Comparison of Thin-Layer Chromatography and Competitive Immunoassay Methods for Detecting Fumonisin on Maize†

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The fumonisin mycotoxins are secondary metabolites of *Fusarium moniliforme*, which are common worldwide in maize. Thin-layer chromatography (TLC) and competitive indirect immunoassay (CI-ELISA) methods were compared for detection of fumonisin B_1 in maize. Corn from the 1991 Missouri maize variety trials (322 samples) was collected, milled, subsampled, and analyzed independently by two laboratories using different screening methods. Fifty-two percent of the samples tested negative for fumonisin B_1 at 1 ppm by both methods. By TLC, an additional 14% of the samples had less than 1 ppm of fumonisin B_1 and more than 1 ppm by CI-ELISA. TLC found 34% of the samples were from 1 to 10 ppm and only 1% of the samples were above 10 ppm of fumonisin B_1. CI-ELISA found 28% of the samples contained fumonisin levels between 1 and 10 ppm and 20% had greater than 10 ppm of fumonisin. In the remaining samples, CI-ELISA consistently reported higher fumonisin levels than TLC (160/322), while TLC was higher in only 10 of 322 samples. The discrepancy may be due to fumonisin B_1 alone being detected by TLC, while CI-ELISA measures total fumonisins. Both methods are well suited for rapid screening of maize samples for fumonisin contamination.

Keywords: *Fumonisin; maize; TLC; immunoassay*

INTRODUCTION

The fumonisins are produced by *Fusarium moniliforme* (Bezuidenhout et al., 1988; Gelderblom et al., 1988) and *Fusarium proliferatum* (Ross et al., 1990); their natural occurrence was first reported in corn from Transkei, Republic of South Africa (Sydenham et al., 1990). In the United States, fumonisin-contaminated corn screenings have been associated with cases of equine leukoencephalomalacia (Marasas et al., 1988; Norred et al., 1989; Plattner et al., 1990; Ross et al., 1990; Wilson et al., 1990) and swine pulmonary edema (Harrison et al., 1990; Ross et al., 1990), but only limited information is available on the natural occurrence and levels of incidence of the fumonisins (Rottinghaus et al., 1992; Murphy et al., 1993). Preliminary survey data suggest fumonisin is worldwide in distribution and is present in maize-based food items. Sydenham et al. (1991) found fumonisin in human corn-based food products from Egypt, Peru, Canada, South Africa, and the United States. Fumonisin has also been identified in Brazilian feeds (Sydenham et al., 1992) and in corn-based products from Switzerland (Pittet et al., 1992).

Analytical methodologies for detecting fumonisins in corn and corn products include thin-layer chromatography (TLC) (Gelderblom et al., 1988; Plattner et al., 1990; Ross et al., 1991; Rottinghaus et al., 1992), high-performance liquid chromatography (Alberts et al., 1990; Gelderblom et al., 1988; Ross et al., 1991; Shepard et al., 1990; Wilson et al., 1990), gas chromatography (Jackson and Bennett, 1990; Sydenham et al., 1990), gas chromatography/mass spectroscopy (Plattner et al., 1990; Voss et al., 1990; Wilson et al., 1990), liquid secondary ion mass spectrometry (Bezuidenhout et al., 1988; Plattner et al., 1990; Voss et al., 1990), and ELISA (Azcona-Olivera et al., 1992; Shelby and Kelley, 1992).

Thin-layer chromatography and ELISA methodology are the most adaptable for screening large numbers of samples. Therefore, two recently developed procedures—the TLC procedure of Rottinghaus et al. (1992) and the monoclonal antibody-based competitive indirect immunoassay of Shelby and Kelley (1992)—were compared. Our intent in this study was to compare the reliability and utility of these screening procedures for detecting fumonisins and provide data on the natural occurrence of fumonisins in maize from the field.

