# Detection of T-2 Toxin in Different Cereals by Flow-Through Enzyme Immunoassay with a Simultaneous Internal Reference

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In most previously described membrane-based immunoassays a separate negative control assay is always carried out to evaluate the performance of the assay. To overcome this problem, a membrane-based flow-through enzyme immunoassay with an internal control has been developed for the detection of T-2 toxin in cereals (patent pending). An Immunodyne ABC membrane was coated with 2 $\mu$L of goat anti-horseradish peroxidase (HRP) (internal control spot) (1:1000) and 2 $\mu$L of rabbit anti-mouse (test spot) (undiluted) immunoglobulins, and the free binding sites were blocked. In addition to the antibody-coated Immunodyne ABC membrane, the assay also comprises a plastic snap-fit device, absorbent cotton wool, mouse anti-T-2 monoclonal antibodies (Mab), and T-2−HRP conjugate. The color intensity ($\Delta E^*_{ab}$) of the internal control and that of the negative sample showed no difference ($P > 0.05$), whereas there was a significant difference between the internal control and positive samples ($P < 0.05$). The minimum detectable limit that could be visually detected with confidence was 50 ng of T-2 per gram of cereal sample.

**Keywords:** *Internal control; T-2 toxin; flow-through membrane-based immunoassay; cereals*

## INTRODUCTION

T-2 toxin [3-hydroxy-4,15-diacetoxy-8-(3-methylbutyryloxy)-12,13-epoxy-$\Delta^9$-trichothecene] belongs to a group of mycotoxins called trichothecenes produced by *Fusarium* species, namely *F. sporotrichioides* and *F. nivale*. This trichothecene is well-known for causing alimentary toxicity. This disease, which reportedly killed thousands of people in Russia in the period of 1942−1947, was associated with ingestion of overwintered grains that had become infected with *Fusarium* species producing T-2 toxin and related trichothecenes (Minervini et al., 1994). Maximum tolerated levels for T-2 toxin in food exist only in Russia (100 ng/g for cereals) (Boutrif and Canet, 1998).

A number of methods have been developed to date for the detection of T-2 toxin in cereals; however, most of them involve sophisticated equipment including HPLC, GC-MS, and some ELISAs (Barna-Vetro et al., 1994). In addition to being expensive, these methods are time-consuming as they also require complicated sample cleanup. Because of the toxicological interest generated by T-2 toxin, there has been an acceleration in the development of simple and time-saving methods for T-2 detection (De Saeger and Van Peteghem, 1996).

This paper, therefore, describes the development of a flow-through membrane-based enzyme immunoassay for the detection of T-2 in cereals that can be used in the field, through a visual determination of assay results also incorporating an internal negative control.

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## MATERIALS AND METHODS

**Apparatus.** The main features of note in this assay are the flow-through device comprising the plastic top and bottom members (Trosley Equipment, Dover, U.K.), 2 cm$^2$ Immunodyne ABC membrane, pore size = 0.45 $\mu$m (Pall Europe Ltd., Portsmouth, U.K.), and absorbent material (100% cotton wool) (Figure 1). GC-MS equipment model and type used were as follows: Hewlett-Packard HP 6890 series GC system, mass selective detector HP 6890 series. An HP 19091S-433, HP-5MS 5% phenyl methyl siloxane capillary column (30 m × 250 $\mu$m × 0.25 $\mu$m) was used.

