Sensitive Enzyme-Linked Immunosorbent Assay for the Mycotoxin Zearalenone in Barley and Job's-Tears

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An easy, sensitive, competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) for zearalenone in barley and Job's-tears was established. To improve sensitivity of the assay system for zearalenone, a solid phase antigen was prepared by affinity purification. This assay system uses a purified antigen and specific monoclonal antibodies for zearalenone. Consequently, the detection limit of zearalenone by CI-ELISA was increased to 0.2 ng/mL (equivalent to 10 ng/g in barley and Job's-tears). Zearalenone in barley and Job's-tears samples could be determined in the range of 25–1000 ng/g using method A (methanol–water and dichloromethane extraction) and method B (methanol–water alone) as sample preparation. The average recoveries of zearalenone from barley samples were observed to be 106% (mean CV, 10.3%) by method A and 98% (mean CV, 7.5%) by method B; also, those of zearalenone from Job's-tears samples were observed to be 96% (mean CV, 9.4%) by method A and 102% (mean CV, 6.5%) by method B. There was little or no difference between method A and method B for the recovery. For the benefit of a facile sample preparation, unknown amounts of zearalenone in barley and Job's-tears samples were assayed by method B. The results obtained by CI-ELISA were closely correlated with those of high-performance liquid chromatography.

Keywords: *Enzyme-linked immunosorbent assay; purified antigen; zearalenone; barley; Job's-tears*

INTRODUCTION

Zearalenone [6-(10-hydroxy-6-oxo-*trans*-undecenyl)-resorcylic acid] is a nonsteroidal estrogenic compound produced by several *Fusarium* species that colonize a number of agricultural commodities (Ichinoe et al., 1983). Zearalenone has estrogenic and growth-promoting activities (Stob et al., 1962), and it causes mycotoxicoses in farm animals. Worldwide surveys (Tanaka et al., 1988) have found grains contaminated with this at toxic levels.

Current analyses of zearalenone in foods and feeds have been carried out by thin layer chromatography (Scott et al., 1978; Gimono et al., 1983; Swanson et al., 1984), gas–liquid chromatography (Bennett et al., 1985), high-performance liquid chromatography (HPLC) (Bagneris et al., 1986; Roybal et al., 1988), and gas chromatography–mass spectrometry (Plattner and Bennett, 1985).

However, these methods are unfavorable for routine screening of a large number of samples and require complicated procedures for cleanup and quantitation. A radioimmunoassay (Thouvenot and Morfin, 1983) and enzyme-linked immunosorbent assays (ELISA) (Liu et al., 1985; Warner et al., 1986; Dixon et al., 1987; MacDougald et al., 1990) have been described. We previously reported the preparation of a highly specific anti-zearalenone monoclonal antibody obtained by immunizing mice with a novel type of antigen in which the hapten is linked to a carrier protein through the C-5 position in a zearalenone molecule (Teshima et al., 1990).

In the present paper, we describe an approach to improve the sensitivity of zearalenone analysis by indirect ELISA (CI-ELISA), including the use of a solid phase antigen obtained by affinity purification and specific monoclonal antibody, and its application to the determination of zearalenone in barley and Job's-tears (*Coix lachryma-jobi* var. Mayuen), without cleanup procedure of samples, and comparison with HPLC.

MATERIALS AND METHODS

Materials. Zearalenone was purchased from Makor Chemical (Jerusalem, Israel). Bovine serum albumin (BSA), 4-methylumbelliferyl β-galactoside, and ovalumin (OVA) were bought from Sigma Chemical Co. (St. Louis, MO). Sheep anti-mouse IgG–β-galactosidase conjugate was supplied by Amersham International Plc. (Amersham, U.K.) and cyanogen bromide activated Sepharose 4B by Pharmacia Fine Chemicals (Uppsala, Sweden). The monoclonal antibody of zearalenone was obtained from the ascites fluid of mice injected with hybridoma cells (clone 7-1-144) as previously reported (Teshima et al., 1990). Other general reagents were of analytical grade. Barley samples were harvested from fields in Japan. Job's-tears samples, which are commonly used for health food and herbal drugs in Japan, were purchased from markets in Tokyo.

