Production of Monoclonal Antibodies for the Specific Detection of Deoxynivalenol and 15-Acetyldeoxynivalenol by ELISA†

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Monoclonal antibodies (MABs) against the mycotoxin deoxynivalenol (DON) were produced using an immunogen consisting of DON conjugated to bovine serum albumin (BSA) through its 15-hydroxyl group (15-DON–BSA). From 80 hybridomas, the four best stabilized hybridoma cell lines secreting anti-DON MABs of subclass IgG_1 were used to prepare ascites in mice. The MABs purified from ascites were used to detect DON by direct competitive ELISA. The MABs were very specific to 15-acetyl-DON and DON but had negligible binding to 3-acetyl-DON, T-2 toxin, sambucinol, and neosolaniol. The effective range of detection was 0.05–20 μg/mL of DON or 15-acetyl-DON. These antibodies also showed a high stability toward grain extracts containing up to 40% methanol.

Keywords: *Deoxynivalenol; 15-acetyldeoxynivalenol; ELISA; monoclonal antibodies*

INTRODUCTION

Deoxynivalenol (DON, vomitoxin, Figure 1), a trichothecene mycotoxin produced by various *Fusarium* spp. (Greenhalgh *et al.*, 1986), occurs in naturally infected corn and small grains.

The analysis of *Fusarium* mycotoxins is normally done by employing physicochemical methods such as thin layer chromatography, high-pressure liquid chromatography, gas chromatography, and GC/mass spectrometry. These methods are expensive and time-consuming and require extensive sample preparation as well as specialized equipment. Therefore, monitoring of mycotoxins in large numbers of samples by these methods is restricted. To overcome this difficulty and to provide a rapid, sensitive, and simple analytical tool for detecting mycotoxins, immunoassay techniques have been developed using both polyclonal and monoclonal antibodies (Pestka *et al.*, 1980; Gendloff *et al.*, 1984, 1987; Ram *et al.*, 1986; Casale *et al.*, 1988; Mills *et al.*, 1990; Usleber *et al.*, 1991; Azcona-Olivera *et al.*, 1992; Nicol *et al.*, 1993).

Monoclonal antibodies (MABs) have an important advantage over polyclonal antibodies in immunoassays in that they offer an unlimited supply of standardized monospecific antibodies. The first report for producing MABs against DON was by Casale *et al.* (1988). They developed a procedure for the selective derivatization of the 3-OH of DON with succinic acid, yielding 3-*O*-hemisuccinyldeoxynivalenol, which was attached to bovine serum albumin (BSA) through its free carboxyl group. This conjugate was then used as an immunogen. The resulting monoclonal antibody reacted well with DON and 3-acetyl-DON and somewhat less well with other trichothecenes, including 15-acetyl-DON.

While 3-acetyl-DON is the main acetylated DON produced by Eurasian isolates of *Fusarium graminearum*, 15-acetyl-DON dominates in North American isolates (Miller *et al.*, 1983; Greenhalgh *et al.*, 1986; Mirocha *et al.*, 1989). Therefore, it seemed appropriate to obtain MABs that would detect 15-acetyl-DON as efficiently as DON by using an immunogen in which DON was linked to a protein through its 15-OH position.

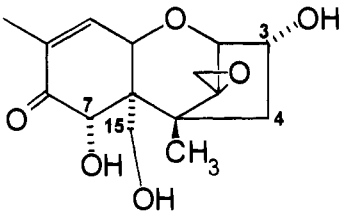

Figure 1. Structure of deoxynivalenol.

MATERIALS AND METHODS

Deoxynivalenol (DON) was obtained from large scale fermentations of *Fusarium culmorum* in liquid culture and purified as described earlier (Greenhalgh *et al.*, 1986). 15-Acetyl-DON (15-AcDON) was prepared by selective hydrolysis of 3,15-diacetyl-DON, which was obtained by acetylation of DON or 3-acetyl-DON (3-AcDON) (Savard, 1991).

Synthesis of DON–Protein Conjugates. *15-Acetyl-3-TBDMS-DON.* A solution of 15-acetyl-DON (256 mg), *tert*-butyldimethylsilyl (TBDMS) chloride (200 mg), and imidazole (190 mg) in *N*,*N*-dimethylformamide (DMF, 8 mL) was stirred overnight at 60 °C under nitrogen (Corey and Venkateswarlu, 1972). The next day, most of the DMF was blown off with nitrogen, and the residue was partitioned between water (20 mL) and ethyl acetate (3 × 20 mL). The organic fraction was washed with water (20 mL), dried over anhydrous sodium sulfate, filtered, and concentrated to dryness under vacuum. Open column chromatography on silica gel with ethyl acetate/hexane (1:3) yielded the pure product (308 mg, 90% yield).

