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**ISOLATION AND CHARACTERIZATION OF NEW
FUMONISINS FROM *FUSARIUM MONILIFORME* NRRL 13616
AND DEVELOPMENT OF NEW ANALYTICAL TECHNIQUES**

Sabrena E. MacKenzie, B. Sc.

A thesis submitted to the Faculty of Graduate Studies in partial
fulfillment of the requirements for the degree of

Master of Science

Department of Chemistry

Carleton University

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The undersigned hereby recommend to the faculty of Graduate Studies the acceptance of this thesis submitted by Sabrena E. Mackenzie entitled :

Isolation and characterization of new fumonisins from *Fusarium moniliforme* NRRL 13616 and development of new analytical techniques

in partial fulfillment of the requirements for the degree of Master of Science.

Thesis Supervisor

Thesis Co-supervisor

Chairman of the
Chemistry Department

ABSTRACT

A crude fungal extract of *Fusarium moniliforme* NRRL 13616, grown in liquid culture, was found to produce the new fumonisin, iso-FB₁ (**6**). The full characterization of iso-FB₁ is presented here. In addition, many other minor fumonisins have been observed in the same extract which produced iso-FB₁. Two of these minor fumonisins, FC₃ and 6-OH iso-FB₁ have been partially characterized and assigned the tentative structures **8** and **9**.

Two new methods for separating the minor fumonisins have been examined. The first method involved a reversible derivatization with maleic anhydride. This method proved successful in isolating several minor fumonisins, however, none of the compounds isolated were present in concentrations sufficient for characterization. The second method involved HPLC analysis of underivatized fumonisin using UV detection. Although it was possible to detect an FB₁ standard in this manner, the amount required to produce even a minimal response from the detector makes this an unsuitable method for isolating the minor fumonisins from a crude extract.

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LIST OF ABBREVIATIONS

DON	deoxynivalenol
ZEN	zearalenone
IARC	International Agency for Research on Cancer
WHO	World Health Organization
ELEM	Equineleukoencephalomalacia
PPE	Porcine Pulmonary Edema
TLC	thin layer chromatography
HPLC	high performance liquid chromatography
OPA	<i>ortho</i> -phthaldehyde
GC	gas chromatography
MS	mass spectrometry
EI	electron impact
CI	chemical ionization
FAB	fast atom bombardment
NMR	nuclear magnetic resonance
COSY	homonuclear correlation spectroscopy
HETCOR	heteronuclear correlation spectroscopy
NOE	nuclear overhauser enhancement

INTRODUCTION

Throughout history man and fungi have been closely associated. The role of fungi in the production of bread and alcoholic beverages has long been well known. More recently fungi have been used in the production of enzymes, organic acids, vitamins, antibiotics, and steroids ¹. Unfortunately, fungi also play a substantial role in human and animal disease as well as crop and food spoilage.

Toxigenic fungi have been known to contaminate crops, both in the field and during storage, since the beginning of agriculture. Many types of foods and feeds are affected causing both acute and chronic toxicoses. Mycotoxicosis is defined as poisoning of humans or animals which follows the entry into the body of toxic substances of fungal origin ¹. The compounds responsible for these poisonings are called mycotoxins.

Mycotoxins are secondary metabolites - compounds generally considered to be non-essential for the growth of individual cells of the organism. There is some debate over the purpose of secondary metabolites to the producing organism, however, it is known that enzymes involved in secondary metabolism are of low specificity ². This lack of specificity usually results in the production of families of structurally related metabolites such as the aflatoxins and fumonisins.

Although mycotoxins have long been a problem, and there have been many serious outbreaks of disease which can be attributed to these compounds, it has only been in the last four decades that there has been significant attention given to the identification of the

toxins. In 1960 there was an outbreak of Turkey X disease in England in which 100,000 turkeys, 14,000 ducklings, and thousands of partridge and pheasants died after consuming contaminated peanut meal. This incident triggered worldwide concern about mycotoxins and led to the discovery of aflatoxin, one of the most potent natural toxins³.

Since that time mycotoxins have come to be viewed as a very serious agricultural and medical threat. Fungi which produce mycotoxins infect many crops which are staple foods for both animals and humans. For this reason, there has been increased research activity into mycotoxins in recent years, focussing on the isolation, characterization, and toxicity of these compounds.

I Most Important Mycotoxins

Although many mycotoxins have been discovered, to date there are only five which are considered to be major problems for health and agriculture; deoxynivalenol (or its analogue nivalenol), zearalenone, aflatoxin, ochratoxin, and fumonisin (Fig. 1) . These compounds have a wide and varied range of biological effects which can be either acute or chronic. Despite the fact that these economically and medically important fungal toxins are relatively few in number, they cause a great deal of economic hardship as well as many illnesses and deaths worldwide because of their wide distribution in foods and feeds.

Aflatoxin B₁

Nivalenol R=OH

Deoxynivalenol R=H

Zearalenone

Ochratoxin A

Fumonisin B₁

Figure 1. Mycotoxins which have the greatest implications for health and agriculture.

I-1 Deoxynivalenol

Deoxynivalenol (DON) (Fig. 1) is probably the most widely distributed mycotoxin in food and feed throughout the world⁴. The fungi responsible for the production of DON are primarily *Fusarium graminearum* and related species. Nivalenol is produced by certain strains of *F. graminearum* instead of DON depending on the geographic origin of the isolate. Within the group which produce DON, some strains make DON via the 3-acetylated precursor and others produce the 15-acetylated precursor⁵.

DON and nivalenol are members of a group of over 150 structurally related compounds called trichothecenes. Trichothecenes possess the tetracyclic 12,13-epoxy-trichothec-9-ene skeleton of the sesquiterpenoids⁶ (Fig. 1). As with most toxins the severity of toxicoses produced by consumption of DON depends largely on the amount and duration of exposure and the species of animal involved. Although acute toxicoses can occur, the main problem related to the ingestion of DON contaminated feed by livestock is poor performance due to chronic low-level consumption⁷.

Ingestion of DON by humans causes Alimentary Toxic Aleukia, a multi-stage disease characterized by severe mucosal irritation, changes in blood leukocytes and platelets and possibly death by suffocation or haemorrhage^{8, 9(a),(b)}.

DON and nivalenol have recently been examined by the International Agency for Research on Cancer (IARC) which found there to be inadequate evidence that DON and nivalenol are carcinogenic, although their toxicity continues to be a major concern⁴.

Deoxynivalenol is not subject to statutory regulation in any country, however guidelines, advisory levels, and official tolerance levels do exist in some countries including the United States and Canada ¹⁰. In Canada, a guideline of 2 mg/kg is given for the occurrence of DON in uncleaned soft wheat, except for that used to prepare infant foods for which the guideline is 1 mg/kg. A guideline of 1.2 mg/kg is given for uncleaned non-staple foods calculated on the basis of flour or bran⁴. Much less is known about the toxicity of nivalenol, however, it is thought to be similar to that of deoxynivalenol, although DON has greater acute toxicity in lab animals ¹¹. No regulations or guidelines exist for nivalenol in any country.

I-2 Zearalenone

Zearalenone (ZEN) (Fig. 1) is also produced by several species of *Fusarium*, primarily *F. graminearum* and *F. culmorum*. Zearalenone is a nearly universal contaminant of corn crops but is also found on wheat, barley, sorghum, oats, and hay. Production of ZEN is promoted by high humidity and low temperatures. These conditions are also favourable for trichothecene production. Due to the fact that ZEN is produced by some of the same species of *Fusarium*, it is often found in the same samples as either DON or nivalenol¹². Zearalenone is an estrogen analogue which produces potent hyperestrogenic responses in susceptible animals ¹³. The mechanism of action of this toxin involves binding of ZEN to the cytoplasmic estrogen receptor ¹⁴.

Zearalenone was recently evaluated by IARC which found that there was limited evidence in animals for the carcinogenicity of zearalenone⁴. An official tolerance level for zearalenone of 1 mg/kg grains, fats and oils was established in the USSR in 1984. Proposed levels in other countries are 0.2 mg/kg maize in Brazil and 0.03 mg/kg in all foods in Romania. In Canada there are currently no regulations or guidelines in effect with regard to tolerable levels of zearalenone in food or feed because it seldom is found at important concentrations¹⁰.

I-3 Ochratoxin

Ochratoxin A (Fig. 1) is produced by only one species of *Penicillium*, *P. verrucosum*¹⁵ and a few species of *Aspergillus*. *A. ochraceus* is the most important ochratoxin-producing species but there are several other rare species of *Aspergillus* that do produce ochratoxin¹⁶. Although ochratoxin A occurs in many commodities throughout the world, it has been found primarily in north-temperate barley and wheat growing areas^{17,18}.

The most recent evaluation by the IARC has determined that there is sufficient evidence for the carcinogenicity of ochratoxin A in tested animals. There was not conclusive evidence to warrant this rating with respect to humans, therefore, it has been included in the 2B group of compounds which is defined as "possibly carcinogenic to humans"⁴.

In 1990, existing or proposed regulations for ochratoxin A were available in several

countries ranging from 1 to 50 $\mu\text{g/kg}$ in food and from 100 to 1000 $\mu\text{g/kg}$ in animal feed⁴. Also in 1990 a WHO/FAO Joint Expert Committee on Food Additives reviewed the literature on ochratoxin A and recommended a provisional tolerable weekly intake of 112 ng/kg body weight¹⁹. Despite this recommendation there are currently no regulations or guidelines in Canada concerning ochratoxin A.

I-4 Aflatoxin

Aflatoxins (Fig. 1) are the most well known of the mycotoxins. They were first discovered over 30 years ago²⁰ and there has been extensive research on this family of compounds since that time. Aflatoxins are known to be produced by three species of *Aspergillus*. *A. flavus* and *A. parasiticus* are the most common but aflatoxins are also produced by the rare species *A. nomius*²¹.

The aflatoxin-producing species of *Aspergillus* are found throughout the world in areas with hot, humid climates. Contamination of dietary staples such as peanuts, corn, and cottonseed meal is widespread in these areas, therefore, exposure is likely to be chronic⁴. Contamination can occur both pre- and post-harvest. The problem of exposure to aflatoxin is not limited to those countries with warmer climates, but is worldwide, as countries with colder climates import their staples from areas where aflatoxin levels are high.

The IARC has recently re-evaluated aflatoxins in terms of their carcinogenicity.

