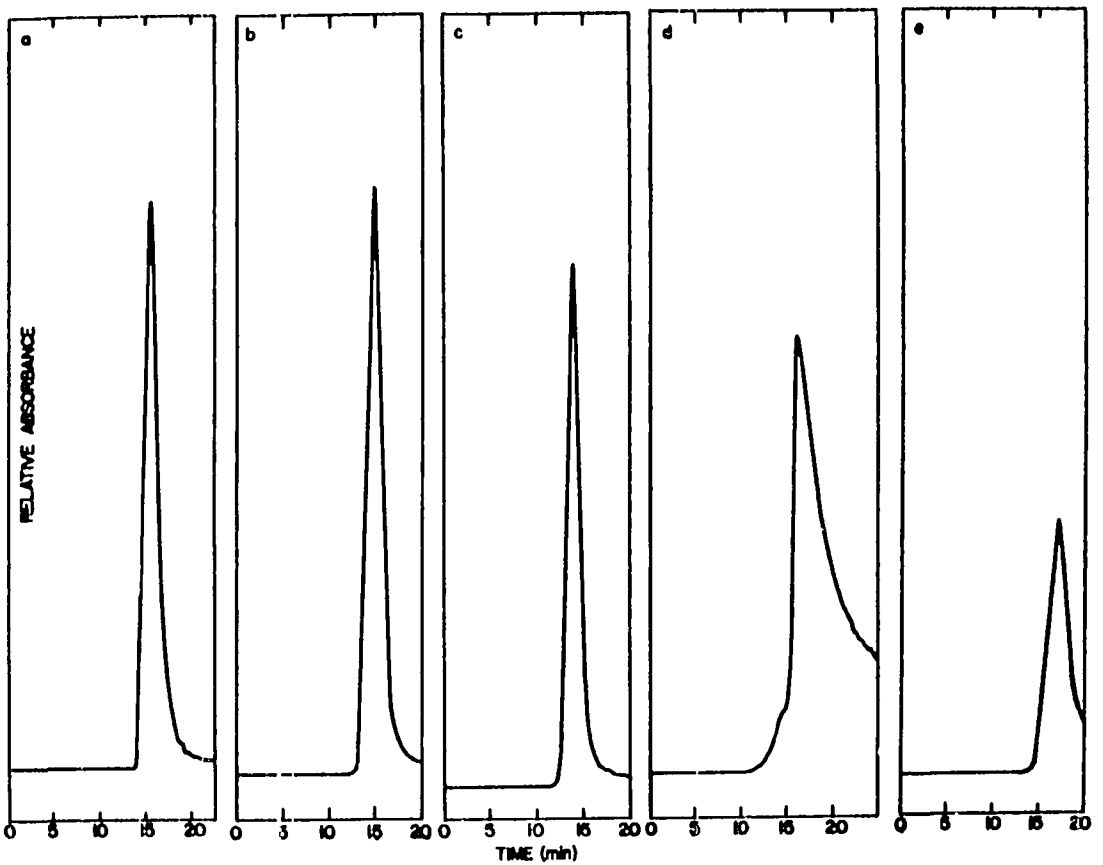


Figure 10. Elution behavior of citrinin in chloroform-base mobile phase: (a) 100% $CHCl_3$, (b) 10% $CH_3CN/CHCl_3$, (c) 30% $CH_3CN/CHCl_3$, (d) 30% $CH_3CN/CHCl_3$ after preequilibration with 50% CH_3CN, (e) 100% $CHCl_3$ after prequilibration with 50% CH_3CN

back to 100% $CHCl_3$, tailing was still observed. In addition, retention times for test solutes could not be reproduced. Furthermore, the original bed height could not be regenerated, even after pumping $CHCl_3$ through the column for several hours. These trends are indicative of irreversible sorption on the gel.

This is inconsistent with other work [67] where it was suggested that partitioning occurred in a wide range of modifier concentrations, and performance could be regenerated after exposure to 50% modifier. The data presented here indicate that partitioning probably occurs at modifier concentrations less than 40%. At high concentrations the mechanism is more likely sorption.

Undoubtedly, all three mechanisms may be operating simultaneously for all solvent systems. With enough data, one could imagine a relationship describing the relative importance of each mechanism as a function of the difference in solubility parameter of mobile and stationary phase.

It appears that the partitioning process is occurring inside the pores rather than on the surface of the gel. The poly(styrene) standard elutes in the void volume and shows no signs of other effects, except in 50% cyclohexane/benzene. Here, the standard was retained somewhat. The void volume in that case is determined by components in a real sample.

It should be noted that the $CHCl_3$ retention values for the test solutes could not be fitted to an absolute calibration curve, i.e., no size or molecular weight parameter produced a linear correlation. Hammett plots for substituted phenols show better correlations at 30% CH_3CN than 0%, indicating polar effects, but the trend is not conclusive.

The data make one skeptical about the validity of the term exclusion chromatography as applied to any solvent system. Residual effects seem to be operating in all cases. For other EC gels, such as Sephadex, where δ gel is unknown, it is not obvious which solvent is appropriate. Many, if not most, EC calibration curves obtained from a homologous series may merely be a summary of polarity effects, since molecular weight and polarity are generally related in a homologous series. Still, it should be emphasized that such curves are useful, because despite whatever functional group interactions are occurring, they are the same for each member of a series.

Figure 11 illustrates a "proper" calibration curve for phthalate esters on Styragel with a $CHCl_3$ mobile phase. However, one cannot claim that this is non-interactive chromatography (EC).

Finally, it should be emphasized that these solvent effects are disadvantageous only if pure EC is desired.

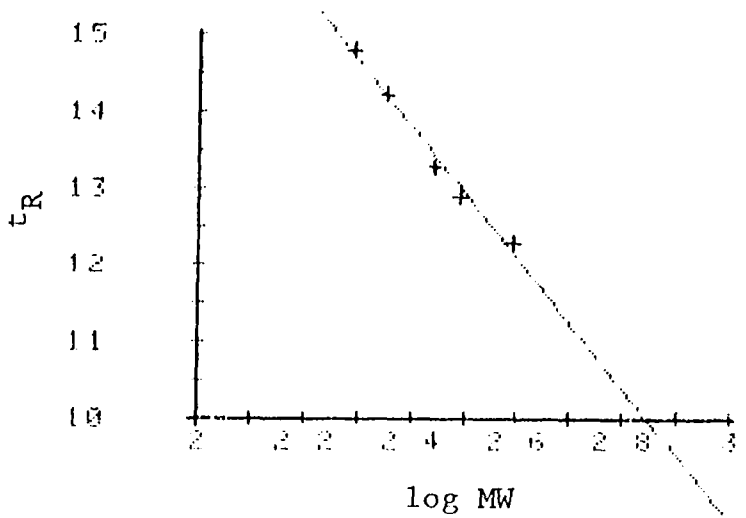


Figure 11. Retention time (t_R) vs. log molecular weight (MW) for a series of phthalate esters on Styragel. Conditions: column, 1.27x23.0 cm; flow rate, 1.00 mL min^{-1}; mobile phase: 100% $CHCl_3$

There are abundant examples that illustrate the advantages of non-exclusion effects in achieving difficult separations.

Mixed Exclusion Chromatographic Solvent Systems

Since both $CHCl_3$ and THF function independently and well as EC solvents, a mobile phase mixture of the two was investigated. Figure 12 illustrates the results. Aniline and phenol were included in the experiments to indicate possible hydrogen-bonding effects.

It is interesting that relatively non-polar solutes such as benzene and toluene show changes in retention for different mobile phases. Clearly, hydrogen-bonding is not the only factor affecting retention. The decrease in retention for phenol in the presence of THF containing solvents is consistent with other observations [53], indicating a hydrogen-bonded complex.

There is no obvious explanation for the change in retention with 60% $CHCl_3$. It does not represent any simple solvent ratio.

Exclusion Chromatography of Mycotoxins

Figure 13 presents data for mycotoxins under the same conditions used to obtain the data given in Figures 6 and 7. The data show the same general trends as the test solutes. However, the multiple functional groups present make

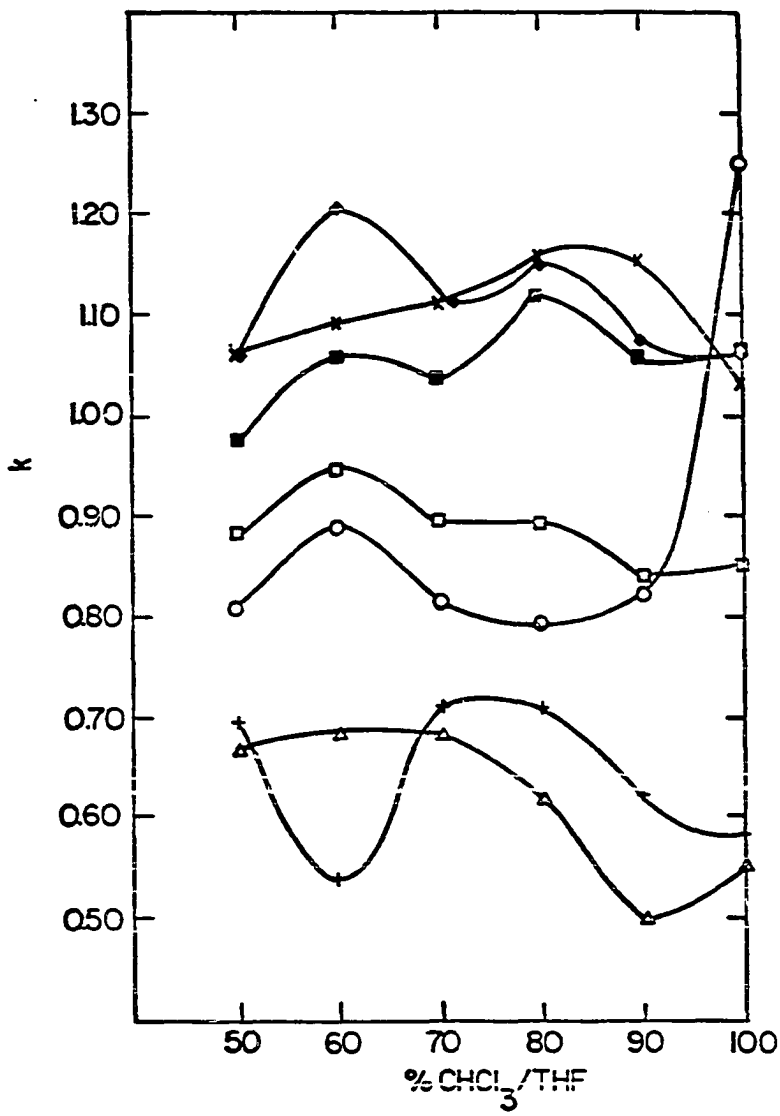


Figure 12. Experimental k vs. % chloroform in tetrahydrofuran (THF) for $CHCl_3$/THF mixtures: (♦) benzene, (x) toluene, (■) aniline, (□) acetone, (○) phenol, (+) aflatoxins, (△) penicillic acid

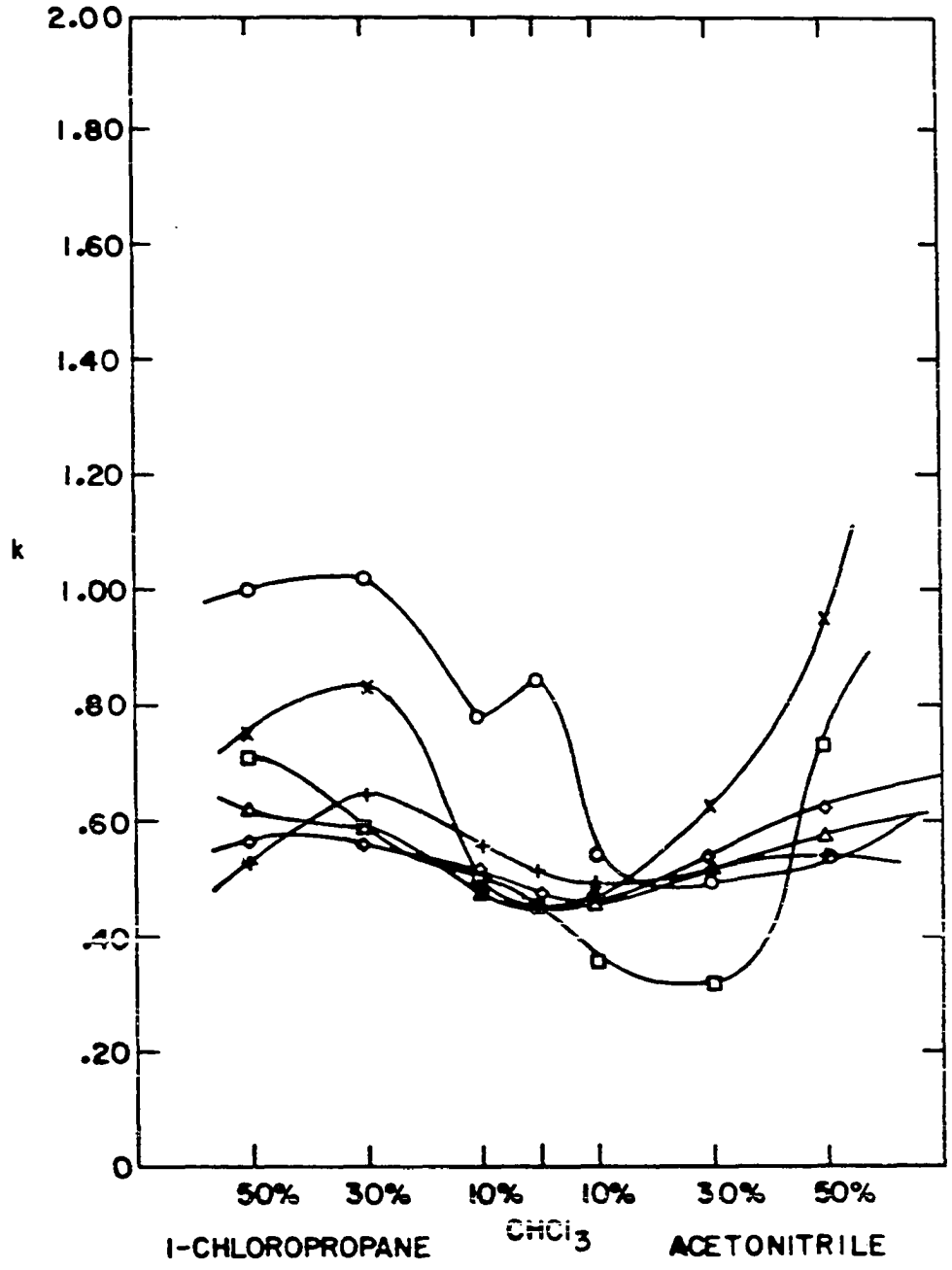


Figure 13. Experimental k vs. % modifier, chloroform-based mobile phase: (○) patulin, (x) sterigmatocystin, (□) ochratoxin A, (△) citrinin, (◇) aflatoxins, (+) penicillic acid

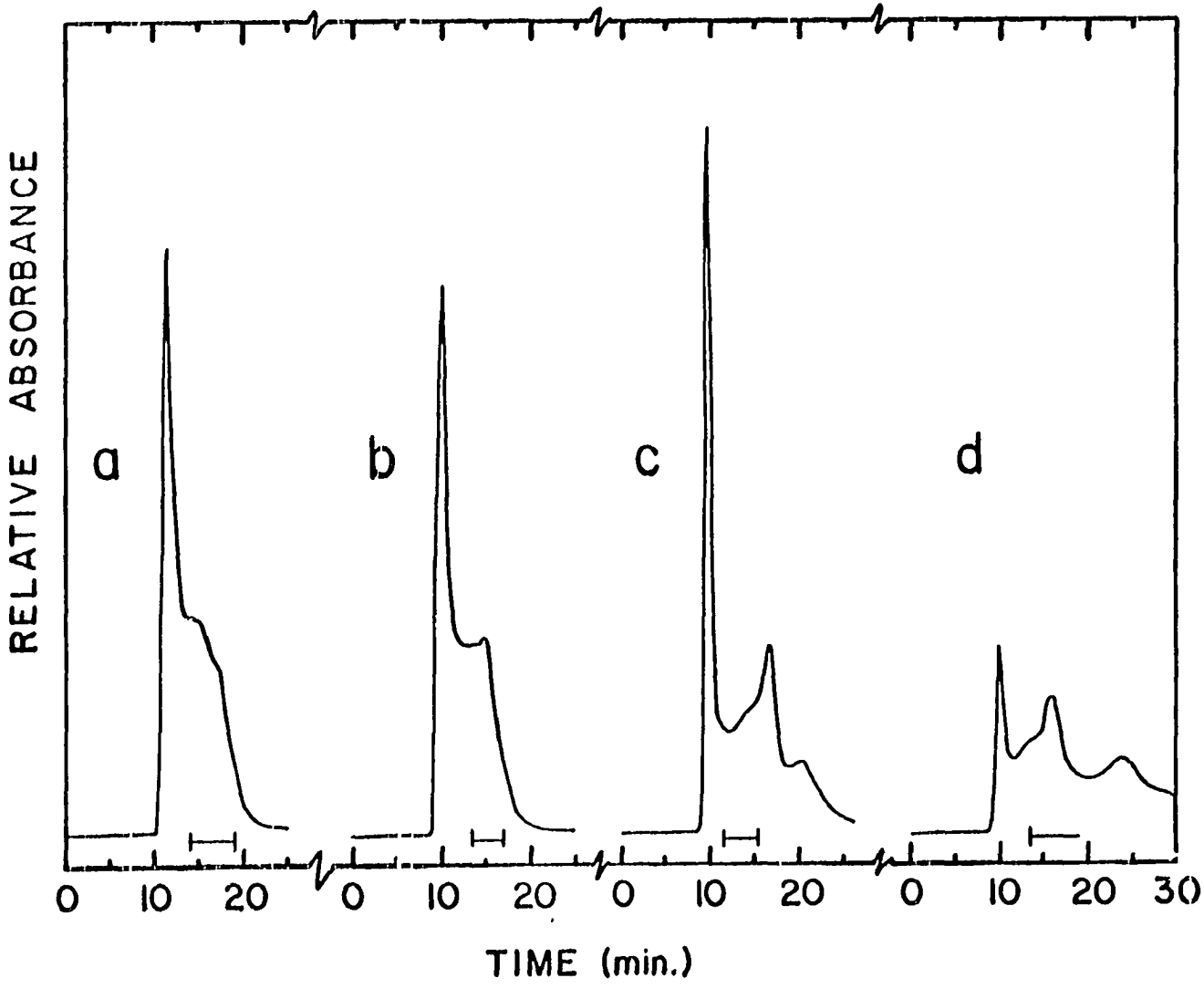
quantitative analysis difficult and produce greater relative changes in retention. The mycotoxins are, in general, polar organic molecules and show the expected trends in a liquid-liquid partitioning system, i.e., decreased retention in a more polar mobile phase.

The reverse-phase system appears best for separation of the mycotoxins in a real sample. The compounds tend to elute in a similar range, allowing a "class" separation of polar materials from non-polar materials.

The effect of the polarity of the mobile phase on a chloroform extract of refuse-derived fuel is shown in Figure 14. First, note that a large fraction of the material eluted in the void volume. This represents the molecules (MW >500) too large to be included in the gel pores. It is also the most highly colored fraction (brown in this case). This illustrates one mechanism operating with the gel and affords a very simple method for reducing the complexity of the matrix.

As the mobile phase becomes more polar (CH_3CN concentration increasing), the less polar components spend more time in the stationary phase and elute after the mycotoxins. The brackets indicate the elution volumes of the mycotoxins. The optimum separation appears to occur with a mobile phase containing 30% CH_3CN. Apparently, a large portion of the absorbing material is less polar than the mycotoxins. In

Figure 14. Chloroform extract of an RDF sample eluted with varying concentrations of CH_3CN in the mobile phase. The brackets indicate the elution times of the mycotoxins: (a) 100% $CHCl_3$, (b) 10% CH_3CN, (c) 30% CH_3CN, (d) 50% CH_3CN. The elution time for citrinin is indeterminate for this mobile phase


RELATIVE ABSORBANCE
a
b
c
d
0
10
20
0
10
20
0
10
20
0
10
20
30
TIME (min.)

general, then, elution occurs in order of decreasing polarity when CH_3CN is present in the mobile phase.

Note that for mobile phases containing 50% CH_3CN, some material elutes very slowly from the column. In fact, some colored components could not be removed from the column with this solvent system. Sorption, not partitioning, must be the principal mechanism. This is further evidence that liquid-liquid partitioning may not occur over a wide range of solvent modifier concentrations. The retention volume of the last eluting component (i.e., the peak capacity of the column) changes in Figure 14. This is contrary to what is expected of a stationary phase which operates by exclusion only.

