Development of a Sensitive ELISA for the Determination of Fumonisin B_1 in Cereals

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Monoclonal fumonisin B_1 antibodies with high titer were raised by using FB_1−glutaraldehyde−keyhole limpet hemocyanin immunogen prepared by a short cross-linker reagent (glutaraldehyde). Mean cross-reactivities of the selected monoclonal antibody for FB_1, FB_2, and FB_3 were 100, 91.8, and 209%, respectively; no reactivity was found with hydrolyzed fumonisin. A direct, competitive enzyme-linked immunosorbent assay for the quantitative determination of FB_1 in cereals has been developed with this antibody. Fifty percent acetonitrile-based solvent with some additives was used for extraction of cereals, and the diluted extracts were used without cleanup in the test. The mean within-assay and interassay coefficients of variation for the standard curve were <10%. The measuring range of this test is 10−500 ng/g, with a detection limit of 7.6 ng/g FB_1. The toxin recovery from cereals infected with 50−200 ng/g of FB_1 varied between 61 and 84%. According to the comparable results of naturally infected maize samples, this test proved to be suitable for the rapid screening of food and feed samples for the presence of FBs.

Keywords: *Fumonisin B_1; FB_1−KLH; ELISA; cereals*

INTRODUCTION

Fumonisins are toxic metabolites produced by naturally occurring *Fusarium* fungi in corn, in particular *Fusarium moniliforme* and *Fusarium proliferatum*. Chemically, fumonisins are characterized by a 20-carbon backbone, 2 tricarballylic acid (TCA) groups, 1−3 hydroxyl groups, and a single amino group. The TCA chains may be removed by alkaline hydrolysis, resulting in the partial (lacking one TCA) or full (lacking both TCA groups) hydrolysis products (HFB_1 and HFB_2) (Beier et al., 1996) (Figure 1). There are at least four naturally occurring fumonisins known as FB_1, FB_2, FB_3, and FB_4. Fumonisin B_1 is the most abundant in contaminated foods and feeds, but the presence of the other fumonisins in cereals is very frequent as well (Maragos et al., 1997). The most investigated one is FB_1, which can cause leukoencephalomalacia in horses, pulmonary edema in pigs, nephrotoxicity, liver cancer in rats, and esophageal cancer in humans (Rheeder et al., 1992).

FBs have been measured mainly in maize and different corn products but have been occasionally detected in rice, wheat noodles, spices, beer, and raw milk (Dutton, 1996; Pittet, 1998). FBs regularly contaminate maize in Hungary as well; case reports were published earlier (Fazekas et al., 1996, 1997).

Fumonisins are analyzed typically by chromatographic methods (TLC, LC and LC-MS, GC-MS, and HPLC), which are expensive and time-consuming and need appropriate instrumentation and/or trained personnel (Dutton, 1996; Maragos et al., 1997). Although the presence and sometimes high content of fumonisins in cereals are a great problem all over the world, only Switzerland has proposed legislation for FB_1; the "acceptable" limit was determined as 1000 μg/kg (Boutrif and Canet, 1998; Pittet, 1998). Owing to their reliability, simplicity, and low cost, immunoassays such as ELISA are acceptable and commonly used analytical methods for the determination of mycotoxins in foods and feeds. In the past few years some ELISA tests has been developed using polyclonal or monoclonal antibodies with different sensitivities and detection limits (Usleber et al., 1994; Azcona-Olivera et al., 1992; Elissalde et al., 1995; Yu et al., 1996).