Preparation of Solid Phase Antigen. To prepare a solid phase antigen for CI-ELISA, zearalenone (0.5 mg) dissolved in ethanol (0.1 mL) was mixed with a solution of OVA (70 mg) in 1.5 mL of 0.2 M sodium bicarbonate (pH 9.6), and 1% formaldehyde (5.0 μL) was added to this solution. The mixture was stirred at room temperature for 3.5 h, and the resulting solution was dialyzed against methanol–water (1:9 v/v) (1 L × 2) and 10 mM sodium phosphate buffered saline, pH 7.2 (PBS) (1 L), for 2 days at 4 °C.

The mole ratio of zearalenone to OVA was determined by measuring the change in the UV absorption (315 nm) of the OVA after conjugation.

Immobilization of Monoclonal Antibody. To use for the purification of solid phase antigen, immobilization of the antibody was prepared according to the method of Tanaka et

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0021-8561/95/1443-0946$09.00/0

al. (1985b). Anti-zearalenone antibody (ascites fluid, 7-1-144) (1.0 mL) was mixed with 1.0 mL of ammonium sulfate (50% w/v), allowed to stand at room temperature for 10 min, and then centrifuged at 2500 rpm for 20 min. The precipitate was dissolved in 1.0 mL of distilled water and dialyzed against 0.05 M phosphate buffer, pH 7.2 (1 L). The dialysate containing "crude globulin" was diluted with 5.0 mL of 0.1 M sodium bicarbonate buffer, pH 8.0, containing 0.5 M sodium chloride. The solution was mixed with 200 mg of cyanogen bromide activated Sepharose 4B gel that had been washed with 100 mL of 1 mM hydrochloric acid solution and stirred at room temperature for 3 h. Unbound material was washed away with 5.0 mL of sodium bicarbonate buffer containing 0.5 M sodium chloride, and the remaining active groups were reacted with 0.1 M Tris-HCl buffer solution, pH 8.0. The suspension underwent three cycles of washing to remove noncovalently absorbed protein, with each cycle consisting of 5.0 mL of 0.1 M acetate buffer, pH 4.0, containing 1.0 M sodium chloride. The immobilized antibody obtained was stored at 4 °C prior to use.

Affinity Purification of Solid Phase Antigen. Five hundred microliters of zearalenone–OVA conjugate solution (1 mg/mL) was mixed with 125 μL of 5-fold-diluted immobilized antibody in 0.05 M phosphate buffer, pH 7.2, and allowed to stand at 25 °C for 1 h. Zearalenone (6 μg) in methanol (25 μL) was added to the reaction mixture and then incubated at 25 °C for 2 h. The resulting suspension was centrifuged at 2500 rpm for 15 min, and the supernatant was dialyzed against methanol–water (9:1) (3 L × 3) for 2 days. The dialysate was diluted with PBS (containing 0.1% ascorbic acid) to 0.5 mg of protein/mL, and purified zearalenone–OVA conjugate was obtained as a solid phase antigen.

Extraction of Zearalenone from Grain Samples. *Method A.* A grain (barley or Job's-tears) sample (50 g) was ground and mixed thoroughly, and the accurately weighed sample (5 g) was shaken with 25 mL of methanol–water (60:40 v/v) and centrifuged at 2500 rpm for 10 min. The supernatant (1.0 mL) was concentrated *in vacuo* to 0.5 mL, extracted with dichloromethane (2 mL × 2), and washed with water (0.5 mL). The organic phase was evaporated *in vacuo*, and the residue was dissolved in 5.0 mL of methanol–0.1 M phosphate buffer, pH 7.2 (1:9 v/v). The resulting solution (50 μL) was assayed by CI-ELISA.

Method B. A ground grain sample (50 g) was mixed thoroughly, and the accurately weighed sample (5 g) was shaken with 25 mL of methanol–water (60:40 v/v) for 15 min and then centrifuged at 2500 rpm for 10 min. The supernatant was diluted 1 to 10 with 0.05 M phosphate buffer, pH 7.2 (containing 0.1 M HCl and 0.1 M glutamine). This solution (50 μL) was assayed by CI-ELISA.

Recovery. Zearalenone dissolved in methanol was added to a ground grain sample to give final concentrations of 25, 50, 100, 250, 500, and 1000 ng/g. The grain sample (5 g) was shaken with 25 mL of methanol–water (60:40 v/v) for 15 min and centrifuged at 2500 rpm for 10 min. The following procedures were carried out according to methods A and B as described under Extraction of Grain Samples, and the resulting solution was subjected to CI-ELISA.