Styragel can be used in a manner that one can control the relative contribution of three different separation modes on one stationary phase by choice of mobile phase. In this case, partitioning and exclusion were combined to achieve better sample preparation than was possible by other methods. Since the stationary phase is relatively nonpolar, it will be most useful in the reverse-phase mode. The column packing is relatively inexpensive and reusable, even on a semipreparative scale, if suitable precautions are taken. This procedure should be useful for many difficult separation problems, especially when judicious use is made of specific interactions (i.e., hydrogen-bonding).

MODIFIED FLORISIL FOR RAPID SAMPLE PREPARATION

Introduction

Although the Styragel column described in the previous chapter provided a good separation of the sample extract, the amount of material collected (~1 mg) exceeds the capacity of the Thin Layer Chromatography plates used for quantitation. The background fluorescence levels were also higher than desirable. Additional sample preparation steps were deemed necessary.

Since a small amount of material was present in the aflatoxin fraction, a short chromatographic column immediately following the Styragel column seemed appropriate. It was also necessary to minimize the time and extent of sample handling involved.

Since the Styragel column involved liquid-liquid partitioning as a separation mechanism, an adsorption material (i.e., liquid-solid interaction) was chosen for the second column. Initially, small (5-20 mm) columns of various adsorbents were packed into disposable Pasteur pipettes. The adsorbent was supported on either side by a small amount of diatomaceous earth. The aflatoxin containing fraction was allowed to flow through these columns. Acidic alumina, neutral alumina, and silica were

selected as adsorbents. With 30% $CH_3CN/CHCl_3$ as the eluent, the aflatoxins passed through the silica, along with most of the components in the sample. Both alumina columns retained some fluorescing material, while the aflatoxins passed through. The overall background level was reduced, but not significantly.

It has been suggested that mixtures of common adsorbents could alter the selectivity compared to a single adsorbent [72].

A 2:1 (w/w) mixture of charcoal/diatomaceous earth gave a clear eluate, but retained the aflatoxins so strongly they could not be subsequently removed. Mixtures of charcoal/alumina behaved as the charcoal columns. Mixtures of alumina and Florisil would tightly sorb the aflatoxins when 30% $CH_3CN/CHCl_3$ was passed through the column. Much of the interfering material passed through the column. The aflatoxins could then be removed to varying degrees by certain solvents. In all cases, acetone or H_2O/acetone mixtures eluted the aflatoxins in a smaller volume than any other solvent. Furthermore, Florisil above gave better recovery of the aflatoxins and was studied further.

Chemical Properties of Florisil

Florisil is a mixture containing approximately 84.0% SiO_2, 15.5% MgO, and 0.5% Na_2SO_4 by weight [73]. The adsorbent possesses permanent acidic properties, and chemisorption is common. Therefore, tailing is often observed. The chemisorptive properties, and retention, are very sensitive to the amount of water present. It has been suggested that at least 1% (w/w) H_2O should be present for the most reproducible properties [74]. This may be accomplished by first activating the adsorbent at 400°C for 16 hours, followed by addition of H_2O and equilibration on a wrist action shaker for 48 hours. The solvents most often used for eluting materials are pentane, methylene chloride, and benzene.

Florisil has been suggested for use in the analysis for aflatoxins [75]. After packing, the adsorbent is deactivated with 1% acetic acid/hexane, dried, and deactivated with 5% (w/w) H_2O. However, this is designed for relatively high levels (>1 ng) of aflatoxins. In the present study, aflatoxins could not be recovered when added to a Florisil column at levels less than 500 pg. Deactivation with 10% H_2O or 1% acetic acid, or treatment of packed columns with 1% acetic acid/$CHCl_3$, 1% H_2O/acetone, 4% H_2O/acetone, or 5% acetic acid/hexane gave recoveries in the 40-70% range. Elution of the aflatoxins with 50%

H_2O/acetone or various combinations of H_2O, methanol, acetic acid, and acetone also gave poor recoveries.

Chemical Deactivation of the Adsorbent

The chemisorption by Florisil is probably due to formation of an organo-Mg complex. The likely points of interaction are indicated in Figure 15. The carbonyl interaction seems most reasonable, considering that acetone disrupts the complex and elutes the aflatoxins.

An attempt was made to mask, or at least reduce, the extent of Mg-interaction with the aflatoxins. Four different chelating agents were mixed with Florisil in equimolar amounts corresponding to the Mg present (3.8 mmol/g). Thus, 3.8 mmol ethylenediaminetetraacetic acid (EDTA), 8-hydroxyquinoline, tartaric acid, or oxalic acid dihydrate were dissolved or suspended in acetone. Each solution was added to 1 g Florisil, suspended in acetone, and stirred several minutes. The supernatant liquid was removed and the solid dried. Each combination was examined for the ability to retain aflatoxin when applied in 30% $CH_3CN/CHCl_3$, and the ability to release the aflatoxins with 4% H_2O/acetone.

The adsorbent treated with 8-hydroxy-quinoline produced a large amount of green fluorescence, indicating chelation with Mg. A significant amount of material leached from the column, and this combination was not studied further. The

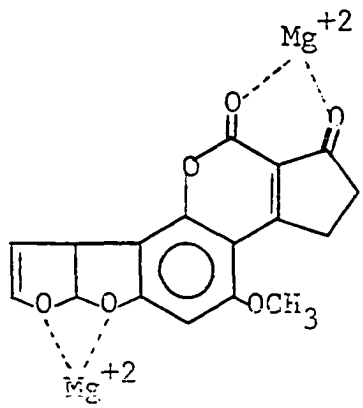


Figure 15. Proposed points of interaction between Mg^{+2} and aflatoxin B1

EDTA-treated adsorbent destroyed the fluorescence of the aflatoxins and for this reason was not studied further. Tartaric acid and oxalic acid-dihydrate worked well, qualitatively, but oxalic acid was chosen partly because of its solubility in acetone.

This oxalic acid-modified Florisil showed some residual chemisorptive properties and it was necessary to deactivate further with H_2O. Samples of Florisil deactivated with 5%, 7.5%, and 10% (w/w) H_2O showed better recoveries when packed in a 6 x 9 mm column. The 7.5% deactivated column was chosen. Furthermore, when the column length was increased to 22 mm, the recovery of all aflatoxins was >90% when 380 pg each was injected.

The purpose of the oxalic acid was to complex the "most active" Mg sites in the Florisil. Obviously, some Mg atoms will be more accessible to components in the mobile phase, while others will be previously complexed or occluded in the matrix (i.e., "less active"). The equimolar amount of oxalic acid added would assure that all Mg atoms are complexed if necessary. However, it is unlikely that all oxalic acid would be complexed. In fact, some acid is recovered in the supernatant liquid, although the amount has not been quantitated.

In unmodified Florisil samples, aflatoxins were sorbed at the top of the column. When deactivated with oxalic

acid and H_2O, aflatoxins would elute slowly through the column while the fraction was being collected. Thus, while collecting a 3.5 mL fraction, some aflatoxins would elute from a Florisil column less than 15 mm long. This extent of deactivation was considered necessary to prevent losses at low levels (<500 pg).

It should be noted that aflatoxins elute from the Florisil in the order G2, G1, B2, B1. This is the order expected after elution from the Styragel column (i.e., reverse phase). Thus, losses due to early elution from the Florisil column are only of concern for G2.

After collection of the aflatoxin fraction on Florisil, it was possible to elute interfering compounds without losing aflatoxins. Thus, more background fluorescence and interfering peaks were removed with 1.2 mL 20% methanol/ $CHCl_3$. Aflatoxins were completely removed with 5.0 mL 4% H_2O/acetone.

Column Design

Figure 16 illustrates the design of the Florisil column. The two pieces are machined from a Teflon rod. The packing is held in place by two pieces of 270 mesh brass screen which have been soldered to small washers. The lower screen fits snugly into the packing compartment. The upper screen fits into a lip at the top of the packing compartment. A tight seal is made between the rim of the lower section and

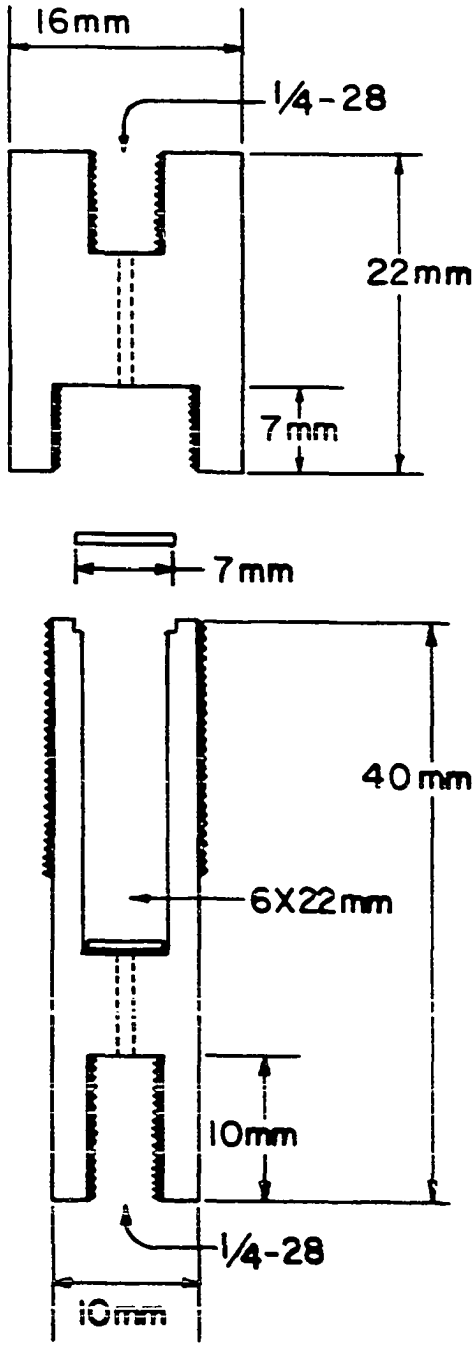


Figure 16. Schematic diagram of the Florisil column

the upper cap. The external connections accept standard Cheminert® fittings. Florisil is dry-packed into the column with tapping. Each column accepts approximately 300 mg Florisil.

Figure 17 illustrates the two valves used for stream switching between the Styragel and Florisil columns. Each valve is a 3-way slider-type designed to work with standard Cheminert fittings. The valves are mounted above each other, immediately following the detector. When Valve-1 is in position "A", the mobile phase passes to a waste container. Position "B" diverts the mobile phase to Valve-2, which must be in position "A", for collection of the aflatoxins containing fraction. After the fraction has been collected, Valve-1 is moved to position "A" and Valve-2 can be moved to position "B". This connects the Florisil column to a 5 mL syringe for elution of interfering components (1.2 mL 20% methanol/$CHCl_3$) and then aflatoxins (5 mL 4% H_2O/acetone).

Thus, while the remaining material is eluting from the Styragel column, the sample is undergoing a second separation. The aflatoxins are collected in a 10 mL pear-shaped flask and the solvent evaporated. Losses due to transfer between glassware and column are eliminated, and exposure to solvents is minimized without increasing the time required.

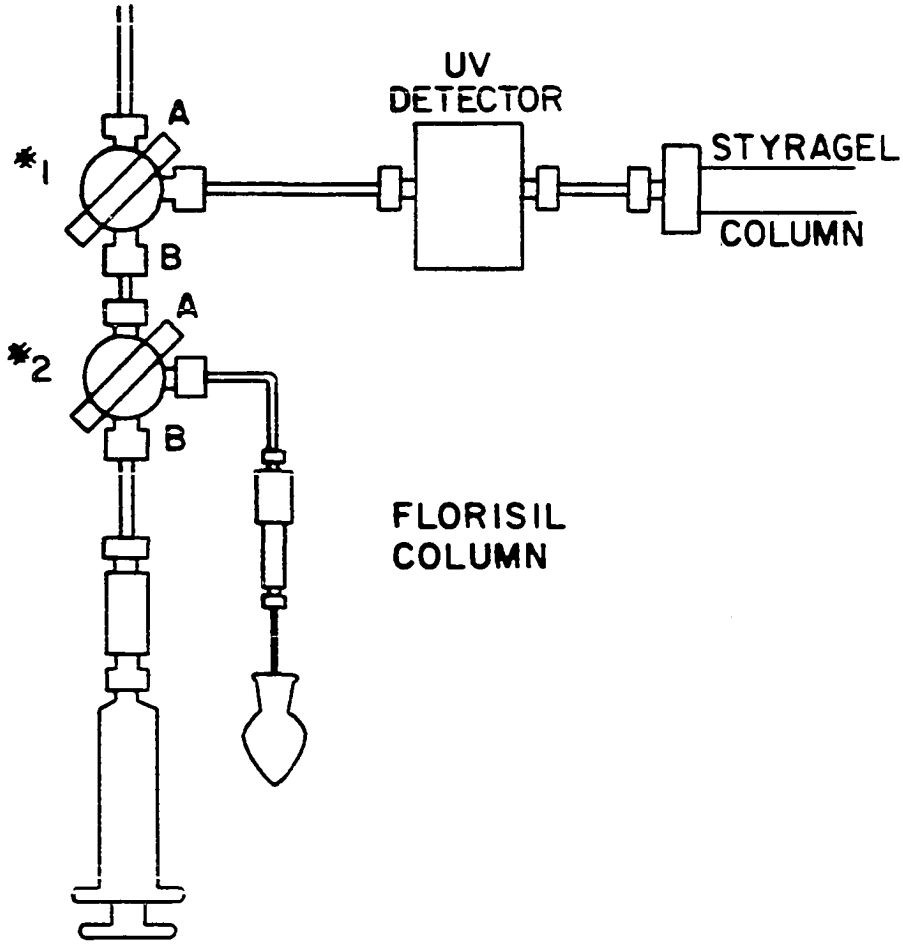


Figure 17. Schematic diagram of stream switching valves between the Styragel and Florisil columns

NEW TECHNIQUES FOR THIN LAYER CHROMATOGRAPHY

Introduction

Thin Layer Chromatography (TLC) was first proposed as a "new chromatographic technique" in 1951 [76]. The term TLC was first applied in 1956 [77]. Like other chromatographic techniques, TLC has enjoyed a period of extensive growth.

The technique involves coating a flat surface, usually glass, with a thin layer of adsorbent (silica gel, alumina, Florisil, cellulose, etc.). A sample dissolved in a suitable solvent is applied to the plate and is allowed to flow through the adsorbent, usually by capillary action. Components in the sample will migrate, in the direction of flow, a fraction of the distance of the leading edge of solvent. This fraction is designated, R_F, where

$$R_F = \frac{\text{distance solute has moved}}{\text{distance solvent front has moved}}. \qquad (10)$$

By definition, $0<R_F<1$.

R_F is related to other chromatographic terms by equations 11-13,

$$R_F = \frac{1}{1 + k} \qquad (11)$$

$$k = K \frac{A_S}{V_M} \qquad (12)$$

$$R_M = \log\left(\frac{1-R_F}{R_F}\right) = \log k. \qquad (13)$$

where k is the distribution constant, A_S the area of adsorbent, and V_M the volume of the solvent.

The mechanism causing separation in adsorption chromatography is competition between solute and solvent for adsorption sites on the surface of the stationary phase [78]. Whether or not a solute molecule will be displaced by a solvent molecule depends on the relative energies of adsorption of each.

More detailed treatments are provided in the classic works of Stahl [79] and Snyder [80].

As the mobile phase mixture proceeds up the plate, the polar components will be preferentially sorbed to the surface. This occurs until that component is removed from the mobile phase. As fresh solvent is continually fed in at the bottom of the plate, a second frontal line of polar solvent develops which follows the main mobile phase front. Fronts are designated α-, β-, γ- etc. Each front moves at a given fraction of the rate of the α-front.

Thus, the area "above" any given front will be depleted of the component forming that front. After development, if a solute is not located below the last demixing front, then the polar mobile phase components causing the fronts below the solute are not necessary.

It should be noted that maximum resolution in TLC occurs for R_F values in the range 0.2 - 0.5 [81].

Application of the Sample

The application of the sample onto a TLC plate is important since this determines the minimum spot size and, hence, resolution. The sample is usually applied in a volume of 2-10 µL with commercially available disposable glass micropipets or a 10 µl syringe with a #3 needle (flat tip).

Micropipets are easier to handle and operator error due to non-reproducible filling of a syringe is not a problem [82]. However, the micropipets are filled by capillary action and when chloroform ($CHCl_3$) solutions are applied, as is usually the case here, the weight of the solutions is too great to allow complete filling by capillary action. If a 5 µL pipet is "filled" in a nearly horizontal position and then placed vertically, nearly half the solution will drain out, forming a drop on the tip. Application of a small spot is difficult in this case. In addition, there is a possibility that the pipet may not always fill completely. Evaporation at the tip may also create problems.

Delivering 5 µL of solution with a 10 µL syringe was found to be more reproducible. Using 1 ng aflatoxin B1 and the fluorescence system to be described later, the percent relative standard deviation (% RSD) for six developed spots was 8.9% and 2.3% for application with a micropipet and

syringe, respectively. Analysis of the applied spots before developing gave similar results. Hence, syringes were used for all remaining work with standards.

Pre-Development of the Spot

In TLC, band broadening will always reduce the resolution between two components by increasing the width of the spots in the direction of development. To improve resolution, the sample spot should be as narrow as possible in this direction. It is important to note that this does not change the chromatography (i.e., R_F values remain unchanged), but merely the "apparent" resolution due to the decreased width of each sample spot. This was first demonstrated by Fessler and Galley [83]. After application of the sample, the plate was dipped in a solvent for which $R_F = 1$ for all components (e.g., methanol), and allowed to develop for 1 minute. All components in the sample followed the solvent front, producing a narrow band immediately above the location of the original circular spot.

The same effect has been accomplished by commercially available "Pre-Adsorbent" TLC plates [84]. In this case, the pre-adsorbent zone has a low activity such that $R_F = 1$ for all components in any solvent. During development, the sample is compressed to a narrow band while in the pre-adsorbent zone, and enters the main adsorbent area as such.

Unfortunately, the width of the band cannot be controlled due to the low activity of the pre-adsorbent. If the entire spot cannot subsequently be analyzed because of instrumental limits, a higher detection limit may result. An adaptation of the method of Fessler et al. [83] was used instead.

After application, the TLC plates were pre-developed 15 seconds in 1:1 (v/v) methanol/$CHCl_3$. The result for aflatoxins is shown in Figure 18. The decrease in spot width increases resolution after development.

For real samples, the amount and chemical nature of the applied material required different solvents for pre-development. The methanol/$CHCl_3$ mixture would not pass "through" the spot, but flowed "around" it, causing distorted spots. A mixture containing a more non-polar solvent was necessary. The non-polar solvents appeared to "wet" the spot area, allowing better solvent flow through the spot during pre-development and main development stages. A 3:7 (v/v) mixture of ethyl ether/acetonitrile was chosen for real samples. The solvent mixture was allowed to flow just beyond the visible edges of the spot. This required about 10 seconds, after which the plate was dried. R_F = 1 for aflatoxins in this mixture. Some material showed little or no movement in ethyl ether/acetonitrile. However, this material also showed little movement in the main developing solvent mixture and did not interfere in the analysis.

Figure 18. Effect of pre-development on the fluorescence scan profile of aflatoxins: (a) after application to the plate, (b) after pre-development (methanol/ chloroform)

Background Fluorescence and Removal of Impurities

The level of background fluorescence on the TLC plates varied from plate to plate. An intense fluorescent band was always found migrating with the β-solvent front (*i*-amyl alcohol front). The intensity of fluorescence decreased slowly behind the front and created a changing baseline in the region of the plate containing the four aflatoxins. The level and rate of change was unpredictable and prevented analysis at higher amplifier sensitivities (less than $1x10^{-8}$ A full scale). The impurity could have been present in either the solvent or the plate. Under long-wave UV radiation (360 nm), the material fluoresced yellow.

All components in the developing solvent were purified, except formic acid, which showed no fluorescence. After purification, no residual fluorescence was noted in concentrated solutions of each of the solvents. Furthermore, the fluorescent band and tailing were not significantly reduced when purified solvents were used in the developing mixture.

Figure 19 further indicated that the impurity was present on the TLC plate. Following the application of 5 µL purified methanol to a plate, a fluorescent scan indicated that the methanol had "eluted" some material. The "peaks" appeared visually as a circle of radius slightly smaller than the circle corresponding to the methanol applied to the plate. The lower fluorescence level within the spotted

Figure 19. Fluorescence scan across an untreated TLC plate following application of 5 µL purified methanol

region indicated that the impurity was indeed in the plate, not the solvent. A similar experiment with cleaned plates produced no change in background level.

Single or double development of a TLC plate with purified methanol moved the impurity to the upper half of the plate, but significant tailing remained in the region of interest. Continuous overnight development in methanol produced plates with little change in background intensity. The impurity could be seen as a dark yellow band at the top of the plate.

After removal from methanol, the plates were dried in a vacuum desiccator (<0.1 mm Hg) for 1 hour in the presence of a heat lamp. This was preferred to oven drying, which often produced background "spikes", probably from physical disturbances on the plate during heating.