Some years ago our institute started a research program to develop sensitive ELISA tests and reagent kits for the routine detection of mycotoxins that are frequent contaminants in Europe. As a result, four

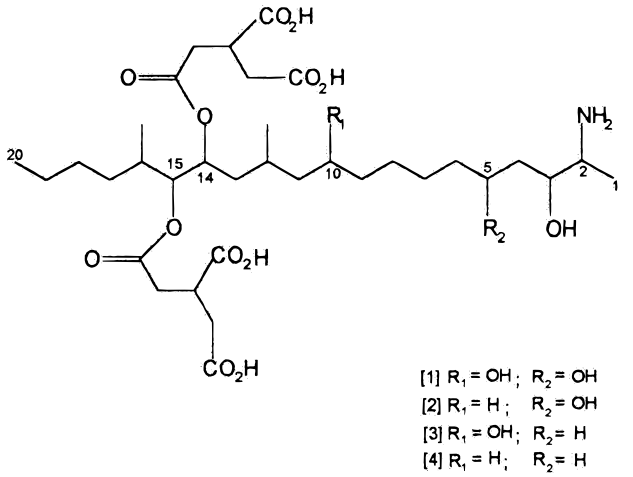

Figure 1. Structure of the fumonisins: (1) fumonisin B_1; (2) fumonisin B_2; (3) fumonisin B_3 and fumonisin B_4.

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ELISA tests and reagent kits for the measurement of *Fusarium* T-2 and F-2 toxins and also for the determination of ochratoxin A and aflatoxin B_1 in cereals have been completed (Barna-Vetró et al., 1994, 1996).

Our goal with this study was to continue this program with the development of a sensitive monoclonal antibody based direct, competitive ELISA test for screening fumonisin B_1 in different cereals. Our work demonstrates that an immunogen (FB_1−GA−KLH) prepared with the short cross-linker glutaraldehyde is able to generate monoclonal antibodies as sensitive and of as high a titer as other immunogens with long cross-linker reagents.

MATERIALS AND METHODS

Reagents. Fumonisin B_1, ϵ-maleimidocaproic acid *N*-hydroxysuccinimide ester (EMCS), *S*-acetylmercaptosuccinic anhydride, and keyhole limpet hemocyanin (KLH) were purchased from Sigma-Aldrich and glutaraldehyde (GA), horseradish peroxidase (HRP), and sodium borohydride from Reanal. Bovine serum albumin (BSA) was from Serva (BRD) and sodium periodate from Fluka (BRD). All other chemicals used were of analytical grade.

FB_2 and HFB_1 were a gift from Jupiter Yeung, National Food Processors Association (1401 New York Ave N.W., Washington, DC 20005), and FB_3 was generously provided by Ronald D. Plattner (USDA-ARS−NCAUR, Peoria, IL).

Preparation of FB_1−KLH Immunogen by GA (Avrameas and Ternynck, 1969). Conjugation of FB_1 to KLH was performed by GA reaction with some modification. KLH (10 mg) was dissolved in 12 mL of 0.8% NaCl, 1.3 mL of GA (2%) was added, and the mixture was incubated for 1 day at room temperature. The excess GA was dialyzed for 2 days and then centrifuged. FB_1 (1 mg) was dissolved in 0.4 mL of methanol and dropwise added to the activated protein, and the mixture was incubated for 20 h at room temperature. Finally, the unreacted aldehyde groups were blocked by the addition of 12 mg of sodium borohydride, and the mixture was stirred for 4 h. The conjugates were then dialyzed in phosphate-buffered saline (PBS) for 3 days and stored at −20 °C.

Preparation of FB_1−BSA Conjugate by EMCS (Szurdoki et al., 1996). Four and a half milligrams of *S*-acetylmercaptosuccinic anhydride was dissolved in 0.1 mL of dimethylformamide (DMF), and 15 mg of BSA in 3 mL of 0.2 M phosphate buffer (pH 8.0) was added dropwise; the mixture was stirred for 1 h at 4 °C and then dialyzed for 2 days in PBS.

Two milligrams of EMCS was dissolved in a mixture of 0.2 mL of methanol plus 0.2 mL of phosphate buffer (pH 8.0) and then 2 mg of FB_1 in 0.2 mL of methanol was added; the mixture was stirred for 18 h at 4 °C. The solution was diluted with 1 mL of distilled water and stirred for 2 h at the same temperature. Excess EMCS was removed by extraction with hexane/ethyl acetate (2:1). The water component contained the FB_1−EMCS complex.

