tions after 6 months' storage in the dark. One can notice a slight decrease in fluorescent intensity compared to those on the left. Also notable in both groups is that the cyclohexane and heptane B_1 spots are tighter than the benzene or toluene spots. The third TLC strip, on the right with the lone spot, is that of standards after 3 months of exposure to normal lighting in the laboratory. The lone spot is that of the benzene system. Complete photodegradation of B_1 occurred in the cyclohexane, heptane and toluene systems, even though the absorbance of these solutions indicated a level of B_1 greater than 1 μg/mL. The superiority of benzene as a solvent for aflatoxin is clearly evident from this exposure test to light.

Replacement of benzene by heptane or toluene in preparing aflatoxin standards appears feasible, provided that exposure to light is minimized. Of the 2 solvents, heptane would be a better choice, as it has a higher vapor exposure safety level. Laboratories not equipped to comply with OSHA regulations for benzene vapors could thus substitute heptane for benzene. Laboratories that have adequate controls to meet OSHA regulations for benzene vapors could continue to use this solvent for aflatoxin standards.

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Enzyme-Linked Immunosorbent Assay for T-2 Toxin

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed for the rapid quantitation of T-2 toxin, a tricothecene mycotoxin produced by members of the genus *Fusarium*. T-2 toxin was first converted to the T-2 hemisuccinate (T-2 HS) and then conjugated by the water-soluble carbodiimide method to either bovine serum albumin for use as an immunogen or to horseradish peroxidase for use as an enzyme marker. T-2 antiserum was air-dried onto polystyrene microtissue culture plates and the ELISA conducted by simultaneously incubating standards of T-2 toxin and the T-2 HS-peroxidase conjugate. Competition curves were prepared by determining total bound enzyme. The ELISA took about 2 hr to complete and allowed minimal detection of T-2 at levels of 2.5 pg/assay. Average recoveries from samples of wheat flour spiked with T-2 toxin in the 1.0-30 ppb range were 95 ± 25% and those for corn meal spiked in the 5.0-30 ppb range were 98 ± 19%. The results suggested the ELISA is a simple and convenient alternative for the screening of T-2 toxin in food and feeds.

INTRODUCTION

T-2 toxin and other related tricothecene mycotoxins are secondary metabolites produced by the fungal genera *Fusarium*, *Trichoderma*, *Myrothecium* and *Stachybotrys* (1). T-2 toxin has been associated with certain mycotoxicoses (1,2), including alimentary toxic aleukia (3), and has recently been suggested to be carcinogenic (4). Because T-2 toxin and the fungi that produce it are found in a number of agricultural commodities (5,6), it presents a potential risk to human and animal health. While a number of analytical methods for T-2 toxin have been developed, including biological assays, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas liquid chromatography-mass spectrometry (GC-MS) (7,8), these either lack sensitivity or specificity or require expensive instrumentation, thus precluding their use in the routine screening of foods and feeds. Recently, this problem was overcome by the development of a radioimmunoassay (RIA) for T-2 toxin (9). The RIA is sensitive and specific for T-2 toxin and can be used in the assay of corn, wheat, urine and serum (10,11).

In spite of its many advantages, the RIA still presents problems related to the disposal and storage of radioactive reagents, the necessity for a scintillation counter, and the number of samples that can be conveniently analyzed. A simpler alternative, the enzyme-linked immunosorbent assay (ELISA), has been applied to the assay of aflatoxin B_1, aflatoxin M_1, and ochratoxin A in our laboratory (12-14). In this report, we describe the development of a solid phase-microtest plate ELISA for T-2 toxin and its application to the assay of artifically contaminated wheat and corn.

