1985

The analysis of volatiles in moldy corn and their correlation to mold presence and mycotoxin production

Walter Gaylord Hyde
Iowa State University

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THE ANALYSIS OF VOLATILES IN MOLDY CORN AND THEIR CORRELATION TO MOLD PRESENCE AND MYCOTOXIN PRODUCTION

Iowa State University PH.D. 1985

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The analysis of volatiles in moldy corn and their correlation to mold presence and mycotoxin production

by

Walter Gaylord Hyde

A Dissertation Submitted to the

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INTRODUCTION

Molds have been a long standing companion to mankind through history. At one end of the spectrum, molds are responsible for many processes yielding food products and for certain drugs and medicinals which promote health and prevent illness. However, research has pointed out the important and harmful health effects of molds in the ecosystem, yet they were not recognized as such by the human population. In crop production, molds are responsible for large economic losses through grain spoilage or decreased nutritional value. When consumed, certain mold by-products are deleterious to animal and human health. Indeed, some of the metabolic products rank among the most potent naturally occurring carcinogens known. An important health consideration is that many of the deleterious compounds can be passed to man through the food chain.

Because of the newly realized importance of molds and mold toxins which are present in a variety of biological samples, such as grains, body tissues and fluids, and food products, both raw and finished, it is desirable to know which toxins are present and at what concentration. The sampling techniques and analytical procedures developed to date for toxin determination are time-consuming, subject to extreme sampling variation, and costly. Also, these techniques yield an analytical extract which is not toxin selective but rather a complex mixture of components which can confuse the final analysis in which mold toxins are present.

In addition to toxins, molds give off aromas and volatile organic compounds which might serve to identify specific molds and toxins produced. A technique of identification of these volatile compounds might possibly alleviate some of the difficulties in analyses described above.

The following research was conducted to explore this possibility. The specific goals of the work were: 1) to develop analytical techniques for the collection, separation, identification, and quantification of volatile organic components commonly reported to be associated with moldy feeds and feed components; 2) to develop a controlled laboratory situation for growing molds on grain and collecting any volatiles produced; and 3) to test the correlation, if any, between volatiles found and molds or mold toxins present.

LITERATURE REVIEW

Mycotoxins and Mycotoxicosis

History

Molds are an ubiquitous and varied collection of organisms. According to Tindall (1983), more than 200,000 species of molds are known. Their tremendous diversity is illustrated by the number of ways in which they affect our daily lives. Food products and medicinals are some of the beneficial by-products of mold growth. Conversely, some 50 to 60 molds are known to be pathogenic to animals and man,or to produce toxic metabolites. Several significant events in man's history are attributed to mycotoxicosis, disease processes caused by mold by-products. In the Middle Ages, "St. Anthony's Fire", or ergotism caused by alkaloids of Claviceps purpurea swept France, killing thousands (Goldblatt, 1972). In Russia during World War II, overwintered moldy grain grain was consumed and caused Alimentary Toxic Aleukia, a disease of dermal necrosis, leucopenia, and bone marrow degeneration (Joffee, 1965). Several molds were identified as contributing to the problem, including Fusarium sp. and Cladosporium sp. Disease outbreaks in horses in the 1940s were ascribed to Stachybotrus infestation of moldy hay (Forgacs, 1965). In 1960, Turkey "X" syndrome swept England killing thousands of turkeys (Blount, 1961), and affecting ducklings and other farm animals (Asplin and Carnaghan, 1961). Aspergillus flavus was then identified in peanut meal, one of the feed components. In the U.S., an outbreak of trout hepatoma was related to toxin-contaminated cottonseed meal used in their diet (Wolf and Jackson, 1963; Halver, 1969). These incidents spurred investigative work into the nature of the mold toxicity, and resulted in the discovery of fluorescent Aspergillus by-products, the aflatoxins.

Many molds are of concern to the food industry because of their ubiquitous distribution and the deleterious effects of their metabolic toxins (Bullerman, 1979), including members of the genera Aspergillus, Penicillium, Fusarium, Alternaria, Trichothecium, Cladosporium, Byssochlamys, and Sclerotinia. The molds are of extreme concern due to the loss of quality of stored grains (Christensen and Kaufman, 1969). Molds are generally classified into three groups based on the conditions under which they will grow. These are: 1) field fungi which occur in the field before the grain is harvested, of which Fusarium, Alternaria, and Cladosporium are members; 2) storage fungi which grow during grain storage, of which Aspergillus and Penicillium are members; and 3) advanced decay fungi which thrive on extensively damaged grain, including Fusarium and others. Fusarium roseum and Aspergillus parasiticus were of particular interest to the present study as representatives of these groups and because the toxins produced are of great economic and health interest in the U.S. These toxins are responsible for a variety of diverse lesions and clinical signs depending on the species involved, on the age, sex, health, and nutritional state of the animal, and also on the type and degree of exposure.

Aflatoxins and aflatoxicosis

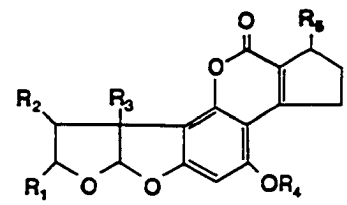
Aspergillus flavus and Aspergillus parasiticus produce secondary metabolites called aflatoxins (the "a" from Aspergillus and the "fla" from flavus). Of the aflatoxins reported, four are of primary interest— B_1, B_2, G_1, and G_2. The B toxins are so named because they fluoresce with a bluish color when irradiated with ultraviolet light (UV) (365nm), whereas the G toxins fluoresce with a greenish hue due to impurities co-extracted with the toxin. The numbers denote their order of chromatographic separation using normal phase techniques. Maggon et al. (1977) and Venkitasubramanian (1977) discussed primary and secondary

metabolism and concluded that during optimum growth of the mold, primary metabolism occurs with little or no aflatoxin formation. As phosphate, nitrogen, or trace elements become limiting, primary processes break down resulting in an accumulation of primary metabolites such as pyruvate, malonate, acetate and amino acids. These products, in excess, stimulate secondary metabolic processes such as the polyketide bisynthetic pathway which promotes aflatoxin over fatty acid synthesis.

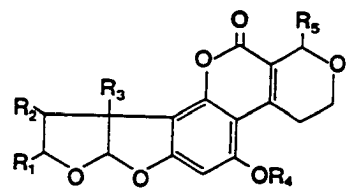
In addition to the four primary aflatoxins, Holzapfel et al. (1966) and Masri et al. (1969) reported the isolation of M_1 and M_2, metabolites of B_1 which are excreted in the milk of lactating animals. The M refers to the excretion matrix, milk, and the subscript refers to the order of elution using normal phase chromatographic techniques.

The aflatoxins are difuranocoumarin derivatives (Figure 1) related to coumarin (Buchi and Rae, 1969). B_1 is the most prevalant and toxic, followed by G_1, B_2, and G_2. Sensitivity to the aflatoxins is species related with ducklings being the most sensitive at lower levels, followed by trout, turkey poults, piglets, sows, calves, cattle, horses, and sheep (Allcroft, 1965; Borker et al., 1966).

Due to the chronic levels usually encountered in real-world situations, it is difficult to ascertain the effects due to aflatoxicosis. Few studies of long-term, low level exposure have been initiated under well-controlled circumstances. Incidents in the field are often due to ingestion of moldy grain, which might contain more than one toxin. Finally, effects of aflatoxicosis are often overshadowed by secondary infectious and noninfectious disorders. Many biological effects have been ascribed to the aflatoxins. Many excellent reviews have been published on the effects of aflatoxin exposure in different species



Aflatoxin	R_1	R_2	R_3	R_4	R_5
B_1	H	H	H	CH_3	=O
B_2	H_2	H_2	H	CH_3	=O
B_{2a}	OH.H	H_2	H	CH_3	=O
Aflatoxicol(Ro)	H	H	H	CH_3	−OH
M_1	H	H	OH	CH_3	=O
M_2	H_2	H_2	OH	CH_3	=O
P_1	H	H	H	OH	$-CH_3$
Q_1	H	H	H	CH_3	−OH



Aflatoxin	R_1	R_2	R_3	R_4	R_5
G_1	H	H	H	CH_3	=O
G_2	H_2	H_2	H	CH_3	=O
G_{2a}	OH.H	H_2	H	CH_3	=O
GM_1	H	H	OH	CH_3	=O

Figure 1. Some aflatoxins and derivatives

(Bullerman, 1979; Tindall, 1983; Bodine and Mertens, 1983; Hoerr and D'Andrea, 1983; Edds and Bortell, 1983). Chronic exposure to aflatoxins has been associated with reduced feed efficiency, liver damage, immunosuppression, impaired blood clotting, increased susceptibility to stress, reduced reproductive performance, abortion, fetal anomalies, and reduced milk production. In addition, B_1 is one of the most potent natural carcinogens known, producing tumors in animals exposed to low levels over moderate periods of time (Ciegler, 1975). Table 1 (Bullerman, 1979) lists some biological and toxicological properties of the aflatoxins.

Trichothecene toxins

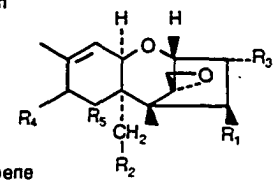
The Fusarium molds also produce secondary metabolites of great concern, mainly because of their economic significance in the agricultural community. The estrogenic toxins, zearalenone and zearalenol, and the trichothecenes, T-2 toxin, vomitoxin (deoxynivalenol or DON) and diacetoxyscirpenol (DAS), are the most harmful of the Fusarium toxins to animal nutrition and health. Extensive reviews of the Fusarium toxins have been written by Bamburg and Strong (1971), Bamburg (1976), Christensen and Mirocha (1974), and by Rodericks et al. (1979).

The trichothecenes are biologically active fungal metabolites produced primarily by Fusarium but also produced by Trichoderma, Cephalosporium, Verticimonosporium, Stachybotrys, and Myrothecium. Trichothecenes are a closely related family of sesquiterpenoids, consisting of a trichothecane ring with a double bond at carbon 9-10 and an epoxy group at carbon 12-13. Hence, they are sometimes referred to as 12,13-epoxy trichothecenes. Naturally occurring trichothecenes are classified by their structure into four groups, illustrated in figures 2-3. Tamm (1979) reported that they are colorless, mostly crystalline, optically active solids which are soluble in moderately polar organic

Table 1. Some biological and toxicological properties of several aflatoxins (Bullerman, 1970).

Toxin	Producing organism	Acute toxicity: LD50 (mg/kg)	Acute toxicity: Animal	Acute toxicity: Route admin.	Effects in animals: Animals affected	Effects in animals: Pathological effects	Effects in animals: Commodities found contaminated
B_1	Aspergillus	0.5-10	Several				
	flavus parasiticus	0.36	Duckling	oral	Birds Duckling Turkey poult Pheasant chick Mature chickens	Hepatotoxin Liver damage Hemorrhage Intestinal tract	Peanuts Corn Wheat Rice Cotton seed
B_2	Same	1.7	Duckling	oral	Quail	Kidneys	Copra Nuts
G_1	Same	0.78	Duckling	oral	Mammals Young pigs Pregnant sows	Bile duct Hyperplasia	Various food Milk Eggs
G_2	Same	3.45	Duckling	oral	Dogs Calves Mature cattle Sheep	Carcinogen Liver tumors	
M_1	Same (also excreted by animals after consumption)	0.32	Duckling	oral	Cat Monkey Man Fish		
M_2	Same	1.23	Duckling	oral	Laboratory animals		

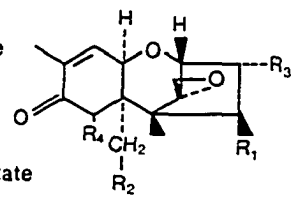
Structure I



	Substituents (R) in Formula				
	R_1	R_2	R_3	R_4	R_5
Calonectrin	H	OAC	OAC	H	H
Diacetylcalonectrin	H	H	OAC	H	H
Trichodermol	OH	H	H	H	H
Pentahydroxyscirpene	OH	OH	OH	OH	OH
Trichodermin	OAC	H	H	H	H
Diacetylverrucarol	OAC	OAC	H	H	H
Verrucarol	OH	OH	H	H	H
Scirpentriol	OH	OH	OH	H	H
Diacetoxyscirpenol	OAC	OAC	OH	H	H
T-2 Tetraol	OH	OH	OH	OH	H
T-2 Toxin	OAC	OAC	OH	OIV	H
HT-2 Toxin	OH	OAC	OH	OIV	H
Neosolaniol	OH	OAC	OAC	OH	H

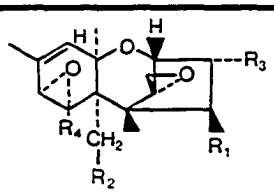
OAC = Acetate OIV = Isovaleryl

Structure II



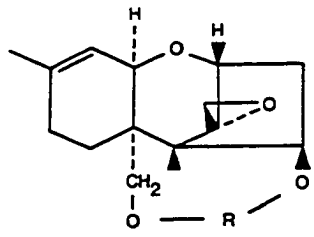
	Substituents			
	R_1	R_2	R_3	R_4
Trichothecolone	OH	H	H	H
Trichothecin	O-iso-crotonyl	H	H	H
Nivalenol	OH	OH	OH	OH
Nivalenol Diacetate	OAC	OAC	OH	OH
Fusarenone	OAC	OH	OH	OH
Deoxynivalenol	H	OH	OH	OH

Structure III



	Substituents			
	R_1	R_2	R_3	R_4
Crotocol	OH	H	H	—
Crotocin	O-iso-crotonyl	H	H	—

Figure 2. Scirpene compounds



Structure IV

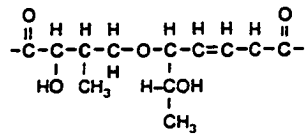
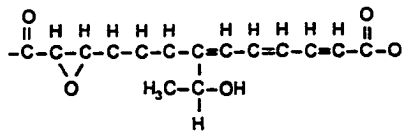
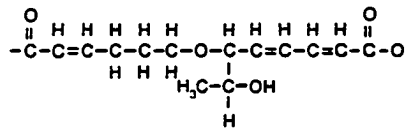
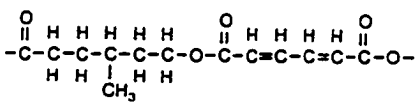
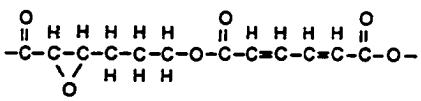
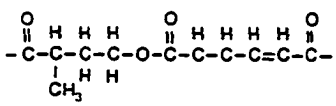
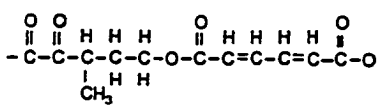
	Substituents (R)
Roridan A	
Roridan D	
Roridan E	
Verrucarin A	
Verrucarin B	
Verrucarin J	
2' dehydro Verrucarin A	

Figure 3. Scirpene compounds

solvents and only slightly soluble in aqueous solutions. Over 40 naturally occurring trichothecenes have been studied and to some degree characterized. T-2 toxin and related trichothecenes are produced principally by Fusarium species and have been implicated in a disease called moldy corn toxicosis, which is characterized by a collection of symptoms including feed refusal, depressed weight gain, digestive problems, diarrhea, and in prolonged or acute cases, death (Bamburg, 1976; Bamburg and Strong, 1971). Smalley (1973) reported pathological findings for these compounds which included hemorrhagic lesions in the stomach, heart, intestines, lungs, bladder, and kidneys.

Trichothecene compounds were first reported in the search for antibiotics and reported by Brian and co-workers (Ueno, 1979) in 1946. Diacetoxyscirpenol (DAS) was isolated in 1961 by Brian and co-workers (Uneo, 1979). Historically, however, trichothecenes have played an influential roll. Ueno (1979) reviewed Woronin's 1891 work on the eastern Siberian staggering grains or "Taumelgetreide" episode in which humans and animals ingesting affected grain suffered from nausea, vomiting, and visual disturbances. The animals also exhibited feed refusal. Woronin isolated Fusarium, Gibberella, Helminthosporium and Cladosporium species. Also, trichothecenes have been implicated as the causative agent in the USSR during 1942-1947 when over 10% of the population of Siberia was stricken with a syndrome including vomiting, skin inflammation, diarrhea, leukopenia, hemorrhage, necrotic angina, sepsis and depletion of bone marrow (Forgacs and Carll, 1972). Overwintered millet, wheat, and barley were the sources of the problem, even in baked goods. Additionally, Aust et al. (1963) and Albright et al. (1964) reported on problems in Illinois when moldy corn was fed to cattle. Gilman et al. (1966) reported the isolation of a toxic compound

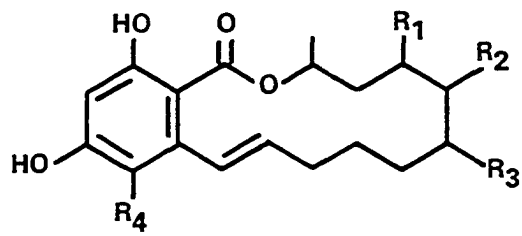
from moldy corn in Wisconsin, which caused edema and hemorrhage of rat skin when applied dermally.

According to Ueno (1979), quite a wide range of fungi are capable of producing the different types of trichothecenes, with about 20% of isolates actually producing the toxins. Because of their world wide distribution, Ueno (1979) cited the Fusarium toxins as possibly the most important of natural mycotoxins.

Symptoms of trichothecene toxicity usually include feed refusal, vomiturition, diarrhea, hemorrhage, edema and necrosis of the skin, hemorrhage in mucus membranes of the stomach and intestine, degradation of hematopoietic tissues and a decrease in white blood cells and platelets, and hemorrhage of the brain meninges, and nervous disorder (Ueno, 1979). In general, young and immature animals are more susceptible than adults with no sex difference observed. Also, Ueno et al. (1971) reported hemorrhage in the intestine, cellular necrosis and karyorrhexis in the actively dividing cells of the thymus, spleen, ovary, and testes of dosed mice and rats and hemorrhage in the intestine, lung, and brain, along with destruction of bone marrow in T-2 dosed cats. He also reported vomiturition and emesis occurring shortly after dosing in dogs, cats, and ducklings regardless of the route of exposure. Wyatt et al. (1972) reported skin necrotization and oral lesions in birds fed T-2 toxin. Ueno and Kubota (1976), Ueno (1979), and Bamburg and Strong (1971) all concluded that the Fusarium toxins are not carcinogenic. However, Ueno et al. (1971) found that nivalenol and fusarenon-X induced respiratory-deficient mutants in yeast cells.

Zearalenone and related compounds

The Fusarium fungi, especially Fusarium roseum, also produce zearalenone and related compounds (Figure 4). These compounds are usually found in corn,



	R_1	R_2	R_3	R_4
Zearalenone	H_2	H_2	=O	H
Zearalenol	H_2	H_2	OH	H
6',8'-Dihydroxyzearalene	OH	H_2	OH	H
8'-Hydroxyzearalenone	OH	H_2	=O	H
7'-Dehydrozearalenone	H	H	=O	H
5-Formylzearalenone	H_2	H_2	=O	CHO

Figure 4. The zearalenone group

wheat, sorghum, barley and oats. The estrogenic characteristics of the zearalenone group are due to their ability to bind estrogen receptor sites (Powell-Jones et al. 1981). Low concentrations in feed are reported to cause hyperestrogenic syndrome in swine (swelling in the teats and vulva), infertility and reduced litter size in swine exposed to these toxins (Mirocha et al., 1971). Zearalenone can be transmitted to piglets in sows milk, causing estrogenism in the young pigs (Harwig and Munro, 1975).

Analysis of Toxins

The analysis of mold toxins in commodities has been an area of intense research due to the serious economic and health implications of toxin-contaminated food. The variety of methods published and the vast scope of the literature attest to the difficulty of the analysis task at the levels of interest and under actual conditions found in real samples from the field. Most procedures published fall into either a screening method or a confirmatory method. Generally, the screening procedures tend to be quickly finished, less sensitive and subject to more sample matrix interferences and can result in false positives or false negatives in some samples. The confirmatory methods, on the other hand, tend to be sensitive but time-consuming, with the false positives and false negatives minimized by additional sample cleanup steps.

Aflatoxin analysis

The Turkey "X" disease in the 1960s prompted early research into methods of analysis for aflatoxins. The bright yellow green fluorescence (BYGF) test was one of the first indicators used to determine if grain was contaminated with aflatoxins (Marsh et al., 1969). In this test, individual kernels of suspect grain are examined under long-wave (365 nm) ultraviolet (UV) light for the

characteristic BYGF emitted by certain metabolic products of mold growth. Marsh et al. (1969) postulated that the BYGF was caused by the action of a heat-labile peroxidase on kojic acid, another secondary mold product. While the test does not directly test for the toxins, Fennell et al. (1973) found good correlation between mold presence, toxin contamination, and BYGF. However, Shotwell et al. (1975) and Barabolak et al. (1978) concluded that while the correlation might lend itself to distinquishing between contaminated and non-contaminated grain, the correlation was not valid between level or number of positive BYGF grains found and actual toxin level found on analysis. Shotwell et al. (1974) observed that corn should be cracked before testing as the BYGF could be under the grain coat, thus diminishing or obscuring its fluorescence. In general, the BYGF test was introduced as a field screening test for quickly assaying positive mold contamination. Chemical analysis usually consisted of solvent extraction of the aflatoxin in the sample, one or more clean-up steps to remove co-extracted interferences from the extract, and finally some form of chromatography to separate the components in the extract for interpretation. Many solvents have been reported in the literature. Chloroform was used in the first method to be adopted as a standard procedure by the Association of Official Analytical Chemists (1980) and has been used by Beebe (1978) and Manabe et al. (1978). Seitz (1975) and Seitz and Mohr (1977) replaced chloroform with methanol to reduce exposure to this toxic carcinogen. Stahr et al. (1977, 1980) reported the use of acetonitrile:water (90:10) for routine feeds and grain analyses.

Chromatographic separation techniques used were based on the need for a rapid response. Pons et al. (1973) and Velasco (1972) introduced minicolumn methods for the rapid screening of commodity samples for aflatoxin contamination. Minicolumns of silica gel (Pons et al., 1973; Shannon et al., 1973;

Shotwell and Stubblefield, 1973), florisil (Velasco, 1972), and alumina, (Shotwell and Stubblefield, 1973), have all been reported plus various combinations of them. The mini-column is subject to false positive results due to the presence in some corn samples of a blue-fluorescing compound which can be mistaken for aflatoxins (Shotwell et al., 1968; Issaq et al., 1977; Gallagher and Stahr, 1980). Also, the differentiation of the aflatoxins present in a sample and their level of contamination is not practical.

Thin layer chromatography (TLC) has been used for the routine analysis of aflatoxins more than any other chromatographic technique. This method allows for complete separation of the aflatoxins from each other and from other potentially interfering components, is capable of detection limits down to nanogram levels and below, and is economical. Both normal phase and reverse phase TLC methods have been reported (Association of Official Analytical Chemists, 1980; Barabolak et al., 1978; Lee and Catalano, 1981). Stahr and Domoto (1982) reported the use of reverse and normal phase high performance TLC (HPTLC) for the analysis of aflatoxins and other mycotoxins. Jain and Hatch (1982) demonstrated that 2-dimensional TLC could separate aflatoxins in samples extracts with unusually high levels of interferences. Stahr et al. (1977, 1980) reported the use of a multimycotoxin screening procedure for the routine determination of aflatoxins in a variety of sample matrixes.

High pressure liquid chromatography (HPLC) has been used recently for aflatoxin analysis offering advantages of automation and greater resolution than TLC but costing much more (Beebe, 1978; Manabe et al., 1978; Cohen and LaPointe, 1981; Devries and Chang, 1982). Both normal phase and reverse phase methods have been offered. Sample interferences and clean-up prior to analysis are particularly important to prevent HPLC column degradation due to irreversibly

bound components that gradually change the makeup of the column resulting in changes in chromatographic performance. The methods cited used UV or fluorescence detectors.

Trichothecene analysis

The trichothecene toxins have been analyzed extensively with liquid-liquid partition followed by extract clean-up and chromatographic separation of the components extracted. Pathre and Mirocha (1979) reported the use of chloroform, ethyl acetate and acetonitrile/potassium chloride/water as extracting solvents for DAS at the 90% or greater level; chloroform, ethyl acetate, diethyl ether, ethanol and acetonitrile/potassium chloride/water as extracting solvents for T-2 toxin at the 80% or greater level; and methanol and ethanol for deoxynivalenol (DON) extraction. However, Vesonder et al. (1976) and Mirocha et al. (1976) reported that ethyl acetate, or 95% methanol or ethanol were unsuccessful in extracting DON. They used water or 40% aq. methanol to achieve a satisfactory extraction. Nakano et al. (1974) used a mixture of 100 ml hexane and 200ml methanol-1% sodium chloride (55:45) to extract DON with an overall recovery of 85%. Work by Stahr et al. (1977, 1979) has shown that acetonitrile/water (90/10) is useful for the quantitative extraction of trichothecenes of known diagnostic significance from grains and prepared feeds.

Most workers use a clean-up step after liquid-liquid extraction to remove potentially interfering components of the extraction prior to chromatographic separation. Hsu et al. (1972) and Eppley et al. (1974) reported the use of silica gel as a clean-up absorbent for T-2 toxin extracts. Florisil was also used for the same purpose by Pathre and Mirocha (1979). Mirocha et al. (1976) reported the use of ferric gel as an absorbant but with low recoveries when used with polar trichothecenes. Stahr et al. (1983a) reported the successful application of silica

gel columns for clean-up, as well as the routine use of an aqueous ferric gel complex to remove sample interferences with minimal effect on extract toxin levels (Stahr et al., 1977, 1978, 1980). While most methods have been applied only to feeds and grains, Stahr et al. (1983b) reported the analysis of trichothecenes in tissues of dosed animals.

Thin layer chromatography (TLC) and gas liquid chromatography (GLC) are used for the actual identification of individual components in a sample extract and for their quantitation. Thin layer chromatography is often used as a screening tool due to its low cost and fast response time. However, it is very much subject to co-extracted sample interferences. Mirocha and Pathre (1973) and Szathmary et al. (1976) used successive TLC developments with different solvent systems to selectively chromatograph first the interferences, then the trichothecenes.

Gas chromatography was used by Bamburg (1969) to analyze T-2 toxin as its trimethylsilyl ether. Pathre and Mirocha (1979) reported on Tanaku and co-workers findings that the silations of T-2 with bis-trimethylsilylacetamide (BSA) or hexamethyldisilazane (HMDS) were not quantitative. They chose a mixture of N-trimethylsilylimidazole (TSIM) and trimethylchlorosilane (TMCS) in pyridine which gave completely derivatized toxins. Mirocha et al. (1976) added BSA to this mixture to derivatize DON. Acetylation has also been used to derivatize the polar trichothecenes to easily chromatographed products. Pathre et al. (1976) analyzed them as the trifluoroacetyl products but had to remove or neutralize the free acid in the final extract prior to GLC. Stahr et al. (1977) and Stahr and Hyde (1980) have noted problems of derivatization, incomplete reactions, new reaction by-products acting as additional interferences, and more time consuming steps to the analysis. They describe the routine analysis of

underivatized T-2 and DAS using conventional GLC techniques at sample concentrations of tenths parts per million (ppm). Stahr et al. (1983a) presented work with "generic" analysis of some groups of trichothecenes wherein the total trichothecene group present in the sample is hydrolyzed to the base triol or tetraol, then reacetylated for GLC as one or two products. While identification of the original components was lost, a measure of total class exposure was possible, with previously unmeasurable traces adding to give a measurable result.

Analysis of Volatiles

Headspace analysis and related techniques

Headspace analysis (Drozd and Novak, 1979) refers to a series of techniques whereby volatile organic components of a solid or liquid sample are allowed (forced) to come to equilibrium with the gas molecules surrounding and within the sample itself. The gas being equilibrated with is referred to as the sample headspace gas, since in most cases it is above a contained sample. Concentration techniques (Dressler, 1979) are usually assumed to be a part of headspace sampling. Various methods are available for the concentration of headspace volatiles allowing for the collection and concentration of the equilibrated volatiles, and their stable storage until subjected to quantitative methods of analysis. Headspace techniques offer advantages over direct analysis of the primary sample: 1) these techniques usually are more successful in representing the original level of highly volatile, low boiling point organic compounds; 2) since the volatiles are separated from the original sample by volatility, other potentially interfering compounds of the original sample such as proteins, sugars, lipids, salts, and pigments are left behind; and 3) as mentioned before, the collection of the volatiles results in the concentration of the compounds of

interest, allowing many techniques to be applied to their identification and quantitation. Much of the research of volatiles in feeds and foodstuff came from earlier work with organic contamination of the atmosphere, and solvent contamination of the hydrosphere. The methods varied in the techniques used to trap the volatiles from the sample headspace gases, and in the ways used to subsequently reverse or untrap them.