Ten milligrams of *S*-acetylmercaptosuccinylated BSA in 2.5 mL of PBS was added to 1.5 mL of solution containing 0.33 M hydroxylamine in 0.2 M phosphate buffer (pH 7.3) and 20 mM ethylenediaminotetraacetic acid (EDTA). The solution was stirred at room temperature for 30 min. Two milligrams of FB_1−EMCS was added dropwise to this solution and stirred under nitrogen for 18 h at room temperature and then dialyzed exhaustively in PBS and stored at −20 °C.

Preparation of FB_1−Peroxidase Conjugate (Wilson and Nakane, 1978). Eight milligrams of HRP was dissolved in 2 mL of distilled water, 0.2 mL of 0.1 M sodium periodate was added, and the mixture was stirred for 20 min at room temperature and then dialyzed overnight in 1 mM sodium acetate buffer (pH 4.4). The pH of the activated HRP was adjusted to 9−10 with 0.1 M NaOH, and the HRP was mixed with 2 mg of FB_1 dissolved in 0.5 mL of distilled water; the mixture was stirred for 3 h at room temperature. The reaction was stopped with 0.2 mL of a sodium borohydride solution (4 mg/mL) and incubated overnight at 4 °C. Finally, the conjugate was dialyzed against 0.1 M PBS and stored at −20 °C.

Immunization. Female Balb/c mice (2 months old) were immunized subcutaneously with 50 μg/50 μL FB_1−GA−KLH conjugate emulsified with equal volumes of Freund's complete adjuvant (CFA) on the first day. After 1 month, mice received a second injection intraperitoneally with the same amounts of conjugates, emulsified with Freund's complete and incomplete adjuvants mixture (CFA/IFA = 1:3, v/v). On day 50, the mice were bled and serum titers were assessed by indirect competitive (ic) ELISA. The third immunization was on day 57 with the same dose of conjugate and adjuvant. Final immunizations were 4 days prior to fusion (on day 70) intravenously.

ic-ELISA. Microplate wells (Dynatech M129B) were coated with 100 μL of FB_1−EMCS−BSA (5 μg/mL) overnight at room temperature. Plates were washed three times with distilled water. Fifty microliters of serially diluted murine serum was added to each well and coincubated with 50 μL of 1 μg/mL FB_1 for 1 h at room temperature. Wells containing serially diluted serum with PBS (0 μg/mL FB_1) were used as control. Unbound antibody was removed by washing five times with distilled water, and then 100−100 μL of goat anti-mouse Ig−HRP conjugate (developed in our laboratory) in PBS−Tween 20 (0.1%) was added to each well and incubated for 1 h at room temperature. Plates were washed five times with distilled water, and 150 μL of tetramethylbenzidine (TMB)/H_2O_2 substrate per well was added and incubated for 15 min at room temperature. The color reaction was stopped with 50 μL of 6 N sulfuric acid, and the OD_{450nm} was measured by an automated microplate reader (Labsystems Multiscan PLUS, Helsinki, Finland).

The ic-ELISA was used partly to identify cell cultures containing anti-FB_1 antibody in fusion and cloning wells. The wells were coated with FB_1−EMCS−BSA. Fifty microliters of PBS (FB_1-free blank) was added to one well and 50 μL of FB_1 (500 ng/mL) to the other well. To both wells 50−50 μL aliquots of a single diluted culture supernatant were added, and the mixtures were incubated for 1 h at room temperature. The assay was finished as described above.

Hybridoma Production. Spleen cells (1×10^8) from a mouse producing antiserum for FB_1 were fused with Sp2/0-Ag14 (from American Type Cell Collection CRL-1581) murine myeloma cell line using PEG 1600 according to the method of Oi and Herzenberg (1980). Until 14 days after fusion, hybridomas were selectively grown in hypoxanthine−aminopterine−thymidine medium. At the half-monolayer state, the antibody-secreting hybridomas were detected by both ic-ELISA and direct competitive (dc) ELISA. Positive hybridomas were grown in hypoxanthine−thymidine medium.