EXPERIMENTAL

Materials

Horseradish peroxidase (type VI), bovine serum albumin (BSA), RIA grade, Tween 20, 1-ethyl-3,3-dimethylaminopropyl-carbodiimide (EDPC), 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS) and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). Complete and incomplete Freund adjuvants were purchased from Difco Laboratories (Detroit, MI). T-2 and T-2 hemisuccinate (T-2HS) were prepared according to previously published methods (9,15). Standard preparations of T-2 were kindly supplied by E.B. Smalley of the University of Wisconsin. All other chemicals were of reagent-grade quality or better. Sep-Pak™ C_{18} cartridges were obtained from Waters Associates (Milford, MA). Albino rabbits were purchased from Klubertanz's Rabbit Farm (Edgerton, WI) and tested to be *Pasteurella* negative before use. Corn meal and wheat flour were purchased from a local grocery store.

Production of Hyperimmune Serum

Antisera specific for T-2 was prepared by multiple site injections of albino rabbits with a T-2 HS-BSA immunogen in the manner described by Chu et al. (9). The resulting hyperimmune serum was purified by the ammonium sulfate method of Herbert et al. (16) and titer was determined by RIA (9).

Preparation of T-2 HS-Peroxidase Conjugate

The method for T-2 HS-peroxidase preparation was essentially that used for aflatoxin B_1 oxime-peroxidase (12).

Briefly, 900 μg of T-2 HS dissolved in 1.0 mL ethanol and 3.0 mL H_2O was mixed with 150 mg EDPC, and 3 mg horseradish peroxidase in 1.0 mL of 25% aqueous ethanol was then added. The mixture was stirred for 30 min at room temperature and another 150 mg EDPC was added. The reaction was continued overnight at 4 C and the product dialyzed against 3 changes of 0.01 M sodium phosphate buffer (pH 7.5) for 3 days. The T-2 HS-peroxidase (0.4 mg/mL) was then lyophilized in small quantities.

Enzyme-Linked Immunosorbent Assay

The ELISA procedure was based on that described for aflatoxin B_1 by Pestka et al. (12) in which polystyrene microtissue culture plates (Falcon 3040) pretreated with BSA and glutaraldehyde were used as the solid phase. Purified antisera was diluted in 0.1 M phosphate-buffered (pH 7.5) normal saline (PBS) and 50-μL aliquots were air-dried in each well under a current of forced air (25-40 C). Microplates prepared in this manner could be stored over desiccant for as long as 3 months with no loss in reactivity. Before use, the plates were washed 3 times by filling each well with 0.2 mL of PBS containing 0.05% (vol/vol) Tween 20 (PBS/Tween) and aspirating out the contents. To decrease nonspecific binding, 0.2 mL of PBS containing 1% (wt/vol) BSA was incubated in each well for 30-60 min at 37 C and the plates were washed 2 more times.

For antisera titration, T-2 HS-peroxidase conjugate (0.4 mg/mL) was diluted (1:50) in PBS containing 5% (wt/vol) BSA and 50-μL aliquots were added to microplate wells previously treated with serial dilutions of either antisera or preimmune sera. The plates were incubated for 1 hr at 37 C, washed 4 times in PBS/Tween, and then reacted for 30 min with 0.1 mL of ABTS-hydrogen peroxide substrate (12). The reaction was stopped by the addition of 0.1 mL hydrofluoric acid/edetic acid stopping reagent and total bound enzyme was determined spectrophotometrically (12).

For the competitive ELISA, 25 μL of standard T-2 toxin or sample diluted in PBS/methanol (9:1) was added to microplate wells that were previously coated with 50 μL of a 1:400 dilution of antiserum in PBS. This was followed by the addition of a 1:25 dilution of T-2 HS-peroxidase in PBS containing 5% (wt/vol) BSA. Plates were incubated and bound enzyme determined as just described.

Extraction of Corn and Wheat Samples

Samples of corn meal and wheat flour were spiked with T-2 toxin in the 0.2-30 ppb range and extracted with methanol and defatted with hexane according to the method of Lee and Chu (10). In some cases, the extracts were subjected to a Sep-Pak C_{18} column chromatography step (10). The extracts were then dried under a current of N_2 at room temperature, dissolved in methanol, and adjusted to a 10% methanol/PBS (vol/vol) concentration.