CI-ELISA. Fifty microliters of zearalenone–OVA (2 μg/mL) in 0.15 M sodium carbonate buffer, pH 9.6, was added to each well of a 96-well microtiter plate and incubated at 4 °C overnight. Unbound conjugate was removed from the plate with four washes of 0.1 M phosphate buffer saline containing 0.5% Tween 20 (PBS/Tween). To block the unoccupied solid phase to minimize nonspecific binding, 0.2 mL of 0.1% casein solution in PBS (PBS/casein) was added, and the mixture was incubated for 1 h at room temperature. The wells were washed with 0.2 mL of PBS/Tween four times, and 50 μL of a 50000-fold diluted monoclonal antibody (ascites fluid, 7-1-144) in PBS/casein was incubated with 50 μL of a sample solution (or standard solution of zearalenone) at room temperature for 1 h. The solution was removed and each well washed as before. Fifty microliters of a solution of sheep anti-mouse-IgG–β-galactosidase (1:1000 dilution in PBS/casein) was added to each well, and the plate was incubated at room temperature for 1 h. Unbound antibody–enzyme conjugate solution was removed, and each well was washed as before. The wells were incubated for 1 h at 37 °C with 100 μL of PBS solution of 0.1 mM 4-methylumbelliferyl β-galactoside. The fluorescence intensity of liberated 4-methylumbelliferone was measured by a Titertek Fluoroskan reader (Flow Laboratories Inc.).

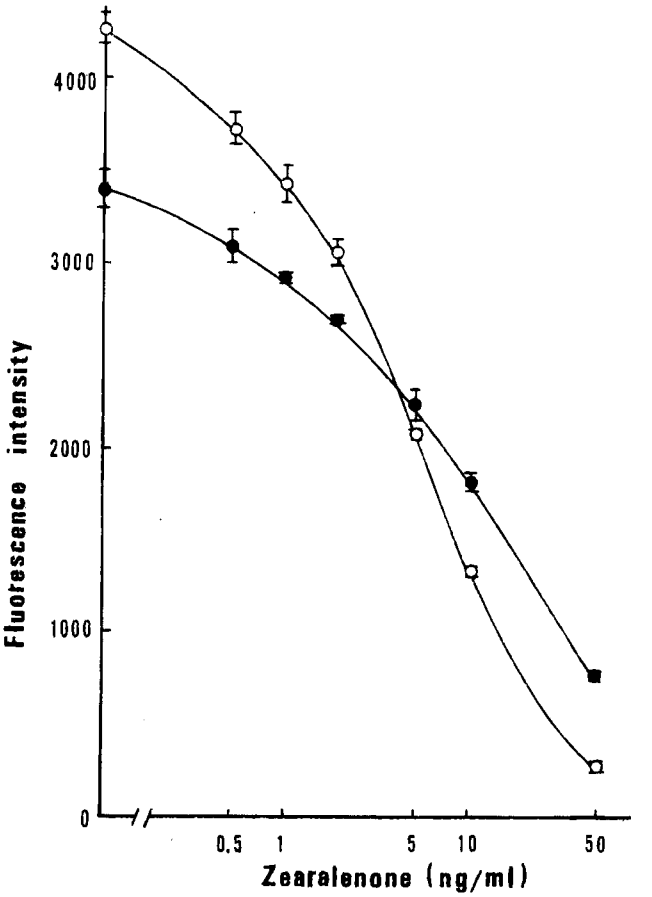

Figure 1. Comparison between standard curves of zearalenone using zearalenone–OVA conjugate obtained with purification (○) and without purification (●) (n = 5).

HPLC. Zearalenone was determined in 200 grain samples by HPLC on a reversed-phase column using acetonitrile–water as a mobile phase and a fluorescence detector according to the method of Tanaka et al. (1993).

RESULTS

Standard Curves of Zearalenone by CI-ELISA. Typical standard curves of zearalenone were constructed with 50000-fold diluted antibody in PBS/casein using purified and unpurified solid phase antigens. The results shown in Figure 1 indicate that the standard curve of zearalenone obtained with the former was apparently different from the latter. The standard curve of zearalenone using a purified solid phase antigen was more sensitive and precise, being in the range of 0.5–50 ng/mL.

