Comparison of Extraction Buffers for the Detection of Fumonisin B_1 in Corn by Immunoassay and High-Performance Liquid Chromatography

Ellen S. Kulisek* and Jan P. Hazebroek

Analytical Biochemistry, Trait and Technology Development, Pioneer Hi-Bred International, Inc., N.W. 62nd Avenue, P.O. Box 1004, Johnston, Iowa 50131

The Associatian of Official Analytical Chemists approved method for quantification of fumonisin B_1 (FB_1) in corn meal or corn-based food products includes extraction into methanol (MeOH)/water (3:1, v/v). Disposal of the extraction medium can pose safety and environmental problems. To secure a rapid and inexpensive screen for FB_1 contamination, a sensitive competitive ELISA using a rabbit polyclonal antibody was developed. This assay was used in a comparative study measuring the extraction efficiency of FB_1 in aqueous or organic solvent buffers using 16 field corn samples. An aqueous phosphate buffer was found to be suitable for extracting FB_1, thus eliminating the need for organic solvents. HPLC and ELISA determinations compared well in fortified samples at known concentrations between 1 and 50 μg/mL of extract. Overestimation at levels >50 μg/mL were common. The characteristics and application of the ELISA for screening purposes are discussed.

Keywords: *Fumonisins; ELISA; immunoassay; HPLC; extraction buffer comparison; corn*

INTRODUCTION

Fumonisins are secondary metabolites produced by *Fusarium moniliforme, Fusarium proliferatum*, and other related species (Chen et al., 1992; Thiel et al., 1991; Nelson et al., 1992). As evidence mounts worldwide implicating these mycotoxins in animal and human diseases (Marasas et al., 1988; Kellerman et al., 1990; Sydenham et al., 1990; Rheeder et al., 1992; Chu et al., 1994), there has been a parallel effort to develop and/or commercialize accurate quantitative assays (Shelby et al., 1994; Tejada-Simon et al., 1995; Schneider et al., 1995; Rice et al., 1995; Yeung et al., 1996; Selim et al., 1996; Sutikno et al., 1996; Yu et al., 1996; Shephard, 1998). Although chromatographic methodologies are considered the industry standard for accuracy and precision, they require considerable sample preparation, which makes them undesirable for high sample throughput. The successful production of both polyclonal (Azcona-Oliveira et al., 1992a; Usleber et al., 1994; Yu et al., 1996; Sutikno et al., 1996) and monoclonal (Azcona-Oliveira et al., 1992b; Shelby et al., 1992; Fukuda et al., 1994; Elissalde et al., 1995; Chu et al., 1995) antibodies against these small analytes has enabled the development of highly sensitive and rapid ELISA formats. In addition, these biological tools are finding their way into other technologies such as immunoaffinity columns (Scott and Trucksess, 1997), fiber optic immunonsensing (Thompson and Maragos, 1996; Maragos, 1997a; Yu and Chu, 1998), immunoaffinity/capillary electrophoresis hybrid systems (Maragos, 1997b), immunoaffinity/liquid chromatography (Trucksess et al., 1995; Maragos et al., 1997), and surface plasmon resonance (Mullett et al., 1998). The ELISAs, however, have a tendency to yield determinations that can be severalfold higher than those obtained by HPLC analysis (Shelby et al., 1994; Tejada-Simon et al., 1995; Sydenham et al., 1996; Sutikno et al., 1996).

We sought to develop a sensitive quantitative ELISA with polyclonal antibodies that would be inexpensive to perform and could be run easily in laboratories at Pioneer Hi-Bred International's research locations for screening purposes. To avoid hazardous waste disposal, we also desired to identify a suitable aqueous extraction buffer. Therefore, we compared several extraction buffers to determine whether it was necessary to use an organic solvent for this application.

SAFETY

Fumonisins are toxic compounds and should be handled with extreme caution.

MATERIALS AND METHODS

Materials. Goat anti-rabbit alkaline phosphatase conjugate used for indirect antisera titrations was purchased from Promega, Madison, WI. All assay development and ELISAs were performed with Nunc-Immuno Plate MaxSorp microtiter plates (NalgeNunc International, Rochester, NY). Purified FB_1 was a gift from J. D. Miller, Agriculture Canada. An aliquot was sent to the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Ames, IA, to confirm the concentration by HPLC prior to assay development and methodology comparisons. Purified FB_2, FB_3, and hydrolyzed FB_1 (HFB_1) were gifts from R. Eppley, FDA. Due to the hazardous nature of these compounds, all fumonsisins should be handled with considerable safety precautions. All other chemicals were of reagent grade or better.