RESULTS

Titration of T-2 HS-BSA by ELISA

Although estimates of the molar ratio of aflatoxin B_1 to horseradish peroxidase can be determined spectrophotometrically (12), the absence of absorption maxima for T-2 above a 250-nm wavelength precludes such a determination. Therefore, only the capacity of the T-2 HS-peroxidase conjugate to specifically bind to antibody was determined in this study. An antiserum specific for T-2 HS-BSA which had a titer of 204 by RIA (9) was used. The ELISA titration was done by drying a dilution series of antiserum onto a BSA-pretreated polystyrene microplate and then determining the binding of the T-2 HS-peroxidase to the individual wells. The last well in a dilution series to yield absorbance visually distinct from that of the preimmune serum control was chosen as the titer endpoint. A typical titration assay is shown in Figure 1. Wells prepared with preimmune sera had an average absorbance of 0.05 whereas the hyperimmune serum had a maximal absorbance of 1.13 at the 1:80 dilution with a titer endpoint of 1:1280. When an unmodified horseradish peroxidase control was added

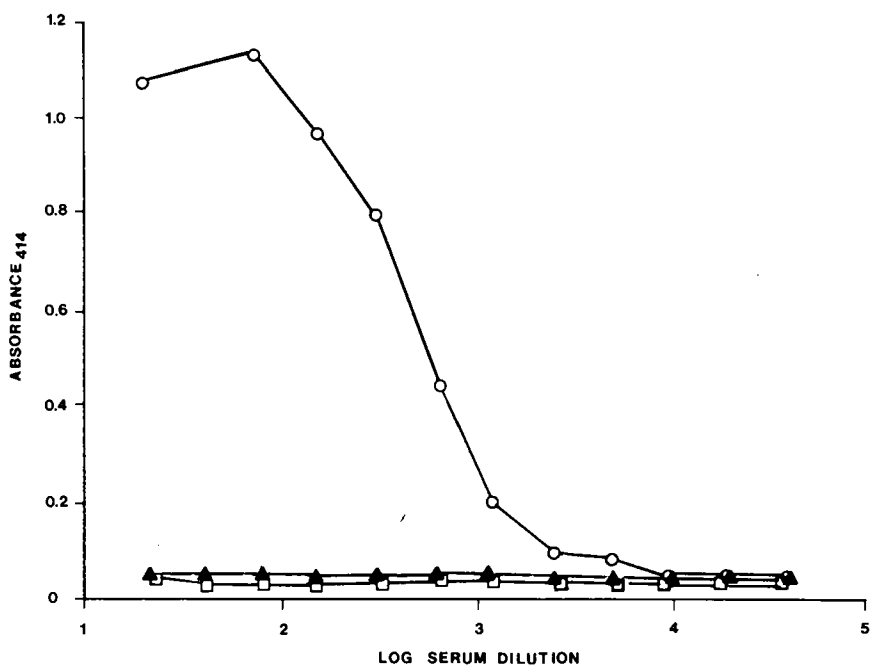

FIG. 1. ELISA titration curve for T-2 antiserum. Titration performed as described in Experimental. Symbols: (○) T-2 antiserum assayed with T-2 HS-peroxidase conjugate (8 μg/mL); (□) T-2 antiserum assayed with unconjugated peroxidase (8 μg/mL); and (▲) preimmune serum assayed with T-2 HS-peroxidase conjugate (8 μg/mL).

to the dilution series of hyperimmune serum at a concentration (8 μg/mL) equivalent to that used for the T-2 HS-peroxidase, only negligible binding (0.04 absorbance units) was detected. The results of the titration assay indicated that the T-2 HS-peroxidase conjugate bound in a specific manner and could be used for titering the T-2 antisera and suggested that it could be used in a competitive-type assay for T-2.