Naturally occurring mixtures of aflatoxins as well as aflatoxin B₁ alone were classified as class 1 human carcinogens ⁴. This means that there is sufficient evidence that these compounds do cause cancer in humans. Exposure to aflatoxins is widely recognized to be a very serious health risk to both humans and animals. For that reason, most countries have regulations governing tolerable levels of aflatoxins in food and feed ¹⁰. In 1987, at least 50 countries had existing or proposed regulations for aflatoxins in foodstuffs ^{10, 22}. The maximum limits range from none detectable to 50 µg/kg of food for either the sum of aflatoxins B₁, B₂, G₁, and G₂ or for aflatoxin B₁ alone ; 5 µg/kg is the most common maximal limit.

I-5 Mixtures of Mycotoxins

In naturally contaminated grain various toxins occur together and may cause combined toxic effects, additive and/or synergistic. This can be as a result of one species of fungi producing more than one toxin. *F. graminearum* often produces both deoxynivalenol and zearalenone. As well, in many places the co-occurrence, on commodities, of different species of toxigenic fungi, which produce different toxins, is often observed ²³.

For example, various studies compare the toxicity of pure DON with DON from naturally infected wheat or maize. DON from naturally infected feed has a higher toxicity than pure DON even when no other toxin was detected in the chemical analysis of the feed ^{24, 25, 26}. This increased activity of DON may be due to co-contamination with

some unknown toxin, or a non-toxic compound enhancing the toxicity of DON. One example of the latter was published by Dowd et al ²⁵. The metabolites 7,8-dihydroxycalonectrin (1), sambucinol (2), and culmorin (3) were not significantly toxic to two species of caterpillar when fed in diets at naturally occurring concentrations. When mixed with DON at natural ratios, these compounds were potently synergistic with DON. These examples of the interactions between mycotoxins are illustrated in Figure 2. The interactions between mycotoxins are complicated and difficult to quantify.

Abbreviations : DON: deoxynivalenol, CUL: culmorin,
 DHCAL : 7,8 - dihydrocalonectrin,
 SAM: sambucinol

Figure 2. Synergism between DON and other *Fusarium graminearum* metabolites to *Heliothis zea* (species of caterpillar)

II Classification

II-1 Genus *Fusarium*

Fumonisin is produced by species of the genus *Fusarium*. *Fusarium* species belong to the form class Deuteromycetes, also known as the Fungi Imperfecti. *Fusarium* species are common field fungi with a worldwide distribution and are routinely found growing on grasses and cereal crops such as oats, wheat, rye, and corn. They are probably the most important toxin-producing fungi of the northern temperate regions and are commonly found on cereals grown in the temperate regions of America, Europe, and Asia¹¹. *Fusarium* are responsible for a variety of diseases of plants, livestock, and man including Fusarium head blight, Porcine Pulmonary Edema, Equine Leukoencephalomalacia, and Alimentary Toxic Aleukia.

Many compounds with known or suspected toxic effects have been isolated from the *Fusarium* species, the structures of a number of the more well known compounds are shown in Figure 3²⁷. The profile of toxins produced varies from one *Fusarium* species to another, and there is also some variation within species. Table 1 shows the toxin production of different *Fusarium* species grown in cultures of rice, cereals, or agar²⁸. The interest in the mycotoxin-producing species of *Fusarium* is expanding globally due to an increase in the number of mycotoxins that are both economically and medically threatening that have been isolated recently.

Figure 3. Some metabolites produced by *Fusarium* species.

Species*	Toxin production**
<i>F. acuminatum</i>	T-2, HT-2, DAS, MAS, MON, NEO
<i>F. avenaceum</i>	MON, FUS C
<i>F. crookwellense</i> (= <i>F. cerealis</i>)	NIV, FUS X, ZEN, FUS C
<i>F. culmorum</i>	DON, ZEN, NIV, FUS X, FUS C, A-DON
<i>F. equiseti</i>	DAS, ZEN, FUC
<i>F. graminearum</i>	DON, ZEN, A-DON, NIV, FUS X, FUS C
<i>F. oxysporum</i>	MON
<i>F. poae</i>	DAS, MAS, NIV, FUS X, T-2, HT-2, FUS C
<i>F. proliferatum</i>	FUM, MON
<i>F. subglutinans</i> (= <i>F. sacchari</i>)	MON
<i>F. sambucinum</i>	DAS, MAS
<i>F. torulosum</i>	WOR
<i>F. semitectum</i> (= <i>F. incarnatum</i>)	ZEN
<i>F. sporotrichioides</i>	T-2, HT-2, DAS, NEO, FUS C
<i>F. tricinctum</i>	FUS C
<i>F. moniliforme</i> (= <i>F. verticillioides</i>)	FUM, FUS C

* Within brackets are alternative names.

** Bold letters indicate important toxin production. Abbreviations: A-DON: acetyldeoxynivalenol, DAS: diacetoxyscirpenol, DON: deoxynivalenol, FUC: fusarochromanone, FUM: fumonisins, FUS C: fusarin C, FUS X: fusarenon X, MAS: monoacetoxyscirpenol, MON: moniliformin, NEO: neosolaniol, NIV: nivalenol, WOR: wortmannin, ZEN: zearalenone, T-2: T-2 toxin, HT-2: HT-2 toxin

Table 1. Toxin Production by *Fusarium* species.

Taxonomy of the genus *Fusarium* is complex and difficult to apply, in part because of the use of different taxonomic systems by different mycologists. This issue is further complicated by the variability of *Fusarium* species in culture and the fact that they can degenerate, particularly under conditions of repeated subculturing on common laboratory media²⁹. This situation has led to a great deal of confusion in the literature on *Fusarium* mycotoxicology for several reasons. Several *Fusarium* toxins have been named for misidentified producing species. Also, elaborate chemical and pathological studies have been reported in the literature and attributed to incorrectly named species. As well, different fungi are lumped together under one name, while in other instances the same fungus is known by a variety of different names, as is the case with the fungus used in this study, *Fusarium moniliforme*²⁹.

II-2 *Fusarium moniliforme*

In 1881 Saccardo described a fungus found on corn kernels in Italy as *Oospora verticillioides*. Around the same time widespread field outbreaks of an animal disease associated with the consumption of mouldy corn occurred in the United States. The fungus which was implicated as the cause of this disease was described as *Fusarium moniliforme* by Sheldon. Although it was soon pointed out that this was, in fact, the same fungus which had already been identified as *O. verticillioides* by Saccardo, the name was not changed back to *O. verticillioides* and the new combination *Fusarium verticillioides* (Sacc.) Nirenberg was not widely accepted²⁹. However, recently there has a publication by O'Donnell et al. which presents a strong argument in favour of *F.*

*verticillioides*³⁰. As a result there has been some confusion over this matter in the literature. Although there continues to be some debate over the issue, *F. moniliforme* Sheldon is currently the name accepted by several authorities on the topic^{29,31} and will be the name used in this study. The concentration and type of mycotoxins and other secondary metabolites that are produced by this fungus vary between strains of *F. moniliforme*. The most common groups of compounds produced by *F. moniliforme* are the fusarins and the fumonisins. Fusarins are C-24 mixed-function compounds and have been shown to be products of combined amino acid and polyketide biosynthesis²⁷. Of the five fusarins that have been characterized (fusarin A, C, D, E and F), only fusarin C has been reported to be mutagenic. The other fusarins have either shown no activity or have not yet been investigated³².

III Fumonisins

III-1 History of the fumonisins

Fumonisins are the most recently discovered of the major mycotoxins. Fumonisins are produced by *F. moniliforme* and *F. proliferatum* and several uncommon *fusaria*^{33,34,35}. Fumonisins were first isolated in 1988 by a South African research group who were investigating the cause of elevated occurrence of human esophageal cancer in parts of Southern Africa³⁶. Shortly thereafter fumonisin B₁ was also isolated as macrofusine by another research team looking for the cause of a well known disease of horses, equine leukoencephalomalacia (ELEM)³⁷. Since that time several other fumonisins have been isolated (Fig. 4)^{36,38,39,40,41,42}.

Figure 4. Structures of fumonisins isolated to date.

Fumonisinins are a family of C-20 long-chain amino polyols esterified with two tricarboxylic acid side chains. Eight fumonisin structures have been identified to date as part of three series. The A-series fumonisinins, FA₁ and FA₂, are acetylated on the amino group while the B-series, FB₁, FB₂, FB₃, and FB₄ have a free amine. The C-series fumonisinins, FC₁ and FC₂, lack the amino-end terminal methyl group possessed by the other two types. The fumonisinins within a series differ only in the number and position of free hydroxyl functions. For example, FB₁ has hydroxyl groups at positions 3, 5, and 10 while FB₂, FB₃, and FB₄ are missing the hydroxyls at positions 10, 5, and both 5 and 10 respectively. Fumonisinins bearing the same number have the same arrangement of hydroxyl groups. For instance, fumonisin A₁ is the acetylated version of fumonisin B₁.

The A-series, consisting of N-acyl derivatives, may be produced during the isolation,²⁷ however, more recent reports have put more weight on a natural origin. The most abundant of the fumonisinins is usually FB₁. However, a few isolates have been found to produce more FB₂ than FB₁. The amounts of FB₂ and FB₃ found are usually about 15-25 % that of FB₁, with other fumonisinins produced in even smaller quantities³. The biosynthesis of fumonisinins appears to involve formation of a polyketide from acetates and the addition of glutamic acid, methionine, and serine⁴³.

III-2 Natural Occurrence of the Fumonisin

F. proliferatum and *F. moniliforme* are the primary producers of fumonisins and are also the most common fungi associated with corn, therefore fumonisins are widely distributed in corn and corn products. These fungi can be recovered from most maize kernels, including those that appear healthy^{44,45,46}. These species cause Fusarium kernel rot which is promoted by warm, dry growing seasons and/or insect damage⁴⁷. Significant accumulation of fumonisins in corn occurs in warm, dry years when weather conditions favour fusarium kernel rot. There is also a strong relationship between insect damage and *fusarium* kernel rot. A field survey demonstrated that the incidence of the European corn borer increased *F. moniliforme* disease and fumonisin concentrations⁴⁸. These studies indicate that both temperature stress and insect damage are positively correlated with the increase in fumonisin concentration in corn crops.

III-3 Toxicity of the Fumonisin

Fumonisin B₁ is the most abundant representative of the fumonisins¹³, therefore most of the toxicological studies have been focussed primarily on it. The other fumonisins are thought to have similar modes of action. Fumonisin are the causative agents of several toxicoses in animals that result from the ingestion of mouldy corn or corn-based feeds. The diseases observed vary considerably among the different species of animals affected.

