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Evidence for the Natural Occurrence of Fumonisin B_1, a Mycotoxin Produced by *Fusarium moniliforme*, in Corn

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Fusarium moniliforme, a common fungal contaminant of corn, was recently shown to produce a group of mycotoxins, the fumonisins, in culture. Moldy home-grown corn collected from an area of the Transkei, southern Africa, was analyzed for the presence of the fumonisin mycotoxins. Fumonisin B_1 (FB_1) was detected in the sample extract, as independently prepared derivatives, by two high-performance liquid chromatographic procedures. A capillary gas chromatographic–mass spectrometric procedure was used to confirm the identity of the tricarballylic acid moiety, present in the esterified hydrolysates of the fumonisins. This is the first conclusive report of the natural occurrence of FB_1 in corn.

Fusarium moniliforme Sheldon, a common fungal contaminant of corn throughout the world (Booth, 1971), has been implicated in animal and human diseases (Marasas et al., 1984b). Various strains of the fungus are known to be highly toxic (Kriek et al., 1981a,b) and carcinogenic (Marasas et al., 1984a; Jaskiewicz et al., 1987) in animals. Since *F. moniliforme* has been associated with human esophageal cancer risk in the Transkei, southern Africa (Marasas, 1982; Marasas et al., 1981, 1988a) and in China (Li et al., 1980; Yang, 1980), recent investigations have focused on the characterization of the carcinogenic compounds produced by this fungus.

The mutagenic activities exhibited by various strains of *F. moniliforme* in the *Salmonella* mutagenicity test resulted in the characterization of the potent mutagen, fusarin C (Wiebe and Bjeldanes, 1981; Gelderblom et al., 1984a; Gaddamidi et al., 1985). However, the lack of carcinogenicity of fusarin C (Gelderblom et al., 1986) makes it unlikely that this mutagen is involved in the carcinogenic effects of the fungus. Recently, several strains of *F. moniliforme* were found to exhibit cancer-promoting activity in a short-term cancer initiation/promotion bioassay in rats using diethylnitrosamine (DEN) as a cancer initiator and the induction of γ-glutamyltranspeptidase positive foci in the liver as end point (Gelderblom et al., 1988b). With use of this bioassay as a monitor for cancer-promoting principles, the fumonisins were isolated (Gelderblom et al., 1988a) and chemically characterized (Bezuidenhout et al., 1988) from culture material of *F. moniliforme* MRC 826, previously shown to be hepatocarcinogenic to rats (Marasas et al., 1984a; Jaskiewicz et al., 1987). In addition to its cancer-promoting ability, fumonisin B_1 (FB_1; Figure 1), the major compound, also exhibits toxic effects in rats similar to that of the culture material of *F. moniliforme* MRC 826 (Gelderblom et al., 1988a). Recently Marasas et al. (1988b) induced the equine neurotoxic disease leukoencephalomalacia (LEM) in a horse by intravenous injection of FB_1 isolated from strain MRC 826.

A sample of home-grown corn from a high esophageal cancer risk area of the Transkei, southern Africa, has previously been shown to be naturally contaminated with at least four *Fusarium* mycotoxins, i.e. moniliformin, zearalenone, deoxynivalenol, and fusarin C (Thiel et al., 1982; Gelderblom et al., 1984b). This paper details the chemical analysis of this corn sample for the presence of FB_1.

EXPERIMENTAL SECTION

Analytical Standards. FB_1 was extracted from *F. moniliforme* MRC 826 as previously described (Gelderblom et al., 1988a). The purity of the analytical standard was assessed by thin-layer chromatography (TLC; see TLC analyses). Visual inspection of the plate showed the presence of a minor contam-

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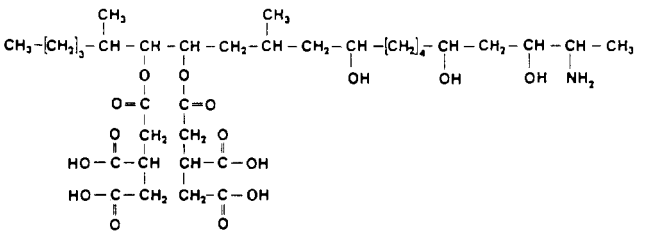

Figure 1. Chemical structure of fumonisin B_1.

inant at a slightly higher R_f value to that of the FB_1 spot, in the position of fumonisin B_3 (FB_3), another fumonisin mycotoxin whose chemical structure has still to be verified. When compared with the FB_1 used by Gelderblom et al. (1988a), the purity of the analytical standard used in this investigation was found to be >92%.