After cloning by the limiting dilution method, ascites was made with the cells that had produced supernatant fluids with the best sensitivity. Mature male Balb/c mice were injected intaperitoneally with 1×10^7 hybridoma cells. After 10−14 days, the ascites fluids were collected, centrifuged, and stored at −20 °C until use.

dc-ELISA. The hybridoma supernatants were screened partly in dc-ELISA; the method was the same as we used earlier for the detection of antibodies against ochratoxin A (OA) (Barna-Vetró et al., 1996). Briefly, before the assay, supernatants were diluted with PBS (1:2). From each supernatant 100−100 μL was added to two microplate wells previously coated with 150 μL of rabbit Ig anti-mouse Ig (IgM plus IgG plus IgA, developed in our laboratory, 15 μg/mL) and incubated for 1 h at room temperature. After washing, 50 μL of PBS buffer (zero toxin concentration, B_0) was pipetted to one well and 50 μL of 500 ng/mL FB_1 in PBS (which means 25 ng/well) to the other. Immediately thereafter, 50−50 μL of FB_1−HRP conjugate in working dilution was added to each well and incubated for 1 h at room temperature. After four washing steps, the enzyme activity was determined as before. Those antibodies were selected where OD_{450nm} at 0 toxin concentration (B_0) was >0.5 and the percent of inhibition at 25 ng/well toxin level was >90%.

The dc-ELISA was modified for measuring FB_1 in cereals. Briefly, microplate wells (Immunoplate F-8, Maxisorp, Nunc, Denmark) were coated with anti-mouse Ig rabbit IgG globulin as previously, and then 120 μL of diluted anti-FB_1 ascites fluid was pipetted to each well and incubated for 18 h at room temperature. After washing with distilled water, plates were dried and stored in a foil bag for up to several months at 4 °C. Fifty microliter FB_1 standards (in PBS) (0.5−25 ng/mL) or extracted samples were coincubated in wells with 50 μL of FB_1−HRP (1:140000) conjugate in PBS−Tween 20 (0.1%) for 1 h at room temperature. After four washing steps, the peroxidase activity was measured as before.

The standard curve of FB_1 was obtained by plotting $\log_{10}$ concentration (x-axis) against B/B_0 (y-axis): B/FB_0 = OD of standard or sample/OD of blank (no toxin added), where optical density (OD) is the mean value.

The FB_1 concentrations in sample extracts were assessed by using the calibration curve and are expressed in nanograms per gram by multiplying the nanogram per milliliter value by 20.

Recovery of FB_1 from Artificially Infected Cereals. To 5 g of finely ground cereals (wheat, maize, rye) was added 50−200 ng/g of pure FB_1 1 day prior to extraction. Thereafter, the samples were shaken for 2 h with 20 mL of extraction solvent (composed of 50 parts acetonitrile, 39 parts water, 10 parts 0.5% KCl, and 1 part 6% H_2SO_4). The extracts were centrifuged for 30 min at 4500 rpm and the supernatants diluted 1:5 with PBS−Tween 20 (0.1%), shaken well, and then centrifuged again. From the clear supernatant 50 μL was used directly in the assay.

Preparation of Naturally Infected Samples for dc-ELISA. To 5 g of finely ground maize samples was added 20 mL of extraction solvent, and the mixture was agitated for 2 h at room temperature. All details were as described earlier.

