Zearalenone in Corn, Wheat, and Feed
Enzyme-Linked Immunosorbent (Agri-Screen) Method

First Action 1994
Final Action 1997

(Applicable to detection of zearalenone in corn, wheat, and pig feed at ≥800 ng/g.)

See Tables 994.01A and 994.01B for the results of the interlaboratory study.

A. Principle

Detection of zearalenone is based on competitive binding enzyme immunoassay (EIA) using specific monoclonal antibodies. Zearalenone is extracted with methanol water (70 + 30). Extract is filtered and mixed with equal volume of zearalenone enzyme conjugate. Samples and controls are placed into microtiter wells coated with zearalenone-specific monoclonal antibodies. Zearalenone present in sample attaches to specific antibodies adsorbed on well. Wells are washed to remove unbound zearalenone and zearalenone enzyme conjugate and activated enzyme substrate is added. After stop solution is added, concentration of zearalenone in sample depends, inversely, on intensity of color development and is determined visually or spectrophotometrically (approximate levels of zearalenone are interpolated from standard curve).

B. Antibody Specificity

Anti-zearalenone antibodies bind zearalenone analogues, especially α-zearalenol. The cross-reactivity is similar for zearalenone and α-zearalenol (100% and 107%, respectively). Cross-reactivities to other zearalenone analogues are: β-zearalenol 29%, α-zearalanol 35%, β-zearalanol 25%. Standard curves constructed with zearalenone standard solutions (3000 ng/g) provide quality control measure for analytical stability.

C. Apparatus

(a) Shaker.—Wrist-action shaker, Burrell or equivalent.
(b) Vials.—4 mL; Teflon-lined screw-cap vials.
(c) Filter paper.—Whatman 2V rapid-flow filter paper, or equivalent.
(d) EIA reader.—Photometer with 650 nm filter, capable of reading microtiter wells (Biotek Model EL308 available from Biotek Instruments, Inc., or equivalent).
(e) Micropipet (optional).—Capable of accurately delivering 100 µL solution (Rainin Pipetteman, or equivalent). Multichannel pipets are acceptable. Pre-set pipetting syringes and tips are available in kit form.

D. Reagents

(a) Zearalenone enzyme conjugate.—Lyophilized.
(b) Conjugate diluent.—Distilled H₂O.
(c) Antibody coated microtiter strip plates.—Polystyrene microtiter strip plates, each containing 8 or 12 wells coated with monoclonal antibodies to zearalenone.
(d) TMB peroxidase substrate (solution A).—Tetramethylbenzidine (Sigma Chemicals, St. Louis, MO 63178, USA), 0.4 g/L citrate buffer (pH 4.0). Store at 4°C.
(e) TMB peroxidase substrate (solution B).—30% H₂O₂/L, 1.5 mL/L citric acid buffer (pH 4.0). Store at 4°C.
(f) Stop solution.—Add 1 mL 3% red dye (FD&C No. 40), 3.5 mg hydrofluoric acid, 10.5 g sodium citrate, 6 mL 1M NaOH, and 400 mg NaEDTA to 1 L distilled H₂O.
(g) Controls.—Methanol water (70 + 30) extracts of zearalenone free corn, wheat, and feed, spiked with zearalenone (500 ng/g). Store in Teflon-lined, screw-capped vials at 4°C. Solutions are stable for several weeks.
(h) Methanol solution.—70%. Mix (v/v) reagent-grade methanol with distilled H₂O (70 + 30).
(i) Standard solutions.—Teflon-lined, screw-capped amber vials containing toxin-free corn, wheat, and feed extracts (methanol water) spiked with zearalenone at levels (ng/g): 0, 200, 500, 1000, 1500, and 3000. Prepare standard curve at time samples are tested.