Effect of Assay Buffer on Standard Curve in Grain. Standard curves of zearalenone in both barley and Job's-tears samples showed improved accuracy and sensitivity (each 10 ng/g detection limit) by the use of assay buffer containing glutamine and Tris-HCl in comparison with 0.05 M phosphate buffer and were constructed in the range of 0–1000 ng/g (Figure 2).

Recovery. Known amounts of zearalenone spiked to barley and Job's-tears samples were extracted, the above-mentioned procedures were performed according to the two methods described under Extraction of Zearalenone, and the solution was applied to CI-ELISA.

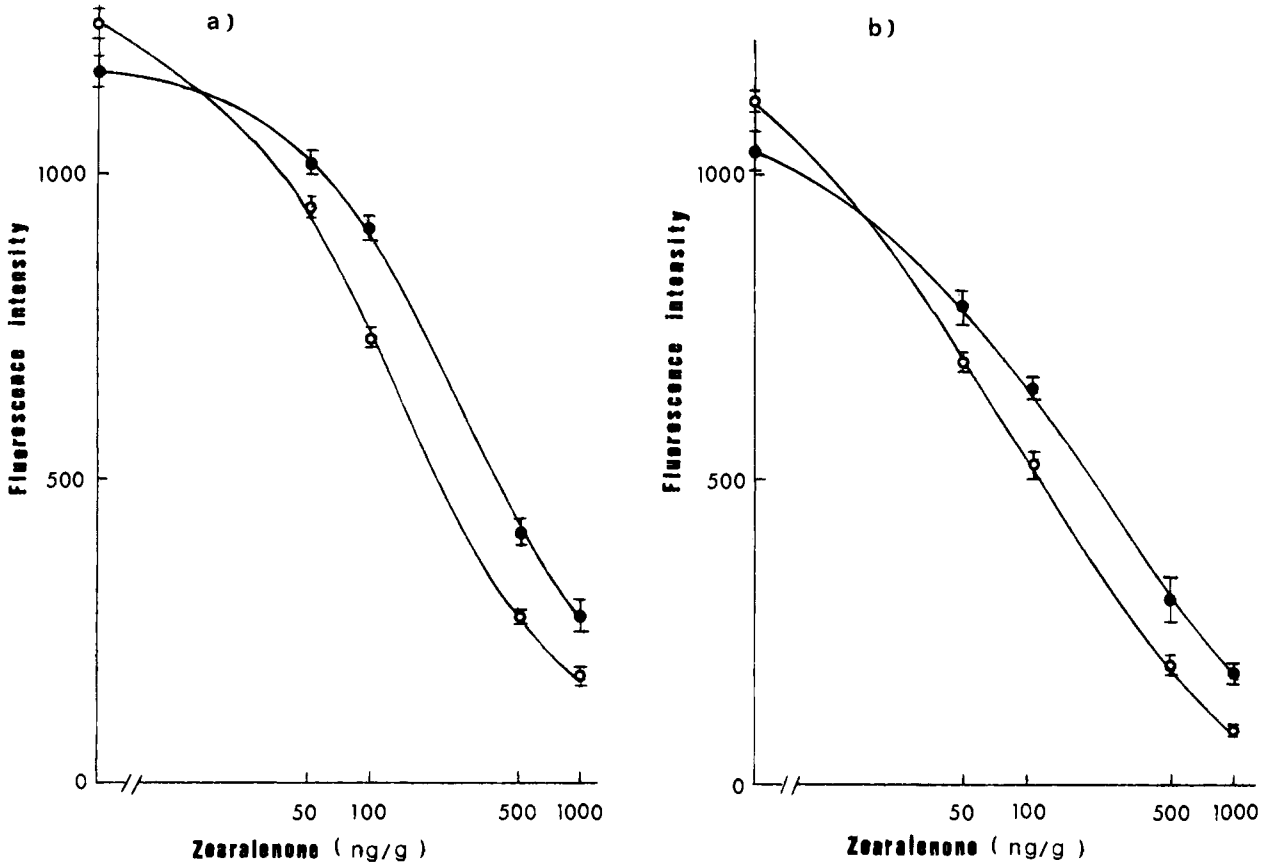

Figure 2. Effect of assay buffer on standard curves of zearalenone in barley (a) and Job's-tears (b) samples by CI-ELISA using method B (●, 0.05 M phosphate buffer, pH 7.2; ○, 0.05 M phosphate buffer, pH 7.2, containing 0.1 M glutamine and 0.1 M Tris-HCl) (n = 5).