The horse is the species which is the most sensitive to the toxic effects of fumonisin poisoning. Equine leukoencephalomalacia (ELEM) is a disease of Equidae long thought to be associated with the ingestion of mouldy feed contaminated with *F. moniliforme* and has now been conclusively demonstrated to be caused by fumonisins^{49,50}. ELEM involves a massive liquefactive necrosis of the cerebral hemispheres. The symptoms begin with lethargy, head pressing, and loss of appetite, and over a period of days progress to convulsions and death⁵¹. Porcine pulmonary edema (PPE) is an unusual disease in pigs which is characterized by recumbency and death. At necropsy the pigs are found to have severe pulmonary edema and hydrothorax⁵¹. PPE has been shown to be caused by both pure fumonisin and *F. moniliforme* culture material and maize containing fumonisin^{52,53}. At lower exposure both kidney and liver damage has been reported in swine⁵⁴.

Fumonisin have also been shown to be nephrotoxic, hepatotoxic and hepatocarcinogenic to laboratory rats⁵¹. Exposure to fumonisin-contaminated corn has recently been directly linked to the elevated esophageal cancer rates in the Transkei⁵⁵.

Fumonisin are considered to be poor initiators and are neither mutagenic nor genotoxic^{56,57}, but are potent cancer promoters⁵⁸.

There is currently not enough information to determine with certainty if fumonisins are human carcinogens. In 1993 the IARC monograph 56 working group examined the data on fumonisins. They concluded that there was sufficient evidence in experimental animals for the carcinogenicity of cultures of *F. moniliforme* that contained significant

amounts of fumonisins and that there was limited evidence in experimental animals for the carcinogenicity of FB₁. Overall, it was decided in 1993 that fumonisins were possibly carcinogenic to humans ⁴.

Fumonisins appear to be stable in most processed foods ^{59,60}. There is no technique presently available for detoxifying fumonisin-contaminated corn and corn products. Guidelines for safe levels of fumonisins in food and feed in Canada or elsewhere do not currently exist ⁴.

III-4 Biological Activity of the Fumonisins

The structural similarity of fumonisin B₁ (4) to sphinganine (5) led Wang et al.⁶¹ to hypothesize that fumonisins may disrupt sphingolipid metabolism. At the moment there are considerable data supporting the hypothesis that fumonisin-induced disruption of sphingolipid metabolism is an important step in the cascade of events leading to altered cell growth, and cell injury observed in vitro and toxicity observed in vivo.

Although the mode of action of fumonisins cannot yet be traced from the initial disruption of sphingolipid metabolism through to the toxic and carcinogenic endpoints observed in many species the correlation between the two is well established ⁶².

In order to understand how disruption of sphingolipid metabolism might contribute to the disease conditions associated with the consumption of fumonisins, it is first necessary to understand how sphingolipids are biosynthesized under normal conditions. Although the pathways of sphingolipid metabolism have not been as well studied as other classes of lipids, typically, biosynthesis proceeds via the reactions described below and shown in Figure 5 ⁶³.

The first is the condensation of serine with palmitoyl - CoA by serine palmitoyltransferase. The resulting 3-ketosphinganine is reduced to sphinganine using NADPH. Sphinganine is acylated to dihydroceramides (also called N-acylsphinganine) by ceramide synthase (sphingosine (sphinganine) N-acyltransferase) using various fatty acyl CoA's. Headgroups (e.g. phosphorylcholine, glucose, etc.) are subsequently added to the 1-hydroxy group. It is not clear when the 4,5-trans-double bond of the sphingosine backbone is added, except that it is after acylation of the amino group. Thus, free sphingosine is not an intermediate of *de novo* sphingolipid biosynthesis. Sphingolipid turnover is thought to involve the hydrolysis of complex sphingolipids to ceramides (n-acyl sphingosine), then to sphingosine ⁶³.

Typically, free sphinganine and sphingosine are present in normal tissues and cells in trace amounts ⁶³. This is expected since free sphinganine is a metabolic intermediate in the sphingolipid biosynthetic pathway and free sphingosine is generated primarily as a

consequence of sphingolipid turnover or degradation⁶⁴ and thus, neither should accumulate under normal conditions. Fumonisin, however, potently inhibits sphingosine (sphinganine) N-acyl transferase (ceramide synthase) which catalyzes the acylation of both sphinganine and sphingosine⁶¹ and disrupts sphingolipid metabolism (Fig. 5)⁶³. The term "disruption of sphingolipid metabolism" includes inhibition of sphingosine biosynthesis, depletion of complex sphingolipids, increase in free sphinganine, a decrease in reacylation of sphingosine derived from complex sphingolipid turnover and degradation of dietary sphingolipids, an increase in sphingoid base degradation products (i.e. sphingosine (sphinganine) 1-phosphate, ethanolamine phosphate, and fatty aldehydes), and an increase in lipid products derived from the increase in sphingoid base degradation products⁶⁵. Since the natural concentration of many intermediates and end-products are altered by fumonisins, it can be expected that there will be a diversity of alterations in cellular regulation and, therefore, a number of different endpoints to fumonisin toxicity which are difficult to trace back.

Figure 5. Pathways of sphingolipid metabolism showing sites of disruption by fumonisin.

The difficulty of establishing a complete mechanism(s) of action from ingestion of fumonisins to the appearance of disease conditions is further complicated by the multitude of functions involving complex sphingolipids. The inhibition of sphingosine (sphinganine) N-acyltransferase can be considered to be the first step in a cascade of events which can lead to the various diseases associated with fumonisins. The involvement of sphingolipids as cell membrane components, especially in brain and nervous tissue, could explain the effects of fumonisins on horses. ELEM results in extensive degeneration of brain tissue. Sphingolipids are also involved in cell-cell communication, cell growth, differentiation, and transformation, and they inhibit protein kinase C. The influence of fumonisins on some of these steps may play a role in their apparent carcinogenicity and other pharmacological effects³. Thus, fumonisin-induced disruption of sphingolipid metabolism could contribute both directly and indirectly to the diseases known, or thought, to be caused by the consumption of fumonisin.

IV Detection Methods for the Fumonisins

Fumonisins are white hygroscopic powders in their pure form. They have not yet been crystallized. Fumonisins are soluble in water, acetonitrile-water and methanol but stable in water and acetonitrile-water (1:1) and unstable in methanol. Fumonisins exhibit

no UV absorption except for end-absorption. The Chemical Abstracts name for fumonisin B₁ is 1,2,3-propanetricarboxylic acid,1,1-[1-(12-amino-4,9,11-trihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediyl] ester. Fumonisin B₁ is also known as macrofusine and has a molecular mass of 721 amu and a chemical formula of C₃₄H₅₉NO₁₅³⁶.

IV-1 Thin layer chromatography

Thin layer chromatography (TLC) has been a widely used technique for the initial screening of fumonisins either directly from liquid culture or after extraction from a solid culture. There have been many solvent systems reported for both normal phase silica TLC and reverse-phase (C-8 or C-18) TLC. Because fumonisins do not have a chromophore, after development the TLC plate must be sprayed (often followed by heating) with a reagent so that a coloured or fluorescent derivative is formed and the fumonisins are visualized.

IV-2 High Performance Liquid Chromatography

The method most commonly used for analysis of fumonisins, is high performance liquid chromatography (HPLC) with fluorescence detection. A number of fluorescent derivatives have been reported to be useful for this purpose including fluorescamine⁶⁶, naphthalene-2,3-dicarboxyaldehyde/potassium cyanide⁵⁹, 4-fluoro-7-nitrobenzo-2-oxa-

1,3 diazole⁶⁰, 9-fluorenylmethyl chloroformate and o-phthaldialdehyde (OPA). The OPA method is the most widely used and has been the subject of international collaborative trials⁶⁷.

IV-3 Gas Chromatography

Hydrolysis of samples to the aminopentol chain followed by gas chromatographic (GC) analysis of the trimethylsilyl or trifluoroacetate derivative by flame ionization detection or mass spectrometry has been reported⁶⁸ but is not widely used.

IV-4 Immunochemical

There have also been reports of immunochemical methods using direct and indirect assays^{69,70,71}. Detection limits using these methods have been reported to be 5 $\mu\text{g/kg}$.

V Separation Methods and Structural Determination

V-1 Chromatography

Separation of the fumonisins from the other components of a fungal culture is carried out via a variety of chromatographies. Many different types and sizes have been used and

generally more than one type of adsorbant is required to obtain pure fumonisin. Separation of the fumonisin-containing fraction from the remainder of the culture involves silica columns combined with strong anion exchange ⁷², a c-18 reverse phase column ⁵³, or some combination of both ⁷³. There have also been a few reports of immunoaffinity columns being used for isolation of analytical amounts ⁵⁵. Once the fumonisin fraction has been separated from the other components of the culture the individual fumonisins can be separated from each other with some type of reverse phase chromatography .

V-2 Mass Spectrometry

As an analytical tool for fumonisins, mass spectrometry was initially only used as a detector following gas chromatographic separation of hydrolysed fumonisins ⁶⁸. MS has since been used in a variety of ways . Fast atom bombardment has been used to acquire protonated molecular ions ⁷⁴. Particle beam interface has been reported to produce both chemical ionization and electron impact spectra, which provides more structural information ⁷⁵. Most recently the electrospray interface has led to the greatest advance in the use of MS for fumonisin determination because, coupled to an LC, it allows detection of fumonisins after separation by the LC system without the need for derivatization ⁷⁶.

V-3 Nuclear Magnetic Resonance Spectroscopy

The main tool for characterizing the fumonisins is currently nuclear magnetic resonance (NMR) spectroscopy. Fumonisins have a number of characteristic features which aid in the structural elucidation of new fumonisins and compounds which appear to be fumonisin-like by a variety of NMR techniques. In addition to the information obtained from ^1H and ^{13}C spectra, two dimensional NMR experiments such as $^1\text{H}/^1\text{H}$ homonuclear (COSY) and $^1\text{H}/^{13}\text{C}$ heteronuclear correlation (HETCOR) spectra can yield very specific structural data.

The ^1H spectrum displays several key signals or groups of signals common to fumonisins (Fig. 6) . The terminal methyl group on the amino-end of the molecule appears as a sharp doublet at 1.27 ppm. Its absence is characteristic of the C-series. The two methyl groups on the carbon backbone (C-21 and C-22) are each sharp doublets around 0.9 ppm. The other terminal methyl group is also in this region of the spectrum, however it is a triplet and slightly further upfield.

The two groups of signals arising from the protons on the carbons to which the side chains are attached are very easy to identify as they have a characteristic pattern. The proton on C-15 gives rise to the signal occurring at about 4.9 ppm and is composed of two doublets. The proton on C-14 is always further downfield at about 5.2 ppm and consists of three doublets. The identification of these two groups of peaks is made easier by the fact that they are the only signals in this region of the fumonisins spectra.