Tricarballylic acid (1,2,3-propanetricarboxylic acid, TCA) was purchased from Fluka Chemicals, Buchs, Switzerland. Only one component could be detected by capillary gas chromatography–flame ionization detection (GC-FID) analysis of the esterified derivative of this standard.

Fungal Culture. Corn cultures of *F. moniliforme* MRC 826 were incubated for 2 weeks at 25 °C followed by 2 weeks at 15 °C after which they were freeze-dried, ground, and stored at 4 °C prior to analyses (Gelderblom et al., 1984b).

Corn Samples. A sample of moldy corn ears of the 1978 crop was obtained from a farm in the Butterworth district, Transkei, during July 1978 (Thiel et al., 1982). Visibly *Fusarium* infected ears were selected and hand-shelled. The kernels (a mixture of *Fusarium*-infected kernels, healthy kernels, and kernels infected with other fungi) were retained as sample M-84 (Thiel et al., 1982; Gelderblom et al., 1984b). Two subsamples, one containing predominantly healthy kernels, and the other predominantly *Fusarium*-infected kernels, were selected from sample M-84 and retained as samples M-84/C and M-84/F, respectively. Hand-selected kernels from first-grade, commercial South African corn together with a sample of commercially available corn meal were used as control samples. Each sample was ground and stored at 4 °C prior to analyses.

Chemical Analyses. *1. Extraction.* Samples of corn (25 g, 5 g for culture samples) were extracted with CH_3OH–H_2O (3:1, 50 mL), by blending for 5 min and filtering. A 25-mL aliquot of the filtrate was evaporated to dryness at 50 °C, dissolved in CH_3OH–H_2O (1:3, 25 mL), and partitioned with $CHCl_3$ (2 × 50 mL). The aqueous phase was evaporated to dryness and resuspended in CH_3OH–H_2O (1:3, 10 mL, 50 mL for culture samples). Aliquots of these extracts (2 mL for HPLC and 1 mL for GC) were further purified by application to a Sep-Pak C_{18} cartridge, washing with CH_3OH–H_2O (1:3, 10 mL), and eluting the toxin with CH_3OH–H_2O (3:1, 10 mL). The eluate was evaporated to dryness.

2. TLC Analysis. Aliquots of the purified sample extracts (in CH_3OH–H_2O, 1:3) were spotted on Silica Gel 60 TLC plates (Merck). The plates were developed in $CHCl_3$–CH_3OH–CH_3COOH (6:3:1), dried, sprayed with a solution of 0.5% *p*-anisaldehyde in CH_3OH–CH_3COOH–H_2SO_4 (85:10:5, v/v), heated for 5 min at 110 °C, and visually inspected. FB_1 showed as a brown-purple spot with an R_f ~0.25.

3. Maleyl Derivatization and High-Performance Liquid Chromatographic (HPLC) Analyses. The purified extracts were maleylated and analyzed by HPLC according to the method of Siler and Gilchrist (1982), with minor modifications. Briefly, the purified extract residues were dissolved in 0.05 M Na_2CO_3 (pH 9.2, 5 mL) and treated with an excess of 1 M maleic anhydride solution in dioxane (3 × 10 μL), while the pH was adjusted to 9 with 0.1 M NaOH, following each addition of maleic anhydride. Following a reaction time of 10 min, the mixture was adjusted to between pH 6 and 7 with use of 0.1 M HCl. The derivatized extracts were then evaporated to dryness at 50 °C and redissolved in CH_3OH–H_2O (1:1, 2 mL). HPLC separations were performed on a C_{18} reversed-phase column using a Waters Model 510 pump, and the UV absorption of the eluate was monitored with a Waters 481 variable-wavelength detector. The detailed chromatographic conditions are summarized in Table I. Data were collected with a Waters 745 data module, and quantitative determination of FB_1 was by comparison of the peak areas in the samples to that of the peak area of a similarly derivatized FB_1 standard. Sample extracts spiked with FB_1 were similarly chromatographed.