Polyclonal Antibody and Antisera Titration. FB_1 was conjugated to keyhole limpet hemocyanin (KLH) or ovalbumin (OVA) using glutaraldehyde according to an established

* Author to whom correspondence should be addressed [telephone (515) 270-3697; fax (515) 270-3367; e-mail kulisekes@phibred.com].

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protocol (Fukuda et al., 1994). To a solution of 0.8 mg of FB_1 and 1.0 mg of carrier protein per milliliter was added dropwise an equal volume of 2% glutaraldehyde in 10 mM phosphate-buffered saline, pH 7.4 (PBS), with constant mixing. After 1 h at room temperature, sodium borohydride was added to a final concentration of 10 mg/mL. The mixture was dialyzed against PBS and sent to H. T. I. Bio-Products, Ramona, CA, for antibody production in rabbits. Animals received multiple subcutaneous injections in complete Freund's adjuvant over a period of several days. Further details of the immunization protocol are proprietary to the contract firm. Test bleeds were performed 40 days after primary immunization. Unconjugated purified FB_1 was also included as an immunogen.

Titrations were performed on all test bleeds against their corresponding immunogens, unconjuated FB_1 and KLH or OVA, by indirect ELISA. Briefly, 50 μL aliquots of 1 μg/mL target protein or purified FB_1 in 0.01 M carbonate−bicarbonate buffer, pH 9.8, was coated onto microtiter plate wells and incubated overnight at 4 °C. Plates were then incubated for 1 h with blocking buffer composed of 0.5 M NaCl, 50 mM Tris base, pH 8.0, and 0.5% Tween 20 to block unoccupied sites. Fifty microliters of antisera diluted serially with blocking buffer was applied to each well, and the plates were incubated for an additional hour at 37 °C. Plates were washed five times with blocking buffer using an automatic microtiter plate washer (SLT 96PW SLT, Lab Line Instruments, Research Triangle Park, NC), after which goat anti-rabbit conjugated to alkaline phosphatase was diluted 1:7500 with blocking buffer and applied to all wells. After a 1 h incubation at 37 °C, plates were washed as described above. Signal was visualized by adding 1 mg/mL *p*-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8, that contained 3 mM $MgCl_2$. Absorbance at 405 nm was measured (SpectraMax 250, Molecular Devices Corp., Sunnyvale, CA). Immune antisera were compared to their respective pre-bleeds. All immune antisera were purified prior to assay development by protein A/G column chromatography followed by depletion of KLH- and OVA-specific antibody populations by Research Genetics, Hunstville, AL, using immunoaffinity column purification.

Competitive ELISA. Horseradish peroxidase−FB_1 (HRP−FB_1) conjugate was purchased from International Diagnostic Systems Corp., St. Joseph, MI. One hundred microliters of protein A/G purified rabbit anti-FB_1 at a concentration of 2 μg/mL in carbonate−bicarbonate buffer, pH 9.8, was placed in each well. The plates were sealed and incubated overnight at 4 °C. After five washes with PBS containing 0.05% Tween 20 (PBST), unoccupied sites were blocked by the addition of 300 μL of PBST containing 0.5% Tween 20 followed by incubation for 1 h at room temperature. Fifty microliters of HRP−FB_1 conjugate diluted 1:5000 with PBST was incubated with 50 μL of purified FB_1 standard or sample extract on a platform shaker for 1 h at room temperature. Standards and samples were prepared with the same buffer compositions. After competition, plates were washed five times with PBST and once with deionized H_2O. One hundred microliters of TM Blue Soluble Form (Intergen, St. Milford, MA) was added to each well, and the plates were agitated for 1 h at room temperature as described for the competition step. The reaction was stopped by the addition of 100 μL of 1 M H_2SO_4. Absorbance at 450 nm was measured as above. FB_1 was quantified using a four-parameter curve fit generated by software (Softmax, Molecular Devices Corp., Sunnyvale, CA).

