

Determination of the presence of fusaproliferin and fusaproliferin deacetyl
in 1996 and 1997 Iowa corn samples as detected by thin layer chromatography

by

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TABLE OF CONTENTS

LIST OF FIGURES	iv
LIST OF TABLES	v
ABSTRACT	vi
INTRODUCTION	1
LITERATURE REVIEW	3
MATERIALS AND METHODS	7
Reagents and apparatus	7
Corn sample collection and storage	7
Selection of samples	8
Preparation of corn samples	8
Extraction of corn samples	8
Extraction procedure for 1996 samples	8
Extraction procedure for 1997 samples	10
Thin layer chromatography analysis of extracts	11
High performance liquid chromatography analysis of extracts	12
RESULTS	14
Sensitivity of test methods	14
Statistics	15
Recovery rate of fp and fp-da spiked samples	15
DISCUSSION	17
CONCLUSIONS	20
Presence of fp and fp-da in Iowa corn samples	20
Methodology	20
Further studies	20
REFERENCES	28

LIST OF FIGURES

Figure 1. Fusaproliferin	23
Figure 2. Retigeranic acid	23
Figure 3. Fusaproliferin-deacetyl	24
Figure 4. Fusaproliferin-acetyl	24

LIST OF TABLES

Table 1. 1996 pre-harvest Iowa corn samples positive for fusaproliferin and/or fusaproliferin deacetyl by reverse phase thin layer chromatography (RPTLC).	24
Table 2. 1996 post harvest Iowa corn samples positive for fusaproliferin and/or fusaproliferin deacetyl by reverse phase thin layer chromatography (RPTLC).	25
Table 3. Detection of fusaproliferin and fusaproliferin deacetyl in 1997 post harvest Iowa corn samples ground using a grist mill. Comparison of detection on RPTLC viewed with short wave UV, vs RPTLC and <i>p</i> -anisaldehyde spray viewed with long wave UV, vs HPLC results on 14 samples.	26
Table 4. Detection of fusaproliferin and fusaproliferin deacetyl in 1997 post harvest Iowa corn samples ground using a Wiley mill. Comparison of detection with RPTLC viewed with short wave UV, vs RPTLC and <i>p</i> -anisaldehyde spray viewed with long wave UV, vs HPLC results on selected samples.	27

ABSTRACT

Fusaproliferin is a recently identified sesquiterpene mycotoxin. Randazzo and co-workers first reported it as proliferin in 1993, but later the name was changed to fusaproliferin. This mycotoxin, produced by *Fusarium proliferatum* and *Fusarium subglutinans*, has been found in corn, wheat, and asparagus in Italy. Munkvold and co-workers recently reported the presence of fusaproliferin in Iowa corn (18). This study looked for evidence of fusaproliferin (fp) and fusaproliferin-deacetyl (fp-da) in samples of Iowa corn using a multiple mycotoxin extraction and thin layer chromatography extraction methods. Both the 1996 and 1997 samples contained fp and fp-da. Thirteen of 118 1996 samples extracted were positive for fp and 23 out of 118 samples were positive for fp-da. Of the 1997 corn samples extracted, 16 out of 34 were positive for fp and 5 out of 34 were positive for fp-da. This is the first report of the presence of fusaproliferin-deacetyl in Iowa corn.

INTRODUCTION

Mycotoxins, metabolites of fungi, are found worldwide as contaminants of commodities used as food for animals and man. Significant levels of mycotoxin production by fungal contaminated grains are believed to occur during periods of plant stress. An estimated 20% of cereal crops produced throughout the world contain measurable amounts of mycotoxins (32).

The fungi producing these mycotoxins can be separated into two general groups, those found under field conditions and those found under storage conditions. Growth of the fungi depends upon the moisture, temperature and oxygen required for reproduction (23). Field fungi do not survive well under drier storage conditions; however, the mycotoxins previously produced can still be detected in the feeds. Contamination of feeds can occur at any stage throughout the handling processes of the feedstuffs (harvest, storage, transportation, and formulation into grain rations). High temperatures encountered during extrusion of pelletized rations may reduce the level of selected mycotoxins (aflatoxin, ochratoxin) present prior to the pelleting process, but the levels of other mycotoxins (trichothecenes) can remain high (6, 22).

The *Fusarium* fungi produce the mycotoxin fumonisin B₁ (FB₁), (*Fusarium moniliforme* and *Fusarium proliferatum*) which has been shown to cause pulmonary edema and hydrothorax in swine (8, 30), equine leukoencephalomalacia (ELEM) (30, 36), and has also been linked with esophageal cancer in humans (26). *F. moniliforme* and *F. proliferatum* are among the most prevalent fungi associated with feedstuffs worldwide (14). Because of the difficulty in identifying these fungi, it has been reported that *F. proliferatum* has been

misidentified as *F. moniliforme* (14). Therefore, the occurrence and significance of *F. proliferatum* as a potential pathogen may be higher than previously reported. *Fusarium proliferatum* has been shown to produce fumonisin, fusarin, beauveracin, moniliformin, and fusaproliferin mycotoxins (7, 11, 12, 19, 20). The pathogenic potential of *F. moniliforme* is not discussed in this paper.

Certain mycotoxins have experimentally and clinically been associated with specific health related problems in animals and humans, yet aflatoxin and deoxynivalenol remain the only mycotoxins with fixed tolerance levels in feedstuffs as established by the United States Food and Drug Administration. In addition, the effects of long term low level consumption of mycotoxins in animals and man has not been investigated for all mycotoxins. Long term studies have been conducted for aflatoxin and fumonisin mycotoxins. Additional studies have explored the synergistic effects of mycotoxins *in vivo* (1, 7, 9).