Table 994.01A Interlaboratory study results for determination of zearalenone using visual method

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Mean*, ng/g</th>
<th>Positive response, %</th>
<th>95% Confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower limit</td>
</tr>
<tr>
<td>Corn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17.2</td>
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<tr>
<td>247 ± 27</td>
<td>36</td>
<td>86.5</td>
<td>99.5</td>
</tr>
<tr>
<td>800</td>
<td>96</td>
<td>93.2</td>
<td>100.0</td>
</tr>
<tr>
<td>2570 ± 180</td>
<td>100</td>
<td>97.2</td>
<td>100.0</td>
</tr>
<tr>
<td>Wheat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>215 ± 10</td>
<td>26</td>
<td>8.2</td>
<td>52.2</td>
</tr>
<tr>
<td>300</td>
<td>37</td>
<td>24.3</td>
<td>51.3</td>
</tr>
<tr>
<td>1000</td>
<td>100</td>
<td>87.2</td>
<td>100.0</td>
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<tr>
<td>1027 ± 29</td>
<td>100</td>
<td>93.4</td>
<td>100.0</td>
</tr>
<tr>
<td>Pig feed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>352 ± 34</td>
<td>17</td>
<td>8.2</td>
<td>30.3</td>
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<tr>
<td>500</td>
<td>75</td>
<td>61.1</td>
<td>86.0</td>
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<tr>
<td>1295 ± 29</td>
<td>100</td>
<td>86.8</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Spiked samples (added concentration) or naturally contaminated samples (concentration determined by LC).
Items (a)–(g) are available as Agri-Screen Kit for Zearalenone (Neogen Corp., 620 Lesher Pl, Lansing, MI 48912). Validated, alternative reagents are acceptable.

E. General Instructions

Store test samples, reagents, and kit components at 4–8°C. Do not freeze. Bring reagents and kit components to room temperature (20–25°C) prior to use. Reagents are stable at room temperature 8 h. Return reagents to 4–8°C after use. Assay one set of test samples (6/set) at time. Assay each set twice. Determine results (color development) after stop solution is added.

F. Sample Extraction

Carefully transfer 20 g test portion to 500 mL glass-stopper or screw-cap extraction flask. Add 200 mL methanol-water solution (70 + 30), close lightly, and shake for 3 min on wrist-action shaker, C(a). Filter through Whatman 2V filter paper, C(c). Collect ca 4 mL. Analyze filtrate immediately or transfer to screw-cap vial and store at 4–8°C for £24 h.

G. Preparation of Reagent Solutions

Bring reagents to room temperature (20–25°C) prior to use.

(1) Enzyme conjugate solution: Transfer conjugate diluent, D(b), to vial, C(b). Replace stopper and gently swirl to mix contents until dissolved. Store in refrigerator for ≤7 days.

(2) TMB substrate solution: Mix solution A, [D(d)], with solution B, [D(e)]. Mix 1 tube when 8-well strip is used (2 tubes/12-well). Use same day.

(3) Remove seal from stop solution vial and set aside for later use.

H. Zearalenone Determination Using Visual Method

(1) Remove from packages equal numbers of mixing wells (red-labeled) and antibody-coated wells, D(c), and place in strip holder. Place antibody-coated wells 2 rows behind mixing wells. Reseal packages. Label end of each strip to keep test solutions in order. Use 1 well/test solution. See Figure 994.01A.

(2) Using micropipet transfer 100 µL aliquots of enzyme conjugate solution, G(1), to each mixing well. Discard pipet tip.

(3) Remove caps from control vials and corresponding test solutions. (e.g., Assay corn test solutions with corn control.) Using new pipet tip, add 100 µL control to first mixing well. Mix solutions by depressing and releasing pipet plunger 3 times. Using same pipet tip immediately transfer 100 L mixed solutions to first antibody-coated well.

(4) With new pipet tip, transfer 100 µL test solution to second mixing well. Mix solutions same way as in (c), and transfer 100 µL mixed solutions to second antibody-coated well. Discard pipet tip.

(5) Repeat step (d) for each test solution and control to be assayed.

(6) Incubate mixed solutions with antibody-coated wells for 15 min after last well is filled.