**Reagents.** The reagents used and their preparation have been described previously (De Saeger and Van Peteghem, 1999; Sibanda et al., 1999). Monoclonal antibodies against T-2 toxin were produced and characterized by the Institute for Animal Sciences, Agricultural Biotechnology Center, Gödöllö, Hungary (Gyöngyösi et al., 1994). Cross-reaction with acetyl-T-2 was 12.8%, with HT-2, 3.4%, and with iso-T-2, 2.5%. There was no cross-reaction with T-2 triol, T-2 tetraol, verrucarin A, verrucarol, roridin A, diacetoxyscirpenol, deoxynivalenol, or zearalenone. The anti-T-2 monoclonal antibody was immunoglobulin (Ig) G1 with kappa light chains. The affinity constant was $3.2 \times 10^{10}$ M$^{-1}$. The anti-T-2 immunoglobulin fraction (pure immunoglobulin fraction; protein content = 1 mg/mL) was used for the flow-through enzyme immunoassay. The T-2−HRP conjugate was prepared by the same Hungarian institute as described by Barna-Vetro et al. (1994). Rabbit anti-mouse immunoglobulins (no. Z259; protein concentration = 3.2 g/L; reacting with all mouse IgG subclasses, mouse IgA, and mouse IgM) were from Dako, Glostrup, Denmark. T-2, Tween 20, casein sodium salt (casein), urea hydrogen peroxide (no. U-1753; containing ~35% $H_2O_2$), and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. The buffer used for dilution of anti-T-2 immunoglobulins and T-2-HRP (assay buffer) consisted of phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% Tween 20 and 0.1% casein and was supplemented with 0.05% Proclin 300 (Supelco, Inc., Bellefonte, PA) as a preservative (Sibanda et al., 1999).

10.1021/jf000337k CCC: $19.00 
Published on Web 10/28/2000

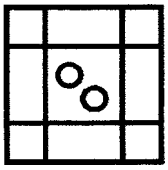

**Figure 1.** $2 \times 2$ cm$^2$ sectioned ABC membrane with a central square spotted with mouse anti-T-2 IgG and goat anti-HRP.

Methanol and water were of HPLC grade. A stock solution of T-2 (1 mg/mL) was prepared in methanol and stored at −20 °C. A working stock solution of T-2 (100 μg/mL) was prepared in 15% methanol PBS−Tween and stored at <5 °C. Working standards were prepared on the day of assay in 15% methanol−PBS−Tween. All T-2 solutions were kept in the dark. The glassware and T-2 waste were decontaminated with sodium hypochlorite solution. The substrate chromogen solution was prepared as described by Dürsch and Meyer (1992) and De Saeger and Van Peteghem (1999).

**Procedure.** *Preparation of Membranes.* The Immunodyne ABC membrane was cut into squares ($2 \times 2$ cm) (Figure 1). Within the central 1 cm$^2$ 2 μL of rabbit anti-mouse (undiluted) and 2 μL of goat anti-HRP (1:1000 in PBS-0.1% casein) antibodies were spotted side-by-side. The membranes were dried at 37 °C for 30 min. The remaining covalent sites of the entire membrane section were blocked by immersing the membranes in PBS containing 2% casein for 30 min. After being dried at 37 °C for 30 min and at room temperature for 30 min, the anti-mouse and anti-HRP coated membranes were stored in vacuum-sealed bags at room temperature in the dark.

*Preparation of Samples.* Cereal (maize, wheat, rye, and barley) grain samples were obtained from the market. Samples for flow-through enzyme immunoassay and GC-MS confirmation were prepared by inoculating sterilized wheat with a *F. sporotrichioides* culture number KF196. These were incubated for periods ranging from 1 to 14 days at ambient temperature.

*Flow-Through Enzyme Immunoassay. (a) Extraction and Treatment of Cereals.* The cereal sample was ground in a household coffee grinder. The mesh size ranged from 0.05 to 2 mm. A 5 g portion was extracted with 15 mL of 80% methanol−water for 15 min and filtered through a Whatman No. 4 filter paper. The filtrate was then diluted with PBS−0.05% Tween 20 to a methanol concentration of 15%. The dilution was filtered through a 0.45 μm filter (Chromafil disposable filter, Macherey-Nagel, Düren, Germany). This filtrate was then used in the immunoassay. Spiked samples were prepared 1 day prior to extraction. Therefore, the appropriate volume of a T-2−methanol solution (20 ng/μL) was added to 5 g of ground cereals. Methanol was allowed to evaporate overnight, and the spiked samples were shaken before extraction.

