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Investigation of analytical methods for scirpene toxins in food and feed

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by

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INTRODUCTION

The most common fungi are of the Fusarium, Penicillum and Aspergillus genera (Padgett, 1974). The Aspergillus genus is replete with toxic and carcinogenic species; especially famous is Aspergillus flavus which produces aflatoxins. Aflatoxins are absorbed and metabolized by the body as foreign compounds according to Chipley, et al. (1974). Since this process has been elucidated, a similar procedure could be inferred for T-2 toxin. Penicillum and Fusarium are lesser known genera but they also produce extremely toxic compounds. They are more commonly found in foodstuff in the north central area of the United States. Fusarium tricinctum and F. roseum are especially prevalent in foods in this area of the United States. High moisture and cold weather are conducive to maximum toxin production by these Fusarium species. Wet grain in the field or in storage is a suitable breeding site for the fungus and, consequently, a source of the mycotoxins produced by the fungi. These fungal toxins become significant factors in animal health in this country and human health in developing countries. The need to analyze for the specific active compounds which produce the mycotoxicosis is apparent. Development of these analyses is the object of this work.

LITERATURE REVIEW

Mold effects on mankind are still being elucidated. One characteristic of molds is the ability to produce mycotoxins, myco for mold and toxins for physical harm produced by the substances. Mycotoxicoses are undoubtedly as old or older than mankind.

Sakaki attempted to associate infected rice with "Kakke" disease in 1891. One of the first recognized cases of mycotoxicosis from cereal grains was characterized by Mujaki in 1909, according to Tsunoda (1970). The disease was associated with infected rice in Japan. The fungus was characterized as Gibberella saubinetu.

In the United States, Mayo (1891) demonstrated an association with Aspergillus glaucus in corn and enzootic cerebritis in horses.

The association of moldy sweet clover and hemorrhagic disease was demonstrated by Schofield (1924). An Aspergillus species was implicated.

In the U.S.A. and U.S.S.R. in the 1920's toxicosis from cereal grains were recognized (Ciegler, 1972). The disease which occurred in Russia during 1942-45 was the most extensive mycotoxin-caused disease ever reported. Symptoms and lesions varied with the length of exposure and amount of toxic food ingested. Millet, which had overwintered due to lack of labor, was the cause of the disease. Five to 85% mortality was observed in various districts. The effects of the disease were necrotic angina, extreme leucopenia, multiple hemorrhage and death.

In the last fifteen years, work in the mycotoxin area has greatly intensified. A key discovery was the source of "Turkey X Disease" by Blount (1961), which was associated with aflatoxin in feed. The aflatoxin was found to be in South American nut meal used in the feed. With the association of aflatoxin and acute disease, the field took on immediate economic significance. Long range effects of aflatoxin were elaborated over the next few years (Wogan, 1965). Thousands of papers each year are now produced on mycotoxins. The subject has been reviewed by numerous authors: Brook and White (1966), Christensen (1971), Armbecht (1970), Ciegler and Lillehoj (1972), Purchase (1971), Scott (1973), Wogan (1965 and 1969), Mateles and Wogan (1967), Ciegler, et al. (1971a and 1971b), Christensen and Mirocha (1974), and Hesseltine (1969).

Hesseltine (1969) provides the best overall summary of disease organism and chemical substance involvement of all the reviews cited. His fold-out has become a classic. It gives a tabular view of all the information available in 1969 of toxicity and causative agent. His review is international in scope and his grasp of the problem leaves little to be desired.

The various reviews cited deal with the distribution and prevalence of mycotoxin. Armbecht (1970) concentrates on animal effects and is strongly aflatoxin oriented. He does have data on effects on large animals and, hence, it is an important article for agriculture and food related work.

Wogan's articles (1965 and 1969) are food related and, hence, are

of interest. They are definitely aflatoxin oriented. Each review author tries to deal with distribution and general significance but concentrates on his particular area of strength.

The reference of Christensen and Mirocha (1974) is an attempt to cover the whole mycotoxin field. The authors discuss aflatoxin, Penicillum toxins and other mold toxins which are found in feed, and they feel are of significance for farm animals. The article is very oriented toward Fusarium species, which is not surprising since the authors have worked in this field so long.

Bamburg (1972) concentrates on trichothecenes and summarizes in a few pages his life's work with these compounds. He discusses analysis and biological effects.

Ciegler and Lillehoj (1972) make a very good summary of the mycotoxin field and try to provide a current perspective and to predict some areas of research which will (and did) become more fruitful. They also predict some residues in tissues from mycotoxins as yet unfound by workers in the field.

Hesseltine (1974) surveys the occurrence of mycotoxins in cereals. His survey is of naturally occurring mycotoxin in international cereal samples. The person who made the determination, the cereal and country of origin is given. Aflatoxin was found in corn, sorghum, rice, millet, barley, copra, wheat, oats, and bermuda grass, and ochratoxin in corn and barley in the United States. Other mycotoxins reported in cereals were Citrinin, Sterigmatocysten, Tenuazonic Acid, Patulin, Penicillic Acid, T-2 toxin, Kojic Acid, Rugulosin and Griseafulvin.

Hesseltine, et al. (1975) surveyed the incidence of aflatoxin in white corn stored in Missouri. They found a substantial amount contained aflatoxin.

Lillehoj, et al. (1975) were able to produce aflatoxin in the field by inoculating growing ears with A. flavus. This established that incidence of aflatoxin could be due to production of toxin during the growing of the corn. It had been assumed that mycotoxin production occurred in storage and therefore, it was strictly a warehousing problem.

The specific diseases associated with mycotoxin exposure are also given in Lillehoj's article.

Stohloff (1972) listed the various committees which deal with methods of analysis for official purposes and how they are coordinated. He presents the evolution of mycotoxin analytical methods from the extraction of a "toxic principle" with chloroform to our present-day knowledge. The FDA philosophy has been to develop individual methods for each mycotoxin. Extraction, cleanup, thin layer chromatography and column chromatography are all described, as well as the various means of quantitation. Polarography, spectrometry, fluorometry and derivatized gas chromatography are presented. Methods for confirmation are also discussed. Colorimetric derivates are presented for aflatoxins, sterigmatocystin, patulin, zearalenone, alternaria toxins, trichothecenes and ochratoxins.

Stohloff proposes mass spectrometry as a preferred method for

confirmation. His concern with suitable standards for analysis is shared by all analysts.

A class of mycotoxins called fusariotoxins are produced by the genus Fusarium. This genus has been characterized according to morphology by a series of workers described below. The class, Fungi Imperfecti, to which Fusarium belongs, is a portion of the Eumycetes or true fungi.

The first Fusarium taxonomy was attempted by Funk in 1809, according to Snyder and Toulson (1965). H. W. Wollenweber and O. A. Reinking (1935) used their system involving the length of the macro conidia spores in 1935 to classify the Fusarium. Snyder and Toulson (1965) proposed the present system. Presently, the Fusarium genus is broken down and grouped into the species by macro and micro conidia size, and morphology of the mycelium and ascospores. The systems of classifications are compared by Tsunoda (1970). There are now fewer species and more varieties of Fusarium (Snyder and Hansen system). This group of fungi tend to mutate quite rapidly, making them difficult to identify.

Characterization by visual acuity leaves something to be desired. However, there does appear to be an association between certain species and the production of certain specific compounds, at least in the laboratory. The diseases thought to be caused by Fusarium toxins are different depending upon the circumstance and condition of growth (Tsunoda, 1970).

Apparently, temperature, moisture and substrate play an important part in the efficiency of production of mycotoxins. Edmunsen (1972) reported a greater production of toxin at low temperatures and in darkness. Bamburg and Strong (1971) also recount similar observations. Eight degrees centigrade is given as the optimum condition for production of T-2 toxin by F. tricinctum.

Fusariotoxins presented by Bamburg and Strong (1971) have been characterized as (a) the 12, 13 epoxytrichothecenes of which T-2 toxin is one and there are 21 others reported in this group. They are elaborated by F. tricinctum and possibly F. nivale, (b) the estrogenic toxins, of which zearalenone is representative, are elaborated by F. roseum graminarium, (c) alkaloidal toxins, Festucine, similar to ergot toxins, and (d) butenolide. Both (c and d) compounds are thought to be elaborated by Fusarium genus. There are undoubtedly many others not yet characterized.

The naming of T-2 toxin was derived from its isolation from F. tricinctum strain T-2 by Bamburg, et al. (1968). Its total characterization by instrumental analysis and synthesis was carried out by Bamburg, and is described by Bamburg and Strong (1971). Proof of structure by mass spectroscopy, nuclear magnetic resonance, infra red analysis and a synthetic degradative study have been done. Nuclear magnetic resonance spectra of 18 of the toxins are given by Bamburg and Strong (1971). Mass spectra of 12 are also given. A summary of the work done over the course of 20 to 30 years in establishing absolute configuration and molecular structure is also given in this article. The trichothecenes

were described as colorless, crystalline, optically active compounds. They were said to be stable under normal laboratory conditions of storage, but may be degraded by heating, oxidation or hydrolysis reactions.

The trichothecenes are sesquiterpenes. Tables 1, 2 and 3 show the structures of these compounds. They have six bridgehead carbons and are named for the compound trichothecene which was the first isolated compound of the series. All of the approximately twenty compounds now recognized contain an olefinic bond and an epoxy group, hence, the name, 12, 13 epoxy trichothecenes is used to describe them.

The "fescue toxin," butenolide, is described by Yates (1971). Butenolide is 4-acetamide-4-hydroxy-2-butenoic acid-lactone. It is associated with T-2 toxin in F. tricinctum cultures and can cause similar clinical signs. In addition, it has been shown to cause "fescue foot" and sloughing of the extremities in livestock.

Festucine is an alkaloid similar to ergot amines and produces a clinically similar disease to ergotism.

Zearalenone and the estrogenic toxins (at least five are known) are well studied. Urry, et al. (1966) elucidated the structure of zearalenone. Mirocha, et al. (1967) studied the effect of zearalenone on livestock and isolated five compounds called F-1 to F-5 estrogenic compounds. McNutt, et al. (1928) and McErlean (1952) characterized vulvovaginitis in swine. More recently, Kurtz, et al. (1969) have tried to histologically characterize changes in the urogenital tract from zearalenone exposure.

This is probably the most common mycotoxin disease observed in

the north central region of the country. Stahr, et al. (1975) reported screening tests which associated zearalenone and field cases. Fortunately, the acute toxicity of zearalenone is not great (Mirocha, et al. 1967) or more diseases would be reported.

Clinical signs of the disease which have been associated with Fusarium toxins are headache, nausea, vomiting, necrotic angina, leucopenia, multiple hemorrhage, sloughing of hooves and extremities, abortion, vulvovaginitis, esterogenism and deaths, (Ciegler, et al. 1971a and 1971b).

Hemorrhage produced by T-2 toxin is described by Bamburg and Strong (1971). Usually non-specific bleeding is observed with mucosal irritation and hemorrhage. The effects of T-2 toxin on poultry are described by Wyatt, et al. (1972, 1973) and Hamilton (1974). They are generally oral lesions, feed refusal or reduction in consumption, slow growth, and cardiac and renal necrosis in addition to lesions cited above.

We became concerned when field cases were observed of massive hemorrhage in livestock at the Veterinary Diagnostic Laboratory. Feed from field cases was found to contain a number of mycotoxins; among them, T-2 toxin and similar compounds (Buck, 1970). Methods of analysis for these compounds in complex matrices were required to help solve field cases.

Human food may also be a source of fusariotoxins as described by Joffe (1965). Bullerman and Ayres (1968) studied the potential for aflatoxin production from fungi isolated from cured meats. They

established the potential of aflatoxin production if these molds grew under conditions of storage or use. This would seem a very likely place for other molds and mycotoxins too, since similar conditions encourage mold growth.

As in the case of the earlier mentioned article by Hesseltine (1974) we are concerned with the distribution of mycotoxins in human foods and potential foods. Ayres, et al. (1970) studied the determination of mycotoxins in foods. The most likely contaminants were surveyed and methods for their analyses are discussed. Other food related research was done by Bullerman and Olivigini (1974) who found Penicillium to be the most frequent toxin producer on cheese stored at 5° - but they also isolated Fusarium, Alternaria and Aspergillus. Armbecht (1970) studied the depth of penetration into cheeses and found the toxin beyond visible mycelium development.

Bullerman (1974) also studied the effects of cinnamon on Aspergillus toxin production. He found that 0.2 to 2% cinnamon retarded Aspergillus growth and toxin production. He speculated that spices may serve other purposes besides seasoning in food. The production of penicillic acid by Penicillium spices in moldy popcorn was also studied by Bullerman (1975). This demonstrates common fungi grow and produce mycotoxins in human food too.

Fusarium molds were found to be ubiquitous. In a study by Padgett (1974) in which panels of cloth were exposed to the atmosphere in different locations, differences were observed. The city (polluted atmosphere) produced less spore fallout than the country side exposures. Of

course, there is less vegetation in the city which could account for differences in the recovery.

Fusarium species are common soil fungi and prominent crop parasites. In one study of Iowa corn samples (Richard, 1968), two hundred and forty-six fungi were isolated, of which seven were Fusaria. When cultured on rice, three species produced toxins which killed ducklings and one was fatal to mice.

In a survey of eastern Canadian field crops, nearly 80% of 3700 seed samples were positive for Fusarium (Gordon, 1952). Only 39% were positive from western Canada.

