

Detection of Zearalenone By Tandem Immunoaffinity-Enzyme-Linked Immunosorbent Assay and Its Application to Milk

JUAN I. AZCONA¹, MOHAMED M. ABOUZIED, and JAMES J. PESTKA^{2*}

Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824-1224

(Received for publication November 20, 1989)

ABSTRACT

A hybridoma-based method utilizing tandem affinity chromatography and enzyme-linked immunosorbent assay (ELISA) was devised to detect zearalenone. A zearalenone specific monoclonal antibody was attached to Sepharose for initial sample clean-up. Zearalenone was eluted with methanol and then quantified by competitive direct ELISA. Average ELISA recoveries from the column for water spiked with zearalenone at levels of 1, 5, 10, 25, and 50 ng/ml were 107, 86, 95, 95, and 92%, respectively, with a mean recovery of 95%. Mean interwell and interassay coefficients of variation were 9.7 and 8.9%, respectively. Average recovery by the method from milk spiked with zearalenone at levels of 1, 5, 10, 25, and 50 ng/ml was 187, 113, 107, 110, and 112%, respectively, with a mean recovery of 126%. Mean interwell and interassay coefficients of variation were 14.5 and 9.1%, respectively. Zearalenone was not detectable in 12 commercial milk samples assayed by the tandem method.

Zearalenone (ZEA) [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)- β -resorcylic acid lactone] is a secondary metabolite produced by members of the genus *Fusarium* after colonization of corn and small grains both in the field and during storage (9,16,24). ZEA elicits estrogenic effects in mammalian reproductive systems and has specifically been associated with field cases of swine hyperestrogenism (12,16). There has also been reported positive evidence for carcinogenicity of this mycotoxin in rodents (18). A recent risk assessment of ZEA estimated a safe intake of 0.05 μ g/kg body weight/day for humans (14). Although no adverse health effects would be anticipated due to ZEA exposure from corn cereals, the toxin could be present in other food sources such as milk and thus could increase exposure estimates.

Zearalenone is metabolized to α - and β -zearalenol, and these metabolites have been detected experimentally at trace levels in cow's milk after oral administration of high zearalenone doses in feed (7,17). The presence of both metabolites in milk is important since α -zearalenol is three times more estrogenic than ZEA while β -zearalenol

has similar activity to ZEA (8,29). Thus, it is important to estimate the incidence of ZEA and its metabolites in milk and milk products because of the worldwide consumption of this commodity.

Conventional methods for analysis of ZEA in foods and feeds include thin-layer chromatography (TLC) (1,5,25), gas chromatography (11,23,26), and liquid chromatography (2,13,22,28). Regardless of the detection limits described, these procedures involve time-consuming extractions and clean-up steps, making the methods inconvenient for the routine screening of large sample numbers. Our laboratory has reported the application of competitive enzyme-linked immunosorbent assays (ELISA's) to detection of ZEA in corn, wheat, grain-based food products, and animal rations using polyclonal rabbit antisera (15,30,31) and, more recently, monoclonal antibodies (4). A new approach to improve aflatoxin analysis includes the use of a monoclonal antibody affinity chromatography column as preparative step prior to determination by liquid chromatography (6).

Here, we report the development of a method for detection of ZEA involving tandem immunoaffinity chromatography and ELISA and its application to milk.

MATERIALS AND METHODS

Materials

All inorganic chemicals and organic solvents were reagent grade or better. Chicken egg albumin (ovalbumin, grade III), Tween 20, 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid) (ABTS), thimerosal, hydrogen peroxide, horseradish peroxidase, zearalenone, pristane, and Sepharose 4B-CNBr were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium and fetal bovine serum were from Gibco Laboratories (Grand Island, NY). Mice were purchased from Charles River Laboratories (Wilmington, MA). Zearalenone-horseradish peroxidase (ZEA-HRP) was prepared as described previously (31). Hybridoma cell line 2G3-6E3-2E2 which secreted anti-zearalenone monoclonal antibody was produced as ascites (4) and purified by ammonium sulfate precipitation (10). Cross-reactivity of that antibody towards zearalenone metabolites and analogues determined by an indirect ELISA was 107, 29, 35, and 25% for α -zearalenol, β -zearalenol, α -zearalanol, and β -

*Visiting post doctoral fellow from the Dpto. de Higiene y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid, Spain.

zearalanol, respectively (4). Protein concentration was determined by the method of Bradford (3) using bovine plasma gamma globulin as standard.

Immunoaffinity gel

Antibody was coupled to Sepharose 4B as described in manufacturer's instructions (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Briefly, 2 g of freeze dried CNBr activated Sepharose-4B (S-4B) were washed and swollen in 1 mM HCl under slight vacuum on a sintered glass filter. The gel was washed with 50 ml of coupling buffer (CB) (0.1 M NaHCO₃, pH 8.3, containing 0.5 M NaCl). After suspending in 10 ml of CB, the gel was mixed with 70 mg MAb in 5 ml of CB and the suspension was mixed end-over-end for 2 h at room temperature. After filtration, the remaining active groups on the S-4B were blocked by placing the gel during 2 h at room temperature in 30 ml of glycine buffer (0.2 M, pH 8.0). This was filtered and the excess of blocking reagent and adsorbed protein was discharged by washing the gel with four alternating cycles of high and low pH; CB, followed by 0.1 M sodium acetate buffer, pH 4.0. Finally the immunogel (\approx 7 ml) was washed with 0.01 M phosphate buffered (pH 7.4) saline (PBS), transferred to 15 ml of PBS containing 0.01% thimerosal, and stored at 4°C until required.