3-TBDMS-DON. To a solution of 15-acetyl-3-TBDMS-DON (308 mg) in methanol (8 mL) was added potassium carbonate (225 mg) and enough water to dissolve it (about 0.5 mL). After 30 min at room temperature, no starting material was visible by TLC. The solution was neutralized with 2 N HCl (700 μL) and concentrated, and the residue was partitioned between water and dichloromethane. The organic solution was dried over sodium sulfate, filtered, and concentrated. The crude product was used directly in the next step.

3-TBDMS-15-hemisuccinyl-DON. 3-TBDMS-DON was added to a 1.7 M solution of succinic anhydride in pyridine (8 mL) (Casale *et al.*, 1988). This solution was stirred at 100 °C for 90 min, and the pyridine was then evaporated under nitrogen at 100 °C. The crude product was chromatographed on silica gel with ethyl acetate/hexane (1:9 followed by 1:3 and 100% EtOAc). The best way to get rid of excess succinate was by

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chromatography on two silica gel preparative plates in succession with ethyl acetate/hexane (1:1). Yield: 255 mg (73.4% from 15-acetyl-3-TBDMS-DON).

15-Hemisuccinyl-DON (15-HS-DON). The 3-TBDMS-15-HS-DON (153 mg) was dissolved in a 1 M solution of $(Bu)_4N^+F^-$ in THF (1.0 mL), and this solution was stirred at room temperature overnight. After concentration under reduced pressure, the crude product was partitioned between water (3 mL, acidified to pH 2 with HCl) and ethyl acetate (3 × 3 mL). The organic fraction was dried over anhydrous Na_2SO_4, concentrated, and chromatographed on silica gel with, first, $CHCl_3$, followed by 5% MeOH in $CHCl_3$ and 10% MeOH in $CHCl_3$. Yield: 71 mg (60% yield).

15-HS-DON–Protein Conjugates. 15-HS-DON was conjugated to BSA, ovalbumin (OA), and horseradish peroxidase (HRP) following the method of Bauminger and Wilchek (1980) with one modification. The conjugates were purified by size exclusion chromatography on Sephadex G-15 (2.5 × 21 cm column) with water at a flow rate of 4 mL/min. Monitoring at 214 nm showed two bands, the first one corresponding to a protein standard previously run. This band was collected and lyophilized.

The presence of DON bound to the protein was confirmed by base hydrolysis of a small sample of the conjugate and GC/MS analysis of the residue and of the nonhydrolyzed conjugate. Typically, 1 mg of the DON–protein conjugate was dissolved in 1 mL of $MeOH/H_2O$ (5:1) and K_2CO_3 (1 mg) was added. After stirring at room temperature for 2 h, the solution was extracted with EtOAc. The extract was dried over anhydrous sodium sulfate, filtered, and concentrated. The absence of free DON, as determined by GC/MS, in the nonhydrolyzed conjugate and its presence in the hydrolysis residue indicated that DON was covalently bound to the protein.

Immunization of Mice. Six week old BALB/c mice were given three intraperitoneal and one intravenous injections at 1 month intervals with the 15-DON–BSA conjugate. The first injection consisted of 250 μg of 15-DON–BSA in 0.1 mL of 0.1 M phosphate-buffered saline, pH 7.4 (PBS), emulsified with an equal volume of Freund's complete adjuvant. Second and third injections were given using Freund's incomplete adjuvant to emulsify the conjugate. The fourth injection, consisting of 100 μg of the conjugate in 0.1 mL of saline (0.85%), was administered intravenously. Three days later, the spleens of injected mice were removed for hybridoma production.

Hybridoma Production and Selection. The procedure for producing hybridomas was as described by Kohler and Milstein (1975). The specific antibody-producing hybridomas were selected by screening the supernatants against DON–OA or OA (control) by indirect enzyme-linked immunosorbent assay (ELISA) as described by Casale *et al.* (1988). Antiserum was considered to contain DON-specific antibody if binding of antibody (as determined by absorbance at 490 nm after 30 min) to the solid-phase DON-OA was greater than that of an OA control.