The most distinctive groups of signals in a proton spectrum for recognizing a

fumonisin-like compound are the signals which arise from the protons on the carbons bearing the hydroxyl groups (Fig. 7) . This aids in identifying the compound as fumonisin-like as well as differentiating between the individual fumonisins. The proton-proton connectivity pattern can be determined by two-dimensional $^1\text{H}/^1\text{H}$ correlation spectroscopy (COSY).

The ^{13}C spectrum also provides valuable structural information. Although not as sensitive, the ^{13}C spectrum is often easier to interpret because no ^{13}C - ^{13}C coupling is observed. Because the resonances due to each carbon in the molecule are discreet, ^{13}C NMR can detect small changes in structure, or impurities, which could be overlooked due to insufficient resolution if only the ^1H spectrum was examined. For example, if the impurities are very similar to the fumonisins in structure or have NMR peaks in the methylene region of the spectrum (2-3 ppm), they are difficult to detect by ^1H NMR ³⁸.

Figure 7. Expanded ^1H spectrum of FB_1 , FB_2 and FB_3

VI Global Concern

The Food and Agriculture Organization of the United Nations estimates that 25 % of the world's food crops are affected by mycotoxins every year ⁷⁷. Although the mycotoxins produced by species of *Fusarium* account for only a portion of this contamination the economic impact of *Fusarium* infestation can be substantial. This results in major economic losses for developed countries. For developing countries, in addition to the economic hardship that can be caused by *Fusarium* contamination of crops there is a much higher risk to human health. The increased health risk is due mainly to a much higher consumption of corn or corn based foods, a lack of testing, and absence of an alternate food source.

VII Fumonisin in Canada

Although occurrence is global, *Fusarium* mycotoxins tend to be more prevalent in the more temperate regions of the world. *Fusarium* fungal growth and mycotoxin production, to a large extent, are dependent on the prevailing weather conditions ⁷⁸. Therefore, fumonisin levels vary from year to year and tend to be elevated in warm, dry growing seasons. The occurrence of elevated levels of fumonisins in Ontario, Canada (a cool maize-growing region) appears to be limited to drought-stressed fields ⁷⁸.

VIII Scope of this Project

Because there can be more toxicity associated with *F. moniliforme* culture extracts than can be attributed to the known fumonisins⁵⁵, it is thought that there are other toxic compounds present. It was the aim of this research work to isolate and characterize as many of these minor fumonisins as possible.

These compounds have proven difficult to isolate as they are present in very small quantities, have very similar structures, and possess no chromophores. Hence, separation is carried out by HPLC without visualization and each fraction must be analysed after elution. This is done by analytical HPLC of fluorescent derivatives. This method is time consuming and results in the loss of some of the isolated compound which is very undesirable when the concentration is already very low. Thus, it was the further aim of this project to determine if another method was available for the chromatographic separation of fumonisins which either utilized a removable, stable, derivative or did not involve derivatization.

RESULTS AND DISCUSSION

I Introduction

The study of the minor fumonisins produced by *F. moniliforme* and the development of better techniques for the analysis of the fumonisins required a large supply of crude fumonisin mixture. This was obtained from a large-scale fermentation of *F. moniliforme* under conditions which optimized fumonisin production by the fungus. Production of secondary metabolites requires specific nutrient limitations as well as certain O₂, pH, osmotic, and sometimes temperature conditions⁷⁹. Fermentation in a liquid medium, as opposed to a solid substrate, reduces the number of contaminants and makes the clean-up procedure easier. The *Fusarium moniliforme* isolate (NRRL 13616) used for this project was a subculture of an isolate originally obtained from corn in Illinois.

II Obtaining the Crude Fumonisin Mixture

After the fermentation was complete the culture material was filtered and the pH was adjusted to 6.0. The culture filtrate was passed through a series of columns to yield a crude mixture of fumonisins. The yield of crude fumonisin mixture is typically about 265 mg/L⁸⁰.

The three most abundant fumonisins, FB₁, FB₂, and FB₃, account for 95-99% of the total fumonisins produced in an *F. moniliforme* liquid culture. These three compounds have already been characterized and were therefore of no interest to this work. Since FB₁ was present in such overwhelming concentration in comparison to the minor metabolites, which were the focus of this project, it obscured these minor metabolites and made them very difficult to detect. For this reason, it was first necessary to remove the FB₁ from the mixture of fumonisins.

The mixture of fumonisins was dissolved in acetonitrile:water (28:72) and chromatographed on a C-18 preparative HPLC column using a gradient of acetonitrile:water (Table 2), and fractions collected. Fumonisin B₁ elutes isocratically at 33% acetonitrile, before any of the other known fumonisins, and most of it was separated from the remainder of the fumonisin mixture. An aliquot of each of the fractions was applied to a silica-gel TLC plate, developed, and sprayed to visualize the fumonisins. The fractions containing mainly FB₁ were combined. The mixture of fumonisins, now containing much less FB₁, will be referred to as the "crude mixture". The remaining FB₁ was still the major component of the fumonisin mixture, but many other peaks were now visible in the chromatogram of the OPA-derivatized crude mixture.

Table 2. Gradient used to remove the majority of FB₁.

<u>Time</u>	<u>Acetonitrile</u>	<u>Water</u>
0	25	75
3	28	75
10	33	67
50	33	67
55	100	0
68	100	0

Table 3. Gradient used for analysis of OPA-derivatized fumonisins.

<u>Time</u>	<u>Solvent A*</u>	<u>MeOH</u>
0	90	10
10	90	10
40	30	70

* Solvent A = 68% MeOH / 32% 0.1 M sodium phosphate, pH adjusted to 3.35 with phosphoric acid

Table 4. Gradient used for analysis of diazomethane-derivatized fumonisins by LC-MS.

<u>Time</u>	<u>Solvent A**</u>	<u>Solvent B***</u>
0	80	20
12	20	80

** Solvent A = 0.05 % TFA in water:water:MeOH (1:1:1), pH 3

*** Solvent B = MeOH:CH₃CN (1:1)

III New Fumonisin From *Fusarium moniliforme*

III-1 Isolation and Purification of iso-FB₁

Because of their low concentration in the mixture, the minor fumonisins were not visible by TLC. For this reason, the minor fumonisins could not be separated based on the TLC method available for the separation of the three major fumonisins. A derivatization with OPA and analysis by analytical HPLC replaced the TLC method used to separate FB₁, FB₂, and FB₃.

A 0.5175g sample (SM26) of the crude fumonisin mixture was dissolved in 5 mL of acetonitrile:water (1:1) and loaded onto a C-18, reverse phase preparatory column. The same gradient of acetonitrile - water that had been used to remove most of the FB₁ (Table 2), flowing at 20 mL/min. was used to separate the FB₁ that was still present and fractionate the remainder of the sample. The first 44 minutes of the gradient were collected together in a round bottom flask while the rest of the sample was collected in one minute fractions labelled SM26-45, SM 26-46, etc.

TLC analysis was carried out and confirmed that the FB₁ had been collected in the initial 44 minute portion and the remaining fractions were relatively free of FB₁. Each of the other 24 fractions (SM26-45 to SM26-68) were individually derivatized with OPA and analysed by analytical HPLC using a C-8 reverse-phase column with a binary gradient (Table 3) flowing at a rate of 1 mL/min and a fluorescence detector.

Sample SM26-45 contained one large peak at a retention time similar, although not identical to that of an FB₁ standard. Fractions SM26-46 to SM26-50 also contained the same main peak in addition to some small impurities. This compound eluted 1.4 minutes after an FB₁ standard under these conditions. Fractions SM26 46-50 were combined to increase the concentration.

The combined sample was derivatized with diazomethane then analysed by LCMS. The HPLC system, with a C-18 reverse phase column from which the sample was eluted with a gradient consisting of acetonitrile, water, and methanol in the presence of 0.05% trifluoroacetic acid (Table 4), was interfaced, via a particle beam, with the mass spectrometer to yield chemical ionization spectra.

The mass spectrum revealed that this compound had the same molecular ion as FB₁. Co-injection into the LCMS system of the SM26 46-50 combined sample with an FB₁ standard showed two distinct peaks which confirmed that although this compound had the same molecular mass as FB₁, it was definitely a different compound. NMR analysis of the sample was inconclusive. Although it was possible to determine that this compound had the characteristic signals of a fumonisin and appeared to be similar to FB₁, no further structural information could be obtained because the sample was too small and too impure.

Another aliquot (SM37) of ~ 0.5 g of crude sample was dissolved in 5 mL of 28% CH₃CN. The same basic procedure was used as for the samples labeled SM26 with a few exceptions. The gradient was altered slightly so that the concentration of acetonitrile was 28% at the beginning of the run rather than 25 %. As well, the column had different

dimensions. For the SM26 run a C-18 RP column with an internal diameter of 20 mm was used, a column with the same packing and length but an internal diameter of 30 mm was used for SM37 to obtain better resolution. The fractions were collected in slightly different ways. Fractions 0-30 were collected together rather than 0-44 because there could have been a compound, or compounds, eluting immediately after FB_1 that was being included with the FB_1 fraction. All fractions were derivatized with OPA and analyzed under the same conditions as the SM26 samples. The fraction SM37-38 was fairly clean, with only one major peak. This fraction eluted under similar conditions (% acetonitrile) as the compound contained in SM26(46-50) and corresponded approximately to the fraction SM26-6. The difference in retention times was likely due to the differences in column size and gradient programming.

Sample SM37-38 was analyzed by the OPA method and had the same retention time relative to an FB_1 standard as SM26 (46-50). Both SM26 (46-50) and SM37-38 elute 1.4 minutes after FB_1 under the conditions described for OPA analysis. Based on this info, the two compounds are likely the same.

Sample SM37-38 was analyzed by direct probe electron impact (EI) MS. This sample showed a fragmentation pattern very similar to that of FB_1 (Fig. 8). The notable difference was the absence of two peaks at 236 and 254 in this sample, which were present in FB_1 . This information indicated that this compound was probably an isomer of FB_1 which differed in such a way that it lost water more quickly than FB_1 . Because the molecular ion was not visible in the EI spectrum, the sample and an FB_1 standard were each analyzed by fast atom bombardment (FAB) spectrometry. The FAB spectrum

indicated that this compound did have the same M^+ as FB_1 (Fig. 9) . At this point, it appeared very likely that SM26 (46-50) and SM37-38 were the same compound, which was a structural or stereochemical isomer of FB_1 . The compound was therefore designated iso- FB_1 .

The small size of the sample prevented analysis by NMR . The same method used to obtain SM37-38 was repeated four additional times to ensure that the method was reproducible and to increase the amount of this compound. All fractions were derivatized and analyzed by the OPA method. All fractions which appeared (by OPA) to contain iso- FB_1 were combined even if they were not completely pure.