Standard Curve for T-2 Toxin by Competitive ELISA

Because optimization experiments for other mycotoxin ELISA (12-14) indicated that antiserum dilutions in the range of 50% of the maximal absorbance obtainable in a titration assay were suitable for competitive ELISA, a 1:400 dilution of T-2 antiserum was used to set up a standard curve for T-2. To facilitate solubility, T-2 toxin standards were first dissolved in methanol (10 μg/mL) and then diluted in 10% methanol in PBS on the day of use. The standards (25 μL) and the T-2 HS-peroxidase conjugate (25 μL, 16 μg/mL) were then simultaneously incubated and total bound enzyme determined. A typical competitive, standard curve is illustrated in Figure 2. The results indicate that the conjugate binding response was maximal between 2.5 and 250 pg/assay with the minimal detectable level being 2.5 pg/assay which was significantly different than 0 at the 97.5% confidence level. Maximal competition for binding with the T-2 HS-peroxidase conjugate occurred at 2,500 pg of T-2 toxin/assay, indicating that ca. 25% of the peroxidase still bound to the solid-phase antibody. These results suggested that the T-2 HS-BSA antiserum also recognized components of the T-2 HS-peroxidase, perhaps the hemisuccinate or a T-2 HS-lysine residue, which were not present on the free T-2 molecule.

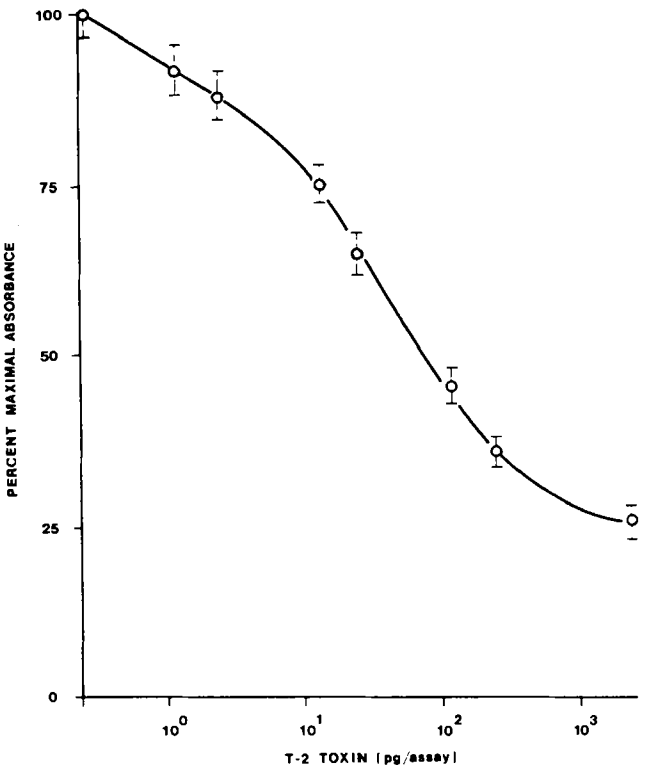

FIG. 2. ELISA standard curve for T-2 toxin. Results represent average of quadruplicate analysis on a single ELISA microplate. As 25-μL samples were used per well, 2.5 pg/assay is equivalent to an original sample concentration of 100 pg/mL.

Effect of Wheat and Corn Extracts on the Binding of T-2 HS-Peroxidase

A previous investigation in our laboratory (10) indicated that extracts of wheat and corn cause significant interference in the T-2 RIA and that a preliminary Sep-Pak C_{18} treatment could be used to remove this interfering material. Therefore, the effects of wheat and corn extracts on the maximal binding of the T-2 HS-peroxidase conjugate were determined both before and after Sep-Pak treatment. The results, as indicated in Table I, showed that extracts equivalent to 25 mg (1.0 g original sample extract/mL) of wheat or corn samples caused substantial interference in the ELISA without significant improvement by the clean-up step. The inhibitory effects of these extracts could be minimized somewhat by further dilution of the samples. When extracts equivalent to 3.13 mg wheat sample and 1.25 mg corn sample were used, the inhibiting effect was less than 3% after Sep-Pak treatment.