The pre-cleaning step changed the nature of the surface because of residual methanol molecules, but the change (decrease) in resolution was not significant. All cleaned plates were deactivated to a reproducible level by storage in an atmosphere containing 48% relative humidity (saturated KSCN solution).

Design and Use of a Sample Applicator for TLC

When applying samples to a TLC plate, the size and exact location of the spot were difficult to control. Evaporation

of the solvent was the rate-limiting step. The ability to place the material in a pre-determined and reproducible position for analysis was considered an unneccesary variable. Several commercial "applicators" were available, but they could not control both spot size and location. Most consisted of an application region flushed with air (or N_2) and a ruled edge for spot location. The fixed position applicators were more expensive and only suitable for syringe or pipette, not both. An applicator was needed which could rapidly evaporate solvent, allow for reproducible positioning of the sample, and permit use of syringe, pipette, or other sample introduction technqiues.

Design

This applicator was designed to accept 5x20 cm TLC plates. A 5-10 cm channel was milled in a clear 7.5x14 cm Lexan® block. The slot depth matched the thickness of the TLC plate, including the adsorbent layer. Three holes were drilled in the block, approximately 13 mm from the bottom of the milled channel. The center of each hole corresponded to assigned micrometer settings on the fluorometer, allowing exact placement of each sample for analysis. Two brass inserts were machined to fit into each hole as shown in Figure 20. The top insert allowed a pipette or syringe needle to contact the surface of the plate at the center of the hole. The lower insert helped direct the gas flow to

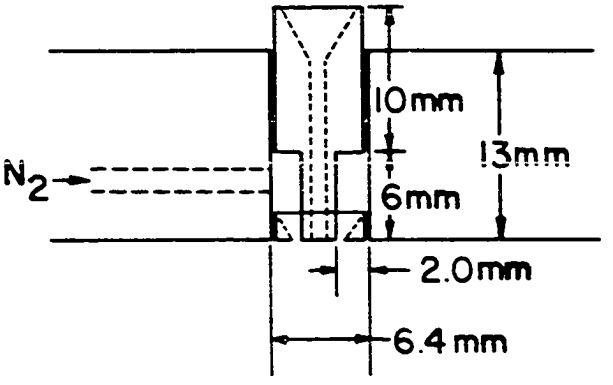


Figure 20. Schematic diagram of upper and lower brass inserts for TLC applicator

the center of the spotting area. Gas flow was provided to each application area through a manifold machined in the block. Standard fittings allowed connection to a compressed N_2 tank.

The entire assembly was mounted on a larger Lexan block, and fastened vertically. This allowed application of $CHCl_3$ solutions with 5 µL pipettes in a horizontal position, thus preventing draining of the pipette before spotting.

For real samples, it was difficult to work with small (i.e., <30 µL) volumes due to evaporation. Larger volumes required multiple applications which often damaged the adsorbent layer and syringe. Dilution of the sample to a standard volume and application of a small fraction would unnecessarily limit the ultimate powers of detection.

Further modification was necessary to allow application of the entire sample. The aflatoxin fraction was collected in a pear-shaped 10 mL flask with a 14/20 standard taper neck. A machined Teflon plug fit snugly into the neck of the flask. A 1 mm O.D. Teflon tube passed through the plug to the bottom of the flask. The other end of this tubing was coiled into three loops. The free end could then be placed into the brass insert and positioned immediately above the surface of the plate. A second length of tubing was inserted into the Teflon plug. The free end was fitted with a 1 mL syringe (Leur tip). This allowed a slight

positive air pressure to force the sample through the tubing, directly onto the TLC plate.

This modification worked best when the entire sample volume could be drawn into the coiled section of the tubing. Because nitrogen flow evaporated the solvent at a slower than expected rate, it was necessary to apply the sample as a series of small (3-5 µL) aliquots by momentary application of a small positive pressure with the syringe. An aliquot could be applied every five seconds. Thus, a 150 µL sample could be applied in 3-4 minutes, producing a small spot with no damage to the adsorbent layer.

Spotting solvent

The size of the sample spot is influenced by many variables, but the most important is probably solvent polarity. Each sample application can be considered a small scale chromatographic system. The solvent will "elute" the sample to an extent determined by the relative polarity of solvent and sample, and strength of adsorbent. Therefore, the polarity of the solvent should be as low as possible to produce a smaller spot.

Aflatoxins are known to sorb to glass. A more polar solvent was recommended for complete removal. However, the need for a small spot size required a compromise. After removal of the solvent in the aflatoxin fraction (5 mL 4% H_2O/acetone), 100 µL 1.5% acetone/$CHCl_3$ was added to the

flask. This assured complete recovery from the walls of the flask and any residual Florisil which might have been flushed from the column. An additional 50 µL of hexane was added to reduce the overall polarity of the solvent. This 150 µL mixture was then applied to the plate as described previously. A check of the procedure with standards showed no losses. Figure 21 illustrates the spot shapes obtained using these techniques. Note the difference in spot width when $CHCl_3$ is the solvent (Figure 21c), compared to 40% methanol/$CHCl_3$ (Figure 21b).

In addition, the applicator is useful for protecting O_2 or H_2O labile compounds from decomposition. Aflatoxins B1 and G1 are H_2O sensitive, which requires solvent evaporation with N_2 flow in the applicator, both after pre-development and development.

Developing Solvent

A variety of solvent mixtures have been suggested for separating the four aflatoxins [5]. Individual TLC plates and laboratory conditions vary to such an extent that a universal developing solvent is not practical. A test of all suggested solvent systems indicates that a mixture of chloroform, *iso*-amyl alcohol, trichloroethane, and formic acid provides the best separation.

Further experiments revealed that trichloroethane did not significantly affect resolution. The best resolution

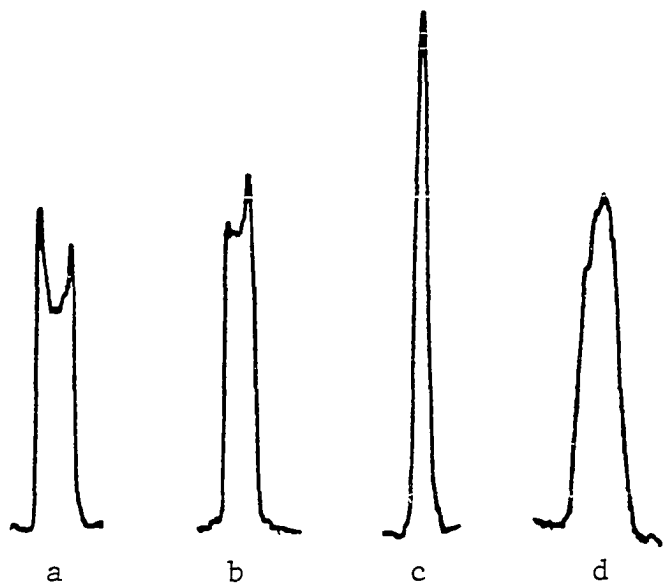


Figure 21. Fluorescence scan profiles of 500 pg each of the aflatoxins obtained using the TLC sample applicator: (a) in 5 µL 40% methanol/$CHCl_3$, no N_2 flow, (b) in 5 µL 40% methanol/$CHCl_3$, N_2 flow, (c) in 5 µL $CHCl_3$, N_2 flow, (d) in 150 µL (100 µL 1.5% acetone/$CHCl_3$, 50 µL hexane), N_2 flow

was obtained using $CHCl_3$/*iso*-amyl alcohol/formic acid (95+4+1). Plates were developed for 1 hour in cylindrical containers in an atmosphere saturated with solvent vapor (filter paper liners). After initial solvent removal with the TLC sample applicator, the plates were dried 30 minutes in a vacuum desiccator (<0.1 mm Hg). Failure to remove the developing solvent completely resulted in additional background fluorescence.

Few generalizations could be drawn concerning optimization of separation. Increasing concentrations of formic acid increased the resolution between the B-group and G-group of aflatoxins. However, B1/B2 and G1/G2 resolution was poorer. *Iso*-amyl alcohol had the opposite effect.

For real samples, a blue fluorescing component appeared near B1. Addition of 5% or 10% hexane to the mixture improved the separation. The eventual use of an internal standard required some compromises in resolution. However, the best resolution for the five peaks and most complete separation from interfering components was obtained using $CHCl_3$/hexane/*iso*-amyl alcohol/formic acid (89.4 + 5 + 4 + 1.6). Values of R_F for the aflatoxins and internal standard are given in Table VI.

The developing solvent mixture is not stable for more than one day. Resolution deteriorates and R_F decreases, presumably due to formation of *iso*-amyl formate.

Table VI

Values of R_F for aflatoxins and internal standard in chloroform/hexane/*iso*-amyl alcohol/formic acid (89.4 + 5 + 4 + 1.6)

B1	0.32
B2	0.25
G1	0.22
G2	0.16
Int. Std.	0.14

Fluorescent Derivative of Sterigmatocystin

Sterigmatocystin exhibits weak natural fluorescence. An aluminum derivative, however, produces a more intense yellow fluorescence. An ethanol solution of $AlCl_3 \cdot 6H_2O$ is sprayed on the plate and fluorescence is observed after heating [5]. The intensity of fluorescence is sensitive to the amount of $AlCl_3$ added, but too much causes diffusion of the spots.

A better solution is saturated $AlCl_3 \cdot 6H_2O$ in $CHCl_3$/methanol (90+10). After heating at 130°C for 5 minutes, the spot is smaller. If aflatoxins are to be determined on the same plate, a slight loss of fluorescence intensity will be noted after heating. However, the loss of fluorescence intensity is less at 130°C than if the plate is heated for a longer time at a lower temperature.

ANALYSIS OF TLC PLATES BY LASER FLUORESCENCE

Apparatus

The TLC fluorescence scanning system consists of a pulsed N_2 laser which emits light at 337.1 nm. The laser output is focused to a sharp line with a cylindrical quartz lens. The incident radiation strikes the plate at an angle of approximately 45°. Fluorescence normal to the plate is focused onto the entrance slit of a 1/4-meter monochromator, which is set at 440 nm. The current produced by the photomultiplier tube is amplified and directed, through an RC time constant circuit, to a digital voltmeter, an X-Y recorder, and an integrator. A block diagram of the system is given in Figure 22.

The TLC plate is held in a screw-driven slide assembly on the bottom of an enclosed box containing the focusing and light collecting optics. The laser is focused on the TLC plate, which is scanned manually across the beam. The screw drive also turns a 10-turn helical potentiometer. The voltage drop across the potentiometer, provided by a 9V battery, drives the X-axis of the X-Y recorder. This allows scanning in either direction and permits the operator to stop/start the scan when necessary.

Figure 23 describes the integrator developed for this system. An optical interrupter mounted on the screw of the

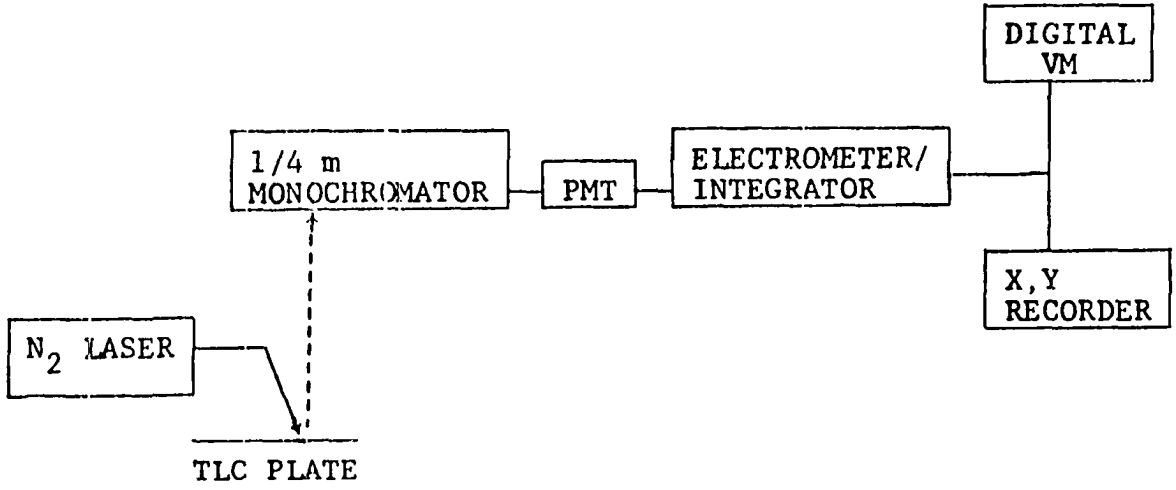


Figure 22. Block diagram of the TLC fluorescence scanning system

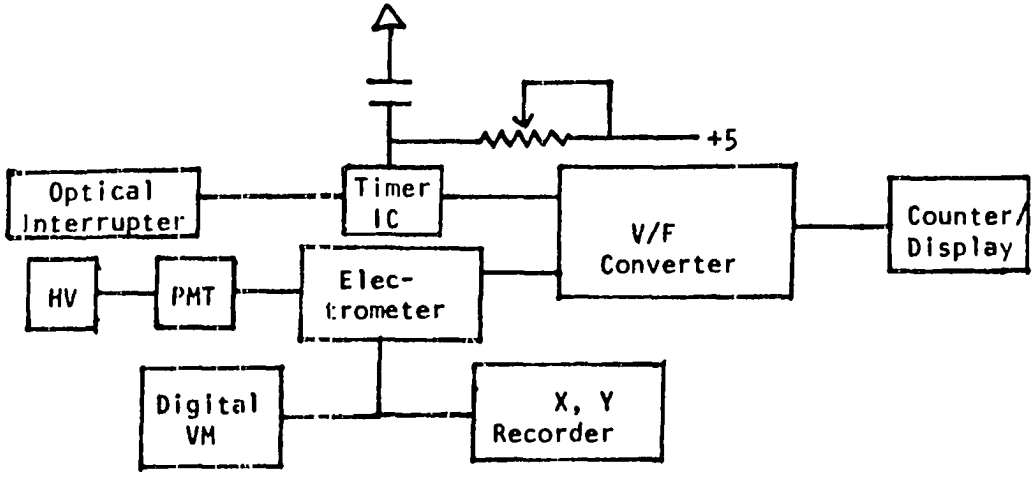


Figure 23. Block diagram of the integrator

plate drive signals the integrator every 1/4 turn. This turns the timer integrated circuit (IC) "on", allowing the V/F converter and counter to collect data from the voltage output of the amplifier. The length of time the IC is "on" is determined by the RC-constant of the IC, which can be varied with a 10-turn potentiometer. The result is a histogram of data points, the sum of which is proportional to the area of the peak. Approximately 25-30 points are collected for an average spot on a TLC plate. The X-axis for this system is position instead of time. Since the integrator only "counts" when "triggered" the scan can be stopped at any position (time) without loss of data. This equipment was designed and constructed in collaboration with Mal Iles (Spectrochemistry Group, Ames Laboratory - USDOE).

Optimization of Parameters

The laser output was restricted by a 5 mm diaphragm before focusing. This provided a sharper focused image on the TLC plate. Smaller diaphragm widths produced sharper images, but with no improvement of the signal/noise ratio.

All optical components were adjusted to maximize the signal reaching the photomultiplier. The collection optics were focused with 430 nm light from a Hg lamp.

A considerable amount of electrical noise was present when using the most sensitive amplifer scale (1×10^{-9} A full scale). The noise was time related, and therefore not

a problem with spatial irregularities on the TLC plates. Operation of the laser at different nitrogen pressures failed to produce any improvement.

The background and noise levels were measured for varying source intensity using neutral density filters. A constant ratio would indicate noise in the source, due to a linear dependence of both variables on source intensity. However, the noise was found to be an approximate square-root function of the intensity, indicating that the noise was in the photomultiplier tube. The tube was replaced with one less sensitive to red light which did not require cooling (RCA 1P28).

The pulse rate of the laser (20 Hz) and the noise made it difficult to maintain a proper baseline with the offset on the amplifier. A 500 ms time constant was placed in the circuit after the amplifer. This influenced the pen on the X-Y recorder, digital voltmeter, and the integrator. Stable baselines were obtained, but the scan rate was reduced to 0.5 - 1 minute per peak. Typical reproducibility values (% RSD) for a set of developed spots were 1.7, 1.5, 2.7, and 1.6% for aflatoxins B1,, B2, G1, and G2, respectively. These reproducibility values include the fact that photo-decomposition decreased the peak area after each scan. Actual reproducibility values (i.e., not including decomposition) should be lower.

Determination of Aflatoxin Standards

Figure 24 illustrates the fluorescence emission curves for the 4 aflatoxins, the internal standard, and background (λ_{ex}=337.1). The wavelengths of maximum emission for B1, B2, G1, G2, internal standard, and background are 428, 426, 442, 438, 432, and 405 nm, respectively. All subsequent data were collected at 440 nm, which provided the best compromise between the fluorescence of the aflatoxins and background.

Figure 25 represents typical scans of developed TLC plates at 3 different concentrations of aflatoxins. The direction of development is to the left in the figure. The individual peaks are, left to right, B1, B2, G1, G2. Note that 10 pg appears to be the detection limit for this system. At the amplifier sensitivity used to detect 10 pg, plate irregularities become the limiting factor. Also, photodecomposition occurs to such an extent that, below 100 pg, only one scan across the spots is allowed. Subsequent scans show significant (i.e. >25%) loss.

In Figure 25a, the baseline is easily adjusted to zero with the amplifier offset. The resolution is sufficient so that the integration is stopped/started at the valley between peaks. However, as shown in Figures 25b and 25c, below 500 pg the change in baseline over the region of interest is too great, relative to peak heights, to allow

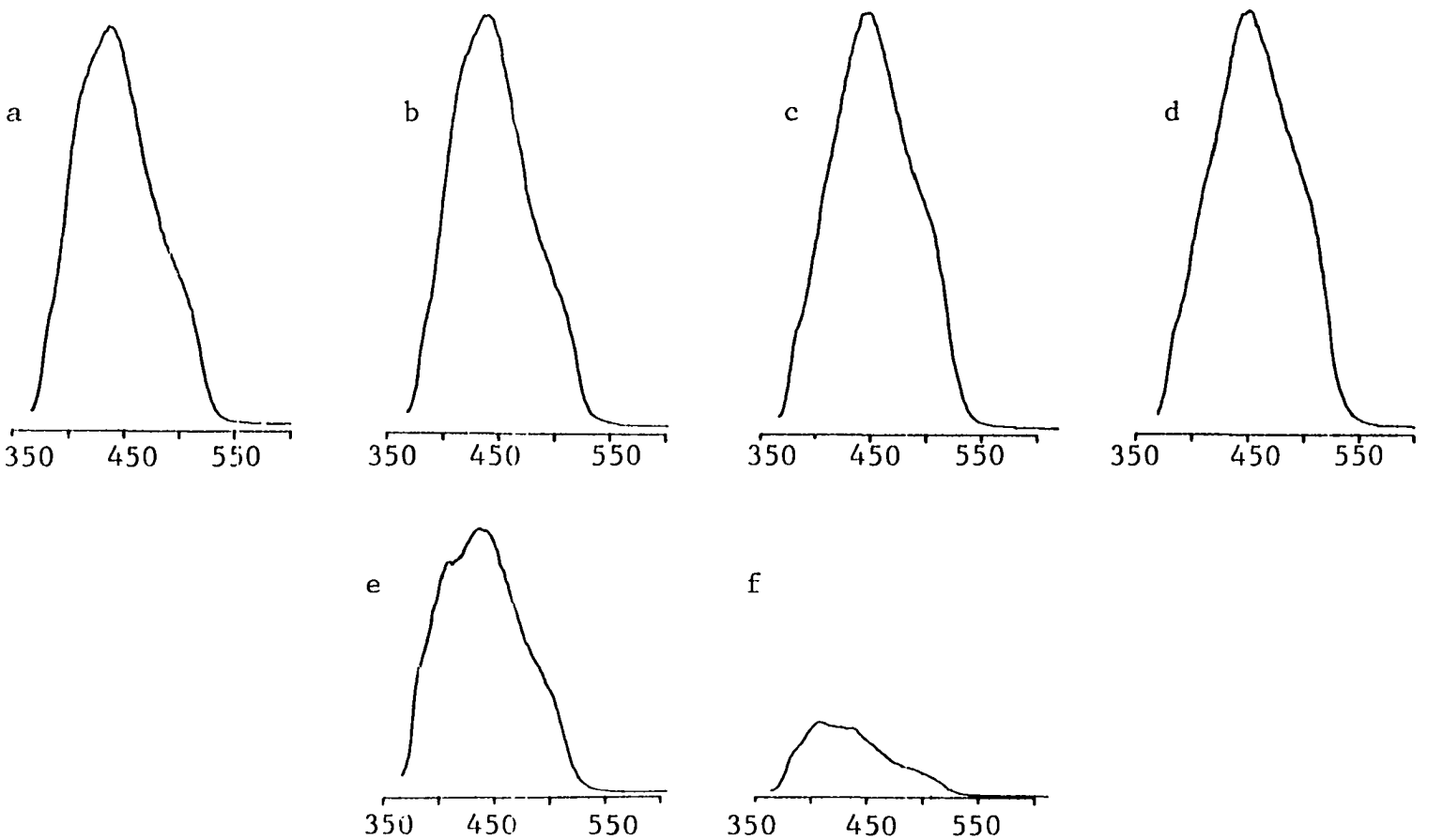


Figure 24. Intensity of fluorescence vs. wavelength (nm) on a TLC plate: (a) aflatoxin B1, (b) B2, (c) G1, (d) G2, (e) internal standard, (f) plate background