Many workers used cryogenic condensation to collect and concentrate volatiles in air samples or volatiles flushed from aqueous solution. Nawar et al. (1960) described passing sample gases through a "U" tube held in liquid nitrogen, resulted in condensation of sample volatiles, including moisture. They used fractional distillation to separate the volatiles. Bellar et al. (1963) reported moisture problems inherent to cryogenically trapping organic volatiles from air or moist samples. Schultz et al. (1971) and Heatherbell and Wrolstad (1969) reported that several desiccants, including calcium sulfate, calcium hydroxide, calcium carbide, and molecular sieve resulted in partial adsorption of the organic volatiles thereby decreasing their desirability as a solution to the moisture problems of cryogenic collecting.

Various absorbents have been used to trap and store volatiles from air or sample headspaces. Saalfeld et al. (1971) described the use of activated charcoal. However, many volatiles are not easily desorbed from this substrate. Saunders and Gammon (1967) described vacuum techniques for desorbing charcoal which resulted in more complete desorption of polar volatiles. Steam desorption of activated charcoal traps was described by Chiantella et al. (1966) and by Saunders (1965). Bertsch et al. (1974) reported Herbolsheimer's and co-workers use of solvent extraction with n-decane to remove chlorinated hydrocarbons from charcoal traps. Jennings and Nursten (1967) found that carbon disulfide was

most efficient in extracting volatiles from charcoal. Grob (1971) reported the use of carbon disulfide to extract more than 100 compounds ranging from C_7 to C_{20} from charcoal.

A later approach to volatiles sampling was the application of thermally-stable polymers and support-bonded chromatographic phases. Schultz et al. (1971) used the porous polymer, Chromosorb 101[RT] to retain the organic volatiles in his sample while letting the moisture pass through. Rook (1972) developed a static gas headspace analysis method wherein 10 ml of water were heated for 12 hours after which the headspace gas was forced through a trap of silica gel. The organics were eluted from the trap and analyzed by GC. Jennings et al. (1972) used Porapak[RT] (Waters Associates, Bedford, MA), a porous polymer, to test the volatiles contents of alcoholic beverages. Kaminski (1982) used Porapak[RT] as a trapping material for the collection of volatiles from moldy grain. Schultz et al. (1971) reported that some of the resins, including Chromosorb 101[RT], consistently gave a background profile, even after extensive conditioning. Sakodynskii et al. (1974) presented a review of physical properties of poly(p-2,6-diphenylphenylene oxide or Tenax[RT] as compared to other resins. Mieure and Dietrich (1973), Bertsch et al. (1974), and Novotny et al. (1974) used Tenax[RT] GC phase (Applied Science Laboratories, Inc., State College, PA) in a trap to collect and concentrate sample volatiles. The Tenax[RT] acted as an absorbent trap for the volatiles and concentrated them as a narrow band. After volatile collection, the Tenax[RT] was heated and the volatiles were eluted from the resin bed with inert gas. Glaze et al. (1973), Junk et al. (1974), Ryan and Fritz (1978, 1980), and Marshall and Fritz (unpublished data, Ames Laboratory, Iowa State University, Ames, IA) described the use of XAD[RT] resins (crosslinked polystyrene divinylbenzene macroreticular resins, Rohm and Haas, Philadelphia, PA) which

effectively absorbed and concentrated organic components while rejecting water. Because of their thermal stability, these resins could then be heated to strip the absorbed volatiles. Marshall and Fritz (unpublished data, Ames Laboratory, Iowa State University, Ames, IA) also described the stability and lack of background profile of Tenax^{RT}, when properly conditioned. Their work pointed out that XAD ^{RT} and Tenax^{RT} complemented each other, with the XAD ^{RT} exhibiting superior trapping and retention of volatiles from water, while the Tenax ^{RT} showed superior thermal stability upon rapid heating during thermal desorption for GC introduction. Kissinger and Fritz (1976) reported a novel approach to volatiles analysis with resin collection on acetylated XAD ^{RT} resin followed by stripping with pyridine. Chriswell et al (1976) showed that the pyridine solvent could be completely eliminated when injected on a copper chloride/chromosorb column. The obvious advantage is a lack of a solvent peak to interfere with the very early eluting volatile peaks.

Insalaco and Dymerski (1983) described a variation of resin trapping whereby air sampling cartridges were thermally desorbed at reduced atmospheric pressures (< 5 torr) resulting in increased recoveries of most volatiles tested, especially the lighter molecular weight, purgable components. Ryan and Fritz (1980) and Marshal and Fritz (unpublished data, Ames Laboratory, Iowa State University, Ames, IA) described instrumental modifications and techniques for thermally desorbing trapped volatile components from a resin concentration tube with introduction to capillary GC columns resulting in much greater component resolution and sensitivity. The sample trap was actually desorbed into a precolumn or micro-trap in line with the capillary GC column where the volatiles were again adsorbed. This step was accomplished at flows of 10-30 ml/min. The micro-trap was then switched in-line with the capillary column, at flows of 25-

50 cm/sec. and rapidly heated. Because of its micro dimensions and low flow rate, minimal band broadening and component dilution in the carrier were observed and excellent component resolution was reported.

Kolb et al. (1981) and Westendorf (1985) describe multiple headspace analysis techniques for successive approximation quantitation of samples. The static headspace of a sample is repeatedly injected, (termed stepwise gas extraction), and the concentration decrease was noted. Using regression equations, the authors calculated the original concentration in the sample headspace, while automatically correcting for any sample matrix effects.

Use of induction heating

Very little has been reported using induction furnace heating for chemical analysis. Stahr and Hyde (unpublished data, Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA) applied induction heating to a variety of biological samples--feed, liver, soil and roughages--to determine its applicability for the release of bound mercury. Stahr, Hyde, and Lerdal (unpublished data, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Ia.) preliminarily investigated the release of volatile organic compounds from feeds using induction heating and later patented the furnace device used (USA patent 4,314,027). Hyde et al. (1983) reported on techniques for induction stripping of volatiles from feeds with resin trapping and subsequent analysis.

Volatiles in foods

Many workers have analyzed the volatiles in a variety of foods. Much of the early work concentrated on normal or fresh foodstuffs and on freshly baked or processed food products, in an attempt to identify and isolate compounds responsible for a food's flavor. Nawar et al. (1960) used cryogenic condensation

to collect volatiles from butter oil, corn oil, potato chips, wine, shrimp and apple juice. Porous polymer trapping techniques were used by Jennings et al. (1972), followed by dry ice volatile condensation to determine volatiles from alcoholic beverages. Hougen et al. (1971) analyzed headspace volatiles of numerous cereal grains and found simple alcohols and carbonyls in the C_2-C_7 range. They used sample heating to equilibrate the volatiles and noted artifact peaks as a result of the heating process. Legengre et al. (1978) investigated volatiles in rice, bran and corn cereals by directly steam distilling the volatiles onto a cooled GC column (poly-m-phenoxylene, Applied Science Laboratories, State College Park, State College, PA. on Tenax^{RT}). Flath et al. (1978), and Flath and Takashashi (1978) used reduced pressure distillation of corn silk followed by diethyl ether extraction to recover volatiles and to identify which ones acted as insect attractants. They found 70% of the recovered components to be C_2-C_9 alcohols with 15-20% of these C_6-C_9 alken-ols. McKeary and Hougen (1977) used volatile profiles obtained from kernel corn by steam distillation/methylene chloride extraction to differentiate between varieties of corn. Wiseblatt and Kohn (1960) investigated volatiles from freshly baked bread. Rao et al. (1978) also looked at fresh bread with the conclusion that raw wheat did not have low molecular weight alcohols as a major class of volatiles but that baked bread did.

Volatiles as a measure of grain quality

Very few workers have tried to apply volatiles testing to the determination of grain quality or deterioration. Dravnieks and Watson (1973) and Dravnieks et al. (1973) attempted using GC profile analysis to distinguish between corn with musty or sour aromas from normal "healthy" corn. No identification of individual volatiles was attempted, but rather a statistical analysis of peaks was used to predict "good" or "bad" grain. All peak areas within previously defined retention

time windows were totalled and tested for correlation to human sensory evaluation of "good" or "bad" grain. Kaminski et al. (1973) and Kaminski (1982) isolated cryogenically condensable volatiles from grains inoculated with bacteria and molds. The volatiles were characterized, using classical organic chemical reactions, to be carbonyls, alcohols, and so forth. Levels of volatiles were found to increase as early as the first day post inoculation. Alcohol levels were higher in mold-inoculated grains than bacteria-inoculated specimens. Gas chromatography was used to identify 1-octene-3-ol as a consistent volatile component of Aspergillus-infected wheat. The sample used was equivalent to 100 grams original material. Abramson et al. (1980) used ambient air sampling of naturally inoculated barley, wheat, and oats and verified several volatiles previously reported but found no octenols.

Several workers have investigated the parameters that affect the efficiency of volatiles equilibrium in water solutions or in real samples. Nawar (1966) found that the medium being sampled very definitely affected the release characteristics for volatiles in the sample. The degree of lipid content, the types of volatiles present, the salt and sugar levels in the sample all affected the efficiency of volatile equilibration into the gaseous headspace. As sample osmolarity increased, so did the coefficient of equilibration for most volatiles. Buttery et al. (1969) experimentally verified the theoretical prediction that higher molecular weight homologs in a chemical series C_1-C_{10}: n-acids, n-alcohols, n-ketones, n-ethers, and n-paraffins were more volatile from aqueous solution than the lower molecular weight members. For example, the static air/water partition coefficient for octanol was 3 times that for butanol under identical conditions. Franzen and Kinsella (1974) investigated the effect on

organic volatility that protein addition had. They found decreased volatility due to increased solubility of many of the volatiles with the protein-water complex.

Summary

Aflatoxins, trichothecenes and the zearalenone group toxins have been shown to be of great concern to human and animal health as well as being an important detrimental economic factor in the agricultural sector. This has established a need to be able to identify toxin presence and quantitate toxin levels in feed commodities.

Chemical tests have been developed to identify and quantitate toxin levels but these tests are subject to many sample matrix interferences, are time-consuming and expensive to perform.

Preliminary work indicated that molds produce other products in addition to the toxins, especially volatile organic compounds, which might correlate with the toxin production. Work on air and water contamination by organic components was done using various headspace analysis techniques for organic volatile collection and concentration. From the work that has been done, it was concluded that techniques using these same principles should be applicable to the collection of volatiles from moldy feeds, allowing rapid analysis while largely eliminating sample interferences. The development of such techniques would permit studies of the correlation of observed volatiles to toxin levels found in moldy grains.

MATERIALS AND METHODS

Materials

Solvents, chemicals and reagents

All organic compounds used for analytical studies were ≥ 98% purity as confirmed by GLC. Sources and compounds are as follows:

Fisher Chemical Company, Springfield, NJ.

2-methyl-1-propanol

Baker Chemical Company, Phillipsburg, NJ.

pentane, cyclohexane, 1-heptanol

Eastman Kodak Chemical Company, Rochester, NY.

2-butanal, heptane

Matheson, Coleman, and Bell Manufacturing Chemists, Cincinnati, OH.

1-butanol, 2-butanone

Aldrich Chemical Company, Milwaukee, WI.

butraldehyde, 1-pentanol, 2-hexanone, hexanoic acid, 2-heptanone, hexanal, heptanal, 1-octene-3-ol, 2-octene-1-ol, 3-octanol, octyl aldehyde, nonyl aldehyde, 2-nonanone, 5-nonanone

All organic solvents used in sample extracts were distilled in glass or equivalent. In-lab GLC purity checks were performed on each lot.

All aqueous solutions were prepared using de-ionized water from a Millipore Milli-Q deionization system with activated charcoal cartridge for removal of trace organics, Millipore Corp., Bedford, MA.

All gases for GLC analysis were obtained from Air Products, Tamaqua, PA.

High purity helium used for GC-MS was purchased from Matheson Gas, East Rutherford, N. J.

Mycology supplies

A slant culture of Aspergillus parasiticus (NCCR* 2999) was obtained from Dr. Abou-Gabal, Veterinary Microbiology, Iowa State University, Ames, IA. Authentic identification was verified using lacto-phenyl cotton blue mount techniques and light microscopic examination for taxonomic features specific to this genus and species. A slant culture of Fusarium roseum (Peska W-8, R-6576) was obtained from Dr. Pat Hart, Michigan State University, MI. (via Dr. H. M. Stahr, Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA). Authentic identification was verified using lacto-phenyl cotton blue mount techniques and light microscopic examination for taxonomic features specific to this genus, species. Also, pure cultures were grown on s abouraud dextrose agar (SDA) to check gross cellular morphology for characteristics of these two species.

Sabouraud dextrose agar medium was prepared by Ms. Bev Fowles, Veterinary Diagnostic Labortatory media preparation section, according to industry-accepted methods (Appendix).

Culture vessels used in the last two studies were manufactured to specification by Harold Hall, Iowa State University chemistry glass shop (Figure 5). A female 71/60 standard taper ground glass joint was added to a 1000 ml. round bottom flask. The male 71/60 fitting was reduced to 24/40 female and fitted with a 3" bushing. Two gas connections, for sweep gas in and out, were added to the 1000 ml round bottom flask. Glass rods fitted with Teflon paddles allowed stirring of the large, dry corn mass when rotated at 28 rpm. using low speed, high torque Bodine^{RT} electric motors.

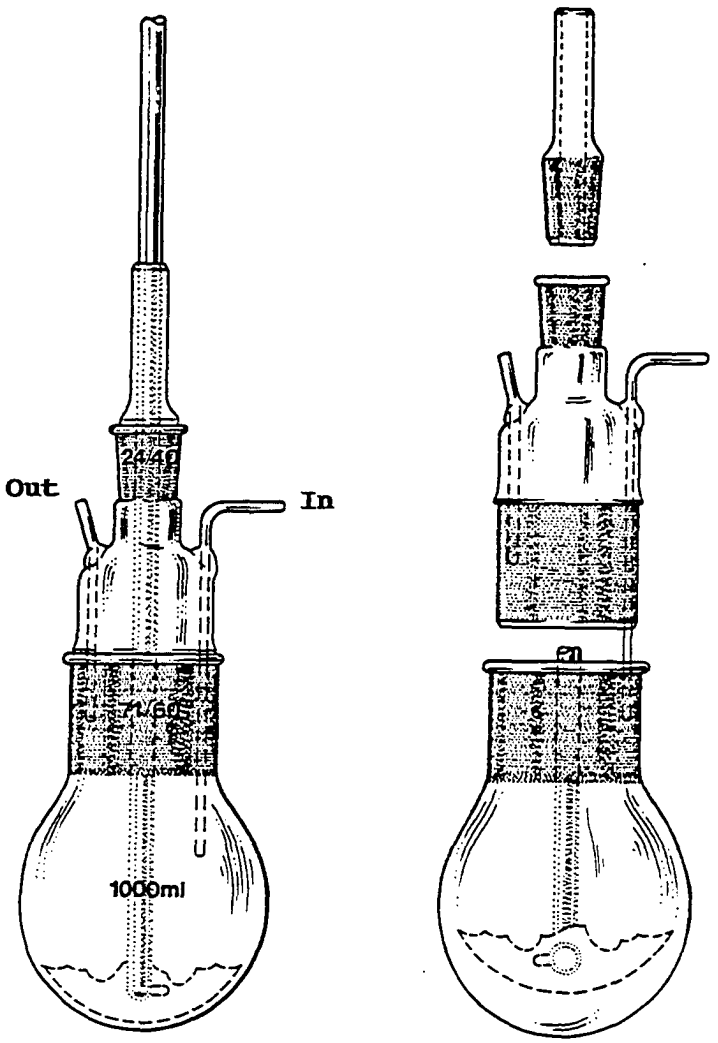


Figure 5. Closed cell model culture vessel, with sweep gas and stirring

Trial 3, closed cell with stirring, used this apparatus for the collection of the dynamic headspace over time. The 71/60 ST joint allowed easy removal of the oversize stirring paddle. The bushing provided a gas-tight seal to the ground-glass stir-rod.

Gas chromatographic materials and supplies

A Bendix model 2300 gas chromatograph with flame ionization detector (FID) and 1 millivolt (MV) stripchart recorder were used to perform the routine GLC. The packed column injector was modified to accommodate the insertion of a 1/4" OD stainless steel resin trap and seal (Figure 6). Also, carrier gas flow to the injector was interrupted with an on-off balltype valve for carrier gas stop-flow operation.

A DB-Wax (polar phase Mega-bore^{RT}) capillary GLC column, 15 m X 0.53 mm fused silica with 1.0 micron phase loading; deactivated fused silica capillary, 3 m X 0.53 mm; and Valco zero-dead volume union for connecting them were obtained from J & W Scientific, Rancho Cordova, CA.

Free fatty acid column phase (FFAP) was purchased from Analabs, North Haven, CT.

Gas Chrom Q, 80/100 mesh and Tenax, 80/100, were obtained from Applied Science Laboratory, State College, PA.

OV-17 phase on Gas Chrom Q, 100/120, was purchased from the Anspec Company, Ann Arbor, MI.

Hamilton syringes for sample introduction to the gas chromatographs were obtained from Hamilton Company, Reno, NV.

Precolumn resin collection

Equipment used for the precolumn resin work was made available by Dr. J. S. Fritz, Ames Laboratory, Iowa State University, and has been described in detail (Ryan and Fritz, 1980). A Tracor 560 GLC was modified for use with capillary columns and for sample introduction from a precolumn desorption unit rather than the conventional heated injector. The essential elements of the precolumn desorption unit (Figure 7) are the Tenax^{RT} trap, the precolumn capillary, and

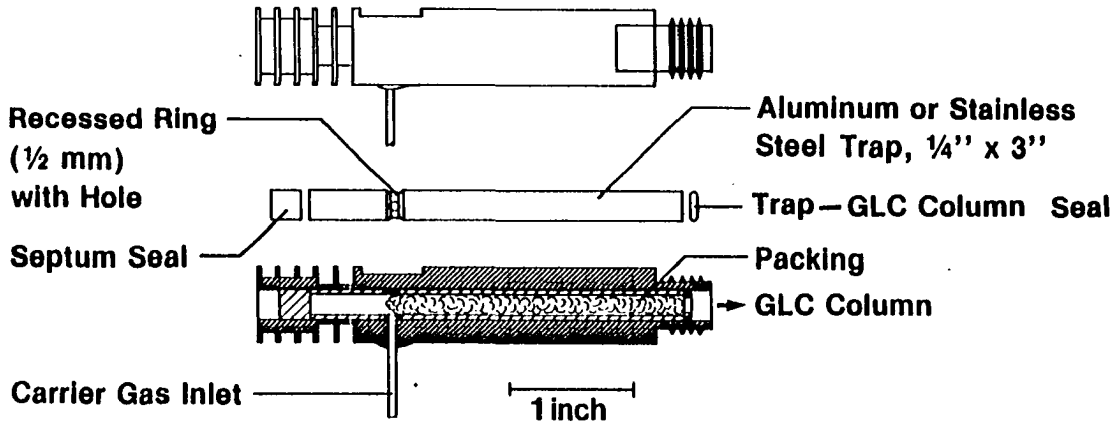


Figure 6. Modified gas chromatograph injector and resin trap assembly

The injector (top schematic) was drilled to 1/4" ID to accomadate the stainless steel resin trap assembly (middle schematic). The trap was packed with 0.1 gm Tenax[RI] and retained with glass wool plugs. The trap in-place is shown (bottom schematic) with the seal to the GLC column made from septum material pierced with a 1 mm capillary for unrestricted flow.

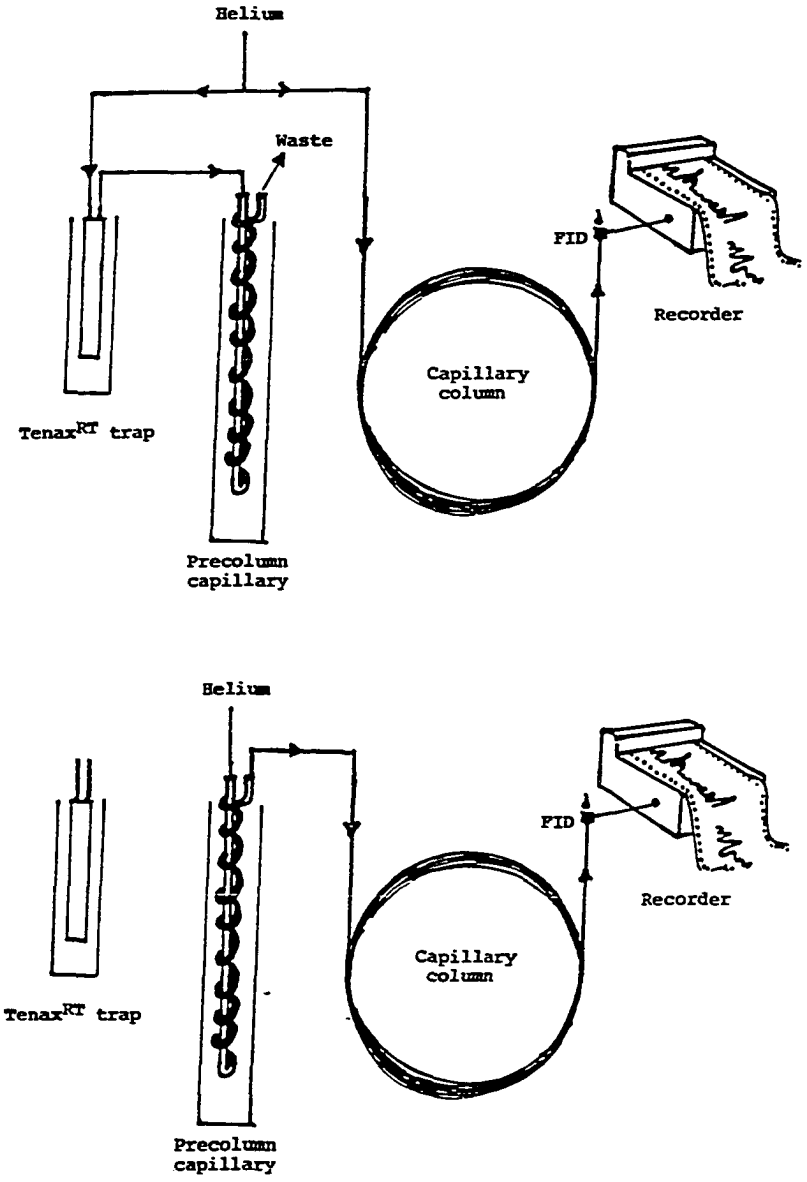


Figure 7. Trap-to-precolumn capillary GLC (Fritz group, Ames Laboratory)

The top schematic shows the connections for Tenax^{RT} trap desorption to the precolumn capillary. The bottom schematic shows the switch-over to precolumn thermal desorption onto the GLC capillary column for separation and identification.

selective switching that allows altering the connections between them. During the collection mode, the Tenax^{RT} trap is heated, with the desorbed volatiles contained therein transfered to the liquid CO_2-cooled precolumn capillary (top schematic). After the trap is totally desorbed to the precolumn capillary, the connections are switched, using rotory switching valves, to the configuration (bottom schematic) allowing the precolumn, when heated, to desorb the volatiles to the GLC capillary column.

Cryogenic-focused capillary equipment

A tee column sheath was fabricated from 1/4" copper refrigeration tubing and connected to the Mega-bore capillary and liquid carbon dioxide (CO_2) with Swagelok ^{RT}fittings (Figure 8). Liquid CO_2 was delivered to the cryogenic sheath through a ball-type on-off valve. The liquid CO_2 encountered a restricting orifice upon entering the tee, and the resulting pressure drop allows adiabatic cooling within the sheath.

Resin trap GC-MS equipment, materials and supplies

A Finnigan 4500 GLC-Mass spectrometer equipped for electron ionization analysis with positive ion collection was used for all mass spectral data evaluation. All data were collected and interpreted using a Teknivent model 29K mass spectral data acquisition system from Teknivent, St, Louis, MO.

Induction equipment

The induction heating was accomplished using a Leco model 521 induction supply from Leco Lab. Equipment Company, St. Joesph, MI. The furnace used for the actual sample heating was built to specification by Andy Wunderlich, Physics Instrument Shop, Iowa State University, Ames, IA (Figure 9).

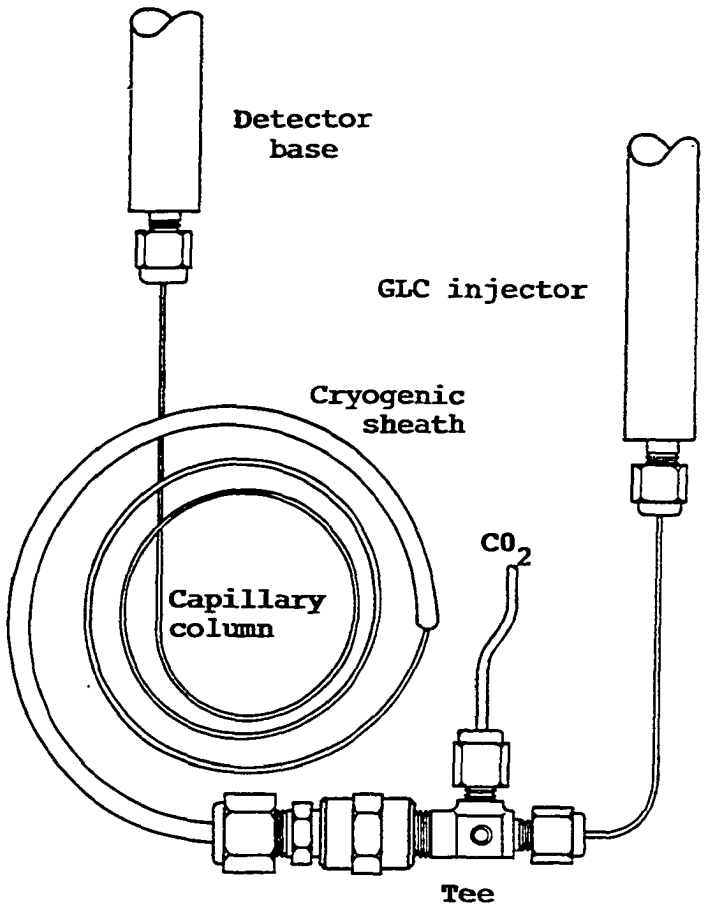


Figure 8. Cryogenic focus Mega-bore capillary GLC

The liquid CO_2 is introduced into the Tee through a resticting orifice, whereupon the liqiud expands to a gas, causing adiabatic cooling. The entire apparatus fits inside the GLC oven. Extended cooling of the Tee will lower the oven temperature by 5-20 °C. This could be minimized by venting the sheath outside the oven.

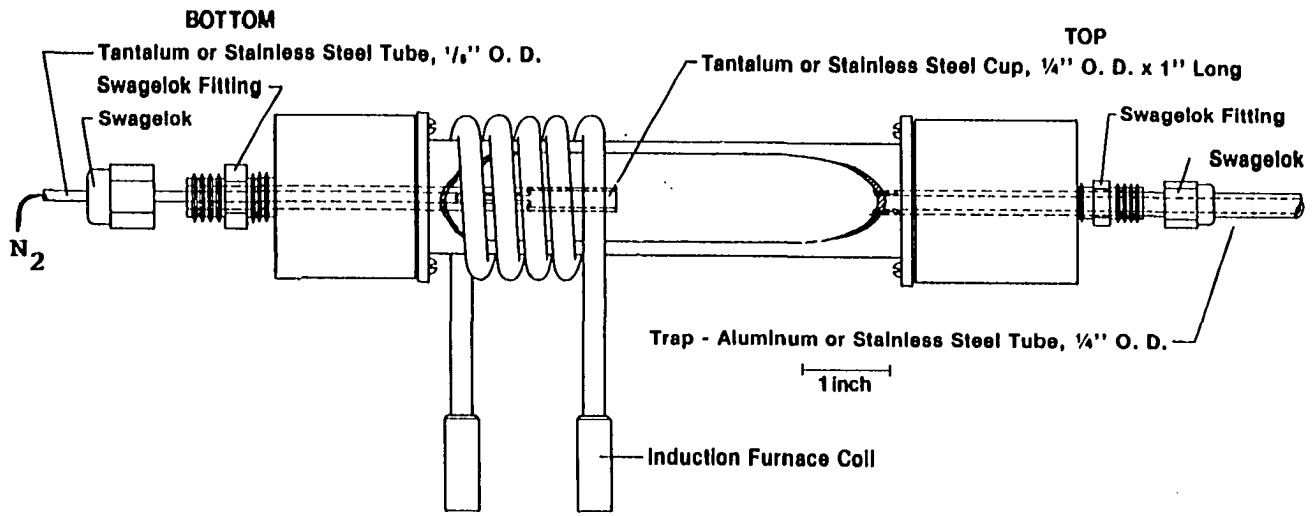


Figure 9. Induction heating assembly

The furnace allows a sample to be heated rapidly while volatile compounds are swept from it by nitrogen gas. The sample is placed in the tantalum or steel cup, a mesh retainer screen fitted to retain the sample, and the cup is inserted into the furnace as shown. The figure is showing the furnace on its side rather than vertically as in operation.