The combined sample consisting of all of the fractions containing iso- FB_1 was cleaned up further by repeating exactly the same HPLC purification method used to obtain SM37-38 . These fractions were labeled as SM51-0....68. This clean-up yielded fractions SM51-(35-38) which contained a relatively large amount of iso- FB_1 (as determined by OPA) and appeared to be very clean. Comparison to a mixture of FB_1 , FB_2 , and FB_3 standards and co-injection with FB_1 confirmed that it was not one of these known compounds. The four fractions were combined and characterized.

Figure 8. Comparison of LCMS(EI) of FB_1Me_4 and $\text{iso-FB}_1\text{Me}_4$.

Figure 9. Comparison of FABMS of iso - FB₁ and FB₁.

III-2 Structural Elucidation of iso-FB₁

Structure 6 (Fig. 10) was assigned to this compound, designated iso-FB₁, based on the following analysis. Fast atom bombardment mass spectrometry (FABMS) data showed that 6 had a molecular weight of 721 amu (Fig. 9), identical to that of FB₁. MSEI (Fig. 8) showed a fragmentation pattern that was very similar to that of FB₁ but not identical. From the MSEI it was possible to learn that the difference in structure likely occurred in the C1 to C5 section. The absence of the two peaks at 236 and 254 indicated the rapid loss of two water molecules suggesting a change involving the hydroxy functions positioned on C3 and C5 in FB₁.

As suspected from the mass spectrometric analysis, nuclear magnetic resonance investigations confirmed that iso-FB₁ and FB₁ had similar structures. The noteworthy differences occurred in the chemical shifts associated with the C-1 to C-6 region of the molecule (Table 5, p. 47)⁸¹.

A large doublet was observed at 1.30 ppm in the proton spectrum of 6 (Fig. 10, p.48). This is characteristic of a signal associated with a terminal methyl group. The chemical shift for this peak was not significantly different from the signal corresponding to H-1 in the proton spectrum for FB₁. Based on this evidence the signal at 1.30 ppm in 6 was assigned to H-1. The COSY (Fig. 11, p. 49) indicated that H-1 was coupled to the proton responsible for the signal at 3.43 ppm which must, therefore, be H-2. This is a sizable down field shift from the 3.14 ppm signal reported for H-2 in FB₁ which may indicate that an electron withdrawing group, such as a hydroxyl, may now be closer to position 2 than in FB₁. Again referring to the COSY, H-2 is also coupled to the proton giving rise to the signal found at 3.55 ppm, indicating that to be H-3. This upfield shift from the value of 3.74 ppm observed for H-3 in FB₁, but still within the range that would be expected for a proton of a secondary alcohol, indicates that C-3 in 6 also bears a hydroxyl group.

Further inspection of the coupling in the COSY spectrum showed that the signal at 3.65 ppm in the ¹H spectrum was associated with the proton on C-4. This is a large downfield shift in the position of the signal for this proton as compared to the signal for H-4 located at 1.55 ppm in FB₁. This chemical shift places it in the region of the spectrum where a secondary alcohol should be found as opposed to the signal for H-4 in FB₁, which indicates a -CH₂- group.

Conversely, the signal corresponding to H-5 in **6** was shifted far upfield from 3.84 ppm observed in the spectrum for FB₁ to 1.45 ppm, indicating that it was no longer associated with a hydroxyl function. The signal associated with H-10 exhibited the same chemical shift and couplings as seen for H-10 in the proton spectrum of FB₁. Other identifiable ¹H NMR signals matched those of FB₁ closely.

The information obtained from the ¹H and COSY spectra indicated that compound **6** was a structural isomer of FB₁ which has hydroxyl functions on C-3, C-4, and C-10 rather than on C-3, C-5, and C-10 as in FB₁. The ¹H NMR spectrum also indicated that the remainder of the molecule was identical to FB₁.

After the peaks in the ¹H spectrum were assigned, it was then possible to correlate the protons to the associated carbons using a ¹H/¹³C correlation experiment (Fig. 12, p.50). The proposed structure, **6**, is further supported by the ¹³C spectrum (Fig. 13, p.51). The signals that arise from C-1 and C-2 in **6** are found at 14.2 ppm and 51.8 ppm, respectively, which differ from the corresponding values of 16.0 ppm and 53.7 ppm reported for FB₁.

The peak associated with C-3 is shifted slightly downfield from 70.3 ppm in FB₁ to 73.2 ppm in iso-FB₁ because it is now adjacent to another hydroxyl group. The signal arising from C-4 shows a very large downfield shift from 41.7 ppm to 72.2 ppm, as expected, because it now carries a hydroxyl function. Conversely, the signal for C-5 shows a very large upfield shift from 68.4 ppm to 34.2 ppm because it is now a simple methylene; the peak associated with C-6 shows a smaller upfield shift from 39.1 ppm to 30.6 ppm as it is now two carbon atoms away from the hydroxyl function rather than just one. No other signals showed deviations in chemical shifts which were significantly

different from those observed in the ^{13}C spectrum of FB_1 .

The presence of positive NOE's (7) observed between H-1 and H-2, between H-2 and H-3 and H-4, but not between H-3 and H-4, in addition to consideration of the bond angles suggested by the J-values indicated the relative stereochemistry of H-4 to be *cis* with respect to H-3. Assuming that the absolute stereochemistry of **6** at the amino function is the same as that of FB_1 ³⁸, the absolute stereochemistry of this metabolite is as shown below.

7

Although it seemed highly unlikely that this compound was a product of the isolation procedure, a sample of the culture filtrate from which iso- FB_1 was originally obtained was spiked with pure iso- FB_1 . A peak with the same retention time as iso- FB_1 was observed in a crude culture filtrate analysed by HPLC after OPA derivatization (Fig.14, p.52). Addition of isolated iso- FB_1 to this culture filtrate produced an increase in the size of this peak. This is positive evidence that iso- FB_1 is a genuine metabolite of *F. moniliforme* and not an artifact of the isolation procedure.

<u>Position</u>	<u>^{13}C</u>	<u>^1H</u>
1	14.2	1.30 (d, $J_{1,2} = 6.8$)
2	51.8	3.43 (dq, $J_{2,3} = 4.3$)
3	73.2	3.55 (dd, $J_{3,4} = 4.0$)
4	72.2	3.65 (m, $J_{4,5} = 12.6, 4.5$)
5	34.2	1.45; 1.55
6	30.6	1.34 (br m)
7	26.3	1.38; 1.54
8	26.5	1.32; 1.52
9	38.9	1.40 - 1.60
10	70.0	3.62 (m)
11	44.5	1.08; 1.50
12	27.0	1.83 (m)
13	37.0	1.45; 1.66
14	72.7	5.16 (ddd, $J_{14,13} = 11.3$; $J_{14,15} = 3.7$)
15	78.9	4.94 (dd, $J_{14,15} = 3.7$; $J_{15,16} = 8.0$)
16	34.9	1.70 (m)
17	32.9	1.08; 1.43
18	29.8	1.18; 1.33
19	23.9	1.22; 1.38
20	14.4	0.89 (t, $J_{20,19} = 7.1$)
21	20.8	0.96 (d, $J_{21,12} = 6.7$)
22	16.1	0.93 (d, $J_{22,16} = 6.8$)
23	173.5	
24	37.3	2.45; 2.71
25	39.7	3.14
26	38.0	2.48; 2.64
27	177.2	
28	178.7	
29	173.2	
30	37.0	2.59; 2.79
31	39.6	3.14
32	37.7	2.51; 2.70
33	176.7	
34	178.2	

Table 5. NMR assignments of iso-FB₁ in CD₃OD (δ , ppm, J , Hz)

Figure 10. ^1H spectrum of iso-FB₁.

Figure 11. COSY of iso-FB₁

Figure 12. $^1\text{H}/^{13}\text{C}$ HETCOR of iso-FB₁.

Figure 13. ¹³C spectrum of iso-IB₁.

Figure 14A *F. moniliforme* culture filtrate.
14B *F. moniliforme* culture filtrate spiked with iso-FB₁.

III-3 Other Fumonisin Isolated From *Fusarium moniliforme*

In addition to iso-FB₁, other new compounds were obtained from the same crude mixture of fumonisins. Because these compounds were only present in small amounts and not pure, they could not be fully characterized. However, tentative structures, **8** and **9**, are proposed from the data for two of these compounds. Both compounds were isolated from the same preparative HPLC separation that yielded iso-FB₁, SM37.

(A) Characterization of FC₃

The first of these compounds (**8**) was found in fraction 52 of sample SM37 and was therefore labelled SM37-52. SM37-52 was derivatized with OPA and analysed by HPLC in the same manner described for iso-FB₁. SM37-52 eluted 0.7 minutes before FB₃ in the OPA system which suggested that it may be similar to FB₃ in structure.

SM37-52 was derivatized with diazomethane and analysed by HPLC-MS, in chemical ionization mode, as described for iso-FB₁. An M⁺ ion of 748 was observed as well as the M⁺ ion for FB₃ at 762. This meant that compound **8** had a molecular weight 14 mass units lower than FB₃, indicating the loss of a methyl group. Inspection of the ¹H and ¹³C NMR spectra confirmed that fraction SM37-52 was a mixture of FB₃ and a similar compound.

The ¹H spectrum of the mixture (Fig. 15, p.58) was not well resolved but it was possible to determine that the hydroxy function on C-10 was present at 3.61 ppm as

expected²⁸. The signal for H-3 was observed at 3.76 ppm rather than 3.44 ppm as for FB₃²⁸. This would be expected if the terminal methyl group was missing. It is similar to the chemical shift difference reported between H-3 in FB₁ (3.74 ppm)²⁸ and H-2 in FC₁ (4.00 ppm)⁴². The H-1 signal which is expected at 1.26 ppm for FB₃ was observed for this mixture but was much reduced in size, suggesting that the compound other than FB₃ was missing this amino terminal methyl group. This new compound was assigned the name fumonisin C₃ (FC₃).

This theory was further supported by the ¹³C NMR spectrum (Fig. 16, p.59). The ¹³C spectrum for SM37-52 was generally similar to FB₃ with a few key exceptions (Table 6, p.60). There were two peaks in the ¹³C spectrum of SM37-52 which could not be assigned to FB₃. One of these peaks was at 46.1 ppm. This was likely to be C-1 in FC₃ which corresponds to C-2 of FB₃, also visible in the spectrum at a chemical shift of 53.4 ppm. A similar relationship has been observed between the chemical shifts of C-2/C-1 of FB₁ (53.7)²⁸ and FC₁ (44.5)⁴².