Bamburg, et al. (1968) have demonstrated that ethyl-acetate will extract trichothecenes and 3/2/1 toluene/ethyl acetate/acetone, respectively, will separate them on thin layer chromatograms. Other solvents and conditions have been detailed (Bamburg and Strong, 1971). Stohloff (1972) has described other solvent systems which can also be used. Ikediobi, et al. (1971) have shown that gas chromatography could be used to analyze for scirpene compounds by making the silyl ether derivatives of the hydroxy groups.

Chicken embryo testing for aflatoxin was demonstrated by Verett (1964), using 4-day old chicken eggs. The aflatoxin was injected in the air sac and yolk in separate exposures. The lethal dose of fifty percent of the eggs (LD50) was 0.1 ug per embryo. The test has been collaboratively studied and has become part of the official methods of the Association of Official Analytical Chemists (A.O.A.C.).

Extracts of food containing fungi which produce toxins were

reported by Diener, et al. (1975) to cause chicken embryo deaths. Stahr, et al. (1975) described the effectiveness of T-2 and thin layer bands from suspected feeds in chicken embryo studies. Bamburg and Strong (1971) described phytotoxicity, insect assays, and a rat skin test for trichothecenes. Instrumental analysis by NMR and Mass Spectroscopy were also used by Stohloff (1972) and Bamburg and Strong (1971) to help identify the Fusariotoxins. Mass Spectrometry was used for mycotoxin identification by Stahr, Henderson, and Junk and was reported in the symposium, edited by Buck (1970).

The importance of mass spectra to confirm the presence of low levels of toxic substances is generally recognized. Stohloff (1972) cited mass spectroscopy as the only way to independently establish the identity and level of toxins. It will not, of course, detect configuration isomers but the molecular formula and major fragments are invaluable in analyzing for an unknown toxin. Numerous substances have been analyzed by this technique at submilligram levels. Biros (1971) reported work with pesticides at submicrogram levels. Mirocha (1974) differentiated a non-toxic substance from a field case with his system.

Interpretation of spectra when thousands of peaks are produced per mass spectral analysis is a very significant problem. This process usually requires more time than the collection process itself. For this reason use of computers has been so widely adopted. Heller (1972) developed a system and implemented it with the aid of the National Institutes of Health. It is now available through commercial channels (General Electric Co., 1974). In this system, an interaction search is made of stored

mass spectral data. The operator sits at a teletype or other data logging device and interacts with the computer through this telecommunication system. The operator gives the computer the highest mass number per each 14 units of mass and the computer "sorts" through 40,000 compounds to select compounds with a common mass spectral fragmentation. Pohland and Sphon (1974)[1] have collected mass spectral data on mycotoxins and have made a data bank available for workers. These spectra have been accumulated from many sources. They have spectra for certain Aspergillus and Penicillium toxins well catalogued. Zearalenone, zearalenol, T-2 toxin and diacetoxyscirpenol are the only Fusarium toxins which have been completed by these workers.

Mirocha, et al. (1975) make a computer search system available at the University of Minnesota. The commercial mass spectral search system (General Electric Co., 1974) based on Heller's work is available and will soon contain the mass spectral data of mycotoxins.

Another approach in solving this information explosion is the use of selective ionization techniques. These methods are chemical ionization, field desorption, ultraviolet or non-electron impact ionization techniques (Fales and Damico, 1972). The field desorption technique is especially attractive for large molecules.

Schulten and Beckey (1972) in Germany and Rinehart and Cook (1974) in the U.S. have published work using a field desorption source to greatly simplify mass spectra. This technique produces a larger

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[1]A. Pohland and J. A. Sphon, F.D.A., Washington, D. C. Private Communication. 1974. Mycotoxin Mass Spectral Data Bank.

concentration of the molecular ion from a compound and other simple fragments. This field desorption system consists of a carbon emitter specially grown and constructed to allow very small quantities of substance to be placed on the expanded surface for introduction into the source. This process is described by Schulten and Beckey (1972). The carbon surface is grown on tungsten wire emitter. The carbon comes from the decomposition of hydrocarbon gases on the wire itself at elevated temperatures. The emitters and sources are available commercially from Varian Instruments; however, they are quite expensive. The carbon surface appears to be constructed of needle-like projections and extremely low currents cause the production of ions from nanogram quantities of sample. This is felt to be the reason that simple fragments are obtained in the mass spectrum with the singly charged molecular ion usually predominating.

Large currents do not tear the molecule apart nor do high concentrations of other molecules result in molecular changes in the vapor produced.

Compounds such as complex toxins and antibiotics have been successfully analyzed by this technique. Sodium acetate and guanine are described by Schulten (1972) and adducts with desoxyribonucleic acids are also listed. Rinehart and Cook (1974) describe analysis of antibiotics such as neomycin B (615 molecular ion) and novabiocin (612 molecular ion). These workers demonstrate the analysis of more than one compound in the presence of others in simple mixtures. Other workers have applied this technique to sequencing amino acids in protein and the direct analysis

of complex toxic protein substances (Schulten, 1974). The source has limitations in that quite often sodium or potassium atoms are "added on" to molecular ions by improper cleaning of the sample or substrate.

Fishback (1973) has detailed the Food and Drug Administration's policy on food contamination and levels of concern. Only aflatoxin has a specified level for action by FDA (20 ppb). The levels of other mycotoxins to be considered harmful by FDA await long-term toxicity tests.

The food residue problem from livestock eating mycotoxins and becoming a source for human foods is still a source of conjecture and experimentation.

Cysewki, et al. (1968) have shown that aflatoxin is primarily eliminated in urine of pigs fed the substance. Armbecht (1970) cites studies in which ducklings were killed by eating livers from other ducklings which had been exposed to toxic doses of aflatoxin. Krogh (1970) has shown that 1/100 of the dose level in feed is the common level to anticipate in milk in feeding studies with cows. Armbecht (1970) also states that no detectable aflatoxin was found in meat from swine which were killed with toxic doses of aflatoxin. Of course, the methods used were those which were designed to detect unreacted parent compound, not tissue reacted species produced from that compound.

Shanks (1972) has reported the rapid elimination of C^{14} labeled aflatoxin from rabbits and suggested no residue problem was expected from aflatoxins. However, Ciegler (1972) has predicted and Chipley, et al. (1974) have found that the aflatoxin in tissues is bound and not available to the usual techniques of extraction used for foodstuffs.

Aflatoxin is said to occur as a glucouronide and/or a protein adduct in tissue. Chipley's method uses B-glucouronidases to cleave the chemical bonds tying up the toxin and B-glucouronide allowing the compound to be released as a non-polar extractable entity.

Krieger, et al. (1975) studied the hydroxylation of aflatoxin B_1 by a microsomal preparation from livers of Rhesus monkeys, and established that aflatoxin is reacted in the liver in the same way other lipid materials are handled by the natural hepatic systems in general detoxification. After reaction the hydroxylated glucouronide or sulfate may be eliminated by the body by the same processes which allow natural lipid molecules used by the body to be eliminated.

How scirpenes and other toxins are handled by the physiological systems is not fully elucidated. The non-persistence of T-2 toxin in animals' digestive systems indicates a favorable elimination of harmful scirpenes. Bamburg and Strong (1971) have cited Kosuri's (1969) work showing increased glucouronides in T-2 feeding studies as evidence for similar adduct formation to that of aflatoxin by T-2 and other scirpenes.

Wallace and Mirocha (1975)[1] have synthesized radioactive T-2 toxin and began feeding studies to determine the distribution and residue from T-2 toxin ingestion. They reported very little accumulation in the animals. Their completed work is not yet available. Bamburg and

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[1] E. D. Wallace and C. J. Mirocha, University of Minnesota. Private Communication. 1975.

Strong (1971) reported less than 5% of the T-2 toxin was absorbed.

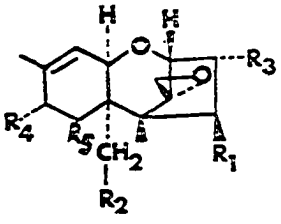
Some of the outstanding needs for further work in this area are presented by Bamburg and Strong (1971). They believe there is a need for rapid screening methods and specific confirmatory techniques for the processing of samples to allow a broader view of their occurrence. This will be necessary if correlations are to be made of the occurrence in foods and feeds and human and animal disease. Another need is for safe disposal of contaminated grains. There is, at present, no well-developed process to rid the owner of Fusarium damaged commodities of his liability. The hydrolysis products, tetraols, are about one-half as toxic as the parent compounds, scirpene esters (Bamburg and Strong, 1971). Further chemical reaction by oxidation should make detoxification possible. The U. S. Department of Agriculture has a process for detoxification of produce containing aflatoxin, which uses ammoniation (Stanley, 1975) and produces innocuous products from relatively labile aflatoxin contaminated products. The scirpenes, after hydrolysis, would be more susceptible to the hypochlorite reaction process described by Natara, et al. (1975), which should oxidize the tetraols to relatively innocuous products.

These are but a few selected articles which bear directly on mycotoxins in foods and feeds and our ability to analyze for them. In one year, 1973, the writer was privileged to receive the Food and Drug Administration's annual compilation of references. Over 1000 references were listed.

TABLE 1

Structures of Scirpene Compounds

Structure I

Compound Name	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[a]	OH	OAC	OAC	OH	H

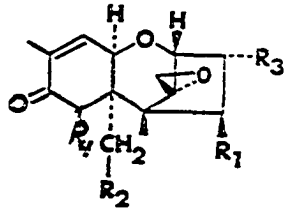
OAC = Acetate OIV = Isovaleryl

[a]Y. Ueno (1970). Bamburg and Strong (1971).

TABLE 2

Structures of Scirpene Compounds

Structure II

Compound		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

Structure III

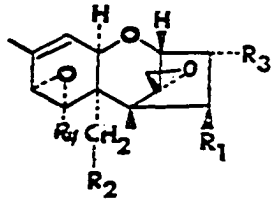
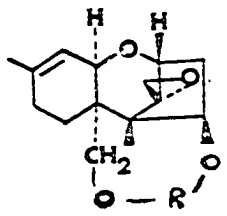
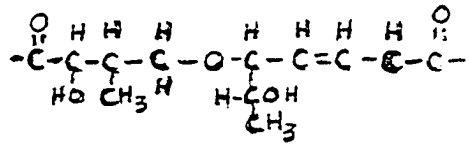
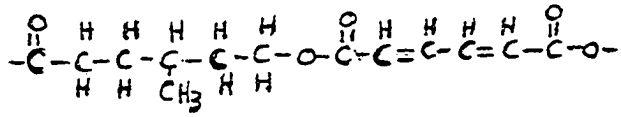
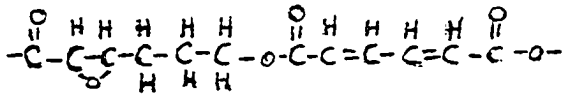
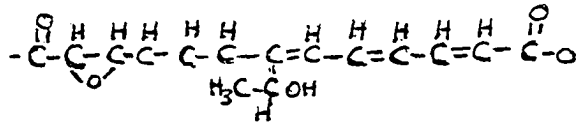
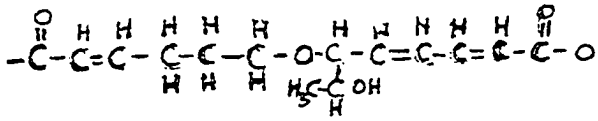
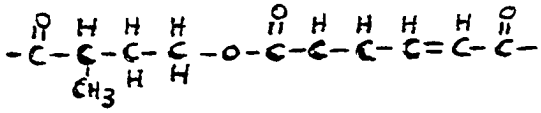
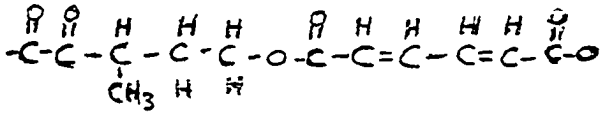
Compound		Substituents			
		R_1	R_2	R_3	R_4
Crotocol		OH	H	H	--
Crotocin		O-iso-crotonyl	H	H	--

TABLE 3

Structures of Scirpene Compounds

Structure IV

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

EXPERIMENTAL PROCEDURES

Methods of Biological Testing

Chicken embryo tests were run according to the A.O.A.C. (12th Ed.) Methods of Analysis, paragraph 26.073, based on Verett's (1964) work. Toxic materials were dissolved in ethanol (95% - 5% water) and 20 ul or less of the solution were placed in the air sac of the chicken egg. The hole was then sealed with Scotch(R) tape. It was necessary to use 4-day old eggs to be certain the embryos were viable before administering the toxic materials. A modified commercial incubator belonging to the Genetics Department, Iowa State University (I.S.U.) was used. The incubator automatically turned the eggs and was moisture controlled to help assure hatchability. Controls gave better than 95% hatchability in this system. Leghorn eggs were obtained from Veterinary Medical Research Institute (V.M.R.I.), I.S.U. One group of ducklings was obtained from Owings Hatchery, Storm Lake, Iowa. Another group of ducklings was obtained from Ridgeway Hatchery, Inc., LaRue, Ohio. Fish were obtained from the Department of Animal Ecology, I.S.U. They were six blue gills and catfish. Field harvested blue gills were 4 inches long and catfish were 6 inches long.