Competitive Indirect ELISA (CI-ELISA). The procedures for competitive ELISAs were adapted from the method described by Azcona-Olivera *et al.* (1992). To each well of a 96 well microtiter plate (Gibco/BRL, Burlington, ON, Canada) was added 100 μL of 15-DON-OA (5 μg/mL in 0.15 M carbonate–bicarbonate buffer, pH 9.6) and incubated at 4 °C overnight. Unbound conjugate was removed by washing five times with 0.1 M PBS/Tween 20 (0.05%, PBST). Nonspecific binding was decreased by incubation of 300 μL/well of 1% OA in PBS at 37 °C, and the plates were washed four times with PBST. Fifty microliters of DON standard (1 μg/mL in 1:9 methanol/water) mixed with 50 μL of the supernatant fluid from the MAB-producing cell lines was then added to each well. The plates were incubated at 37 °C for 3 h or overnight at 4 °C and washed five times with PBST. One hundred microliters of goat anti-mouse IgG peroxidase conjugate (1 μg/mL in Tris-HCl buffer, pH 7.4) was added to each well, incubated at 37 °C for 2–3 h, and washed five times with PBST. The bound peroxidase was determined by o-phenylenediamine dihydrochloride (OPD) substrate (0.4 mg/mL, 200 μL/well) in citrate buffer, pH 5.0. Absorbance was recorded at 490 nm after 30 min.

Competitive Direct ELISA (CD-ELISA). All CD-ELISA tests were done using MABs purified from ascites. Production of ascites and purification of MABs were as described earlier (Liddell and Cryer, 1991). The CD-ELISA procedure was adapted from those of Dixon *et al.* (1987) and Casale *et al.* (1988). One hundred microliters of suspension containing MAB (100 μg/mL) in carbonate/bicarbonate buffer, pH 9.6, was added to each well and air-dried at 35 °C overnight. Plates were washed five times with PBST and unbound solid-phase sites blocked by incubation with 300 μL/well of 1% OA in PBS for 1 h at 37 °C. The plates were washed four times with PBST. A mixture of 50 μL of serially diluted DON standard or spiked extracts in 1:9 methanol/water and 50 μL of 15-DON–HRP (2 μg/mL) in 1% OA in PBS was then added to each well and incubated at 37 °C for 1 h. The plates were washed eight times with PBST. Bound peroxidase was then determined by reaction with *o*-phenylenediamine and absorbance recorded as described above for CI-ELISA. The quantitation of deoxynivalenol was performed by measurement of the inhibition of binding of the MABs to the solid-supported DON–OA.

Spiked Corn and Wheat Samples. To 1 g samples of ground corn or wheat were added different volumes of a 40 μg/mL solution of DON in 1:9 methanol/water. The total volume of solvent was made up to 5 mL and the samples underwent end-over-end mixing for 1 h. After centrifugation at 2000 rpm for 5 min, the samples were filtered and the filtrate was analyzed by CD-ELISA and GC as described in Scott *et al.* (1983).

RESULTS

Synthesis of DON Conjugates. DON (Figure 1) was conjugated to three different proteins, bovine serum albumin (BSA) for the production of an immunogen, ovalbumin (OA) for use as a coating protein for the identification of DON-sensitive antibody-producing cell lines by indirect ELISA, and horseradish peroxidase (HRP) for analysis by direct ELISA.

A method was devised to attach DON to these proteins through its 15-hydroxyl position. This procedure involved blocking of the 3-OH position but not the 7-OH because of its low reactivity (Savard, 1991). Although the 3-Ac group can be selectively removed from 3,15-diAc-DON, our attempts to selectively remove a 3-hemisuccinyl group from 3,15-diHS-DON failed. We then resorted to a protection-deprotection scheme.

Having obtained 15-AcDON by deacetylation of 3,15-diAcDON, the 3-OH was protected by reaction with *tert*-butyldimethylsilyl (TBDMS) chloride, and the 15-Ac was removed by mild hydrolysis with potassium carbonate, yielding 3-TBDMS-DON. Succinylation of the 15-OH was then performed on this crude material with succinic anhydride in pyridine. Removal of the silyl protecting group was then performed with fluoride ion, for an overall 40% yield of 15-HS-DON from 15-AcDON. The only problem with this last reaction was the removal of excess tetrabutylammonium fluoride, which had a tendency to smear during silica gel chromatography. Partition from the aqueous solution with ethyl acetate instead of dichloromethane solved this problem. While ethyl acetate was not as good a solvent for the desired product, it did not extract the ammonium fluoride. As a result, repeated extraction with ethyl acetate followed by chromatography on silica gel produced pure 15-HS-DON.