Determination of FB_1 in Naturally Infected Samples by HPLC. For this experiment, 11 maize samples were collected from one area (Heves County) of Hungary in 1998. The samples were homogenized and stored at −20 °C until use. HPLC analysis of FB_1 and FB_2 was done according to the method of Shephard et al. (1990) and Sydenham et al. (1992) as follows. Finely ground maize samples were extracted with MeOH/water (3:1 v/v), and the crude extracts were cleaned using solid-phase strong anion exchange (SAX) cartridges (Varian, Harbor City, CA). The fumonisins were separated from the SAX cartridges with 1% acetic acid in methanol. Derivatization of fumonisins was performed with *o*-phthaldialdehyde (OPA) and mercaptoethanol. OPA derivatives of fumonisins were separated on a LiChrospher RP 18, 150 × 4 mm, 5 μm analytical column, and the mobile phase was acetonitrile/water/acetic acid (50:50:1). The contents were estimated with a fluorometric detector with excitation at 335 nm and emission at 450 nm. For quantitative determination a calibration curve was prepared. The validity of our method was gauged as follows: recovery rate was 75% at 200 ng/g FB_1 and FB_2 concentration and 71% at 1000 ng/g FB_1 and FB_2; detection limit was 20 ng/g for FB_1 and 50 ng/g for FB_2; the signal-to-noise ratio was 10:1 for FB_1 and 5:1 for FB_2 FB_3 as well.

RESULTS AND DISCUSSION

The quality of the specific antibody (affinity, avidity, cross-reactivity) is one of the most important factors for the development of sensitive assays. High-quality monoclonal antibodies can be generated only by using appropriate immunogen (carrier protein and conjugation method), good immunization protocol, and screening procedures for hybridomas. As the FB_1 molecule has one amino group on its C_2 position, its coupling to different carrier proteins [OVA, BSA, HSA, KLH, or cholera toxin (CT)] (Beier et al., 1996) is very easy. Different cross-linker reagents are available for coupling, for example, glutaraldehyde, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Fukuda et al., 1994), sulfo-SMPB (Kamps-Holtzapple et al., 1994), and succinimidyl 6-(*N*-maleimido) hexanoate (MHS) (Szurdoki et al., 1996). According to Elissalde et al. (1995) the use of a longer cross-linker (∼16 atoms) between the hapten and carrier can result in antibodies with higher affinity and assays with greater sensitivity than those obtained using a shorter cross-linker (i.e., glutaraldehyde). An alternative method for coupling fumonisin B_1 to protein is one of its carboxylic acid groups by a mixed anhydride method, which resulted in a sensitive ELISA test for fumonisin B_1, whereas immunogen prepared through the $-NH_2$ group produced only a low titer of antibody (Yeung et al., 1996).

For the preparation of our monoclonal FB_1 antibodies the following strategy was used: one conjugate was prepared using shorter (GA) and another one with longer cross-linker reagents (EMCS). FB_1−GA−KLH was used as the immunogen, and FB_1−EMCS−BSA was used as the plate-coating antigen in the ic-ELISA. We planned to change the immunogens in the next experiment if FB_1−GA−KLH resulted in antibodies with poor affinity.