Recovery of T-2 from Spiked Wheat and Corn Samples by ELISA

To test the efficacy of monitoring T-2 in wheat flour and corn meal, samples were spiked with standard T-2 in the range of 0.2-30.0 ppb. The toxin was recovered by methanol extraction and defatted with hexane. As RIA recoveries of T-2 toxin in wheat and corn samples were improved after Sep-Pak treatment (10), ELISA were done both before and after clean-up. For wheat samples, extracts equivalent to 3.13 mg wheat were added per well (Table II). The best recovery of added toxin could be made on the Sep-Pak-treated samples at the 1.0 ppb level with an estimate of 86 ± 35% being obtained. For the untreated wheat samples, the minimal level was 5.0 ppb in which 83 ± 38% recovery was determined. Recovery data of T-2 from corn meal (Table III), in which extracts equivalent to 1.25 mg

TABLE I

Effect of Wheat and Corn Extracts on T-2 HS-Peroxidase Binding to ELISA Solid-Phase

	Inhibition (%)			
	Wheat		Corn	
g extract/mL	With Sep-Pak treatment	Without Sep-Pak treatment	With Sep-Pak treatment	Without Sep-Pak treatment
1.0 (25)[a]	18	19	34	36
0.5 (12.5)	9	15	28	31
0.25 (6.25)	6	7	15	20
0.125 (3.125)	0	3	–	–
0.100 (2.5)	–	–	7	10
0.050 (1.25)	–	–	2	8

[a]Numbers in parentheses indicate total mg of sample extract/assay well.

TABLE II

ELISA Recovery of T-2 from Artificially Contaminated Wheat Flour Samples[a]

Added T-2 toxin (ppb)	Recovery			
	Before Sep-Pak		After Sep-Pak	
	(ppb)	(%)	(ppb)	(%)
0.2	0.9 ± 0.6	445 ± 275	0.5 ± 0.1	245 ± 30
1.0	1.5 ± 1.7	153 ± 166	0.9 ± 0.4	86 ± 35
2.5	3.1 ± 2.1	122 ± 85	2.4 ± 0.7	96 ± 26
5.0	4.2 ± 2.0	83 ± 39	4.8 ± 1.4	96 ± 28
17.5	14.9 ± 2.5	85 ± 14	18.1 ± 2.5	103 ± 14
30.0	27.5 ± 9.9	92 ± 33	27.5 ± 5.3	92 ± 18
Mean recovery	87[b]		95[c]	
Average standard deviation	29		25	
Coefficient of variation	32.9		26.2	
Number of assays	6		10	

[a]Extractions performed on single set of samples. Extracts equivalent to 3.13 mg sample were added per well. Results represent mean of 2 ELISA performed on different days.

[b]Statistical analysis for 5.0-30 ppb samples only.

[c]Statistical analysis for 1.0-30 ppb samples only.

corn were used, showed estimates at the 5.0 ppb level of 109 ± 18% and 108 ± 39% for treated and untreated extracts, respectively. In general, the Sep-Pak treatment appeared to remove interfering materials from the extracts as well as decrease sample variation. The results also indicated that, although some sensitivity in the ELISA was lost by dilution of the sample, the method was still effective in detecting T-2 at the 1.0 and 5.0 ppb level, respectively, in wheat and corn.

