Rapid Detection of Fumonisin B_1 in Corn-Based Food by Competitive Direct Dipstick Enzyme Immunoassay/Enzyme-Linked Immunofiltration Assay with Integrated Negative Control Reaction

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Membrane-based competitive enzyme immunoassays (EIA) in dipstick and immunofiltration (ELIFA) format for the detection of fumonisin B_1 (FB_1) were developed. A nylon membrane was coated with anti-FB_1 antibodies and with anti-horseradish peroxidase (HRP) antibodies. A FB_1–HRP conjugate was used both as the labeled antigen for competitive assay of FB_1 and for noncompetitive binding to the anti-HRP antibodies (negative control). Both tests had visual detection limits for FB_1 in the range of 7.5–10 ng/mL (buffer solutions) and 40–60 ng/g (corn-based food samples). The tests were applied to corn-based food samples, and the results were compared with those of microtiter plate EIA and high-performance liquid chromatography. With assay times of 60 min (dipsticks) and 10 min (ELIFA), these rapid tests should be useful to qualitatively detect FB_1 in corn-based foods.

Keywords: *Mycotoxin; fumonisin; detection; corn; enzyme immunoassay; HPLC*

INTRODUCTION

Fumonisins are a group of mycotoxins discovered in 1988 from cultures of *Fusarium moniliforme* (Gelderblom et al., 1988). Frequent occurrence of fumonisins as natural contaminants in corn-based foods and feeds has been reported for many countries [for reviews see, e.g., Scott (1993) and Bullerman and Tsai (1994)]. The most abundant of these, fumonisin B_1 (FB_1), was found to induce leukoencephalomalacia in horse (Marasas et al., 1988), pulmonary edema in swine (Colvin and Harrison, 1992), and hepatic cancer in rats (Gelderblom et al., 1988, 1994). A possible role of high levels of fumonisins in corn in the etiology of human esophageal cancer is under discussion (Sydenham et al., 1990).

The known toxic effects, as well as frequency and contamination height of fumonisins in corn, present a potential threat to human and animal health (Thiel et al., 1992), which suggests the need for inexpensive screening methods to monitor for the presence of these toxins in foods and feeds. Immunochemical approaches have been shown to be useful tools for the screening of cereals for mycotoxins (Chu, 1992; Pestka, 1994), complementary to physicochemical methods. Monoclonal (Azcona-Olivera et al., 1992; Fukuda et al., 1994; Shelby et al., 1994), polyclonal (Usleber et al., 1994), and anti-idiotype/anti-anti-idiotype (Chu et al., 1995) antibodies have been produced against fumonisins; some of these (Azcona-Olivera et al., 1992; Usleber et al., 1994; Chu et al., 1995) have been used in microtiter plate EIA for the detection of fumonisins in foods or feeds. A membrane-based test for FB_1 at the parts per million level has also been described (Abouzied and Pestka, 1994).

The objective of this study was to prepare sensitive visual test systems for the qualitative detection of FB_1 in corn grit, popcorn, and cornflakes, which could be performed in a less equipped laboratory or in a non-laboratory environment. Integrated positive control reactions are quite common for noncompetitive immunoassays developed in clinical chemistry and can be found in, e.g., most modern types of commercial pregnancy tests (Hicks, 1993). In food hygiene, in particular for haptens, such tests are still the exception [e.g., Idexx Laboratories Inc. (1994)]. Here we describe an approach to integrate negative controls in two rapid competitive EIAs for fumonisins by using anti-peroxidase antibodies. The results obtained by these tests for naturally contaminated food samples were checked by microtiter plate EIA and by high-performance liquid chromatography (HPLC) to confirm the threshold detection level determined for artificially contaminated samples.

EXPERIMENTAL PROCEDURES

Materials. Safety Note: Fumonisins are suspected carcinogens and should be handled with care.