4. Extraction, Fluorescamine Derivatization, and HPLC Analysis. Samples of corn (25 g) were extracted with CH_3OH–H_2O (3:1, 50 mL) by shaking for 30 min. The extracts were filtered, and a 5-mL aliquot of the filtrate was evaporated to dryness at 50 °C. The residue was partially redissolved in CH_3OH–H_2O (7:3, 1 mL) and fully solvated following the addition of CH_3OH (1 mL). The solution was transferred to the top of a prepared chromatographic column (10-mm i.d.) containing activated silica gel (2 g, 70–230 mesh; Merck) suspended between two layers of anhydrous Na_2SO_4 (1 g), in CH_3OH. The column was washed with CH_3OH (15 mL), the toxins were eluted with 0.1% CH_3COOH–CH_3OH (20 mL), and the eluate was evaporated to dryness. The purified residues were redissolved in 0.05 M $NaHCO_3$ (pH 8.6, 1 mL), and an aliquot (25 μL) was diluted to 500 μL in a plastic microfuge tube (1.8-mL capacity) with the same $NaHCO_3$ solution. While vortex mixing, fluorescam-

Table I. Chromatographic Conditions for the HPLC and GC Analyses of Fumonisin B_1 (FB_1) and Tricarballylic Acid (TCA) Derivatives

parameter	maleyl deriv of FB_1	fluorescamine deriv of FB_1	esterified TCA	
chromatographic procedure	HPLC/UV	HPLC/fluorescence	GC–FID	GC–MS (EI)
column	Waters Novapak C_{18} (4 μm), 150 mm × 4.6 mm (i.d.)	Phenomenex Ultracarb ODS 30 (7 μm), 250 mm × 4.6 mm (i.d.)	DB-5 (0.25-μm film), 30 m × 0.32 mm (i.d.)	DB-5 (0.25-μm film) 60 m × 0.32 mm (i.d.)
mobile phase	0.05 M KH_2PO_4–CH_3OH (3:7, pH 3.5)	0.1 M acetate buffer (pH 4.0)–CH_3CN (1:1)	helium	helium
flow rate	1 mL/min	1 mL/min	35 cm/s	30 cm/s
injection vol, μL	5 or 10	20	1	1
splitless (on time), min			0.5	0.5
injector temp, °C			140	140
detector temp, °C			280	280
temp profile				
init temp, °C			50 or 3 min	70 for 1 min
final temp, °C			280	280
rate, °C min^{-1}			15	15
detector fuel gases			air, 300 mL/min; H_2, 30 mL/min	
detector wavelengths, nm	230	390 (excitation), 475 (emission)		
detector sensitivity	0.010 AUFS	20-nm slit width	2^7	
mass range, *m/z*				50–350

Table II. Levels (μg/g) of Fumonisin B_1 (FB_1) and Tricarballylic Acid (TCA) Assayed as Their Maleyl and Butyl Ester Derivatives, Respectively

sample[a]	FB_1 measd as maleyl deriv[b]	TCA calcd as due to FB_1 (maleyl deriv results)	TCA measd as Bu ester deriv[c]
hand-selected control corn	<10[d]	<4.9	<0.5
commercial corn meal	<10	<4.9	13
M-84/C	<10	<4.9	21
M-84	44	22	101
M-84/F	83	41	164
MRC 826	9280	4530	6400

[a] Key: M-84/C = healthy corn kernels; M-84 = moldy corn kernels; M-84/F = *Fusarium*-infected corn kernels; MRC 826 = culture material of *F. moniliforme* MRC 826. [b] Determined by HPLC/UV; detection limit approximately 10 μg/g. [c] Determined by GC–FID; detection limit 0.5 μg/g. [d] Subsequent analysis indicated that this was a contaminant peak eluting in the position of FB_1.