Methods

Preparation of inoculated feed for volatiles production

Dry, yellow-dent, whole kernel corn was purchased from a local grain elevator. Using a Wiley mill [RT] no. 1, the kernel corn was ground to pass a #200 mesh screen, then mixed by hand to obtain a homogeneous mixture. Samples were analyzed for aflatoxins, scirpenes, and estrogenic mycotoxins (Materials and Methods, Chemical Analysis of Mold Toxins). Moisture content was determined by weighing 10 gm of the homogeneous ground corn into a pretared container, then drying for 24 hours at 105 °C and reweighing. The weight difference was due primarily to sample moisture. Sabouraud dextrose agar plates were inoculated with spores and mycelium from authentic slant cultures of Aspergillus parasiticus and Fusarium roseum. These were harvested for preparation of the aqueous spore suspension when sporulation peaked, as evidenced by color and gross morphologic colony appearance. Mycelium and spores from plate cultures were scraped into pre-measured volumes of autoclaved water and blended at high speed to break the conidia and mycelium from each other. Portions of the aqueous suspensions were diluted to 10^{-4}, 10^{-5}, and 10^{-6} in water. A spore count was determined from these dilutions, using SDA plates incubated at room temperature. Final spore concentrations, based on the plate reading in the range of 30-300 spore colonies, were in the range of 10^{4}-10^{6} spores per ml.

Actual cultures were prepared by weighing 200gm corn into the culture vessel, autoclaving for 20 min at 15 psi to sterilize the feed, then inoculating with 20ml aqueous mold spore suspension. At time of inoculation (day 0), stirring, sweep gas flow (6 ml/minute nitrogen), and volatiles collection with a

Tenax^{RT} trap were all initiated. At the conclusion of an entire volatiles experiment (20-25 days), all grain materials suspected of toxin contamination were thoroughly wetted with bleach/water (50/50) and allowed to soak for 24 hours prior to disposal.

Resin traps and culture vessel headspace volatiles trapping

Traps of stainless steel tubing, 1/4" OD X 2 15/16" length (Figure 6) were prepared by the Physics Instrument Shop, ISU. Tenax ^{RT} resin, 0.1 g of 80/100 mesh, was used as the trapping medium. Silanized glass wool plugs were used to retain the resin in place. The assembled traps were conditioned prior to use by inserting them into the modified gas chromatographic injector at 230 °C for at least 10 minutes, with nitrogen carrier flowing. Actual sample collection of volatile organic compounds was accomplished as compressed cylinder air flowed into the culture pot headspace, mixed with any volatile compounds, then exited through the Tenax^{RT} trap where the organic compounds were adsorbed and concentrated. The amount of volatiles collected depended on the length of sampling and the metabolic activity of the mold in culture.

Chemical analysis of mold toxins

Two methods of analysis were used depending on the amount of sample to be analyzed (50 grams (gm) or 1 gm).

VDL multi-mycotoxin method (50 gm): Fifty grams of ground feed was weighed into a blender and 200 ml acetonitrile/water 90/10 was added. This was blended at medium speed for 4 minutes, then allowed to settle prior to filtration. One hundred ml were filtered through Whatman #1 filter paper and reserved. Bleach was added to the remaining sample fluid to degrade the toxins prior to disposal. One hundred ml petroleum ether were added to the reserved

sample extract in a separatory funnel, mixed and vented. The ether layer was discarded. Ferric gel was prepared by adding 10 ml 10% aqueous ferric chloride to 100 ml water, then titrating to pH 4.6 with 4% sodium hydroxide. The resulting gel was added to the separatory funnel and sample extract. This was gently mixed, allowed to separate and 100 ml removed. The remaining sample/gel was discarded. The reserved sample was returned to the separatory funnel, 100 ml. methylene chloride was added , and mixed. After separation, the methylene chloride layer was saved and concentrated to dryness using nitrogen gas. Routine TLC separation and observation were accomplished using Whatman KC18F plates (reverse phase C_{18} with fluorescent indicator, Whatman Clifton, NJ). Ten percent of the extract was spotted on the plate with appropriate standards and developed in ethanol/water/acetic acid 65/35/1. The aflatoxins and zearalenone group were visualized using long wave UV (365nm) and observing appropriate fluorescence. If positive, the spots were scraped for fluorescent confirmation and quantitation. The scirpenes were observed by overspraying the plate first with aluminum chloride, then anisaldehyde, followed by heating. If the sample was especially troublesome, due to co-extracting materials, TLC was accomplished using EM Science Silica Gel 60 plates (normal phase/no indicator, EM Science, Cincinnati, OH) and chloroform/acetone 90/10 developing solvent.

Mini-extraction (1 gm): One gm sample was weighed into a blender and 100 ml acetonitrile/water 90/10 added. This was blended for 4 minutes. All the extraction fluid was filtered and reserved. This was defatted as in the VDL method. To the defatted sample extract, 50 ml 2% sodium chloride and 50 ml water were added, followed by mixing. To this, 50 ml methylene chloride were added, mixed, allowed to separate, and reserved. Another 25 ml methylene chloride similarly added, mixed and reserved. The methylene chloride extract

was concentrated to dryness using nitrogen gas. The total extract was analyzed as described in the VDL method for the presence of toxins.

All fluorometry was accomplished using an Aminco-Bowman dual monochrometer fluorometer (American Instrument Company, Silver Springs, MD). The aflatoxins were confirmed using 360nm excitation and 430nm emission. Zearalenone was confirmed using 320nm excitation and 440nm emission.

Volatiles analysis of cultured feed samples

Prior to daily volatiles collection by induction heating, the furnace sweep gas flow was checked and corrected to 300 ml/min. Also, the Leco induction supply grid and plate currents were set to 150 milliamperes (ma) DC. Each day, at approximately the same time, the headspace resin trap was removed for GLC analysis and 1.5-2.0 g cultured feed was taken from the culture vessels. Of this, 1 gm was reserved for chemical toxin analysis and the rest was used for induction volatiles analysis. An analytical single pan weighing machine was used to weigh 0.2 gm which was then placed into the sample cup of the induction furnace. A metal mesh screen was installed to prevent sweep gas from dislodging the sample. The cup was inserted into the furnace chamber and the Swaglok^{RT} sealing assembly secured. A previously conditioned Tenax^{RT} resin trap was installed at the furnace outlet and secured. Nitrogen sweep gas at 300 ml/min was started and the induction heating period initiated. It consisted of 3 cycles, each of 8 seconds on and 52 seconds off. After collection, the resin trap was removed and wrapped in aluminum foil until desorption-GLC analysis. The sample cup was removed, the old sample disposed of, and the apparatus allowed to cool for the next sample.

Packed column chromatographic techniques and conditions

Routine chromatographic separation of the components trapped on Tenax RT resin was accomplished using a modified Bendix RT 2300 GLC equipped with a flame ionization detector (FID). All routine separations were achieved using 10% free fatty acid phase (FFAP). Free fatty acid phase was prepared by dissolving FFAP in chlorinated solvent, adding it to Gas Chrom Q (80/100 mesh) and slowly removing the solvent under vacuum with a roto-evaporator. Columns were prepared using solvent-washed copper tubing and silanized glass wool. Column performance was monitored daily for chromatographic degradation by injection of a standard mixture of compounds of interest. GLC conditions were;

Oven Temp	135°C
Injector Temp	200°C
Detector Temp	240°C
Carrier Gas	Nitrogen (50 cc/min.)
Hydrogen Flow	30 cc/min.
Air flow	1 standard cubic feet per hour

When a trap was to be analyzed, the carrier flow was shut off using the ball valve. The injector cap and septum were removed and any previously analyzed trap withdrawn from the injector. The new trap was inserted and the septum and injector cap replaced. After several seconds to allow the new trap to thermally equilibrate, the ball valve was opened which allowed the carrier gas to sweep the adsorbed trap contents onto the GLC column. Zero time was marked on the strip chart when the ball valve was opened. Retention times and intensities of resulting peaks were observed.

Capillary GLC techniques

Two different capillary techniques were applied to the analysis of volatiles collected on resin traps: 1) precolumn resin collection from the resin trap, followed by thermal stripping to the GLC capillary (work with the Fritz-modified Tracor GLC); and 2) cryogenic focusing of the volatiles as they were thermally stripped directly onto the leading edge of a large-bore capillary column.

Conditions for the precolumn-resin work were:

Oven temp	80-160°C temp. programmed, 5°C/min.
Injector	200°C
Detector	FID
Detector temp	220°C
Carrier	Helium (Zero Grade)
Carrier Flow	1 ml/min.

The analysis sequence consists of desorption of the collected sample volatile trap onto the precolumn followed by precolumn capillary desorption onto the capillary column. A typical analysis consisted of several stages of operation, each with differing parameters of time, temperature and gas flow.

Step 1: A trap ready for analysis was inserted into the cooled trap heater (22°C) and secured to the sweep gas lines with 1/4" SwagelokRT assemblies. At this time, the sweep flow is off. Carbon dioxide coolant flow is initiated to the precolumn resin capillary to achieve and maintain 10 °C. Also, the GLC oven is cooled to 80°C, readying it for the chromatographic separation of this sample.

Step 2: When the precolumn has stabilized at 10 °C, sweep gas flow is started, and heating of the original resin trap assembly to 215 °C starts. The actual rate of heating is approximately 55 °C per minute. The desorbed volatiles

are swept from the original trap onto the cooled precolumn capillary where they are resorbed. The sweep gas then exits to waste.

Step 3: At the end of the desorption-readsorption period, the original resin heater is deactivated and cooled with CO_2 to 22°C. Using multi-port valves, the precolumn capillary is switched in line and backward to the GLC capillary column inlet. Precolumn cooling to 10 °C is still maintained at this point. The precolumn now experiences reversed carrier flow with respect to flow during collection. The carrier flow exits the precolumn and enters the head of the GLC capillary column.

Step 4: Cooling of the precolumn is discontinued and rapid heating of the precolumn is initiated, from 10°C to 260°C. At this same time, the GLC program is started and separation of the collected sample volatiles over time is observed.

Step 5: After completion of the chromatographic separation, the precolumn heater is deactivated and the precolumn capillary cooled with CO_2 to 10°C for the next sample, restarting with step 1.

Mass spectral techniques

All mass spectral (MS) analyses were done with the Finnigan 4500 GLC-MS in EI, positive ion collection mode. The glass jet separator GLC-MS interface used effectively reduced the GLC carrier flow (25-35 ml/min at atmospheric pressure) to the low pressures necessary for EI reactions (< 10-6 torr).

Mass spectrometer GLC conditions were:

Oven temp	varied with the column used
Injector temp	230°C
Carrier Gas	Helium
Carrier Flow	25-35 ml/min

Mass spectrometer conditions were:

Separator temp	245°C
Ionizer Vacuum	<10-6
Ionizer temp	140°C
Accelerating Voltage	70 EV

The injector of the GLC-MS was modified as described for the Bendix 2300 to allow insertion of a 1/4" OD resin trap into the injection port. Because of the high vacuum on the GLC column, no stopflow was attempted during GLC-MS analysis. Time 0 was counted from the moment of septum cap replacement. Several techniques were used in evaluating what compounds were present in the feed volatile chromatograms. After subtracting the column background from a spectra of interest, it was normalized to the base peak. This corrected spectra was compared to a library of locally generated volatile standard spectra for similarities, using the Teknivent computer data management system. If this comparison failed to provide a suitable identification, then the spectra was manually compared to spectra in the EPA/NIH (1980) collection of over 15,000 EI spectra (EPA/NIH, 1980). If no suitable identification was found, the spectra was searched using the Chemical Information System, Inc. mass spectral search system (Baltimore, MD), with a collection of over 45,000 EI mass spectra. These latter searches were performed using probability base matching, a process that reverse compares the unknown spectra to the standard library spectra to see if the library compound is a part of the unknown.

Statistical methods of analysis

All statistical evaluation of data was accomplished with the Statistical Analysis System (SAS), a collection of statistical programs under license from the SAS Institute, Inc., Cary, N.C., running at the Iowa State University Computation Center.

The SAS procedure CORR was used to test the degree of relationship between the volatiles levels found and toxin levels found.

The procedure GLM was used to test different models of regression for the curves found to be statistically significant using the procedure CORR.

The program used to evaluate correlation between toxin level found and volatile compound presence is listed in the Appendix.

RESULTS AND DISCUSSION

Gas Chromatographic Method Development

Packed column GLC

The separation of low molecular weight volatile compounds was achieved using gas liquid chromotography. While many column phases could accomplish this, primarily two support phases were tested because of their ready accessibility and familiarity to the principal investigator. Free fatty acid phase and OV-17 phase were evaluated for separation characteristics when used to chromotograph a laboratory mixture of expected volatiles. Alcohols, especially the octene-ols were of particular interest because of literature reports concerning their presence in moldy feed.

Ten percent FFAP on Gas Chrom Q ^{RT} (80/100 mesh) and 3% OV-17 on Gas Chrom Q^{RT} (100/120 mesh) were evaluated. Columns of each phase were prepared, conditioned overnight, then tested using a model volatile mix. While both columns were quite capable of separating the mixture, repetitive injections showed the FFAP to be more reproducible than the OV-17, with respect to component retention time and peak measurement. Using the same injection techniques in both cases, the OV-17 seemed more affected by the water present. Injections made back-to-back produced nonreproducible results as compared to injections made over longer time periods. Free fatty acid phase, however, seemed unaffected by sample moisture as evidenced with either immediately adjacent injections or widely spaced ones.

Figure 10 shows an example of an isothermal chromotographic separation on 10% FFAP of a volatile standard mixture, while Table 2 illustrates the degree of reproducibility achieved with this column material. Retention was measured

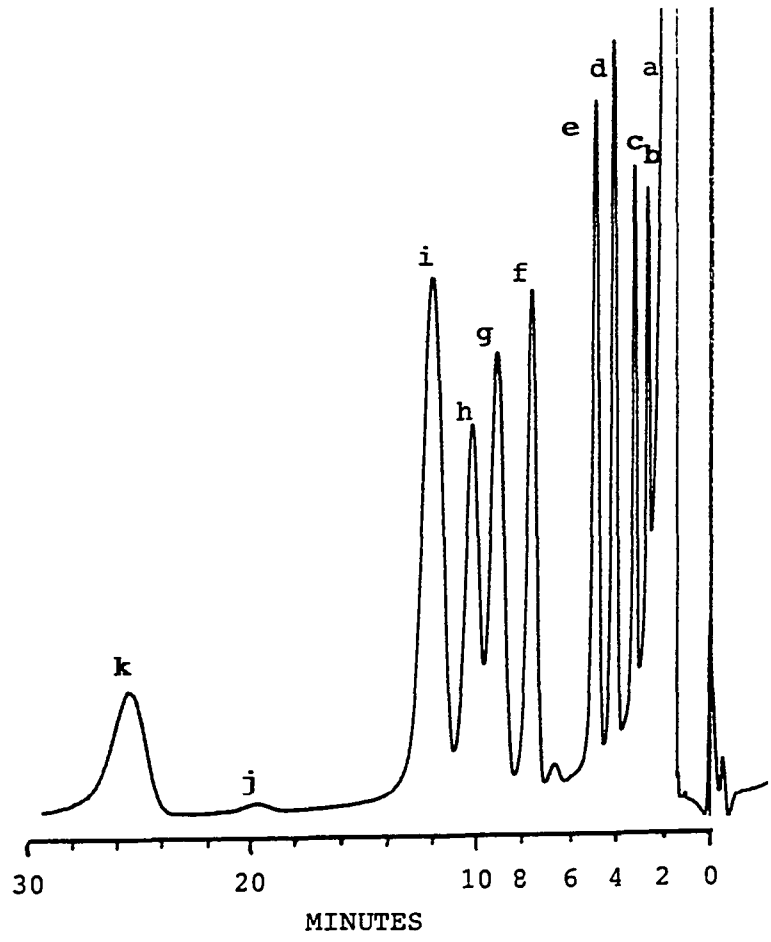


Figure 10. Gas chromatogram of a model volatile standard mixture in water

10% FFAP, 135°C isothermal, 20 cm/min

a solvent
b 2-pentanone
c 2-hexanone
d methyl butanol
e 1-pentanol
f 1-hexanol
g 3-octanol
h 2-octanol
i 1-octanol and 1-octene-3-ol, merged
j impurity in 2-octene-1-ol
k 2-octene-1-ol

Table 2. Same-day measurement of peak retention, area and height reproducibility using 10% FFAP, isothermal, 135 °C

	1-pentanol	1-hexanol	2-octanol	1-octene-3-ol 1-octanol	2-octene-1-ol
Retention (mm)	19.6	30.0	41.9	46.8	98.9
RSD (%)[a]	1.5	3.2	8.0	2.2	2.1
Peak area	85.0	98.3	110.8	250.5	118.2
RSD (%)	1.5	3.1	2.9	4.7	4.3
Peak height	94.4	74.6	50.0	81.7	25.4
RSD (%)	1.5	2.7	4.6	3.6	5.2

[a] RSD = relative standard deviation expressed as a % of the mean.

from injection in millimeters (mm), peak height in mm and peak area in mm^2 (mm pk ht x mm pk width at 1/2 pk ht). The values given are averages of 6 injections of a model volatile mixture containing, 1-pentanol, 1-hexanol, 1-octanol, 2-octanol, 1-octene-3-ol, and 2-octene-1-ol. The injections were made with a Tenax^{RT} trap in place in the GLC injector so that the trap served merely as an extension of the 10% FFAP GLC column. Since the injections were made within the same day, without removing the trap, its contribution to each chromatogram is identical and minimized, and the chromatographic reproducibility approaches the ideal. Reproducible retention times were particularly important because of their use in identifying observed peaks. Most of the relative standard deviation observed were in the range of 2-5% which indicated very acceptable reproducibility within a laboratory.

Temperature programming profiles were investigated and found to substantially increase resolution between homologous volatile components. Figure 11 demonstrates the separation of a much greater range of volatile components when the column was thermally programmed from 80 °C to 160°C at 10°C per minute. Volatiles, including the light alcohols and low molecular weight ketones, were separated very well. These components were lost in the solvent front when using isothermal conditions necessary for the elution of the C_8 alcohols within an acceptable time.

In comparing isothermal and temperature programming GC results (Figures 10 and 11), note that a range of particular interest, the eight carbon alcohols, were separated with greater resolution using temperature programming. However, temperature programming detracted from the reproducibility of retention time as a measure of a compound's identity. Also, it added substantially to the length of time per analysis, since the oven had to be cooled and equilibrated between

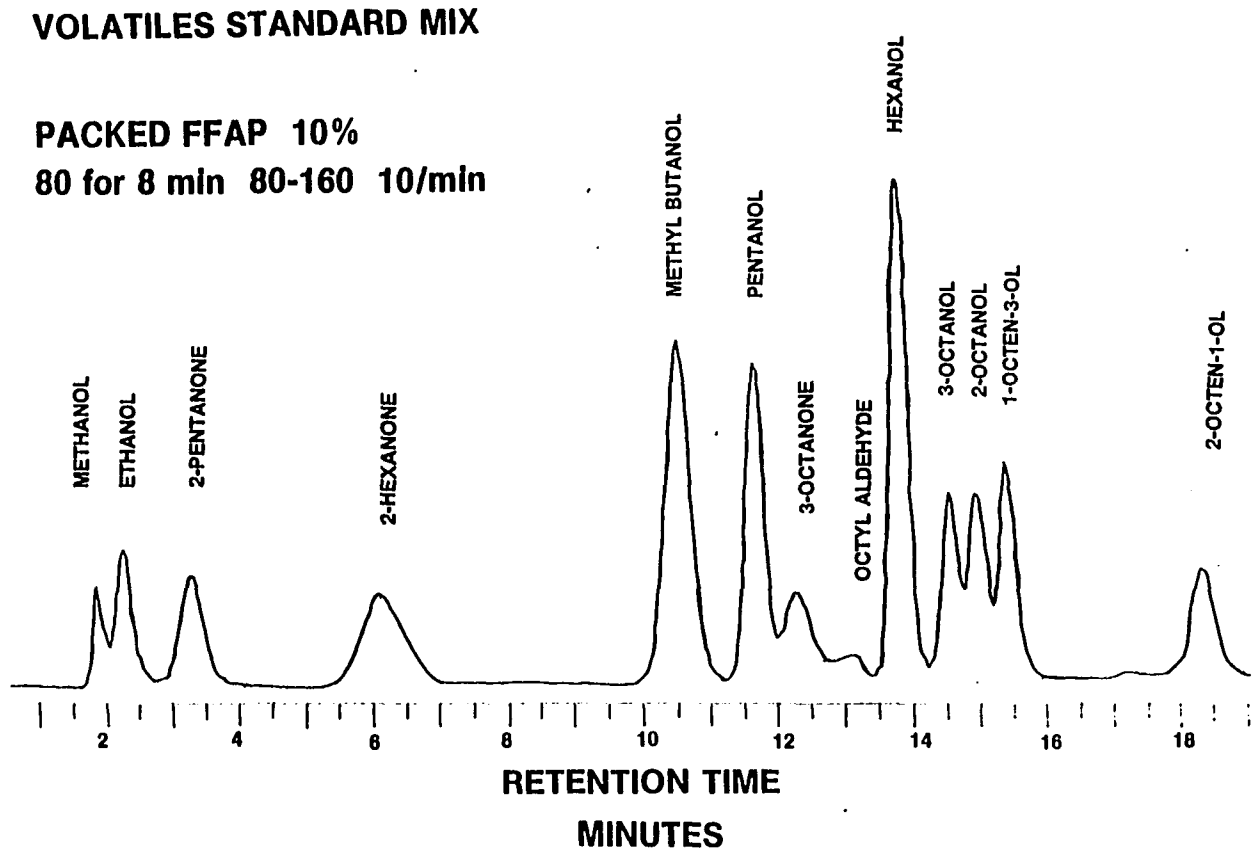


Figure 11. Gas chromatogram of a model volatile standard mixture in water, temperature programming 10% FFAP, 80°C for 8 min, then 80°-160°C, 10°C/min., Finnigan 4500 GC/MS/DS

samples. Since the target volatile C_6- C_9 alcohols were of primary interest, isothermal GLC was pursued. Isothermal GLC parameters were studied in an attempt to optimize them for C_4 and heavier model organic compounds. Numerous temperatures were analyzed for the best compromise between time of analysis and degree of resolution with 120°-135°C accepted as the standard isothermal temperature for separating the C_6-C_9 alcohols depending on column size used. Many of the smaller or more polar compounds suffered poor resolution but larger or less polar compounds eluted in a reasonable time.

Packed column chromotography offers several advantages to the analysis of "real world" samples. It was generally forgiving of sample interferences, allowed large amounts of sample to be injected without overload and was used for hundreds of samples before chromographic performance was affected. However, one of the hopes of this work was to see what volatiles were produced and for this goal optimum component resolution was thought advantageous since retention time was an important identification parameter when mass spectrometry was not available. Early studies of the volatiles using FFAP with MS yielded some peaks with merged components. When using GLC/FID detection, this merging was often unapparent.

Capillary GLC technique

One of the principal advantages of capillary GLC is the number of theoretical plates available, which results in superior component resolution. Because of this increased resolution, a much sharper component peak is eluted to the detector, usually resulting in a lower level of detection. The application of head space resin techniques to capillary GC proved to be the most difficult adaptation of the analytical method development. Carrier flow rates used in these two techniques are optimumly quite different.

When thermally stripping a resin trap, high temperatures and moderate flows are desired to remove the volatiles rapidly and in as tight a band as possible. This rapid removal decreases the length of time the resin is exposed to high temperatures thus minimizing resin degradation and sample decomposition while allowing even strongly retained species to be desorbed. Typical flows used often mate well with packed GLC, 20-45ml/min. carrier gas. Capillary GLC achieves its advantageous resolution with very small flows, typically 0.5 to 1 ml/min. Also, integral to maximum resolution is concise sample introduction.

The difficulty arises when a sample is eluted from a resin trap at 20-50 ml/min. but taken up by the capillary column at 1 ml/min. or less. If a typical sample is desorbed in approximately 20 sec. at 35 ml/min. then the sample actually occupies 11.67 ml volume. This would be introduced to the capillary column over 11 minutes time, resulting in tremendous band broadening and a large decrease in resolution of components.

Various procedures were attempted to interface these two divergent techniques. By the use of normal capillary gas flows, but with the entire GC oven at 22°C, resin retained samples were desorbed onto the column. Final chromotograms of the standard volatiles mix were less resolved than the packed column results. The injector temperature 160°C was thought to be promoting band broadening by allowing chromotographic activity, e.g., the internal column temperature due to heated carrier gas still sustained chromotographic separation resulting in component movement in the column while resin desorption was not complete resulting in band broadening.

GLC with Carrier gas stop-flow was investigated to see if the volatiles could be desorbed from the resin with no flow, then rapidly swept from the resin gaseous dead volume onto the capillary at capillary flow rates. This technique

improved the resolution achieved with a model mixture of organic compounds but still not beyond that obtained with an optimum packed column.

Precolumn resin trapping interfaced with capillary GLC

Unique techniques to solve these problems had been developed and investigated for applicability to various classes of organic compounds found in water (Ryan and Fritz, 1978, 1980; Marshall and Fritz, unpublished data, Ames Laboratory, ISU, Ames, IA). Very little difference exists between problems associated with headspace sampling for volatile organic content and water sampling for dissolved organic contamination.