FB_1 was purchased from Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany. Fumonisin B_2 (FB_2), prepared as described by Voss et al. (1993), was a gift from William J. Chamberlain, U.S. Department of Agriculture, Richard B. Russell Agricultural Research Center, Athens, GA. Fumonisin B_3 (FB_3) was obtained from PROMEC, Tygerberg, South Africa. Rabbit antiserum against FB_1, precipitated with ammonium sulfate, and FB_1–horseradish peroxidase (HRP) conjugate were used as described earlier. In direct competitive microtiter plate EIA, the relative cross-reactivities of the antibodies with FB_1, FB_2, and FB_3 have been found to be 100%, 24%, and 55%, respectively (Usleber et al., 1994). Goat anti-HRP antibodies were purchased from Sigma-Aldrich. A preactivated nylon membrane which binds proteins covalently (Immunodyne, 0.45 μm pore size) was purchased from Pall GmbH, Dreieich, Germany. All chemicals and solvents used were of at least analytical grade; methanol was of HPLC grade.

Dipstick EIA. To prepare dipsticks, a section (ca. 12 × 1 cm) of the membrane was fixed on a transparent plastic (overhead projector) sheet (ca. 12 × 10 cm) by a double-sided adhesive layer (Tesafix No. 5338, BDF AG, Hamburg, Germany). The coating of the membrane with antiserum was achieved using a semiautomated spraying instrument originally designed for thin-layer chromatographic applications (Camag Linomat IV, Camag, Berlin, Germany). The sheet was fixed on the motor-driven table of the instrument. Anti-FB_1 antiserum was diluted 1:50 with phosphate buffered saline

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(PBS; 0.01 mol/L phosphate buffer, pH 7.2, containing 0.1 mol/L NaCl) and filled in a 100 μL precision syringe. The antiserum solution was applied to the membrane as a fine line (1−2 mm thickness; 6 μL of solution/cm) under nitrogen flow and constant forward−backward movement of the table (ca. 2 cm/s). The antiserum flow rate was about 0.5 μL/s; thus, about 24 runs were required to complete line coating. The syringe holder of the instrument was moved 4−5 mm, and then a second parallel line, using anti-HRP antiserum solution (1:4500 in PBS), was sprayed onto the membrane accordingly. Then the sheet was cut into strips (ca. 0.7 × 10 cm) and dried at 35 °C for 30 min. To block the free protein binding sites of the membrane, the dipsticks were immersed in PBS containing 2% sodium caseinate for 30 min. The test strips were dried again and stored dry at room temperature.

To perform the assay, each dipstick was incubated for 30 min in a 12 mL plastic test tube containing 1 mL of buffer solution (25% methanol in PBS) or extract solution, to which 20 μL of the FB_1−HRP conjugate had been added at a peroxidase concentration of 100 ng/mL. The dipstick was then washed thoroughly (20 min) by stirring in a beaker filled with wash solution (distilled water containing 0.15 mol/L NaCl and 0.25% Tween 20), followed by a second wash step in PBS for 1 min. Finally, the dipstick was incubated for 2−3 min in a test tube containing 1 mL of enzyme substrate/chromogen solution (1 mmol/L 3,3′,5,5′-tetramethylbenzidine and 3 mmol/L H_2O_2, in 0.2 mol/L potassium citrate buffer, pH 3.95; Gallati and Pracht, 1985). The total test time was about 60 min.

ELIFA. To perform the ELIFA, a previously described (Schneider et al., 1994) test device was used, which consists of a plexiglass base, an absorbent cellulose layer, membranes coated with antiserum, and a plexiglass lid with eight holes. Pieces of the Immunodyne membrane (ca. 3 × 3 cm) were coated by pipetting two dots (4 μL each) of diluted anti-FB_1 antiserum (1:10 with PBS) and anti-HRP antiserum (1:500 with PBS). Blocking of free protein binding sites was performed as described for dipsticks. To perform the assay, coated membranes were fixed in the test device. The membrane was prewetted with 150 μL of PBS. Then, standard or sample solution (150 μL), wash solution (200 μL), FB_1−HRP solution (50 μL; 1 μg/mL PBS), and wash solution (2 × 200 μL) were sequentially dropped onto the membrane at 1−1.5 min intervals between each step, to allow the liquid to be absorbed by the cellulose layer. Finally, 50 μL of enzyme substrate/chromogen solution was added. After 1 min, the color reaction was stopped by adding 100 μL of wash solution. The total test time was approximately 10 min.