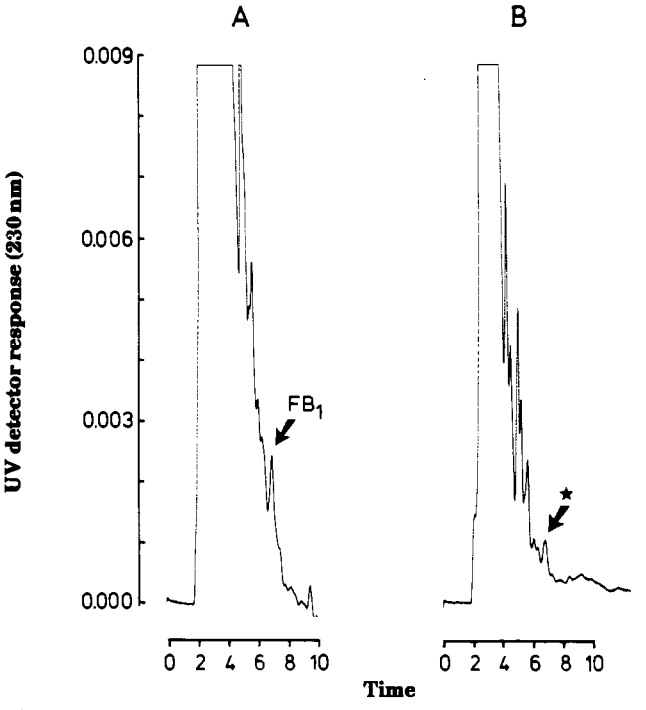

Figure 2. HPLC chromatogram of (A) the maleyl derivative of sample M-84 and (B) a similarly prepared extract of hand-selected corn kernels showing the presence of a contaminant peak (★), which elutes in the chromatographic position of fumonisin B_1 (FB_1).

ine (50 μL, 2 mg/100 μL of acetone; Aldrich) was added to the tube. Mixing was continued for 1 min, and the mixture was then centrifuged for 1 min at 9000*g*. Aliquots (20 μL) of the derivatized extracts were separated by reversed-phase HPLC (Table I) using a Waters 510 pump coupled to a Perkin-Elmer 650S fluorescence detector.

5. Hydrolysis, Derivatization, and Gas Chromatographic Analyses. Purified sample extracts were hydrolyzed (under N_2) with 6 M HCl (2 mL) at 95 °C for 3 h. Each sample was then cooled and an aliquot of each subjected to esterification and acylation according to the method of Labadarios et al. (1984). Briefly, an aliquot (500 μL) of each hydrolysate was transferred to an appropriate tube, and the excess acid was removed by freeze-drying under reduced pressure. Esterification of the residue was performed under nitrogen with isobutyl alcohol containing 3 M HCl (250 μL) and heated at 100 °C for 45 min. The acidified isobutyl alcohol was removed by freeze-drying under reduced pressure and the residue acylated with heptafluorobutyric anhydride (100 μL) by heating for 10 min (under nitrogen) at 150 °C. The samples were then cooled in ice, freeze-