Competitive ELISA Standard Curve Prepared in Wheat and Corn Extract

An alternative approach to minimize the interference in samples to be analyzed would be to incorporate an appropriate amount of "clean sample extract" in the standard solutions for the competitive ELISA. We have also tested this approach and the results in Figure 3 illustrate competition curves which were prepared in PBS, unspiked wheat extract (3.13 mg/well), or unspiked corn extract (3.13 mg/well). Although equivalent sensitivities were obtained for PBS and wheat extract, the limits of detection appear to be diminished by the presence of corn extract. Similar results were obtained for Sep-Pak-treated sample extracts. The effect of corn extract may be due to the binding of T-2 HS-peroxidase to interfering materials, or to the actual presence of T-2. The actual presence of T-2 is impossible to verify as analytical methods achieving this sensitivity are unavailable. Thus, the feasibility of using food extracts in standard curves will require further verification using extracts from agricultural commodities carefully selected to be free of mold contamination, as well as a comparison of different varieties to ensure that variations in interfering material content do not exist.

DISCUSSION

A number of reports have described the acute and chronic toxic effects of T-2 toxin in man and animals (1-3) and, in one instance, the mycotoxin has been shown to cause tumors in rats (4). Because T-2 toxin has been shown to occur in agricultural commodities, it is imperative that a simple and sensitive procedure be developed to monitor the toxin in human foods and animal feeds. The results presented here demonstrate that the ELISA is the simplest assay developed so far for T-2 with a minimal detection limit in standard buffer of 2.5 pg/assay. The practical limits for wheat were 1.0 ppb or greater (95 ± 25%) and those for corn were 5.0 ppb or greater (98 ± 19%) when the food extracts were subjected to a Sep-Pak clean-up step. Without the clean-up step, the average recoveries were 87 ±

TABLE III

ELISA Recovery of T-2 from Artificially Contaminated Corn Meal Samples[a]

Added T-2 toxin (ppb)	Recovery			
	Before Sep-Pak		After Sep-Pak	
	(ppb)	(%)	(ppb)	(%)
0.2	2.2 ± 1.6	1100 ± 780	0.6 ± 0.3	250 ± 98
1.0	3.8 ± 2.3	375 ± 230	1.2 ± 0.7	124 ± 69
2.5	5.4 ± 2.7	217 ± 110	3.7 ± 1.3	146 ± 54
5.0	5.4 ± 1.9	108 ± 38	5.5 ± 0.9	109 ± 18
17.5	17.1 ± 6.4	98 ± 37	13.4 ± 2.6	76 ± 15
30.0	37.4 ± 12.7	125 ± 42	33.0 ± 6.8	110 ± 23
Mean recovery	10[b]		98[c]	
Average standard deviation	39		19	
Coefficient of variation	35.6		19.0	
Number of assays	9		9	

[a]Extraction performed on single set of samples. Extracts equivalent to 1.25 mg sample were added per well. Results represent mean of 3 ELISA performed on separate days.

[b]Statistical analysis for 5.0-30.0 ppb samples only.

[c]Statistical analysis for 5.0-30.0 ppb samples only.