Chemicals and Glassware Used

All chemicals were of analytical reagent grade, if available; if not available, "nano-grade"(R) (Mallinkrodt) or "Spectro"(R) (Fisher) were used. Glassware was of a type used for pesticide analyses (Kontes Glass Co., Vineland, N. J.). Nitrogen from Chemistry Stores, I.S.U.,

was used to concentrate extracts. High purity nitrogen from Matheson Chemical Co., Joliet, Illinois, was used for the carrier gas for gas chromatography. Aflatoxins and diacetoxyscirpenol were obtained from Calbiochemical Co. T-2 toxin was supplied by Dr. Harlan Burmeister, N.R.R.L., U.S.D.A., Peoria, Illinois. Zearalenone was obtained from Commercial Solvents Corporation, Terra Haute, Indiana. The Fusarium compounds fusarenon x and neosolaniol were obtained from Professor Y. Ueno, Microbial Chemistry, Faculty of Pharmaceutical Science, Science University of Tokyo, Tokyo, Japan. Trichothecin, verrucarol and verrucarin were obtained from Professor C. Tamm, Institut. fur Organische Chemie, Der Universitat Basel, St. Johanns - Ring 19, 400 Basel, Switzerland. Roridan C, diacetylcalonectrin and calonectrin were obtained from Dr. W. B. Turner, Imperial Chemical Industries, Lmtd., Pharmaceutical Division, Mereside Alderly Park, Macclesfield, Cheshire SKP4TG, Great Britain.

All glassware was cleaned by soaking in chromic acid cleaning solution and rinsing until free of acid solution. An alternate procedure with annealing at 500° C was used successfully.

Gas Chromatography and Other Instrumental Techniques

GLC analyses were done on a Bendix Biomedical Gas Chromatograph Model 6000. Columns were made by the I.S.U. glass shop. Commercial supports and liquid phases were obtained from Applied Science Laboratories, State College, Pennsylvania. Thin layer chromatography was done on Silica gel "G" plates made from Silica gel "G" obtained from Brinkmann Instruments, Waterbury, New York. Disposable pipettes for spotting

samples were obtained from Burroughs Corporation, Research Triangle, North Caroline.

For field desorption studies, a Varian 709 mass spectrometer was used. This equipment was located in the Mass Spectroscopy Laboratory at the University of Illinois, Champaign, Illinois, under the direction of Dr. Carter Cooke.

Mass spectrometry of extracts and standards was done by the solid inlet probe with the MS 902 Atlas High Resolution Mass Spectrometer. The sample inlet tubes were made from pyrex capillary tubes which were annealed to clean them. This allowed disposable sample holders to be used and to reduce contamination between samples. A Packard Scintillation Spectrometer Tricarb Model 3375 was used for tritium counting. Packard Permaflor scintillation fluid was used as a diluent for scintillation counting. Nuclear Magnetic Resonance (NMR) Spectra were produced of the compounds by the Chemical Instrument Services group using the Varian High Resolution NMR Spectrometer. Commercial gas chromatography/mass spectrometry determinations were made by Schrader Analytical Laboratories, Detroit, Michigan.

RESULTS AND DISCUSSION

Development of Methodology

Attempts to recover added amounts of T-2 toxin from mixed feed samples and tissues, using the published procedure of Ikediobi, et al. (1971), were unsatisfactory. Their procedure was developed for use with cereal grains. Some of the problems encountered in addition to recoveries are as follows: (a) The time to analyze a sample was long (2 days). (b) Vacuum concentrators are necessary to concentrate ethylacetate. They are expensive and slow. (c) Silica columns are slow and require frequent standardization for use as cleanup tools. (d) Trisilyl ether derivative techniques produce artifact GLC peaks from feed components. Derivative solutions must have an internal standard to correct for the completeness of conversion of the compound to the ether. Silyl ether derivatives cause silicon dioxide to coat the flame detector and cause the detector to lose sensitivity. The detector must be cleaned and few samples can be analyzed at one time. (e) Linear programming does not lend itself to quantitation because of the lack of reproducible temperature effects and column bleed.

To attempt to correct these limitations, the following procedural steps were developed. Acetonitrile/water extraction of the samples allows defatting and decolorization of the extracts, producing a much cleaner extract. The T-2 toxin is partitioned into chloroform which allows much easier concentration of the extract. The complete procedure is given under "T-2 toxin procedure". Finally, high temperature (250-270°C)

GLC was investigated to overcome the need for silyl ether preparation and temperature programming.

T-2 toxin procedure

A procedure for the analysis of T-2 toxin and five other mycotoxins was developed and reported at the 1974 A.O.A.C. meeting, Washington, D. C. It is given in the Appendix. It was applied to feeds and commodities from the field. Recoveries of added standards of aflatoxin and zearalenone were made and results reported at the meeting. The text of the method is described here.

The method is given in stepwise fashion as follows:

1. Acetonitrile extraction of feed by blending
2. Defatting with petroleum ether
3. Decolorization with ferric gel (Velasco, 1974)
4. Partition into chloroform
5. Evaporation under nitrogen
6. Redissolution in a fixed volume (300 ul) of the 98/2 benzene acetonitrile
7. A thin layer chromatography step with 3/2/1 toluene, ethyl acetone or 90/20/2, Xylene, isopentyl alcohol, methanol or other appropriate solvent
8. Quantitation by fluorescence for aflatoxin, sterigmatocystin ochratoxin and zearalenone and by fluorescence quenching for rubratoxin.

To quantitate T-2 toxin, gas liquid chromatography (GLC) can be used without derivatization. T-2 toxin gives a peak at 14 minutes

retention time at 280° on a 3% Dexsil 300 (3% OV-1 gas chrom Q) column. The column was 1/4 inch in diameter and 6 feet long. Injector temperature was 250° C and a carrier gas flow was 80 ml/minute. Sensitivity was in the order of 0.1 ug for T-2 toxin and diacetoxyscirpenol using the flame detector on the Bendix model 6000 GLC. Retention times for the scirpene toxins are shown in Table 4. Gas chromatograms are shown in Figures 1 and 2. A spectrum from a mass spectrometer verified that this was T-2 toxin. It is shown in Table 5. Also, 3% OV-1 on gas chrom Q was found to give sharper peaks and greater sensitivity than 3% Dexsil 300 and a 10-minute retention time for T-2 toxin at 250° C. Other scirpene toxins were obtained from sources listed in experimental section. They were chromatographed and are shown in the chart for scirpene retention times (Table 4).

It was found to be necessary to add the ferric gel step to decolorize the extracts. Attempts to eliminate the gel caused a band to obscure T-2 toxin on TLC plates and to reduce the sensitivity of GLC because a matrix peak eluted very closely to that of T-2 toxin.

This method, which worked for feed, was applied to tissue, but only 10-20% of the added T-2 toxin could be recovered as evidenced by the GLC analysis. Subsequently, a procedure based on the A.O.A.C. method for Vitamin A assay (1975) with boiling concentrated NaOH was used. An 85% recovery was obtained without liver but in the presence of liver only 20% recoveries were obtained. A study to optimize T-2 toxin standard and tissue weight was then performed. The results are shown in Table 6.

TABLE 4

Retention Times of Some Trichothecenes[a]

Compound	Retention Time[b]
Roridan C	0.11
Verrucarol[c]	0.20
Diacetylcalonectrin	0.25
Calonectrin	0.27
Verrucarin[d]	0.35
Tricothecin	0.37
Diacetoxyscirpenol	0.40
Neosolaniol	0.67
T-2 Toxin	1.00
Under the above conditions Roridan A and Fusarenon-X did not chromatograph.	

[a]Underivatized standards: GLC, 6 ft., 1/4" O.D. glass column packed with 3% Dexsil 300, column temperature 270°C, flor 50 ml/min N_2, inlet 270°C.

[b]Relative to T-2 toxin retention time of 23 minutes.

[c]Hydrolysis product of Roridan E.

[d]Due to improper labeling by the supplier of this toxin, the authors do not know which verrucarin it is.

FIGURE 1

DIRECT GLC OF SCIRPENES

DIRECT GLC OF SCIRPENES

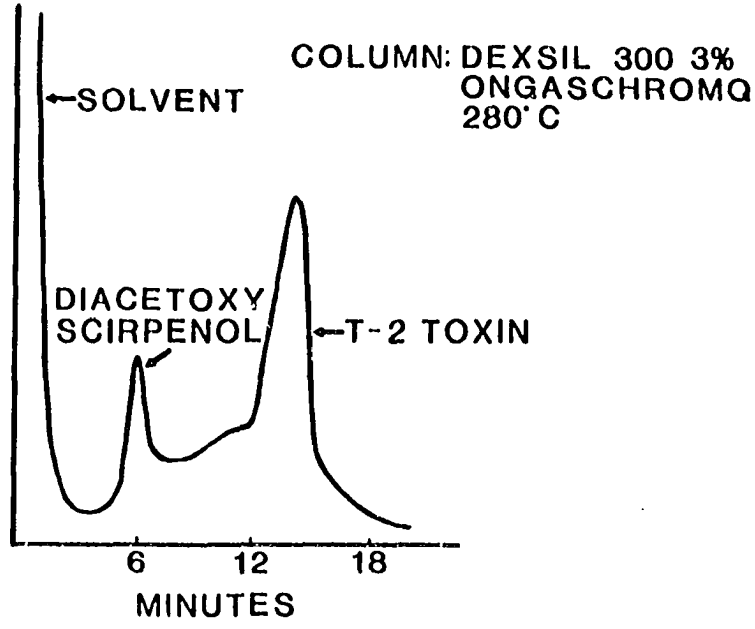


FIGURE 1

FIGURE 2

DIRECT GLC OF SCIRPENES

DIRECT GLC OF SCIRPENES

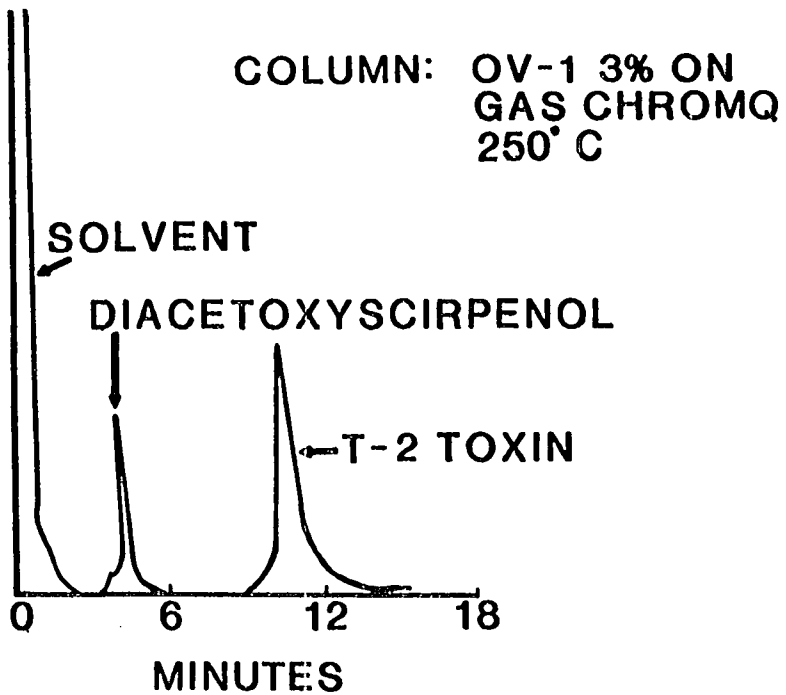


FIGURE 2

TABLE 5

Mass Spectral Data for T-2 Toxin GLC Peak[a]

GLC Peak (Atomic Mass Units) (AMU)	Solid Probe (Atomic Mass Units) (AMU)
43	43
60	60
85	85
121	121
180	180
364	364
382	382
407	407

407 = T-2 Toxin (466 Amu) minus Acetate (59 Amu)

364 = T-2 Toxin (466 Amu) minus Isovalerate (102 Amu)

[a]GC/MS Analysis by Schrader Laboratories (20 eV. Electron Ionization).

TABLE 6

Recovery of T-2 Toxin from Liver Tissue

Weight of Tissue, (gms.)	T-2 Toxin Added, (ug.)	Recovery of T-2 Toxin (ug.)
5	5	4.8
10	10	7.0
50	50	10.0
100	100	18.0

Tritiation of T-2 toxin and radioactive recoveries

An attempt to synthesize radioactive T-2 toxin was made to validate the tissue method. Twelve mg of T-2 toxin were dissolved in benzene and placed in contact with metallic sodium on a steam bath in a sealed, glass stoppered container. The sample was allowed to contact sodium overnight. The sodium was removed and the solution was then exposed to 200 microliters of tritiated water (2 millicuries of activity). This was allowed to remain in contact overnight. The mixture was acidified (4 ml 2N HCl) and the extract was extracted with chloroform (30 ml total volume) three times. A 100 microliter aliquot was spotted on TLC (thin layer chromatogram Silica Gel G), developed in a 3/2/1 (toluene/ethyl acetate/acetone) solvent system. The band was measured and GLC analysis was made. Results are reported below as data for Trial 1 H^3 T-2 toxin synthesis.

An isotope exchange synthesis was next attempted. Twelve mg of T-2 toxin were dissolved in acetonitrile and allowed to contact 400 ul tritiated water (4 millicuries) for four weeks. Samples were removed and checked at 2 and 14 days and at 4 weeks. Fifty ul each time were removed, dissolved in 100 ul acetonitrile/water (90/10) and partitioned according to the analytical procedure given above. The T-2 toxin band was visualized as described above, and eluted from the gel with 10 ml methanol. The filtered solvent was placed in a scintillation vial and concentrated to near dryness. A 20 ml portion of scintillation fluid was added. A similar preparation from the TLC plate above the T-2 toxin band served as a control. The samples were counted (2 times, 10 minute inter-

vals) and the results are shown for isotope exchange tritiation of T-2 toxin. Recoveries of radio-labeled T-2 toxin were then done with liver samples.