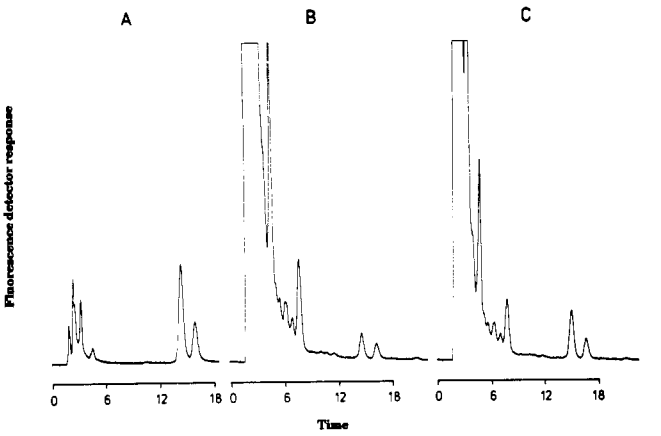

Figure 3. HPLC chromatogram of (A) the fluorescamine derivative of 65 ng of fumonisin B_1 (FB_1), (B) a similarly prepared derivative of sample M-84/F, and (C) the same sample extract spiked with derivatized FB_1.

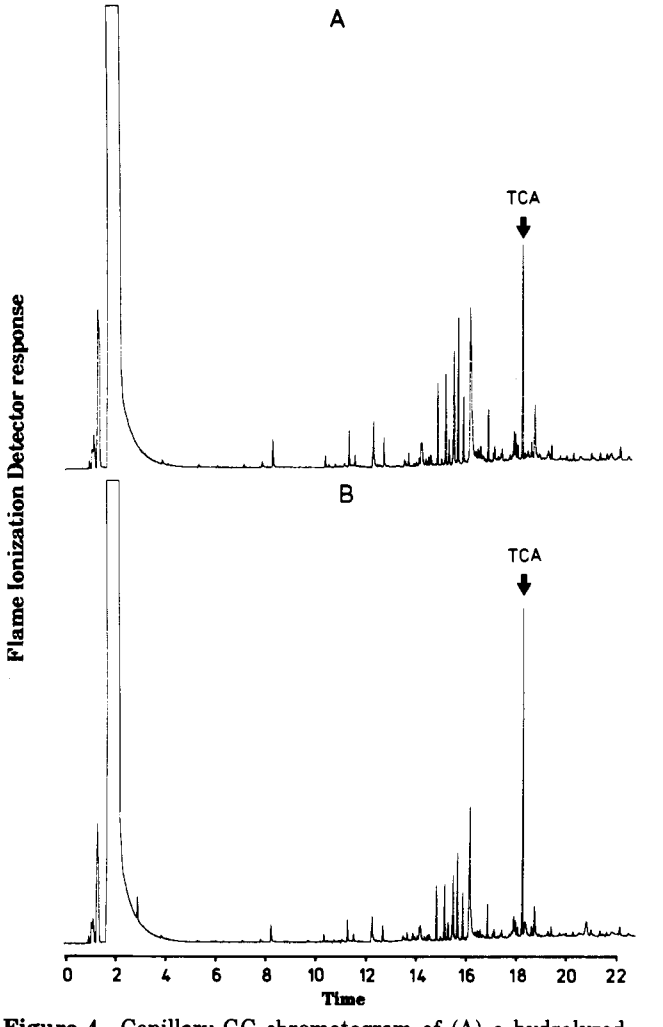

Figure 4. Capillary GC chromatogram of (A) a hydrolyzed–esterified extract of M-84/C showing a peak eluting at 18.3 min and (B) the same sample extract spiked with esterified tricarballylic acid (TCA).

dried, and dissolved in ethyl acetate (100 μL). Capillary gas chromatographic separations of 1-μL aliquots of each derivatized hydrolysate were performed on an apolar capillary column connected to a Carlo Erba Mega 5300 gas chromatograph fitted with a flame ionization detector and a Waters 745 data module. The prevailing chromatographicconditions are outlined in Table I. Each sample was analyzed in triplicate, and samples spiked with FB_1 were similarly treated. Quantitative determinations were done by comparison of the peak areas of