This study was designed to explore the incidence of the recently discovered mycotoxin fusaproliferin (fp) and fusaproliferin deacetyl (fp-da) in a sampling of the 1996 and 1997 Iowa corn crop. The effectiveness of a multi-mycotoxin extraction process and the use of thin layer chromatography (TLC) as a screening test for the presence of fp and fp-da in corn samples were also investigated (33).

LITERATURE REVIEW

Fusaproliferin (Figure 1) was first reported as proliferin in 1993 by Randazzo and coworkers but was later changed to the current name (25, 31). This mycotoxin was initially isolated from *Fusarium proliferatum* (Matsushima) Nirenberg, a member of the *Fusarium* section, *Liseola*, which is found worldwide as a plant pathogen, most notably as a cause of ear rot and stalk rot in corn (13, 14, 18). Fusaproliferin is also produced by *Fusarium subglutinans* (11, 20).

Santini and coworkers suggested through their investigations of the similarities of the molecular structures of fusaproliferin and retigeranic acid (Figure 2) that both compounds are derived from the precursor geranyl farnesol (25). Previous studies indicate steroidogenesis can occur by a sesterterpene pathway (of which retigeranic acid is a precursor) as well as the cholesterol pathway (2, 34).

Fusaproliferin, a sesterterpene mycotoxin, was the first such carbon-25 structure isolated from *Fusarium* species (24, 26). Prior to this discovery, the largest numbers of sesterterpenes were isolated from marine sponges. These naturally produced compounds are reported to act as natural feeding deterrents, while others inhibit nitric oxide synthase induction in marine macrophages stimulated with lipopolysaccharides, or have anti-inflammatory properties via the inhibition of the phospholipase-A enzyme (2, 3, 4, 24, 34). The effects of sesterterpene mycotoxins in mammals may have similar effects.

The production of mycotoxins depends on unspecified stress conditions occurring to the plant as well as the ambient temperature, oxygen, and moisture content of the grain (23). The optimum temperature for germination of *F. proliferatum* (3 different isolates) was 30° C

with a water activity (a_w) range of 0.994-0.98. At 0.88 a_w germination occurred, but was slow and at 0.85 a_w , no germination occurred. Marginal a_w levels are considered in the 0.92-0.90 range (17). Water activity is a term that expresses the measure of the availability of water to microorganisms. Pure water has an a_w of 1.0. In a previous study Marin *et al.* (16) reported optimal growth at a pH 5.5 over a relatively wide temperature range of 20-35° C. These studies indicate *F. proliferatum* will grow over a relatively wide temperature and moisture range (15, 16, 17).

The original *F. proliferatum* isolate found to produce fusaproliferin was obtained from corn grown in the northern part of Italy. The growing seasons are cooler and wetter in northern Italy than in the southern parts of the country. Miller and coworkers conducted a study of mycotoxin production by *F. moniliforme* and *F. proliferatum* in the 1993 Ontario, Canada corn crop. Of the strains tested all produced fumonisins and fusarins. In addition, some strains of *F. proliferatum* produced moniliformin (18). Detectable levels of fumonisins were found in corn from areas where drought and slightly above normal temperatures (during corn ear development) were followed by cool, wet conditions (18). Fusaproliferin and fumonisin B₁ (FB₁) have been found in the same corn sample (20, 29). Investigations into the specific environmental conditions that favor mycotoxin production and the specific mating populations of *F. proliferatum* which produce fusaproliferin continue.

Fusaproliferin (fp) was produced by two of six different mating populations of *Gibberella fujikuroi* (*Fusarium* section *Liseola*) during testing for the production of fp and beauveracin (19). Asparagus fields infected with stalk rot in Connecticut, Massachusetts and Michigan produced *F. proliferatum* from the same mating population that produced fp as

identified by Moretti and coworkers (5). Fusaproliferin has also been isolated from corn, wheat and asparagus (19, 20).

Munkvold and co-workers were the first to report fusaproliferin production by Iowa strains of *F. subglutinans* and *F. proliferatum* and beauvericin production by strains of *F. proliferatum* isolated from corn. This study also concluded that eight of the strains of *F. proliferatum* tested produced fumonisins, beauvericin, and fusaproliferin (20).

Experiments utilizing culture material of *F. proliferatum* are present in the literature (7, 9, 10, 21, 35, 37). Researchers conducting studies with known FB₁ levels have suggested the results experienced may have been due to additional toxins not measured, or to a synergistic effect between two toxins (1, 9, 10, 21, 35,). Studies in chicks exposed to *F. proliferatum* culture material in the rations have been reported with conflicting results. Nagaraj and Wu conducted a study in chicks using a strain of *F. proliferatum* known to produce fumonisin and moniliformin. Chicks can normally tolerate up to 65 ppm moniliformin in their diets. In the above study, the moniliformin level was 30.6 ppm and the highest fumonisin level was 44.7 ppm. The toxicity seen at the lower levels was attributed to the possibility of the culture material containing additional toxic factors (21).