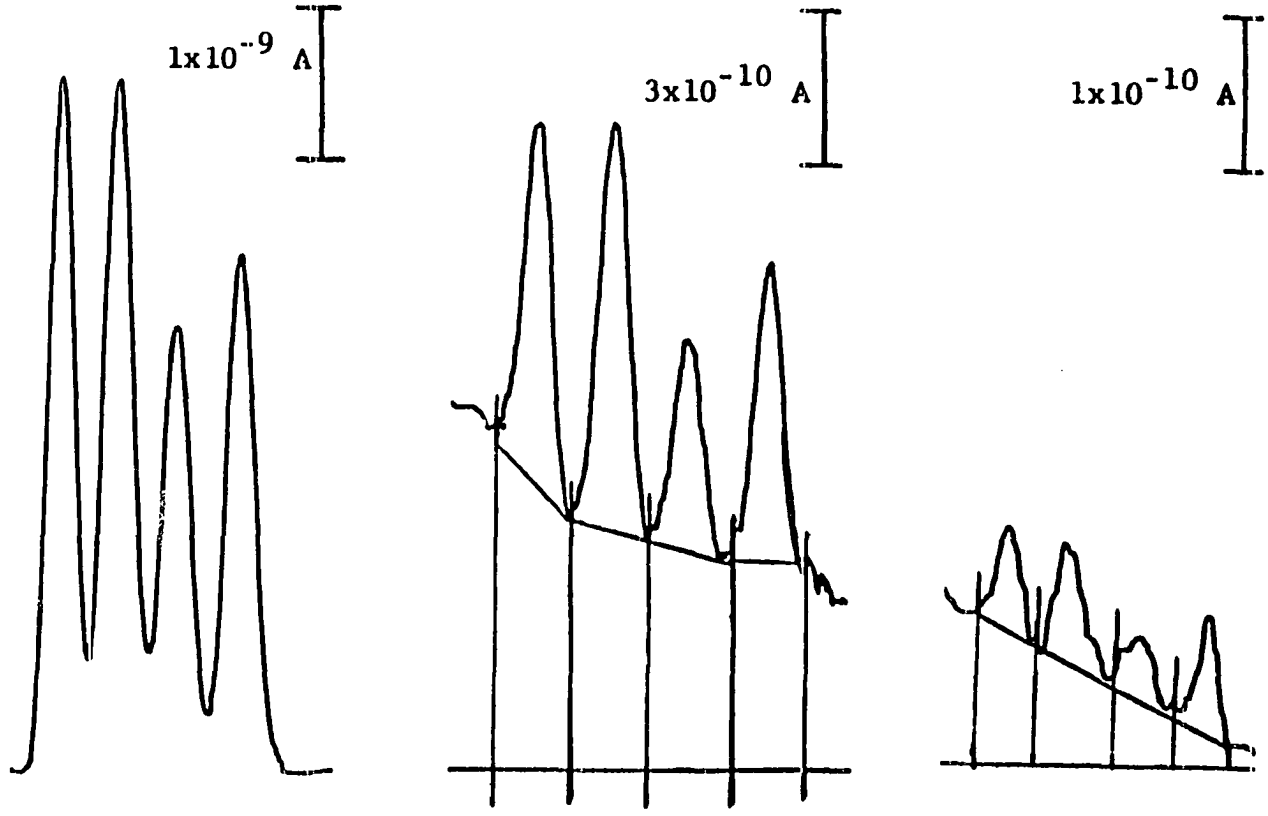


Figure 25. Typical fluorescence scans of aflatoxins on developed TLC plates. Direction of development is to the left. The peaks represent, l to r, B1, B2, G1, G2: (a) 1 ng each, (b) 100 pg each, (c) 10 pg each

proper use of the offset.

Figure 25b and 25c illustrate the method of background correction used. The offset is adjusted so that the lower background level, usually at lower R_F than G2, has a positive value. The stop/start points for the integration of each peak are indicated by the vertical lines. The horizontal line representing "zero" is also shown. The area of the resulting trapezoid is then measured. In a separate experiment, it has been determined that the conversion factor for this system is 4.15 integrator areas/mm^2. This number is then subtracted from the area obtained from the scan to obtain the peak area.

A calibration curve for the 4 aflatoxins using these techniques is shown in Figure 26. The curves appear linear over almost two orders of magnitude. There is some curvature above 1000 pg. The %RSD for each set of data points ranges from less than 5% for higher concentrations to 25% for the lower concentrations.

Fluorescence Enhancement

It has been reported recently that TLC fluorescence could be enhanced by spraying the plate with viscous solvents (i.e., paraffin oil/hexane, glycerol/methanol) [85]. Fluorescence enhancement factors were reported to be greater than 10 for some compounds. It was not possible to spray uniformly a reproducible layer onto the plate.

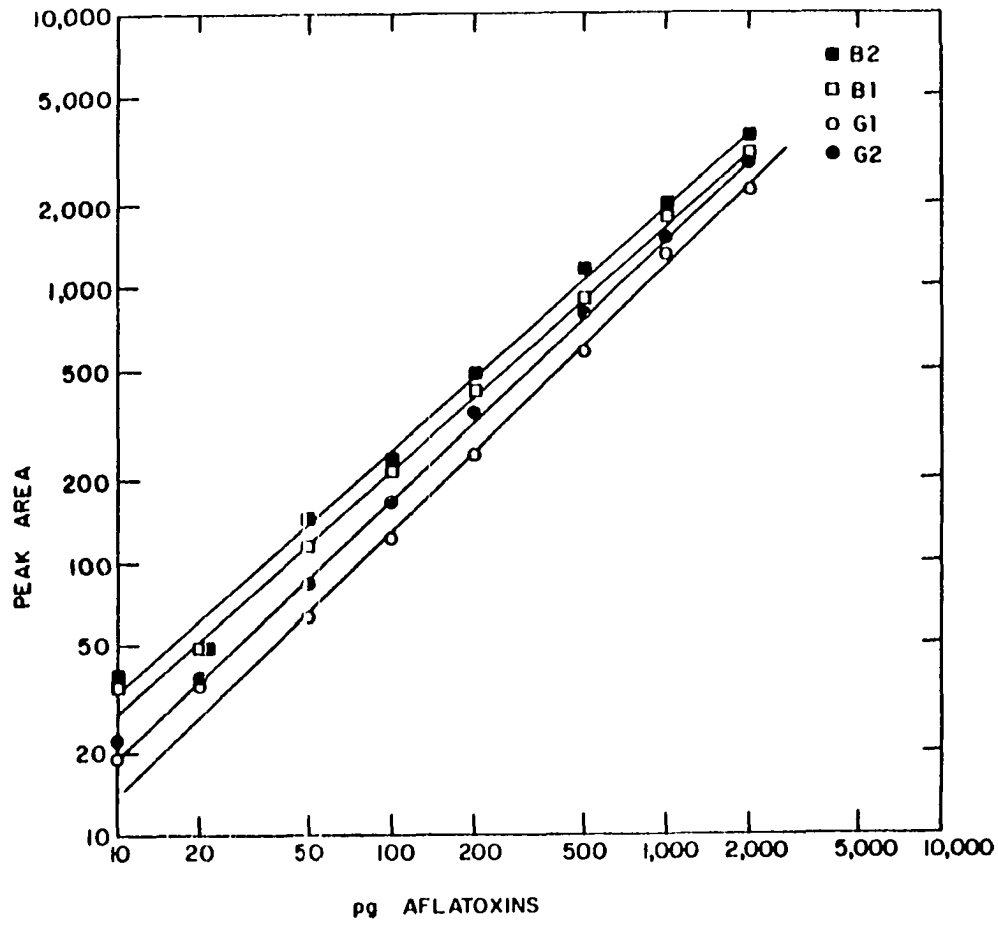


Figure 26. Calibration curve for aflatoxin standards on developed TLC plates

Instead, plates were dipped in 5-50% solutions of paraffin oil/hexane until the plate coating appeared uniform. After initial solvent removal, the plates were dried and analyzed. Figure 27 illustrates the enhancement for two aflatoxins. The maximum occurred near 30% paraffin oil. An example of the enhancement is given in Figure 28.

The mechanism of enhancement is not obvious. The peak shape did not change and there were no changes in the emission spectrum.

Fluorescence quenching by O_2 has been often observed [86]. The paraffin oil could function by excluding O_2 from the surface of the plate. The system was adapted so that N_2 could be flushed over the plate at the point of excitation. Cooling of the N_2 on the plate with liquid N_2 produced no additional fluorescence.

Fluorescence enhancement factors for nitrogen are given in Table VII. Nitrogen alone provides 60-70% of the enhancement produced by paraffin oil. However, it is not clear whether this indicates a different mechanism or lower O_2 exclusion efficiency for this particular case. If a small amount of O_2 is allowed to mix with the N_2 flow, the fluorescent intensity is reduced to the level observed in air. Pure O_2 almost completely quenches the fluorescence of the sterigmatocystin-Al derivative, while the fluorescence of the aflatoxins is decreased by a factor of two.

Table VII

Fluorescence enhancement factors for the four aflatoxins and the sterigmatocystin-Al derivative (SM_{Al})

	Enhancement Factor		
	N_2	30% Paraffin Oil	20% Glycerol
SM_{Al}	3	3.3	7.3
B1	1.3	2.4	0.8
B2	1.3	2.2	1.1
G1	1.5	2.3	0.7
G2	1.5	2.7	1.1

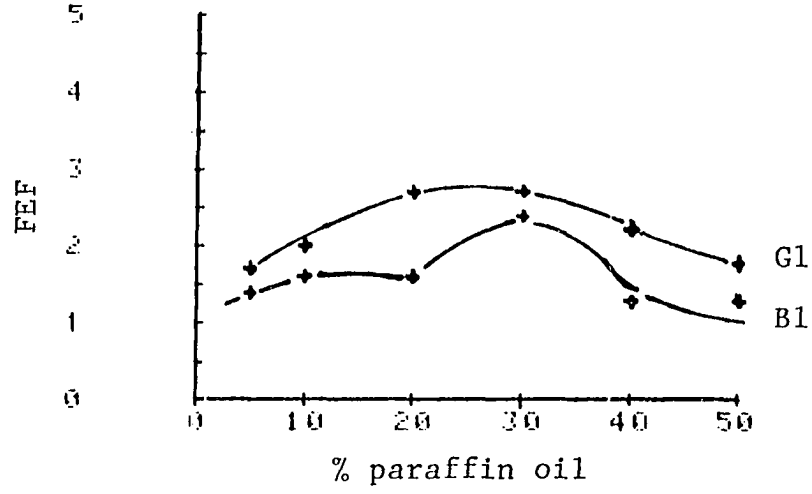


Figure 27. Fluorescence enhancement factors (FEF) for aflatoxins B1 and G2 on TLC plates dipped in paraffin oil/hexane solutions

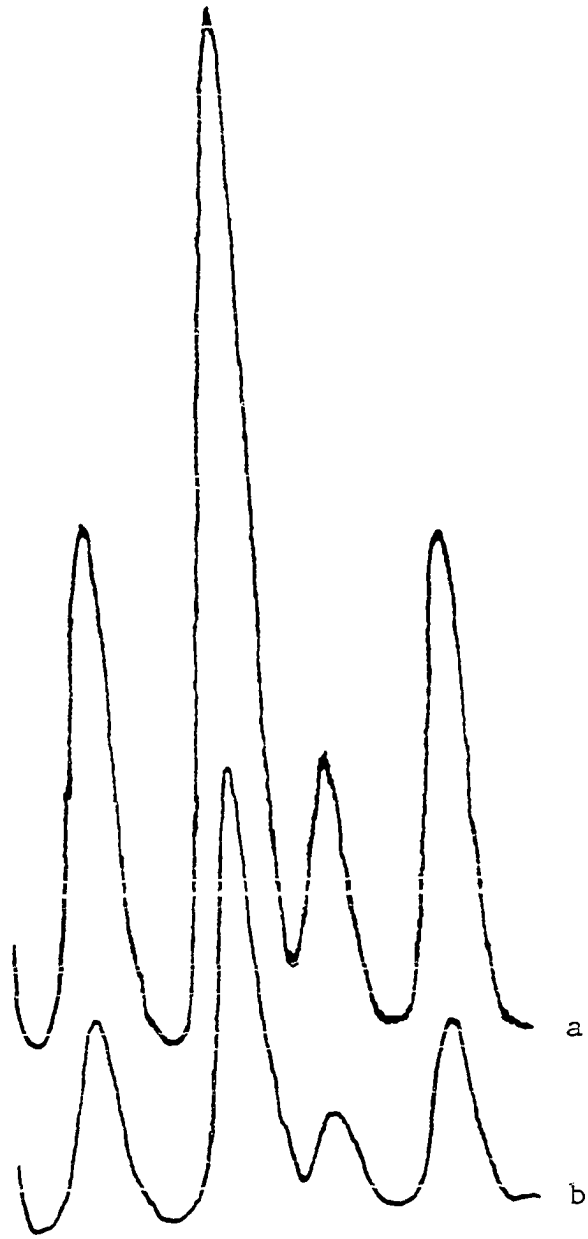


Figure 28. Fluorescence scan of 4 aflatoxins on a developed TLC plate: (a) after exposure to 30% paraffin oil/hexane, (b) before exposure

These data seem to indicate that the paraffin oil functions by reducing O_2 quenching.

An attempt was made to correlate the enhancement with fluorescence lifetimes. Photographs of the photomultiplier signal vs. time were recorded from an oscilloscope. Plots of log (intensity) vs. time were made for the time period starting after the laser pulse began to decay (10-15 ns). Assuming a first order decay, the slope of the linear portion would be -k/2.303 where k represents the decay constant. The lifetime, τ, was defined as,

$$\tau = 1/k \tag{14}$$

Results are given in Table VIII. Unfortunately, there appears to be no direct relationship between lifetime and fluorescence enhancement. It should be noted that since the background lifetime is of the same magnitude as the mycotoxins, time-resolved (i.e., gating) techniques offer no improvement in signal/background ratios.

While paraffin oil did increase the fluorescence of the aflatoxins, it also increased the noise and background levels. Its primary advantage here would be mainly to protect developed plates from decomposition and was not studied further.

Table VIII

Fluorescent lifetimes (τ) for mycotoxins

	τ (ns)			
	O_2	N_2	30% Paraffin Oil	20% Glycerol
SM_{A1}	7.2	14.7	15.6	12.8
B1	5.3	8.6	5.3	4.4
B2	4.6	7.4	4.5	7.7
G1	6.6	8.8	8.2	6.0
G2	6.1	8.4	8.0	7.2
Background	5.7	11.4	6.2	4.6

Quenching Phenomena

All previous steps in the analytical scheme have shown essentially quantitative recoveries. However, when 500 pg each of the aflatoxins was added to the sample just before application to the spot, a 50-70% recovery was obtained.

In Figure 29, the peak heights (or areas) are less than the same level of standards developed on the same plate. Notice, however, that the aflatoxins are resolved without interference from matrix components. In fact, the lower background on this section of the plate provided a "window of vulnerability" to analysis.

The recovery was not dependent on the concentration of aflatoxins added to the plate. When up to 2 ng was added to the sample, the recovery remained constant when compared to similarly treated standards. This eliminated any constant mass losses due to chromatography or sample handling problems. In addition, dilution of the sample did not increase recovery.

To eliminate specific chemical interferences, samples were extracted with equal volumes of various reagents before injection onto the Styragel column. Thus, extractions with 0.1 M HCl, 5% NaOH, 1% $KMnO_4$, 5% SO_3^{-2}/OH^-, and 5% EDTA had little effect on recovery. While some individual matrix components were affected by some reagents, the overall background level was unaffected. The absorbance trace of

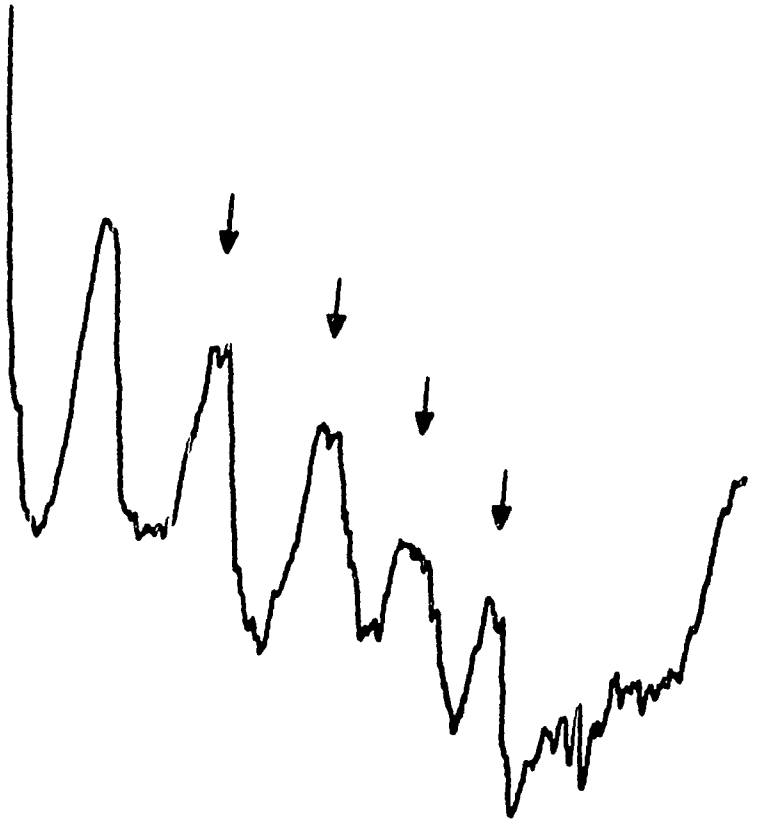


Figure 29. Fluorescence scan of an RDF sample developed on a TLC plate. The sample was spiked with 500 pg each of the aflatoxins (arrows) just before application to the plate

material eluting from the Styragel columns did not change.

The data indicate an absorption type of quenching. Emission spectra of the aflatoxins did not change in the presence of the matrix, eliminating coupling between similar states of background and aflatoxin. It is possible that the background absorption is so high that the incident intensity (I_o) is effectively reduced. The effect would be a "lowering" of the aflatoxin calibration curve, since fluorescence intensity is proportional to I_o. Unfortunately, losses are also observed in spiked peanut butter, where the background fluorescence level is only slightly higher than background. This indicates a change in surface characteristics such as absorptivity and scattering coefficients. Both background absorption and surface phenomena are probably responsible.

Development of an Internal Standard

Fluorescence loss due to matrix effects should be corrected through the use of a internal standard. It is assumed that the added internal standard will be affected by the matrix to the same extent as the aflatoxins. Therefore, the area ratio of aflatoxin/internal standard should remain constant for real sample or standard.

For this analytical problem the selection of an appropriate standard is difficult. The compound must absorb light at 337.1 nm and fluoresce at 440 nm. Its R_F value must be similar to that of the aflatoxins and fall within

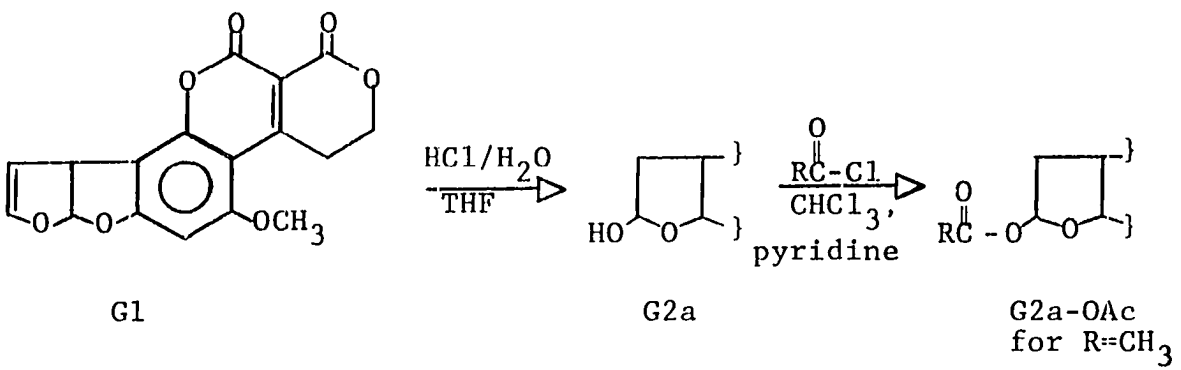
the analytical "window" created by the background fluorescence. An aflatoxin derivative seems most appropriate.