Hydrolysis labeling of T-2 toxin

No GLC peak was observed for T-2 toxin in the concentrated extract of the hydrolysis solution. A radioactivity level of 27 cpm was obtained at the band height of T-2 toxin on the TLC plate. The plate was of silica gel G and was developed in 3/2/1 toluene/ethyl acetate/acetone solvent. An activity level of 2180 cpm was obtained at the origin on the TLC plate. Repeating the analysis with silica gel TLC plates but developing the plates in 4/1 chloroform/methanol solvent, a level of 2180 cpm was observed at an Rf of 0.6. T-2 toxin standard gave an Rf of 0.9 in this solvent. No GLC peak was observed for the extract. The TLC band matched the band obtained for T-2 tetraol made according to the procedure reported by Bamburg and Strong (1971).

Isotope exchange labeling of T-2 toxin

T-2 toxin and tritiated water (2 millicuries) were dissolved in acetonitrile. The radioactivity (RA) increase in the T-2 toxin was followed by partitioning into chloroform and counting the radioactivity of the band the same Rf as T-2 toxin. Silica gel G and 3/2/1 toluene/ethyl acetate/acetone were used. The following results were obtained:

Time of Exposure (days)	TLC Band Activity (cpm)
2	50
14	400
28	3000

The remaining T-2 toxin was partitioned into chloroform and concentrated to dryness. It was redissolved in methanol for analysis by GLC and tissue amending studies. The radioactivity of the T-2 toxin was 1.5 cpm/ug.

Recovery of labelled T-2 toxin

The radioactive T-2 toxin was added at 100 ug per 5 g of liver. One hundred and fifty cpm per sample was obtained in this manner. The same samples were analyzed by GLC.

T-2 toxin recovery by RA counting 90 ± 10%

T-2 toxin recovery by GLC counting 100 ± 5%

Instrumental Confirmation

Nuclear magnetic resonance spectra

Nuclear magnetic resonance spectra were obtained for 12 scirpene compounds. Two are most alike chemically, Neosolaniol and T-2 toxin. They are shown in Figures 3 and 4 and data for the analysis is given in Tables 7 and 8. They differ in only one group, isovalerate instead of an acetate group, as shown in Table 1. Tables 2 and 3 also are illustrative of structural formulas of other groups of scirpene toxins.

FIGURE 3

NUCLEAR MAGNETIC RESONANCE
SPECTRUM OF T-2 TOXIN

NUCLEAR MAGNETIC RESONANCE
SPECTRUM OF T-2 TOXIN

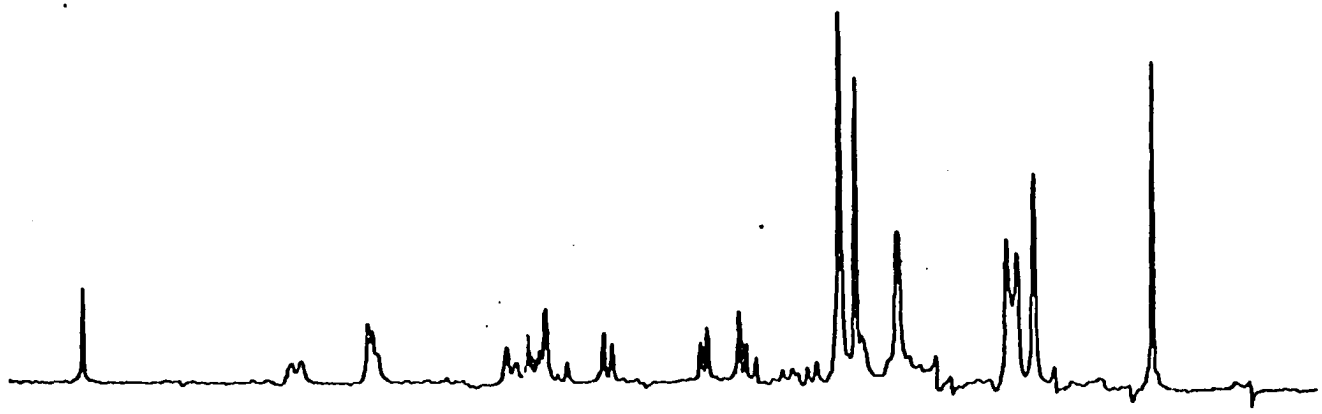


FIGURE 3

FIGURE 4

NUCLEAR MAGNETIC RESONANCE
SPECTRUM OF NEOSOLANIOL

NUCLEAR MAGNETIC RESONANCE
SPECTRUM OF NEOSOLANIOL

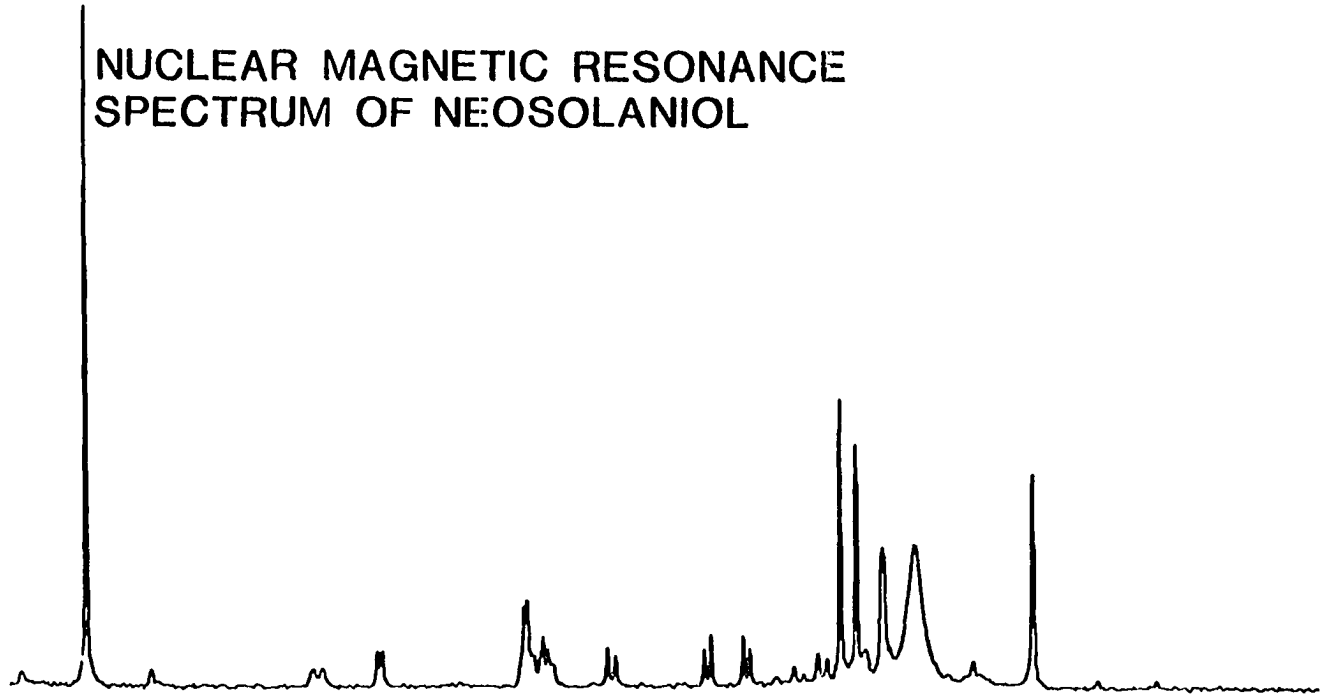


FIGURE 4

TABLE 7

NMR Data for T-2 Toxin

T-2 Toxin in CDCL3 270CT75
P(180) = 0.100 USEC
P(90) = 3.500 USEC

TAU Values
Recovery Time = 3.999 Sec
P3 = 0.100 USEC
D3 = 0.001 Sec
P4 = 0.100 USEC
D4 0.001 Sec
Total Scans = 64

No. of Freq. Domain Points = 2048
SW = 1201.923
DW = 416.000
DE = 416.000
SO = 176.060
TC = 0.000
SF = 90.000
TA = 0.000
TB = 0.000
T1 = 0
T2 = 0
NC = 1

&PP
T-2 Toxin in CDCL3 270CT75

No.	Cursor	Freq.	ppm	Intens.	Area
1	634	653.780	7.264	11676	21740
2	931	479.478	5.327	7544	35210
3	936	476.543	5.294	6708	40318
4	1098	381.469	4.238	6320	38670
5	1117	370.319	4.114	9522	65036
6	1177	335.106	3.723	6470	25518
7	1185	330.411	3.671	5292	16820
8	1275	277.592	3.084	5552	20060
9	1281	274.071	3.045	7268	25160
10	1315	254.117	2.823	9238	44140
11	1322	250.009	2.777	5438	16948
12	1419	193.082	2.145	46160	229060
13	1436	183.105	2.034	38126	175598
14	1480	157.282	1.747	19140	214988

TABLE 7 (cont'd)

No.	Cursor	Freq.	ppm	Intens.	Area
15	1595	89.792	0.997	18308	119698
16	1606	83.336	0.925	16736	81946
17	1624	72.772	0.808	26370	110798
18	1748	0.000	0.000	40286	97332

&F1 = 700 &F2 = -100

TABLE 8

NMR Data for Neosolaniol

Neosolaniol in CDCL3 270CT75
P(180) = 0.100 USEC
P(90) = 3.500 USEC

TAU Values
Recovery Time = 3.999 Sec
P3 = 0.100 USEC
D3 = 0.001 SEC
P4 = 0.100 USEC
D4 = 0.001 SEC
Total Scans = 512

No. of Freq Domain Points = 2048
SW = 1201.923
DW = 416.000
DE = 416.000
SO = 176.649
TC = 0.000
SF = 90.000
TA = 0.000
TB = 0.000
T1 = 0
T2 = 0
NC = 4

&PP
Neosolaniol in CDCL3 270CT75

No.	Cursor	Freq.	PPM	Intens.	Area
1	634	653.193	7.257	337680	780176
2	702	613.285	6.814	8192	21984
3	871	514.103	5.712	8944	39744
4	881	508.235	5.647	9152	47376
5	939	474.196	5.268	18176	64224
6	944	471.261	5.236	18336	47280
7	1094	383.230	4.258	44096	433824
8	1110	373.840	4.153	26176	126064
9	1115	370.905	4.121	20144	107984
10	1176	335.106	3.723	20624	60080
11	1185	329.824	3.664	17424	56720
12	1273	278.179	3.090	20512	55344
13	1280	274.071	3.045	27536	77360
14	1314	254.117	2.823	27024	76016
15	1321	250.009	2.777	21184	63472
16	1350	232.989	2.588	7248	19360
17	1367	223.013	2.477	12608	51984

TABLE 8 (cont'd)

No.	Cursor	Freq.	PPM	Intens.	Area
18	1377	217.144	2.412	8656	29200
19	1381	214.796	2.386	6752	13104
20	1392	208.341	2.314	19024	102096
21	1402	202.472	2.249	16512	64912
22	1417	193.669	2.151	144032	505296
23	1433	184.279	2.047	121360	610288
24	1460	168.433	1.871	72096	578448
25	1493	149.066	1.656	73056	1531696
26	1555	112.680	1.252	15840	230288
27	1616	76.880	0.854	108016	426960

Mass spectral analysis

Fragmentography mass spectrometry was developed to assist in confirmation of TLC extracts. The MS 902 inlet capillary was replaced by disposable glass capillaries. Mass spectral data on nine scirpene compounds was obtained by Douglas Truesdale with the high resolution Mass Spectrometer MS902 Atlas. Data on these compounds is shown in Table 9 for "Mass Spectral Data for Standard Tricothecenes." As little as 10 nanograms was sufficient sample when using high resolution mode and monitoring the most sensitive AMU to .0001 OMU, to confirm the presence of T-2 toxin. The ratio of characteristic ion was made to an unexpected ion from the compound at 2 or 3 masses. If the ratio was characteristic of the compound, it is assumed it was perfect.

Field desorption compared with electron impact mass spectroscopy

Field Desorption (F.D.) mass spectroscopy reference spectra are shown in Figures 5, 6 and 7. There are spectra of T-2 toxin and aflatoxin B_1 at different filament currents (T-2=0,8ma; aflatoxin=12,18ma).

Electron Impact (EI) spectra are shown in Figures 8 through 15. These were all made at 70 eV ionization potential. This process requires more sample than F.D. These spectra are more complex and give a relatively small parent ion (M+).

TABLE 9

Mass Spectral Data for Standard Trichothecenes[a]

Compound	Molecular Weight	Base Peak	Ions Selected for Screening
Verrucarol	266	191	266
Fusarenone X	314	43	306, 336, 354
Verrucarin A	502	502	502
Trichothecin	332	257	332, 257
Calonectrin	336	263	263, 313
Diacetylcalonectrin	278	Sample lost -	no spectrum
Zearalenone	318		318
T-2 Toxin	466	364	364
Roridan A	464	489 ?	489, 533 ?
Neosolaniol	364	364	364, 306
Trichodermol	250	135	250

[a]Obtained by Douglas Truesdale, Chemistry Instrument Services, I. S. U.