Two other group's chick feeding studies included feed amended with culture material of *F. proliferatum* in the rations containing known quantities of moniliformin, fumonisin B₁ and fumonisin B₂. Later, Plattner and coworkers reported the strain (M-5991) of *F. proliferatum* could produce beauvericin. Studies conducted by Javed and coworkers utilized The lowest weight in the chick was observed when the diet included a combination of

moniliformin and fumonisin indicating a synergistic effect between the two mycotoxins(9, 10). In a companion report it was revealed the strain used in the study had the potential to produce beauveracin (10).

Longrieco and coworkers reported purified fp was toxic to brine shrimp larvae (*Artemia salina*) (LD₅₀ of 55 microM), human non-neoplastic B-lymphocytes IARC LCL 171 (Cytotoxic Concentration ((CC₅₀)) of 55 microM), and an insect cell line (lepidopteran SF9, CC₅₀ of 70 microM)(11). Fusaproliferin has also shown teratogenic effects on chick embryos (28). The deacetyl (Figure 3) form of fp was not toxic in the brine shrimp assays; fp-acetyl (Figure 4) was three times more toxic than fp in the assay (27). The toxicity of the three forms of fp was tested using an assay that utilizes a gram negative salt-water phosphorus bacterium as the indicator organism. The ED₅₀ of fp was at 70-80 micrograms/ml; fp-da was at 60 micrograms/ml and fp-acetyl was toxic at 5 micrograms/ml. To give a perspective to the above levels, the ED₅₀ of carbadox and FB₁ are 6.14 micrograms/ml and 130 micrograms/ml respectively using this assay (Personal communication with H. M. Stahr).

The deacetyl form of fusaproliferin (fp-da) (Figure 3) is a natural metabolite which has been reported to occur in the ratio of 1:3 fp-da:fp, while the acetylated form (fp-ac) has not been found in nature (27). Ritieni and coworkers showed the fp-ac was 3 times more toxic in the brine shrimp assay than fp. Their studies indicate the toxicity of fp was lost when the acetyl group was removed. Preliminary cell culture work with rabbit liver enzymes suggest fp is hydrolyzed to fp-da *in vivo* (27). Whether fp-da is a precursor or metabolite of fp is still under investigation (27).

MATERIALS AND METHODS

Reagents and apparatus

- (a) Methylene chloride (MeCl_2)¹ and methanol (MeOH)² were HPLC grade.

Veterinary Diagnostic Laboratory (VDL) personnel redistilled petroleum ether³ and acetonitrile⁴ in glass. Solvents and ferric chloride were analytical chemical grade.

- (b) Latch-Lid Chromatotank⁵

- (c) The fusaproliferin standard was provided by the Dipartimento di Scienza degli Alimenti, Portici (Na), Italy.

- (d) The fp-deacetyl standard was isolated in the Veterinary Diagnostic Laboratory from corn kernels inoculated with a strain of *F. subglutinans*, (Item 2284, Istituto Tossine Micotossine, Bati, Italy) (personal communication H. M. Stahr).

Corn sample collection and storage

All the 1996 samples (119) had previously been used in other studies. The pre-harvest samples were from a study supported by the National Animal Health Monitoring System (NAHMS). The post harvest samples were from the Grain Quality Research Program (GQRP) at Iowa State University. All 1996 samples were previously ground to a uniform consistency and were maintained at -28°C . The specific procedure used to gather these samples is unknown. Samples were maintained in plastic bags.

All the 1997 samples (35) were collected at Iowa elevators as a part of the Grain Quality Research Program at Iowa State University. These whole kernel corn samples were

¹ Di-Chem, Des Moines, IA 50309

² Fischer Scientific, Pittsburgh, PA 15219

³ Phillips Petroleum, Sweeny, TX 77480

⁴ GNI Chemical, Deer Park, TX 77536

⁵ General Glassblowing Company, Richmond, CA 94806

received in cloth bags and stored at -28°C after receipt. After samples were ground, they were labeled, placed in plastic tubs with lids, and stored at -28°C.

Selection of samples

Post harvest 1996 samples were chosen on the basis of previous mycotoxin results. The samples negative for mycotoxins were excluded to increase the possibility of finding fp or fp-da, as these were the first samples tested for their presence. Pre-harvest 1996 samples were selected using a table of random numbers. Post harvest 1997 samples were selected as every fifth sample received.

Preparation of corn samples

The 1997 corn samples were ground utilizing two different methods. Fifteen samples were ground using a gristmill with 16 ounces of clean corn (tested negative by thin layer chromatography for fusaproliferin (fp) and fusaproliferin deacetyl (fp-da)) between each sample and vacuuming between samples. Twenty samples were ground using a mill⁶ with a 2mm screen, cleaning the working parts of the mill between each sample. Corn samples from 1996 had previously been ground to 2mm size particles.

Extraction of corn samples

Extraction of 1996 samples used a modified multiple mycotoxin extraction procedure (33). Extraction of 1997 samples followed the multiple mycotoxin extraction procedure (33).

1996 sample extraction procedure

- a. Place a fifty-gram sample of ground corn in a modified Waring blender with 200 ml warmed acetonitrile-water (90:10) and blend at high speed for 4 minutes.