The synthesis of an internal standard is illustrated in Figure 30. A commercially available derivative is aflatoxin G2a. It is formed by hydration of the double bond in the furan ring of aflatoxin G1. The R_F value for G2a is very low (<0.1) and unsuitable. It is known that alcohols adsorb strongly to silica. This explains the greater retention of G2a, since the rest of the molecule remains unchanged. The retention of the molecule should be reduced (i.e. greater R_F) by modification of the alcohol moiety. This is most readily accomplished through esterification, since esters are not retained as strongly on silica.

Inductive effects

Figure 31 illustrates retention characteristics of series of substituted acetate esters of G2a (G2a-OAc). As expected, G2a-OAc has a greater R_F value. It is now partially resolved from G2.

Using similar arguments, one could predict a change in retention of the ester if its electronic characteristics were altered. By polarizing the ester bonds, the energy of adsorption, and, as a result, retention, would increase (i.e. lower R_F). This can be accomplished by introducing electronegative Cl-atoms. Due to the complexity of the



G1 G2a G2a-OAc for R=CH_3

Figure 30. Synthesis of the internal standard

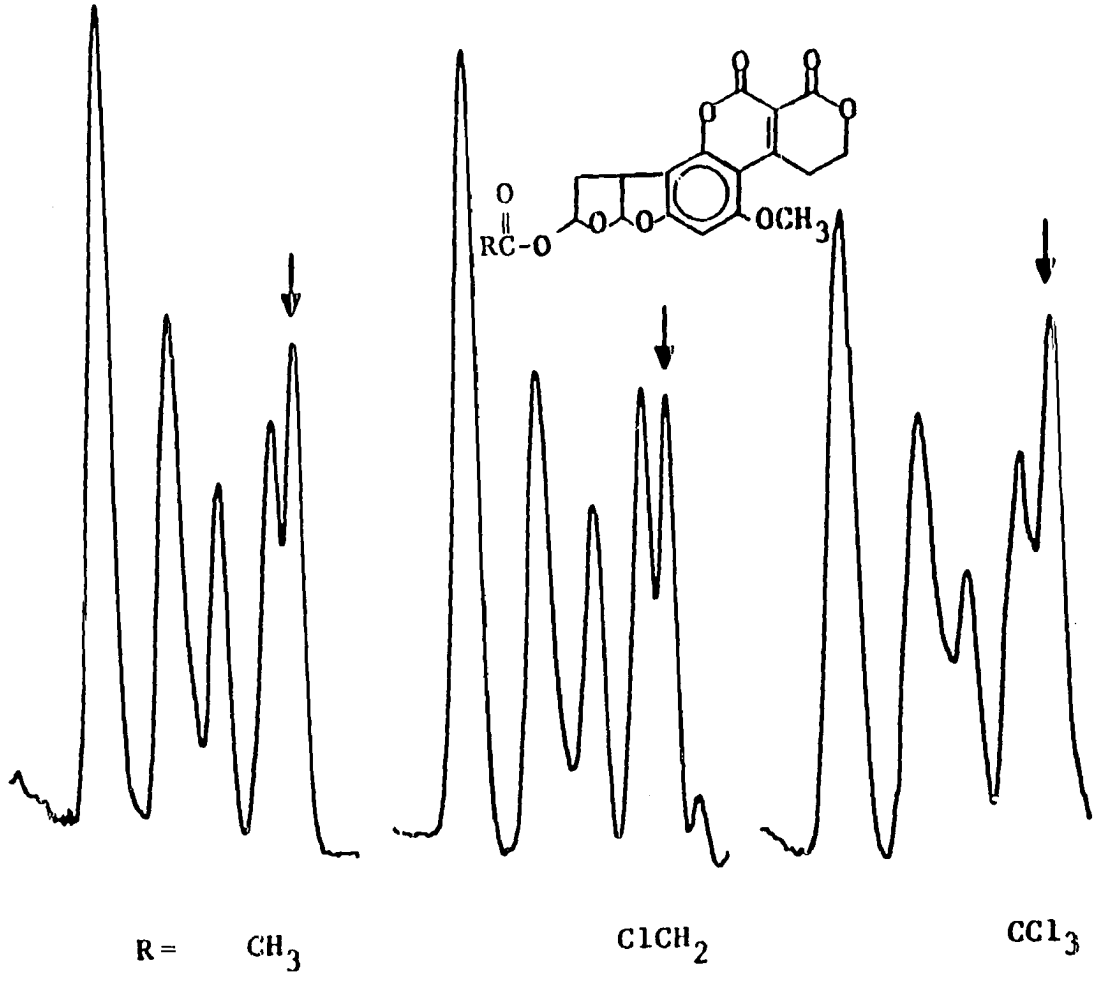


Figure 31. Retention characteristics of substituted esters of aflatoxin G2a

molecule, the effect is not pronounced here, but the distance between G2 and the ester derivative does increase with increasing Cl-substitution. The actual resolution is better than indicated in Figure 31, due to the presence of impurities in the derivative reaction mixtures.

Unfortunately, the mono- and trichloro-esters are unstable and cannot be purified by TLC or HPLC. The aflatoxin derivative, G2a-OAc, is easily purified by preparatory TLC and may be synthesized from either G1 or G2a, depending on availability. It is, however, somewhat unstable in the presence of methanol.

Analysis using the internal standard

The TLC developing solvent was modified, as described earlier, to maximize the G2/G2a-OAc separation. The internal standard was added to all standard solutions and samples at a level equivalent to 200 pg aflatoxin/5 µL. This represented the middle concentration range of the calibration curve.

Figure 32 illustrates results using the internal standard. The lower curve is a TLC scan of developed standards. The upper curve represents the same level of aflatoxins and internal standard added to a real sample. Although a difference in peak heights was expected, the ratio between the area (or height) of the internal standard and the aflatoxins remains constant. All quenching effects are now corrected.

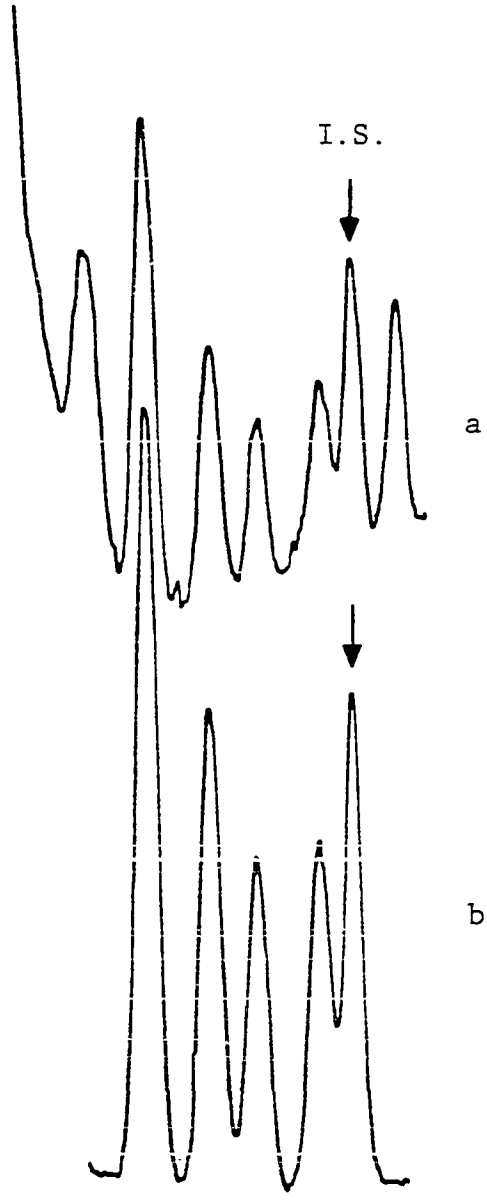


Figure 32. Quenching effects for RDF samples on TLC plates: (a) fluorescence scan of an RDF sample developed on a TLC plate, spiked with aflatoxins and internal standard, (b) identical levels of aflatoxin standards and internal standard developed on the same plate

The B1 ratio is slightly higher in the sample, indicating the possible presence of B1.

Calibration Curves

The logarithmic calibration curves for each of the four aflatoxins are presented in Figure 33. The concentration range is 10 pg to 5000 pg aflatoxin standard applied to the TLC plate. The data and % RSD are given in Table IX. As expected the % RSD is less at intermediate concentrations, where the aflatoxins and internal standard are present in similar amounts.

In Figure 33, the data points show a good correlation with a second-order polynomial. Non-linearity at high concentration could indicate self-quenching or electronic saturation. Theoretical discussions [87] have indicated that TLC fluorescence calibration curves should show non-linearity at higher concentrations. However, Figure 34 indicates that the non-linearity is due to photomultiplier saturation. The data are linear, however, at concentrations below 250 pg (Figure 35).

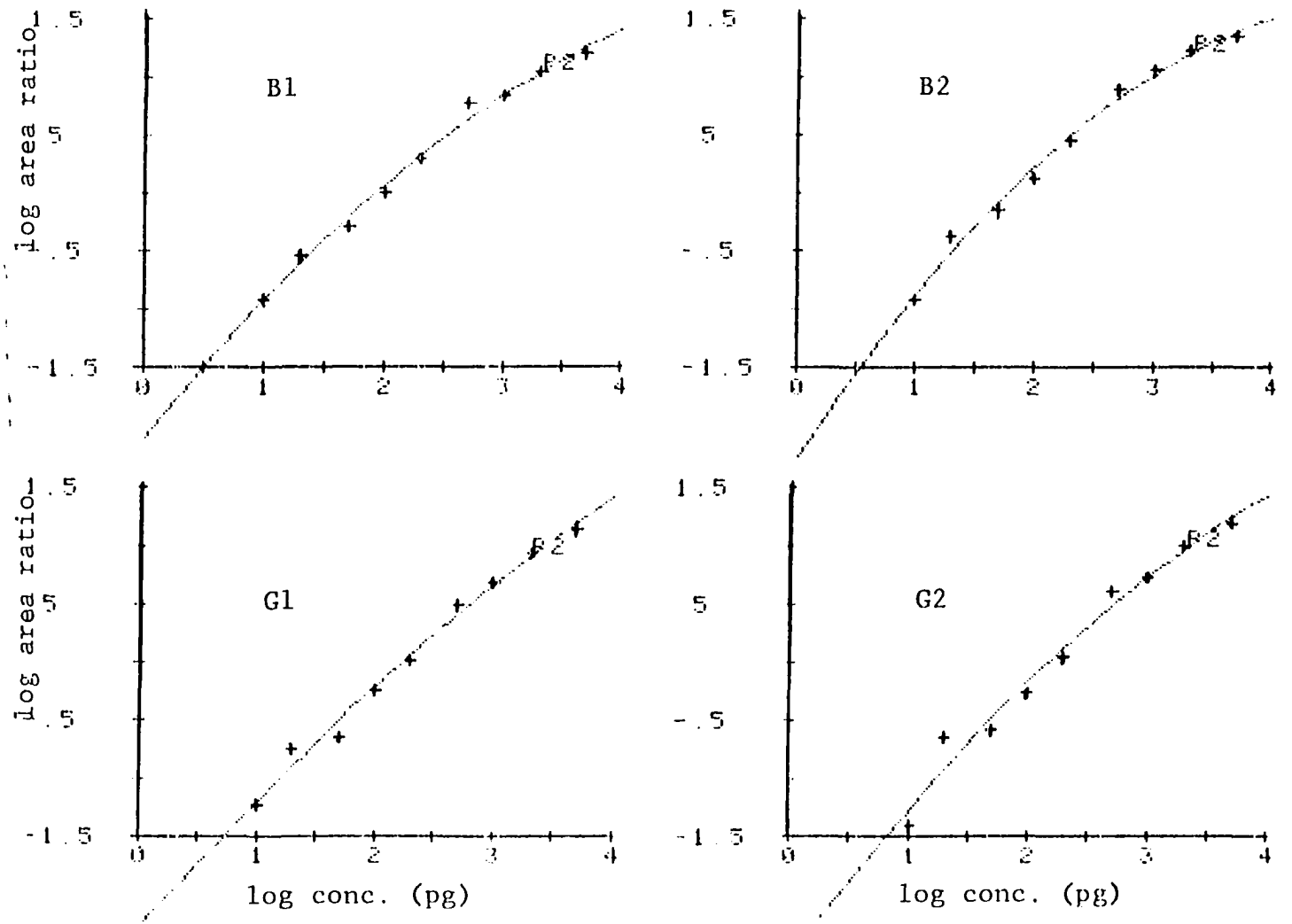


Figure 33. Log (peak area/internal standard) ratio vs. log concentration (pg) calibration curves for four aflatoxins

Table IX.

Data for calibration curves

Concentration (pg)	B1		B2		G1		G2	
	Ratio	% RSD	Ratio	% RSD	Ratio	% RSD	Ratio	% RSD
5000	16.1	4.70	22.2	3.54	13.8	6.99	15.4	7.06
2000	11.0	11.6	16.6	11.6	8.54	11.3	10.0	12.9
1000	6.75	16.0	11.3	12.7	4.96	9.38	5.45	8.38
500	6.04	8.43	8.01	5.78	3.12	13.0	4.03	4.45
200	1.92	4.92	2.89	6.70	1.04	13.5	1.12	1.74
100	.985	8.23	1.36	8.07	.552	6.92	.523	17.6
50	.518	8.62	.717	4.00	.222	12.1	.265	19.6
20	.282	34.6	.413	12.9	.176	27.0	.228	15.1
10	.124	29.8	.119	12.6	.058	34.3	.040	61.2

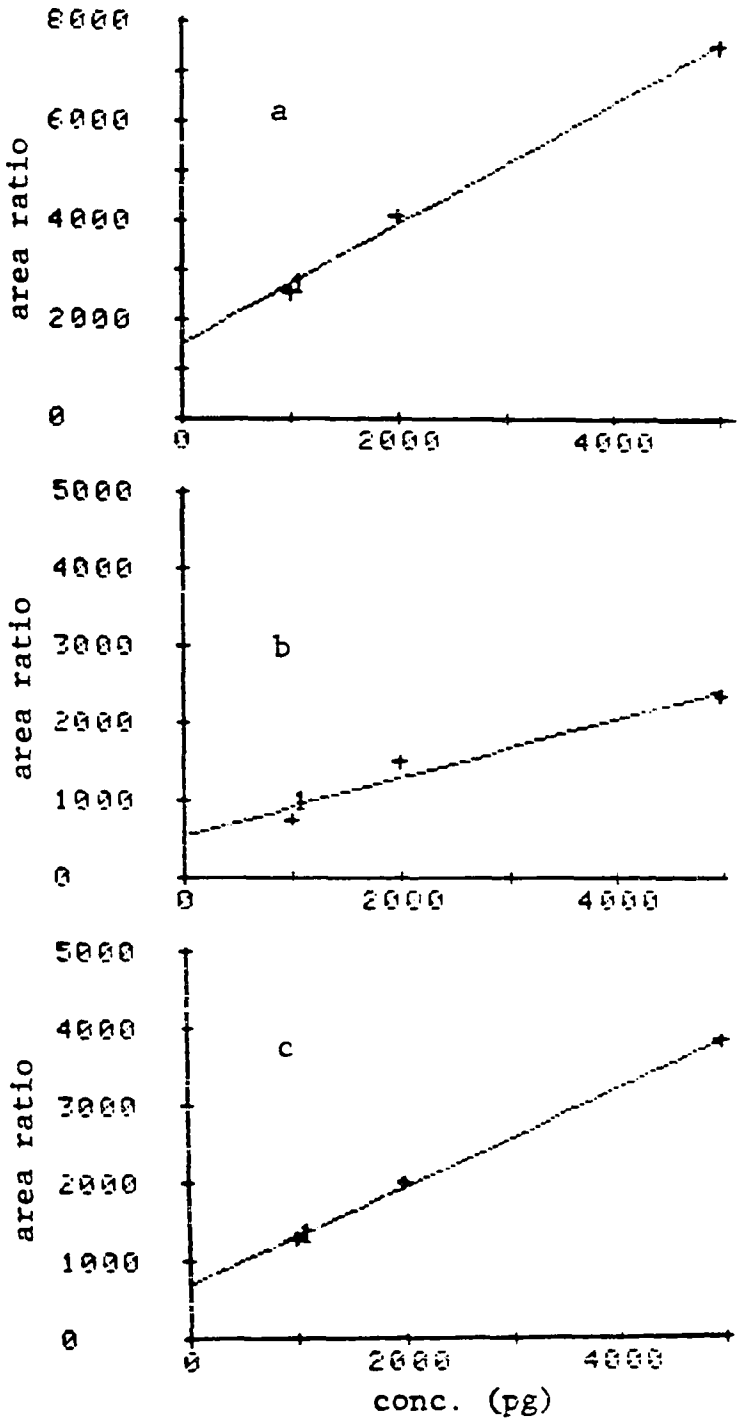


Figure 34. Effect of photomultiplier voltage and amplifer range on non-linearity at high concentrations of aflatoxin B2: (a) 700V, $1x10^{-8}$A, (b) 700V, $3x10^{-8}$A, (c) 600V, $1x10^{-8}$A

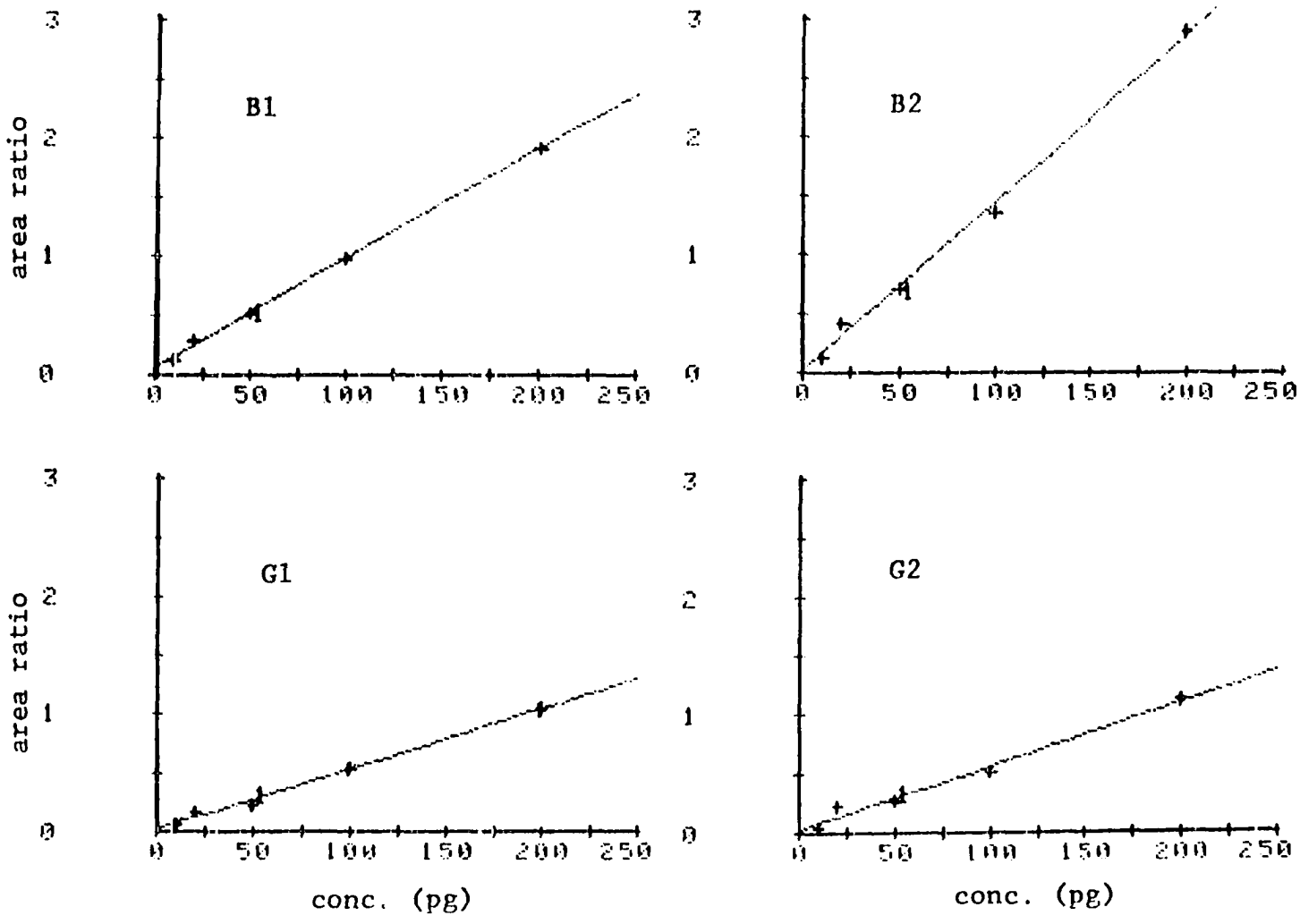


Figure 35. Linear calibration curves for four aflatoxins at low concentrations

ANALYSIS OF REAL SAMPLES

Introduction

An air sample (RDF-1) was collected at the Ames Solid Waste Recovery System during the period (11/18/81 - 11/19/81). The plant had been functioning normally for several days prior to sampling. A second sample (RDF-2) was collected (3/5/82). On that date, the plant first began operations after a one week shutdown. A contaminated ground corn sample was obtained from Dr. John Richards (National Animal Disease Center-USDA, Ames Iowa). A contaminated sample of peanut meal was obtained from Dr. H. M. Stahr (Veterinary Diagnostic Lab, ISU).