Table 1. Recovery of Zearalenone from Spiked Barley by CI-ELISA

zearalenone added, ng/g	method A recovery ng/g[a]	%	CV,[b] %	method B recovery ng/g[a]	%	CV,[c] %
25	32 ± 5.3	128	16.2	26 ± 3.0	105	11.5
50	56 ± 9.3	111	16.6	48 ± 5.2	90	6.7
100	105 ± 5.3	105	5.2	97 ± 7.0	97	7.2
250	255 ± 24	102	9.6	235 ± 16	95	5.8
500	475 ± 13	95	7.7	486 ± 28	97	6.6
1000	972 ± 61	97	6.3	1037 ± 68	103	7.4

[a] Mean ± SD (n = 6; plates). [b,c] Interassay coefficient variations. Mean interassay, CVs were 10.3% (method A) and 7.5% (method B), respectively.

Table 2. Recovery of Zearalenone from Spiked Job's-Tears by CI-ELISA

zearalenone added, ng/g	method A recovery ng/g[a]	%	CV,[b] %	method B recovery ng/g[a]	%	CV,[c] %
25	24 ± 3.5	96	13.5	28 ± 3.1	112	11.0
50	43 ± 4.6	92	10.7	52 ± 3.1	104	6.0
100	95 ± 10	95	10.9	98 ± 6.5	98	6.6
250	254 ± 18	102	7.2	237 ± 13	95	5.4
500	443 ± 32	92	7.3	490 ± 25	98	5.1
1000	970 ± 67	97	6.9	1018 ± 47	102	4.6

[a] Mean ± SD (n = 6; plates). [b,c] Interassay coefficient variations. Mean interassay, CVs were 9.4% (method A) and 6.5% (method B), respectively.

The results are summarized in Tables 1 and 2. The mean recovery of zearalenone from spiked barley was observed to be 106% (range, 95–128%; mean CV of 10.3%) by method A and 98% (range, 90–105%; mean CV of 7.5%) by method B. The mean recovery of zearalenone from spiked Job's-tears was observed to be 96% (range, 92–102%; mean CV of 9.4%) by method A and 102% (range, 95–112%; mean CV of 6.5%) by method B.

Table 3. Levels of Zearalenone in Barley (B) and Job's-Tears (J) Samples by CI-ELISA

sample	ng/g	sample	ng/g	sample	ng/g
B-1	10	J-1	575	J-21	85
B-2	69	J-2	20	J-22	70
B-3	25	J-3	550	J-23	260
B-4	76	J-4	85	J-24	39
B-5	51	J-5	19	J-25	73
B-6	658	J-6	125	J-26	795
B-7	54	J-7	53	J-27	16
B-8	43	J-8	60	J-28	87
B-9	33	J-9	210	J-29	1150
B-10	76	J-10	70	J-30	25
B-11	101	J-11	535	J-31	20
B-12	28	J-12	118	J-32	172
B-13	270	J-13	37	J-33	40
B-14	48	J-14	55	J-34	1395
		J-15	227	J-35	21
		J-16	140	J-36	410
		J-17	65	J-37	564
		J-18	575	J-38	135
		J-19	20	J-39	325
		J-20	550	J-40	130

Determination of Zearalenone in Grain Sample. Levels of zearalenone were measured in 200 grain samples by competitive indirect ELISA using method B. Zearalenone was not detected in 146 grain samples by CI-ELISA; however, levels of zearalenone were determined by CI-ELISA in 14 barley and 40 Job's-tears samples, ranging from 10 to 1395 ng/g as shown in Table 3. The levels of zearalenone detected by CI-ELISA and HPLC in grain samples are illustrated in Figure 3. The

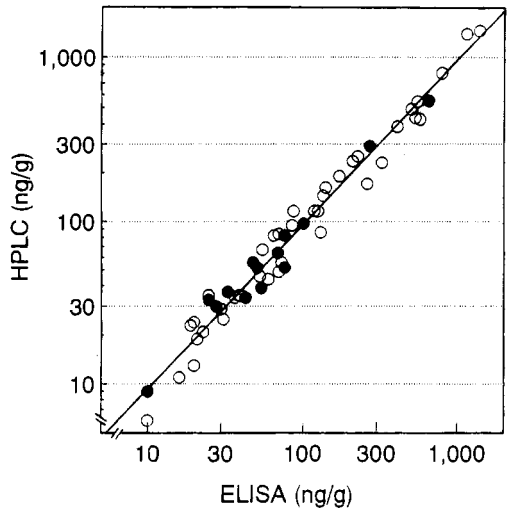

Figure 3. Correlation between CI-ELISA and HPLC of zearalenone in barley (●) and Job's-tears samples (○) ($n = 54$, $r = 0.971$, $y = 1.01x + 0.91$).

overall correlation coefficient r between CI-ELISA and HPLC was 0.971, $y = 1.01x + 0.91$, where y corresponds to values obtained by HPLC.