Sample Preparation. All food samples (popcorn, corn grit, cornflakes) were purchased from retail stores in the area of Munich, Germany. Some of the samples, in particular corn grit (corn semolina), were imported products from Italy. Popcorn and cornflake samples were finely (particle size < 1 mm) ground before extraction in a laboratory mill; corn grit (corn semolina) was used directly.

1. *Extraction of FB_1 from Corn Grit and Popcorn.* Popcorn or corn grit samples (10 g) were mixed with 20 mL of methanol/water (75/25) on a magnetic stirrer for 30 min. The mixtures were filtered through a paper filter (No. 595 filter paper circles, Schleicher & Schuell, Dassel, Germany), diluted 1:3 with PBS, and directly assayed by dipstick EIA and ELIFA. For corn grit and popcorn samples, these unpurified methanolic extracts were also used in microtiter plate EIA (Usleber et al., 1994) after dilution with PBS to a final methanol content of 10%. Additionally, these extracts were purified using strong anion exchange (SAX) cartridges and analyzed by microtiter plate EIA and HPLC according to a procedure described earlier (Usleber et al., 1994).

2. *Extraction of FB_1 from Cornflakes.* For fumonisin analysis of cornflakes by the immunochemical assays, 5 g sample were mixed with 10 mL of methanol/water (75/25) for 30 min on a magnetic stirrer. The mixtures were filtered through a paper filter (No. 595 filter paper circles, Schleicher & Schuell), diluted 1:3 with PBS, and directly assayed by dipstick EIA and ELIFA. For microtiter plate EIA, the unpurified extracts were adjusted to a methanol content of 10% with PBS and further diluted with 10% methanol in PBS if necessary. For preparation of cornflake extracts for HPLC analysis, the following procedure was used: Finely ground cornflakes (5 g) were mixed with 25 mL of acetonitrile/water (50/50) and extracted for 30 min under magnetic stirring. The mixture was centrifuged for 15 min (1500*g*) and the supernatant filtered through a paper filter. An aliquot of this unpurified extract was adjusted to an acetonitrile content of 5% with PBS, further diluted at least 1:3 with acetonitrile/PBS (5/95), and directly assayed by microtiter EIA.

Five milliliters of the extract was mixed with 15 mL of an 1.3% aqueous acetic acid solution. A Sep-Pak C_{18} cartridge (Waters-Millipore, Milford, MA) was prewashed with 5 mL of acetonitrile and 5 mL of acetonitrile/water (25/75). Then the diluted unpurified extract was passed through the cartridge, followed by 5 mL of acetonitrile/water (25/75). Fumonisins were eluted with 3 mL of acetonitrile/water (80/20). The eluate was dried in a rotary evaporator, and the residue was dissolved with 0.5 mL of methanol and finally filtered through a 0.22 μm filter (Millex-GV4, Millipore). An aliquot of this extract was diluted 1:10 with PBS for microtiter EIA; 50 μL was derivatized with 200 μL of *o*-phthaldialdehyde (OPA) solution (Sydenham et al., 1992) for HPLC analysis.