MATERIALS AND METHODS

Materials. All inorganic chemicals and organic solvents were of ACS grade or better. Fumonisin B_1 was obtained from Sigma Chemical Co. (St. Louis, MO) and from Dr. W. C. A. Gelderblom (Republic of South Africa). *Caution: Fumonisin B_1 is a suspected carcinogen and should be handled appropriately.* Cleanup columns (C_{18} Sep-Pak Vac, 3 cm^3) were purchased from Waters (Division of Millipore, Milford, MA). All samples were ground with a Stein mill (Model M-1, Fred Stein Labs, Atchison, KS). TLC C_{18} plates were purchased from Whatman International Ltd. (Maidstone, England). Fluorescamine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Microtiter plates (96 well, Immunolon 4) were from Dynatech Laboratories (Chantilly, VA). Goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (170-6516) was obtained from Bio-Rad Laboratories (Richmond, CA). The

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chromagen, o-phenylenediamine dihydrochloride (P-8287), and phosphate–citrate urea buffer (P-9305) were purchased from Sigma.

Samples. Corn samples (approximately 0.5 kg) were collected, as part of an ongoing mycotoxin monitoring program, from the University of Missouri variety test plots. A diverse group of 10 or 12 hybrids (3 replicates) was collected from each of 9 test sites in Missouri. Samples were labeled by location and hybrid. Dry land test plots were located in Audrain (northeast), Saline (west central), Gasconade (east central), Nodaway (northwest), Vernon (southwest), and Stoddard (southeast) counties. Irrigated test plots were located in Boone (central), Barton (southwest), and Scott (southeast) counties. Samples (322 total) were dried for a week at 60 °C in large forced-air drying ovens. Samples were ground and split for fumonisin analysis.

TLC Extraction and Cleanup. Fumonisin B_1 was analyzed according to the method of Rottinghaus et al. (1992). Fifty grams of finely ground sample was placed in a wide-mouth, polypropylene, screw-cap bottle with 100 mL of acetonitrile/water (1:1) and placed on a wrist-action shaker (Burrel Corp., Pittsburgh, PA) for 30 min. Contents were allowed to settle, and a 10–25-mL aliquot of solvent extract was decanted and filtered. The cleanup column was preconditioned with 5 mL of methanol followed by 5 mL of 1% aqueous potassium chloride (KCl). Two milliliters of filtrate was combined with 5 mL of 1% aqueous KCl and applied to the column. The column was washed with 5 mL of 1% aqueous KCl followed by 2 mL of acetonitrile/1% aqueous KCl (1:9); the eluants were discarded. The fumonisins were eluted with 4 mL of acetonitrile/water (7:3), and the column eluant was evaporated to dryness under a stream of air on a Reacti-Therm heating module (Pierce Chemical Co., Rockford, IL) prior to TLC analysis.

Thin-Layer Chromatography. The sample residue was dissolved in 100 μL of acetonitrile/water (1:1), and 10 μL was spotted on a C_{18} TLC plate along with 10 μL of fumonisin B_1 standards (5, 10, and 100 ppm) dissolved in acetonitrile/water (1:1). The TLC plate was developed in methanol/4% aqueous KCl (3:2), air-dried, and sprayed with 0.1 M sodium borate buffer (pH 8–9) followed by fluorescamine (0.4 mg/mL in acetonitrile). After 1 min, the plate was sprayed with 0.01 M boric acid/acetonitrile (40:60). The TLC plate was air-dried at room temperature and examined under longwave ultraviolet light. Fumonisin levels were estimated by visual comparison (bracket results) with standards.

Confirmation. The remaining sample residue (4.5-g equivalents) and fumonisin B_1 standards (100 μL of 10 ppm) were hydrolyzed with 2 N KOH for 1 h at 60 °C (Plattner et al., 1990). The reaction mixtures were each extracted with 1 mL of ethyl acetate, the ethyl acetate was taken to dryness, and the residues were dissolved in 100 μL of acetonitrile/water (1:1). Ten microliters was spotted on a C_{18} TLC plate. The TLC plate was developed in methanol/4% aqueous KCl (4:1) and air-dried. The plate was sprayed as above and reexamined under longwave UV light.