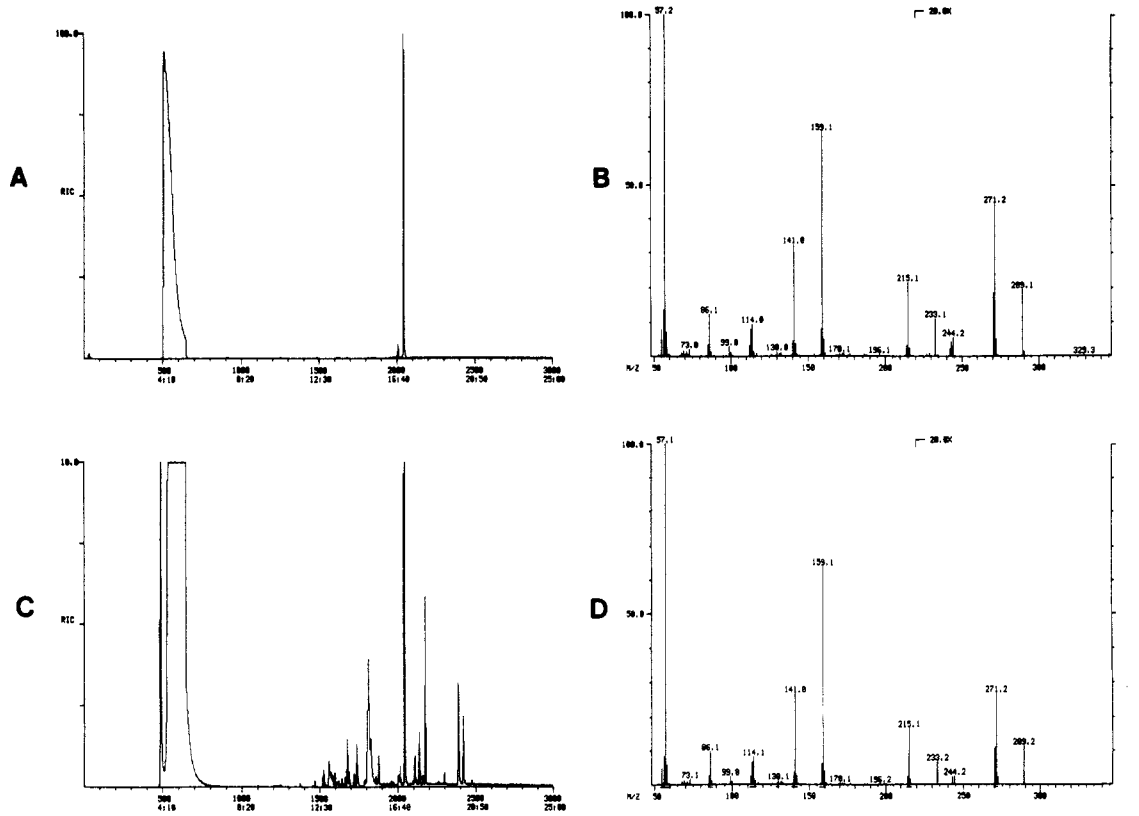

Figure 5. (A) Total ion chromatogram (TIC) of esterified tricarballylic acid (TCA) showing a major peak eluting at 17.1 min, (B) the mass spectrum (MS) of esterified TCA, (C) TIC of a hydrolyzed–esterified extract of M-84/F also showing a major peak at 17.1 min, and (D) MS of the peak eluting at 17.1 min shown in (C).

the derivatized TCA moiety, against a calibration curve of similarly derivatized TCA standards (found to be linear over the range 25–150 ng). Capillary gas chromatographic–mass spectrometric data of the TCA butyl ester peak present in the samples were obtained on a Finnigan MAT 4500 gas chromatograph–mass spectrometer under the conditions specified in Table I.

RESULTS AND DISCUSSION

Extracts of a fungul culture (*F. moniliforme* MRC 826), two samples of control corn, and the three Transkeian corn subsamples were prepared and analyzed by HPLC for the presence of FB_1, as its maleyl derivative. The results generated are given in Table II. The chromatogram obtained from a 5-μL aliquot of a corn sample extract (M-84), as its corresponding maleyl derivative, is shown in Figure 2A. Base-line separation of the FB_1 peak was not possible due to the presence of substrate matrix interferences.