Evaluation of Dipstick EIA and ELIFA. Both test systems were visually evaluated by comparing the color development of the specific reaction zone (coated with anti-FB_1) with that of the negative control zone (coated with anti-HRP). Owing to the competitive EIA test principle, a positive sample results in reduced or completely inhibited color development of the anti-FB_1-coated area, whereas the negative control zone showed the same color intensity in each test, regardless of the amount of toxin present in the sample. The minimal detection limit was defined as the lowest concentration level of FB_1 that was scored "positive" in five tests by six test persons. The test sensitivity for FB_1 in buffer solutions was determined by analyzing serial dilutions of toxin standards (in 25% methanol/PBS) in a concentration range from 1 to 100 ng/mL. For the determination of the visual detection limit for FB_1 in sample materials, popcorn and corn grit samples (FB_1 negative by HPLC) were artificially contaminated with the toxin in a concentration range from 10 to 1000 ng/g, extracted as described above, and analyzed by dipstick EIA and ELIFA.

RESULTS AND DISCUSSION

Rapid visual immunochemical techniques have successfully been established in clinical chemistry more than a decade ago (Valkirs and Barton, 1985). Such tests, which offer the possibility to perform a simple presence/absence test in a nonlaboratory environment, have also found increasing interest in various areas of food hygiene. For mycotoxin analysis, tests have been designed as single-analyte (Schneider et al., 1991; Usleber et al., 1993) or multianalyte (Schneider et al., 1995) dipstick EIAs or as line immunoblots (Abouzied and Pestka, 1994).

Owing to the competitive test principle, all of these tests require a negative control simultaneously performed to discriminate "reduced color development" of positive samples. In the tests described so far, the negative control is performed as a second, separate test. Because such rapid tests have their major advantages when small numbers of samples (or even only one sample) have to be analyzed, a technique was studied in which a negative control is integrated within each test, to further simplify the test protocol. Goat anti-HRP antibodies were used to coat a second line or dot of each test membrane. These antibodies bind a constant amount of the FB_1−enzyme conjugate, which, after addition of the chromogen/substrate solution, results in a "maximum color development" zone near the specific reaction zone (Figure 1). The use of anti-

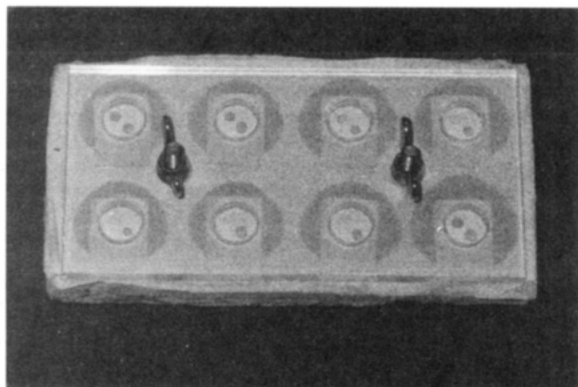

Figure 1. Analysis of serial dilutions of FB_1 buffer solutions (25% methanol/PBS) by ELIFA with integrated negative control zone (lower right dot in each well). Upper row (from left): 0, 3.1, 6.25, and 12.5 ng of FB_1/mL. Lower row (from left): 25, 50, 100, and 0 ng of FB_1/mL. Original size of the test device is 16 × 8 cm. Original color of dots is blue.

HRP antibodies as an integrated negative control was found to be a simple and reproducible way to provide a negative control within each test, thus reducing the test protocol, as well as the materials required.

Although specific anti-FB_1 antibodies and anti-HRP antibodies seem to compete for the same FB_1–HRP conjugate in the first place, this did not affect these visual tests in our experiments. Several reasons may account for this. First, even in a high-quality hapten–HRP conjugate, there may be a certain percentage of unlabeled HRP, which is preferentially bound by the anti-HRP antibodies in a constant ratio. Second, to obtain sufficient color development for visual evaluation, the immunoreagents have to be used in relatively high concentrations. Even in a solution free of fumonisins, only an unknown percentage of the FB_1–HRP is bound by the specific anti-FB_1 antibodies; another part is available for binding to the anti-HRP antibodies. Third, as visual evaluation is far from being exact, slight variations of the color development of the negative control would not be registered. Fourth, in a theoretical extreme case (no unlabeled HRP, high concentrations of toxin present in a sample), the amount of HRP bound to the negative control would only increase, and the test would still give a "positive" result.