Hybridomas. Several hundred hybridomas were first screened by indirect ELISA for the presence of antibodies against DON. About 80 hybridomas giving positive reactions were further tested by indirect competitive ELISA. Ten hybridomas that gave 50% inhibition or better with 50 ng of DON/assay were cloned

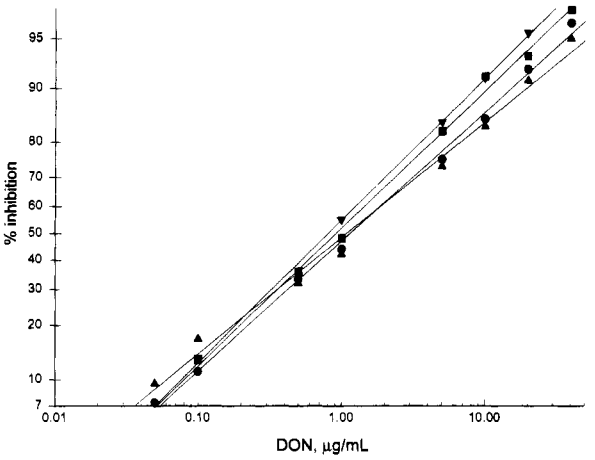

Figure 2. Sensitivity of antibodies from four cell lines against a range of DON concentrations by competitive direct ELISA. Concentrations of 100 μg/mL of antibody were used for all lines. The lowest detectable amount of DON was 2.5 ng/assay or 0.05 μg/mL, corresponding to 0.25 ppm for a typical assay in which 1 g of grain is extracted with 5 mL of solvent and 50 μL is used for the assay. Different symbols are used for each cell line.

three times by limited dilution. Four MAB-producing cell lines were selected for expansion and used to produce ascites. MABs (type IgG_1) purified from ascites were used in all subsequent experiments to detect DON employing competitive direct ELISA.

Characterization of Monoclonal Antibodies. *Isotyping of Antibody.* The monoclonal antibodies used for ELISA were IgG_1 with κ-light chain, as determined with a mouse subclass identification kit (Gibco BRL Life Technologies Ltd., Gaithersburg, MD).

Sensitivity. Quantitation of DON concentrations with purified MABs showed that 5 μg of MAB/mL was necessary for an accurate estimation of DON concentration. The MABs produced by these cell lines were all able to detect as little as 0.05 μg/mL (2.5 ng of DON/assay) (Figure 2), this value being the 7% inhibition level, the lower practical limit of the logit scale. This concentration is equivalent to 0.25 ppm for a typical assay in which 1 g of grain is extracted with 5 mL of solvent and 50 μL is used for the assay.

Selectivity. Most detailed work was done using one cell line, 511H4D11E8G. The derivatization of DON at the 15-OH produced highly selective antibodies as expected (Figure 3). The MAB reacted well with both DON and 15-AcDON, while it was approximately 170 times less sensitive to 3-AcDON and did not recognize T-2 toxin, sambucinol, or neosolaniol within our experimental parameters.

Solvent Compatibility. Since DON and other trichothecenes are best extracted by solvent mixtures containing a certain proportion of organic solvent (Scott *et al.*, 1983; Kostiainen *et al.*, 1989; Lauren and Greenhalgh, 1987), the compatibility of our MABs with methanol was also measured (Figure 4). Although increasing the proportion of methanol in the extracting solvent resulted in a slightly lowered sensitivity, the assay could be performed satisfactorily using an extraction solvent consisting of up to at least 40% methanol, the highest concentration tested.

Analysis of Spiked Samples. To determine the accuracy of the ELISA test, spiked corn and wheat extracts were analyzed by ELISA and GC (Figure 5). The results show the ELISA test to give a satisfactory analysis for levels of DON from less than 1 to 40 ppm. Comparison of the ELISA and GC data yielded a correlation factor R^2 of 0.981.