Headspace samples were collected in resin traps, as before. These traps were thermally desorbed at optimum flow rates, 20-40 ml/min. onto a macro capillary resin precolumn held at 10°C by a liquid CO_2 spray. Effluent from the precolumn was vented to waste. Because of the subambient temperatures, the organic components re-absorbed onto the leading edge of the precolumn resin capillary in a tight sample bond. When original resin trap desorption was complete, gas flow was routed such that this trap was flushed to waste and the precolumn resin capillary was put backwards in line with the head of the capillary column. Flow was then at capillary optimum rate and heat was applied to the precolumn resin trap. The tight sample band at the leading end of the precolumn was rapidly desorbed off and in a reverse direction (since the connects were made backwards). Because the precolumn capillary was packed, void space contributing to band broadening was vastly minimized. If heating was rapid enough, with good heat transfer to the resin bed, a tight sample band "injection" to the chromotographic capillary column could be made. Figures 12-14 illustrate the potential of this technique using different classes of organics dissolved in water. This technique required controlling several "stages", each consisting of

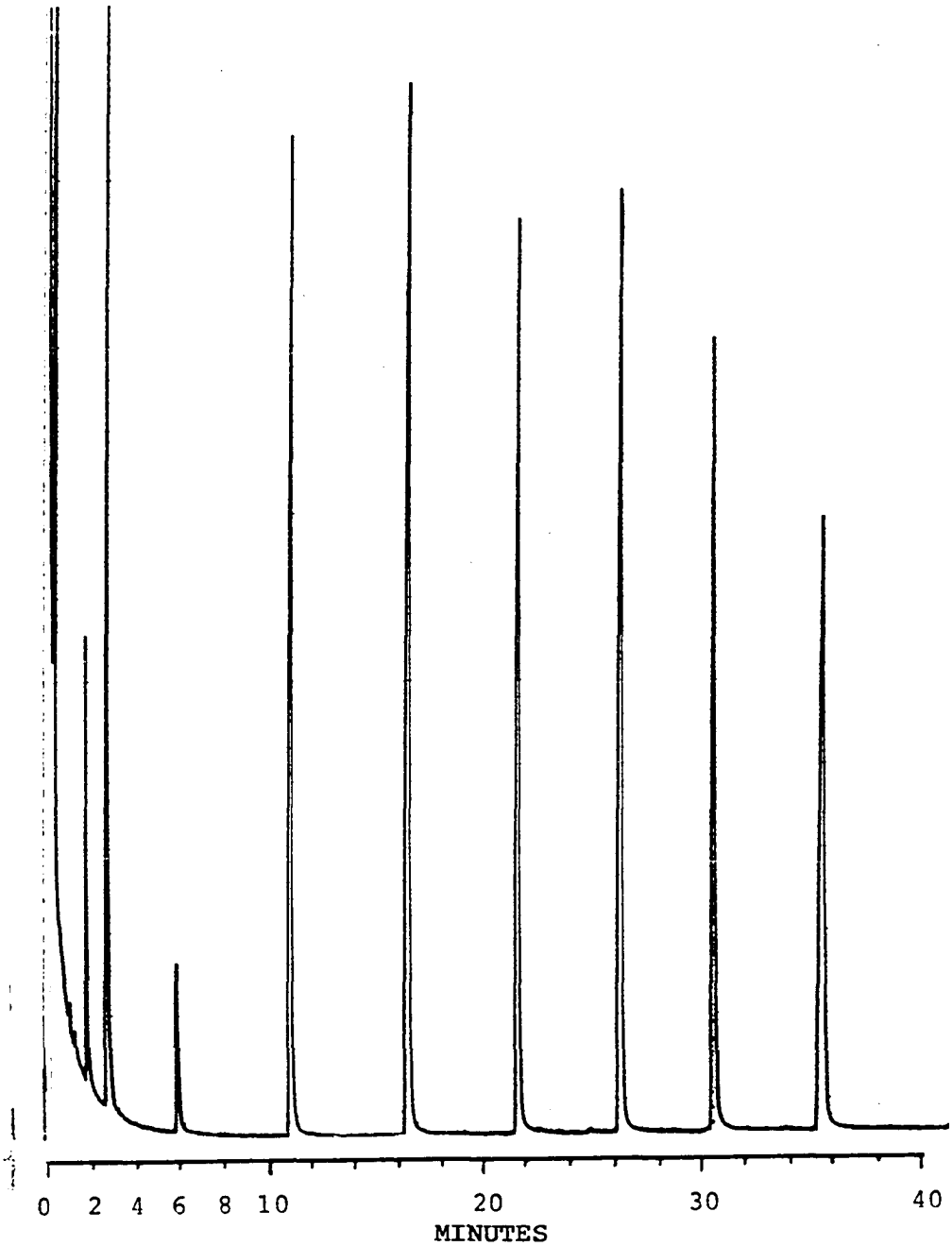
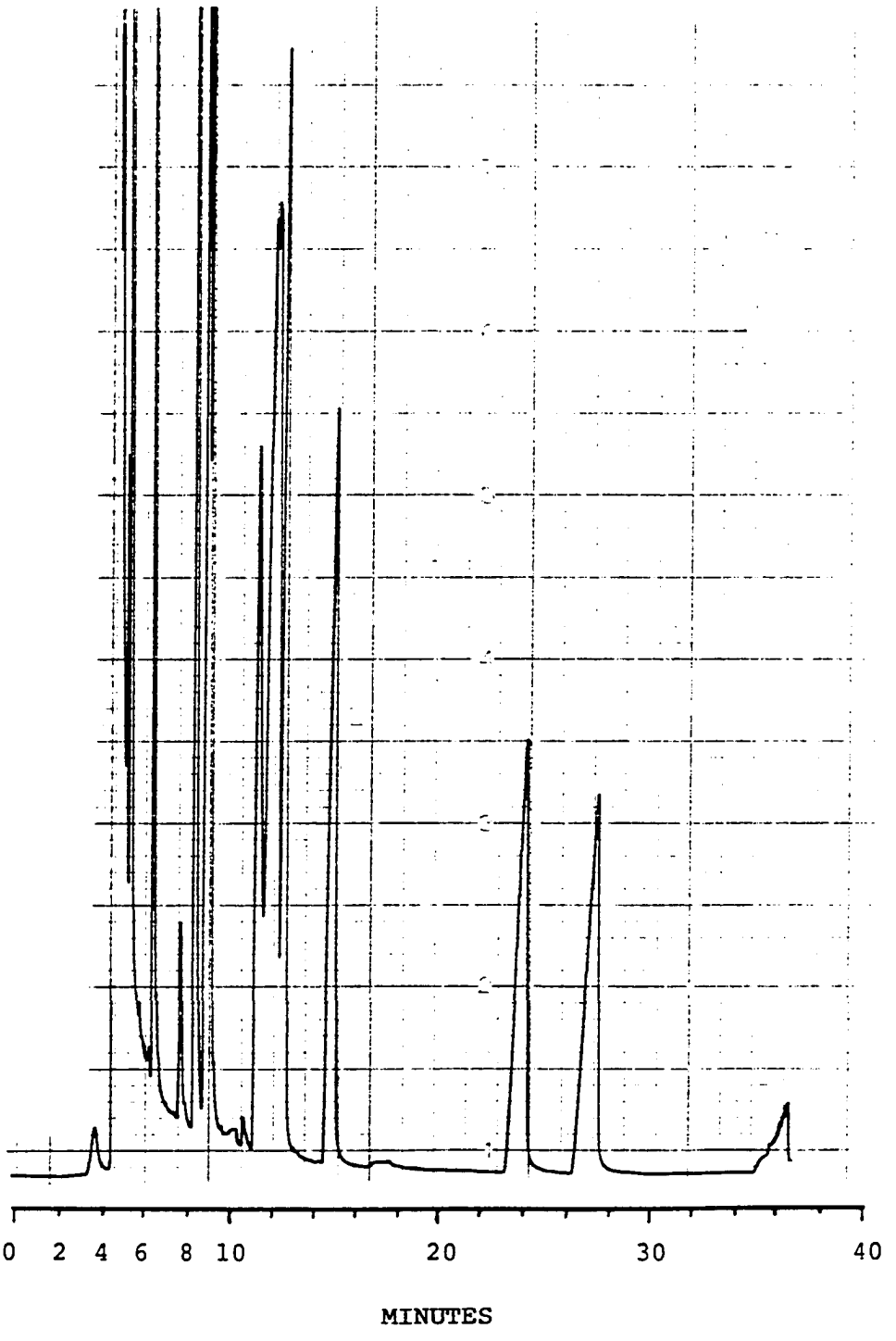


Figure 12. Gas chromatogram of n-alkanes C_9-C_{18} by trap-precolumn capillary SE-54, 30m, 80°C for 8 min, then 80°-160°C, 10°C/min.


0
2
4
6
8
10
20
30
40
MINUTES

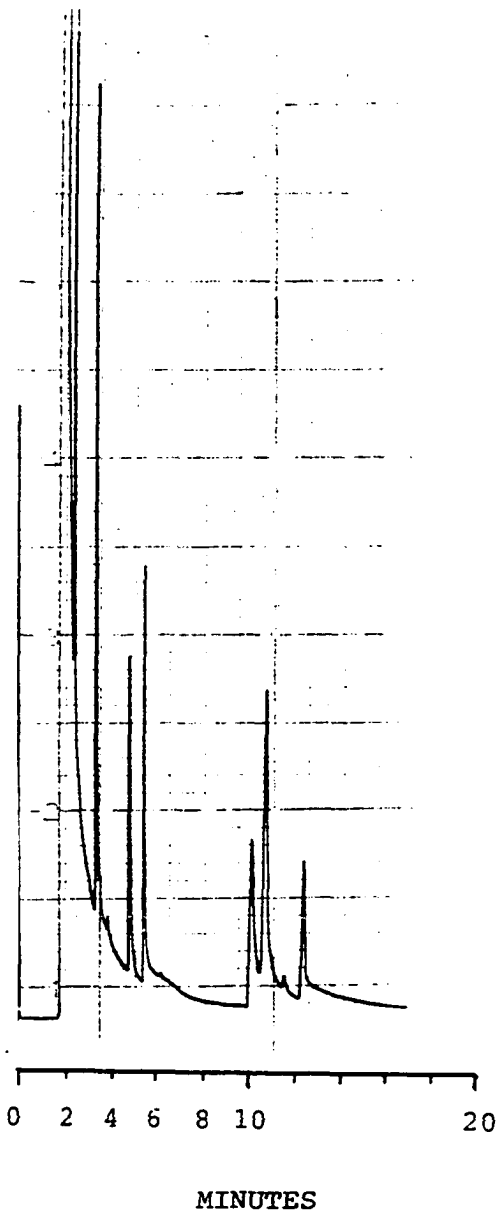


Figure 14. Gas chromatogram of C_4-C_9 alcohols by trap-precolumn capillary SE-54, 30m, 80°C for 8 min, then 80°-160°C, 10°C/min.

device temperatures, carrier gas flow route and rate, CO_2 flow, and duration of each stage (see Materials and Methods: Precolumn Resin Collection). Time per analysis was approximately 30 minutes plus the time of chromotographic separation yielding a total analysis time of one hour per sample.

In future studies, this technique should be utilized for routine analysis as well as investigated as an inlet device to mass spectrometry. The results warrant the additional time of analysis and additional equipment expense. The technique was extremely adaptable to automation with the stages controlled by commercially available instrumentation, such as purge-and-trap controllers.

Cryogenically focused capillary GLC

The solution advanced by Dr. J. Fritz and his co-workers to the resin desorption capillary interface problem was not rapidly or inexpensively amendable to most analytical laboratories, requiring specialized and user-modified instrumentation. However, facets of the work contributed to another solution. Using cryogenic cooling of the leading edge of a mega-bore capillary column offered many potential advantages. Mega-bore capillary (internal diameter 0.53 mm) readily supported packed column flow rates of 20-35 ml/minute while maintaining a superior resolving capability to packed columns. By using the first few turns of the capillary column itself as the cryogenic equivalent to Fritz's precolumn, it was possible to redeposit the thermally desorbed volatiles on the GC mega-bore capillary column itself.

Figure 15 illustrates volatile standard separation using methanol-dry ice as a cryogenic coolant for a coil of deactivated mega-bore capillary (1 meter). The GLC oven was opened (deactivating the oven heaters) and the coils immersed in methanol dry ice. The resin trap to be analyzed was then inserted into the GLC injector as in the routine packed column technique. The desorbed volatives were

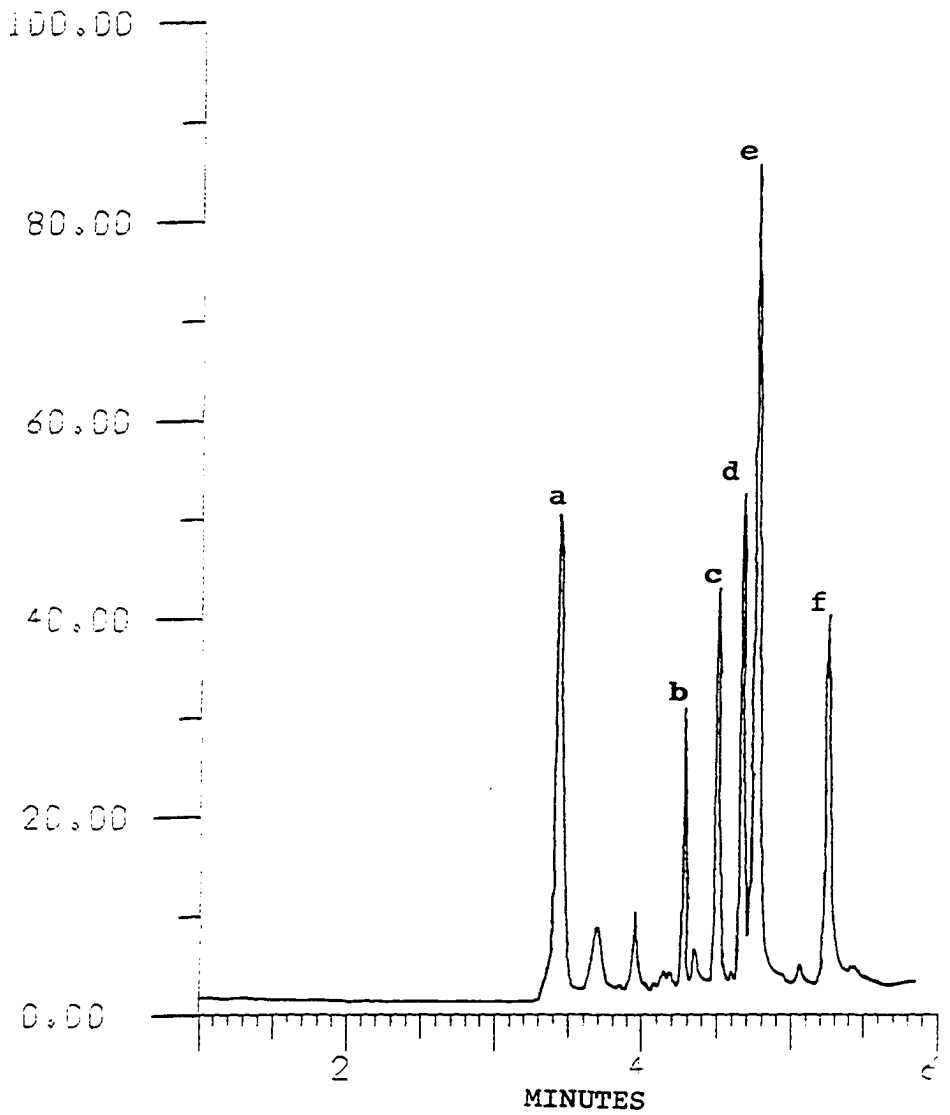


Figure 15. Gas chromatogram of C_4-C_8 alcohols by cryogenic-focused capillary

DB-WAX Mega-bore^{RT} 0.53mm X 15m, 0 °C for 3 min. then 125°C isothermal

a 1-pentanol
b 1-hexanol
c 1-heptanol
d 1-octanol
e 1-octene-3-ol
f 2-octene-1-ol

recollected in the cooled coil. To test the completeness of recollection, comparison of direct standard injection on column and from a resin were made with results showing 100% retention of C_2-C_9 organics in the coil. At the methanol-dry ice temperatures, time of cryogenic collection didn't seem to affect component resolution with three minutes minimun tested and a maximum of six minutes showing no degradation of component resolution, which indicated that at this temperature there was so little or no chromotographic mechanism operating that time differences marked in seconds made no difference.

However, retention time with standards was non-reproducible due to the necessity of cooling the GLC oven for access to the column coil and the difference in time from analysis to analysis in transferring the coil from the cryogenic bath to the GLC oven and starting the GC heating. Actual samples analyzed using this technique were restricted to the GLC-mass spectrometer so component identification could be determined by a combination of relative retention time and component mass spectral examination.

By eliminating the need to open the GLC oven for access to the column coil this deficiency could be eliminated. Figure 8 (Materials and Methods) shows the final design, of such a cooler or cryogenic focus. In this design the trap is desorbed by the GLC injector onto the mega-bore capillary. The leading twelve inches of the capillary runs through a 1/4-inch copper tube coiled to fit the capillary and ending in a tee. The tee allows entry of liquid CO_2 through a flow restrictor into the sheath, where expansion to a gas takes place resulting in adiabatic cooling of the sheath. and capillary column. The tee is capped off with a disc of silicon rubber septum material allowing only the capillary column through and forcing the CO_2 to exit through the long axis of the sheath. The 1/4-inch copper is fit to the final leg of the tee. This design allows desorption with

recollection at subambiant temperatures while the GLC is closed and oven operating, resulting in more reproducible retention times for accurate FID retention time peak identification. However, the capillary lost resolving power for the model volatile mixture over a few weeks of routine use. The sample moisture was thought to be responsible for this, and could be avoided in the future by sweeping dry nitrogen gas through the traps prior to GLC analysis.

Resin Trap Design and Performance

Tenax^{RT} resin was chosen as a suitable trapping material because of the extensive work already reported on its application to organic compound retention and concentration in air, water and foodstuff analysis. Its thermal stability and lack of background were advantageous to this work.

Stainless steel tubing 1/4-inch OD was used to construct traps which allowed the normal carrier flow into the GLC injector to preheat along the sides of the trap when in place then enter the trap and flush through it and into the head of the GLC column. Initial attempts with standards yielded extremely long retention times and exaggerated bond broadening until a seal was installed at the base of the trap between it and the head of the GLC column (Figure 6). The seal forced all carrier to flow as mentioned before. Without this seal, some of the carrier was not eluting the trap, resulting in a much slower desorption of volatives therein. The overall length of the trap was chosen to adapt to the injector assemblies of the two gas chromotographs used in this work. The final capacity of the traps was 0.1 gm of 100/120 mesh Tenax ^{RT} with silanized glass wool plugs to retain the resin bed in place during volatiles collection and analysis. All newly prepared traps were solvent washed to eliminate oils from machining, were packed with Tenax^{RT} and were conditioned prior to use.

Ideally, the Tenax^{RT} traps would adsorb and hold the volatiles in a tight bond until thermally desorbed. However, since Tenax ^{RT} itself is a suitable chromatographic material, this is not the case. Volatile components, while being adsorbed on the resin from the sample headspace, still were chromotographically active and could chromatographically spread through the trap over long periods of time. Marshall and Fritz (unpublished data, Ames Laboratory, ISU, Ames, IA.) predicted breakthrough from non-overloaded, extended sampling time (Figure 16). Here, the elution volume is plotted versus elution temperature with 0.2gm Tenax^{RT} resin for a number of organic compounds. From this graph, it is evident that organic species do chromatograph even at low temperatures and that depending on the temperature of the Tenax ^{RT} and the compound being considered, volatile breakthrough could occur. Since collection of a 24-hour dynamic headspace was anticipated, it was important to understand the nature of the chromatographic process occurring in the traps, especially: 1) The loading capacity or the upper level of volatile which could be retained, and 2) The eluting volume (V_R) necessary for volatile compound breakthrough. System loading capaci' ; was checked by plotting sample size analyzed versus peak area and observing a break from the expected response curve. The trap and detector were shown to be quantitative and linear at least to 100 ug. for all volatiles tested from the early eluting 2-pentanone to the much later eluting 2-octene-1-ol (Figure 17). Generally speaking, chromatographically active species had lower load limits and lower V_R values than less active components.

To test breakthrough, aliquots of a standard alcohol mix (C_5-C_8 in water) were introduced by syringe onto the leading edge of a trap, then eluted with air for 24 hours at 6 ml/min. (the conditions of extended-headspace sampling). Peak area of the trap chromotogram after 24 hour pseudo-collection was compared

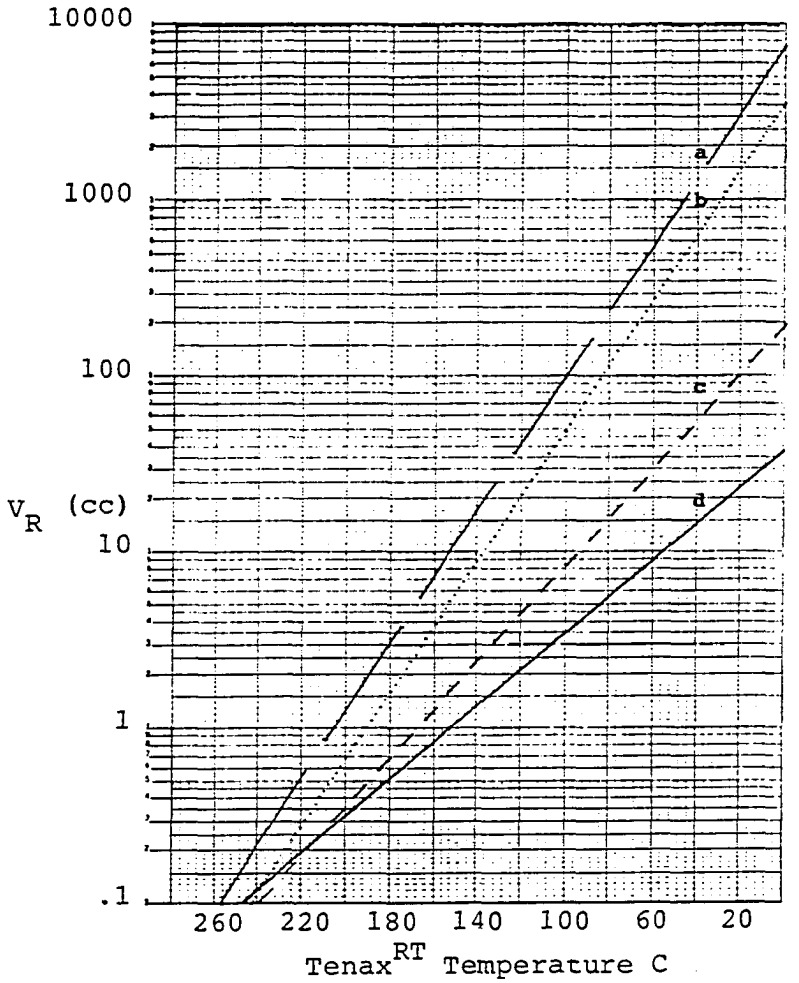


Figure 16. Elution volumes (V_R) for selected organic compounds from Tenax RT as a function of elution temperature (Marshall and Fritz, unpublished data, Ames Laboratory, Iowa State University, Ames, IA)

a	2-methyl cyclohexanone	bp 165°C
b	2-heptanone	bp 151.5°C
c	2-methyl pentanol	bp 120°C
d	n-butanol	bp 117.3°C

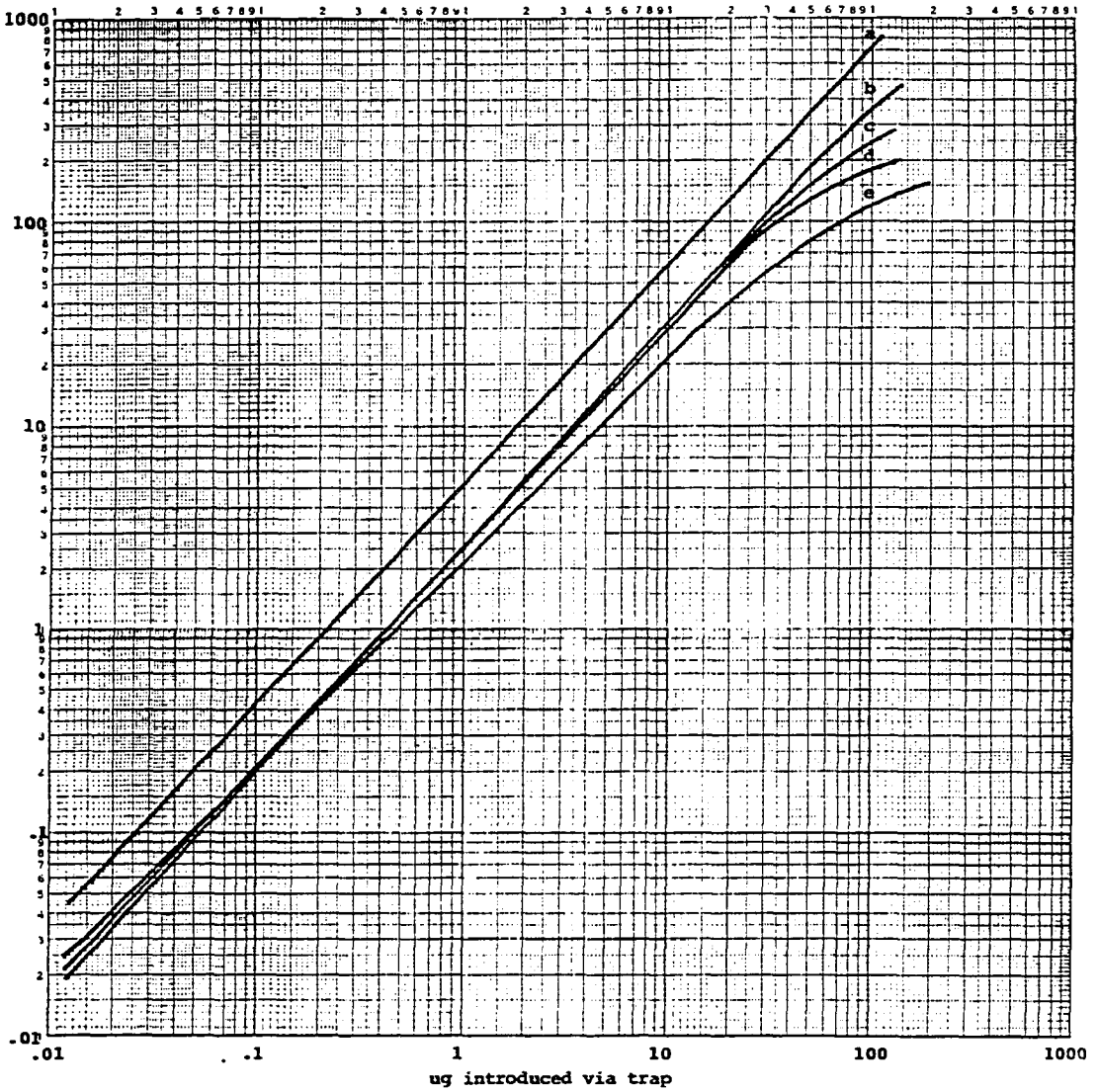


Figure 17. Standard curves of selected model volatile compounds by Tenax RT trap-GLC

10% FFAP, 135°C isothermal, 20 cm/min

a merged 2-octanol and 1-octene-3-ol
b 2-octene-1-ol
c 1-heptanol
d 1-hexanol
e 1-pentanol

with a freshly injected and analyzed trap (Table 3). These results indicate that the lighter or more chomatographically-active components such as 1-pentanol do chromatograph and even "break through" over extended collection times of 24 hours at room temperature. However, the compounds of interest were retained quantitatively (> 90%). By lowering the trap temperature during collection, breakthrough of even the early-eluting components could be minimized, but condensation of water vapor would also increase.

Resolution (R_s) of experimentally obtained peaks can be defined as a function of peak separation (W) and individual peak width (W_x, W_y) (Eq. 1).

$$\text{Eq. 1} \quad R_s = 2W/(W_x + W_y)$$

Figure 18 illustrates that bond broadening due to 24 hour collection times was not excessive as evidenced by very little loss of resolution.

In an attempt to increase the trap capacity and prevent breakthrough of early eluting volatile compounds, a carbon/Tenax^{RT} combination was tested since many components have a higher affinity for carbon than Tenax^{RT}. A commercially available carbon adsorbent was used (Carbopak^{RT}, Supelco Company, Bellefonte, PA.). Figure 19 illustrates the dramatic loss of resolution, from 0.9 to 0.59 due to the addition of carbon to the trap and the chromatogram showed no general improvement in early peak retention. Therefore, this work used traps containing 0.1 gm Tenax^{RT} as the sorbent.

The reproducibility of 6 prepared traps as compared to 6 trials with one tube (Table 4) showed close agreement of retention time, peak area, and peak height. When using a number of different traps over different days, the reproducibility or relative standard deviation (RSD) was greater than that observed for 6 trials with one trap in a single day. This was attributed to inconsistent packing of the Tenax^{RT} in the traps. However, the degree of reproducibility observed, 3-10% RSD

Table 3. The effects of extended trap sampling on the recovery of volatile standards

	1-pentanol	1-hexanol	2-octanol	1-octene-3-ol 1-octanol	2-octen-1-ol
24-hour pk area	95	113	115	252	105
Direct injection	126	124	109	234	101
% retained	75%	91%	106%	108%	104%

Figure 18. Comparison of resolution effects due to 24-hour eluted trap introduction to GLC versus direct injection

10% FFAP, 135°C isothermal, 20 cm/min
Resolution (R_s) of two chromatographic peaks (X and Y) is experimentally determined using width or peak separation (W) and individual peak base widths (W_x and W_y) and the relationship $R_s = 2W/(W_x + W_y)$. Rs increases as a function of improved resolution.