Using FB_1−GA−KLH immunogen, all treated animals (n = 6) produced specific FB_1 antibodies with ∼50% inhibition in the presence of free FB_1 (1 μg/mL). Spleen cells from hyperimmunized mouse were fused with Sp2/0-Ag14 myeloma cells, and the resulting hybridoma supernatants were analyzed parallelly by ic-ELISA and dc-ELISA. The use of dc-ELISA avoided the selection of nonspecific antibodies against the carrier protein as only the FB_1-specific monoclonal antibody reacted. With this selection strategy, 16 hybridomas were obtained that all recognized the unconjugated FB_1. From these active hybridomas four cells were cloned and recloned by limiting dilution methods and retested again by dc-ELISA. The resulting antibodies were very sensitive; the inhibitions at 500 ng/mL of free FB_1 toxin level (25 ng/well FB_1) were >90%. Ascites fluid containing the monoclonal antibody (1D6F11E3) was used in all subsequent experiments. The subclass of monoclonal antibody was identified with isotype-specific immunoglobulins (Serva, BRD), and the antibody proved to be IgG_1, with kappa light chains. The affinity constant of the antibody was 1.3×10^{10} L/M, determined according to the method of Beatty et al. (1987). The specificity of the antibody toward fumonisins and other mycotoxins was checked by dc-ELISA. The cross-reactivity values were calculated as (IC_{50} of FB_1/IC_{50} of compound) × 100. (IC_{50} is defined as the concentration of toxin required to inhibit color development by 50%.) The relative cross-reactivities with FB_1, FB_2, FB_3, and HFB_1 were found to be 100, 91.8, 209, and 0%, respectively. Our anti-fumonisin antibody did not recognize the zearalenone, T-2, and deoxynivalenol mycotoxins that are commonly found in grains. The high cross-reactivity data indicated that the acidic side-chains of the intact molecule (at C-14 and C-15) might play a part in the antigen−antibody interaction, confirming the statements of Beier et al. (1996) and Elissalde et al. (1995). Their other findings that the absence of a hydroxyl group at C-5 in the case of FB_3 decreased the affinity of the monoclonal antibodies by an average of 3-fold was not observed in our studies. Our antibody recognized FB_2 and FB_1 equally well and FB_3 much better; a similar antibody has not been published yet in the literature. Therefore, our immunoassay would be expected to give a determination

Table 1. Recovery of FB_1 Mycotoxin from Artificially Contaminated Cereals[a]

FB_1 added (ng/g)	rye flour FB_1 detected (ng/g)	maize FB_1 detected (ng/g)	maize flour FB_1 detected (ng/g)	wheat FB_1 detected (ng/g)
200	156 ± 21 (78%)	123 ± 21 (62%)	151 ± 10 (76%)	145 ± 27 (72%)
100	83 ± 15.9 (83%)	63 ± 11 (63%)	63 ± 7.0 (63%)	78 ± 14 (78%)
50	42 ± 9.9 (84%)	31 ± 7.1 (61%)	42 ± 11 (84%)	35 ± 8.7 (70%)

[a] Each sample was infected with different FB_1 toxins in three parallel experiments and then extracted, and each extract was assayed in three replicates ($n = 9$). Recovery percent is detected FB_1 (ng/g)/added FB_1 (ng/g) × 100.

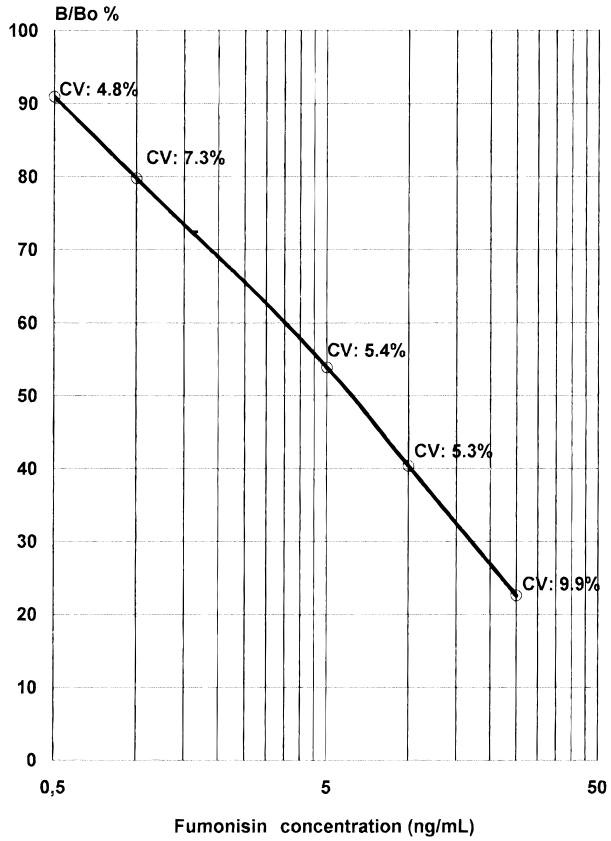

Figure 2. Dose−response curve of FB_1. The 50% inhibition value of FB_1 was 5.4 ng/mL with a detection limit of 0.38 ng/mL.