⁶ Wiley mill

- b. Transfer 100 ml of the liquid (filtered through #1 Whatman filter paper) into a 250 ml separatory funnel fitted with a Teflon stopcock.
- c. Add 100 ml petroleum ether to the filtered liquid, shake by hand 3 times, and vent between mixes.
- d. Decant off the (lower) acetonitrile-water layer. After discarding the petroleum ether layer, place the acetonitrile-water layer in the original separatory funnel.
- e. Add 100 ml of deionized water⁷ to the acetonitrile-water layer.
- f. Add 2 ml a 20% NaCl solution to the acetonitrile-water layer.
- g. Fifty ml of methylene chloride (MeCl_2) was added to the acetonitrile-water, shaken and vented between mixes.
- h. Drain the MeCl_2 layer was into a 250 ml Erlenmeyer flask.
- i. Add an additional 50 ml of MeCl_2 to the acetonitrile-water layer, shake, and vent between mixes.
- j. Drain the MeCl_2 layer into the 250 ml Erlenmeyer flask.
- k. Evaporate the MeCl_2 layer on a low temperature steam bath under nitrogen or air effusion just to dryness.
- l. Three ml of methanol (MeOH) was used to redissolve and transferr the extract to a 2-dram glass vial with an aluminum-coated cap.
- m. Take the extract to dryness on a low temperature steam bath under nitrogen or air effusion to dryness.
- n. Extracted samples were stored at -28°C until preparation for thin layer chromatography development.

⁷ Millipore filtered water

1997 sample extraction procedure

- a. Place a 50 gram sample of ground corn in a modified Waring blender with 200 ml warmed acetonitrile-water (90:10) and blended at high speed for 4 minutes (33).
- b. Transfer 100 ml of the liquid (filtered through #1 Whatman filter paper) into a 250 ml separatory funnel fitted with a Teflon stopcock.
- c. Add 100 ml petroleum ether to the liquid, shake by hand 3 times, and vent between mixes.
- d. Decant off the (lower) acetonitrile-water layer. After discarding the petroleum ether layer, place the acetonitrile-water layer in the original separatory funnel.
- e. Add the decolorizing reagent to the acetonitrile-water layer. The decolorizing reagent is a 100 ml solution of 15% ferric chloride titrated with 4% aqueous sodium hydroxide while constantly stirring until a pH of 4.6 is attained.
- f. After adding the above solution to the acetonitrile-water layer, shake for 3-5 seconds, vent and allow to separate.
- g. Decant off 100 ml of the acetonitrile-water layer.
- h. Rinse the separatory funnel with hot tap water and place the acetonitrile-water layer back into the original funnel.
- i. Add 2 ml a 20% NaCl solution to the acetonitrile-water layer.
- j. Add 50 ml of methylene chloride (MeCl_2) to the acetonitrile-water layer, shake and vent between mixes.
- k. Drain the MeCl_2 layer was into a 250 ml Erlenmeyer flask.

- l. Add an additional 50 ml of MeCl_2 to the acetonitrile-water layer, shake, and vent between mixes.
- m. Drain the MeCl_2 layer into the 250 ml Erlenmeyer flask.
- n. Evaporate the MeCl_2 layer on a low temperature steam bath under nitrogen or air effusion just to dryness.
- o. Add 3 ml of methanol (MeOH) to redissolve the extract and transfer to a 2-dram glass vial with an aluminum-coated cap.
- p. Take the extract to dryness on a low temperature steam bath under nitrogen or air effusion to dryness.
- o. Extracted samples were stored at -28°C until preparation for thin layer chromatography development.

Thin layer chromatography (TLC) analysis of extracts

- a. Samples were spotted on thin layer plates using a hand held micropipetter and calibrated, disposable glass pipettes.⁸
- b. Add 100 μl MeCl_2 - MeOH (50:50 v:v) spotting solvent to the dry extracted samples.
- c. The volume spotted was calculated to be equivalent to 1 gram of the original sample.
- d. One microgram each of fp and fp-da standards were included on each plate.
- e. One gram equivalent of spiked sample (fp and fp-da) was included on each plate.
(Samples were spiked at 1 microgram/gm of sample.)
- f. Samples were spotted on Whatman® Reverse Phase C18 F TLC plates.

⁸ Clay Adams, Parsippany, NJ, 07054

- g. Plates were placed in a latch lid chromatotank and developed in ethanol-water-acetic acid (65:35:1 v:v:v).
- h. Plates were allowed to air dry completely, then they were viewed under short wave UV.
- i. Plates were then sprayed with 5% *p*-anisaldehyde (AlCl_3) in MeOH (31), which caused the fp, and fp-da to take on a deep rose to lavender color when heated.
- j. Sprayed plates were viewed with a long wave UV lamp; the fp and fp-da fluoresced an orange color.

Because of the limited quantities of fp and fp-da standards, a spiked sample was not included with every group of five corn samples. The spiked sample included on the plate was from previous spike recovery efforts if a spiked sample was not run with the group of samples being spotted on the TLC plate.

To calculate the levels of fp and fp-da found in the positive extracted samples, a 1 gram representative sample of each extract was taken to dryness, then 100 microliters of the MeOH/ MeCl_2 (50:50 v:v) was added to the extract. Serial two fold dilutions were carried out until the fp and/or fpd could not be seen on the TLC plate. The levels reported under the TLC sections in (Tables 1 through 4) were calculated from the above endpoints.

High performance liquid chromatography (HPLC) analysis of extracts

The first group of 14 1997 samples extracted were all evaluated by HPLC to determine the correlation of positives detected with the *p*-anisaldehyde spray and long wave UV with short wave UV screening with HPLC results. Ten percent of the next group of 15 1997 samples positive under longwave UV with *p*-anisaldehyde spray which showed fluorescence for fp or fp-da were respotted on TLC plates. Fluorescent spots at the same R_f

as the fp and fp-da standards were scraped off the TLC plate and eluted from the silica gel overnight with MeOH. The supernatant liquid layer was decanted off into clean 2-dram glass vials with a foil-lined top and blown to dryness using nitrogen or air effusion. These extracts were analyzed using HPLC methodology, developed by personnel in the Chemistry Section of the Veterinary Diagnostic Laboratory at Iowa State University.