Comparison of Extraction Buffers. Sixteen field corn samples were used to compare efficiencies of various buffers to extract FB_1. Approximately 10−20 g of seed was ground in a coffee grinder. Fifteen milliliters of extraction buffer was added to 3 g of ground seed in a 20 mL glass vial, and the mix was agitated for 30 min at room temperature. The buffers tested were deionized H_2O, PBS, PBST, 70% aqueous methanol (MeOH) (extraction buffer used with the Neogen ELISA kit), and 50% aqueous acetonitrile (AcN). Particulates were allowed to settle for 30 min at room temperature. Supernatants were aspirated and maintained at −20 °C until analysis. Subsamples of each extract were analyzed by both the in-house ELISA and by a contract laboratory using the AOAC-accepted HPLC-based method (Rice et al., 1995). For the ELISA, samples were diluted with PBS to achieve FB_1 concentrations that interpolated within the linear part of the standard curve. The corn seed samples were also analyzed using the Neogen ELISA according to kit protocol. All data incorporated the corn meal weight-to-buffer ratio of 1:5 as the first dilution factor. FB_1 amounts are reported as micrograms per gram.

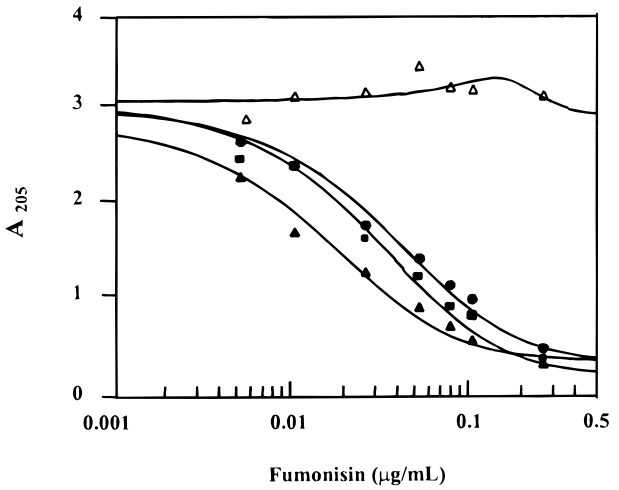

Figure 1. ELISA parameters of FB_1 (▲), FB_2 (●), FB_3 (■), or HFB_1 (△) spiked into PBS extracts of fumonisin-free corn meal.

HPLC and ELISA methodologies for FB_1 quantitation were compared further by adding known amounts of purified FB_1 to PBS extracts of corn meal determined previously to be free of fumonisins by HPLC following 70% aqueous MeOH extraction. Percent recoveries were determined by both HPLC and ELISA on subsamples of identical aliquots.

RESULTS AND DISCUSSION

Rabbits immunized with FB_1 conjugated to either KLH or OVA achieved high titers (>10000) to both the carrier protein and FB_1, whereas there was no evidence of an immune response from animals injected with purified FB_1. FB_1 as a coating antigen proved to be an adequate target for distinguishing the relative specific titer from the immune response to the protein carrier. After the first course of immunization, animals immunized with KLH−FB_1were boosted with OVA−FB_1, and OVA−FB_1-immunized animals were boosted with KLH−FB_1. Rabbits immunized with purified FB_1 were switched to KLH−FB_1. A single KLH−FB_1-immunized rabbit was chosen for assay development on the basis of its ability to maintain a high titer to purified FB_1 following KLH- and OVA-specific antibody depletion by immunoaffinity column chromatography.

We developed a direct competitive ELISA with horseradish peroxidase conjugated to purified FB_1 as the competing molecule. The assay has a dynamic range of 0.010−0.625 μg/mL, with an IC_{50} of 0.05 μg/mL. The effect of sample matrix was tested by incorporating corn meal extract free of FB_1 into diluents used to prepare FB_1 assay standards. The results were identical to those obtained in the absence of corn meal extract. This lack of matrix effects was due to the large dilutions required to achieve optical densities in the linear part of the standard curve. The most reliable predictive range is 0.025−0.25 μg/mL. Relative cross-reactivities for FB_1, FB_2, and FB_3 at IC_{50} were 0.017, 0.04, and 0.025 μg/mL, respectively (Figure 1). HFB_1 was not detected. No other compounds were tested.