The aflatoxin concentration in each sample was calculated using equation 15,

$$\text{Conc.(ppb)} = \frac{X \cdot V}{V_{inj} \cdot W} \qquad (15)$$

where X is the amount of aflatoxin found on the TLC plate (pg), V the volume to which the extract was diluted (mL), V_{inj} the volume injected onto the Styragel column (190 µL in this case), and W the weight of the sample (g).

RDF Samples

Each RDF sample (2.0g) was extracted with 50 mL of methanol. After filtration and precipitation, the samples were diluted to 10 mL with 30% $CH_3CN/CHCl_3$. Representative

elution profiles from Styragel for sample and blank are shown in Figure 36. A standard addition experiment was performed with RDF-1. Additional 2.0 g samples were spiked, before extracting, to provide 20, 50, 100, and 200 pg of each aflatoxin per 190 µL injection onto the Styragel column. Since the entire injected sample is applied to the TLC plate, the spiked levels transfer automatically to the developed TLC plate.

The results are shown in Figure 37. The calculated intercept is 261 pg aflatoxin B1. Using the calibration curves and the ratio from the unspiked sample, a value of 246 pg B1 in the sample is obtained. These concentrations correspond to 6.87 and 6.47 ppb B1, respectively. The agreement indicates that the matrix is not interfering with the analysis and that the recovery of the added standards was good. The standard addition curves for the other aflatoxins showed zero or near-zero intercepts.

A representative example is given in Figure 38. Figure 39 shows a higher level of B1 present in RDF-2. This sample aliquot contains 645 pg B1 (17.0 ppb). The % RSD for this sample is 19.6%. The peak appearing next to the internal standard in both cases is not G2, but an artifact of the analytical method that could not be eliminated.

A blank sample (4.0 g) was treated similarly. Figure 40 shows 115 pg B1 (1.51 ppb) for this sample.

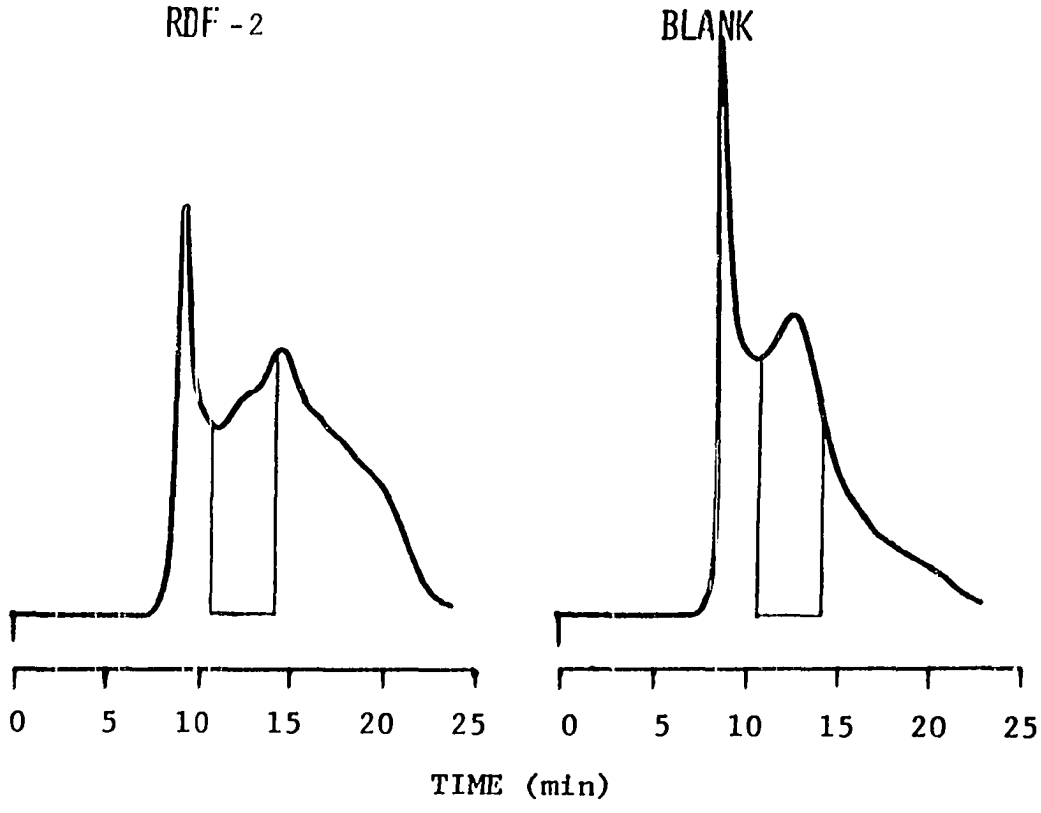


Figure 36. Elution profiles from Styragel for RDF-2 and Blank, showing the aflatoxin containing fraction

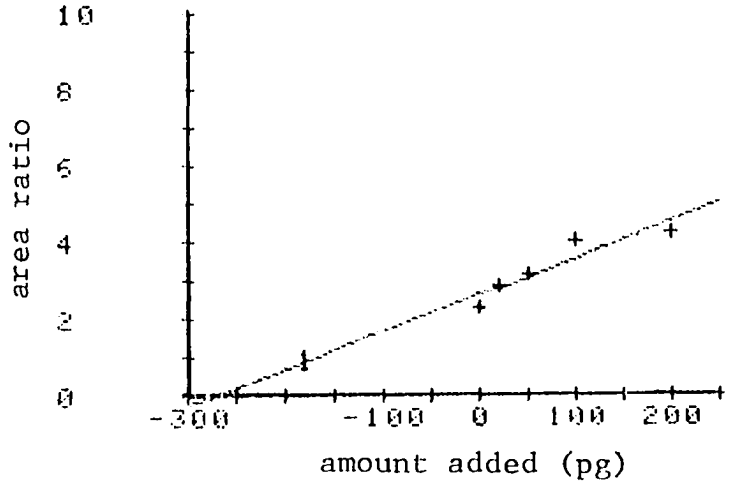


Figure 37. Standard additions experiment for RDF-1. The intercept is 261 pg aflatoxin B1

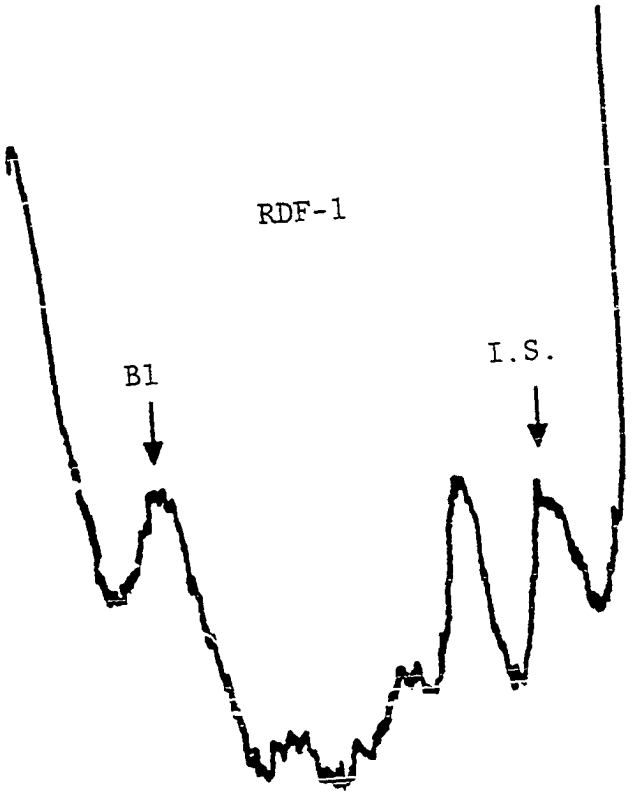


Figure 38. Fluorescence scan of a developed plate showing 6.47 ppb aflatoxin B1 in RDF-1

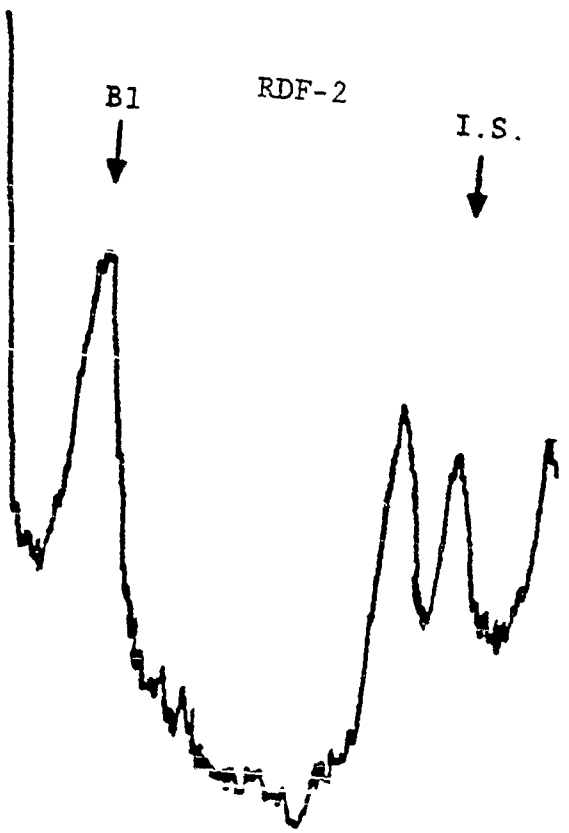


Figure 39. Fluorescence scan of a developed plate showing 17.0 ppb aflatoxin B1 in RDF-2

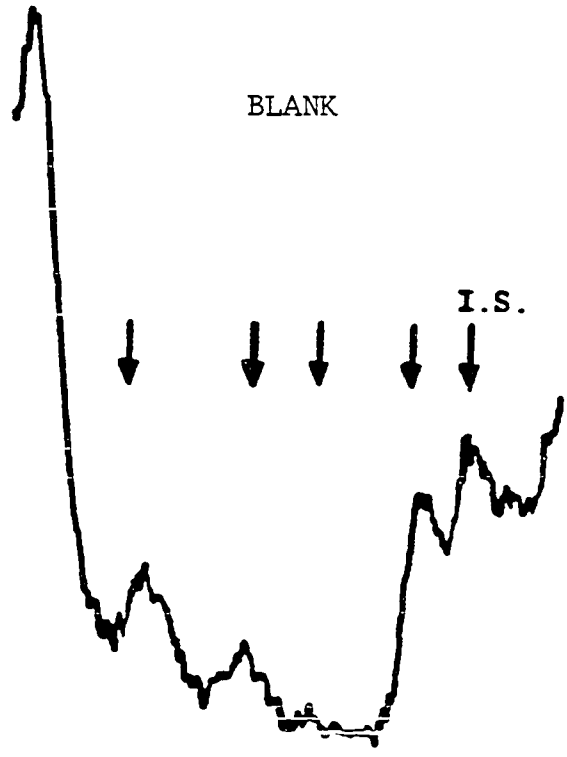


Figure 40. Fluorescence scan of a developed plate showing what could be 1.51 ppb aflatoxin B1 in the Blank. The arrows indicate the expected positions for the four aflatoxins

Corn

A 10.0 g sample of ground corn was extracted using the CB method [5] (5 mL H_2O, 50 mL $CHCl_3$) and diluted to 25 mL. Figure 41 shows the elution from Styragel and the resulting TLC fluorescence scan. Even though the Florisil column was not used for this sample, there are no interferences, and the background level is low. The levels obtained for B1, B2, G1, and G2 are 245, 22.3, 6.2, and 2.0 ppb, respectively. The B1 level may actually be higher, due to saturation problems at this level.

Peanut Meal

A 10.0 g peanut meal sample was treated in the same way as the corn sample. Figure 42 shows the results. The Florisil column was also not used for this sample. The background level is low, but one component interferes with the analysis of G2. However, the other aflatoxins may be easily determined. Levels of 20.4, 2.51, 2.18, and 3.46 ppb are found in this sample.

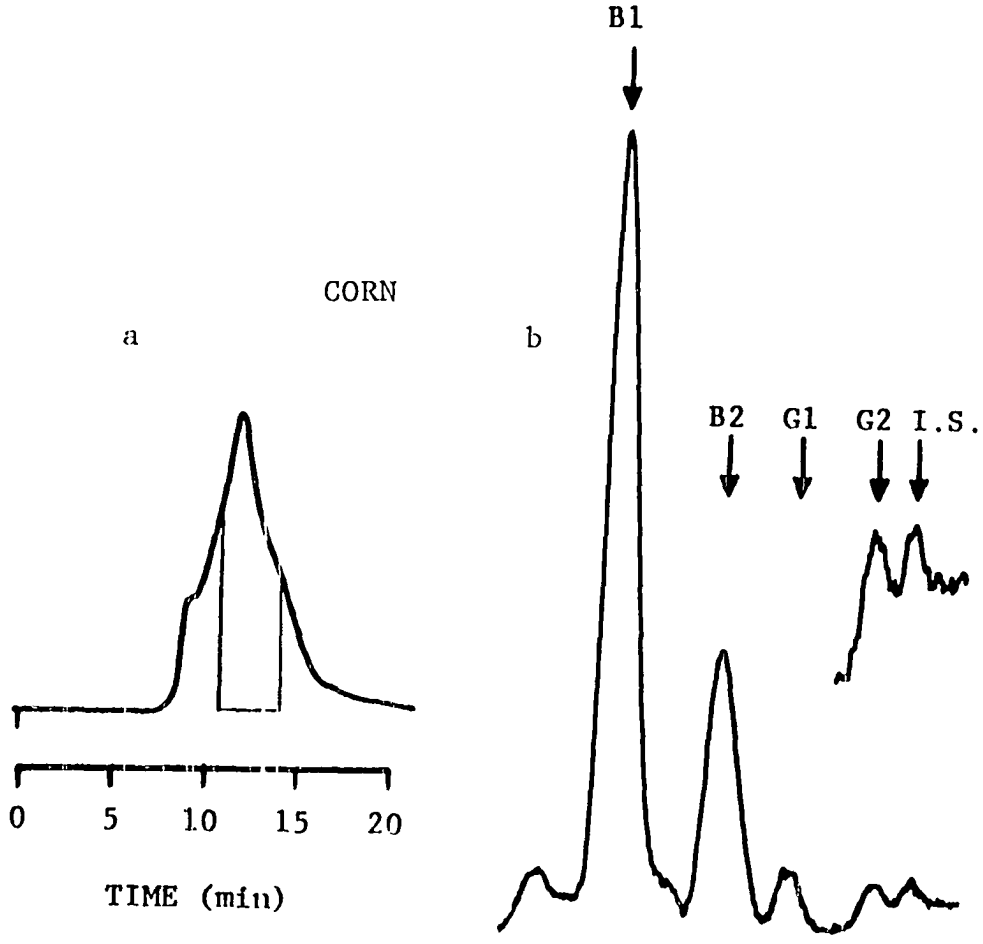


Figure 41. Contaminated corn sample: (a) Styragel elution profile, (b) TLC fluorescence scan showing aflatoxins present in the sample

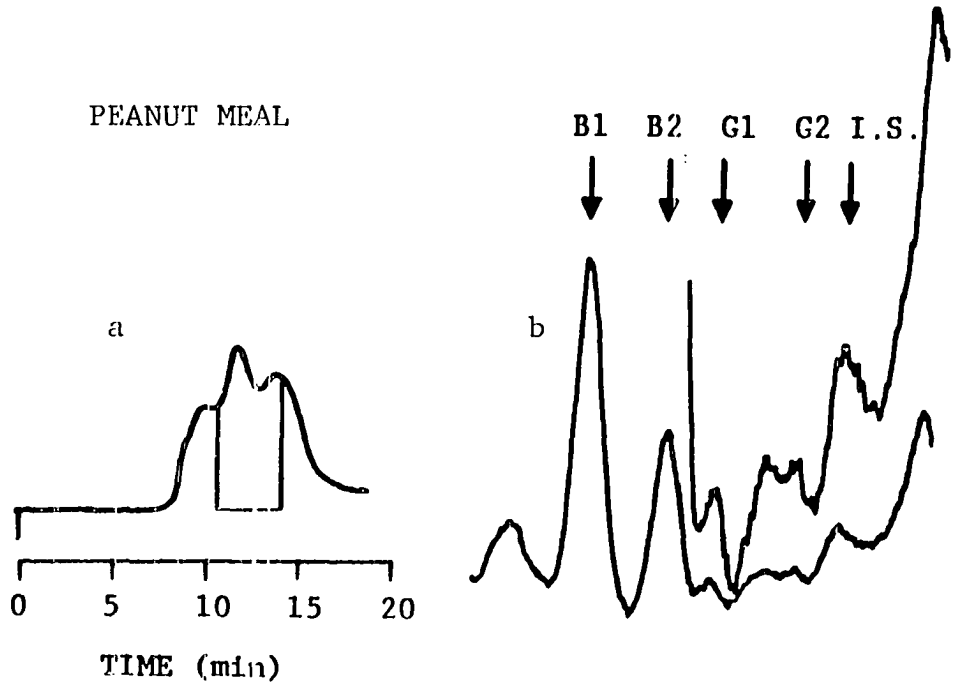


Figure 42. Contaminated peanut meal: (a) Styragel elution profile, (b) TLC fluorescence scan showing aflatoxins present in the sample

CONCLUSIONS

RDF Samples

The presence of aflatoxin B1 in the air of the ASWRS is not surprising. As mentioned earlier, high levels of A. flavus are found in the air. The fact that RDF-2 levels are much higher is predictable. The garbage being processed that day had been in the holding area for a week, during which time the temperatures remained above freezing. The presence of a multitude of organisms was indicated by the increased temperature of the center of the pile.

Chemical Confirmation of Aflatoxin Contamination

The only question remaining is whether or not the peaks labelled B1 in Figures 38 and 39 actually are aflatoxin B1. At this point, the sample has undergone three chromatographic separations and a precipitation step. An interfering component must have an identical R_F-value, absorb light at 337.1, and fluoresce at 440 nm. For these reasons it is likely that the peak is B1.

In addition, the peak shape is similar to the standards and does not change in the standard additions experiment. The peak interfering with G2 was shown to be distinct from G2 in the additions experiment.

Finally, an RDF sample was exposed to H_2O and a small (5-10 µL) amount of trifluoroacetic acid, and heated 10 minutes at 40-50°C. This reaction is an accepted confirmatory test, as it converts B1 to B2a. After removal of the H_2O followed by TLC development, the peak corresponding to B1 had disappeared. An identically treated standard produced the same results. Thus, the presence of B1 in the RDF samples is confirmed.

The labelled peak appearing in the blank is also probably B1. This could represent a "normal" background level, since A. flavus is quite common, but more information is needed.

Critical Evaluation of the Method

Advantages

Use of the Styragel column is the most important step in the analytical method. The investigation of solvent effects has led to a better understanding of the mechanism of separation. This, in turn, produced better separations by taking advantage of the combined exclusion and partitioning processes. The fact that the column is reusable is probably its biggest advantage.

The modification/deactivation of Florisil represents an improvement over existing methods. Care must be taken to avoid premature elution of aflatoxins, but this adsorbent allows recovery at levels below 500 pg. Existing

modifications are concerned with ng levels of aflatoxins. Column design, including the stream-switching values, reduces operator handling and sample exposure. For replicate analyses, the Florisil column can be repacked before the next sample elutes from the column.

The absolute detection limits for the TLC plates are similar to the lowest reported values. The laser fluorescence system is the only one of its kind. The low relative concentration detection limits are possible due to the ability to apply the entire Styragel/Florisil sample to the plate.

In general, the sample preparation eliminates uncertainties between individually packed columns and adsorbents, and provides an overall reduction in time involved. The sample obtained could be analyzed by any other method at this point.

Problems

The uncertainties in the quantitation of developed TLC samples may be the major drawback of the method. The levels for this method range from 5 to 20% for all but the lowest concentrations.

The integrator is the likely source of error. Even though all electronic equipment was carefully shielded, R_F radiation from the laser often caused the integrator to count at random intervals. The integrator stop/start decisions and background correction were performed manually.

An interface to allow computer collection and manipulation of the data should solve this problem. The ultimate limit of variation may be determined by the pulse-to-pulse reproducibility of the laser, which is presently within a few percent.

Sample decomposition may be a problem. Data from RDF-1 were collected during a two-week period, while RDF-2 was analyzed in a few days giving a smaller % RSD. While no clear trends were evident, the aflatoxins are known to be light sensitive and the complexity of the sample matrix makes the possibility of chemical reactions likely.

Modified Florisil had a finite useful lifetime. After 1-2 weeks, the adsorbent was less active, probably due to atmospheric moisture. The decrease in activity was such that aflatoxins were lost during collection. Best results were obtained with the adsorbent stored in a glass-stoppered flask wrapped with paraffin. Warming in front of a heat lamp before opening reduced the rate of decomposition.