Figure 2B shows the chromatogram obtained from a 10-μL aliquot of an extract of hand-selected control corn kernels as the maleyl derivative. While the degree of matrix interference is much lower than that observed in Figure 2A, a small peak was observed at the chromatographic position of FB_1 (corresponding to <10 μg/g of FB_1; Table II). A similar peak was also observed in the sample of commercially available corn meal as well as in extracts of three other control corn samples (data on the latter samples are not included). The presence of this peak in several control corn samples indicated the possibility that it was an interfering compound found intrinsically in corn.

Therefore, a number of matrix-related factors clearly demonstrate the limitations of the maleyl derivative procedure for the determination of FB_1 in naturally contaminated corn samples. Using the maleyl derivative procedure, the average corrected recovery (in triplicate) of FB_1 from hand-selected control corn spiked at 100 μg/g was found to be 66.3% with a standard deviation of 1.4%.

Further evidence for the presence of FB_1 was obtained by the extraction, fluorescent labeling, and chromatographic separation of extracts of the Transkeian corn samples. Figure 3A shows the chromatogram of a fluorescamine-derivatized FB_1 standard, where two well-resolved peaks may be seen at the retention times of 14.3 and 16.5 min, respectively. Figure 3B shows the chromatogram obtained from a similarly derivatized extract of sample M-84/F, and Figure 3C, the same extract spiked with derivatized FB_1. HPLC separation of a preformed fluorescamine–primary amine complex often results in dual peaks for primary amines, as was the case for FB_1 (Figure 3A). These peaks result from the formation of the acid alcohol and the lactone derivatives of the fluorescent complex both exhibiting identical fluorescent characteristics (Perrett, 1985; Rosenthal, 1985). Due to the necessity for optimization, this fluorescamine derivatization method could not be used quantitatively, and hence, its application to the naturally contaminated corn subsamples merely yielded supportive evidence for the presence of FB_1.

Only a single peak could be detected by GC–FID in a hydrolyzed, esterified, and acylated FB_1 standard, under the conditions outlined in Table I (GC–FID data). Esterification of an aliquot of the FB_1 hydrolysate (without the subsequent acylation step) resulted in the detection of the same chromatographic peak. This peak was also observed in an esterified derivative of an authentic TCA standard. These observations suggested that the FB_1 had been successfully hydrolyzed but that in the process only the esterified TCA moiety could be detected and not the anticipated aminopentol moiety.

By the hydrolysis/esterification procedure, the levels of the TCA moiety present in each sample were deter-

mined and the results are given in Table II. Figure 4A shows the GC–FID chromatogram obtained from a hydrolyzed–esterified extract of M-84/C, while in Figure 4B the same extract spiked with similarly esterified FB_1 standard is illustrated. A well-resolved peak corresponding to the isobutyl ester of TCA can be observed at a retention time of 18.3 min (Figure 4A,B).

In order to verify the identity of the TCA moiety present in the Transkeian corn samples, hydrolyzed–esterified extracts of each sample were analyzed by GC–MS (chromatographic conditions are given in Table I). Figure 5A details the total ion chromatogram (TIC) of the esterified TCA standard, with a single peak eluting at a retention time of 17.1 min, the mass spectrum (MS) of which is shown in Figure 5B. Figure 5C displays the TIC of the hydrolyzed–esterified extract of M-84/F, where a well-defined peak can also be seen at 17.1 min. The MS of this peak is given in Figure 5D. The excellent agreement between the two spectra (Figures 5B,D) verified the presence of TCA in the hydrolyzed–esterified extract of the Transkeian corn sample.

The TCA contribution due to the FB_1 (determined as its maleyl derivative), present in each sample, was calculated, and the results are given in Table II. The levels of TCA determined experimentally (by GC–FID) were invariably higher in each sample than the corresponding TCA content contributed by the FB_1. In the case of the hand-selected control sample, the peak observed in the chromatographic position of FB_1, as its maleyl derivative (Figure 2B) was undoubtedly a contaminant peak since no corresponding TCA was detected in the sample. Since a similar sized peak was also observed in the maleylated extracts of the commercial corn meal and M-84/C samples, the levels of TCA determined experimentally in these samples could not be directly attributed to the presence of FB_1.