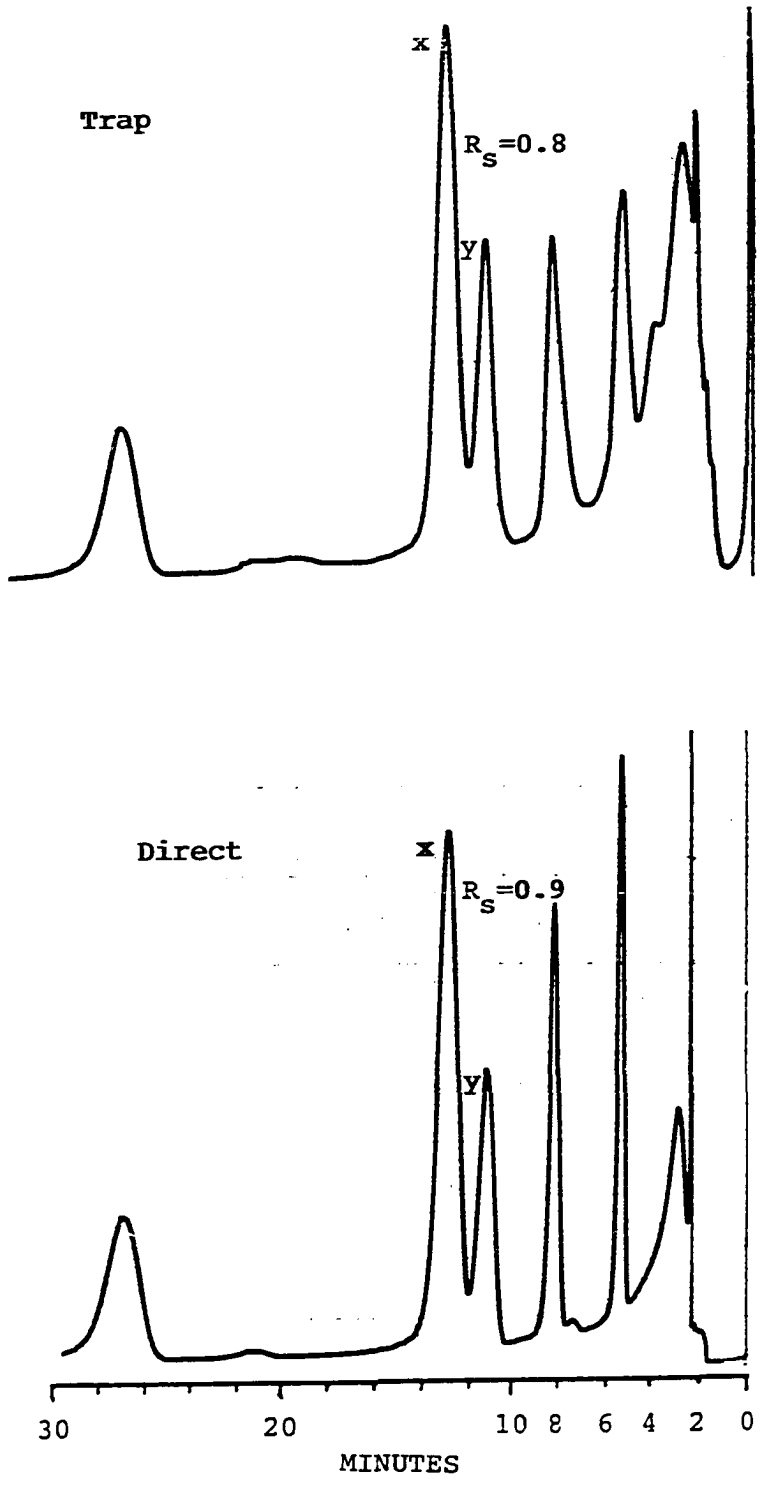

Trap
x
R_s=0.8
y
Direct
x
R_s=0.9
y
30
20
10
8
6
4
2
0
MINUTES

Figure 19. Comparison of resolution effects due to Tenax ^{RT} versus Tenax^{RM}/carbon trap introduction to GLC

10% FFAP, 135°C isothermal, 20 cm/min
Resolution (R_s) of two chromatographic peaks (X and Y) is experimentally determined using width or peak separation (W) and individual peak base widths (W_x and W_y) and the relationship $R_s = 2W/(W_x + W_y)$. Rs increases as a function of improved resolution.

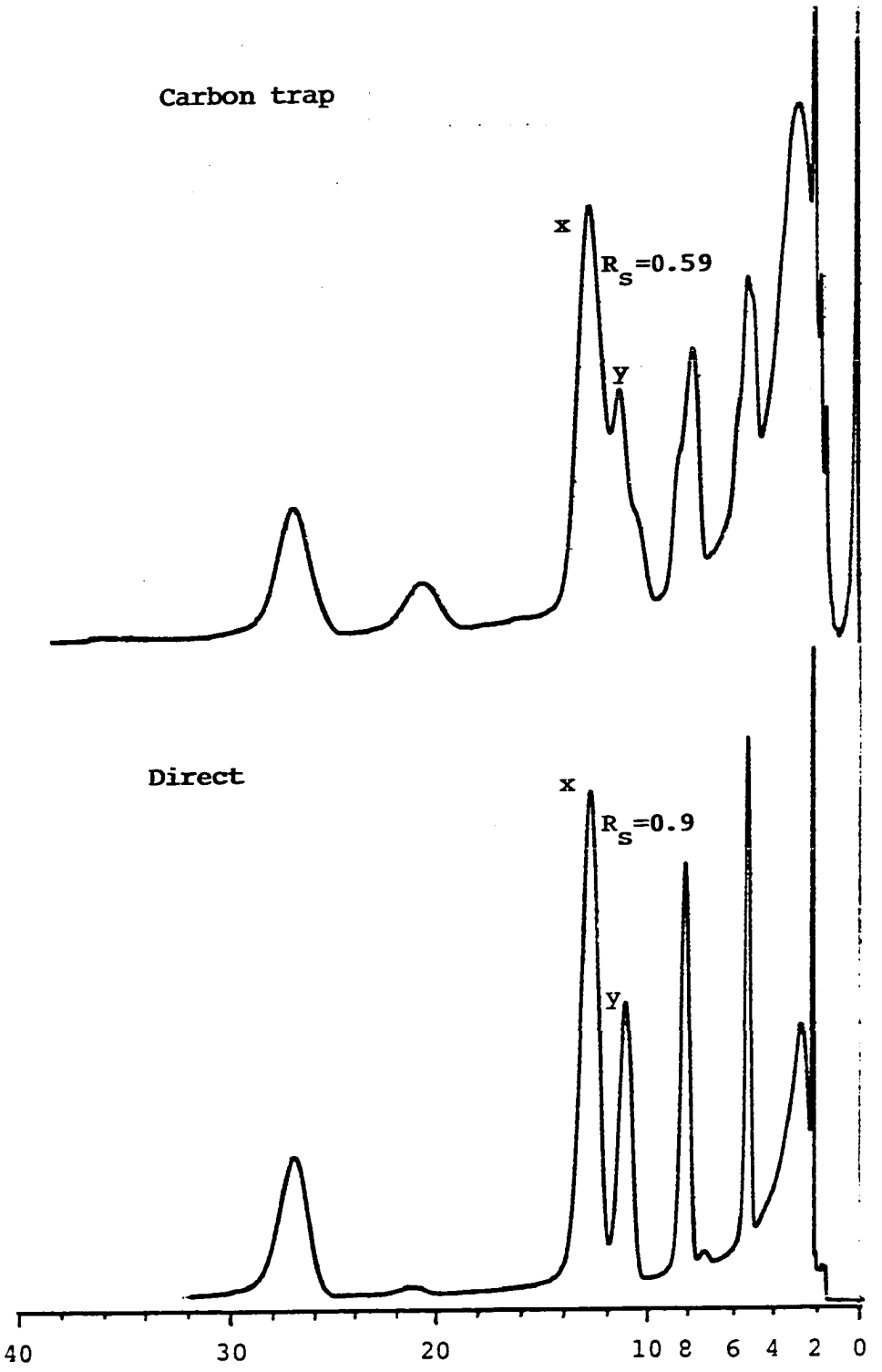

Carbon trap
x
R_s=0.59
y
Direct
x
R_s=0.9
y
40
30
20
10
8
6
4
2
0

Table 4. Reproducibility of trap-introduced volatiles by GLC

SAME DAY	1-pentanol	1-hexanol	2-octanol	1-octene-3-ol 1-octanol	2-octene-1-ol
Retention (mm)	19.6	30.0	41.9	46.8	98.9
RSD (%)[a]	1.5	3.2	8.0	2.2	2.1
Peak area	85.0	98.3	110.8	250.5	118.2
RSD (%)	1.5	3.1	2.9	4.7	4.3
Peak height	94.4	74.6	50.0	81.7	25.4
RSD (%)	1.5	2.7	4.6	3.6	5.2

DIFFERENT DAYS-DIFFERENT TUBES	1-pentanol	1-hexanol	1-octene-3-ol 2-octanol	1-octanol	2-octene-1-ol
Retention (mm)	18.9	28.8	38.6	44.4	92.9
RSD (%)	4.4	3.6	3.3	3.2	3.4
Peak area	78.1	85.6	97.3	219.0	102.3
RSD (%)	7.3	5.4	9.7	6.3	7.7
Peak Height	78.1	58.4	41.4	66.9	18.9
RSD (%)	7.3	10.0	10.4	8.7	10.7

[a] RSD = relative standard deviation expressed as a % of the mean

was acceptable and in accordance with variation of other laboratory procedures involving GLC measurement from day to day.

Peak height and peak area measurements are the most common means used to quantitate detector response to an eluting compound. Comparisons of peak height and peak area showed peak area to be as linear at every component-concentration tested. At levels below 350 ng., peak height was as accurate as peak area and much faster to calculate.

Using peak area, direct injection standard curves were compared to standard introduction via traps. Standards introduced from the trap averaged 90% of the direct injection standards with a RSD of ±5%. Daily volatiles standard injections were made to monitor any changes in detector sensitivity to volatile compounds with time.

Standard Volatiles from Feed Using Induction Heating

Induction furnace heating was investigated for its thread stripping capability because of its rate of heating and cooling and the ease of sample volatiles collection from it. Because of the rapid rate of heating, power to the induction coil had to be pulsed or cycled to prevent surface scorching of feed in contact with the cup wall while allowing heat to penetrate the feed as a whole.

Figure 20 shows the final cup temperature achieved using 5-10 second heating cycles. As expected, studies of cycle time versus standard recovery for different sample matrices showed that sample moisture and composition played an important role in the liberation of volatiles without sample scorching.

When no sample matrix was present, standard volatiles were quantitatively transferred from cup to column at cycle times of 3 or more seconds. However, when standards were added to a blank sample (ground corn with moisture between 20-25%) cycle time became very important. Two processes were

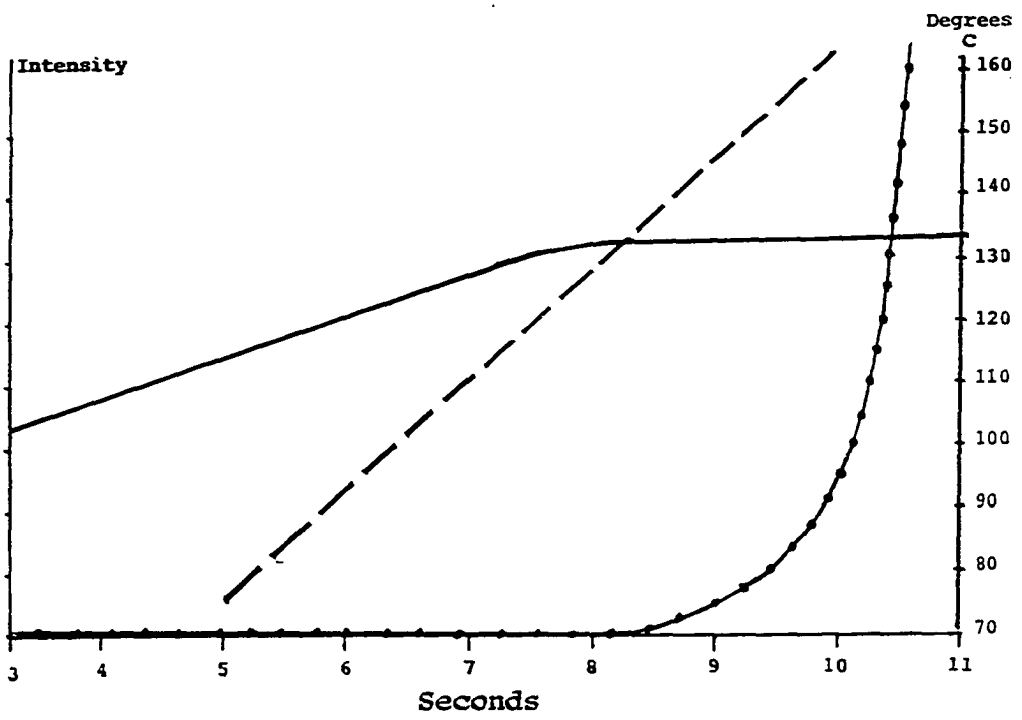


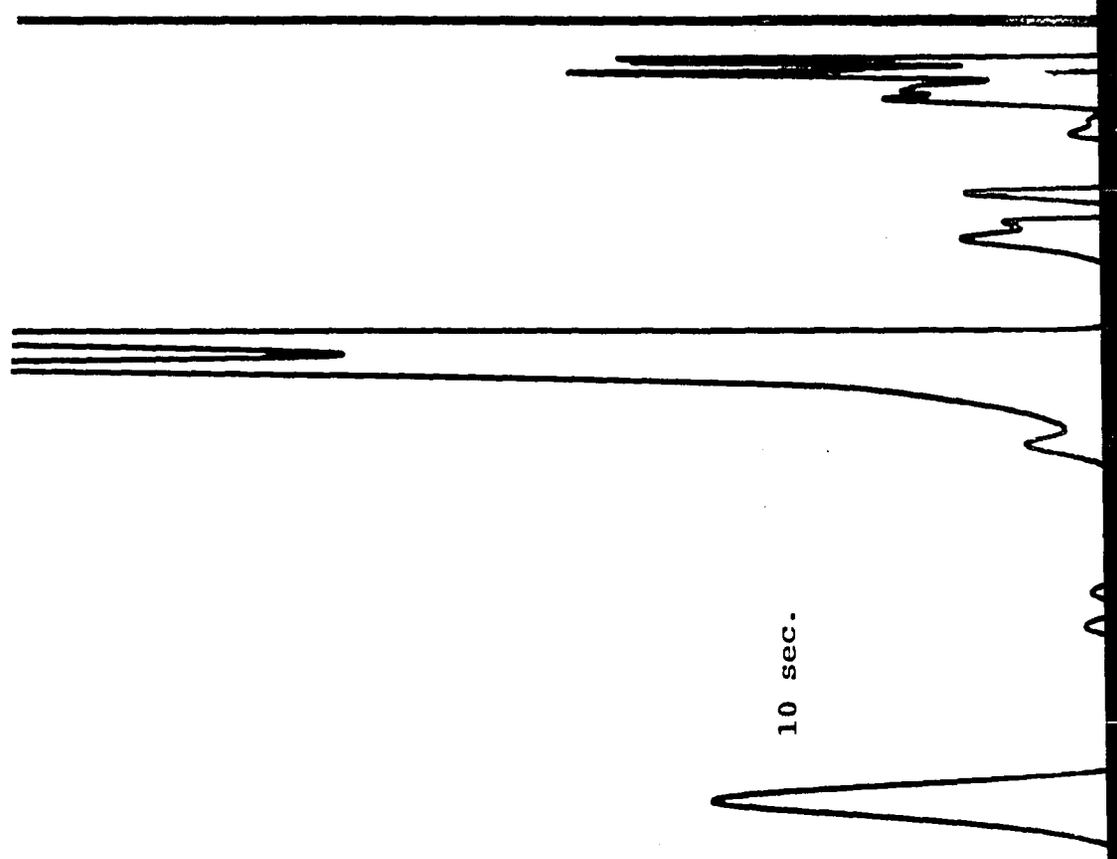
Figure 20. Heating cycle time effects on sample cup temperature, volatile standard recovery, and appearance of scorch products

— — — Sample cup temperature °C
——— Volatile standard recovery
—•—•— Scorch product intensity

occurring, thermal stripping of volatiles with subsequent transfer out of the feed; and excessive heating at the sample cup/sample interface leading to scorching and the appearance in the volatile profile of scorch products. As heating cycle time increased, volatile transfer increased. However, scorching products also increased. Figure 20 demonstrates cycle time effect on standard recovery from a feed matrix and also its effect on the appearance of scorch products. Eight seconds was chosen as the cycle time to be used in the inductive-heating because volatile standards were quantitatively transferred yet scorching products were minimized. The sample cup temperature (Figure 20) at 8 seconds cycle time was approximately 122-130°C. Figure 21 illustrates the appearance of the scorch products, thought to be di-methyl furan and other substituted furans.

Feed Analysis for Volatiles and Toxins

After developing techniques for collecting and analyzing volatiles, the next goal was to apply this methodology to the collection of volatiles actually produced as molds developed in ground corn. As reported by several workers (Review of Prevous Work), grains, including corn, have volatile profiles even when "clean" or mold free. Different batches of "clean" corn obtained from a local mill and from the Iowa State University feed mill were quite different in the background profiles obtained (Figure 22). No attempt was made to identify peaks in the corns with substantial background, since their history (storage, growth, treatment and handling) were unknown. However, chemical analysis of two "clean" corns with non-blank volatile profiles, did reveal ppb traces of aflatoxin. For the studies, only corn with very low initial backgrounds was used (Figure 22 a). Prior to inoculation with mold spores, all feeds used were autoclaved to inhibit other organisms, bacteria, yeasts and molds from contributing to the


10 sec.

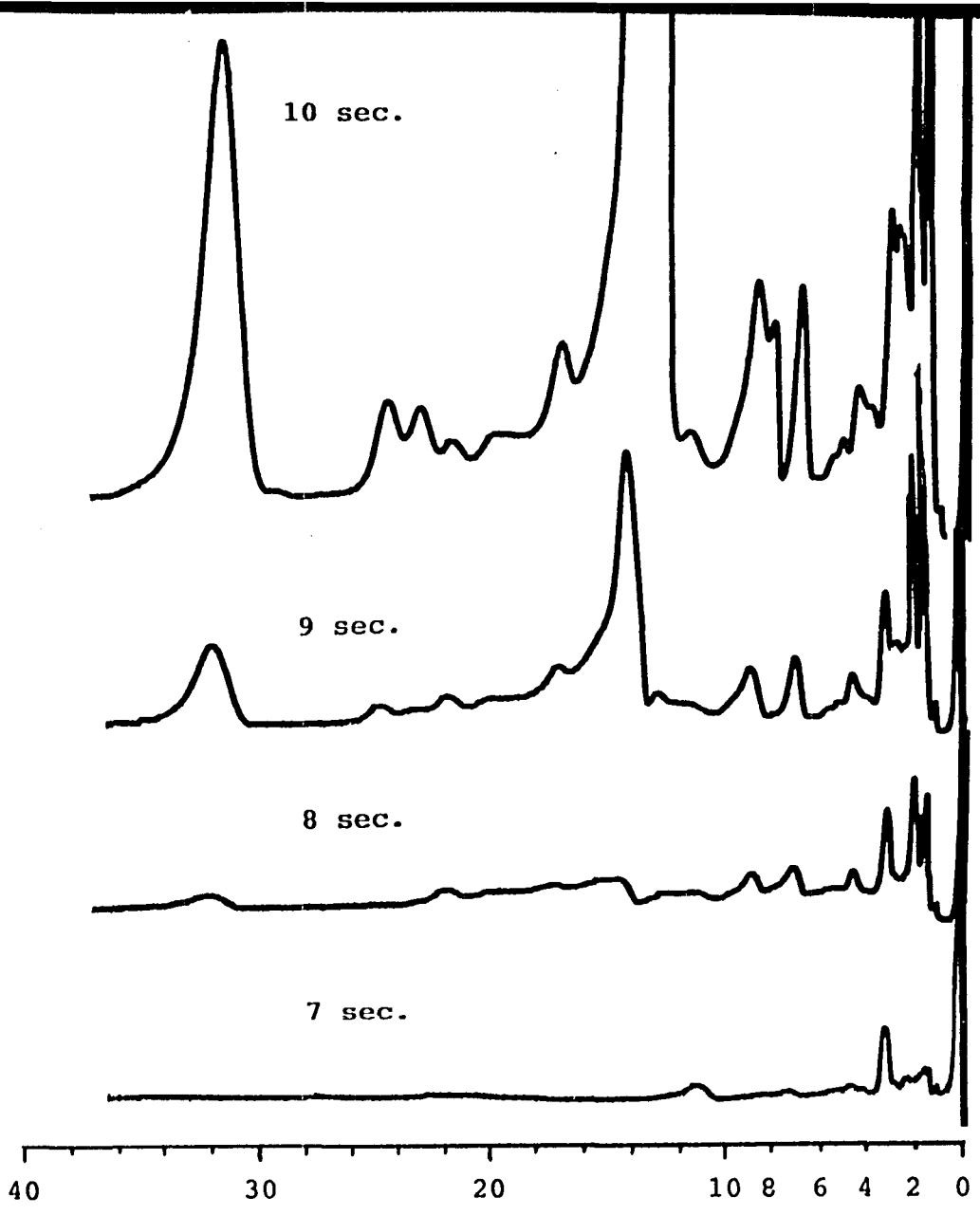


Figure 21. Scorch products in the volatile profile as a function of heating cycle time

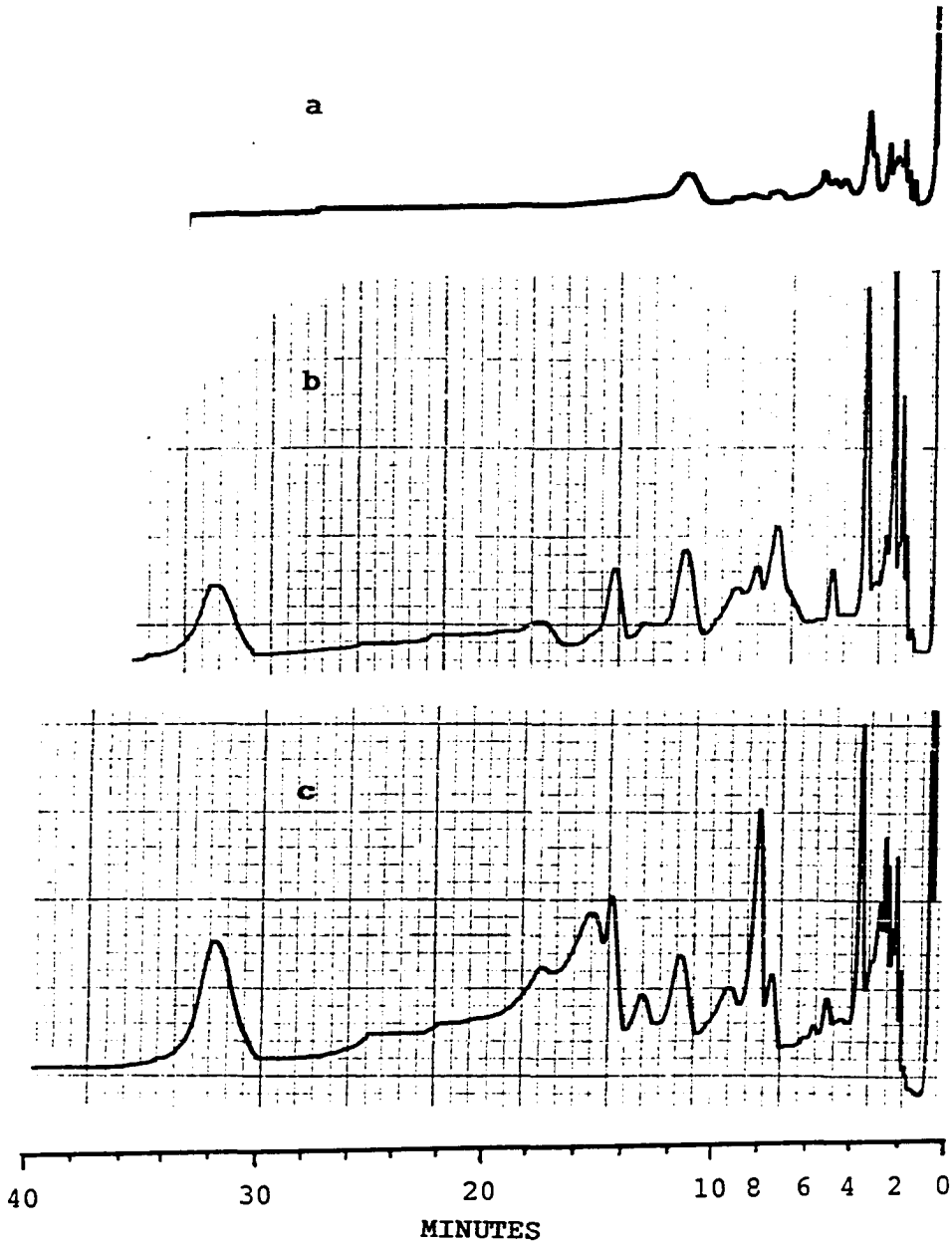


Figure 22. Volatile profile from 3 corn samples

10% FFAP, 135°C

volatile profiles later obtained. However, the autoclaving process was capable of contributing significantly to the initial blank feed volatile profile. Two different autoclaves were utilized in the course of this work. The lower chromatogram (Figure 23) clearly shows additions to the chromatogram of a clean corn (upper chromatogram) autoclaved in the autoclave later found to be used for sterilizing bacteriological refuse and garbage prior to its disposal. Also, the container used to hold grain during the autoclaving was a possible contributing source with Nalgene^{RT} contributing early components as compared to glass. With appropriate care, corn was ground and autoclaved, then tested to insure an acceptable blank corn for mold inoculation and volatiles collection.

The reproducible culturing of molds in feed unexpectedly presented special problems which were not completely solved during this work. To test the hypothesis that molds produce volatiles which directly correlate to toxins produced, requires that subsamples of homogeneously ground corn, inoculated with aliquots of an aqueous mold spore suspension simultaneously and held under identical conditions of temperature, oxygen/carbon dioxide levels, light and humidity, would produce volatiles and toxin on similar time scales and with similar intensities. This assumption was not supported by the experimental data. In attempting to understand the cause of the nonreproducibility of volatile profiles and levels of toxin analyzed, different experimental culture models were used.

Trial 1: open cell model

The first model consisted of weighing 50 gm of homogeneously ground corn into 100 ml glass beakers, inoculating with 5 ml of either Aspergillus or Fusarium aqueous spore suspension, covering with aluminum foil, and incubating at room temperature. A control feed was inoculated with distilled water and

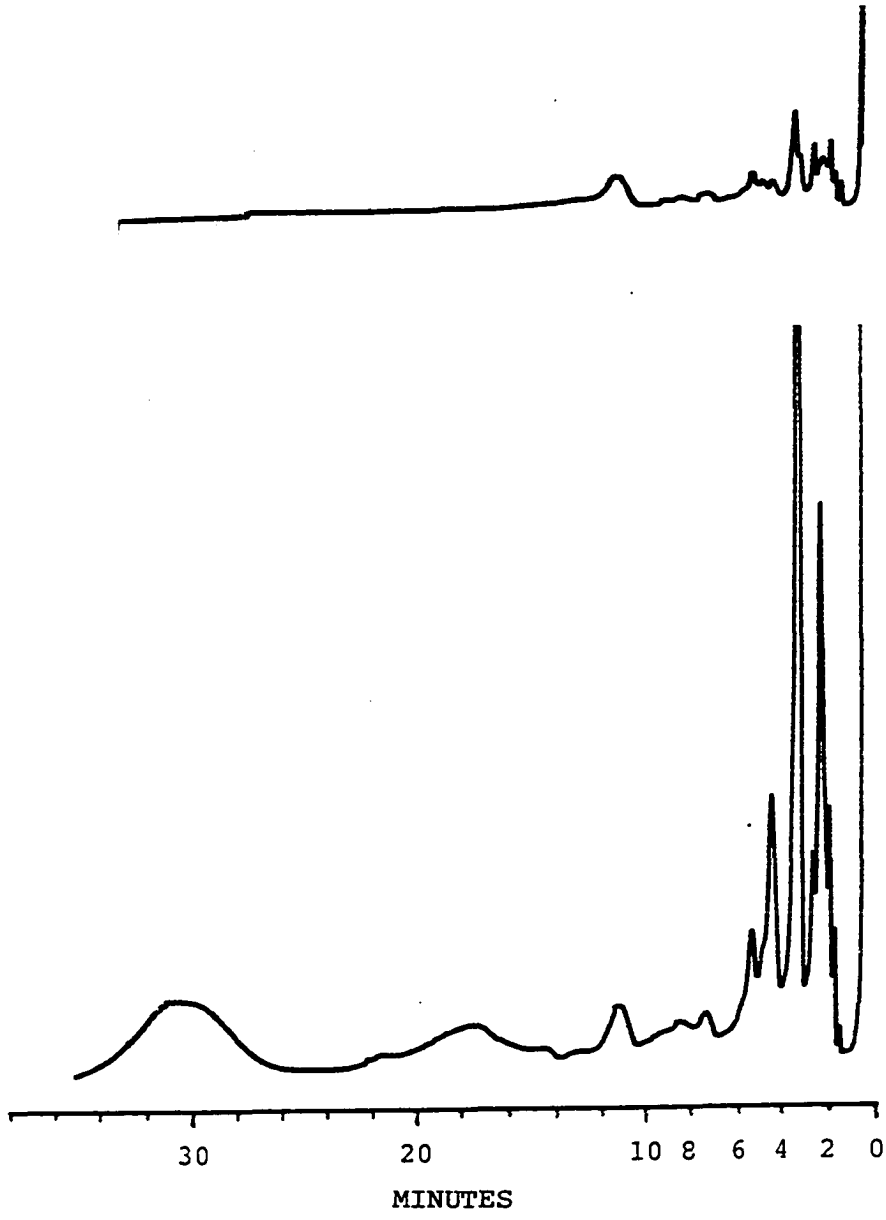


Figure 23 Volatile profile from autoclaved corn

10% FFAP, 135°C

similarly treated. Each day, the headspace was sampled for 10 minutes, then 0.1 gm samples were analyzed for volatiles, headspace and induction, and the remaining 49.9 gm analyzed for toxin level. This preliminary experiment was conducted over a period of 12 days with visible sporulation evident from day 4 and increasing with time. Aflatoxin levels were first observed by TLC on day 4 as well and also increased with time (Table 5). Samples collected for volatile analysis yielded no detectable levels in the headspace and very low volatile concentrations, at levels of less than 100 ppb, in the inducted samples (Figure 24). Peak intensities in 45 peak windows were statistically evaluated for correlation to aflatoxin level. The chromatograms over time, when analyzed statistically, yielded several peaks in the Aspergillus culture which had correlation coefficients of greater than 0.5 to aflatoxin content. Peak 30 had a correlation coefficient of 0.86 (perfect correlation between 2 variables is 1.0) and matched in retention time a contaminant found in 2-octene-1-ol under indentical conditions. However, the concentration of the compound was too low to give a mass spectrum of any quality. Only low-molecular weight, high intensity mass ions were distinguishable above the noise level. However, the low MW ions recorded for the unknown peak were also part of several volatile model compounds, including the octene-ols. The Fusarium inoculated feeds failed to produce either detectable Fusarium toxins or volatiles with any trend.