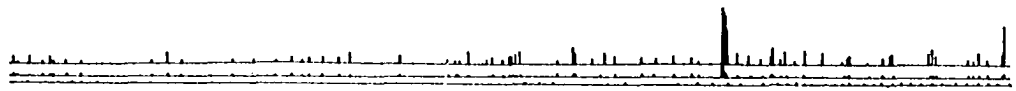
FIGURE 5

FIELD DESORPTION MASS SPECTRUM OF T-2 TOXIN

T-2 TOXIN

1" = 10"

0ma



364

FIGURE 5

FIGURE 6

FIELD DESORPTION MASS SPECTRUM OF T-2 TOXIN

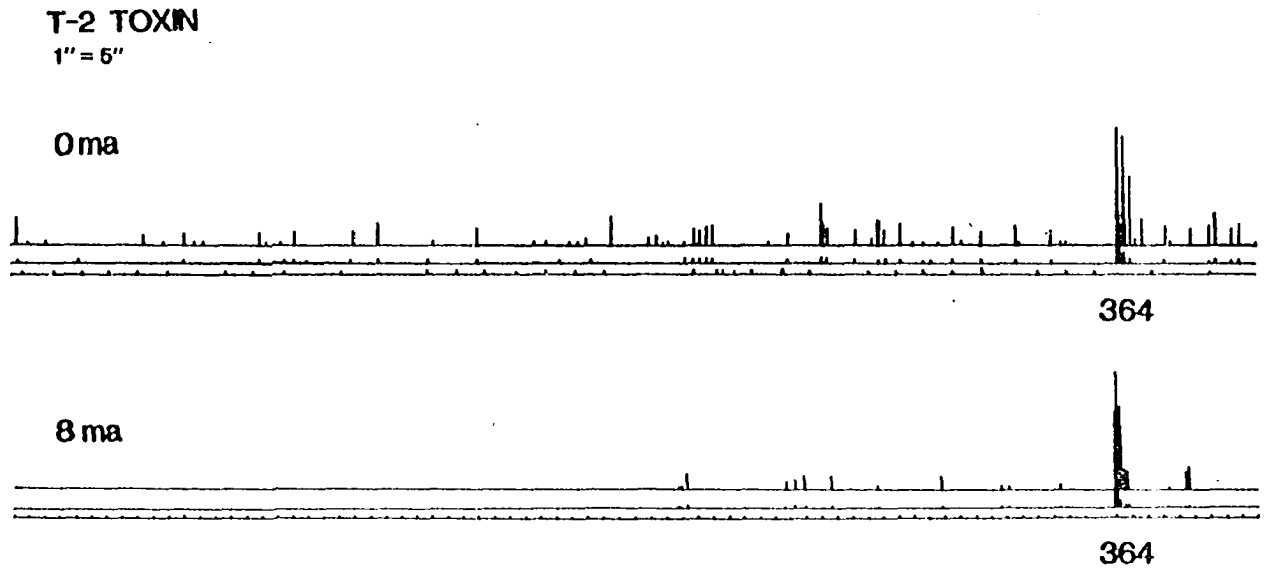


FIGURE 6

FIGURE 7

FIELD DESORPTION MASS SPECTRUM OF AFLATOXIN B_1

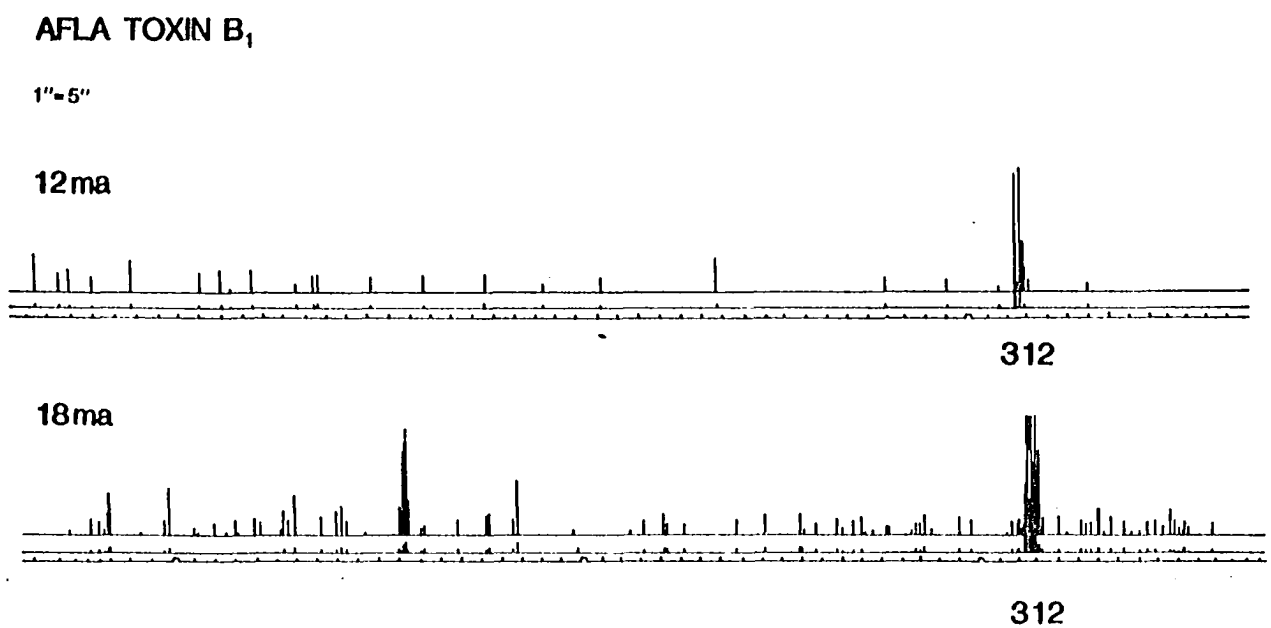


FIGURE 7

FIGURE 8

ELECTRON IONIZATION SPECTRUM OF AFLATOXIN B_1

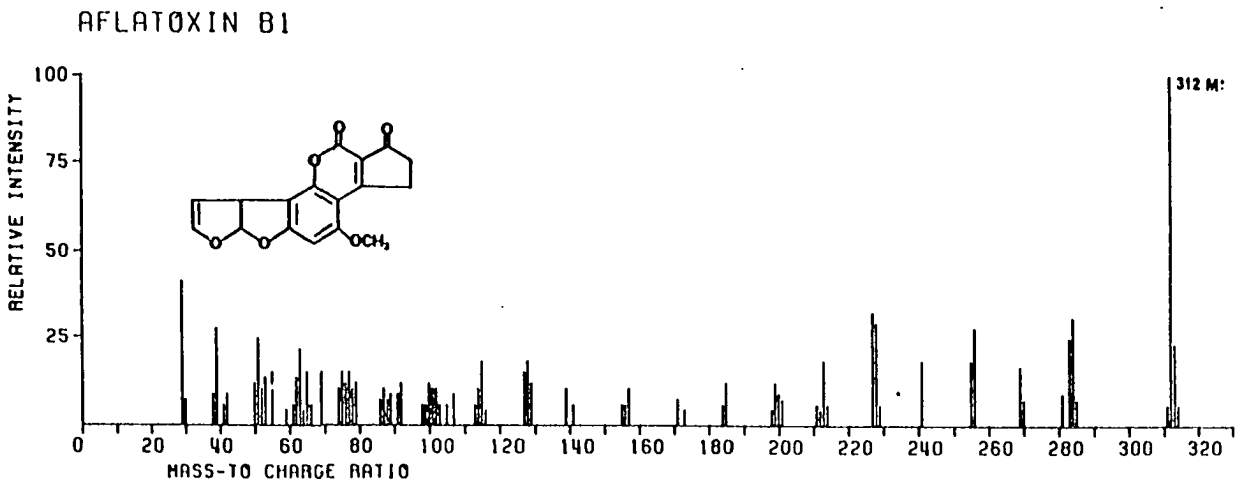

AFLATOXIN B1
RELATIVE INTENSITY
100
75
50
25
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
MASS-TO CHARGE RATIO
312 M+
OCH3

FIGURE 8

FIGURE 9

ELECTRON IONIZATION MASS SPECTRUM OF AFLATOXIN M_1

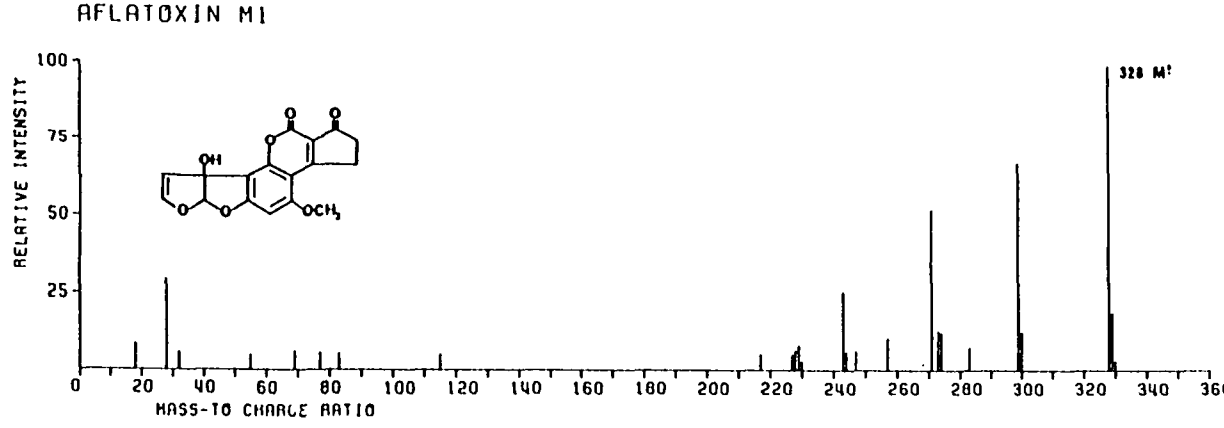

AFLATOXIN M1
RELATIVE INTENSITY
100
75
50
25
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
MASS-TO CHARGE RATIO
328 M+
OH
OCH3

FIGURE 9

FIGURE 10

ELECTRON IONIZATION MASS SPECTRUM OF AFLATOXIN M_2

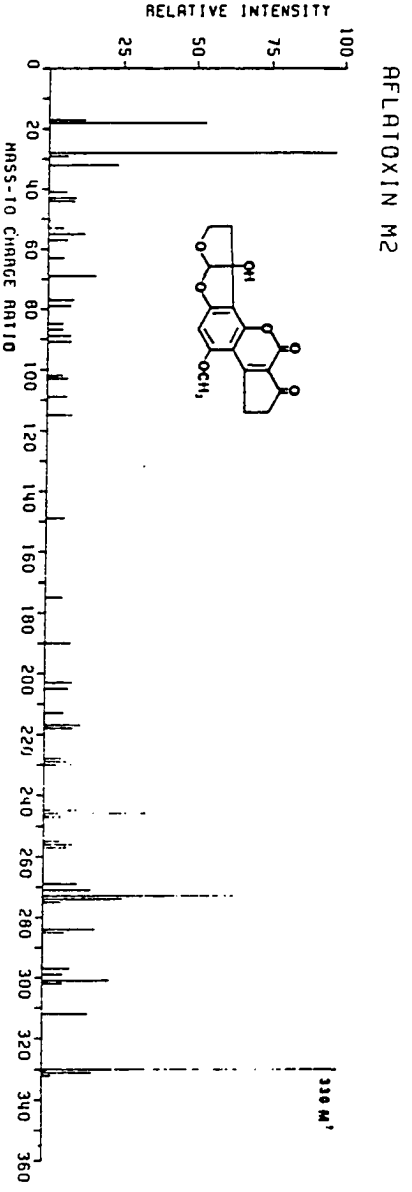

AFLATOXIN M2
RELATIVE INTENSITY
0
25
50
75
100
MASS-TO CHARGE RATIO
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
330 M'
OH
OCH3
O
O
O
O

FIGURE 10

FIGURE 11

ELECTRON IONIZATION MASS SPECTRUM OF AFLATOXIN P_1

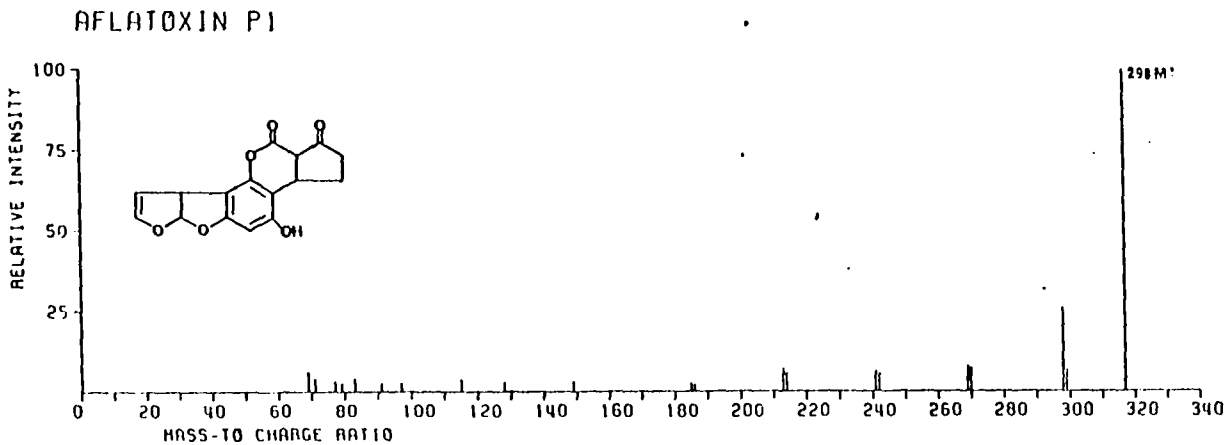

AFLATOXIN P1
RELATIVE INTENSITY
100
75
50
25
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
MASS-TO CHARGE RATIO
298M+
O
O
O
O
OH