The Shimadzu SPD-6A UV Spectrophotometric detector was coupled with a Perkin Elmer C₁₈ silica (3mm X 3 cm) column and with a Shimadzu Sil-9A auto injector. The HPLC conditions for fp and fp-da included MeOH/H₂O (75:25 v:v) as the eluent system with a flow rate of 1.0 ml/min. The wavelength used to quantify the fp and fp-da was 263 nm. The retention time of an authentic standard of fp was 1.9 minutes; fp-da was 1.4 minutes. Single analytes were run. (personal communication P. Imerman)

RESULTS

Sensitivity of test methods

Fusaproliferin and fp-da standards were spotted on reverse phase TLC plates. Under short wave UV fp was detectable to 0.32 micrograms; fp-da was detectable to 0.17 micrograms. After spraying the TLC plate with *p*-anisaldehyde spray and viewing under long wave UV, fp was visualized at 0.069 micrograms and fp-da at 0.049 micrograms. The limit of detection of the HPLC method used in this study was 50 nanograms.

Of the 1996 samples tested by RPTLC, 13 of 118 samples tested were positive (with indicator spray) for fp only, 23 of 118 samples were positive for fp-da only, and 8 of 118 samples were positive for both fp and fp-da (Tables 1 and 2). Thirty eight percent of the samples positive by RPTLC were tested by HPLC (Tables 1 and 2). Of the 1997 samples tested 16 of 34 were positive for fp and 5 of 34 were positive for fp-da. None of the 1997 samples contained both fp and fp-da. Twenty-one of these samples positive by RPTLC (76%) were tested by HPLC (Tables 3 and 4).

Because of the initial problem with the correlation of TLC results and HPLC results, fourteen of the 1997 samples were viewed under short-wave UV (254 nm), sprayed with *p*-anisaldehyde spray, viewed under long wave UV (366 nm), and checked by HPLC. Fluorescent bands which were representative of R_fs consistent with fp and fp-da, were sent for HPLC analysis (Table 3). Five samples ground using the grist mill that had the potential for cross contamination (3489, 3416, 1110, 60035) were eliminated from the final 1997 statistical analysis.

Statistics

The null hypothesis (H_0) was there is no correlation between HPLC and TLC results. Comparing HPLC results with the calculated TLC levels of fp, using the least squares line, $y = 1.676 + 0.9122x$, resulted in a coefficient of correlation (r) of 0.954 and a coefficient of determination (R square) of 0.91. Upon comparison of the fp-da values, using the same methodology, $y = 2.8097 + 0.9516x$, $r = 0.75$ and R square = 0.57. These results indicate there is a direct relationship between the HPLC and TLC results, however, the fp data has a better fit than the fp-da data. Using the analysis of variance (ANOVA) on the fp data for rejection of H_0 ; ($F = 70.74$ and $F_a = 6.6610$) $F > F_a$ therefore, you reject H_0 , concluding there is a direct relationship between HPLC and TLC results.

Using ANOVA to look at the number of samples positive for fp and fp-da in the pre and post harvest 1996 samples, H_0 : there is no statistical difference between the mean values (of fp) positives in the pre and post harvest samples. The pre-harvest fp mean was 0.8386 ppm; post harvest mean was 0.421 ppm. As calculated $F = 0.85$ and $F_a = 3.922$, therefore, H_0 is not rejected and there is no statistically significant difference between the means of fp in pre and post harvest samples. For fp-da, $F = 6.72$ and $F_a = 3.992$, $F > F_a$, therefore H_0 is rejected. There is a statistically significant difference between the fp-da pre-harvest mean (0.7613) and the post harvest mean (0.16997).

Recovery rate of fp and fp-da spiked samples

Problems were encountered with the consistency of recovery rates. Percent recovery of spiked samples (spiked at 1 microgram per gram of sample) of fp and fp-da ranged from 40% to greater than 100%. The fp and fp-da standards were relatively stable in methanol for approximately 10 months. After this time, the concentration of the fp standard began to

decrease, while the fp-da standard concentration continued to concentrate. Rechecking the standards closer to the time spike recovery efforts are conducted may help with the consistency. An average of 60% recovery was calculated from the spike recoveries conducted for both fp and fp-da (fp - 40%, 50%, 60% and fp-da - 40%, 50%, 70%).

DISCUSSION

Results from this investigation indicate fp and fp-da were both present in samples of the 1996 and 1997 Iowa corn crop and the multiple mycotoxin extraction process as described can be used for extraction and detection of these compounds. The initial fp and fp-da standards were analyzed by Mass Spectrometry (MS), however because of limited funds, no positive extract samples were analyzed by MS to confirm the same compound was present. This MS confirmation of the presence of the two compounds in a representative number of positive samples is imperative in the evaluation of this TLC method. If a compound other than fp or fp-da migrates close to the same distance on the TLC plate it could create the potential for false positives using this method.