Future Work

More information is needed about solvent effects on Styragel. Physical parameters for the gel must be evaluated, and retention characteristics in more varied solvent systems should be studied. Retention characteristics of other mycotoxins should be studied in new solvent systems.

The preliminary aflatoxins data for corn and peanut meal indicate that the method may be applicable to a variety of matrices. This would represent an improvement over existing methodology, which requires separate procedures for each sample matrix. Separation and determination of other mycotoxins also need to be studied.

The availability and popularity of Liquid Chromatographic (LC) systems indicates that LC will soon completely replace TLC for analysis. The adaptation of these sample preparation procedures to LC quantitation is inevitable.

However, some questions remain unanswered about surface effects in TLC. Investigations of solvent effects on spotting and fluorescence enhancement by paraffin oil would be interesting.

Health Risk Assessment

It is of interest to evaluate the exposure level of workers to aflatoxin B1. An air sampler passing 1400 L min^{-1} for 8 hours will sample 6.72×10^{5}L. A total of 4.7 g is collected, giving an average level of 7.0×10^{-6} g L^{-1} particulates in the air. Assuming 15 ppb (15 ng/g) B1 in the sample gives 1.0×10^{-4} ng L^{-1} B1 in the air. An average person inhales 6L min^{-1} [88] or 2880 L day^{-1} (8 hours). The total exposure to B1 is then 0.29 ng day^{-1}. However, only approximately 25% (w/w) of the particulates are respirable (i.e. reach the lungs), reducing the exposure

level to 70-80 pg day^{-1}.

An average worker is not likely to inhale a toxic amount in a lifetime; however, the long-term mutagenic effects are unknown.

EXPERIMENTAL SECTION

General Considerations

NMR spectra were recorded using a Varian A-60 instrument. UV-VIS spectra were recorded with either a CARY 219 or a Perkin-Elmer 320 spectrophotometer.

All glassware in contact with any mycotoxins was cleaned in a KOH/alcohol bath. Clean glassware were rinsed with dilute acid, distilled water, acetone, and then air dried. No carry-over problems were observed.

Purification of Solvents

All solvents were filtered through a fine glass frit (4-5 μm) or 0.5 μm Teflon filter. The following solvents were A.C.S. grade and used as received: toluene, benzene, phenol, 2-butanone, nitrobenzene, aniline, chloroacetic acid, trichloroacetic acid. The following were reagent grade and used as received: formic acid, ethyl ether, trifluoracetic acid. Pesticide-grade hexane was used as received.

Chloroform

Chloroform as received contained approximately 0.5% (v/v) ethanol, which was unacceptable for chromatographic studies. Ethanol was removed by passing chloroform through a column of neutral alumina (activity Grade I) (25 g/500 mL)

[89]. A blue fluorescing impurity was removed by distillation under nitrogen.

Acetonitrile

Acetonitrile (2L) was refluxed over $KMnO_4$ 24 hours and distilled under nitrogen through a Vigreux column. The first 10% of the distillate was discarded. The remaining distillate was passed through a column containing 100 g acidic alumina (activity Grade I). The purified solvent showed a UV-cutoff below 190 nm.

Acetone

Acetone (2L) contained a blue fluorescing impurity and was refluxed several hours over $KMnO_4$. This was followed by distillation through a Vigreux column under nitrogen.

Methanol

Several small pieces of Na metal were dissolved in 2L methanol, followed by several g iodine. After refluxing several hours the solvent was distilled [89].

Iso-amyl alcohol (3-methyl-1-butanol)

Several small pieces of Na metal were dissolved in *iso*-amyl alcohol and the solvent was distilled immediately. This removed an unidentified yellow impurity.

Tetrahydrofuran (THF)

THF was purified by refluxing one hour over CaH_2, followed by distillation under nitrogen [89].

Pyridine

Pyridine was dried over KOH overnight, followed by distillation over barium oxide [89].

Other solvents

1-chloropropane, acetyl chloride, and benzoyl chloride were distilled under nitrogen.

Thin Layer Chromatography

TLC plates were obtained as 20x20 cm plates, prescored to 5x20 cm (Anasil O, TLP099, Analabs, Inc., North Haven, CT). The 250 μm coating consisted of 15 μm particles with a 60Å pore diameter and 300 m^2/g surface area with an organic binder.

Plates were purified by allowing them to develop in methanol overnight. After removal, the plates were dried in a vacuum desiccator for one hour in the presence of a heat lamp. Storage was in an atmosphere of 48% relative humidity (saturated KSCN solution).

Exclusion Chromatography

Styragel, a poly(styrene)-divinyl benzene copolymer, was obtained from Waters Associates (Milford, MA). The gel has a 37-75 μm particle size distribution and 60Å pore size. The exclusion limit was near 500 μ.

As mentioned previously, the chromatographic system consisted of a pump, injector, column, and detector. The

stationary phase (10.0g) was allowed to swell in $CHCl_3$ and packed as a balanced density slurry in $CHCl_3$/toluene. After settling, the packing was compressed to form a 12.7 mm x 23.0 cm column. Agreement of capacity factors, k, calculated for identically packed columns, was within $\pm$ 0.03. All changes in mobile phase composition occurred in increments of 10%.

The capacity factor, k, was calculated using equation 16,

$$k = (V_R - V_o)/V_o \qquad (16)$$

where V_R is the retention volume of the solute and Vo is the void volume, represented by the retention of a polystyrene standard.

The equation for k normally used in exclusion chromatography (EC) has pore volume, V_i, in the denominator rather than V_o. Equation 16 is used here for two reasons. First, the system is operating in a non-EC mode for much of this work and equation 16 is valid for these conditions. Second, it is unlikely that any solute can achieve total permeation without interacting with the gel, and thus measurement of V_i is often a subjective experiment [90].

Florisil

The design and operation of the Florisil column has been discussed.

To prepare large amounts, 10.0 g of Florisil (Fisher, F-101) was suspended in 75 mL acetone and stirred. Stirring was continued for 10 minutes after addition of 4.85 g oxalic acid $\cdot 2H_2O$ in 30 mL acetone. The suspension was allowed to settle and the supernatant liquid removed. The remaining solid was dried overnight at 80°C, yielding 10.3 g. The adsorbent was deactivated with 7.5% (w/w) H_2O and equilibrated for 24 hours on a wrist action shaker.

Synthesis of the Internal Standard

Preparation of acid chlorides

Acid chlorides were prepared from the corresponding acid using the method of Brown [91]. The acid (0.1 mol) and benzoyl chloride (0.2 mol) were heated and distilled through a Vigreux column under nitrogen. By this procedure it was possible to obtain 30-35% yields for chloroacetyl chloride and trichloroacetylchloride. NMR spectra were consistent with expected products (chloroacetyl chloride ($CDCl_3$) - δ4.5 ppm (s), no acid protons; trichloroacetyl chloride, no spectrum, no acid protons, some $CHCl_3$ present from decomposition of starting material).

Preparation of acetate derivative of aflatoxin G2a (G2a-OAc) from G2a

One mL of a 10 ppm by weight solution of aflatoxin G2a (Sigma Chemicals) in $CHCl_3$ was cooled to 0°C. One drop

dry pyridine was added. Two drops of acetyl chloride (or other acid chloride) were added during a 20 minute period. The solvent was removed as quickly as possible, the residue dissolved in $CHCl_3$, and purified by preparatory TLC in 10% $CH_3CN/CHCl_3$. The derivative, identified by its R_F (slightly less than that of G2), was scraped from the plate and eluted with 50% $CH_3CN/CHCl_3$. After evaporation, the derivative was dissolved in $CHCl_3$.

Preparation of G2a-OAc from G1

One mL of a 10 ppm solution of G1 in THF was warmed to 50°C and 2 drops concentrated HCl added. After 5 minutes, a small amount of anhydrous Na_2SO_4 was added and the solution filtered. One drop acetyl chloride was then added and the solvent removed. Purification was also by preparatory TLC. An attempt to prepare G2a-OAc directly by solvo-mercuration failed.

Other derivatives could not be purified by TLC or LC and were not studied further.

Preparation of Mycotoxin Standards

The four aflatoxins, sterigmatocystin, ochratoxin A, penicillic acid, and patulin were obtained from Aldrich Chemical Co. (Milwaukee, WI). Citrinin was a gift from Eivind Lillihoj (USDA Regional Research Laboratory, Peoria, IL).

All mycotoxins were obtained as solids and dissolved in an appropriate solvent for UV standardization. Aflatoxin stock solutions were 10 ppm each. All other solutions were 100 ppm.

A series of dilutions containing all four aflatoxins was prepared. Three concentrations were prepared for each decade. The concentrations were adjusted to provide a given amount in a 5 μL volume. Thus, the standards labelled "100", "200", and "500" actually represented 20, 40, and 100 pg/μL solutions of each aflatoxin.

Calibration curves

All solutions were diluted with a $CHCl_3$ stock solution containing the internal standard. This eliminated the uncertainty in adding the internal standard separately. The calibration curve data were obtained by applying 5 μL to the plate with a 10 μL syringe. The area for each aflatoxin was divided by the area of the internal standard for that particular sample. This eliminated problems caused by irregularities in the plate or day-to-day variations in laser output. Calibration curves were obtained by plotting the area ratio vs. amount applied to the plate.

Operating Parameters

The nitrogen laser was built in the Ames Laboratory. A power supply provided 10 kV and 7 mA. The discharge chamber was filled with nitrogen at a pressure of 90 Torr,

and discharged at a rate of 20 Hz. A beam with 8 x 30 mm dimensions and a pulse width of 10-15 ns was produced.

The photomultiplier tube (RCA 1P28) was operated at 700 V. The signal was amplifed by a MODEL 135 electrometer (Princeton Applied Research).

The Analytical Method for Aflatoxins in Refuse Derived Fuel

Collection of the Sample

An air sample is obtained by collection on a quartz fiber filter or similar material. The sample is scraped from the filter, mixed thoroughly, and a weighed aliquot removed.

Extraction

The sample is placed in a glass-stoppered flask with 50 mL methanol and shook on a wrist-action shaker for 1 hour. The sample is removed by filtration.

Precipitation

The methanol is removed with a rotary evaporator and the residue suspended in 30% $CH_3CN/CHCl_3$. After thorough mixing, the solution should be filtered to remove particulates larger than 5 µm. The sample is then diluted to a predetermined volume.

Exclusion Chromatography

The sample is injected onto the Styragel column, which uses a 30% $CH_3CN/CHCl_3$ mobile phase at 1.00 mL/min. The fraction containing the aflatoxins is diverted to the Florisil column by means of Valve-1. The elution time of the

aflatoxins has been determined previously by injection of standards, and is usually 11-14 minutes after injection.

Florisil

After the fraction has been trapped on Florisil, interfering components are removed by injection of 1.2 mL 20% methanol/$CHCl_3$ through the Florisil column via Valve -2. Aflatoxins are eluted into a 10 mL pear shaped flask with 5.0 mL 4% H_2O/acetone. The solvent is removed with a rotary evaporator.

TLC

The sample is dissolved in 100 µL 1.5% acetone/$CHCl_3$, then 50 µL hexane is added. A 5 µL aliquot of the internal standard is added and the entire solution is applied to a TLC plate with the sample applicator.

The spot is pre-developed in a 30% ether/acetonitrile solution and dried with the applicator. The plate is developed 1 hour in formic acid/*iso*-amyl alcohol/hexane/$CHCl_3$ (1.6 + 4 + 5 + 89.4). After preliminary drying in the applicator, the plate is dried in a vacuum desiccator for 30 minutes.

Fluorescence analysis

The plate is placed in position and scanned across the focused laser. The amplifier offset and range switches are used to put the background "on scale". The output is monitored with an X-Y recorder. The starting and stopping

points for each peak are noted on the recorder, and the integrator reset for each peak. Background correction is performed manually and background area is subtracted from the recorded integral. From the area ratio of peak to internal standard, the amount of aflatoxin present is determined using the calibration curves. Relative concentration is determined using equation 15.

To prevent confusion due to variations in TLC development, it is advisable to develop one set of standards only on each plate. By scanning the standards first, the "location" of aflatoxins is determined in the real samples. Identical R_F values for sample and standard provide additional confirmation of aflatoxins.

PART II. ISOLATION AND IDENTIFICATION OF THE SPORE PIGMENT IN *ASPERGILLUS FLAVUS*

INTRODUCTION

Fungal pigments are of interest to mycologists for use in fungal taxonomy. The color and other macroscopic characteristics are often the first properties used in identifying an organism. Thus the identification of pigments is of interest.

Fungal pigments have been studied extensively. The known pigments in the Basidiomycetes have been summarized [92]. An extensive survey of fungal pigments [93] is concerned with mycelial pigments only.

The majority of pigments are orange, red, or blue, with many variations. The chemical compounds responsible for these colors are diverse, with the quinones and carotinoids predominating. Within each of these groups many specific compounds have been identified (i.e., at least 60 quinones and 20 carotinoids). Pyrones, polyenes, sesquiterpenes, tropolones, aryl carbonyls, and N-heterocycles have also been identified.

Spore pigments have not been investigated to any considerable extent. While much is known about the gross composition and characteristics of fungal spores [94], the

chemical nature of the pigments has been largely ignored. The brown and black pigments have been identified as melanins, which are presumably large polyaromatic molecules. Some work has considered the function of these pigments in protecting the spore from lysis [95-97] and ultraviolet radiation [98]. Fats, lipids, and saccharides have been identified in the interior of the spore [99-101] while the cell wall has been found to contain polysaccharides, phosphates, protein, and nucleic acids [102].

Aspergillus flavus

The mycelium and conidiophores of Aspergillus flavus are hyaline (transparent). However, the spores, a characteristic olive green, are useful for taxonomy. The prolific nature of this genus makes it easy to culture and recognize.

In the smooth conidia (spores) of Aspergillus the pigment is present in diffuse form [103]. Little information is available concerning the chemistry of the green pigment. The maximum spectral reflectance of A. flavus spores is in the yellow (560-575 nm) region [104]. The correlation between the appearance of an electron spin resonance [ESR] signal and accumulation of pigments in A. flavus spores indicates a stable free radical [105] is present, but not necessarily in the pigment. Exposure of mature green conidia of several species of Aspergillus to ammonia fumes changes the green color to yellow-brown. The change is

reversible and the green may be restored by exposure to acetic acid or HCl fumes. A correlation has been proposed between the degree of acidity and the shade of green [106]. In other species of Aspergillus, the green color is produced only in the presence of Cu(II) and halogen ions. Green conidia are reported to contain larger amounts of copper [107,108].

EXTRACTION OF THE PIGMENT

The pigment was not readily released from the spore walls. Many traditional techniques were unsuccessful, as documented below.

Spore Disruption

Spores were suspended in distilled water and shaken thoroughly in the presence of 100 μm glass beads. After several minutes, this succeeded in "cracking" open most (>90%) of the spores. Unfortunately, the pigment remained with the spore wall fragments and any attempt at filtration resulted in a clear filtrate and clogged filter medium.

An attempt to disintegrate the spores further by either a high-speed blender or ultrasonic agitation also failed. Use of a vortex mixer and glass beads [109] caused some additional breakage, but still left relatively large fragments.

Enzymatic Hydrolysis

The enzyme chitinase was obtained to hydrolyze the chitin in the spore walls. Spore fragment suspension and enzyme were mixed in a pH 6.0 buffer and stirred several days. The chitinase was expected to liberate 3.5 mg glucosamine/mg enzyme in 48 hours.

After one week at 22°C, large spore fragments were still present. In solutions containing acetate buffers,

fresh mycelium was present, indicating germination of unruptured spores.

Spore walls are known to have protective layers which inhibit chitinase [96]. However, spore suspensions treated with 5% Triton X-100 or acetone were not susceptible to hydrolysis by chitinase.

Chemical Degradation

Direct extraction failed with several common organic solvents, including chloroform, tetrahydrofuran, acetone, hexane, ethanol, and water. Extraction with 10% Triton X-100 gave a yellow filtrate, but refluxing caused decomposition of the detergent.

The pigment might be covalently bound to the spore wall. Spore suspensions were refluxed in 0.5 M HCl and 0.5 M NaOH. The HCl extraction produced a clear filtrate. The NaOH solution, however, was red and little material remained on a 0.5 μm filter. The alkaline solution fluoresced green. Upon acidification with HCl, the solution became green and a precipitate appeared.

Buffers

The solid obtained by alkaline extraction retained some red color and less severe extraction techniques were investigated. A 0.25 M, pH 9 carbonate buffer refluxing for 1.5 hours produced a brown spore mass and red filtrate, which became green in acid. However, the buffer appeared to decompose and was considered unsuitable.

A 0.2 M, pH9 phosphate buffer gave a brown spore mass and green acidified filtrate after a two hour extraction. Upon cooling, small crystals of an olive green solid precipitated. The solid was most readily collected on a 0.5 μm filter. The solid was not soluble in most organic solvents, including ethyl acetate, chloroform, acetone, methanol, acetonitrile, water, tetrahydrofuran, hexane, and toluene. The precipitate was only soluble in dimethylsulfoxide (DMSO) or N,N-dimethylformamide (DMF). The DMF solution became red, however, indicating an acid-base reaction. Therefore, the pigment appears to be a fairly strong acid.

PURIFICATION OF THE PIGMENT

The extracted pigment showed little movement on most common chromatographic sorbents.

No movement was observed on silica with the following mobile phases - 40% $CH_3OH/CHCl_3$, CH_3CN, CH_3OH, 50% $DMF/CHCl_3$. On acidic alumina (Activity Grade V, 15% w/w H_2O), no movement was observed with mobile phases consisting of acetic or formic acid. Dilute HCl stripped the column, but no pigment was recovered. An attempt to chromatograph the pigment on Styragel using previously discussed partion/exclusion effects [110] failed using 30% $CH_3CN/CHCl_3$ or 30% $DMSO/CHCl_3$. The pigment was eluted on diatomaceous earth with 60% $DMSO/CHCl_3$, but the material was red. However, acidification resulted in a color change back to green. The presence of HCl in the mobile phase (0.1 M) prevented the color change.

Pigment movement and resolution were better, however, on Styragel, which has a more controlled particle size. A mobile phase consisting of 35% $DMSO/CHCl_3$ with 0.05 M HCl moved the pigment readily on a 14x85 mm gravity packed column. The main component eluted quickly and was followed by a smaller band which comprised less than 10% of the sample. A small amount of material remained at the top of the column.

The age of the spores determined the relative amount of the first two bands. Older spores (i.e., harvested a longer period of time after accumulation of pigment) showed greater amounts of the minor component. This may be a decomposition product.

STRUCTURE ELUCIDATION

Spectroscopic Data

UV-VIS

In acidic solution (2.37 mg pigment in 2.0 mL DMSO and 1.0 mL 0.1 M HCl), the pigment shows a broad absorption band in the 600-680 nm region (A = 0.75 at 620 nm). The solution absorbence increases steadily at shorter wavelengths. In basic solution (1.29 mg pigment in 2.0 mL DMSO and 1.0 mL 0.1 M NaOH), the spectrum shows an absorption of 0.3 to 0.4 in the 900-700 nm range with steadily increasing absorbence at shorter wavelengths. A dilution of this solution to 100 mL shows a peak at 480 nm (A = 0.15) with steadily increasing absorbence. For both acidic and basic solutions, further dilutions revealed no characteristic absorption patterns.

NMR

An acceptable ^{1}H FT-NMR could not be obtained. Only broad peaks were observed, suggesting the presence of a free radical. However, no ESR spectrum was observed. The ^{1}H data obtained were: δ 2.49 (s), slightly broadened (DMSO); δ3.62 (s), slightly broadened (H_2O); δ7.16 (s), broad, small; δ8.29 (s) broad, small. A ^{13}C spectrum revealed one very small peak at 79.3 ppm.

IR

An FT-IR spectrum of pigment evaporated on a NaCl disk gave the following peaks: 3600-3200 cm^{-1}, broad; 3100-2900, weak; 1646, strong 1534; 1411, weak; 1233, weak; 1025; 948, weak; 663, weak.

Emission Spectroscopy

No metals are present except in trace amounts.

Mass Spectrometry

A mass spectrum of the purified pigment was obtained by direct insertion into the electron beam. As the probe was heated, the intensities of the high mass clusters shifted to higher and higher mass. This suggested that a probe distillation of a complex mixture was taking place. The highest mass reported was 717.64.

The high mass region is dominated by weak clusters which resemble hydrocarbons. Prominent peaks appear at 692, 677, 664, 603, 577 and 425. The lower mass region is dominated by the base peak at 57, and peaks at 69, 71, 84, 91, 98, and 233.