Competitive ELISA. The CI-ELISA was conducted on 1-g samples weighed into disposable plastic 25-cm^3 cups. Samples were extracted by pipetting 5 mL of phosphate-buffered saline (pH 7.2) plus 0.5% Tween 20 in PBS (PBST) directly into the sample cup, stirring briefly, and allowing the sample to settle for 30 min. Meanwhile, microtiter plates were coated with a dilution of fumonisin B_1–bovine serum albumin conjugate (approximately 1 μg/mL) in 0.05 M sodium carbonate buffer (pH 9.6). Coating was complete in 30 min at room temperature. Wells were then washed five times with PBST by means of a hand-held wash bottle. Fifty microliters of PBST sample extract was pipetted directly from the sample cups into the coated microtiter wells using large-orifice disposable pipets. Monoclonal antibody (Mab8H3) developed in our laboratory against fumonisin B_1 (Shelby and Kelley, 1992) was diluted in PBS–Tween to approximately 5 μg/mL, and 50 μL was added to the wells containing samples. Following a 15-min incubation at room temperature, the wells were rinsed with PBST as before, and a 1:1000 dilution of goat anti-mouse IgG (H+L) horseradish peroxidase conjugate in PBST was added. This was allowed to incubate at room temperature for 15 min and washed with PBST, as before. The chromagen, o-phenylenediamine dihydrochloride, was diluted in phosphate–citrate urea buffer (Sigma P-9187) at 1 mg/mL, and 100 μL was added to the wells and incubated for 15 min. The reaction was stopped by the addition of 50 μL of 3 M sulfuric acid. Optical density was measured at 490 nm using a Dynatech MR 580 ELISA plate reader. For semiquantitative ELISA a standard curve was generated on the upper horizontal axis of each plate, by diluting fumonisin B_1 in PBST and making 1:2 dilutions from 100 to 0.1 ppm. A regression equation of log ppm vs A_{490} was computed for each plate, and absorbance values were converted to parts per million of total fumonisin.

Table 1. Fumonisin Concentration in Missouri 1991 Corn Samples Determined by the TLC Method of Analysis

		fumonisin category		
Missouri county	location	N/D	1–10 ppm	>10 ppm
Saline	west central	59	31	
Nodaway	northwest	52	48	
Vernon	southwest	37	59	3
Audrain	central	83	14	
Boone	southwest	52	46	2
Barton	southeast	64	33	3
Stoddard	southeast	76	24	
Scott	southeast	100		
Gasconade	east central	79	21	

RESULTS

A survey was carried out in cooperation with the Agronomy Department at the University of Missouri to determine the frequency and levels of occurrence of fumonisin B_1 in the 1991 Missouri corn harvest. A total of 322 samples from University test plots were collected and analyzed for fumonisin B_1 by TLC and CI-ELISA screening procedures. Results of the TLC survey are presented in Table 1. The highest incidence of FB_1 was in Barton, Vernon, Boone, and Nodaway counties, which are located in the central to western part of the state, while in the eastern part of the state, FB_1 incidence was low, with no FB_1 being detected at 1 ppm in samples from Scott county. Fumonisin B_2 was observed at low levels in some samples, but no attempt was made to measure its concentration.

Mean fumonisin levels for all samples was 4.4 ppm for the ELISA method of analysis. The two tests differed in that the TLC was interpreted by categories, while the ELISA yielded numerical values. This made correlation analysis impossible due to the non-numerical nature of the TLC data. A typical standard curve for an ELISA plate (Figure 1) shows an absorbance inflection at about 0.1 μg/mL in buffer. This translates to 0.5 μg/g for the maize sample, allowing for the 1:5 sample buffer ratio. When the data are grouped by TLC category and compared, the tests agreed in 152 of 322 samples, while the ELISA test indicated higher total fumonisin in 160 of 322 samples and lower total fumonisin in only 10 of 322 samples (Table 1; Figure 2). The greatest discrepancy between analyses was in the <1 ppm TLC category. Here ELISA indicated >1 ppm in 65 of 86 tests, but the ELISA mean was only 2.0 ppm, suggesting that the discrepancy is minor.