The exclusion of the ferric oxide gel decolorization step in the 1996 samples resulted in higher background color in those samples and could explain the original problems with compound identification by TLC but not by HPLC. The first fourteen 1997 samples extracted were ground using a grist mill which increases the potential for cross contamination of samples. However, the specific purpose of this first group of samples was to test the correlation of the RPTLC results and the HPLC results. A Wiley mill was used for the second set of 20 1997 samples. The use of this type of mill decreases the potential for cross contamination between samples. The correlation between RTLC and HPLC results were improved for the 1997 samples (Tables 3 and 4).

The early 1996 extracts were setting in methanol at -28° C for 6 months before they were analyzed because of lack of HPLC methodology. Yet after this time, 7 of these samples were positive for fp and fp-da, indicating (along with the stability of the standards) that the compound is relatively stable in methanol for 6-10 months. After the 10-month point, both

compounds showed significant evidence of deterioration (personal observation).

Studies previously cited indicate mating populations of *Giberella fujikuroi* (*Fusarium* section *Liseola*) which include *F. proliferatum*, have been found in asparagus and corn crops in the United States (5, 19). In addition, fusaproliferin has been isolated from asparagus in Italy (26).

Experiments utilizing culture material of *F. proliferatum* are present in the literature (6, 8, 9, 20, 33, 35). Researchers conducting studies with known FB₁ levels suggested results might have been due to additional toxins not measured or to a synergistic effect between two toxins. Studies incorporating *F. proliferatum* culture material in feeding trials have later discovered the strain they were using was capable of producing a new mycotoxin (beauveracin) which was being investigated (6, 8, 9). Studies utilizing *F. proliferatum* culture material in feed trials should test for the presence of moniliformin, fusaproliferin, beauveracin, fusarin, and fumonisin to determine what levels are present before beginning a study. An alternative to feeding culture material is to feed pure compounds, however, this adds additional time and expense to feeding trials. However, by feeding the pure compound of interest, the investigator can rule out interference with additional potentially toxic compounds.

The similarities in structure of fp and retigeranic acid and evidence steroidogenesis can take place by the sesterterpene pathway may lead to possible further investigation of the metabolism of fp and fp-da by the body. One must consider the possibility that metabolites could be involved in steroid synthesis, or be potential blockers of steroid pathways, thereby mimicking steroidal effects on the body.

Evidence indicating *F. proliferatum* can appear on feeds other than corn is an indication that fusaproliferin has the potential to enter the food chain (humans and animals) at various points (5, 19).

CONCLUSIONS

Presence of fp and fpd in Iowa corn samples

This study had a two fold purpose: to determine if fusaproliferin and fusaprolifein deacetyl are present in Iowa corn, and to determine the usefulness of the TLC methodology described, as a screening procedure for the detection of these two compounds. Both of these purposes were successfully achieved. However, only the future measurement of toxicity of these compounds will determine if the TLC method described in this study is a useful tool as a screening test at the limits of detection described.

Methodology

The ferric oxide gel decolorization step is essential to eliminate background color which can interfere with the accuracy of the described spray detection method. This is supported by the correlation of the HPLC results and the *p*-anisaldehyde sprayed TLC results in the 1996 versus 1997 assays. Additional work needs to be conducted on the spike recovery efforts and to determine the amount of analyte that is lost by removal of samples from the TLC plate. This method is successful in the extraction and recovery of fp and fp-da from corn samples.

Further studies

Further studies in cell culture to try to identify how fusaproliferin is metabolized and initial animal studies feeding pure compound need to be undertaken to determine the toxicity level of this mycotoxin. If the toxicity level is at or above the 60 ppb range, the TLC methodology described can be used successfully as a screening method. At present evidence for toxicity being less than a microgram per gram is non existent. If the toxicity level is

determined to be at low ppb levels, or fp is found to be synergistic with other mycotoxins at low levels, additional screening methods with increased sensitivity need to be developed.

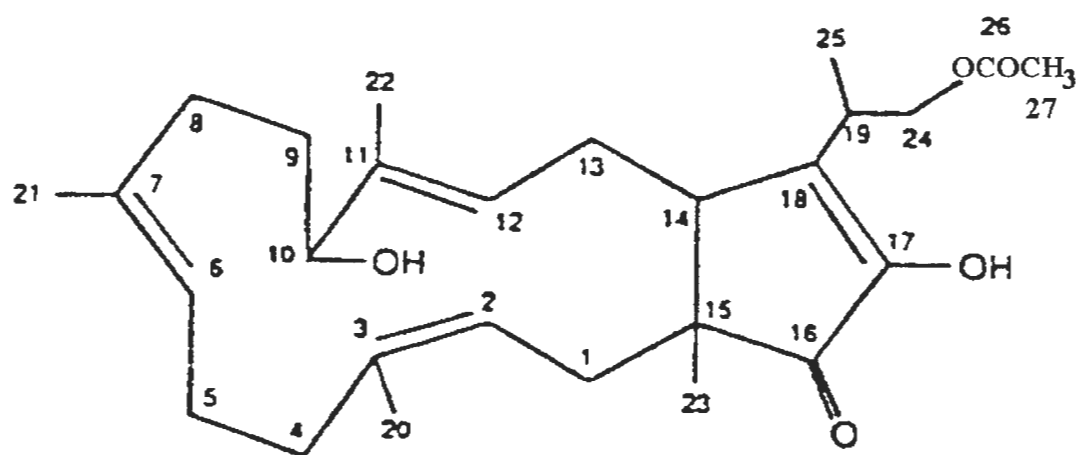


Figure 1. Fusaproliferin (from Santini *et al.* 1996)