Degradation Products

The mass spectrometric data for the $KMnO_4$ degradation of the spore pigment, obtained with a direct insertion probe Finnigin 4000 instrument, are partially summarized in Figures 43-44. In Figure 43, a component in the first liquid chromatographic fraction is identified as decyl hexanoate

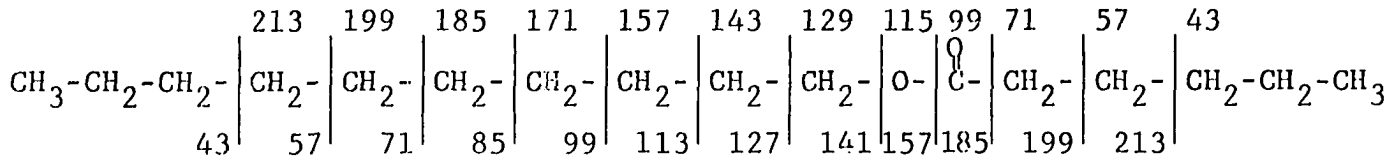


Figure 43. Fragments observed for decyl hexanoate from the earliest eluting liquid chromatography fraction

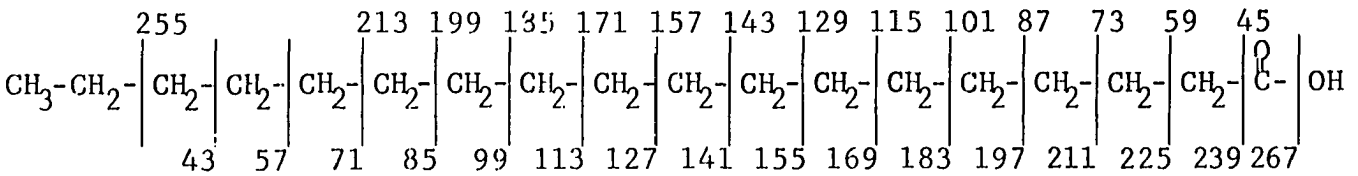


Figure 44. Fragments observed for stearic acid from the second liquid chromatography fraction

(MW = 256). The mass spectrum is dominated by the hydrocarbon clusters, and all masses indicated are observed in the spectrum. At high mass, the indicated peaks are two to three times as intense as adjacent mass units. The base peak in the spectrum is at m/z 43. The molecular ion's intensity is 1.7% of the base peak. Since a fragment at m/z 256 is also observed in the spectrum of the pigment, it is not clear whether this represents a decomposition product or merely one component that has been carried through intact. The latter case is more likely since an ester is not likely to be formed by permanganate oxidation.

In Figure 44, the mass units observed in the second LC fraction indicate the presence of stearic acid, a C_{18} carboxylic acid. The molecular ion at m/z 284 is less than 1% of the base peak at m/z 43. Upon further heating of the probe, an ion appears at m/z 312, indicating the presence of a C_{20} carboxylic acid.

The third LC fraction, collected as a tail on the second elution band, shows patterns similar to the second fraction. A prominent ion at m/z 77 and others at m/z 91 and 122 indicate the possible presence of an aromatic ring, but no complete structure could be established.

Conclusions

The spectral data for the pigment indicated that the molecule was primarily hydrocarbon. The identified fragments confirmed this. The C_{18} chain was probably attached to the rest of the molecule through a double bond or an aromatic ring.

Unfortunately, the identified fragments do not account for the color of the pigment. The chromophore is probably a central, substituted aromatic ring which cannot be identified in the mixture.

Future Work

A better purification step is necessary. If the present "purified" material is a mixture, as the mass spectrum suggests, the components must be separated and identified on a more efficiently packed Styragel or μStyragel column.

Since the pigment appears to be primarily a hydrocarbon, on a molar hydrogen basis, any attempted degradations must also separate the hydrocarbon fragments from the chromophore. If the C_{18} fragments can be removed, which would not be difficult in the $KMnO_4$ degradation, the structure of the chromophore might be readily determined.

EXPERIMENTAL SECTION

Purification of Solvents

Dimethyl sulfoxide (DMSO)

DMSO was stored overnight over NaOH. The solvent was then distilled at reduced pressure over BaO [89].

N,N-Dimethylformanide (DMF)

DMF was stirred over KOH for one hour, then distilled over BaO [89].

Culture Methods

Previously identified spores of Aspergillus flavus sclerotia were suspended in water and inoculated into six 2800 mL Fernback culture flasks. Each flask had been sterilized and contained 150 mL of Czapek's medium (Difco Laboratories, Detroit, MI).

At 22 °C, white mycelium was visible in a few days. Sporulation was noted by the appearance of a light yellow color after one to two weeks. The characteristic olive green color formed two to four weeks after inoculation. The color appeared stable for about three weeks, after which time the green slowly deteriorated to brown.

Spore Harvest

The spores could be liberated by suspending them in either 40% ethanol/H_2O or 0.85% (w/v) NaCl containing 0.1%

(v/v) Twreen 80 (polysorbate 80). Approximately 100 mL of solution was swirled in each flask to dislodge the spores. This spore suspension was removed by aspiration and the procedure repeated.

The suspension was filtered through a 0.5 μm filter. The filtrate appeared yellow, but this was a component from the medium, not the spores.

Extraction

Dry spores were weighed into a flask and refluxed in a 0.2 M, pH8 phosphate buffer for two hours. Approximately 10 mL of buffer was used for 100 mg spores.

After cooling, the solution was filtered through a 0.5 μm filter, producing a red filtrate. The solution turned green upon acidification with concentrated HCl. A green solid precipitated upon cooling and was collected on a 0.5 μm filter. Approximately 10 mg pigment could be obtained from 1 g spores.

Purification

Approximately 6.0 g of Styragel were slurried in a 35% DMSO/$CHCl_3$ solution containing 0.05 M HCl. The slurry was gravity packed into a 14 mm i.d. column. The pigment, dissolved in 70% DMSO/$CHCl_3$, was added to the column and eluted with the DMSO/$CHCl_3$/HCl mixture. The main component eluted first, with a minor component eluting immediately afterwards.

The collected fraction was diluted with three to four volumes of $CHCl_3$ and cooled. The green precipitate was collected on a 0.5 μm filter.

$KMnO_4$ Degradation

A few mg of purified pigment were dissolved in 20 mL H_2O containing 0.5 g K_2CO_3. A 3% (w/v) solution of $KMnO_4$ was added dropwise at 110°C until the purple color remained for more than a few seconds. After stirring 5 minutes, the solution was cooled and filtered.

The filtrate was acidified with concentrated HCl and extracted with $CHCl_3$. The $CHCl_3$ solution contained a small amount of a light yellow solid.

The mixture appeared to be complex when a H_2O solution was analyzed by LC, but two major elution bands and a tailing region were collected for mass spectrometric analysis. Conditions: 25.0 cm x 4.6 mm Zorbax C8 column (DuPont) operated at 1.00 mL/min with 40% H_2O/methanol, 50 μL injection volume, absorbence monitored at 254 nm.

REFERENCES

1. Goldblatt, L. A. J. Am. Oil Chem. Soc. 1977, 54, 302A.

2. Alexopoulos, C. J. "Introductory Mycology", 2nd ed.; Wiley: New York, 1962; Part IV.

3. Sargeant, K.; Sheridan, A.; O'Kelly, J.; Carnaghan, R. B. A. Nature 1961, 192, 1096.

4. Mycotoxic Fungi, Mycotoxins, Mycotoxicoses. An Encyclopedic Handbook, Vol. I "Mycotoxic Fungi and Chemistry of Mycotoxins", Wyllie, T. D.; Morehourse, L. G., Eds.; Marcel Dekker: New York, 1977; Chapters 1-4.

5. "Official Methods of Analysis of the A.O.A.C." Horwitz, W., Ed.; Association of Official Analytical Chemists: Washington, D.C., 1980; section 26.

6. Velasco, J. J. Am. Oil Chem. Soc. 1972, 49, 141.

7. Pons, W. A. J. Assoc. Off. Anal. Chem. 1976, 59, 101.

8. Panalaks, J.; Scot, P. M. J. Assoc. Off. Anal. Chem. 1977, 60, 583.

9. Chelkowski, J. Photochem. Photobiol. 1974, 20, 279.

10. Takahashi, D. M. J. Chromatogr. 1977, 131, 147.

11. Haddon, W. F. J. Assoc. Off. Anal. Chem. 1977, 60, 107.

12. Haddon, W. F.; Wiley, M.; Waiss, Jr., A. C. Anal. Chem. 1971, 43, 268.

13. Berman, M. R.; Zare, R. N. Anal. Chem. 1975, 47, 1200.

14. Bradley, A. B.; Zare, R. N. J. Am. Chem. Soc. 1976, 98, 620.

15. Diebold, G. J.; Zare, R. N. Science 1977, 196, 1439.

16. Shannon, G. M.; Shotwell, O. L. J. Assoc. Off. Anal. Chem. 1976, 59, 963.

17. Stack, M. E.; Nesheim, S.; Brown, N. L.; Pohland, A. E. J. Assoc. Off. Anal. Chem. 1976, 59, 966.

18. Egmond, H. P. V.; Paulsch, W. E.; Deyll, E.; Schuller, P. L. J. Assoc. Off. Anal. Chem. 1980, 63, 110.

19. Stoloff, L. J. Assoc. Off. Anal. Chem. 1981, 64, 373.

20. Heathcote, J. G.; Dutton, M. F. Tetrahedron 1977, 25, 1497.

21. Lenenberger, U.; Gauch, R.; Baumgartner, E. J. Chromatogr. 1978, 161, 303.

22. Stray, H. J. Assoc. Off. Anal. Chem. 1978, 61, 1359.

23. Golinski, P.; Chelkowski, J. J. Assoc. Off. Anal. Chem. 1978, 61, 586.

24. Josefsson, E.; Möller, T. J. Assoc. Off. Anal. Chem. 1979, 62, 1165.

25. Stoloff, L. J. Assoc. Off. Anal. Chem. 1980, 63, 247.

26. Chalam, R. V.; Stahr, H. M. J. Assoc. Off. Anal. Chem. 1979, 62, 570.

27. Marti, L. R.; Wilson, D. W.; Evans, B. D., J. Assoc. Off. Anal. Chem. 1978, 61, 1353.

28. Ciegler, A.; Kurtzman, C. P. J. Chromatogr. 1970, 51, 511.

29. Pero, R. W.; Howan, D.; Owens, R.-G.; Snow, J. P. J. Chromatogr. 1972, 65, 501.

30. Takeda, Y.; Isohota, E.; Amano, R.; Uchiyama, M. J. Assoc. Off. Anal. Chem. 1979, 62, 573.

31. Gorst-Allman, C. P.; Steyn, P. S. J. Chromatogr. 1979, 175, 325.

32. Grinero, A. J. Assoc. Off. Anal. Chem. 1979, 62, 579.

33. Lee, K. Y.; Poole, C. F.; Zlatkis, A. Anal. Chem. 1980, 52, 837.

34. Josefsson, E.; Möller, T. J. Assoc. Off. Anal. Chem. 1977, 60, 1369.

35. Bly, D. D. Science 1970, 168, 527.

36. Kremmer, T.; Boross, L. "Gel Chromatography. Theory, Methodology, Applications"; Wiley: New York, 1979; Chapter 1.

37. Brook, A. J. W.; Munday, K. C. J. Chromatogr. 1970, 50, 307.

38. Brook, A. J. W.; Hously, S. J. Chromatogr. 1969, 42, 112.

39. Strenli, C. A. J. Chromatogr. 1971, 56, 219.

40. Determan, H.; Lampert, K. J. Chromatogr. 1972, 69, 123.

41. Prakash, V.; Naudi, P. K. J. Chromatogr. 1975, 106, 23.

42. Gelotte, B. J. Chromatogr. 1960, 3, 330.

43. Nefedov, P. P.; Lazareva, M. A.; Belenskii, B. G.; Frenkel, S. Y.; Koton, M. M. J. Chromatogr. 1979, 170, 11.

44. Strenli, C. A. J. Chromatogr. 1971, 56, 225.

45. Bergman, J. G.; Duffy, L. J., Stevenson, R. B. Anal. Chem. 1971, 43, 131.

46. Einarsson, R.; Zeppezaurer, M. J. Organomet. Chem. 1974, 71, 277.

47. Suzuki, N.; Saitoh, K.; Shibukawa, M. J. Chromatogr. 1977, 138, 79.

48. Popl, M.; Fähnrich, J.; Stejskal, M. J. Chromatogr. Sci. 1976, 14, 537.

49. Takahagi, H.; Seno, S. J. Chromatogr. Sci. 1974, 12, 507.

50. Cazes, J.; Gaskill, D. R. Sep. Sci. 1967, 2, 421.

51. Freeman, D. H.; Killion, D. J. Polym. Sci., Polym. Phys. Ed. 1977, 15, 2047.

52. Cazes, J.; Gaskill, D. R. Sep. Sci. 1969, 4, 15.

53. Klimisch, H. J.; Reese, D. J. Chromatogr. 1972, 67, 299.

54. Freeman, D. H.; Angeles, R. M. J. Chromatogr. Sci. 1974, 12, 730.

55. Edwards, G. D.; Ng, Q. Y. J. Polym. Sci., Part C 1968, 21, 105.

56. Freeman, D. H.; Angeles, R. M.; Enogonio, D. P.; May, W. Anal. Chem. 1973, 45, 768.

57. Nakae, A.; Muto, G. J. Chromatogr. 1976, 120, 47.

58. De Ruvo, A. J. Polym. Sci., Polym. Chem. Ed. 1973, 11, 3017.

59. Saitoh, K.; Suzuki, N. J. Chromatogr. 1975, 111, 29.

60. Hausler, D. W.; Hellgeth, J. W.; McNair, H. M.; Taylor, L. T. J. Chromatogr. Sci. 1979, 17, 617.

61. Gaylor, V. F.; James, H. L.; Weetall, H. H. Anal. Chem. 1976, 48, 44R.

62. Gaylor, V. F.; James, H. L. Anal. Chem. 1978, 50, 29R.

63. Yamamoto, Y.; Yamamoto, M.; Ebisni, S.; Takogi, T.; Hashimoto, T.; Izuhara, M. Anal. Lett. 1973, 6, 451.

64. Saitoh, K.; Ozawa, T., Suzuki, N. J. Chromatogr. 1976, 124, 231.

65. Burrell, H.; Immergut, B. In "Polymer Handbook"; Brandrup, J.; Immergut, E. H., Eds.; Interscience: New York, 1966; p. IV-341.

66. Mori, S. Anal. Chem. 1978, 50, 745.

67. Mori, S.; Yamakawa, A. Anal. Chem. 1969, 51, 382.

68. Karger, B. L.; Snyder, L. R.; Eon, C. Anal. Chem. 1978, 50, 2126.

69. Karger, B. L.; Snyder, L. R.; Horvath, C. "An Introduction to Separation Science"; Wiley-Interscience: New York, 1972; p. 50.

70. Snyder, L. R. In "Modern Practice of Liquid Chromatography"; Kirkland, J. J., Ed.; Wiley-Interscience: New York, 1971; p. 138.

71. Karger, B. L.; Snyder, L. R.; Eon, C. J. Chromatogr. 1976, 125, 71.

72. Carlton, J. K.; Bradbury, W. C. Anal. Chem. 1955, 27, 67.

73. "Florisil. Properties, Applications, Bibliography" Floridin Co: Pittsburgh, PA.

74. Snyder, L. R. J. Chromatogr. 1963, 12, 488.

75. Levi, C. P. J. Assoc. Off. Anal. Chem. 1969, 52, 1300.

76. Kirchner, J. G.; Miller, J. M.; Keller, G. J. Anal. Chem. 1951, 23, 420.

77. Stahl, E. Pharmazie 1956, 11, 633.

78. Snyder, L. R. J. Chromatogr. 1971, 63, 15.

79. Stahl, E., "Thin-Layer Chromatography"; Academic Press: New York; 1965.

80. Snyder, L. R., "Principles of Adsorption Chromatography"; Marcel Dekker: New York, 1968.

81. Gniochon, G.; Bresalle, F.; Siouffi, A. J. Chromatogr. Sci. 1979, 17, 368.

82. Emanuel, C. F. Anal. Chem. 1973, 45, 1568.

83. Fessler, J. H.; Galley, H. Nature 1964, 201, 1056.

84. Felton, H. R. "Technical Report No. 8001. The Pre-Adsorbent Phenomenon in TLC"; Analtech, Inc.: Newark, 1980; p. 1-6.

85. Uchiyama, S.; Uchiyama, M. J. Chromatogr. 1978, 53, 135.

86. Parker, C. A. "Photoluminescence of Solutions"; Elsevier: Amsterdam, 1968; p. 236.

87. Hurtubise, R. J. Anal. Chem. 1977, 49, 2160.

88. Ruppel, G. "Manual of Pulmonary Function Testing"; Mosby: St. Louis; 1975; p. 13-15.

89. Gordon, A. J.; Ford, R. A. "The Chemist's Companion"; Wiley-Interscience: New York, 1972; p. 431-436.

90. Hansler, D. W.; Hellgeth, J. W.; McNair, H. M.; Taylor, L. T. J. Chromatogr. Sci. 1979, 17, 617.

91. Brown, H. C. J. Am. Chem. Soc. 1938, 60, 1325.

92. Besl, V. H.; Bresinsky, A.; Kronawitter, F. Z. Pilzk. 1975, 41, 81.

93. Engster, C. H. Z. Pilzk. 1973, 39, 45.

94. Weber, D. J.; Hess, W. M. "The Fungal Spore. Form and Function"; Wiley-Interscience: New York, 1971; Chapter 1.

95. Potgieter, H. J.; Alexander, M. J. Bacteriol. 1966, 91, 1526.

96. Kuo, M. J.; Alexander, M. J. Bacteriol. 1967, 94, 625.

97. Bloomfield, B. J.; Alexander, M. J. Bacteriol. 1967, 93, 1276.

98. Durrel, L. W. Mycopathol. Mycol. Appl. 1964, 23, 339.

99. Sumi, M. Biochemiche Z. 1928, 195, 161.

100. Gunasekaran, M.; Hess, W. M.; Weber, D. J. Can. J. Microbiol. 1972, 18, 1575.

101. Ballio, A.; DiVittorio, V.; Russi, S. Arch. Biochem. Biophys. 1964, 107, 177.

102. Horikoshi, K.; Iida, S. Biochim. Biophys. Acta 1964, 83, 197.

103. Raper, K. B.; Fennel, D. I. "The Genus Aspergillus"; Williams and Wilkins: Baltimore, 1965; Chapter 1.

104. Kulik, M. M.; Johnson, R. M. Mycologia 1969, 61, 1142.

105. Leighton, T. J.; Stock, J. J.; Herring, F. G. Biochim. Biophys. Acta 1971, 237, 128.

106. Thom, C.; Church, M. B. Am. J. Bot. 1921, 8, 103.

107. Takogi, Y.; Sakaguchi, K. I. J. Gen. Appl. Microbiol. 1957, 3, 125.

108. Takagi, Y. J. Gen. Appl. Microbiol. 1957, 3, 269.

109. Van Etten, J. L.; Freer, S. N. Appl. Env. Microbiol. 1978, 35, 622.

110. Bicking, M. K.; Kniseley, R. N. Anal. Chem. 1980, 52, 2164.

ACKNOWLEDGEMENTS

It is necessary, at this point, to make a few non-chemical remarks.

Tradition requires that I first express my deepest gratitude to my family (especially Mom) for support, moral and financial, during my imprisonment in Iowa. At times, they were the only reason I remained. I owe them much, and promise to pay it all back (at \$100 $month^{-1}$).

I must acknowledge Maggie in a separate paragraph. Her presence made an incredible difference in my work habits. Of course, she did not want to be acknowledged here, because she "came along for a free ride the last part of it". However, she still gets my award for "Best Performance in the Role of Supporting a Graduate Student". She also paid off the loan on my car.

I am deeply indebted to Dr. H. J. Svec and Dr. R. N. Kniseley. Dr. Svec kept an open mind and took on a graduate student shunned by everyone else. It is a credit to his temperament and sense of fair play. I hope I haven't disappointed him. He instilled in me a respect for grammatical precision that two years of Latin had not accomplished. His contribution is greater than he would suggest. Dr. Dick Kniseley is also important, in addition to the fact that he provided the money for this project. His incessant "keep smiling" remarks kept me going when nothing made sense (which

was quite often). He was always more than happy to attempt to explain spectrochemical principles to an otherwise muddle-headed separations chemist.

I must also offer brief thanks to G. A. Russell and D. J. Johnson for being honest with me at a time when the commodity was in short supply.

That about covers the beatitudes!

This thesis is really dedicated, along with a sustained Bronx cheer, to all the inhabitants of the Sacred Ivory Tower who proclaimed that this project shall not be completed.

Of course, no acknowledgement would not be complete without mentioning that many others provided technical assistance, including Deanna, suffering through her first thesis, and L. H. Tiffany suffering through her first chemist. I must also thank two good friends for providing something physical to shovel whenever I needed a break from graduate school.

My musical contacts (DP, JC, JM, MB) provided a contrast in my life.

Finally, my thanks to Dr. Jimmy Hershberger, my rhythmically-minded organist who helped me fill my closet with 101 empty six-packs of Heineken. (In the beginning there was the Light....then the Dark.)