Fumonisins are often extracted from corn seed in aqueous methanol. Rice et al. (1995) conducted a percent

Table 1. Comparison of Extraction Buffers on FB_1 Quantitation by HPLC and ELISA[a]

sample	H_2O HPLC	H_2O ELISA	PBS HPLC	PBS ELISA	70% MeOH HPLC	70% MeOH ELISA	50% AcN HPLC	50% AcN ELISA	Neogen ELISA
1	ND	0.11	0.9	0.19	ND	0.13	ND	0.7	0
2	3.7	0.5	5.2	1.5	0.7	1.1	0.8	1.0	1.6
3	6.3	2.25	1.1	2.2	1.7	1.6	1.9	0.2	2.6
4	4.9	1.85	3.5	2.7	1.2	1.5	1.5	1.4	3.4
5	1.1	3	1.2	3.0	1.6	4.0	2.0	6.0	6.3
6	1.8	7.7	2.1	8.5	2.4	8.5	2.9	7.0	9.3
7	3.9	17.5	4.3	13	5.6	14	8.2	15	12
8	4.3	17	5.3	14	6.3	13	10	16	15
9	6.5	16	7.6	21	5.5	20	13	6.3	19
10	11	50	11	44	15	31	20	89	18
11	11	28	13	38	16	23	25	9.8	31
12	13	39.5	14	49	19	28	24	26	45
13	38	62	51	87	32	53	42	39	34
14	30	54	23	112	33	75	44	14	54
15	55	215	42	138	80	156	120	120	89
16	110	370	110	345	93	155	160	90	104

[a] Field corn samples were ground and extracted at a 5:1 (v/w) ratio. Extracts were maintained at −20 °C prior to analyses. Aliquots of subsamples were compared by HPLC and the Pioneer ELISA. The Neogen ELISA was performed as described by the manufacturer. All data are in micrograms per gram.

recovery experiment on *Fusarium* culture material, feed corn, and commercially prepared chicken feed. Their data indicated that 50% aqueous AcN yielded the highest percent recovery in the shortest time (30 min). In the interest of safety and convenience for Pioneer research locations, we desired to identify a nonorganic buffer that would extract FB_1 as efficiently as organic solvents. Sixteen field-grown corn seed samples were extracted in different solvents and analyzed for FB_1 by both HPLC and our ELISA. The 70% aqueous MeOH extracts were also analyzed for FB_1 using the Neogen kit. The amounts of FB_1 extracted from corn seed are summarized in Table 1. Data for extractions in PBST are not shown because the surfactant did not induce significant differences in extraction efficiency.

There was fairly good agreement among all HPLC analyses of individual samples with low (<5 μg/g) FB_1 concentrations. At greater levels of contamination, more FB_1 was found, generally in the 70% aqueous MeOH extracts, and still more in the 50% aqueous AcN extracts. This finding agrees with the results of Rice et al. (1995). Water and PBS extracts yielded very similar results across all FB_1 concentrations, with general agreement among all ELISA data for individual samples with FB_1 levels up to 20 μg/g. More variability was found among the highly contaminated sample extracts. Summarized results reveal that aqueous and organic extractions yielded similar ELISA data for FB_1 concentrations between 0.1 and 20 μg/g, the range encountered normally in field-grown corn seed. Both the Pioneer and Neogen ELISA systems yielded estimates that were greater than the HPLC determinations. It has been speculated that overestimation by ELISA is due to cross-reacting compounds that are structurally related to fumonisin (Tejada-Simon et al., 1995; Sutikno et al., 1996); however, the identities of these compounds have not been elucidated. Clearly, cross-reactivity with FB_2 and FB_3 is involved, although the level of FB_{1-3} combined in typical samples is usually less than the values obtained from ELISAs (Sydenham et al., 1996). Similar interferences with ELISA signals by nontargeted analytes in contaminated sample matrixes have been observed with a radioimmunoassay for aflatoxins (Rauch et al., 1988, 1989). Further research into the identification of cross-reacting analytes with fumonisins in contaminated corn meal would yield valuable information.