It was thought that the low levels of volatiles found in both feed samples might have been due to their volatility. The sample beakers were not sealed but loosely covered with aluminum foil to allow the cultures to breathe.

Sporulation gave visual evidence that the molds were not growing uniformly in the beakers or at the same rate. Since 0.2g of feed was used for testing of the induction of volatile compounds, sampling criteria required a very homogeneous

Table 5. Trial 1 open cell model: Aspergillus toxin B_1 level and peak 30 intensity as a function of time

Day	B_1 level	Peak 30 intensity
1	NDA [a]	NDA
2	NDA	NDA
4	100 ppb	2.0
6	567 ppb	25.0
7	608 ppb	15.0
9	554 ppb	42.0
14	474 ppb	6.5
20	79 ppb	---

[a] no detectable amount

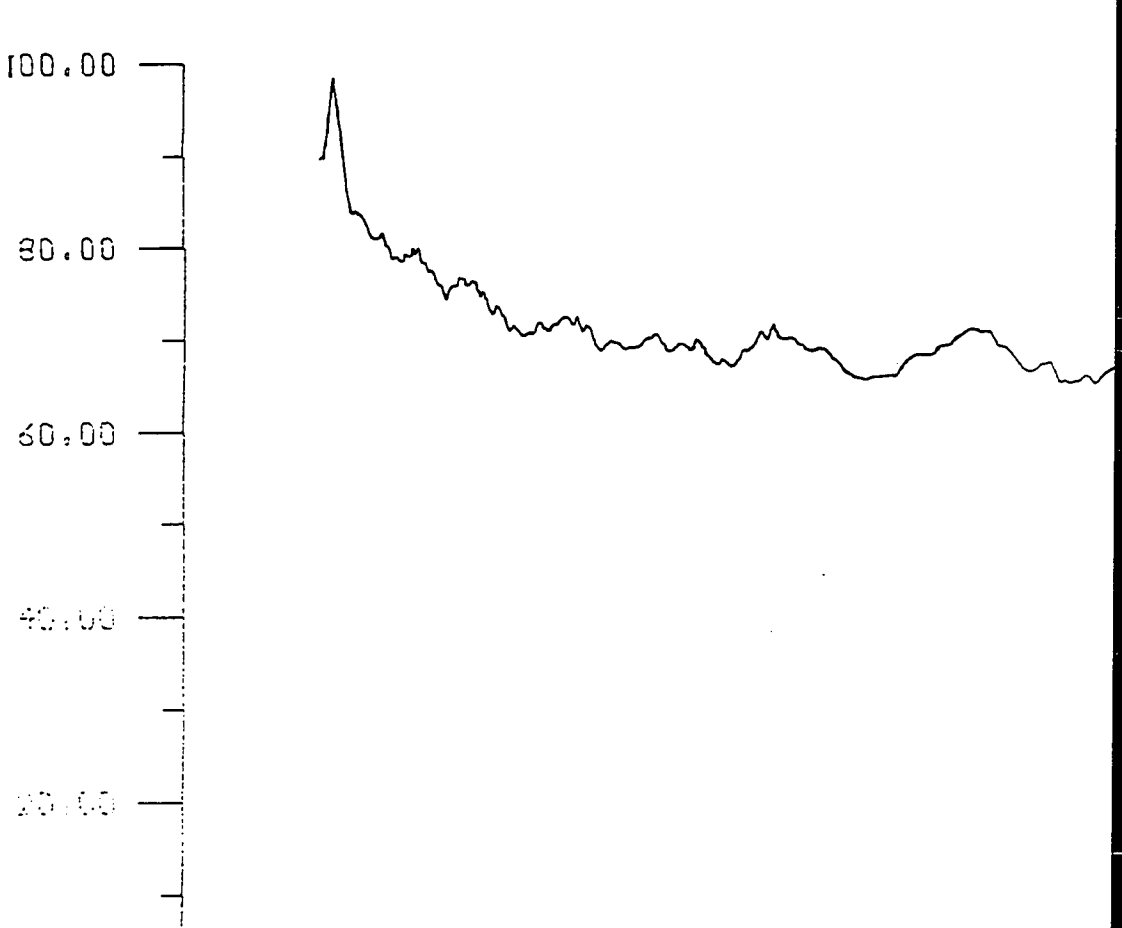
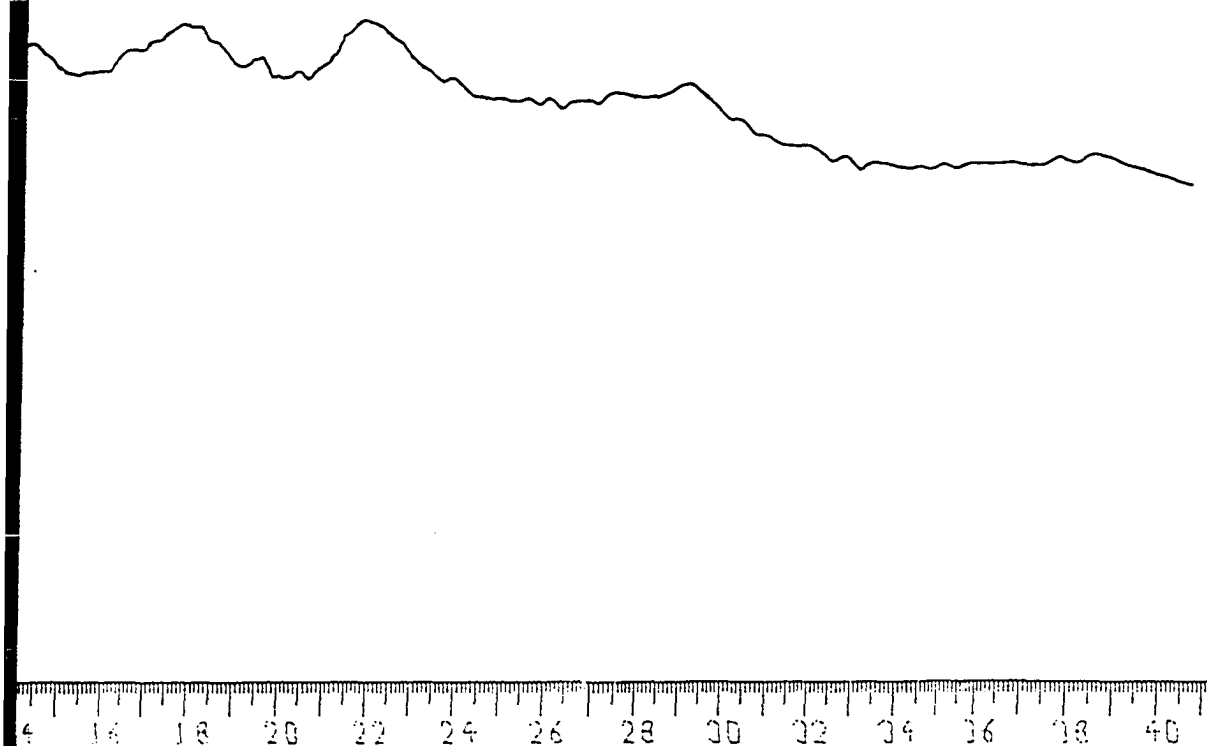


Figure 24. Volatile profile from Aspergillus parasiticus-inoculated corn

trial 1, open cell model, headspace sample day 7
10% FFAP, 125°C



noculated corn

7

sample be used so the small subsample would be representative of the whole sample. This was obviously not true, at least in regard to sclerotia or spore distribution in the feed. This observation raised questions about the degree of homogeneity of the volatiles in the feed.

Mixing in an open vessel was found to further dissipate the already low volatile concentrations in tests with control feed spiked at 5-6 ppm with model volatiles. The absence of mixing prior to sampling, so that volatiles were not further diluted or lost, allowed inhomogeneous sampling to affect levels of volatiles observed and toxins analyzed.

Trial 2: closed cell model-no stirring

In Trial 2, a closed container model was used for total containment of the volatiles produced by molds on corn. Since the molds are aerobic, moist air was supplied (6-9 ml/min.) to support their growth and metabolism. Volatiles were collected from the swept headspace only. Three peaks indicated some degree of correlation with mold growth through time (Figure 25). The retention time of Peak a matched closely that of 1-octene-3-ol, and another peak, c, coincided with a minor peak found with the 2 octene-1-ol standard. Table 6 summarizes the data from this trial. Trial 1 data (Table 5) indicated that toxin and volatile intensity versus time were inverted bell curves for Aspergillus parasiticus. The goal of this trial was to see if larger concentrations of volatiles could be preserved with a "closed cell" approach. Because of a desire to get as complete a record of correlation as possible without any perturbation to the system, the vessel was not opened during the 21 day trial, thus no toxin samples were possible, nor were any of the daily headspace samples sacrificed for mass spectral identification. Volatile peaks resulted which did correlate to development of the mold. Using data from Table 6, plots of actual versus predicted Peak a and c intensities

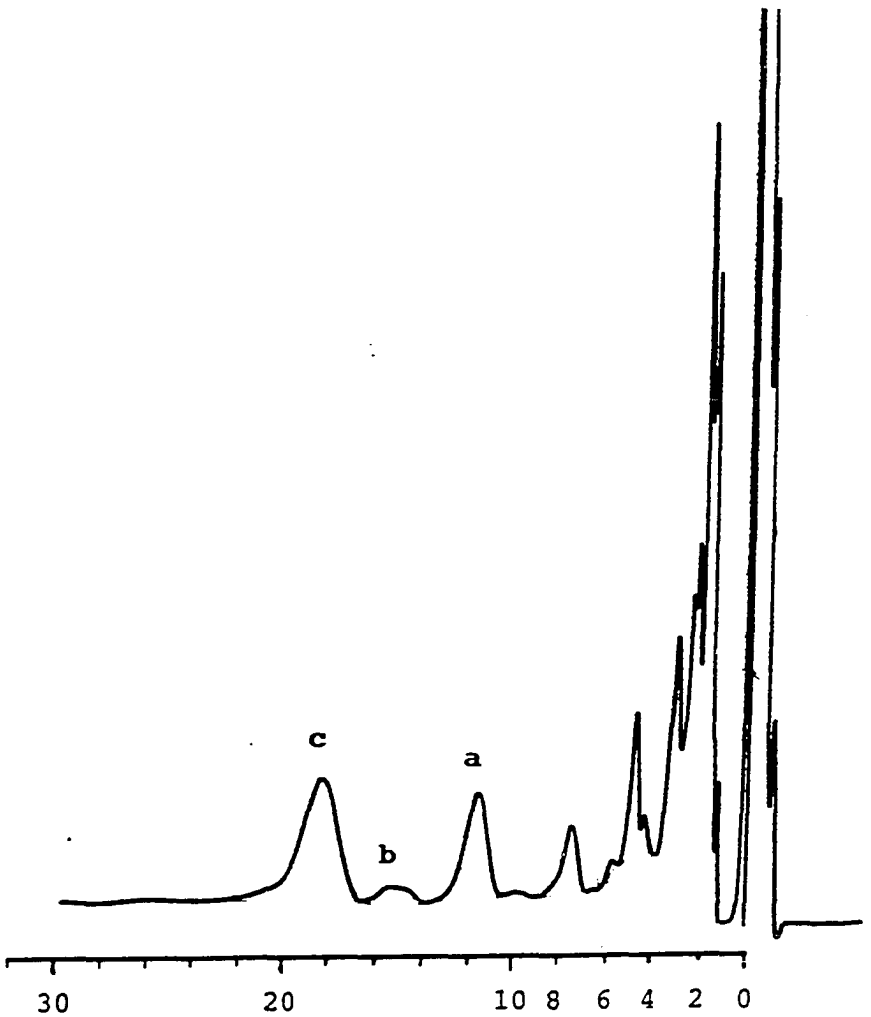


Figure 25. Volatile profile from Aspergillus parasiticus-inoculated corn

trial 2, closed cell model, no stirring, headspace sample day 14
10% FFAP, 135°C

a RT match for 1-octene-3-ol
b unidentified
c RT match to impurity in 2-octene-1-ol

Table 6. Trial 2 closed cell model, no stirring
Aspergillus volatile compound intensities

Day	Peak Ht a	Peak Ht b	Peak Ht c
2	.	.	0.2
3	.	.	2.9
4	2.0	0.4	0.8
5	2.2	0.9	1.8
6	4.0	0.6	1.6
7	4.6	0.7	7.2
8	5.2	0.5	1.2
9	3.5	0.9	3.5
10	6.7	1.0	7.6
11	11.2	1.3	11.8
12	2.5	.	1.2
13	9.8	1.0	12.4
14	15.1	2.6	17.8
15	6.5	1.8	15.1
16	5.8	0.5	4.4
17	9.0	.	15.6
18	6.5	.	7.6
19	3.5	.	2.5
20	3.0	.	1.8
21	7.3	.	6.0

(Figures 26-28) showed the expected inverted curve. Concentration of the volatiles was much higher, resulting in >100ng of each component over much of the collection period. It was not possible to test for correlation between toxin production and particular volatiles however, since no samples were taken.

Trial 3: closed cell model-stirring

Trial 3 consisted of a specially designed and manufactured culture vessel (previously illustrated in Figure 5) which allowed collection of the dynamic headspace and also allowed stirring for sample homogeneity since it was intended to get samples for induction volatile analysis and toxin analysis. The first design achieved several design goals. Stirring with the large form-fitting paddle at 28 RPM actually "folded" the entire mass of the moist feed as it stirred. Because of the large vessel mouth and the flexible paddle design, it was possible to completely remove the paddle for either sample removal or cleaning the apparatus at the end of a run. The bushing allowed introduction of the paddle drive rod without loss of volatiles being constantly swept onto the resin trap. Three of these culture vessels were constructed to allow simultaneous multiple runs.

Stirring a mold culture to obtain a homogeneous sample for analysis introduced an unexpected variable to the results. Heretofore, the aflatoxin had been detected as early as day 4 and peaked around days 7-10, with volatiles following this trend. Also sporulation was evident at about day 3-5 and continuing. When the culture was stirred, all of these signs of metabolism were observed to be much delayed in onset, by 3-6 days.

Both an Aspergillus parasiticus and a Fusarium roseum culture were stirred constantly over a 13 day period. In the Aspergillus parasiticus low concentrations of volatiles were observed in the headspace samples.

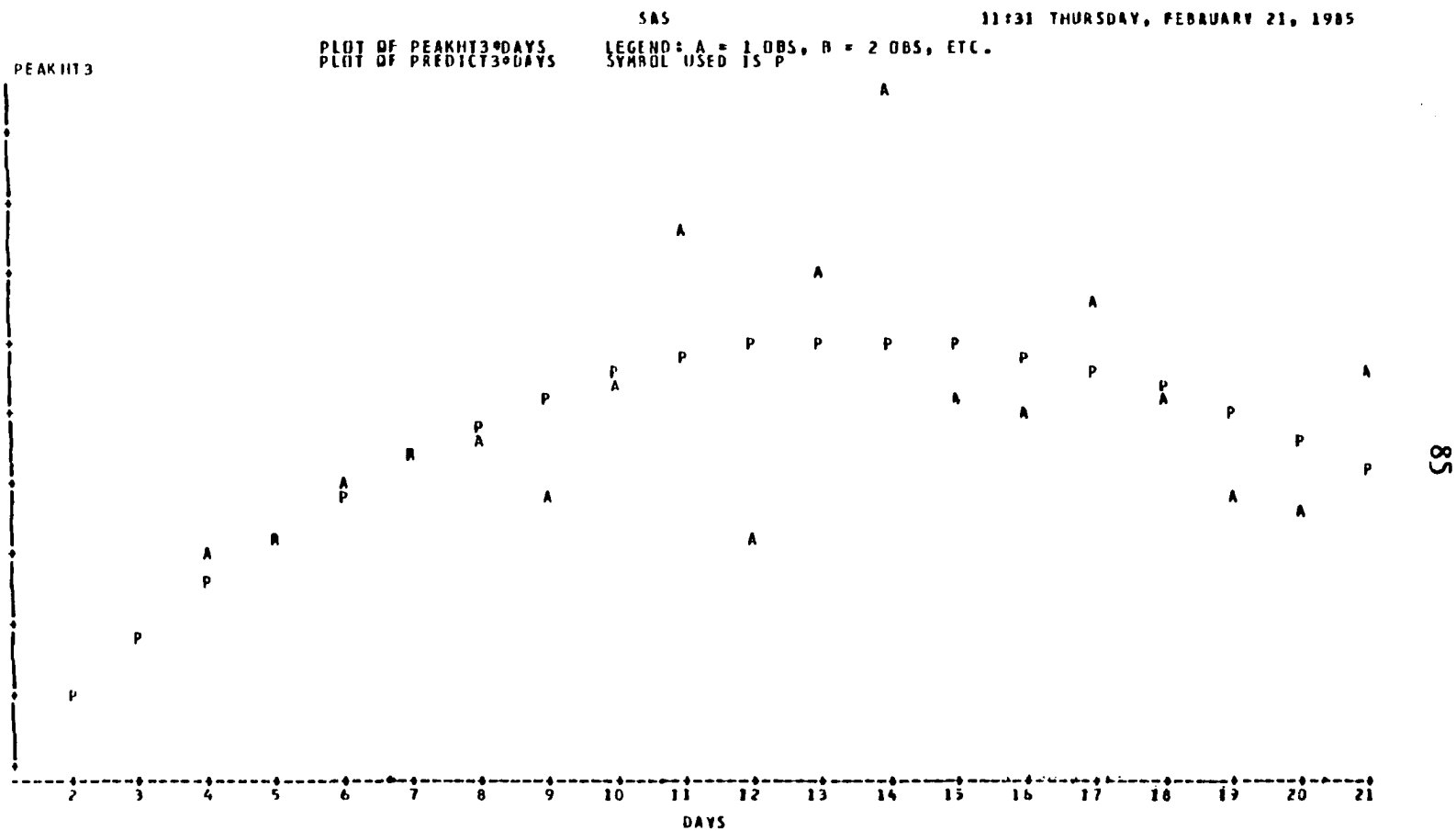


Figure 26. Plot of peak 3 intensity versus time post-inoculation

Actual observations (A) and predicted values (P) of peak 3 intensity are plotted versus days post-inoculation.

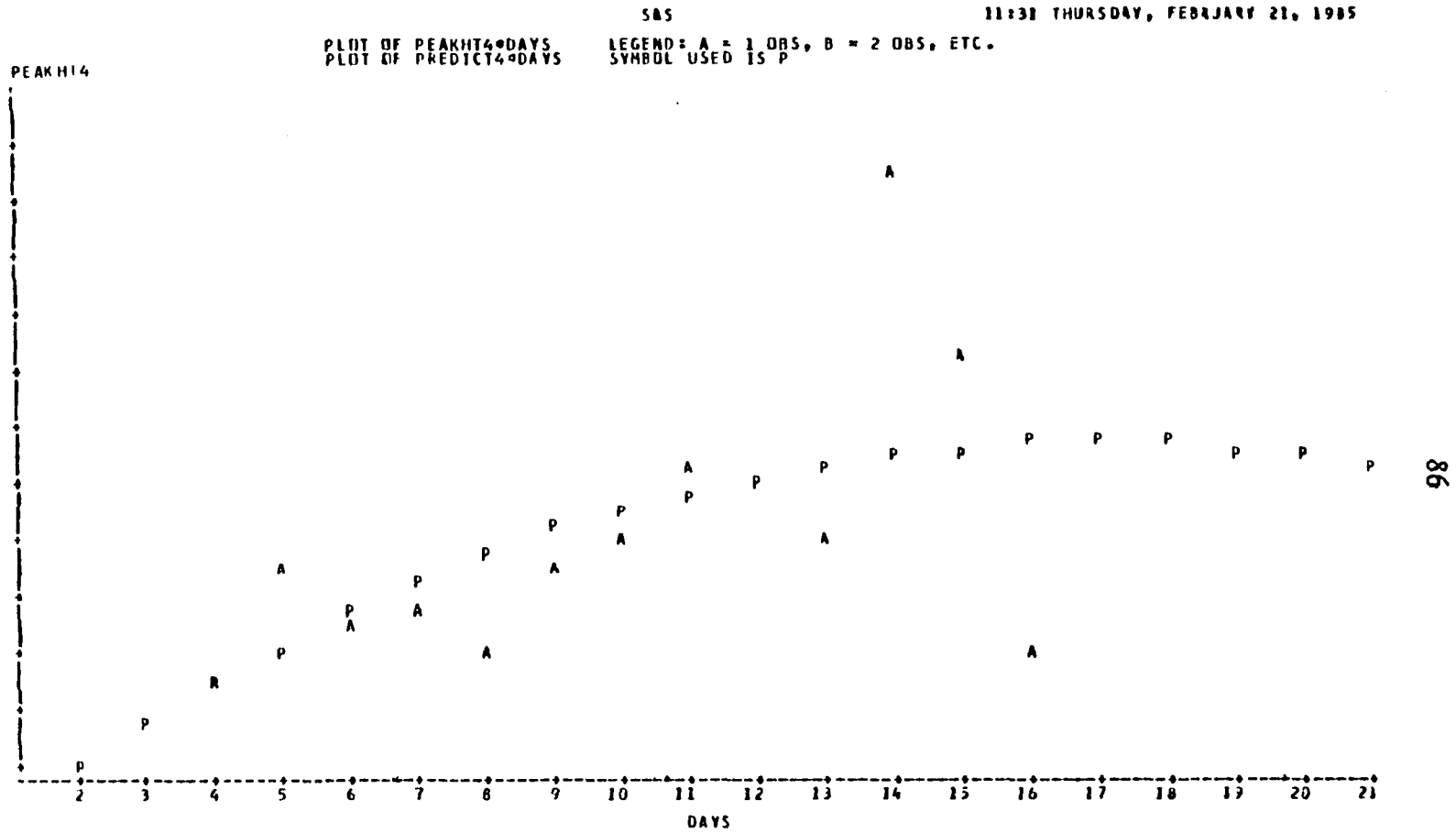


Figure 27. Plot of peak 4 intensity versus time post-inoculation

Actual observations (A) and predicted values (P) of peak 4 intensity are plotted versus days post-inoculation.

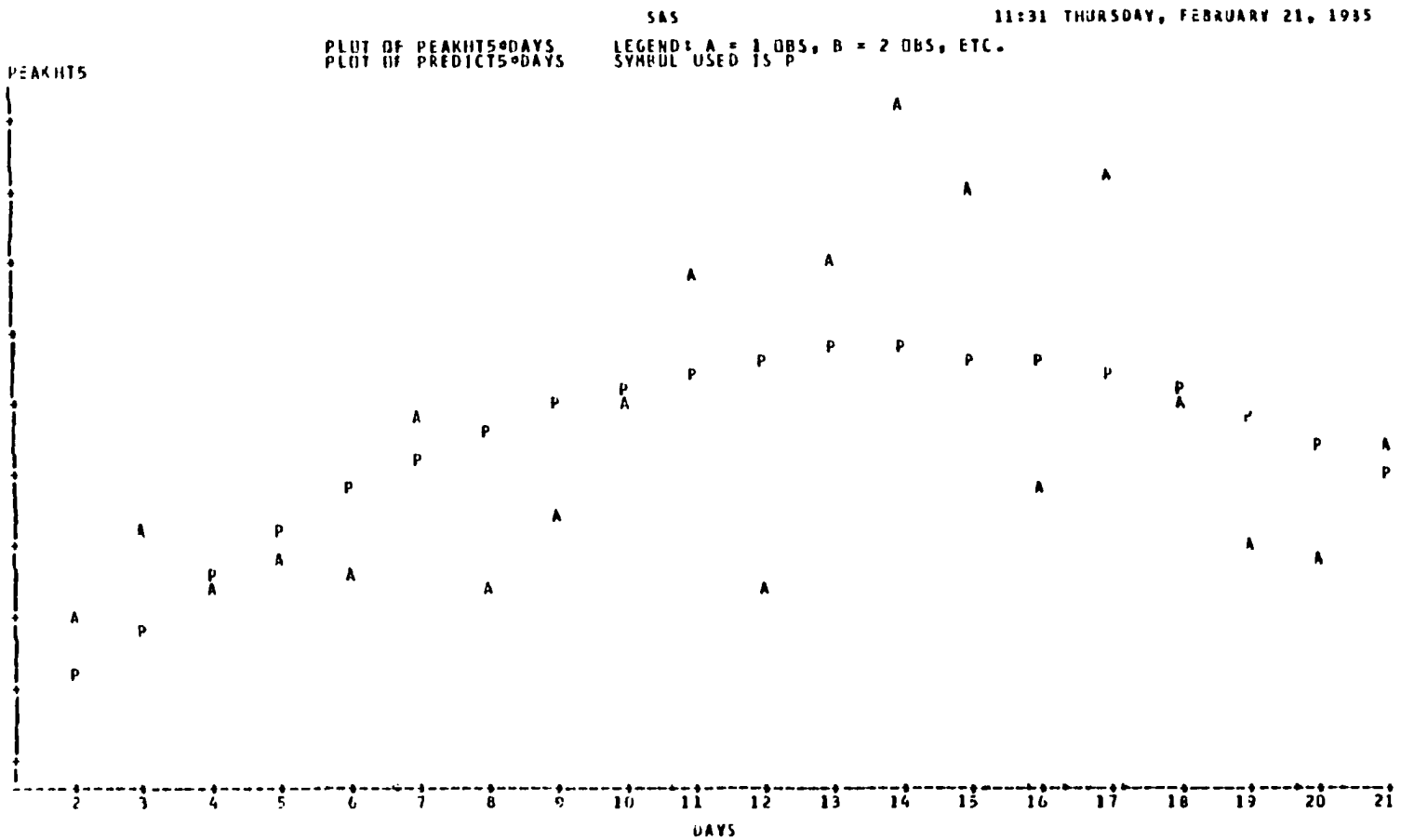


Figure 28. Plot of peak 5 intensity versus time post-inoculation

Actual observations (A) and predicted values (P) of peak 5 intensity are plotted versus days post-inoculation.

Figure 29 illustrates the actual Aspergillus parasiticus culture volatile profiles obtained in the headspace for days 1-13 using 10% FFAP, 135 °C with FID detection and Table 7 contains the data. Peaks a and c corresponded to peaks observed in Trial 2, the incubated/no stirring study previously discussed. Toxin levels were first observed on day 7 and increased dramatically with time. Figure 30 illustrates the relationship between aflatoxin B_1 and G_1 versus time; and headspace volatiles a and c versus time. The plots indicate that volatiles seem to maximize at the initiation point of aflatoxin production. No Fusarium toxins were observed in the Fusarium inoculated culture until day 14, the last day of the study, when 0.7 ppm deoxynivalenol (VOM) was noted; and no volatiles were observed. The control feed tested negative for significant volatiles and toxins throughout this experiment.