DISCUSSION

The specificity of the immunoassay is directly related to the specificity of the antibody used in the assay. Previous tests of cross-reactivity of Mab8H3 showed it to be specific for the intact fumonisin structure and

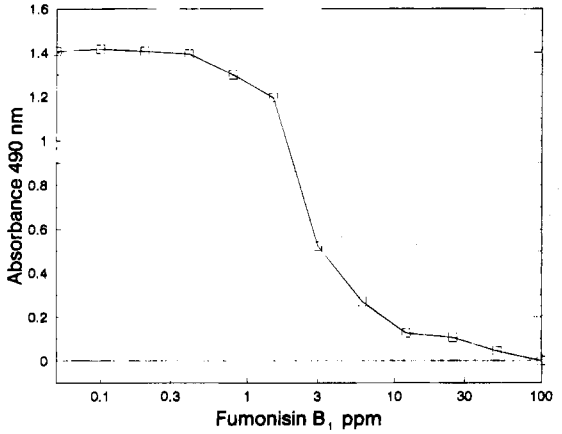

Figure 1. Standard curve of ELISA absorbance at 490 nm vs fumonisin concentration.

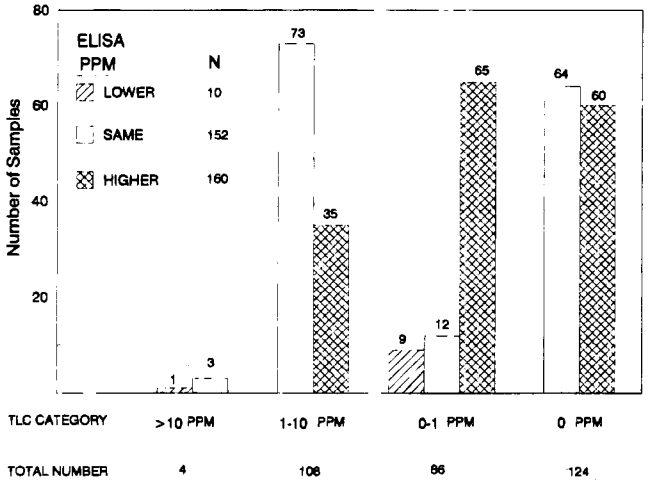

Figure 2. Comparison of ELISA vs TLC methods of detection.

entirely nonreactive with tricarballylic acid and other hydrolytic products (data not shown). Reactivity was essentially the same for FB_1, FB_2, FB_3, and FB_4, while other potential cross-reactive compounds were not recognized. Due to this spectrum of Mab reactivity, the immunoassay could best be described as one for total fumonisins and would be expected to indicate somewhat higher levels than FB_1 alone, as indicated by TLC. The indirect competitive format of the assay differs slightly from a similar immunoassay (Azcona-Olivera et al., 1992) using a similar Mab but not using a fumonisin–peroxidase conjugate in the direct format. We chose to use commercially available second antibody peroxidase conjugates because of the reproducibility of commercial conjugates. The sensitivity range 0.5–10.0 ppm is compatible with both methods and is a common range of values for U.S. maize. Toxicological data suggest that 5 ppm is the level of concern for this toxin in equidae (Riley et al., 1993), but no general level of concern applicable to other animals or humans is available at this time.

The greater values for fumonisin indicated by ELISA are somewhat more than can be accounted for by cross-reactivity of the Mab with fumonisins other than B_1. We believe that the seemingly higher ELISA values are not due to greater sensitivity of ELISA, but rather to the reactivity of the Mab with other non-fumonisin analytes in the sample, possibly undescribed related molecular structures. Current investigations are in progress to determine what other molecular species in maize cultures of *F. moniliforme* are reactive with the antibody.

Both assays are suitable for rapid screening of relatively large numbers of samples for potentially dangerous levels of this toxin. ELISA offers an advantage in terms of time and total cost of conducting the assay; however, it requires a source of the antibody and conjugates which may not be immediately available, while TLC is immediately available to any laboratory with little setup or other investment required. Both methods lack the quantitative accuracy of HPLC but have a definite place in screening and safeguarding the food and feed supply.

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