The dipstick assay required an intensive and relatively long washing to avoid high background color. This was not observed for ELIFA, which had very short reaction times of less than 2 min. When the two membrane-based test formats were compared in terms of sensitivity, virtually no differences were found between dipstick EIA and ELIFA. The minimal visual detection limits for buffer solutions of FB_1 by dipstick EIA and ELIFA were both in a range from 7.5 to 10 ng/mL; concentration levels of 12.5–15 ng/mL completely suppressed color development of the FB_1 zone of both test formats. With these characteristics, the tests were approximately 50 times less sensitive than the corresponding microtiter plate EIA (instrumental evaluation). However, probably owing to the higher concentrations of immunoreagents used in dipstick EIA and ELIFA and to the different reaction kinetics of these tests, membrane-based tests have been found to be very robust against sample matrix interference and high concentrations of organic solvents. Extracts containing 25% methanol, corresponding to 170 mg of sample/mL, could be analyzed without adversely influencing the test systems. With both test systems, dipstick EIA and ELIFA, a detection limit for FB_1 in artificially contaminated corn samples in the range of 40–60 ng/g could be achieved by employing a simple, unified sample preparation procedure.

To check the data obtained by dipstick EIA and ELIFA, several samples (corn grit, popcorn, cornflakes) were additionally analyzed by HPLC and microtiter plate EIA. When the sample preparation method described by Sydenham et al. (1992) was used for corn grit and popcorn samples, the results confirmed the qualitative results of the rapid tests (Table 1). The results for 1994 corn grit and popcorn samples were similar to those obtained in an earlier study during 1992 and 1993 (Usleber et al., 1994). In general, frequent contamination of corn-based foods from the German market with fumonisins was found, although the toxin levels in most products were comparatively low. High levels were found in import products (corn grit) from Italy, which is in agreement with the findings of Doko and Visconti (1994), who reported high fumonisin contamination of corn in Italy. Samples exceeding a HPLC value of 50–100 ng/g were clearly positive in the dipstick EIA and in the ELIFA, the color development was completely inhibited.

In Switzerland, a preliminary tolerance level for fumonisins in food samples of 1 μg/g has recently been suggested (Zoller et al., 1994). A practical screening test has to detect fumonisins at or below the tolerance level. Both tests should therefore be useful as first-action yes/no screening tests in these matrices. If required, the sensitivity of immunochemical tests can also easily be reduced, by using higher amounts of antiserum for coating or higher concentrations of the FB_1–HRP added.

Initial tests with cornflake samples showed that positive results of dipstick EIA and ELIFA agreed with those obtained by microtiter plate EIA for the corresponding unpurified methanolic extracts. However, in extracts purified with strong anion exchange cartridges, little or no toxin was found by microtiter plate EIA and HPLC. As most of these samples contained unknown amounts of other ingredients (e.g., honey, fruit, and chocolate), recovery of fumonisins using SAX cleanup was checked. Toxin recovery was variable and generally very low, ranging from 0% to 15% (data not shown). This is in agreement with others, who found low or varying recoveries of fumonisins from various foods to be a critical point (Scott and Lawrence, 1994). Using artificially contaminated sample material and RP 18 cartridges for extract purification, recoveries (n = 4) for FB_1 at levels of 50 and 500 ng/g were 65.6 ± 10.7% (unpurified extracts, EIA), 63.6 ± 27.2% (purified extracts, EIA), and 64.7 ± 10.4% (purified extracts, HPLC). However, HPLC values for naturally contaminated samples were still significantly lower than those of the immunoassay.