Because of the delaying effect constant stirring had on sporulation and volatiles production, and the goal that the laboratory model should resemble a field situation as much as possible, the culturing method was changed. The cultures in the next 4 batches of Aspergillus parasiticus-inoculated corn were stirred 1 hour for homogeneity just prior to sampling. Samples for induction volatile collection and mass spectral analysis were taken in an attempt to positively identify the volatile compounds found when toxins were found. Very low volatile levels were recorded in these experiments, with little consistent appearance of a, b, or c. The headspace volatile profile of one of 4 experiments (Figure 31) showed the appearance of a and c starting at day 2 and peaking between day 4 and 7 (day 5-6 are missing from the GLC/FID traces in Figure 31 because they were analyzed by GC/MS/DS for their identity). Figure 32 plots component a and c volatile level and aflatoxin level versus time. Aflatoxin

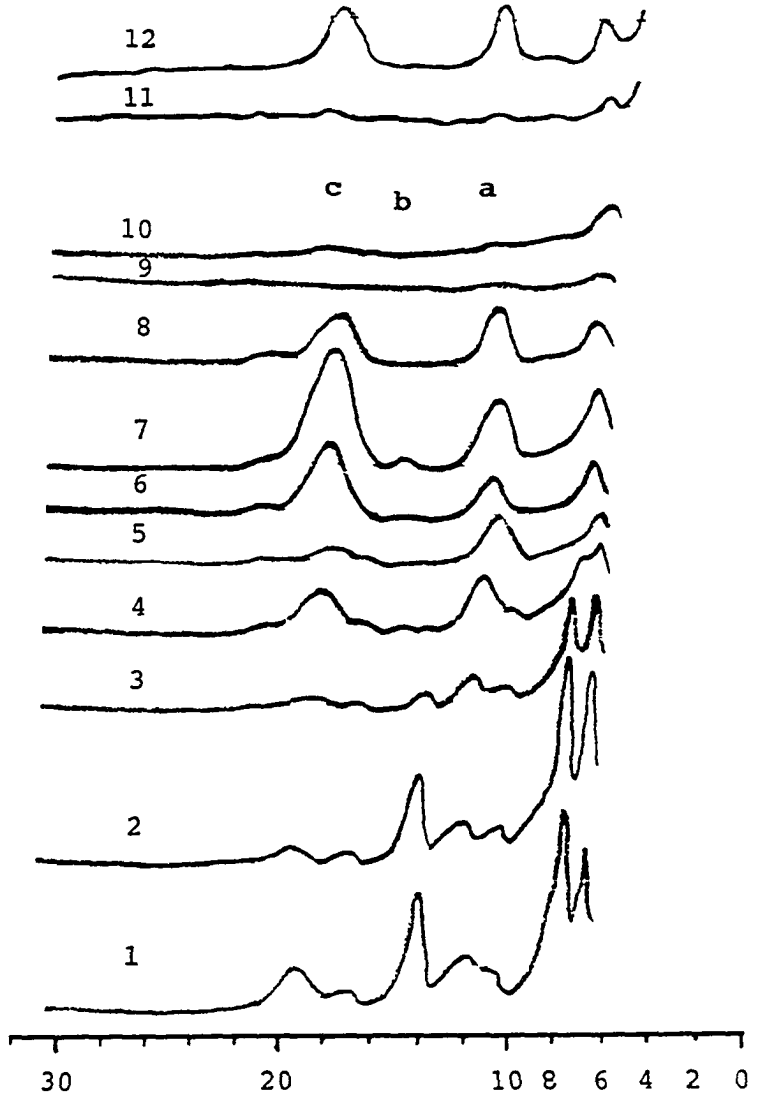


Figure 29. Volatile profiles from Aspergillus parasiticus-inoculated corn trial 3, closed cell model, constant stirring

headspace samples
10% FFAP, 135°C
a RT match for 1-octene-3-ol
b unidentified
c RT match to impurity in 2-octene-1-ol

Table 7. Toxin concentration and volatile level versus time
Trial 3 Closed cell model, constant stirring: Aspergillus aflatoxin levels and volatile intensities as a function of time

Day	B_1 level	G_1 level	Peak a	Peak c
1	NDA [a]	NDA	3.0	5.0
2	NDA	NDA	3.5	3.0
3	NDA	NDA	3.5	1.5
4	NDA	NDA	6.0	5.5
5	NDA	NDA	6.8	1.8
6	NDA	NDA	6.0	10.2
7	10 ppb	trace	9.2	16.7
8	70 ppb	20 ppb	8.1	7.0
9	--	--	1.4	0.0
10	53 ppb	58 ppb	1.0	1.0
11	33 ppm	6 ppm	--	--
12	--	--	--	--
13	--	--	8.5	8.3
14	68 ppm	22 ppm	--	--

[a] no detectable amount

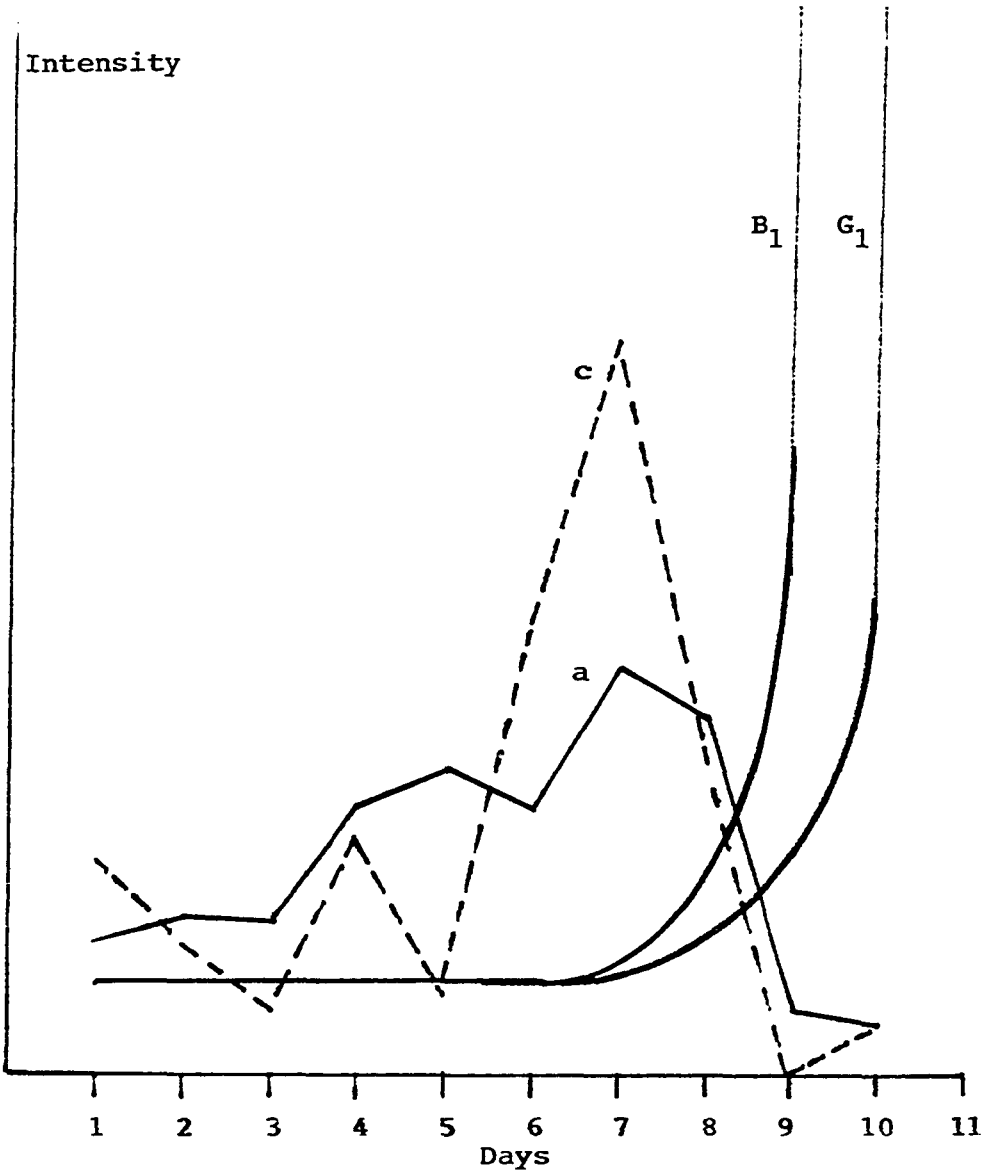
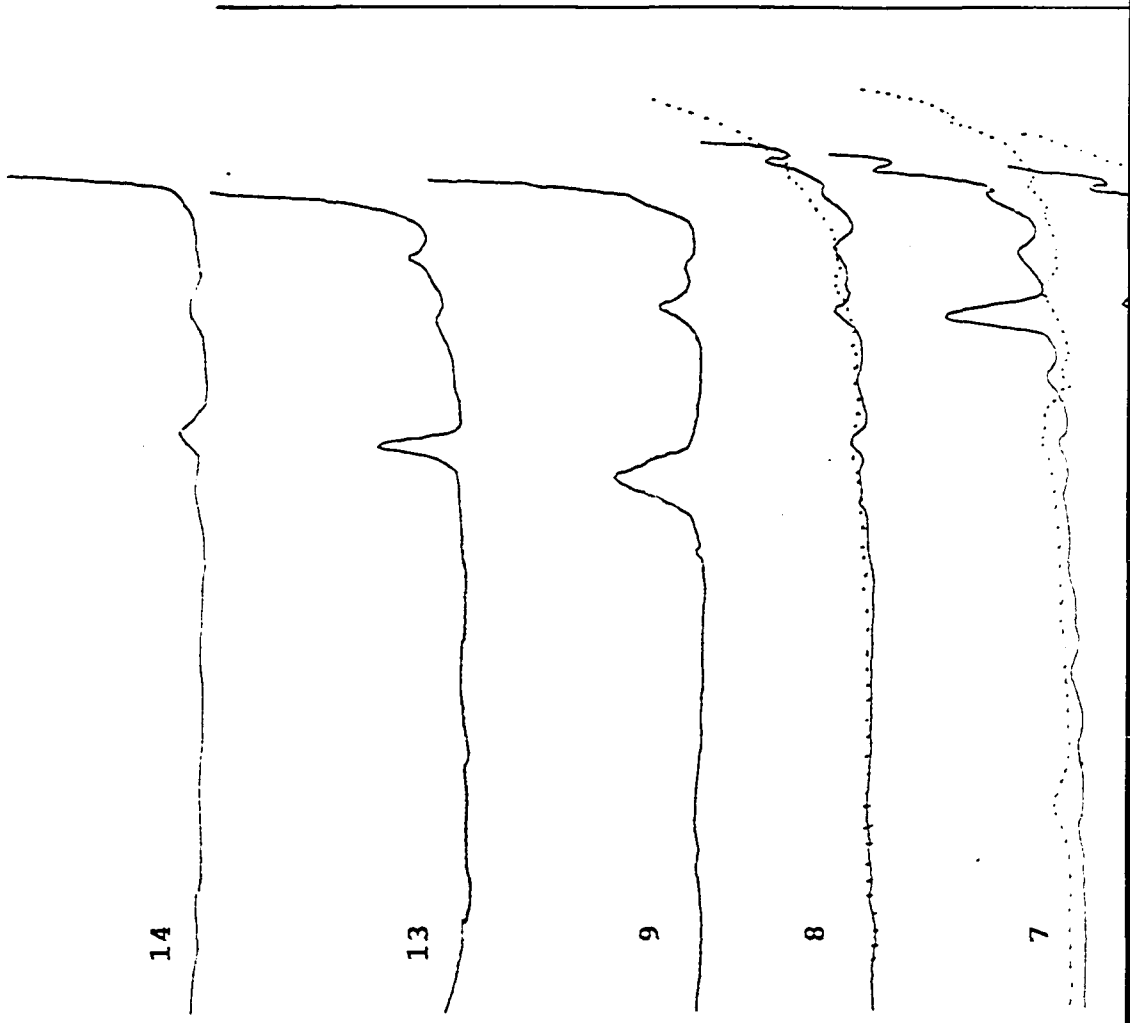


Figure 30. Volatile and toxin level from Aspergillus parasiticus versus time trial 3, closed cell model, constant stirring

The curves illustrate two concurrent processes: the appearance of aflatoxins B_1 and G_1, and the appearance of volatile peaks a and c. The volatiles seem to maximize on day 7 just when the aflatoxins start to appear.


14
13
9
8
7

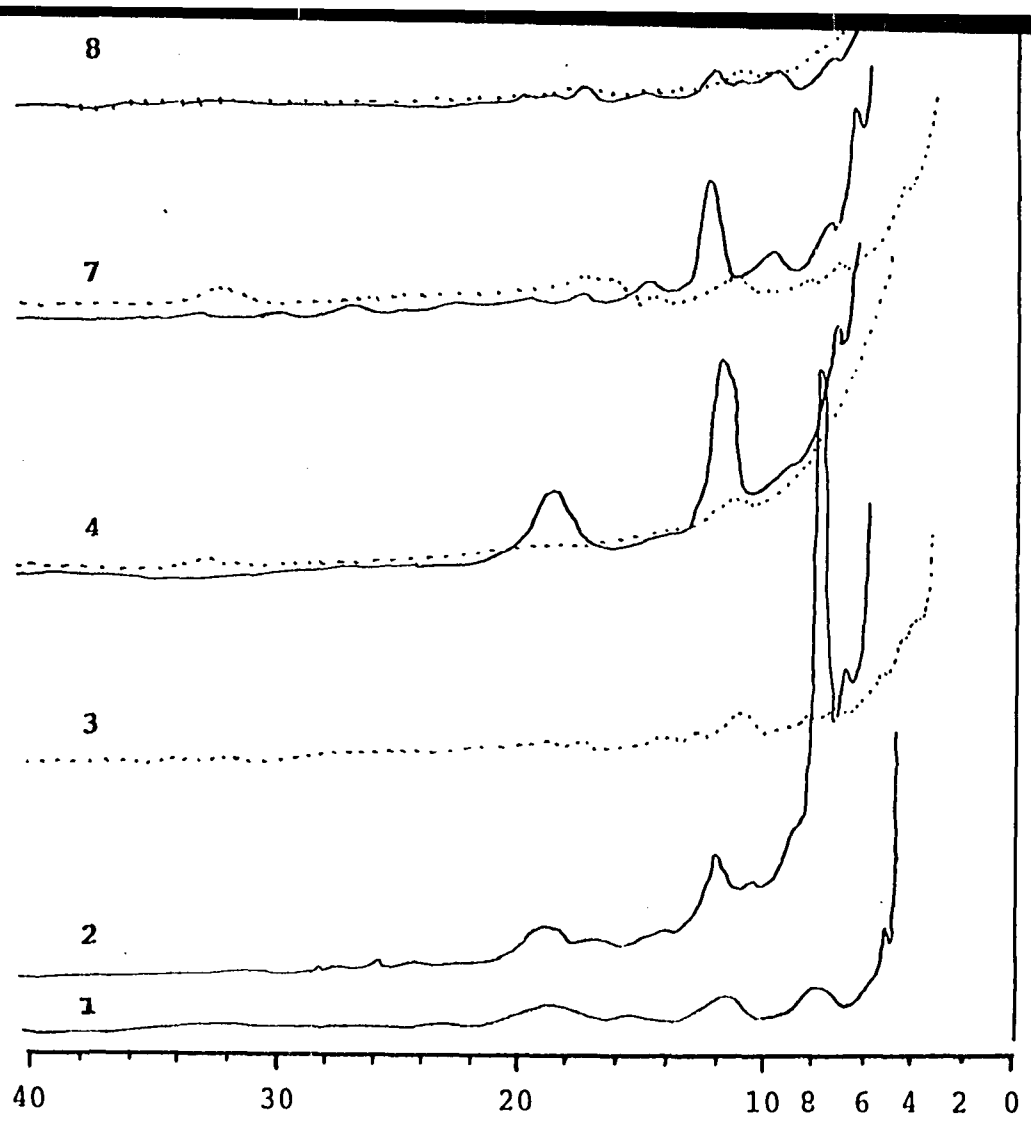


Figure 31. Trial 3 closed cell model 1 hour stirring
Aspergillus parasiticus-inoculated corn
10% FFAP, 135°C, FID ——— headspace ..... inducted

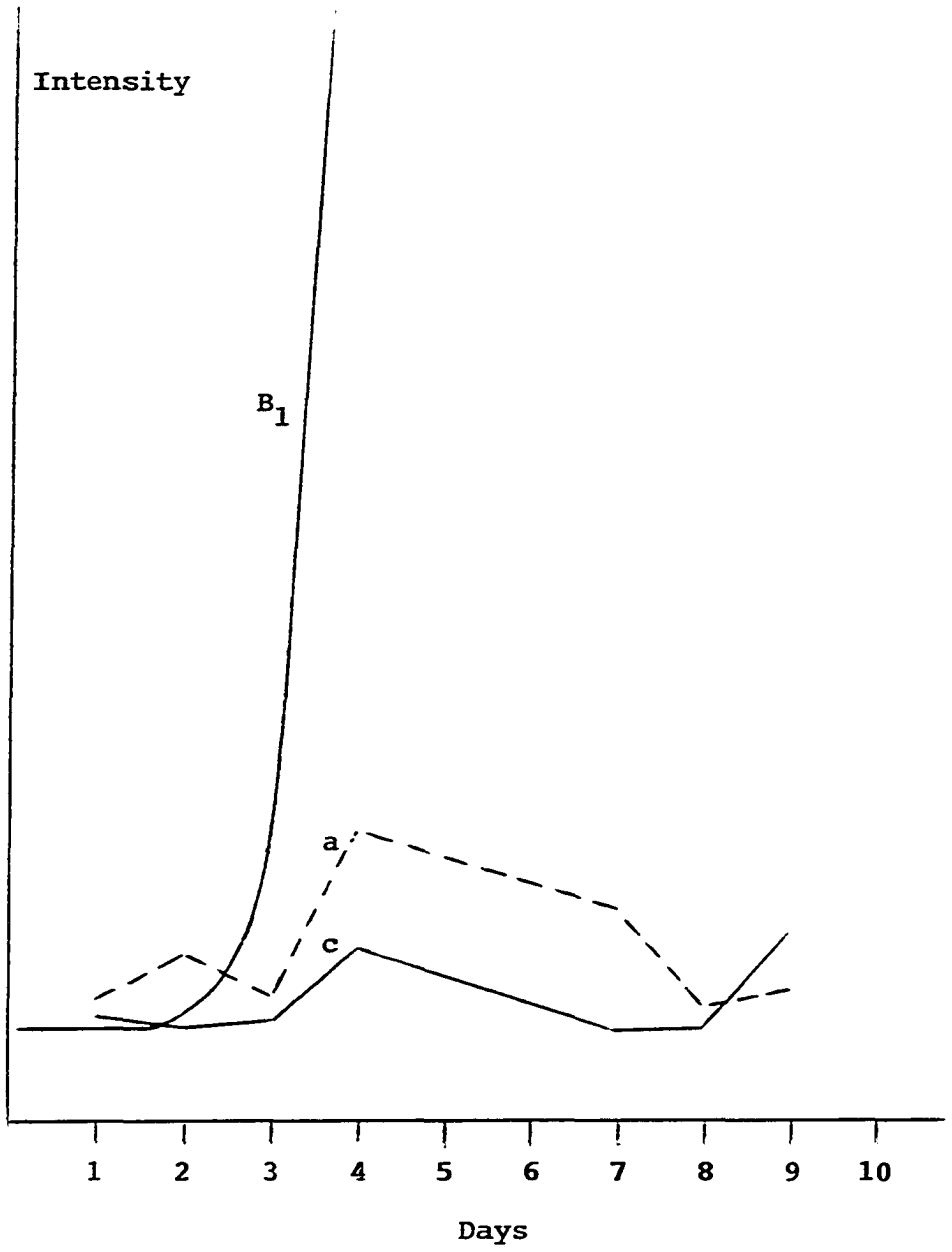


Figure 32. Volatile concentration and aflatoxin level from Aspergillus parasiticus versus time
trial 3 closed cell model, 1 hour stirring

Volatile peaks a and c, and the aflatoxin B_1 level are shown versus time. As noted in the trial 2 study, the volatile concentration seems to maximize at the point of toxin production.

levels also started to dramatically increase at the time of maximized volatile detection, as noted in the previous trial with constant stirring.

Mass spectral analysis of duplicate inducted traps confirmed peak a to be 1-octene-3-ol, agreeing and confirming initial findings in trials 1 and 2. While the levels of a were still quite small, quality spectra were obtained which matched well with spectra obtained from authentic 1-octen-3-ol standard (Figure 33).

Extensive comparisons of the volatiles profiles obtained from the dynamic headspace sampling and the inductive thermal stripping showed that both processes yielded similar chromatograms with the induction chromatogram about twice as intense as the headspace sample for the same day, especially for later eluting volatile compounds (Figure 34-35). The very early eluting volatiles were conversely more intense in the headspace samples. This can be explained in terms of the equilibrium that the volatiles were establishing between the feed matrix/mold vegetation and the air space being constantly swept by the nitrogen gas. The feed matrix acted like a chromatographic adsorbent, in allowing the volatiles to equilibrate between the gas phase and the feed itself. Components in greater concentration in the air phase were more volatile and probably less polar which would also, for similar reasons, be early chromatographic eluting peaks from GLC. Components equilibrating at higher levels in the feed would be more polar components, which would chromatographically elute later under the conditions used in this study. It was not unexpected that the headspace would be more sensitive for the early eluting components than the induction sample since the inducted sample contained the volatile content of only 0.2 gm, while the daily dynamic headspace sample represented the total volatile headspace from the entire 200 gm sample over a sampling period of 24 hours. A larger sample for

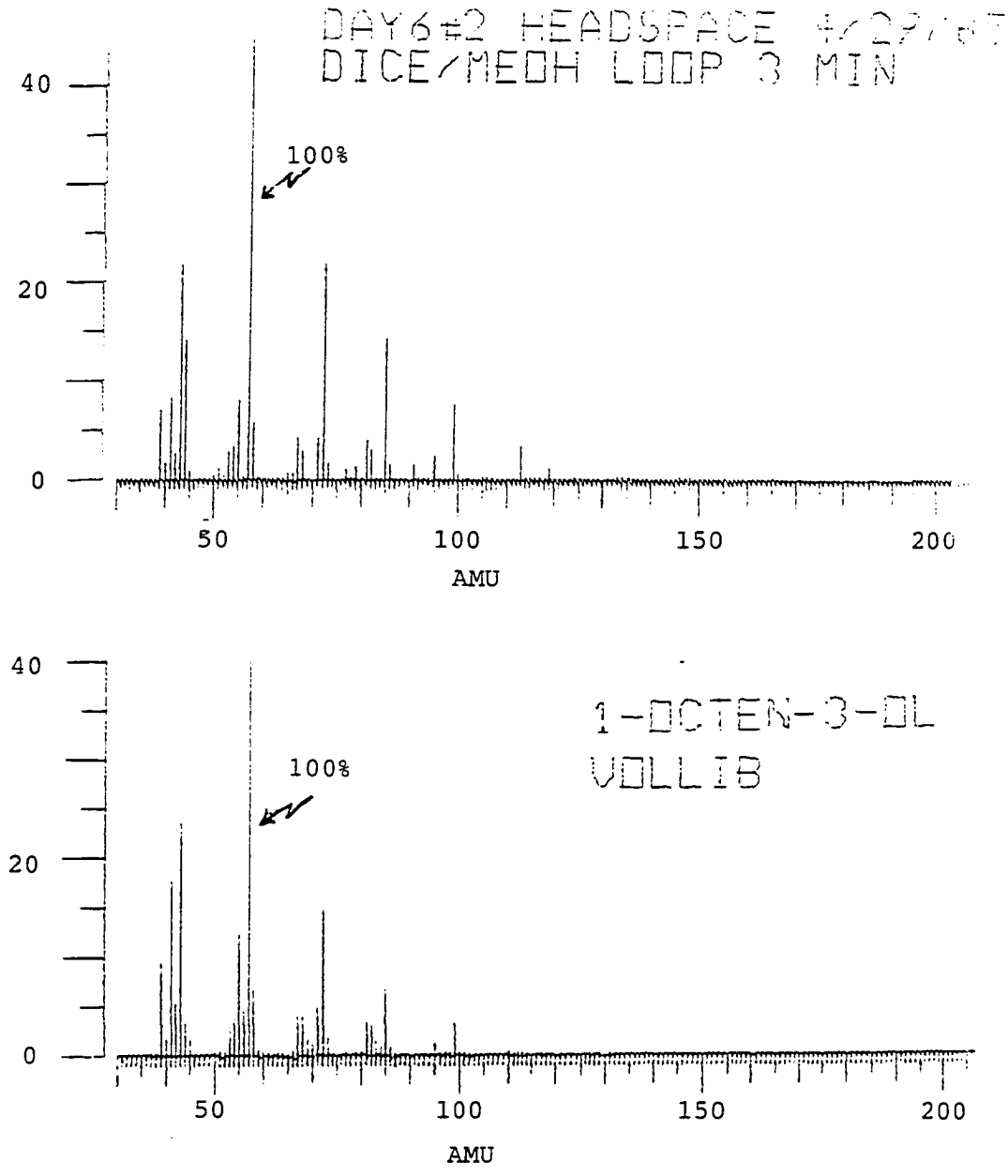
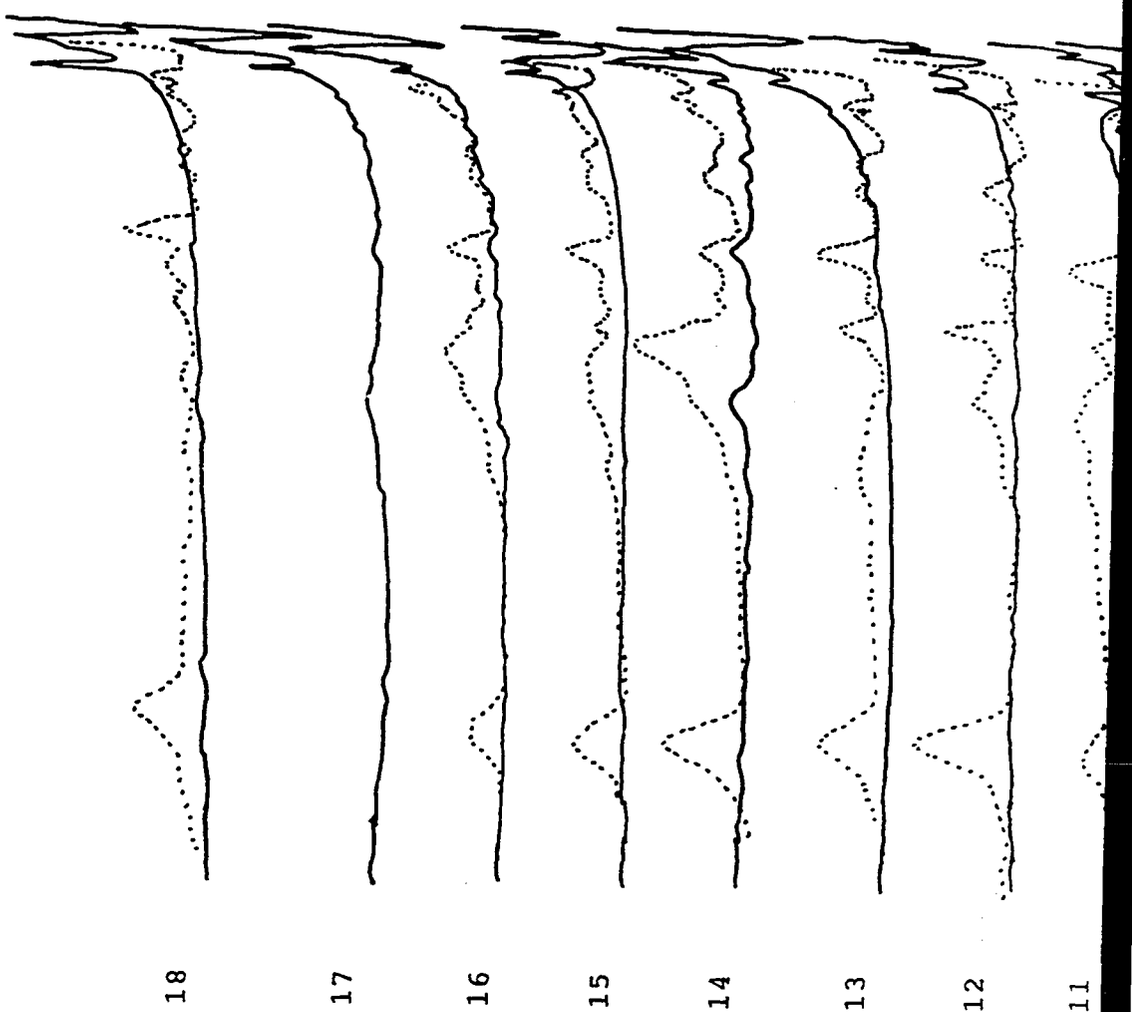