*(b) Immunoassay Procedure.* The bottom member of the plastic test device was filled with 1.5 g of 100% cotton wool. The anti-mouse and anti-HRP coated membrane was placed on the absorbent material. The top member was then fitted onto the bottom member. The membrane was placed on the absorbent pad such that the center of the membrane, where the antibody spots were located, was accessible through the aperture of the top member (Figure 2). Assay reagents were successively dropped onto the membrane through the aperture with a micropipet. First, 100 μL of anti-T-2 immunoglobulin solution (1:1000 in assay buffer) was dropped onto the membrane, followed by 300 μL of wash solution (PBS−0.05% Tween 20), 600 μL of T-2 standard solution or sample extract solution, 100 μL of HRP conjugate (1:250 in assay buffer), and 600 μL of wash solution. The liquid was allowed to be sucked through the membrane by the absorbent material before the next reagent was added. Then, 50 μL of chromogen ($H_2O_2$−TMB) solution was dropped onto the membrane. After 3 min, the dot color intensity was evaluated. The whole assay procedure was performed at room temperature. It took <15 min to analyze one sample. Visual evaluation of the color was done by comparing the dot color intensity of the test with that of the internal control. A negative sample resulted in the same color intensity as that of the internal control, because of the inverse relationship between toxin concentration and color development. Because the color was not stable for long, the reading had to be done within 5 min. A portable colorimeter (Minolta Chroma Meter CR-321, Minolta Co., Ltd., Osaka, Japan) was used to quantify the color of the dots on the membrane (De Saeger and Van Peteghem, 1999). This was done by measuring the difference between the color of the white membrane (as a reference) and the color intensity of the dots on the test membrane. This color difference was expressed as a single numerical value, $\Delta E^*_{ab}$ (Minolta Co., 1994).

**Figure 2.** Components of the flow-through device: (upper row, from left to right) plastic bottom member and absorbent material; (lower row, from left to right) plastic top member and membrane.

*Gas Chromatography−Mass Spectrometry (GC-MS). (a) Sample Preparation.* Eight gram portions of *F. sporotrichioides* inoculated cereal sample were extracted with 40 mL of acetonitrile/water (82:18, v/v) by shaking for 30 min on a wrist-action shaker. The mixture was then filtered through Whatman filter paper. Crude extracts (35 mL) were purified on columns packed with Darco G-60 activated carbon (Serva), natural aluminum oxide 90 active, 70−230 mesh (Merck), and Celite 545 (Serva) in the proportions 4:4:4 (w/w/w). T-2 toxin was eluted with the same solvent mixture (30 mL). The elutes were evaporated to dryness on a rotary vacuum evaporator, and the residue was redissolved in 3 mL of chloroform/acetone (4:1).

*(b) Derivatization and GC-MS Analysis.* Samples were transferred to an autosampler vial. They were then evaporated to dryness under nitrogen and derivatized by acylation with 100 μL of trifluoroacetic acid anhydride at 40 °C for 20 min. The derivative was evaporated under nitrogen and the residue dissolved in 100 μL of 2,2,4-trimethylpentane and analyzed by GC-MS. The chromatographic conditions for the analysis were 80 °C beginning oven temperature, with temperature increasing at a rate of 15 °C/min to an end temperature of 280 °C. The injection port was splitless, and the chromatographic separation was achieved with helium as the mobile phase at a flow rate of 0.7 mL/min. Acylated T-2 toxin was detected under a SCAN mode ranging from 100 to 500 Da with 2.58 scans/min, SIM mode mean peak 401 and additional 568.