DISCUSSION

We have produced monoclonal antibodies that are very specific to DON and 15-AcDON. While our work was in progress, two papers were published in which 15-DON–protein conjugates similar to ours were syn-

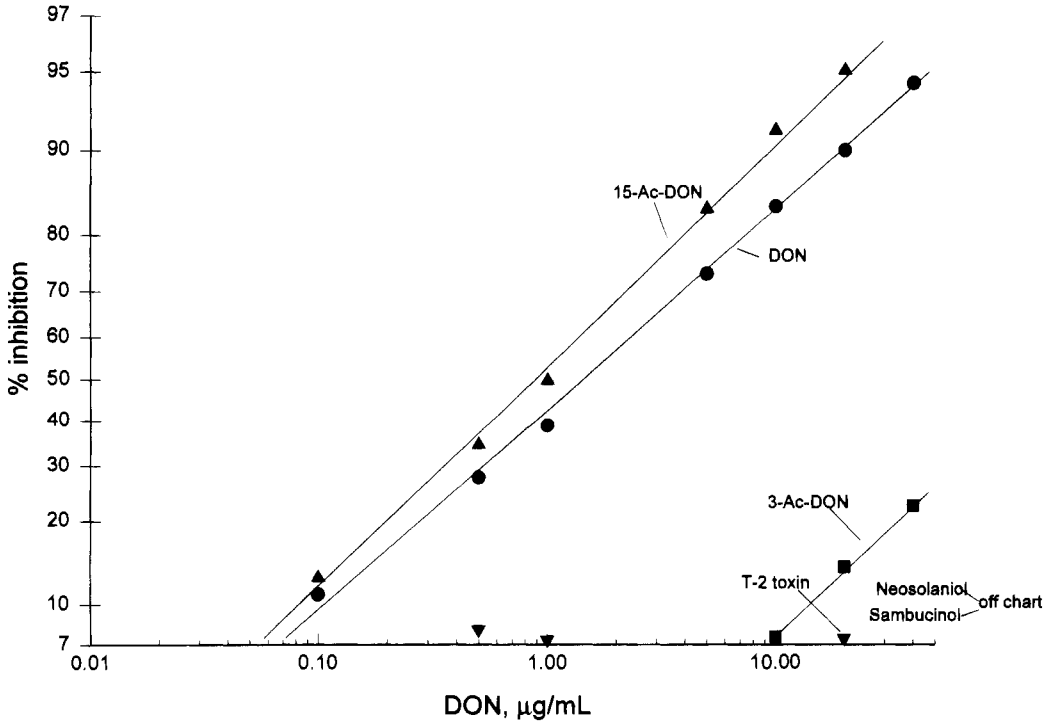

Figure 3. Selectivity of MABs for cell line 511H4D11E8G. As expected from an antibody generated by a DON molecule attached to a protein by its 15-OH group, DON and 15-AcDON have similar reactivities with the antibody. 3-AcDON has a reactivity approximately 170 times lower, while neosolaniol, T-2 toxin, and sambucinol show no reactivity.

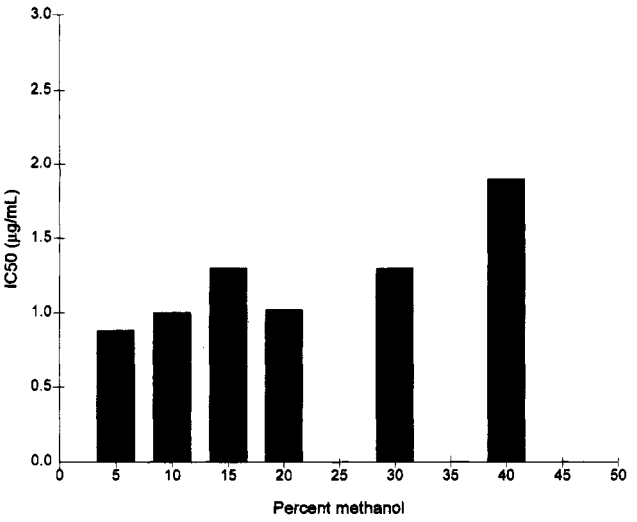

Figure 4. Effect of methanol concentration on assay results. Up to 40% MeOH in the extract (the largest concentration tested) could be tolerated by the antibody without significant effects on the analytical results.