The fact that the TCA levels in five to six samples were higher than could be explained by the presence of FB_1 was not unexpected, as it has already been shown that TCA-containing compounds other than FB_1 (such as fumonisin B_2; FB_2) are produced by *F. moniliforme* (Gelderblom et al., 1988a). In the case of the MRC 826 culture material, the FB_1 accounted for >70% of the total TCA detected experimentally in the hydrolyzed–esterified extract, which agrees with the observation that FB_1 is the major fumonisin produced by this fungus in culture (Gelderblom et al., 1988a). In samples M-84 and M-84/F, FB_1 contributed less to the total TCA than in the culture material, implying that more of the other fumonisins were present in these samples. Attempts to confirm the presence of FB_1 and other related fumonisins (FB_2 and FB_3) in extracts of the Transkeian corn samples by TLC were inconclusive, since the detection limit of the method (500 μg/g for each toxin) was far higher than the levels present in the samples.

None of the chromatographic methods presented should be considered as fully developed analytical procedures for the determination of FB_1 in corn. However, the application of three chromatographic procedures to a naturally contaminated sample provided conclusive evidence for the presence of FB_1 in corn. This evidence suggests that humans in an area of the Transkei, southern Africa, may well be exposed to the cancer-promoting *F. moniliforme* mycotoxins, the fumonisins.

Subsequent to the acceptance of this publication, Voss et al. (1989) reported the cooccurrence of FB_1 and FB_2, in two corn samples associated with outbreaks of equine LEM in the United States.

ABBREVIATIONS USED

DEN, diethylnitrosamine; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3; GC–FID, gas chromatography–flame ionization detection; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; LEM, leukoencephalomalacia; MS, mass spectrum; TCA, tricarballylic acid; TIC, total ion chromatogram; UV, ultraviolet.

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Registry No. FB_1, 116355-83-0.

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Avermectin B_{1a} Metabolism in Celery: A Residue Study

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Radioactivity equivalent to less than 4% of the total avermectin B_{1a} applied to mature and immature celery as a mixture of unlabeled and [^{14}C]- or [^{3}H]avermectin B_{1a} remained in harvested plants. The half-lives of radiolabeled avermectin B_{1a} residues as measured by the dissipation of total ^{3}H radioactivity from celery parts ranged from 5.2 to 12.9 days in celery treated at 0.01 and 0.10 lb of AI/acre. The percentage of total radioactivity in celery parts readily extractable with acetone (range of 57.8-97.1%) generally decreased with increasing postharvest intervals. High-performance liquid chromatography of acetone extracts of celery leaves and stalks produced four discernible peaks of radioactivity, designated as polar metabolites, moderately polar metabolites, avermectin B_{1a}, and the $\Delta^{8,9}$ isomer of avermectin B_{1a}. The percentage of acetone extract radioactivity represented by polar metabolites generally increased with increasing postharvest intervals.

Abamectin (MK-0936) is a macrocyclic lactone pesticide that has been under investigation as an acaricide/nematicide/insecticide in citrus, orchard, and field crops (Price, 1983; Schuster and Everett, 1983; Wright, 1984; Reed et al., 1985; Burts, 1985). It is being developed as a miticide/insecticide to control imported red fire ants and several phytophagus pests on horticultural and agronomic crops. The use of this compound to control the leaf miner (*Liriomyza sp.*) in celery is currently proposed, and the results of this study present data relevant to such use. Avermectin B_{1a} (AVM-B_{1a}) is the major component of abamectin (MK-0936). The specific objectives of the present study were (1) to determine the dissipation rate of this major component from radiolabeled AVM-B_{1a}-treated immature and mature celery plants and (2) to examine the metabolism of AVM-B_{1a} by immature and mature celery plants grown in pots under field conditions after multiple applications.

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