## RESULTS AND DISCUSSION

The goat anti-HRP was first calibrated to achieve a color intensity similar to that of a negative buffer control. $\Delta E^*_{ab}$ values for a negative and an internal control ranged between 12.30 and 16.00. In this assay the visual limit of detection is defined as the least T-2 toxin concentration that inhibits color development. Color development is inversely proportional to T-2 toxin concentration. There were no differences ($P$ value > 0.05) between the color intensities of the internal control and negative buffer or negative cereal samples (rye, wheat, barley, and maize) (Table 1). Internal control and negative control results were reproducible on different days. Therefore, an internal control could be used

**Table 1. Comparison of Internal Control to Negative Controls[a]**

| | buffer | wheat | maize | rye | barley |
|---|---|---|---|---|---|
| internal control($\Delta E^*_{ab}$ mean) | 16.59 ± 1.94 | 14.74 ± 1.45 | 12.83 ± 0.38 | 12.35 ± 0.66 | 14.09 ± 0.92 |
| negative control($\Delta E^*_{ab}$ mean) | 15.63 ± 1.09 | 15.05 ± 1.29 | 12.35 ± 1.16 | 12.84 ± 1.51 | 14.15 ± 1.64 |
| *P* value | 0.243399 | 0.727212 | 0.403865 | 0.525736 | 0.941212 |

[a] $n = 8$ (between-days repeatability).

**Table 2. Comparison of the Internal Control and Negative Sample against the Test Sample with a T-2 Toxin Concentration of 50 or 32 ng/g[a]**

| | wheat | maize | rye | barley |
|---|---|---|---|---|
| | (A) 50 ng/g | | | |
| internal control ($\Delta E^*_{ab}$ mean) | 14.41 ± 0.53 | 12.97 ± 0.33 | 12.68 ± 1.02 | 14.43 ± 0.16 |
| negative sample ($\Delta E^*_{ab}$ mean) | 13.80 ± 0.13 | 12.79 ± 0.18 | 12.52 ± 0.94 | 15.18 ± 0.74 |
| 50 ng/g T-2 toxin ($\Delta E^*_{ab}$ mean) | 6.38 ± 0.05 | 7.5 ± 1.06 | 5.72 ± 0.94 | 8.79 ± 0.15 |
| *P* value | 0.0002 | 0.00564 | 0.00624 | 0.000293 |
| | (B) 32 ng/g | | | |
| internal control ($\Delta E^*_{ab}$ mean) | 14.41 ± 0.53 | 12.97 ± 0.33 | 12.68 ± 1.02 | 14.43 ± 0.16 |
| negative sample ($\Delta E^*_{ab}$ mean) | 13.80 ± 0.13 | 12.79 ± 0.18 | 12.52 ± 0.94 | 14.95 ± 0.74 |
| 32 ng/g T-2 toxin ($\Delta E^*_{ab}$ mean) | 7.94 ± 0.2 | 6.93 ± 1.06 | 7.12 ± 0.54 | 10.79 ± 0.33 |
| *P* value | 0.000536 | 0.000275 | 0.01672 | 0.005374 |

[a] $n = 5$ (number of assay runs per sample).

**Table 3. Recovery of T-2 Toxin Spiked in Wheat Samples Calculated with Reference to T-2 Toxin in Buffer[a]**

| spiked T-2 toxin | 16 ng/g | 32 ng/g | 48 ng/g | 50 ng/g | 75 ng/g |
|---|---|---|---|---|---|
| buffer ($\Delta E^*_{ab}$) | 7.59 | 6.83 | 5.65 | 5.10 | 5.57 |
| wheat ($\Delta E^*_{ab}$) | 7.51 | 6.28 | 5.60 | 5.25 | 5.11 |
| mean recovery (ng/g) | 16.02 | 35.22 | 48.02 | 48.61 | 82.04 |
| RS | 1.0 | 4.6 | 2.4 | 2.0 | 5.3 |
| RSD% | 6.2 | 13.1 | 5 | 4.1 | 6.5 |

[a] $n = 8$ (number of assay runs for each concentration).