The second of the two peaks was observed at 70.0 ppm, the region where a carbon bearing a hydroxyl group can be expected, and was assigned C-2 (C-3 in FB₃). A smaller peak was also observed at 73.0 ppm which is where C-3 for FB₃ was expected. The peak at 73.0 ppm was the same size as the peak at 53.4 ppm suggesting that they belonged to the same compound. Other signals in the spectrum closely matched those reported for FB₃²⁸. Based on the evidence provided by OPA analysis, MS, and NMR the compound which comprises the major part of sample SM37-52 was tentatively identified as FC₃ and assigned structure 8.

8

Following the tentative identification of this compound as FC₃ (Mar 1996), a paper was published (Nov 1996)⁴² which reported the isolation of FC₃ from a different species of *Fusarium*, *F. oxysporum*. The chemical shifts reported closely matched the values reported here and have been used for confirmation.

(B) Characterization of 6-OH iso-FB₁

The second of the two tentatively identified compounds (9) was found in fraction 45 of sample SM37 after it had been separated preparatively, therefore, it was labelled SM37-45. This fraction was derivatized with OPA and analysed by the HPLC method detailed for iso-FB₁. SM37-45 contained a compound with a retention time 0.5 minutes longer than FB₁ and 0.9 minutes less than iso-FB₁. Sample SM37-45 also contained a

large FB₁ peak indicating that it had not been completely separated from FB₁ during the preparative chromatography.

The ¹³C spectrum (Fig. 17, p.61) of fraction SM37-45 suggested that there were two major compounds present because there were two sets of fumonisin peaks. For example, there was a peak at 53.8 ppm as well as one at 51.2 ppm. This is the region where the signal for C-2 is found in the fumonisins and is a good indicator of how many fumonisins are present because no other peaks are expected in this region.

One set of signals corresponded to FB₁ which contains three hydroxy functions on C-3, C-5, and C-10²⁸. Judging by the number of signals in the 68-74 ppm region of the spectrum, the second fumonisin appeared to contain four unsubstituted hydroxy functions. The chemical shift of C-2 in this compound is very similar to C-2 of iso-FB₁²⁸ (Table 7, p.60) suggesting that two of the hydroxyl groups may have been on C-3 and C-4 as in iso-FB₁.

The ¹H spectrum of SM37-45 (Fig. 18, p.62) confirmed the information obtained from the ¹³C spectrum. The signals for the hydroxyl functions on C-3, C-5, and C-10 of FB₁ were present at the expected chemical shifts²⁸. In addition to the signals expected for FB₁ there was an extra set of signals downfield at 3.94 ppm and another superimposed on the left shoulder of the signal for H-3 of FB₁. Also the signal for H-10 seemed to be twice as large as the signal for H-5 suggesting that it may have been present in both compounds. There was a small peak at 3.44 ppm which may have been H-2 as that is very similar to the chemical shift for H-2 in iso-FB₁ (Table 5, p.47).

LC-NMR analysis performed in another laboratory allowed separation of the ¹H

spectrum of the new compound from that of FB₁. This experiment confirmed that this new fumonisin did contain four hydroxyl groups. TOCSY analysis established that the hydroxyl functions were on C-3, C-4, C-6, and C-10 ⁸². Based on all available data the compound which was a major component of SM37-45 was tentatively named 6-OH iso-FB₁ and assigned structure **9**.

Figure 15. ^1H spectrum of SM37-52

Table 6. Comparison of key ^{13}C chemical shifts (in ppm) of SM37-52, FB_3 , and FC_3 .

	<u>C - 1</u>	<u>C - 2</u>	<u>C - 3</u>
SM37-52 (FC_3)	46.1	70.0	-
SM37-52 (FB_3)	16.1	53.4	73.0
FB_3 ²⁸	16.0	53.5	73.2
FC_3 ⁴²	46.1	69.9	-

Table 7. Comparison of key ^{13}C chemical shifts (in ppm) of SM37-45, iso- FB_1 , and FB_1 .

	<u>C-1</u>	<u>C-2</u>	<u>C-3</u>	<u>C-4</u>	<u>C-5</u>	<u>C-6</u>	<u>C-10</u>
SM37-45 (6-OH iso- FB_1)	14.3	51.2	73.2	72.6	36.5	69.2	71.5
SM37-45 (FB_1)	16.1	53.8	70.5	41.7	68.6	39.1	70.0
iso- FB_1 ⁸¹	14.2	51.8	73.2	72.2	34.2	30.6	70.0
FB_1 ²⁸	16.0	53.7	70.3	41.7	68.4	39.1	69.8

Figure 17. ^{13}C spectrum of SM37-45.

Figure 18. ^1H spectrum of SM37-45.

III-4 Conclusions

A comparison of the fumonisins suggests that the primary sites of substitution for the fumonisins are the amino function at C-2 (C-1 of the FC series) and the hydroxyls at C-3, C-14, and C-15 with a more random pattern of hydroxylation at positions 4, 5 and 10. The unconfirmed characterization of two other fumonisins, one possessing an additional hydroxyl at C-6 (6-OH iso-FB₁) and the other similar to FB₃ but missing the terminal methyl group (FC₃), suggests an even wider variety of possible fumonisin compounds.

The pattern of substitution is significant because intact sidechains and the presence of a free amine are requirements for the toxic effects caused by the fumonisins, however, the degree of cytotoxicity does vary depending on the placement of the hydroxyl groups⁸³. Perhaps the production of many fumonisins with slight structural differences by the fungus keeps the affected organism from developing effective defences. The presence of many different fumonisins in a culture likely have additive, and possibly synergistic, toxic effects as well.

In summary, iso-FB₁ (**6**) has been isolated from *F. moniliforme* and characterized for the first time. **6** is a structural isomer of FB₁ with the hydroxyl groups on C3, C4, and C10 rather than C3, C5, and C10 as in FB₁. Additionally, two other new fumonisins have also been isolated from *F. moniliforme* and have tentatively been assigned structures **8** and **9**. They appear to be FC₃ and 6-OH iso-FB₁ respectively.

IV Development of New Isolation Techniques for Fumonisin

As mentioned earlier one of the major problems with the analysis of fumonisins is the necessity of derivatizing the sample prior to the analysis. The most widely accepted derivatizing agent is ortho-phthalaldehyde (OPA) which produces a fluorescent derivative. This is a fairly effective method for both qualitative and quantitative analyses. It is also quite sensitive and has a low detection limit. However, this method can only be used for analytical purposes and not to monitor the preparative chromatography or to purify compounds because the original compounds are not recoverable after derivatization. This is due to the instability of the derivatives and the nature of the degradative products. Having to monitor each chromatographic fraction by analysis of its OPA derivative is very tedious and leads to the loss of material, which is impractical when so little is available.

For these reasons, one of the goals of this project was to find a chromatographic method for fumonisins which either employed a reversible and non-destructive derivatization process or one which required no derivatization step at all, the latter option being the more desirable.

IV-1 Maleic Anhydride

(A) Literature Search

A search of the literature revealed a rapid, reproducible, and fully reversible derivatization which might be suitable for separating the minor fumonisin metabolites from *F. moniliforme*. Siler and Gilchrist⁸⁴ reported a method for the analysis of phytotoxins from *Alternaria alternata* using maleic anhydride and UV detection. This procedure reportedly met all of the requirements stated previously and the reaction took place at an amine site which is similar to the amine function in the fumonisins (Fig. 19)⁸⁵.

In fact, the *Alternaria* toxins were very similar in structure to the fumonisins.

Figure 19. Reversible derivatization of fumonisins with maleic anhydride.

(B) Derivatization of a Standard FB₁ Sample

A 10 mg sample of pure fumonisin B₁ was dissolved in a minimal amount of distilled, deionized water and adjusted to pH 9.2 with a 0.1 M sodium carbonate solution. A 10:1 molar excess of maleic anhydride to fumonisin (approximately 1.36 mg of maleic anhydride for every mg of fumonisin) was used to ensure complete derivatization. The maleic anhydride crystals were added slowly over a period of 5-10 minutes to the buffered fumonisin solution. It was evident that a reaction was taking place as the solution became turbid and effervescent as the pH dropped. The drop in pH was due to the release of maleic acid from the excess maleic anhydride as the reaction proceeded .

After all of the maleic anhydride had been added, the final pH of the solution was between two and three. The reaction mixture was allowed to remain at this pH for five minutes to ensure that the reaction was complete. Before analysis by HPLC, the pH of the solution was adjusted to between six and seven by dilution with the mobile phase buffer (0.1 M sodium phosphate).

According to the literature ⁸⁴ this mixture should be stable for at least four weeks at this pH if stored at less than 4° C, whereas the OPA derivative is only stable for thirty minutes. This is an important feature of this method because the separation of the crude mixture had to be repeated several times because of the low concentration of the minor metabolites.

(C) HPLC Analysis of the Maleated FB₁ Sample

A sample of the maleated FB₁ was chromatographed on a c-8 reverse-phase HPLC analytical column using the gradient outlined in Table 8 (p.73) at a flow rate of 1 mL/min and with a UV detector at a wavelength of 250 nm. Two peaks were detected, the first at 2.6 minutes and the second at 6.0 minutes. Injection of a blank confirmed that the peak at 2.6 minutes was a solvent peak. The peak at 6.0 minutes was collected.

(D) Demaleation of the FB₁ Sample

The pH of the sample collected at the sixth minute of the gradient was found to be between three and four. This is the pH range necessary for the removal of the maleyl group. Demaleation was carried out by incubating the maleated sample at 37° C for 24 hours in the methanol-phosphate buffer mixture in which it had eluted from the column.

After incubation the sample was filtered through glass wool to remove a white precipitate (later determined, by NMR, to be maleic acid). The solvents were then removed under vacuum and the sample was redissolved in methanol and filtered again to remove any further precipitate. The methanol was removed under a stream of nitrogen.

(E) Testing the Method

NMR analysis of this sample showed broad peaks as well as a number of peaks not belonging to the FB₁ spectrum. In order to determine if any or all of the impurities observed in the NMR spectrum of the sample were arising from the maleic anhydride, or the derivatization procedure, another experiment using pure FB₁ was performed. A 5 mg sample of pure fumonisin B₁ was maleated using the method described above. A sample was injected onto the same HPLC system as the original sample using the same gradient. The sample was collected from the detector exit line and divided in two (by volume).

The first half of the sample was freeze dried to remove all traces of water, then taken up in methanol to cause the precipitate to settle out of solution, allowing it to be filtered off. The methanol was removed under vacuum and the sample was lyophilized. The dried sample was dissolved in a minimal amount of distilled, deionized water and loaded onto C-18 sep pak mini-columns, rather than glass wool. The columns were washed with a small amount of water to remove any very polar impurities, such as salts. The maleated FB₁ sample was then eluted from the column using methanol. The methanol was removed under a stream of nitrogen and the sample was again lyophilized.