FIGURE 11

FIGURE 12

ELECTRON IONIZATION MASS SPECTRUM OF AFLATOXIN Q_1

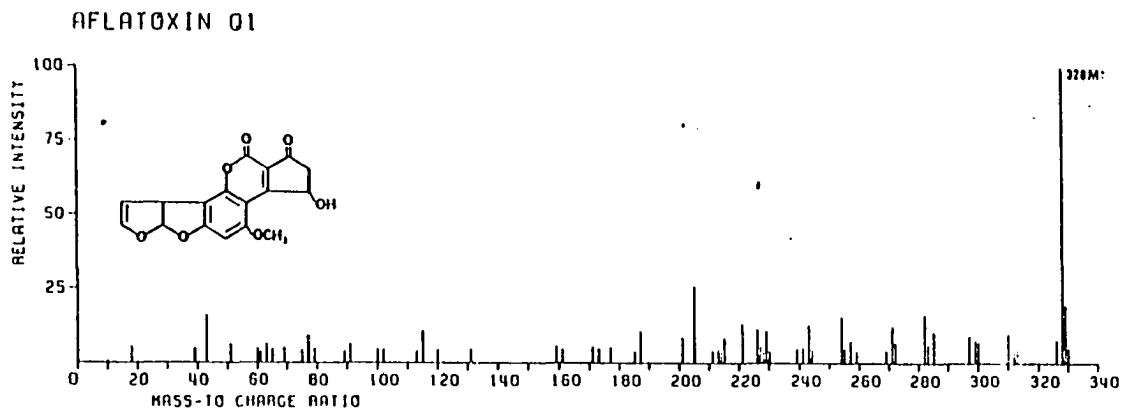

AFLATOXIN 01
RELATIVE INTENSITY
100
75
50
25
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
MASS-TO CHARGE RATIO
OH
OCH3

FIGURE 12

FIGURE 13

ELECTRON IONIZATION MASS SPECTRUM OF T-2 TOXIN

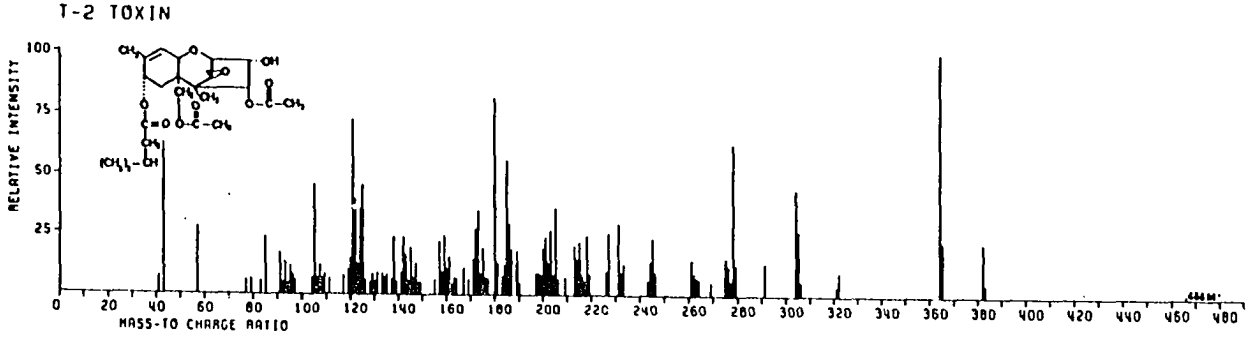

T-2 TOXIN
RELATIVE INTENSITY
100
75
50
25
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
MASS-TO CHARGE RATIO

FIGURE 13

FIGURE 14

ELECTRON IONIZATION MASS SPECTRUM OF DIACETOXYSCIRPENOL

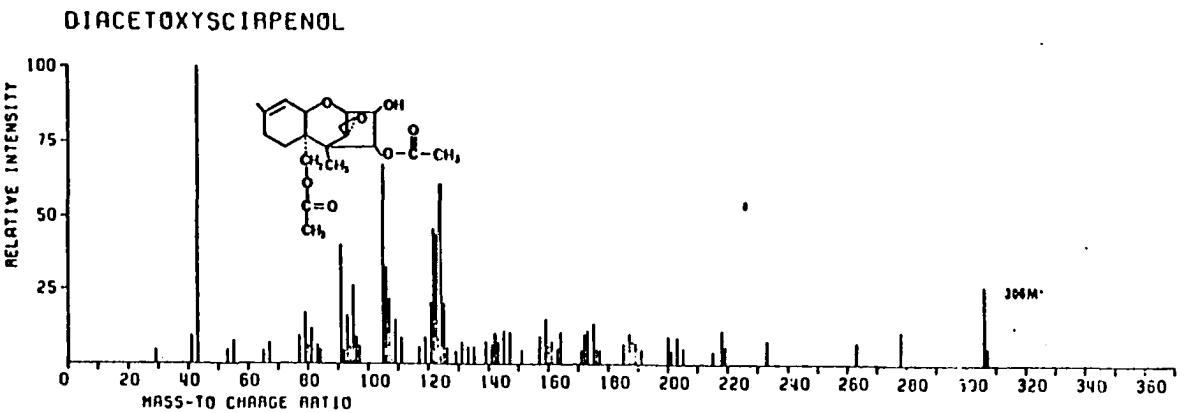

DIACETOXYSCIRPENOL
RELATIVE INTENSITY
100
75
50
25
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
MASS-TO CHARGE RATIO
OH
O-C-CH3
CH2CH3
C=O
CH3

FIGURE 14

FIGURE 15

ELECTRON IONIZATION MASS SPECTRUM OF ZEARALENONE

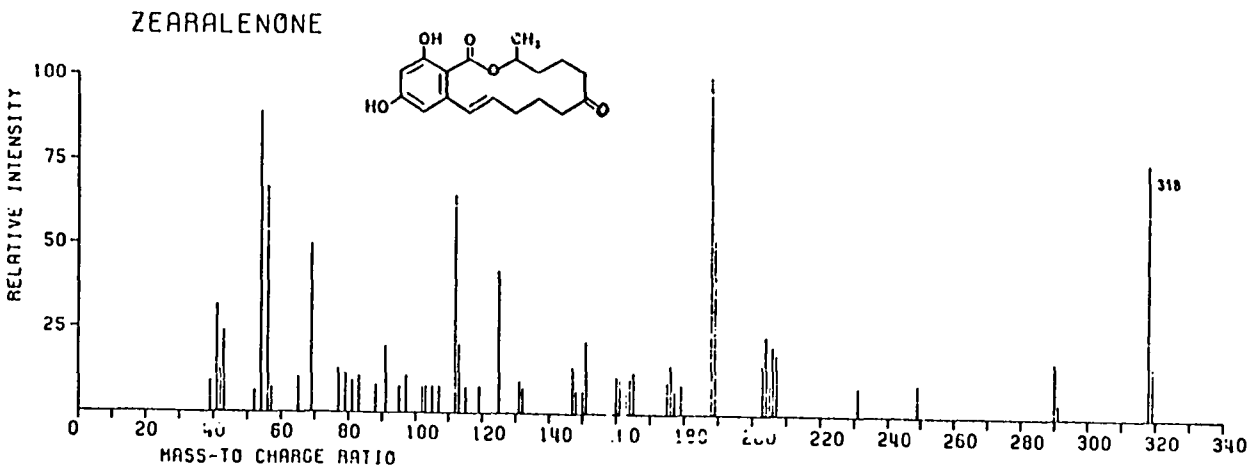

ZEARALENONE
OH
O
CH3
HO
O
O
100
75
50
25
0
RELATIVE INTENSITY
318
20
60
80
100
120
140
220
240
260
280
300
320
340
MASS-TO CHARGE RATIO

FIGURE 15

Infra red spectrum

Infra red spectrum of T-2 toxin was obtained on a Perkin Elmer Model 337 Infracord Infra Red Spectrometer. The spectrum is shown in Figure 16.

Development of Biological Confirmation Tests

Egg (Chicken Embryo) Tests

Levels of T-2 toxin were administered to embryonating eggs and the lethality was observed. Other known toxic substances were also tested to determine their toxicity relative to T-2 toxin using this method. The results are shown in Tables 10, 11 and 12.

Aflatoxin B-glucouronide production and analysis

Ducklings To produce tissues for analysis, three of six five-day old ducklings were administered doses, calculated to be lethal, of aflatoxin B_1 (1 ug in 1 ml of propylene glycol was given to each duckling). A dosing needle was placed on the rear of the duckling's tongue to cause the bird to swallow. The ducklings were observed for three weeks. Two were then necropsied, one control and one experimental. Tissues were analyzed for aflatoxin metabolites (including B-glucouronide) by the method of Chipley (1974)[1]. The remaining ducks were observed for two years. They were allowed to grow to maturity and raised progeny. Twenty ppb aflatoxin B_1 was found in the necropsied ducklings' livers. No detectable amount of

[1] J. R. Chipley, Poultry Department, Ohio State University, Columbus, Ohio. Private Communication. 1974.

FIGURE 16

INFRA RED SPECTRUM OF T-2 TOXIN

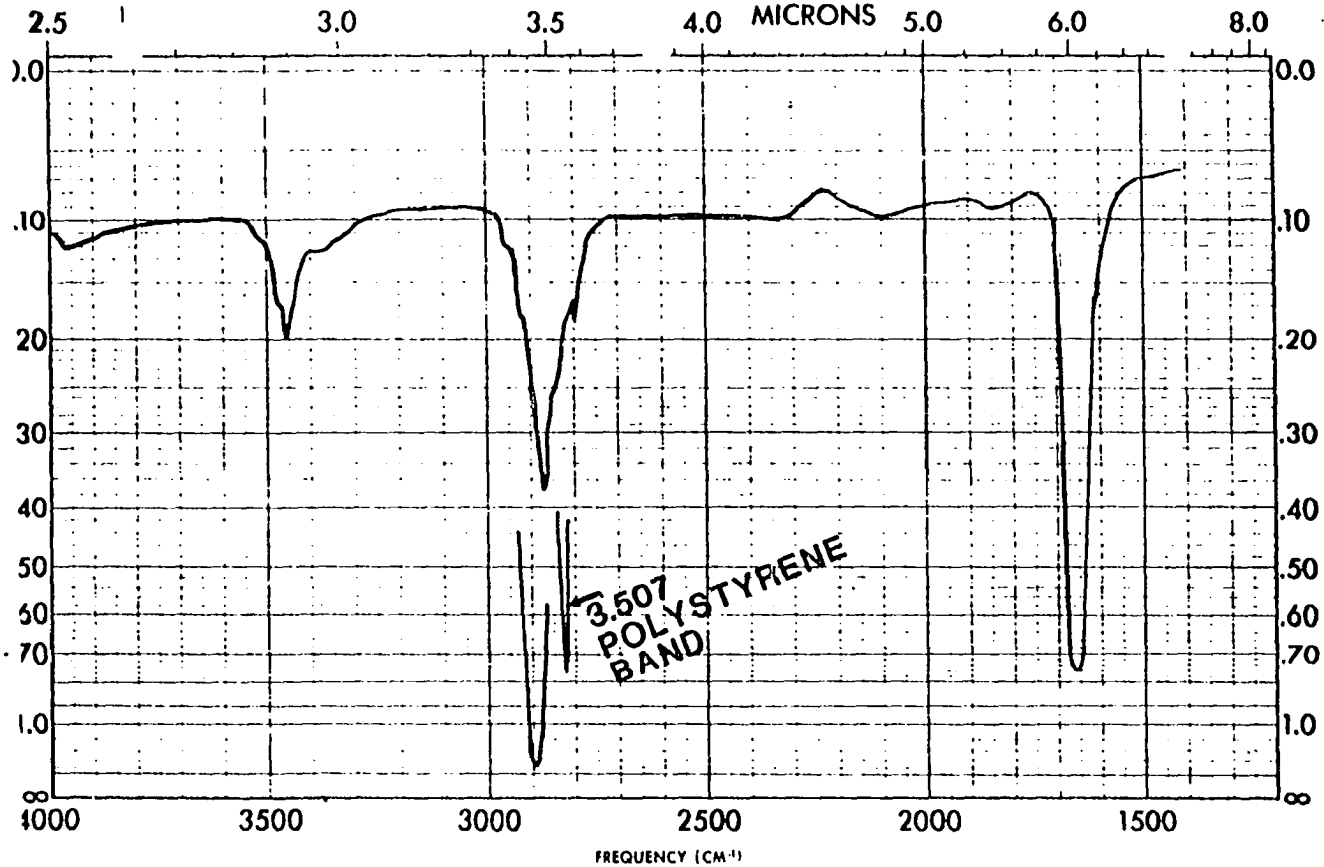

2.5
3.0
3.5
4.0
MICRONS
5.0
6.0
8.0
0.0
.10
20
30
40
50
60
70
1.0
∞
4000
3500
3000
2500
2000
1500
FREQUENCY (CM⁻¹)
3.507
POLYSTYRENE
BAND
SAMPLE T-2 TOXIN
CURVE NO. FIGURE 16
CONC 1000 PPM
CELL PATH 0.10 mm
REFERENCE C CL4
SCAN SPEED SLOW
SLIT N
OPERATOR hms
DATE 11-7-75
ORIGIN
SOLVENT C CL4
REMARKS
PART NO. 337-1207
PERKIN-ELMER

TABLE 10

Egg (Chicken Embryo) Tests

Alfatoxin	
Treatment	Deaths
Controls	0/6
Solvent Controls	0/6
25 ng Aflatoxin B	6/6
100 ng Aflatoxin B	6/6

TABLE 11

Egg (Chicken Embryo) Tests

T-2 Toxin

TRIAL I		TRIAL II		TRIAL III	
Treatment	Deaths	Treatment	Deaths	Treatment	Deaths
Controls	0/6	Controls	0/6	Controls	1/6
.01 ug T-2	0/6	Solvent controls	0/6	0.08 ug	3/5
.1 ug T-2	5/6	0.1 ug/egg	3/6	0.16 ug	4/5
1 ug T-2	6/6				

Totals	Controls and .01 ug	1/30
	0.08 and .1 ug	8/11
	1 ug	12/12

TABLE 12

Egg (Chicken Embryo) Tests

Other Toxic Substances

Other Mycotoxins		Pesticides	
Treatment	Deaths	Treatment	Deaths
Rubratoxin 1 ng	1/6	Furadan 1 ug	0/3
10 ng	3/6	5 ug	0/3
Zearalenone 1 ng	2/6	Dieldrin 10	3/6
10 ng	3/6	50	3/6
Penicillic Acid 10 ng	1/6	Parathion 1.0	3/6
		0.1	3/6
T-2 Toxin 1 ug	6/6		
		T-2 Toxin 0.1	6/6
Control Solvent	0/6		
		T-2 Toxin 0.01	2/6
		Controls (solvent)	0/6

aflatoxin M_1 was found in the livers. Other fluorescent compounds which could have been P or Q aflatoxin were observed.