(7) Wash wells after incubation with distilled H2O. Gently direct H2O stream into each well with wash bottle. Shake out H2O. Repeat wash steps 10X. After last wash tap wells hard enough on paper towel to remove remaining H2O. Do not dry with paper, cloth, or stream of air.

(8) Transfer 100 µL aliquots of TMB substrate solution, G(2) to each antibody-coated well using new pipet tip.

(9) Incubate for 15 min and make preliminary observation. Test well containing low levels of zearalenone (ng/g) will develop blue color within 1–5 min. Positive test solutions (>500 ng/g) will remain clear or light blue. Note blue color develops within 5 min.

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Zearalenone added or determined by LC, ng/g</th>
<th>Mean foundb, ng/g</th>
<th>St</th>
<th>StL</th>
<th>RSDb %</th>
<th>RSDL %</th>
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<tbody>
<tr>
<td>Corn</td>
<td>800</td>
<td>940</td>
<td>30</td>
<td>153</td>
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<td>16.3</td>
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<td></td>
<td>2570 ± 180</td>
<td>3191</td>
<td>350</td>
<td>840</td>
<td>11.0</td>
<td>26.3</td>
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<tr>
<td>Wheat</td>
<td>300</td>
<td>452</td>
<td>70</td>
<td>143</td>
<td>15.5</td>
<td>31.7</td>
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<tr>
<td></td>
<td>1027 ± 29</td>
<td>1482</td>
<td>150</td>
<td>236</td>
<td>10.1</td>
<td>15.9</td>
</tr>
<tr>
<td>Pig feed</td>
<td>500</td>
<td>613</td>
<td>99</td>
<td>167</td>
<td>16.1</td>
<td>27.2</td>
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<tr>
<td></td>
<td>352 ± 34</td>
<td>255</td>
<td>36</td>
<td>146</td>
<td>13.9</td>
<td>57.1</td>
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</tbody>
</table>

a First line lists average results for spiked samples, second line lists average results for naturally contaminated samples. Average for spectrophotometric method was calculated from values determined from standard curve.

Table 994.01B Interlaboratory study results for determination of zearalenone by spectrophotometric method

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Figure 994.01A—Suggested set-up for microtiter wells: visual determination.

Figure 994.01B—Suggested set-up for microtiter wells: spectrophotometric determination.
(10) After 15 min incubation, using new pipet tip add 100 μL aliquots of stop solution, D(f), to each well.

(11) Screen wells on white, matte-finish background. Test solutions showing less pink color than control are negative. Test solutions showing more pink color than control are positive.

Repeat steps (1)–(11). Results from both assays should agree.

Positive results must be confirmed by AOAC Method 985.18 (see Section 49.9.02).

I. Zearalenone Determination by Spectrophotometric Method

Perform as in H(l)–(11) using 12-well microtiter strips of mixing wells and antibody coated wells, D(c) (see Figure 994.01B). Use 1 uncoated well from black marked package to blank microtiter well reader, I(a).

(a) Set blank on microtiter well reader with uncoated well containing only 100 μL enzyme conjugate, G(l), and 100 μL stop solution, D(f).

(b) Determine and record absorbance, A, for standard solutions, D(l), and test solutions. Assign 100% A to 0 ng/g standard solution. Divide A value of test well by A value of 0 ng/g standard solution. Repeat this procedure for all remaining wells. Prepare graph by plotting on logit-log paper A values of standard solutions (%) versus zearalenone concentration (ng/g). Draw best-fit line through points obtained. Read from standard curve approximate level of zearalenone in test samples. Assay test solutions at same time standard curve is prepared for test sample matrix (corn, wheat, or feed).

(c) Repeat assay on test solution and record results.

(d) Average results of duplicate assays to determine approximate level of zearalenone in test sample.

[Note: 650 nm filter provides measure of blue color development. EIA reader equipped with this filter ignores red color. Positive results must be confirmed by AOAC Method 985.18 (see Section 49.9.02).]

Reference: J. AOAC Int. 77, 1500(1994).

Revised: March 1998