Figure 33. Comparison of mass spectra of peak a and of authentic 1-octene-3-ol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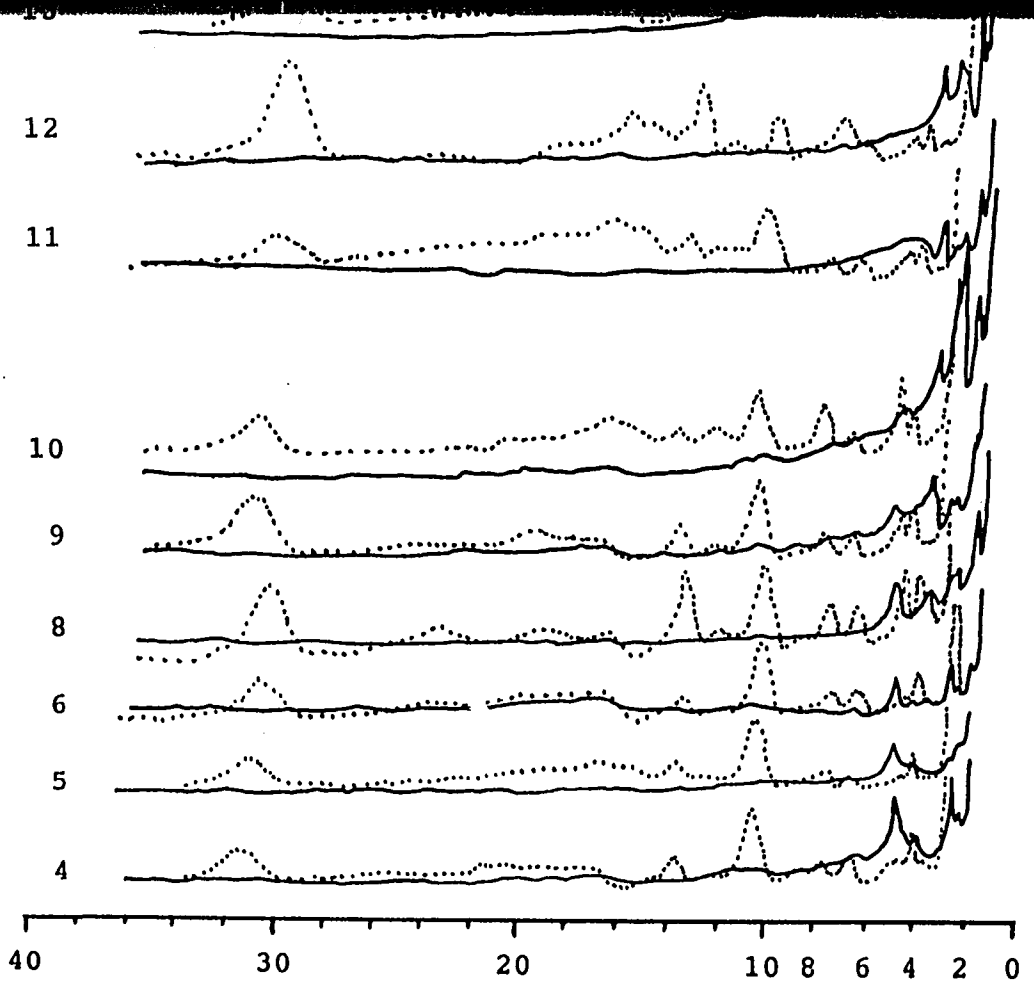
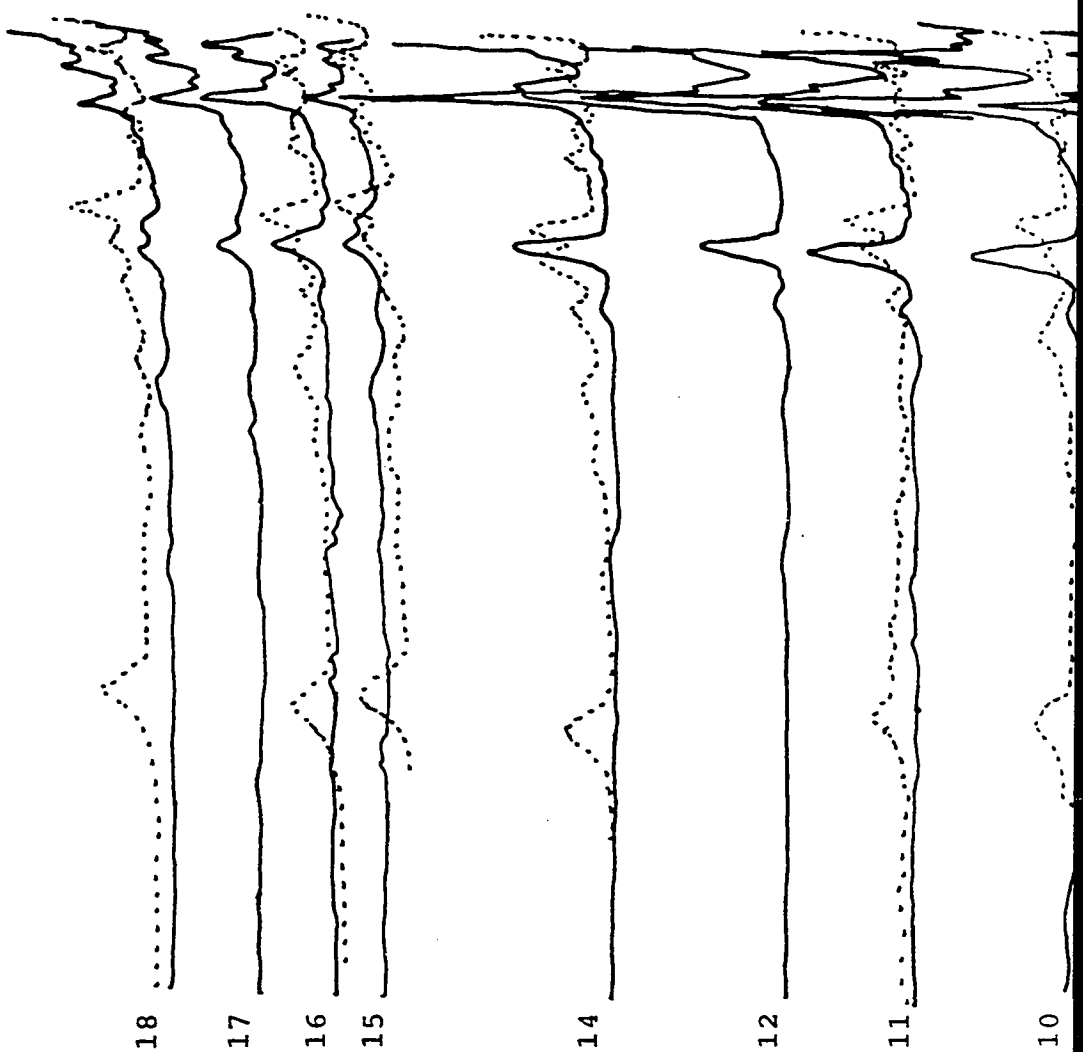


Figure 34. Volatile profiles from Aspergillus parasiticus-inoculated corn trial 3, closed cell model, 1 hour stirring

10% FFAP, 135°C ——— headspace ..... inducted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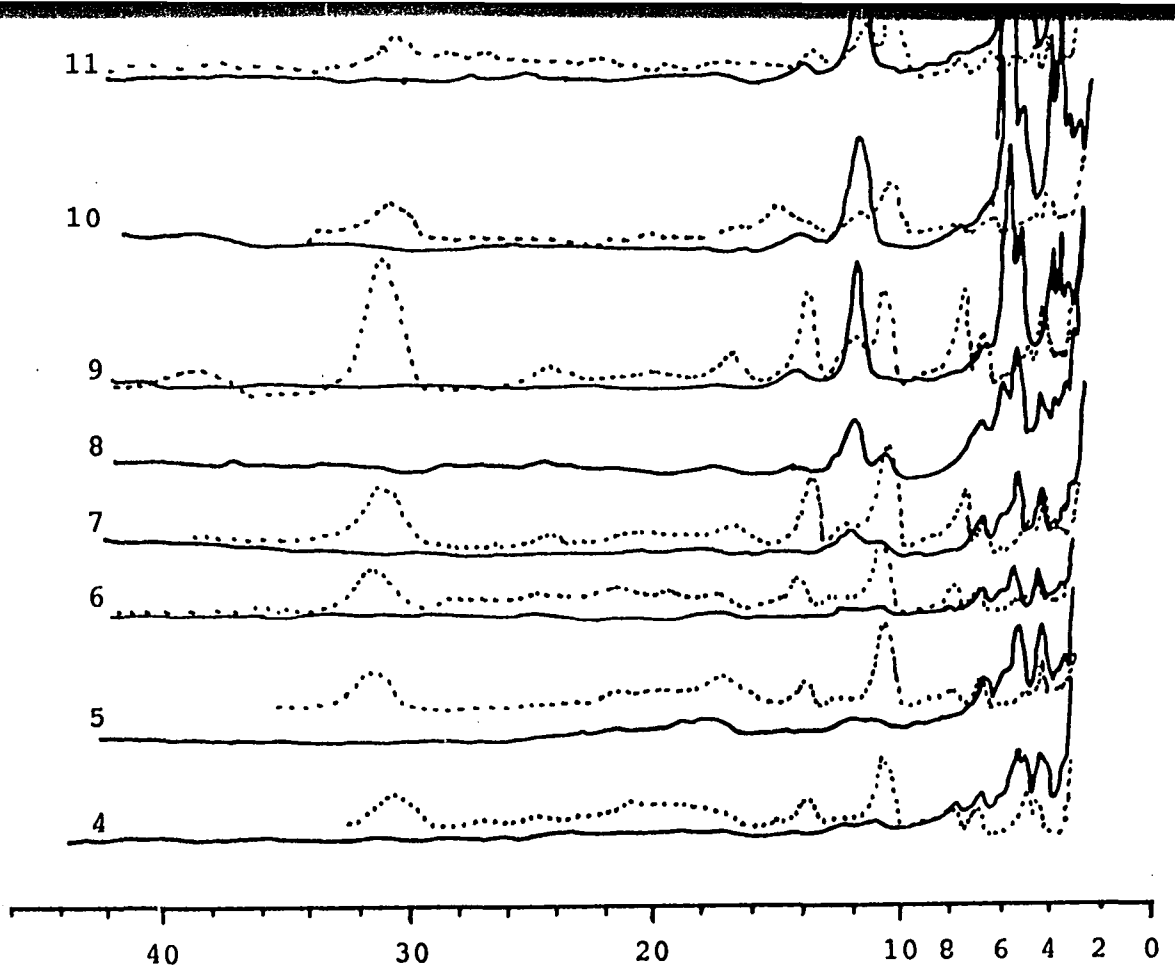

Figure 35. Comparison of volatile profile from inducted and headspace samples of Fusarium roseum-inoculated corn, trial 3, closed cell model, 1 hour stirring

10% FFAP, 135°C ——— headspace ..... inducted

induction or other thermal process for liberating the volatile components would yield a much more intense volatile profile.

In summary, the analytical method of choice for this work was packed column GLC using 10% FFAP isothermally at 135 °C with FID detection, or 120-125°C isothermally with GC/MS/DS detection. This column was impervious to sample moisture, reproducible results were obtained over months of routine use, and stable to the introduction of hundreds of sample injections over that time. Resolution was adequate for the investigations because GC/MS was also available. However, preliminary work with precolumn capillary recollection and with mega-bore cryogenic focusing offered much improved resolution allowing the use of retention time as a primary identification tool.

The results of several laboratory models used to grow fungi on ground corn determined that a closed cell model to contain the volatiles was helpful in increasing the level of volatiles observed, probably because it prevented their loss by volitilization. Stirring constantly was found to retard sporulation and the onset of both volatiles and toxin production in Aspergillus parasiticus.

Finally, work with Aspergillus parasiticus and Fusarium roseum indicated that the Aspergillus would produce a series of 2-3 volatiles, labeled a, b, and c throughout this work, which maximized just at the initiation point of aflatoxin production. One of these, a, was conclusively identified, using retention time measurements and using GL/MS/DS, to be 1-octene-3-ol. Preliminary experiments with Fusarium roseum indicated the presence of only 1-octene-3-ol, with no observable levels of the others.

SUMMARY AND CONCLUSIONS

The goals of this work were to develop sampling and analysis methodology for the collection, concentration and analysis of any organic compounds produced by molds as they developed in grain; to devise a laboratory model culture which could be monitored with such sampling and analysis methodologies; and to see if any correlation existed between the presence of volatiles in a sample and the presence of either specific molds or their toxins.

Sampling techniques using Tenax^{RT} resin were developed which resulted in reproducible and predictable retention of model volatile organic compounds. Performance characteristics of the resin in a trap device were extensively investigated with system linearity to standard model compounds established.

Introduction of the adsorbed trap volatiles onto several gas chromatographic system configurations was studied. Packed FFAP column GLC suffered least chromatographic degradation from sample moisture but also gave the least component resolution. This study relied on a combination of retention times obtained and mass spectral data to identify components. The cryogenically focused megabore capillary column offered superior resolution but was rapidly degraded by moisture in very few samples. The resin-precolumn capillary developed by Fritz and co-workers offered much promise but was not generally available in a commercial package and due to constraints of accessibility was not used extensively during this work. However, improved resolution would allow retention time to be used as a primary means of volatile identication. Either the moisture in the sample traps must be dried prior to megabore cryogenic focusing or the precolumn capillary interface system should be used.

Several laboratory culture models were studied, resulting in the development of a closed cell vessel, allowing limited stirring for homogeneous sampling, yet

allowing complete collection of the headspace volatiles. Final studies comparing headspace volatiles to thermally stripped volatiles indicated that components which elute from a FFAP GLC column early also can be successfully collected in the sample headspace whereas, later eluting components were more completely sampled from the termally stripped sample by inductive coupled heating. Inductive heating was more sensitive than headspace sampling in detecting the octene-ol compounds in Aspergillus parasiticus inoculated corn samples.

Inductive heating was investigated as a means of thermally stripping volatiles from a moldy corn sample. Performance characteristics were determined and optimized for application to laboraory cultures.

Sample size for inductive thermal stripping (or any other heat process used to strip the volatiles) was very important. Two tenths gram of moldy grain was not enough, because of sample inhomogeneity and the variation that inhomogeneity passed on to the results. Larger sample sizes, 1-10 grams, should be tested. Problems due to moisture may possibly be accentuated but volatile levels will be much higher resulting in much better GC/MS component identification.

Volatiles were collected from a Aspergillus parasiticus-inoculated corn in several laboratory experiments. In most, 2 or 3 peaks, designated a, b and c throughout this work, were correlated with the initial appearance of toxin, the sporulation process of the mold, or some other related process. The volatiles did not maintain a correlation to toxin level after initial toxin appearance. While sporulation was not a studied parameter of this work, preliminary observation indicated this was a likely process of direct correlation to the appearance and disappearance of certain volatiles. Also, a study of residence time of volatiles in

grain dried to the point where further mold action is inhibited would be an interesting subsequent investigation.

One volatile, Peak a, was identified in induction and headspace samples as 1-octene-3-ol using resin trap/GLC /MS techniques of sampling and analysis. Identification of peaks b and c was not accomplished during this work but seemed important to distinquish between Aspergillus parasiticus volatiles and Fusarium roseum volatiles since 1-octene-3-ol appeared by itself in at least 1 Fusarium culture but the other peaks were only seen with 1-octen-3-ol in Aspergillus cultures.

Sources of volatiles other than mold growth in corn were identified, such as grain containers used and autoclaving.

This work may have raised more questions than it answered. However, it offers a routine and systematic starting point for future investigations of the relationship between mold production of volatile compounds and production of toxins; and of the feasability of volatiles testing as a measure of grain quality.

SUGGESTIONS FOR FUTURE WORK

Future studies should consider the possibility that certain volatiles are correlated more directly to another process, such as sporulation, than to toxin production. Even if this proved true, a study of the residence of the volatiles under field conditions might prove that the volatiles linger from such a process long enough and at a sufficient concentration, to indicate that mold growth occurred under conditions suitable for toxin production.

The use of the Mega-bore capillary column is easily amenable to most laboratories. The means whereby sample trapped moisture could be dried or otherwise eliminated should be studied, and also the effects of such treatment on the trapped volatiles. Also the precolumn capillary developed by Fritz and co-workers should be applied to this application. Both of these techniques offer the potential of using retention time as the primary or sole means of identifying volatiles observed.

The processes described in this work should be applied to fungi other than just Aspergillus parasiticus and Fusarium roseum. Indeed, the Fusarium work should be extended beyond that which was accomplished here. These principles should eventually be applied to field samples and proven for their worth as indicators of mold and mycotoxin contamination.

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APPENDIX

VDL Multimycotoxin Method

Aflatoxins (B1, B2, G1, G2), Vomitoxin, Scirpenes, T-2 Toxin Diacetoxyscirpenol, Zearalenone, Zearalenol and Sterigmatocystin

Mycotoxins are extracted, defatted and decolorized. The extracts are cleaned up by thin layer chromatography (TLC) and the compounds visualized by ultraviolet (UV) light or colored products. Quantitation of aflatoxins and zearalenone has been done by UV spectroscopy and fluorodensitometry. The scirpenes, vomitoxin and zearalenol are quantitated by gas chromatography (GC).

Reagents and Apparatus

a. extraction solvent: acetonitrile/water, 90/10; acetonitrile, Eastman white label, distilled in glass; water, Millipore, "Milli Q" or equivalent
b. developing solvents: 85/10/5, chloroform, acetone, 2-propanol; 3/2/1,toluene, ethyl acetate, acetone; 3/2/1 + 1; toluene, ethyl acetate, acetone, formic or acetic acid
c. thin layer plates: silica gel G plates; 40 g silica gel plus 95 ml ethanol spread 0.5 mm thick with a DeSaga spreader; Merck silica gel: silica N-HR or G; glass or aluminum backed (Brinkmann Instruments); Quantum, HPTLC and reverse phase (Whatman, Inc.)
d. defatting solvent: 2,2,4-trimethylpentane, ACS grade, or petroleum ether distilled in glass
e. spotting solvent: 98/2, chloroform/acetonitrile
f. decolorizing reagents: 15% ferric chloride ($FeCl_2.6H_2O$), 4% aqueous sodium hydroxide

g. Waring blender: explosion-proof

h. gel preparation apparatus: 50 ml Buret with a teflon stopcock, Corning Model 12-B pH meter (or equivalent)

i. aflatoxin stock standards: 100 ng/ul; add 10.0 ml chloroform to the 10 mg in the standard vial; mix and pipet 1 ml into a 10 ml volumetric flask and dilute to volume with chloroform; evaporate remaining 9 ml in the vial under stream of purified nitrogen, and store under refrigeration (stock solution is stable for 1 year, if frozen) Note: Aflatoxin standards are electrostatic in the dry state. Therefore, extreme caution should be used in handling. (standards from Calbiochem-Behring)

j. aflatoxin working standards: 10 mg/ul; pipet 1 ml of 100 mg/ul stock solution into a 10 ml volumetric flask, evaporate to dryness under N2 and dilute to volume with Nanograde methanol

k. scirpenes, zearalenone, zearalenol, sterigmatocystin and vomitoxin standards: 1 ug/ul; weigh 10 mg dry standard into 10 ml volumetric flask and dilute to volume with Nanograde methanol

l. p-anisaldehyde spray reagent: 85/10/5, methanol/concentrated glacial acetic acid/concentrated sulfuric acid and 0.5 ml p-anisladehyde

m. pH 4.0 buffer: Fisher Scientific (or equivalent)

n. potassium hydroxide: 20%, aqueous

o. Darco G-60 powdered charcoal

p. bleach bath: 10% CloroxR; after using glassware, soak it in the bath 2-3 hours before washing normally

q. steam bath

r. gas chromatograph: equipped with a FID, a 6 ft long x 3 mm ID glass column packed with 10% OV-101 or 3% OV-1 on Gas Chrom Q at 260oC

s. regulated prepurified nitrogen supply

Procedure

1. Weigh 50 gm of feed into a Waring blender. Weigh a duplicate sample into another blender and add a known amount of working standard. Spike with 4-6 ppb for aflatoxins, 1 ppm for zearalenone, and 2 ppm for T-2 toxin, diacetoxyscirpenol (DAS), vomitoxin, zearalenol, and sterigmatocystin.
2. Add 200 ml extraction solvent (acetonitrile/H2O) and blend at high speed for 4 minutes.
3. Decant 100 ml of supernatant through filter paper into a graduated cylinder and transfer into a 500 ml separatory funnel fitted with a teflon stopcock.
4. Add 50 ml petroleum ether, shake, vent and shake 0.5 minutes. Allow layers to separate. Drain and save aqueous layer into 250 ml beaker. Discard petroleum ether. Return aqueous layer to separatory funnel and repeat with an additional 50 ml petroleum ether.
5. To make the ferric gel, place 100 ml of water (Millipore "Q" or equivalent) and exactly 10 ml of the 15% ferric chloride solution into a 250 ml beaker.
6. Titrate this solution with 4% sodium hydroxide, constantly stirring and measuring with pH meter, until the pH is 4.6.
7. Into the separatory funnel containing the sample extract, add the gel, shake, vent and shake for 0.5 minute.
8. After shaking, allow the gel to settle until separation occurs or filter sample if necessary.
9. Drain 100 ml of acetonitrile/H_2O layer into a graduated cylinder. Discard remaining acetonitrile/H_2O into bleach bath. Rinse separatory funnel well with hot water. Return the 100 ml acetonitrile/H_2O layer to the separatory funnel. The separatory funnel may be reused if rinsed thoroughly.

10. Add 50 ml of chloroform to the extract and shake.
11. After the layers have separated, drain and filter the chloroform layer into a 250 ml Erlenmeyer flask and repeat chloroform extraction. (Note: If an emulsion forms, add an additional 50 ml $CHCl_3$ to the extract and drain through an anhydrous Na_2SO_4 filled filter funnel. Glass wool is packed into the funnel constriction to retain the Na_2SO_4. Each sample is handled individually, so contact time is minimized.)
12. Add 0.1 gm powdered charcoal (Darco G-60) to the sample. Filter as soon as possible. The use of glass microfibre paper speeds up the filtering process.
13. Evaporate chloroform extract on a steam bath under a nitrogen effusion apparatus or in a vacuo. Do not overdry.
14. Redissolve the extract, either before or after GLC in 500 ul of the spotting solvent.
15. Spot 10% on a thin-layer plate along with appropriate standards. For aflatoxins spot 5-20 ng; 10-20 ug T-2 and DAS; 5-10 ug vomitoxin; 5-10 ug zearalenone; 10-20 ug zearalenol and 10-20 ug sterigmatocystin. Use reverse phase TLC with 20/30/1.5 solvent for vomitoxin samples.
16. Develop the plate in the preferred solvent system (see Developing Solvents) to within 1-2 cm of the top.
17. Visualize by UV light. T-2 toxin, DAS and vomitoxin are visualized by spraying with p-anisaldehyde spray. Sterigmatocystin is visualized by UV light and sprayed with 20% aqueous potassium hydroxide to increase fluorescent intensity and to change the red color to a more intense yellow.
18. If TLC resolution is poor, reverse phase or high performance plates (HPTLC) may be used.

19. The final sample volume, the volume spotted on thin-layer plates, concentration of standards and sample size are by no means rigorous and may be varied depending upon the particular situation.

Developing Solvents

1. Usually, the first development is done in 3/2/1 and zearalenone and zearalenol are analyzed. Then, if aflatoxins are resolved, the plate is sprayed to visualize T-2 toxin and DAS. If not, continued development is done in 85/10/5.
2. The 3/2/1 system was originally used for separation of tricothecenes, but will give a good separation of zearalenone and sterigmatocystin though the aflatoxins are not separated as well as in the 85/10/5 solvent.
3. Sterigmatocystin can also be resolved in the 3/2/1 + 1 solvent.
4. Additional developments, 2 or more, with 3/2/1 are possible. One additional development in 85/10/5 is possible and sometimes desirable. HPTLC or reverse phase plates are used if separations are still not complete with two developments.

Quantitation of T-2 Toxin, Zearalenol, DAS, and Vomitoxin

1. Determine T-2 toxin, zearalenol, and DAS directly using a flame ionization detector (FID) equipped with a 6 ft x 3 mm ID glass column packed with 10% OV-101 or 3% OV-1 on Gas Chrom Q at 260 °C. Vomitoxin may be determined by reducing column temperatures to 230 °C.
2. Redissolve the evaporated sample extract in 0.1 ml of chloroform and inject 5-8 ul onto the column.
3. On a 3% OV-1 column at 260 °C, retention times for T-2 toxin, DAS, and vomitoxin are 20, 8 and 4 minutes, respectively, and 28, 11 and 5 minutes on

a 10% OV-101 column. Zearalenol eltues in 22 and 31 minutes on the respective columns.

4. This method is used at a sensitivity of 1 ug for typical samples.

Quantitation of Zearalenone

1. Scrape the fluorescent spot at the same Rf as the zearalenone standard from the TLC plate and elute from the silica gel with methanol.
2. Prepare standards of zearalenone between 1.0 and 10.0 ug/ul in methanol. Dilute the sample accordingly.
3. Using Cary14 (or equivalent) UV spectrophotometer, a scan from 250 nm to 350 nm gives a characteristic peak. Calibrate according to Official Methods, A.O.A.C., 12th Ed., Sec. 26.004.
4. Quantitate by measuring the peak height at absorbance maximum, 274 nm.
5. Check the purity of the zearalenone standard, using the following molar absorbtivities: ultraviolet maxima 236 nm (e = 29,700), 274 nm (e = 13, 909) and 316 nm (e= 6,000).

Quantitation of Aflatoxins

1. Quantitate aflatoxins in a manner similar to zearalenone.
2. Scrape the fluorescent spot from the TLC plate and elute from silica gel with methanol.
3. Treat samples and standards in the same manner as described in Sec. 26.004-26.009 of Official Methods, A.O.A.C., 12th Edition.

Accuracy, Precision and Interferences

Recovery of standards added to samples of aflatoxin average 85%. Precision of measurements on the same sample are 14% RSD. Recovery of added T-2 toxin without carbon cleanup and diacetoxyscirpenol averages 85% $\pm$ 10% RSD. For other mycotoxins, the average recovery of standards is 85%. However, not enough

data are available to calculate good RSDs. Authentic standards of known purity are required to even attempt semiquantitative work. Ochratoxin, aflatoxin, zearalenone, zearalenol, rubratoxin, T-2 toxin, and diacetoxyscirpenol meet this criteria.

Sabouraud-dextrose Agar

Ingredients

Neopeptone 10 g

Dextrose 40 g

Agar 15 g

5 g Bact-Agar (to prevent dehydration during storage)

Directions:

1. Add 65 g of dehydrated SDA plus 5 g Bact-Agar to 1000 ml cold distilled water and heat to boiling to dissolve the medium completely.
2. Sterilize in autoclave 15 minutes at 15 psi (120 °C)
3. Dispense 12-15 mls into sterile 100 x 15 mm petri plates.
4. Store at 10 °C.

Statistical Analysis System Program

```
1.  Data Volatile;
2.  Infile Green;
3.  Input Days      Peak Ht3      Peak Ht4      Peak Ht5;
4.  Proc    Print;
5.  Proc    Corr;
6.  Proc    Plot;
7.          Plot   Peak Ht3*DAYS              Peak Ht4*DAYS
            Peak Ht5*DAYS;
8.  Proc    GLM;
9.          Model  Peak Ht3=DAYS      DAYS*DAYS;
10.         Output       OUT=NEW3          P=PREDICT3        R=RESID3;
11. Proc    GLM    DATA=NEW3;
12          Model  Peak Ht4=DAYS      DAYS*DAYS;
13          Output       OUT=NEW4          P=PREDICT4        R=RESID4;
14. Proc    Print  DATA=NEW4;
15. Proc    GLM    DATA=NEW4;
16.         Model  Peak Ht5=DAYS .    DAYS*DAYS;
17.         Output       OUT=NEW5          P=PREDICT5        R=RESID5;
18. Proc    Plot   DATA=NEW5;
19.         Plot   Peak Ht3*DAYS      PREDICT3*DAYS='P'/Overlay;
20.         Plot   RESID3*DAYS/UREF=0;
21. Proc    Plot
22.         Plot   PeakHt4*DAYS       PREDICT4*DAYS='P'/Overlay;
23.         Plot   RESID4*DAYS/UREF=0;
24. Proc    Plot
```

```
25.         Plot   PeakHt5*DAYS       PREDICT5*DAYS='P'/Overlay;
26          Plot   RESID5*DAYS/UREF=0;
```