Fish Fish were administered aflatoxin B_1 at a dose of 1 ug per fish (see experimental procedures). Aflatoxin B_1 in propylene glycol was administered with the dosing needle as described above. The fish were allowed to live for six months, being fed commercial fish food. They were then necropsied and the livers analyzed for aflatoxin. No detectable aflatoxin residues were found in the fish and the fish appeared normal.

Rats Small areas of the skins of weanling female rats and fully grown rats were painted with T-2 toxin according to the method of Bamburg and Strong (1971). Dermal necrosis was observed of the painted spots on the rats. Mature rats exhibited this effect at 10 ug of T-2 toxin per rat and weanling females at 1 ug of T-2 toxin per rat.

Rats were fed 5 ppm T-2 toxin with commercial rat blocks. After day three, feed remained in the feeder. During day four, no feed was eaten. On day five, the rats were necropsied. No effect on the rat tissues was observed. No detectable T-2 toxin was found in the rat livers.

The limit of detectability was 0.2 ppm because of the small liver size.

T-2 toxin feeding of ducklings

To produce tissue residues for analysis, T-2 toxin was fed to

ducklings. The ducklings had a salmonella infection when they arrived and had to be treated. Streptomycin was used as it showed the greatest effect on the disease organism. After two weeks of treatment and one week of recovery, the nine remaining ducks were placed on rations of 1 and 5 ppm T-2 toxin. The diet consisted of T-2 toxin added to Purina poultry starter fed free choice. A slowed consumption of feed was observed after four days in the 5 ppm T-2 toxin group.

After 18 days, the ducks were all alive. At necropsy, there were no mouth lesions. There were mild digestive tract lesions in the 5 ppm group. No detectable T-2 toxin was found in the duck livers or muscles. Microscopic examination of the tissues showed that the 5 ppm livers had fat deposits. The 1 ppm livers were normal.

Culturing of mycotoxic molds to produce toxins for analysis

T-2 toxin was produced from a culture (NRRL 3229) obtained from Northern Regional Research Laboratory, (USDA, Peoria, Illinois). White corn autoclaved at 121° C for 18 minutes was used as a substrate. A culture was suspended in 1 ml of water to produce a broth. Inoculation was done aseptically from this broth prepared from the freeze-dried culture. Three culture conditions were used with 20% moisture corn as substrate; (1) in light, (2) in the dark, and (3) in the dark alternated between refrigerator and room temperature each 16 hours.

After four weeks, flasks were sterilized 18 minutes in a 121° C autoclave. A few kernels were cultured on potato dextrose agar plate to check sterility. The contents of flasks were ground and analyzed for mycotoxins.

The T-2 toxin levels were 3 ppm for the flask in the light, 6 ppm for the flask in the dark, and 10 ppm for the flask which was exposed to dark, warm/cold. A peak which eluted between diacetoxyscirpenol and T-2 toxin on GLC analysis was obtained; it did not match any scirpene standard yet tried.

The light flask produced two fluorescent bands with similar Rf to aflatoxin B_1 and G_1. The spot-like aflatoxin B_1 gave a molecular ion of 270.2513 atomic mass units (A.M.U.). The spot-like aflatoxin G_1 gave a molecular ion of 405.0860 A.M.U. by mass spectral analysis. Formulas consistent with 405.0860 A.M.U. are as follows:

$C_{20}H_{13}N_7O_2$ $C_{22}H_{15}NO_3$ $C_{23}H_{11}N_5O_3$

Discussion of Results

Bamburg and Strong (1971) reported that less than 2% of the T-2 toxin was retained in the animals they tested in feeding studies. This would lead us to believe tissue levels of T-2 toxin would be very low.

If all of the substance (T-2 toxin) were concentrated in one tissue, we would expect several parts per million in this tissue, even from an inefficient process. Rats which resisted eating at levels of 5 ppm in their diet, would still consume enough in the first few days to be detected, if it were concentrated as T-2 toxin. This assumes that it would be available for extraction and analysis. Ducklings did not seem as reticent to eat feed containing 5 ppm T-2 toxin. Neither species exhibited severe lesions at 5 ppm in their diet. The ducklings

did have an exposure to Salmonella infection and an antibiotic, which may be of some significance. Refusal effects were seen immediately in old rats given feed containing 100 ppm T-2 toxin. This feed was submitted for analysis by our laboratory.

The recovery work with liver tissue suggests that a preferential binding or conversion by the liver tissue took place. More work should be done with radio-labeled T-2 toxin at a higher level in order to collect more data to better understand this phenomena. More work with a more sensitive technique for analysis by gas chromatography, such as one technique described by Eppley (1975)[1], should also help understand the interaction. He increased sensitivity of the analysis by using trifluoroacetic anhydride to make an electron capture sensitive compound, the trifluoroacetate ester.

Since our attempt at aflatoxin B-glucouronide analysis was unsuccessful, we attempted to do trichloro and trifluoro acetic anhydride derivatization. No new peaks were obtained on the GLC analysis of the products of this reaction.

Our results do show that only extremely small amounts (less than 100 ppb) are found as residues in tissues from animals fed a comparatively high level (5 ppm). Of course, other compounds could be complicating the toxicity findings and go undetected. For instance, butenolide (Yates, 1971) is not detected by GLC or TLC so far applied

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[1]R. M. Eppley, FDA, Washington, D. C. Private Communication. 1975. Referee's Report on T-2 Toxin. A.O.A.C. Meeting.

and yet causes symptoms similar to those of T-2 toxin in cattle. The GLC test developed does not respond to all scirpene compounds. Feed refusal, and skin painting are effective tests at rather high concentrations (1-5 ppm) of T-2 toxin. The chick embryo test appears to be the most practical bioassay that our laboratory has applied to field cases. Only a source of fertile eggs and a reliable incubator are necessary and the sensitivity is adequate (0.1 ug) for detecting compounds toxic to chick embryos.

Our inability to observe aflatoxin M_1 was explained by the Ohio State workers (Chipley, 1974[1]) as being due to species differences or perhaps to lack of adequate standards and techniques for less than nanogram amounts which might be present.

We did find aflatoxin B_1 residue in the livers. It is also possible that our B-glucouronidase was not as active as the enzyme that the other workers used. An enzyme assay is not equivalent to the use of the enzyme in the actual procedure for aflatoxin glucouronide.

The lack of effect on various species tested by aflatoxin B_1 may be a function of the viscous solvent, propylene glycol, or selection of a particularly hardy group of animals may account for the lack of effect. Propylene glycol has been shown to affect the toxicity of pesticides in work with rabbit feeding trials. It was selected because it was feared ethanol might increase the toxicity of the aflatoxin.

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[1]J. R. Chipley, Poultry Department, Ohio State University, Columbus, Ohio. Private Communication. 1974.

It appears that darkness and cold are suitable conditions for F. tricinctum to produce increased amounts of T-2 toxin. The fluorescent aflatoxin-like spots observed on the TLC analysis for T-2 may be other mycotoxins elaborated by F. tricinctum due to the mutating effect of ultraviolet light. These same spots were observed in other samples received from South Dakota State University. The odd mass molecular ion found by mass spectral analysis suggests a nitrogen containing mycotoxin. The illuminated sample had less mold growth than the samples kept dark. The screening method (given in Appendix) described in our work has been applied to thousands of samples by analysts at I.S.U., and in New York, South Dakota, North Dakota, Alabama, Mississippi, Kentucky and Missouri. Some data for I.S.U. results are given in Table 13.

In some cases where more cleanup is needed, selective thin layer cleanups making use of diethyl ether development, before the conventional one is used, can be done. In other cases, multiple developments or multi-dimensional developments are required. Feed additives such as sulfa drugs and arsanilic acid remain at the origin in 3/2/1 (toluene/ethyl acetate/acetone) and 90/20/2 (xylene, isoamyl alcohol, methanol) solvents.

Adjusting the amount of ferric gel should also be a workable approach for difficult samples.

By using two gas chromatographic columns we have not yet found a sample which gave a false positive test for T-2 toxins as confirmed by Mass spectroscopy. Using a single column (3% Dexsil 300) we did

TABLE 13

Frequency of Mycotoxicoses[a]

Mycotoxins		No. Cases	Confirmed Cases	No. Exposed	Morb	Mort
Zearalenone	S	6	6	380	186	4[b]
T-2 toxin	S	2	2	446	402	2
Aflatoxin	B	1	1	900	8	8[c]
	P	1	1	30,000	0	0[d]
	Man	1	1	1	1	0
Scirpenes	B	1	1	48	48	0
	S	1	1	80	80	0
Diacetoxyscirpenol	S[e]	1	1	-[e]		
Zearalenone-Aflatoxin	S	1	1	2,287	1,000	5
Aflatoxin-T-2	S	1	1	800	200	25
Zearalenone-T-2	Man	1	1	4	4	0
Fusarium sp toxin	E	1		7	3	3
Zearalenone, nitrate	0	1	1	34	17	0[f]
		19	18	34,987	1,949	47

[a]Dr. Gavin Meerdink's quarterly epidemiology report. Veterinary Diagnostic Laboratory, Iowa State University.

[b]Deaths were believed due to other causes.

[c]Toxin in rumen content but cause of deaths not confirmed.

[d]Were deaths but not attributed directly to aflatoxin.

[e]Was not fed; analyzed before feeding due to mold appearance; then diluted.

[f]*Vibrio* sp. isolated from the aborted lambs.

get some false positives which may have been other scirpene toxins. It is not recommended that thin layer alone be used for screening for T-2 toxin. There are spurious feed components which are present with scirpenes, which will respond to TLC and anisealdehyde spray, as scirpene toxins do.

The nuclear magnetic resonance (NMR), infra red (IR), and particularly mass spectrometry are preferred as means of confirmation. The NMR spectrum of T-2 toxin and Neosolaniol are given as reference spectra shown in Figures 3 and 4. Unfortunately, this analysis requires milligrams of sample. Other NMR spectra of scirpene are presented in the Bamburg and Strong (1971) chapter on trichothecenes. The infra red spectrum is shown as reference. It provides chemical functional group and structural information.

The hydroxyl, carbonyl and vinyl groups are shown in the spectrum. They may be used in comparison of unknown isolated compounds to known compounds. This technique also required several milligrams of sample, a thousand times more than is usually found in samples submitted from the field.

Ideally, a chemical ionization source with gas chromatography mass spectroscopy or field desorption mass spectroscopy can be used. Figures 5, 6 and 7 detail the simplification which results when field desorption sources are used as opposed to electron impact sources for mass spectroscopy. The electron impact spectra are shown in Figures

8 through 15. These are from Pohland and Sphon (1974)[1]. The variation in observed fragments increase with filament current as shown in Figures 5, 6 and 7. Figure 5 shows that at zero filament current a spectrum is obtained. The other techniques, NMR and IR, are selective but lack sufficient sensitivity to be used for small samples typically encountered and handled in practical situations. There are limitations to this technique. In two cases, the molecular ion was not observed at all by mass spectroscopy, even by an FD source. This is believed to be due to the lack of instrument sensitivity and operator experience. Sphon (1975)[2] reported molecular ions from T-2 toxin in his work with an FD source and food samples.

A limitation of field desorption is the cleanliness of the analysis system required, due to (nanogram) size samples. The advantages, however, outweigh the disadvantages; they are sensitivity and selectivity.

Using the technique described in this dissertation, samples may be screened by high resolution mass spectroscopy and confirmed in a very practical fashion. This system is slow due to its complexity, but valuable as a necessary adjunct to our screening work. The mass spectral ions selected for monitoring are shown in Table 9.

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[1]A. Pohland and J. A. Sphon, F.D.A., Washington, D. C. Private Communication. 1974. Mycotoxin Mass Spectral Data Bank.

[2]J. A. Sphon, F.D.A., Washington, D. C. Private Communication. 1975. Application of Field Desorption for detection of mycotoxin in foods. A.O.A.C. Meeting.

SUMMARY AND CONCLUSIONS

A method of analysis has been developed for T-2 toxin and validated for feeds and human foods. The method involves extraction by acetonitrile, defatting with petroleum ether, decolorization with ferric gel and partitioning into chloroform. Thin layer chromatography and gas chromatography, using two columns, are recommended for a presumptive test. Mass spectral analysis and chicken embryo tests were done and are recommended for confirmatory tests. Field desorption and/or a gas chromatographic inlet system for mass spectrometry are most satisfactory.