The second half of the sample was incubated as soon as it was obtained in order to remove the maleyl group. After removal of the maleyl group the second half of the sample was dried and cleaned up in exactly the same manner as the first half of the sample.

NMR spectra of both halves of the sample were compared to the spectrum of a pure

sample of FB₁. The NMR spectra were much improved from that of the first FB₁ sample that was treated using this method. The spectra of both the maleated and demaleated samples had sharp peaks and the proper chemical shifts. A few small interference peaks in the spectra of the two derivatized samples were assigned to water, methanol, and maleic acid, which were the impurities that caused the peak broadening in the original sample but had now been largely removed by the sep-pak columns.

The maleic acid peaks at 6.2-6.6 ppm were present in both the maleated and demaleated samples, but were not very large and were much smaller in the demaleated sample as expected. The peaks associated with the maleic acid were smaller than expected because overnight refrigeration of a methanol solution caused the precipitation of maleic acid. Based on the results of this experiment it was determined that this derivatization procedure does not impede the characterization by NMR of treated samples.

(F) Separation of Maleated Crude Mixture

Since the derivatization with maleic anhydride appeared to be successful with a single pure fumonisin it was performed on an impure mixture of fumonisins. A crude sample of about 70 mg was treated and chromatographed using the same procedure described above for FB₁.

The chromatogram of the maleated crude mixture showed a number of large peaks which were collected separately. Identical fractions from multiple injections were combined.

After testing all fractions collected from the column to ensure that they were within the correct pH range, each fraction was demaleated, cleaned up, and concentrated as described previously.

The HPLC chromatograms of some fractions had shown only one peak but the broad shape of these peaks suggested that they may have been hiding other components. It was thought, however, that the main component of each fraction would be present in much higher concentration than any minor component, providing an acceptable NMR spectrum.

The NMR spectra of these fractions showed sharp peaks and some of the NMR characteristics of fumonisins were distinguishable, such as the signals for the intact side-chains. However, as this initial separation was only intended as a trial the amount of crude fumonisin mixture that had been used was very low, consequently, there was not enough material to characterize any individual compounds. It was possible to determine that the fractions were definitely fumonisin-like in structure and that this derivatization method worked for mixtures of fumonisins as well as single pure compounds.

Since this method had proven to be successful for separation of a mixture of fumonisins the separation was scaled up. A 200 mg sample of the crude fumonisin mixture was maleated in the usual fashion. The reverse phase C-8 analytical column was replaced with a reverse phase C-18 semi-preparative column. The flow rate of the solvents was increased from 1 mL/min to 4 mL/min to compensate for the change in column size. The gradient was also modified slightly to accommodate the different column (Table 9, p.73). Several fractions were collected.

Another 200 mg sample maleated and chromatographed under exactly the same

conditions gave an identical chromatogram. The two samples were collected in an identical manner and the corresponding fractions combined. Each fraction was concentrated under vacuum, lyophilized, dissolved in methanol, and filtered to remove the precipitate. Although care had been taken to collect the peaks visible in the chromatogram separately, baseline separation was not achieved with the HPLC so the collected fractions likely contained at least some impurities.

Each of the fractions was rechromatographed under the conditions used to separate the crude mixture in order to determine the purity of the fractions. Initially the fractions were analysed with the same gradient used to separate the crude mixture. However, in order to achieve separation of the individual components of some fractions, the original gradient was altered. Because some of the fractions had been taken from the beginning of the original run, some from the middle, and some from the end some fractions were a mixture of fairly polar compounds and some were a mixture of less polar compounds. Since the mixture in each fraction had originally been observed as a single peak under the initial gradient they obviously eluted under very similar conditions which necessitated fine tuning of the gradients to achieve separation. Each of the fractions contained a few peaks which were reasonably well resolved. Separation of the peaks was achieved in many cases but the very low concentration of each compound made characterization of the separated components impossible although it was possible to establish that they were fumonisin-like in structure.

(G) Evaluation of the Method

Resolution and collection of the individual peaks was certainly made easier by the fact that the maleated compounds were visible during the run. The reversibility of the maleic anhydride derivatization allowed for the recovery of the fumonisins after separation and removed the need for analysis of each fraction which allowed separation to be based on detector response. The derivatization and subsequent separation of the fumonisins went well but trouble was experienced with the characterization of the compounds due to insufficient sample size.

Even with visualization these fumonisin-like compounds proved to be very difficult to separate in some cases. This is due to the fact that the fumonisins are all very similar in structure and interact with the column similarly and therefore elute under like conditions. This obstacle is inherent in the nature of the compounds and is always going to be problematic, which makes this visualization technique a valuable tool for overcoming this problem. Progress in this direction was made with this project as a few minor components of the crude fumonisin mixture, which had eluded all attempts at isolation thus far, were isolated, although not characterized. This method has clearly proven to be a successful way to separate the minor fumonisin metabolites. The derivatization procedure does not interfere with the NMR spectra of the compounds so characterization would be possible if more sample were available.

Table 8. Gradient used for separation of analytical amounts of maleated fumonisins.

<u>Time</u>	<u>Solvent A *</u>	<u>MeOH</u>
0	90	10
3.5	90	10
16	65	35
20	90	10

Table 9. Gradient used for separation of semi-preparative amounts of maleated fumonisins.

<u>Time</u>	<u>Solvent A *</u>	<u>MeOH</u>
0	80	20
3.5	80	20
22	45	55
26	80	20

* Solvent A = 68% MeOH / 32% 0.1 M sodium phosphate, pH adjusted to 3.35 with phosphoric acid

Table 10. Gradient used for separation of underivatized fumonisins.

<u>Time</u>	<u>Acetonitrile (0.1% TFA)</u>	<u>Water (0.1% TFA)</u>
0	25	75
1	25	75
35	70	30
45	100	0
55	100	0

IV-2 Underivatized Method

Although the maleic anhydride derivatization was quite successful at separating the minor fumonisins, a method for detecting (and thus separating) the fumonisins without derivatization would be ideal. The carbonyl groups of the side chains on the fumonisins are detectable by a UV detector at 190 nm so it became a goal of this project to determine if using an HPLC method combined with UV detection was possible for separation of the minor fumonisins.

(A) UV Detection of Underivatized FB₁

A 10 μg FB₁ standard was injected onto a C-18 reverse phase analytical column. A binary gradient of acetonitrile:water (Table 10, p.73) run at a flow rate of 1 mL/min. was used to elute FB₁. Trifluoroacetic acid (0.1%) was added to both solvents in order to sharpen the fumonisin peak.

One of the problems inherent in this method was that the strong absorbance of acetonitrile at 190 nm made it difficult to see any peaks. For this reason the amount of FB₁ standard injected was increased to 200 μg .

A very small peak was detected at 16.5 min., which corresponded to the reported retention time for FB₁ under these conditions ⁸⁶. However, the rise in the baseline of the detector due to the absorbance of acetonitrile was so severe that it was difficult to say for sure if this was an actual peak or just noise in the baseline.

The sample was collected and concentrated as much as possible. After concentration the sample was spotted on a tlc plate with an FB₁ standard. A spot was visible on the TLC plate at the same R_f as the FB₁ standard.

(B) Evaluation of the Method

Due to time constraints and equipment malfunctions this part of the project was not pursued further. The preliminary results obtained indicate that it is possible to detect underivatized fumonisin B₁ using a UV detector at 190 nm. The main problem experienced was the weak absorbance of the fumonisins at this wave length and the strong absorbance of acetonitrile which made the detection limit too high under these conditions. This necessitates use of a large sample which makes it an unsuitable method in its present form for detecting the minor fumonisins.

V Further Work

The minor fumonisins, which have been isolated during the course of this project, but not characterized due to the presence of impurities or an insufficient amount of material need to be characterized. This is necessary in order to give a complete picture of the spectrum of fumonisin metabolites that are being produced by *F. moniliforme*. The maleic anhydride derivatization procedure presented in this paper shows promise toward

this end but needs further exploration. Currently LC-NMR shows the most promise for characterizing impure mixtures containing a low concentration of the minor fumonisin of interest. However, this requires very costly equipment and is therefore unavailable to most researchers.

Safe limits of consumption for both humans and animals need to be established. In order to achieve this, an assessment of the degree of contamination of crops with fumonisin producing *Fusarium* species needs to be established as well as the consumption patterns for affected crops since they vary widely in different areas. The minor fumonisins need to undergo toxicity testing, both individually to assess the degree and nature of their toxicity and in mixtures to determine possible synergistic effects.

EXPERIMENTAL

I Physical Analysis

I-1 Detectors

A Varian 2550 variable wavelength UV detector with an 8 μL cell was used to monitor the maleic anhydride analysis. A Varian 9550 variable wavelength detector with a 15 μL cell size was used to monitor the HPLC runs of underivatized FB_1 . The HPLC analysis of the OPA-derivatized fumonisin samples were monitored by a Shimadzu RF-551 fluorescence detector set at 335 nm (excitation) and 440 nm (emission).

I-2 Mass Spectrometers

(A) Fast Atom Bombardment (FAB)

FAB spectra were recorded on a Kratos Concept II H spectrometer with an acceleration voltage of 8 kilovolts and a fast atom beam of 12 to 15 kilovolts. The analysis was carried out in a glycerol matrix.

(B) Liquid Chromatography - Mass Spectrometry

Samples were prepared for analysis by methylation with diazomethane. Typically, a

solution containing 1 mg of fumonisin in 200 μ L of methanol was treated dropwise with freshly prepared diazomethane in ether until a yellow color persisted. The reaction mixture was then evaporated to dryness under a stream of nitrogen. The resulting residue was made up to volume in methanol.

A gradient of 80% A to 80% B (A = 0.05% trifluoroacetic acid in water:methanol:acetonitrile (1:1:1) (pH 3); B = methanol:acetonitrile (1:1)) over 12 minutes (Table 4) was pumped through an analytical 250 mm x 4.6 mm ID LiChrosorb 5 μ m RP-18 column at a rate of 1 mL/min to elute the fumonisins. A carrier solution of 50 mM aqueous L-serine was added post column at a flow rate of 50 μ L/min by a model 100DM syringe pump.

The sample was introduced for chemical ionization (CI) or electron impact (EI) analysis via a Vestec model 95001 particle beam universal interface. Both EI and CI mass spectra were recorded on a Finnigan MAT 312 instrument coupled to an INCOS data system.