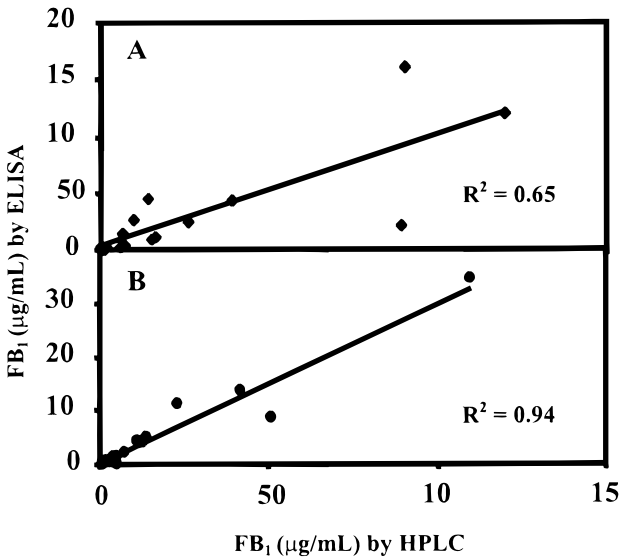

Figure 2. Correlation between FB_1 amounts in the field corn samples shown in Table 1 as determined by ELISA and HPLC. Data are from samples given in Table 1 extracted with 50% aqueous AcN (A) and with PBS (B). All data are in micrograms per gram.

If data were summarized to include all values for the four different extraction buffers, there was a weak correlation ($R^2 = 0.60$) between the amount of FB_1 measured by HPLC and the amount as determined by ELISA. When the data generated from samples extracted in 50% aqueous AcN were excluded, a closer correlation ($R^2 = 0.88$) was found between the ELISA and HPLC results. Indeed, the analogous correlation was not very good for samples extracted in 50:50 aqueous AcN (Figure 2A). The reduced ELISA signal coupled with the higher HPLC values (Table 1) would weaken the correlation between determinations using the two methods. The amount of AcN present in the standards and diluted extracts was well below the level of AcN (25 vol %) that adversely affects ELISA kinetics (data not shown). Further investigations were not conducted to determine how 50% aqueous AcN extraction might be affecting recognition of the target molecule prior to performance of the ELISA. Correlation coefficients were high for individual buffers, with 0.95, 0.94, and 0.98 for H_2O, PBS (Figure 2B), and 70% aqueous

Table 2. Recovery of FB_1 by HPLC and ELISA[a]

sample	FB_1 spike (μg/mL)	HPLC	Pioneer ELISA
1	0	0.34	0
2	0	0.14	0.15
3	0	0.1	0.13/0.1*
4	0	0.68	0.11/0.07
5	1.0	1.64	1.46
6	1.0	3.6	1.28
7	2.0	2.8	1.3/1.94
8	3.0	3.4	1.5/2.2
9	5.0	6.6	2.2/3.12
10	8.0	8.0	4.1/4.9
11	10	11.6	10.3/8.7
12	20	18.4	16.8/19.5
13	30	28	32
14	50	43	44.5/53
15	100	65.0	129/138
16	200	183	188/250

[a] PBS extracts of fumonisin-free corn meal were spiked with known amounts of purified FB_1. Aliquots of subsamples were analyzed by HPLC and ELISA. All data are presented as micrograms per milliliter. The asterisk (*) indicates analyses that were performed on two separate dilutions in the ELISA.

MeOH, respectively. Thus, PBS was chosen as a suitable, nonorganic buffer for sample prepartion. Our observations with both the Pioneer and Neogen ELISAs for fumonisins indicate that higher (800× or 1000×) dilutions of sample yielded unreliable determinations. This was evident with highly contaminated material such as samples 15 and 16 (Table 1). These samples were diluted 1000×. We recommend that dilutions no greater than 500-fold be reported in quantitative terms but be qualified with an appropriate description such as "highly contaminated". This phenomenon with highly contaminated samples is difficult to explain but may be characteristic of ELISA kinetics.

The percent recoveries of FB_1 using either HPLC or ELISA added to a fumonisin-free corn seed extract are summarized in Table 2. Both HPLC and ELISA determinations were reasonably close to spiked levels. We do not have an explanation for the uncharacteristic underestimation by the ELISA at 3−8 μg/mL FB_1. HPLC analysis requires a solid phase extraction cleanup step prior to quantification. It does not appear, however, that this step significantly reduced predicted FB_1 values in all samples. In some cases, the results exceeded 100% recovery. It is difficult to speculate on explanations for these recoveries by HPLC, which do not include contaminated columns between sample runs, interfering compounds, or nonspecific derivatization of molecules. Furthermore, these data indicate that potentially cross-reacting compounds are absent in uncontaminated material.

We developed a competitive ELISA that is being used to screen FB_1 in corn seed extracted in PBS. It is important to ensure that the experimental optical densities are within the most reliable predictive range of the standard curve. We also found that samples diluted extensively often yielded higher interpolated values when compared to HPLC determinations. For these reasons, we recommend that at least two dilutions be run for each sample and that confirmatory analyses by HPLC be replicated twice.

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