Ion monitoring with a high resolution mass spectrometer may be done to increase the sensitivity of this technique to fractions of micrograms of T-2 toxin in thin layer chromatogram band eluates. Other confirmatory procedures should include biological tests on skin. Nuclear Magnetic Resonance and Infrared Spectrometry also may be used for confirmatory purposes, if enough substance is available. Tissue levels resulting from feeding 5 ppm T-2 toxin to animals resulted in levels of less than tenths of ppm.

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of her citizens. Thanks to all the hundreds of millions of people who make her promises real. Thank God for all my blessings.

APPENDIX:

DIAGNOSTIC LABORATORY CHEMICAL ANALYSIS FOR MYCOTOXINS[1]

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[1] Submitted to be published in JAOAC ... 1975.

Diagnostic Laboratory Chemical Analysis for Mycotoxins

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Progress has been made in analyzing for multimycotoxins in diagnostic case samples. The Food and Drug Laboratory method (Stohloff et al.) (1) of extraction has been combined with Velasco's decolorization (2) and an optimum TLC development system to provide an efficient method for screening and quantitation of aflatoxins, zearalenone, and other estercoumarin compounds. Recovery studies have been made, problems encountered including purity of "standards" and interfering bands. Rubratoxin and tricothecenes are analyzed by separate extractions. Tricothecenes are also usually seen by the multimycotoxin extraction, qualitatively. The Veterinary Diagnostic Laboratory, Iowa State University, does several dozen cases a year which are processed by screening, quantitation, and/or instrumental confirmation depending upon the gravity of the case. Zearalenone, aflatoxin, sterigmatocysin, rubratoxin, and T-2 toxin have been observed and confirmed in field cases.

Introduction:

Stohloff (3) has reviewed analytical methods for mycotoxin and traced their development from "The Assay of a Toxic Principle in Certain Groundnut Meals" (4) in 1961 to the separation and quantitation of tricothecenes by Idekiobi, Bamburg and Strong (5) in 1971. People have attempted to determine multiple mycotoxin in mixtures of standards (6) and complex food and feeds (7). However, no one has presented information on a method which will allow the detection of most common mycotoxins which an animal or human might be subjected to from ordinary environmental microflora which are encountered in typical foodstuffs. Pier (8) and his co-workers are attempting studies of synergistic effects of multiple mycotoxins in feeds on animal growth and health. This work is the outgrowth of the Hemorrhagic Disease Symposium (9) at Iowa State University in 1970. At that time, multiple mycotoxins were found in feed samples; however, no one mycotoxin was present at a level which could account for the symptoms. Typical diagnostic case work requires that an elimination be made of multiple exposure possibilities. This is the reason we have worked to develop such a broad spectrum screening system.

Reagents and Apparatus:

A. Extraction solvents: acetonitrile/4% KCL (90/10); ACS grade.

B. Development solvents: xylene/isoamyl alcohol/amylacetate (9/1/1); toluene/ethylacetate/acetone (3/2/1), all are V/V/V.

C. Thin layer plates: Brinkman Industries Inc., Westbury, New York. 0.2 mm MN silica Gel N-HR plastic or glass backed.

D. Defatting solvent: ACS grade--2,2,4 trimethyl pentane (isoctane) or petroleum ether.

E. Spotting solvent: benzene-acetonitrile (98/2).

F. Decolorizing reagents: 10% $FeCl_3$ or 15% $FeCl_3 \cdot 6H_2O$ filtered; 4% aqueous NaOH.

G. Extraction apparatus: Waring-type blender, explosion proofed.

H. Gel preparation apparatus: 50.0 ml buret equipped with teflon stopcock; pH meter, Corning model 12-B or equivalent.

I. Standards: aflatoxins, 0.25-0.5 ug/ml in benzene; zearalenone, 0.1-1.0 mg/ml in benzene; T-2 toxin and diacetoxyscirpenol 0.1-1.0 mg/ml in chloroform; ochratoxins A and B, 10 ug/ml in benzene; sterigmatocystin 10 ug/ml in benzene.

Screening Feeds[1] for Mycotoxins[2] Other Than Rubratoxin

Preparation of Sample Extract:

Weigh out 50 g of feed into a Waring blender. Add 200 ml of extraction solvent (9/1 acetonitrile-4% KCl v/v) and blend at high speed for 1-2 minutes. Filter 100 ml of supernatant through filter paper into graduated cylinder and transfer into 250-ml separatory funnel fitted with teflon stopcock. Defat sample by extracting twice with 50 ml of isooctane or pet ether. Discard isooctane.

Preparation of Gel:

Into a 600-ml beaker, place 100 ml of distilled water and exactly 10.0 ml of the 10% $FeCl_3$ solution. Titrate this solution with the 4% NaOH, constantly stirring and measuring pH with pH meter, until the pH is about 4.6. After several gels have been made this way, an average volume of 4% NaOH can be calculated; this volume can then be added with pH measurement.

Decolorization of the Sample Extract:

Into the beaker containing the sample extract, add the gel and stir for 1-2 minutes. After stirring, allow the gel to settle for a few minutes. Filter through folded filter paper 100.0 ml of the decolorized sample and transfer the filtrate into the 250-ml separatory funnel along with 50 ml of distilled water.

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1 Corn, mixed feeds, cereal grains.

2 Aflatoxins, zearalenone, ochratoxins, tricothecenes, sterigmaticystin.

Extraction of Mycotoxins:

Add 50 ml of chloroform to the filtrate and shake. After the layers have separated, drain the chloroform layer into a 125-ml Erlenmeyer flask and repeat chloroform extraction. Evaporate chloroform on steam bath under N_2 or in vacuo.

Gas Liquid Chromatography:

T-2 toxin and diacetoxyscirpenol may be determined directly using a FID equipped with a 6-foot-1/2-inch glass column packed with 3% Dexsil 300 (Applied Science, Dexsil 300 GC) on chromsorb Q.

The evaporated sample extract should be redissolved in 0.1 ml of chloroform and injected into GLC. At a column temperature of 280°C retention times for T-2 toxin and diacetoxyscirpenol are 13 and 4.4 minutes, respectively.

Sensitivity of this method is about 0.1 ug depending upon the instrument being used and the sample matrix. If the sample volume is 0.1 ml, then 1.0 ppm may be detected (in feeds).

Thin Layer Chromatography:

The sample extract, either before or after GLC, should be redissolved in 400 ul of the spotting solvent and 50 ul spotted on a thin layer plate along with appropriate standards. Develop the plate in the preferred solvent to within 1 to 2 cm of the top.

Aflatoxins, zearalenone, ochratoxins, and sterigmatocystin may be observed by UV light after TLC development. Table 1 gives Rf data and expected fluorescent colors for the above mycotoxins.

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

Developing Solvents:

The 2 developing solvents mentioned above have differing separation characteristics and should be used accordingly.

The 9/1/1 system is quite good for separation of aflatoxins, B_1, B_2, G_1, G_2, but can give interfering bands for other mycotoxins.

The 3/2/1 system was originally used for separation of tricothecenes (10), but will give good separation for zearalenone and sterigmatocystin though the aflatoxins are not separated as well as in the 9/1/1.

Addition of 1% formic acid to the 3/2/1 system will move ochratoxins A and B away from the origin and allow their detection.

When screening for ochratoxins and some other mycotoxin, twice development is necessary; once with 3/2/1 and once with 1% formic acid added.

Quantitation of Zearalenone:

For quantitation of zearalenone, the fluorescent spot at the same Rf as the zearalenone standard should be scraped from the TLC plate and eluted from the silica gel with methanol.

Standards of zearalenone should be between 1.0 and 10.0 ug/ml in methanol. The sample should be diluted accordingly. Using Cary^R 14 or equivalent UV spectrophotometer (calibrate according to Sec. 26.004), a

scan from 250 nm to 310 nm will give a characteristic peak. Quantitation can be done by measuring the peak height at the absorbance maximum, 274 nm.

For checking purity of zearalenone standard, the following molar absorptivities are useful: ultraviolet maxima 236 nm (ϵ=29,700), 274 nm (ϵ=13,909), and 316 nm (ϵ=6,020) (11).

Zearalenone Recovery:

Recovery studies done on 12 different feed samples (mixed feeds, pelleted feeds, corn) have been done.

The feed samples were spiked with zearalenone at a level of 4 ppm and quantitated after TLC. The average zearalenone recovery was 95% with standard deviation of 7%.

Quantitation of Aflatoxins:

Quantitation of aflatoxins should be done in a manner similar to zearalenone. The fluorescent spot should be scraped from the TLC plate and eluted from the silica gel with methanol.

Samples and standards should be treated in the same manner as described in Sec. 26.004-26.009.

Recovery data on several feed samples spiked with aflatoxin B_1, after overcoming problems with purity of standards, was 90%.

Screening for T-2 Toxin and Diacetoxyscirpenol:

Qualitative recovery has been done with T-2 toxin and diacetoxyscirpenol. Samples spiked with these 2 mycotoxins gave characteristic

peaks by GLC.

Thin layer chromatography may also be done for T-2 toxin and diacetoxyscirpenol using the 3/2/1 solvent coupled with p-anisaldehyde spray (3) to give visible spots. This technique leaves much to be desired in that many compounds will react to give similar colors and many interferences.

Screening for Ochratoxins A and B:

Ochratoxins A and B undergo partial breakdown during the extraction decolorization and evaporation of the sample. This is probably due to the acidic conditions which probably leads to ester formation.

Since there is breakdown, quantitation is not possible, but qualitative screening may be done.

Screening for Sterigmatocystin:

Recovery data for sterigmatocystin has not been done, but visual estimation by comparison with standards seems to indicate that recovery is quite good.

Spraying the TLC plate with 20% aqueous KOH increases fluorescent intensity as well as changing the red color to a more intense yellow.

Confirmation

Presence of fluorescent compounds or GLC peaks which match standards is only presumptive evidence of mycotoxins and should not be used as confirmation.

There are a variety of techniques for derivative formation and reagent sprays used with TLC (3), but use of these methods is not proof of identity. The best confirmation is probably the use of TLC or GLC coupled with mass spectrometry.

Rubratoxin Screening in Feeds

Sample Extraction:

Weight out 50 g of feed and blend twice with 150 ml of pet ether; discard the pet ether after filtering each time. Blend the sample with 150 ml of methanol and filter. Discard the feed and evaporate the methanol to dryness under N_2.

Redissolve the extract with 100 ml of ethyl acetate, filter, and discard solid part. Evaporate filtered ethyl acetate to about 0.4 ml under N_2 (12).

Thin Layer Chromatography:

Spot equivalent of 1-5 g of feed on a TLC plate (silica gel G with fluorescent indicator) along with 10 ug of rubratoxin standard (1 mg/ml).

Develop the plate first in 50:50 (V/V) methanol:chloroform to move colored material away from the origin and towards the top of the plate. Depending upon the sample, this development may require several hours.

After the color has developed away from the origin, remove the plate and add 1% formic acid to the solvent and place the plate into the tank again. Allow development of the plate approximately one-half the way up.

Observe rubratoxin, via quenching, under UV light at a Rf of 0.5 in the acid system.

TABLE 1. TLC data for mycotoxins on silica gel G-HR

Mycotoxin	Rf (3/2/1)	Rf (3/2/1 + 1% acid)	Fluorescent Color	Lower Detectability Level
Aflatoxin B_1	0.48		Blue	1 - 5 ng
Aflatoxin B_2	0.45		Blue	1 - 5 ng
Aflatoxin G_1	0.43		Green	1 - 5 ng
Aflatoxin G_2	0.36		Green	1 - 5 ng
Zearalenone	0.69		Bluegreen	0.1 - 0.2 ug
Sterigmatocystin	0.80		Red or yellow[1]	10 - 20 ng
Ochratoxin A		0.64	Blue	10 - 20 ng
Ochratoxin B		0.48	Blue	10 - 20 ng

[1] Yellow fluorescence after spray with aqueous 20% KOH.

Discussion

The recovery data for zearalenone presented above tends to indicate that the procedure presented here is useful and can be used on a variety of samples. This, however, should not be oversimplified by the experimentor by assuming that all sample matrices are alike or that the method will work for each sample.

Each sample that is run should be split and one-half spiked with the appropriate mycotoxin or mycotoxins to insure recovery from that particular matrix. Also, for any sample which is highly colored or a case in which there is some doubt concerning the validity of an answer, standards addition should be done.

Since sterigmatocystin is quite close to aflatoxin structurally, the recovery is probably quite good.

Direct determination of T-2 toxin and diacetoxyscirpenol overcomes the problems of trying to make a silylether derivative, which not only destroys the sample extract and makes it impossible to do other tests, but also eliminates problems of GLC interference due to the reagents. It is assumed that other tricothecenes can be determined in this manner, but as of yet, this laboratory has not obtained standards.

Chemical evidence of a mycotoxin in a feed sample should not always be assumed to be the factor causing the field problem.

Provided that adequate sample is available, toxicity tests may be done using the egg embryo test (Sec. 26.057) or the rat skin test (C. Mirocha, Private Communication). Also, feeding studies using the animal

involved in the field problem should be done if possible. This is to obtain absolute proof for a correct diagnosis.

In most cases, biological testing is impossible due to lack of sample, money, time, and manpower. In absence of this or extensive conformation techniques, mass spectrometry is probably the best technique available provided authentic standards and spectrum are available.

Recommendation:

We recommend that the general referee consider these methods for interlaboratory collaboration.

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