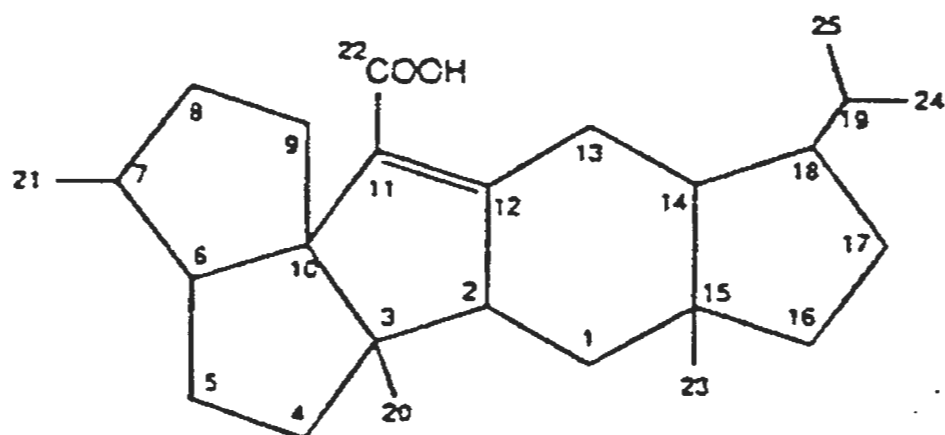


Figure 2. Retigeranic acid (from Santini *et al.* 1996)

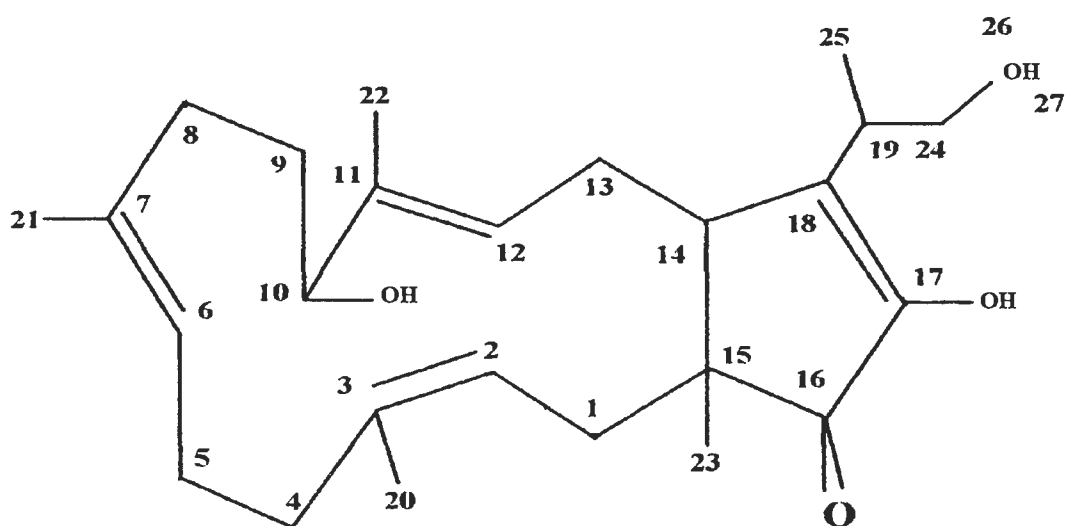


Figure 3. Fusaproliferin deacetyl (from Ritieni *et al.*, 1997)

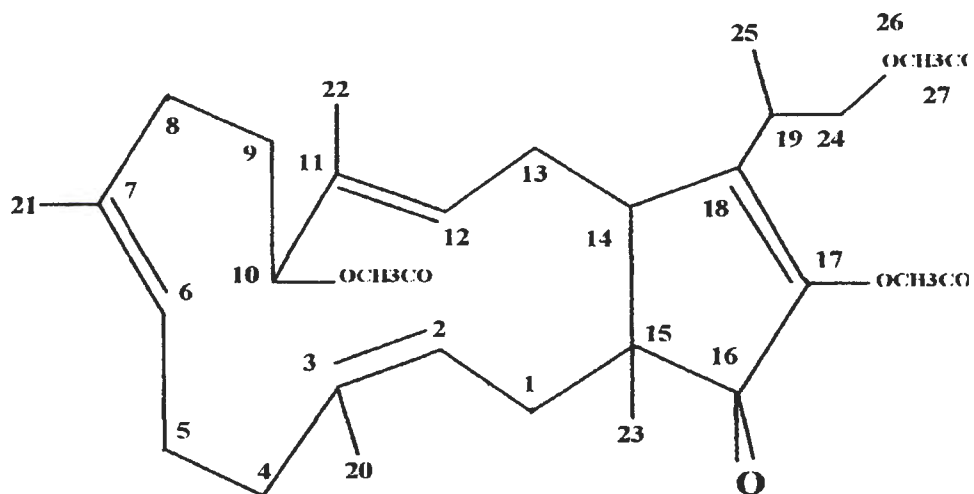


Figure 4. Fusaproliferin acetyl (from Ritieni *et al.*, 1997)

Table 1. 1996 pre-harvest Iowa corn samples positive for fusaproliferin and/or fusaproliferin deacetyl by reverse phase thin layer chromatography (RPTLC).^a