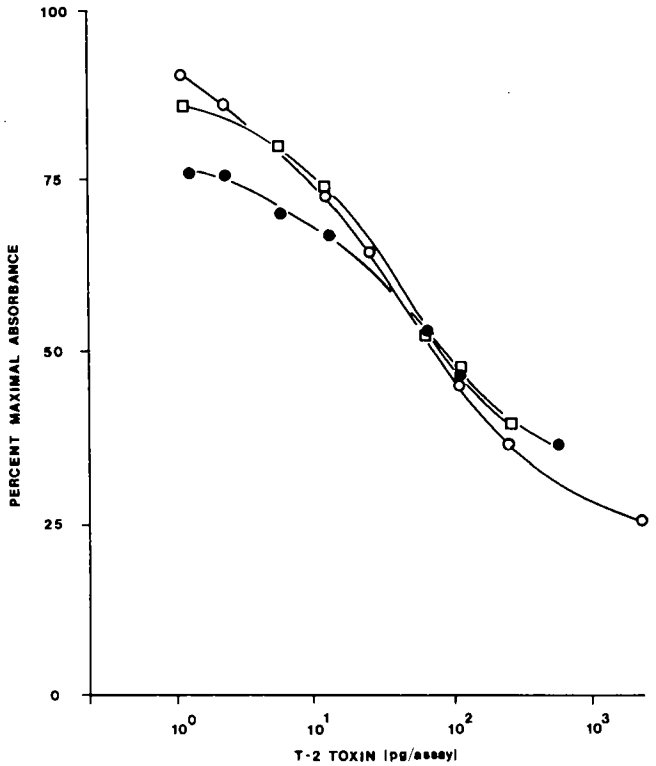

FIG. 3. Effect of corn and wheat extracts on T-2 ELISA standard curve. Extracts were defatted with hexane but not subjected to Sep-Pak treatment. Standard competition curves prepared as described in Experimental. Symbols: (○) standard curve prepared in PBS; (●) standards prepared in PBS containing 3.125 mg corn extract/well; and (□) standards prepared in PBS containing 3.125 mg wheat extract/well.

29% for wheat at the 5.0 ppb or greater level and 110 ± 39% for corn at the 5.0 ppb level or greater. Sensitivity for agricultural commodities might be further increased with an improved column clean-up step or by use of a standard food extract when preparing a standard curve.

While slightly better practical sensitivity with lower variability was obtained previously in this laboratory in corn and wheat by the RIA (10), the ELISA has significant advantages in terms of simplicity, shorter assay time (3 hr vs overnight), absence of radiation hazard, stability of reagents, and the number of samples that can be routinely assayed.

In regard to the specificity of antisera prepared against T-2 HS-BSA, we have previously determined by RIA that the antibody is most reactive for the isovaleroxy moiety of the T-2 molecule with lesser reactivity for other side groups (9). Hence, T-2 HS-BSA antisera bound HT-2 6 times and T-2 triol 46 times less than T-2 with negligible binding to other T-2 analogs. Although the cross-reactivity of this antibody was not determined in the ELISA, we have found cross-reactivity spectra for other mycotoxin ELISA to be generally comparable to those for RIA (12-14). Nevertheless, we recommend that cross-reactivity of T-2 HS-BSA antisera be tested in the ELISA when specificity considerations are critical, e.g., as in metabolism studies of T-2 toxin.

A second point of consideration in the T-2 ELISA is the high coefficients of variation for wheat (26.2%) and corn (19.0%) after clean-up and those for wheat (32.9%) (19.0%) after clean-up and those for wheat (32.9%) and corn (35.6%) without the Sep-Pak step. Similar standard deviations were found in the aflatoxin B_1 ELISA of ELISA of milk (13,17). Possible sources of variation in the ELISA are variability induced by the food extract, variability in interwell protein, adsorption characteristics of the microplate (18) and inaccuracy of measuring a 25-μL volume. We have found that errors in the ELISA can be best minimized by inclusion of Tween 20 in the wash buffer, preincubation of the plate with 1.0% BSA in PBS, inclusion of BSA in the peroxidase conjugate diluent, and by incubation of the standards and samples in alternating rows of 8 across the microplate.

Both the ELISA and RIA have significant advantages over chemical and biological assays in terms of simplicity, cost per assay and, in most cases, specificity. The sensitivity of the T-2 ELISA (2.5 pg/assay) compares favorably to that for TLC, GC, HPLC, GC-MS, rabbit reticulocyte cytotoxicity assay, phytotoxicity assay, and the brine shrimp larvae assay (7,8,19,20). Estimates of T-2 at the 5.0 ppb level by the ELISA can be made even without the Sep-Pak clean-up. The large number (96) of wells on the microplate makes the ELISA ideal for the large-scale screening of agricultural commodities by simple visual detection of grossly contaminated samples. Future investigation will be directed toward applying the ELISA to the monitoring of corn and wheat that is naturally contaminated with T-2 toxin.

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