In contrast, results for spiked samples were in good agreement. Furthermore, high dilutions of unpurified methanolic and acetonitrile/water extracts, corresponding to less than 10 mg of sample/mL of extract, were used for microtiter plate EIA. Therefore, it was not likely that sample matrix interferences did cause false-positive results in the EIA. Additionally, RP 18 extracts analyzed by microtiter plate EIA gave results corresponding with those obtained for unpurified extracts. Similar findings have been reported before (Pestka et al., 1994; Usleber et al., 1994; Chu et al., 1995). The agreement of observations made with three different antibodies is noticeable, and it may indeed be speculated

Table 1. Fumonisins in Corn-Based Food Samples: Comparison of Results Obtained by Dipstick EIA, ELIFA, Microtiter Plate EIA, and HPLC, Respectively

		unpurified extract			purified extract[a]	
sample	country of origin[b]	dipstick EIA[c]	ELIFA[c]	microtiter plate EIA[d]	microtiter plate EIA[d]	HPLC[e]
		All Extractions Performed with Methanol/Water (75/25)				
popcorn[f]	D	−	−	52	47	47
popcorn[f]	D	−	−	35	18	12
popcorn[f]	D	+	+	180	120	100
popcorn	D	−	−	<10	<1	<10
popcorn	D	−	−	<10	<1	<10
corn grit[f]	D	−	−	27	16	20
corn grit[f]	D	−	−	33	25	21
corn grit[f]	D	+	+	350	230	360
corn grit[f]	I	+	+	1300	990	1200
cornmeal	I	+	+	3500	3000	1300 B_1/700 B_2/400 B_3
		Extraction with Acetonitrile/Water (50/50)				
cornflakes	D	−	−	<10	<1	<20
cornflakes	?	+	+	52	29	<20
cornflakes	?	−	+	29	17	<20
cornflakes	?	−	−	30	16	<20
cornflakes	D	+	+	80	60	<20
cornflakes	?	+	+	170	180	50
cornflakes	?	+	+	630	640	110
cornflakes	?	+	+	1600	1500	760 B_1/200 B_2/90 B_3

[a] Purified with SAX (corn grit, popcorn) or C_{18} SPE (cornflakes) column. [b] D, Germany; I, Italy; ?, unknown. [c–e] Results expressed as (*c*) fumonisin positive (+) and negative (−) by visual evaluation, (*d*) FB_1 equivalents (ng/g), (*e*) FB_1 (ng/g); two samples also contained detectable amounts of FB_2 and FB_3 (ng/g, single determinations). [f] For comparison purposes, microtiter plate EIA and HPLC (data for FB_1 only) results are shown as reported earlier (Usleber et al., 1994).

that an unknown, cross-reacting compound with a structure similar to that of the fumonisins (Pestka et al., 1994) could be present in several samples. Combining thin-layer chromatography with immunoblotting (ELISAGRAM), as described by Pestka (1991), could be used to see whether such a cross-reacting compound could be detected.

The maximum toxin levels found in this study in one cornflake sample both by EIA and by HPLC (sum of fumonisins B_1, B_2, B_3 at least >1 $\mu g/g$) should be taken seriously, in particular because, as indicated from their packaging design, most of these foods are intended for daily consumption by children. However, further studies are required to identify the causes for discrepancies between immunochemical and physicochemical tests for fumonisins. The results obtained for cornflakes further show the need to carefully check the applicability of a certain sample extract preparation technique for each individual sample matrix, as demonstrated earlier by Scott and Lawrence (1994).

In conclusion, the tests described here, dipstick EIA and ELIFA, could both be useful as yes/no screening tests for fumonisins in corn grit and popcorn. A "flow-through" immunochemical test system for fumonisins, comparable to the ELIFA described here, has so far not been reported. It should be noted that with an assay time of about 10 min and a total test time (including sample extraction) of less than 1 h, this technique is the most rapid detection method for this toxin. Further work would aim at replacing magnetic stirring for extraction with manual shaking of the sample/solvent mixture for several minutes, which would enable the design of completely self-contained testing devices for on-site testing.

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