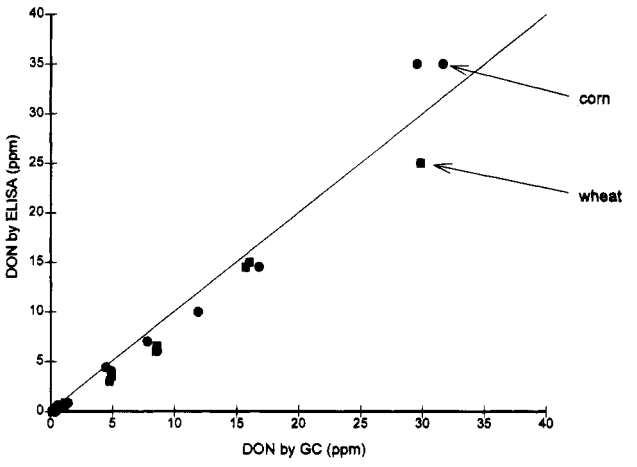

Figure 5. Testing of spiked corn and wheat extracts. Spiked corn and wheat extracts were analyzed by competitive direct ELISA and by GC against standard solutions of DON. No interference by corn or wheat components was observed. The line crossing the figure represents a 1:1 theoretical correlation between the two methods of analysis.

thesized using a different approach. Mills *et al.* (1990) produced polyclonal antibodies that detected DON, but the authors did not test the antiserum against 15-AcDON. Nicol *et al.* (1993) produced MABs that exhibited cross-reactivity with DON, 3-AcDON, and 15-AcDON. However, the immunogen used by the authors was a mixture of 3-DON–MSA (mouse serum albumin) and 15-DON–MSA.

The synthesis described in this paper is also significantly different from the other approaches. While both of the previous papers used enzyme-mediated selective hydrolysis for the preparation of the 15-HS-DON moiety, we used a protection–deprotection scheme. This method was much faster, requiring only 4 days for the preparation of 15-HS-DON as opposed to approximately 10 days for the enzyme-based method. Another significant improvement in the preparation of DON–protein conjugates in general was our use of size exclusion chromatography for the purification of the conjugates instead of dialysis. This modification cut the purification time from 3 days to 15 min and produced protein conjugates free of buffer salts.

The usual methods for the verification of the attachment of a hapten to a protein rely on the measurement of the number of free amino groups left on the protein (Habeeb, 1966). Since this method does not identify the moiety blocking the amino groups, a different method was used. A sample of each DON–protein conjugate synthesized was hydrolyzed in base, and both the conjugate and its hydrolysis product were analyzed by GC/MS. The absence of free DON in the conjugate and its presence in the hydrolysis product confirmed the covalent binding of the DON molecule onto the protein.

Comparison to other DON antibodies, both monoclonal and polyclonal, described in the literature showed that they all have very low cross-reactivity with non 8-ketotrichothecenes. They also have higher reactivity with DON acetylated at the site of attachment to protein in the immunogen than with DON itself. For reliable analytical results, it is important that the difference in reactivity with DON and acetylated DON be as small as possible. Otherwise, the presence of a 10 ppm/1 ppm concentration of DON/AcDON in a sample could be interpreted as 110 ppm overall DON with an antibody with a 10000% cross-reactivity for 3-AcDON such as the one developed by Usleber *et al.* (1991) or 13 ppm with the Nicol *et al.* (1993) antibody if the AcDON was 15-AcDON. By contrast, our antibody showed only a 175% cross-reactivity with 15-AcDON and would yield a result of 11.75 ppm. No comparison could be made with polyclonal 15-DON–BSA-based antibodies since they have not been tested against 15-AcDON (Mills *et al.*, 1990). It should be noted that while polyclonal antibodies may give good sensitivity and cross-reactivity data, it is difficult to get constant antibody binding because of the variability of the antibody obtained using different batches of animals.

The higher sensitivity of antibodies for DON acetylated at the site of conjugation of the immunogen is due to the closer similarity of structure between these two compounds than between DON and the conjugate. Use of 15-DON–BSA as immunogen appears to reduce this difference in sensitivity slightly as the DON moiety of the immunogen is farther removed from the protein and thus exposes a larger part of itself for binding to suitable antibodies.

The monoclonal antibodies and ELISA method described in this paper will be useful in the rapid and routine screening of deoxynivalenol-contaminated grain. Such experiments are underway in our laboratory, and preliminary results indicate that DON can be easily detected in both corn and wheat infected with *Fusarium* and that DON concentrations determined by ELISA and GC have a very high correlation coefficient.

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