of all fumonisins present, not just FB_1. As a result, our dc-ELISA may show a higher value for FB_1 than other instrumental (e.g., HPLC) methods. Although others (Beier et al., 1996; Elissalde et al., 1995) have stated that linkage of FB_1 through its primary amine to a carrier protein is unlikely to produce high-quality antibodies, we found that our immunogen (FB_1−GA−KLH) elicited high-titer, high-affinity antibodies that recognize free fumonisins. The working dilution of our monoclonal antibody was very high (1:100000). In our experiment the monoclonal antibody was coated indirectly onto the microplate using rabbit Ig anti-mouse Ig as the capture antibody. Figure 2 shows the dose−response curve of FB_1 with the detection limit in buffer solution (0 ± 2 SD) being 0.38 ng/mL; the 50% displacement values of B/B_0 (IC_{50}) was 5.4 ng/mL. Sensitivity, defined as the slope of the standard curve at the inflection point (i.e., middle of the range), was 1.19. The correlation coefficient (r) of the linear part of the calibration curve was estimated by the Statistical Graphics program (Statgraphics 5.0) and proven to be 0.91. The within-assay ($n = 10$) and interassay ($n = 30$) coefficients of variation for standard concentration of FB_1 (0.5−25 ng/mL) were <10%.

Table 2. Comparison of FB Concentrations Obtained by ELISA and HPLC

	HPLC		ELISA
sample	FB_1 (ng/g)	FB_2 (ng/g)	FB_1 equiv (ng/g)
1	<20	<50	<10
2	<20	<50	<10
3	<20	<50	<10
4	147	<50	222
5	28	<50	86
6	105	<50	183
7	<20	<50	<10
8	<20	<50	<10
9	<20	<50	<10
10	2444	926	>500
11	<20	<50	25

A very important part of the validation is the detection of matrix effect, which should be minimized by the sample preparation procedure. To extract mycotoxins from different cereals, methanol- or acetonitrile-based organic solvents have been used in different concentrations (50−75%) combined with different cleanup steps (Azcona-Olivera et al., 1992; Yeung et al., 1996). The aim of our work was to simplify the procedure by eliminating sample cleanup. However, although the acetonitrile-based solvent was compatible with the ELISA, we were unable to simplify the procedure because the centrifugation step could not be eliminated (content of the solvent: 50 parts acetonitrile/39 parts distilled water/10 parts 0.5% KCl/1 part 6% sulfuric acid).

Recoveries of FB_1 from artificially infected cereals are summarized in Table 1, ranging from 61 to 84%. The measuring range of our test was 10−500 ng/g with a detection limit of 7.6 ng/g.

To compare the immunoassay method with an established procedure, 11 naturally infected maize samples were extracted and their fumonisin contents analyzed by HPLC and dc-ELISA as summarized in Table 2. The negative toxin content of the samples was confirmed by both methods. In some samples our ELISA showed a higher value for FB_1 than did HPLC. We believe that the higher ELISA values at these samples are due partly to the greater sensitivity of ELISA and partly to the high reactivity of our monoclonal antibody to other fumonisins. This cross-reactivity is particularly advantageous because it enables the simultaneous detection of all three fumonisins in doubtful or suspicious samples. In our case we measure total fumonisins rather than FB_1.

From economic and stability points of view, in our commercialized ELISA kit, the FB_1-specific antibody was precoated on the surface of polystyrene strips, the enzyme-labeled FB_1 was in a stabilized buffer, and all other reagents (washing buffer, substrate/chromogen, and stopping solution) were kept in concentrated form. The stability of these reagents was 6 months at 4 °C. On one microplate the FB_1 content of 40 cereal samples can be determined in duplicate.

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