Sample #	RPTLC- <i>p</i> -anisaldehyde spray		HPLC	
	Fp (ppm)	Fp-da (ppm)	Fp (ppm)	Fp-da (ppm)
009	–	2.8	NA	NDA
032	–	0.5	NDA	NA
050	2.8	–	NA	NA
058	–	0.5	NA	NA
081	2.8	2.0	NA	NDA
119	0.7	–	0.76	NA
120	–	0.5	NA	NA
126	1.2	0.5	1.9	NA
127	5.5	–	NDA	NA
137	–	4.0	NA	NA
144	–	3.9	NA	NA
145	–	1.0	NA	NA
150	22.1	7.8	23.1	1.0
155	–	0.5	NA	NA
181	2.0	2.8	1.4	NA
183	–	1.0	NA	NA
186	–	2.8	NA	NDA
188	2.8	–	1.9	NA
200	1.0	7.8	6.6	6.0
203	1.4	–	NDA	NA
218	–	1.0	NA	NA
223	–	2.0	NA	NA
259	–	2.0	NA	NA
274	–	3.9	NA	NA
284	–	0.5	NA	NA
285	–	2.0	NA	NA
290	–	2.0	+fp ^b	NDA
321	0.7	0.5	NA	NA
325	0.7	–	NA	NA

^a 64 total samples analyzed

^b Sample analyzed for fp-da (NDA) reported as +fp with no level

NDA – No detectable amount

NA – Not analyzed

(–) – Samples negative by TLC

Table 2. 1996 post harvest Iowa corn samples positive for fusaproliferin and fusaproliferin deacetyl by reverse phase thin layer chromatography (RPTLC).^a

Sample #	RPTLC- <i>p</i> -anisaldehyde spray		HPLC	
	Fp (ppm)	Fp-da (ppm)	Fp (ppm)	Fp-da (ppm)
6086	1.4	–	NA	NA
6087	2.8	–	NDA	NA
6930	1.4	2.0	NA	NA
6945	1.4	–	NDA	NA
6946	–	1.0	NA	NA
6949	1.4	–	NDA	NA
6950	–	1.0	NA	NA
6953	0.7	–	NA	NA
2281005	2.8	0.5	NDA	NA
2281007	2.0	–	NA	NA
2282001	5.5	0.5	NA	NA
2381001	–	1.0	NA	NA
2381007	–	2.8	NA	NA
4281001	1.4	–	0.1	NA
4281011	1.4	–	NA	NA

^a 54 total samples analyzed

NDA – No detectable amount

NA – Not analyzed

(–) – Samples negative by TLC

Table 3. Detection of fusaproliferin and fusaproliferin deacetyl in 1997 post harvest Iowa corn samples ground using a grist mill. Comparison of detection with RPTLC viewed with short wave UV, vs RPTLC and *p*-anisaldehyde spray viewed with long wave UV, vs HPLC results on 14 samples.

Sample #	Grind # ^a	Short Wave UV		RPTLC <i>p</i> -anisaldehyde spray & long wave UV		HPLC	
		fp	fp-da	fp	fp-da	fp (ppm)	fp-da (ppm)
50056	1	–	–	+	–	0.06	NDA
50057	2	+	–	+	–	0.1	NDA
109	3	–	+	+	–	0.3	0.03
3489	4	–	+	+	–	0.1	0.05
3416	5	–	–	+	+	0.06	0.04
1110	6	–	–	+	+	0.06	0.04
60035	7	+	+	+	+	0.4	0.04
2478	8	+	+	+	–	0.6	0.05
50004	9	+	–	+	–	0.2	NDA
1501	10	–	–	–	–	NDA	NDA
4294	11	+	+	+	–	0.7	NDA
3142	12	–	+	–	–	NDA	NDA
50029	13	+	–	+	–	0.9	NDA
656	14	+	+	+	+	1.6	0.3

^a Grist mill used for sample grinding

NDA – No detectable amount

(+) - Sample visible on TLC plate

(–) – Sample not visible on TLC plate

Table 4. Detection of fusaproliferin and fusaproliferin deacetyl in 1997 post harvest Iowa corn samples ground using a Wiley mill. Comparison of detection with RPTLC viewed with short wave UV, vs RPTLC and *p*-anisaldehyde spray viewed with long wave UV, vs HPLC results on selected samples.

Sample #	Grind # ^a	Short Wave UV		RPTLC <i>p</i> -anisaldehyde spray & long wave UV		HPLC	
		fp	fp-da	fp	fp-da	fp (ppm)	fp-da (ppm)
50019	15	–	–	–	–	NA	NA
50008	16	+	–	+	–	0.9	NA
50058	17	–	–	1.38	–	NA	NA
50006	18	+	–	+	–	1.4	NA
50044	19	–	–	+	–	0.2	NA
50040	20	–	–	–	–	NA	NA
50007	21	+	–	+	–	0.8	NA
50039	22	–	–	±	–	0.1	NDA
50015	23	–	–	±	–	0.2	NA
50023	24	+	+	0.7	0.5	NA	NA
50024	25	–	–	–	–	NA	NA
50003	26	–	–	0.7	0.5	NA	NA
50014	27	–	–	–	–	NA	NA
50020	27	–	–	±	–	0.1	NA
ID-44	29	–	–	–	–	NA	NA
50016	30	–	–	–	–	NA	NA
2686	31	+	–	0.7	–	NA	NA
4116	32	–	–	–	–	NA	NA
286	33	–	–	–	–	NA	NA
4650	34	–	–	0.7	–	NA	NA

^a Wiley mill used for sample grinding

NDA – No detectable amount

(+) - Sample visible on TLC plate

(–) – Sample not visible on TLC plate

(±) - Sample difficult to see on plate

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