**Table 4. Comparison of Flow-Through Enzyme Immunoassay and GC-MS Results on the Analysis of *F. sporotrichioides* Fermented Samples**

| | flow-through enzyme immunoassay | | |
|---|---|---|---|
| code | internal control | sample spot | GC-MS (ng/g) |
| JAN00I | IC | negative | 35 |
| JAN00II | IC | positive | 50 |
| JAN00III | IC | positive | 120 |
| FEB00VI | IC | positive | 500 |
| FEB00VII | IC | positive | 800 |

directly as a negative control against a sample. Color development in buffer was similar to that of grain samples. There was, therefore, no significant matrix interference.

The spiked samples used in this study had concentrations of 0, 32, 50, 75, and 100 ng/g. The assay proved to be highly sensitive for 75 and 100 ng/g; hence, this work has concentrated mostly on 0, 32, and 50 ng/g. Comparison of assay responses of the internal control and negative sample control against positive sample showed a significant difference ($P < 0.05$) at the 95% significance level (Table 2). Although the visual detection limit was 50 ng/g, there was a significant color reduction at 32 ng/g. Therefore, color development was investigated at 48 ng/g in comparison to 50 ng/g. A faint spot appeared after a while, and a distinction between assay responses for 48 and 50 ng/g could be made by visual judgment. $\Delta E^*_{ab}$ values for barley were relatively high in positive samples, and this was due to high background. However, no blue color appeared on the sample spot after the addition of the chromogen.

Recoveries of spiked T-2 toxin were investigated in wheat samples, and the concentrations used were 0.0, 16, 32, 48, 50, and 75 ng/g. This analysis was made by comparing assay responses between T-2 toxin in buffer and wheat (Table 3). T-2 toxin recoveries ranged from 97 to 110%, and the RSD% values fell within the acceptable limits for immunoassay techniques and are similar to those determined for enzyme immunoassays within and outside this laboratory. Method performance is therefore considered to be acceptable for screening purposes in the field, particularly due to the high recoveries of the assay. Therefore, results from the analysis of naturally contaminated samples will sufficiently represent the actual levels in the sample within a range of 90−100%.

*F. sporotrichioides* fermented samples were analyzed using a GC-MS method after different incubation periods resulting in specific T-2 toxin concentrations, namely, 35, 50, 120, 500, and 800 ng/g. The results obtained when these samples were analyzed with the flow-through enzyme immunoassay corresponded with the concentrations obtained by GC-MS analysis (Table 4). The 35 ng/g sample produced a blue spot, and for those from 50 ng/g and above there was no spot. This demonstrated that the cutoff limit (50 ng/g) for cereal grains can be used with confidence for screening in flow-through enzyme immunoassay.

These results show that this method requires no extensive extraction and purification steps. The test itself is rapid and does not require an additional negative control. The integrated internal control serves the purpose of conferring confidence on the functionality of the assay as well as acting as the standard color intensity reference for test samples. The precision of the test itself shows it can be applied in areas having no laboratories for screening purposes.

## ABBREVIATIONS USED

T-2 toxin, 3-hydroxy-4,15-diacetoxy-8-(3-methylbutyryloxy)-12,13-epoxy-$\Delta^9$-trichothecene; HRP, horseradish peroxidase; Mab, monoclonal antibody; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography−mass spectrometry; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; TMB, 3,3′,5,5′-tetramethylbenzidine; PBS, phosphate-buffered saline.

ACKNOWLEDGMENT

We thank I. Barna-Vetro and L. Solti (Institute for Animal Sciences, Agricultural Biotechnology Center, Gödöllö, Hungary), for providing the monoclonal anti-T-2 antibodies and the T-2-HRP and A. Boulonne for taking the flow-through device photograph.

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Received for review March 14, 2000. Revised manuscript received July 25, 2000. Accepted September 27, 2000. We express our sincere gratitude to the Ministry of the Flemish Community, Economy Administration, Belgium, for funding this research program.

JF000337K