DISCUSSION

Several immunoassays for zearalenone have been developed (Thouvenot and Morfin, 1983; Liu et al., 1985; Warner et al., 1986; Dixon et al., 1987).

It is noted that an antibody generated by immunization with an antigen in which a hapten is conjugated to carrier protein through the C-5 position remote from a lactone ring is capable of discriminating zearalenone from zearalenol and related compounds. Zearalenone–OVA conjugates as solid phase antigen synthesized according to the modified Mannich reaction (Miwa et al., 1977) were a mixture of the C-3 and C-5 positional isomers, whereas the sensitivity of CI-ELISA was influenced by the use of the mixture. To increase the sensitivity of CI-ELISA for zearalenone, the mixture of solid phase antigens prepared according to the Mannich reaction was purified using the immobilized antibody. In low concentration of added zearalenone, zearalenone–OVA conjugate freed from the antibody binding zearalenone–OVA conjugate was collected. This zearalenone–OVA conjugate could be the isomer from which the antibody is displaced most easily with zearalenone. It means a more sensitive assay. By using a purified solid phase antigen, a standard curve of zearalenone was constructed with a 1:50000 dilution of the antibody (ascites fluid) to be improved on the detection limit (0.2 ng/mL) of zearalenone. Typical standard curves of zearalenone in the range from 0.5 to 50 ng/mL are indicated in Figure 1. Although zearalenone–OVA conjugate was labile in aqueous solution, the purified solid phase antigen was stable at −20 °C with the addition of 0.1% ascorbic acid over 12 months.

Liu et al. (1985) reported that competitive ELISA standard curves are affected by extracts of the samples. Affinity column chromatography as sample cleanup step prior to determination by ELISA was described by Azcona et al. (1990). Bennett (1991) demonstrated the reliability of an immunoassay procedure as a screening method for zearalenone at >800 ng/g in corn, wheat, and feeds.

The accuracy of the present method was examined with various known amounts of authentic zearalenone spiked ranging from 25 to 1000 ng/g of grain. Initially, method A used an organic solvent extraction (methanol–water) followed by a second organic extraction (dichloromethane) to remove obstacles interfering with the binding of hapten to antibody. Next, to simplify cleanup procedures, aqueous methanol obtained by extraction of ground grain samples was directly applied to CI-ELISA using the assay buffer containing glutamine and Tris-HCl (method B). Approximately identical recoveries of zearalenone from spiked barley were obtained by methods A and B, as shown in Table 1. Furthermore, recoveries of zearalenone from spiked Job's-tears samples were nearly identical to those of zearalenone from spiked barley samples (Table 2). Thus, we added sufficient glutamine and Tris-HCl to the mixture of antibody and sample extracts to reduce interference in the binding of antigen to antibody. By including sufficient glutamine in the buffer solution level, and also using a purified solid phase antigen and a highly specific antibody, we were able to obtain more sensitive and accurate standard curves for zearalenone in barley and Job's-tears samples.

It has been well documented that Job's-tears products can be contaminated by mycotoxins (Tanaka et al., 1985a; Narita et al., 1992). It has been demonstrated that the present ELISA for zearalenone was sensitive and facile because no cleanup procedure (method B) of barley and Job's-tears samples was used and because a purified solid phase antigen was used. The values obtained by ELISA were well correlated with those obtained by HPLC. The ELISA used here has facilitated the assay procedure and led to a reliable analytical method. The methodology used may be applicable to the screening of other grains for zearalenone.

ACKNOWLEDGMENT

We thank Dr. H. Kamimura, Tokyo Metropolitan Research Laboratory of Public Health, for suggestions and N. Narita and M. Suzuki, Division of Microbiology, for technical assistance.

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Received for review June 24, 1994. Revised manuscript received November 28, 1994. Accepted January 29, 1995.[⊗]

JF930642O

[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1995.