I-3 NMR

^1H and ^{13}C NMR spectra were recorded in methanol- d_4 (CD_3OD), at 500.13 and 125.18 MHz respectively, on a Bruker AM500 operating at 303 K, using a 5 mm normal geometry $^1\text{H}/^{13}\text{C}$ probe. Chemical shifts were referenced to CD_3OD at 3.3 ppm and 49.0 ppm for ^1H and ^{13}C respectively. Confirmation of ^1H chemical shifts and connectivity was made by $^1\text{H}/^1\text{H}$ homonuclear correlation spectroscopy (COSY). Protons attached to

carbons were determined by $^{13}\text{C}/^1\text{H}$ Heteronuclear correlation spectroscopy (HETCOR). As well, ^{13}C multiplicity (DEPT) and NOE difference spectroscopy were performed to aid in structural determination.

II Fermentation

Seed culture was prepared by macerating a 2% malt agar slant of *F. moniliforme* NRRL 13616 in 80 mL of sterile distilled water. An aliquot (2.5 mL) was used to inoculate thirty-two 250 Erlenmeyer flasks each containing 50 mL of a medium consisting of glucose (20 g), KH_2PO_4 (2 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (2 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g) and ammonium chloride (3 g) that had been autoclaved at 121°C for 12 minutes. Amounts given are per liter of distilled water which had been put through a Barnstead Nanopure II system, $18.3\text{ M}\Omega/\text{cm}$. The cultures were put on a rotary shaker (3.81 cm throw, 220 rpm) at 28°C for 48 hours⁸⁰.

Production medium (9.3 litres) consisting of sucrose (40 g), glycerol (10 g), $(\text{NH}_4)_2\text{HPO}_4$ (1 g), KH_2PO_4 (3 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g), and NaCl (5 g) (amounts per litre of ultrapure water as above) were added to a 15 L jar from a New Brunswick Scientific fermenter with pH (Ingold) and O_2 electrodes (N.B. Scientific 900 series). The jar containing the production medium was autoclaved at 121°C for 20 minutes. Net CO_2 in the output air was measured with an ADC 225 MK3 instrument (ADC, Hoddeson, England)⁸⁰.

The seed culture was macerated and centrifuged in sterile 500 mL bottles at 10, 400g. Fifty percent of the actual volume was removed and the macerate re-suspended under aseptic conditions and added to the fermenter⁸⁰.

The culture was grown at 28°C and stirred at 380 rpm. Air was pumped into the fermenter at 3.2 L/min. Foam was controlled with a mechanical foam breaker and the twice-daily addition of 1 mL Dow Corning antifoam B. The initial pH was 6.2, and fell to < 2 after ca. 24 hours, and was not controlled. The fermentation was run for 240 hours⁸⁰.

Physical data on the fermentation (temperature, pH, O₂, CO₂) was acquired using a Hewlett-Packard 3421A data acquisition system, HP 9114B disc drive using custom software running on an HP41CX (developed by Dr. A. Taylor, NRC, Halifax).

Figure 20. Procedure for obtaining a crude mixture of fumonisins from *F. moniliforme*.

III Isolation of Fumonisin (Obtaining the sample)

III-1 Filtration

At the conclusion of the fermentation period the *F. moniliforme* culture material was pushed out of the fermenter and into two or three large Erlenmeyer flasks using air pressure. The culture material was then filtered through Whatman #1 filter paper under vacuum. The mycelium was washed and dried at 40° C under vacuum and the resulting dry weight determined. The filtrate was vacuum-filtered again through Celite 503 in a fine pore sintered glass funnel. After filtration the pH of the filtrate was adjusted to 6 with 1N NH_4OH in order to make it amenable to anion-exchange chromatography ⁸⁰.

III-2 Ion-Exchange Chromatography

In order to save time, four gravity-fed anion-exchange columns were run simultaneously rather than one larger one. The procedure was identical for each of the four columns. For each column, 20 g of DEAE Sephadex A-25 (Pharmacia LKB) was swollen overnight in distilled deionized (dd) water (200 mL). The following day, the gel was poured into a 100 mL column and charged with a formate counter-ion by washing with 0.5 N formic acid solution (300 mL). The column was then rinsed with dd water until the pH of the eluent was above 3 (500-800 mL). The culture filtrate was then loaded onto the column and followed by a dd water wash (300 mL). Elution was then started

with 0.1 N HCOOH . A brown band moving from the top of the column was collected in 20 mL fractions (300-400 mL) ⁸⁰.

III-3 TLC Analysis of the Fractions

Fractions were analyzed by TLC. Samples were spotted on 250 μ m Whatman silica gel plates and allowed to dry. The plates were developed in a solvent system of CHCl₃:MeOH:H₂O:AcOH (60:32:7:1). After development plates were allowed to dry and then sprayed with MeOH:H₂SO₄:AcOH:Anisaldehyde (85:5:10:0.5) followed by heating to visualize the fumonisins and reveal which fractions contained fumonisins, usually the first three brown fractions after the start of the HCOOH. R_f values of 0.25 for FB₁ and 0.31 for FB₂ and FB₃ were obtained. The fumonisin containing fractions were combined, concentrated under vacuum and lyophilized. A typical yield of brownish solid was 1.45 g (total from four columns) ⁸⁰.

III-4 Silica Gel Chromatography

The brownish solid was dissolved in CH₃CN/H₂O (87:13) and chromatographed on an open silica gel 60 (63-200 μ m, 30 g) column (20 mm i.d.) with the same solvent. Five fractions of 11 mL each, followed by 6 mL fractions were analyzed by tlc as above and the fumonisin-containing fractions (8-16) were combined. Typical yield of fumonisin - containing brown oil from this column is about 1 g ⁸⁰.

III-5 Reversed - Phase Chromatography

The brown oil was dissolved in 28:72 acetonitrile : water and chromatographed on a 300 x 30 mm i.d. C-18 preparative HPLC column packed with 50 μm , 120 Å ODS material. The solvent was driven through the column at a flow rate of 20 mL/min by a Shimadzu LC8A pump and controlled by a Shimadzu SCL-6B control station. The gradient shown in Table 2 was used in order to remove the majority of the FB_1 . Fractions were collected in 1 min/tube aliquots by a Gilson FC204 automatic fraction collector. TLC analysis was carried out on these fractions as above. The fractions containing mainly FB_1 were removed . The remaining fractions were combined, concentrated under vacuum , and lyophilized to yield the crude fumonisin mixture .

IV HPLC Fractionation of the Crude Extract

Preparative HPLC was conducted with the same HPLC system used to remove the FB_1 . The crude sample was loaded onto a 300 x 30 mm i.d. or a 300 x 20 i.d. C-18 reverse phase preparative column packed with 50 μm , 120 Å ODS material. The solvent was pumped through the system at a flow rate of 20 mL/min. A binary gradient of acetonitrile:water (Table 2) was used to remove FB_1 and fractionate the sample.

V OPA Method

V-1 Preparation of the OPA solution

The OPA solution was prepared by dissolving 20 mg of OPA in 2.5 mL of 0.1M sodium borate buffer. After mixing, 0.50 mL of MeOH was added followed by 25 μ L of mercapto-ethanol. Solution is stored in a dark vial and is good for one week at 24°C.

V-2 Analysis of OPA Derivatized Fractions by Analytical HPLC

Each sample was derivatized pre-column by a Varian model 9100 autosampler in a ratio of 20 μ L of sample to 180 μ L of OPA reagent. 1 μ L of the derivatized sample was injected onto a 250 x 4.6 mm i.d. C-8 reverse phase column packed with 5 μ m, 80 Å spherisorb from Waters. A binary gradient (Table 3) consisting of solvent A (32% 0.1M phosphate buffer adjusted to pH 3.35 with phosphoric acid, 68% methanol) and Solvent B, MeOH, was used. Solvent was pumped through the system at a rate of 1 mL/min by a Varian model 9012 pump. Data was collected and the system controlled by Varian Star Workstation Software version 4.0.

VI Maleic Anhydride Method

VI-1 Preparation of Maleic Anhydride Derivatives

Maleic anhydride crystals were used to derivatize crude mixtures of fumonisins as well as fumonisin standards in a 10:1 molar ratio. A dry sample of the crude mixture was dissolved in 0.1 M Na_2CO_3 and the pH was adjusted to 9.2. Maleic anhydride crystals were added to the buffered toxin solution slowly over a five minute period. The solution became turbid and the pH dropped as the reaction proceeded. The mixture was allowed to sit at this pH for ten minutes to ensure derivatization had taken place. After ten minutes the pH was adjusted to between six and seven by addition of the sodium carbonate buffer (since solution is acidic after the reaction has taken place). The derivative is stable at this pH for about one month when stored at 4°C.

VI-2 HPLC Analysis of Maleic Anhydride Derivatives

(A) Analytical

An 8 μm , 120 Å reverse - phase c-8 column 250 mm x of 4.6 mm was used to analyze the maleic anhydride derivatives. Solvent was pumped through the column by a Varian Vista 5500 HPLC pump at 1 mL/min. A gradient of 0.1 M sodium phosphate:methanol (38:62) and methanol was used (Table 8) at a flow rate of 1 mL/min.

(B) Preparative

An 8 μm , 120 Å C-18 reverse-phase semi-prep column 250 mm x 10 mm was used to separate the derivatized fumonisins in the crude mixture. Solvent was pumped through the system by a Varian Vista 5500 pump. The flow rate was increased to 4 mL/min and a different gradient than that used for the analytical analysis was used (Table 9), but with the same solvents.

VI-3 Lyophilization

Solvents were removed by a Caframo VV 2000 rotovap. The sample was then frozen by immersing it in methanol containing dry ice. After the sample was frozen (approximately 1 min), it was then lyophilized using a LABCONCO lyophilizer with an Edwards pump and a Cryocool CC-100 immersion cooler.

VI-4 Post Column Sample Clean-up

Lyophilized demaleated samples were dissolved in 1 mL of water and loaded onto C-18 SEP PAK mini columns. The column was washed with 2 mL of water to remove the salt. The fumonisins were then eluted with 2 x 2 mL of methanol.

VII HPLC Conditions for the Underivatized Method

A Phenomenex C-18 reverse phase column packed with sphereclone 5 μ m ODS(2) and having the dimensions 250 x 4.6 mm was used. A gradient of acetonitrile:water was used (Table 10) to elute the compounds. The eluant was monitored by a Kipp and Zonen single channel model BD 112 flatbed recorder.

VIII Standards and Chemicals

All fumonisin standards were provided by the laboratories of Dr. Marc Savard and Dr. David Miller. All solvents used for isolation and purification were glass distilled HPLC grade purchased from Aldrich, Fisher, or BDH.

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APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
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