Immunoaffinity chromatography

Immunoaffinity columns consisted of 1 ml tuberculin syringes containing 200 μ l of Sepharose-MAb slurry and held in place between porous polyethylene frits. Columns were equilibrated with PBS. The affinity chromatography studies were carried out at room temperature.

The immunogel was initially assessed for activity by passing an aliquot of 1 ml of distilled water containing standard ZEA (0-50 ng). Samples were pushed through the column using pressure supplied by a small peristaltic pump with a flow rate of 0.5 ml/min. The column was washed then with 5 ml of 10% (v/v) methanol in PBS (when 5 and 10 ml milk samples were assayed, the volume was increased) after which air was pumped during 1 min. ZEA bound to the immunogel was eluted with 2 ml of methanol followed by passing air for 1 min; this fraction was evaporated and then resuspended in 1 ml of PBS. Quantitation of ZEA in the eluate was determined by ELISA. Columns were re-equilibrated with 15 ml of PBS for reuse.

ELISA

Wells of polystyrene microtiter plates (Immunolon Removawells, Dynatech Laboratories, Alexandria, VA) were coated by air-drying (40°C) with 125 μ l of 1:750 dilution zearalenone monoclonal antibody (MAb) (10 mg/ml) in sodium carbonate-bicarbonate buffer, pH 9.6. MAb-treated plates were washed four times with a microtiter plate washer (Dynatech Miniwash, Dynatech Laboratories, Alexandria, VA) by filling each well with 300 μ l of 0.02% (v/v) Tween 20 in PBS (PBS-Tween) and aspirating the contents. Nonspecific binding was decreased by incubating each well for 30 min at 37°C with 300 μ l of 1% ovalbumin (w/v) in PBS (OA-PBS) followed by four washes of PBS-Tween. Next, a 50 μ l aliquot from samples or 50 μ l of ZEA standard (0 to 50 ng/ml in PBS) and 50 μ l of ZEA-HRP conjugate (diluted 1:800 in OA-PBS) were added consecutively to MAb coated wells. Plates were incubated for 1 h at 37°C and washed eight times with PBS-Tween, and bound ZEA-HRP was determined (19) using an enzyme immunoassay reader (Micro-elisa Minireader II, Dynatech Laboratories, Alexandria, VA).

Milk samples

Twelve samples representing different lots of dehydrated skim milk were purchased from mid-Michigan retail grocery stores. Samples (9.6 g) were reconstituted in 100 ml of distilled water (according to the proportions shown by the producers) and then filtered through Whatman No. 1 filter paper. Spiked samples were prepared by dilution of zearalenone stock (1 mg/ml in methanol) with distilled water and milk filtrates to give final concentrations of 1, 5, 10, 25, and 50 ng/ml. Spiked (1 ml) and unspiked (1, 5 and 10 ml) samples were subjected to tandem immunoaffinity-ELISA as described above.

RESULTS

A competitive direct ELISA was carried out by simultaneously incubating zearalenone with an appropriate dilution of ZEA-HRP over a MAb solid phase. A typical competition standard curve is shown in Fig. 1. Response

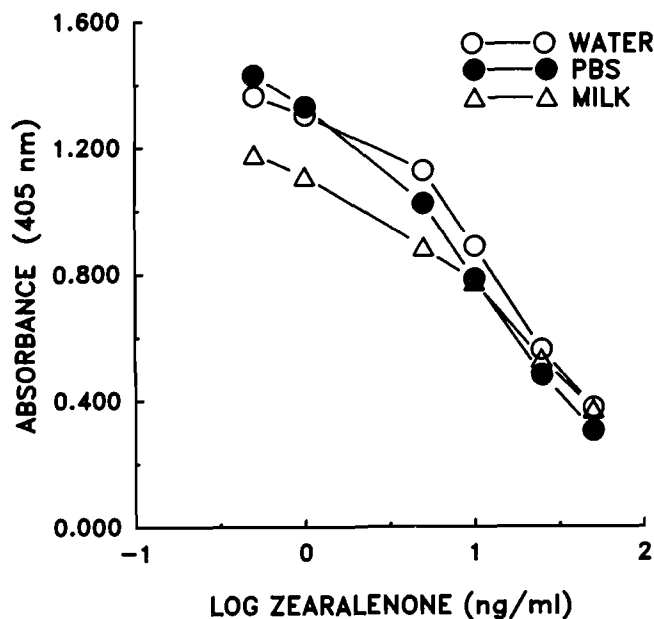


Figure 1. Competitive ELISA standard curve of zearalenone in water, PBS, and milk. Each data point represents triplicate determinations in a single microtiter plate. Standard deviation values were always less than 3%. Milk used in standard curve was previously passed through an immunoaffinity column.

range for this curve was between 0.5 and 50 ng/ml. The detection limit for ZEA (1 ng/ml or 50 pg/assay) was comparable to that previously described for competitive indirect (4,15,20) and direct (31) ELISA's and radioimmunoassay (27). Although the standard curve pattern of zearalenone in milk approximated those patterns obtained in distilled water and PBS, a decrease in absorbance values was observed at lower zearalenone levels in milk compared to the other diluents. This suggested that milk samples required a clean-up step prior to ELISA. An immunoaffinity clean-up procedure was therefore devised.

ELISA ZEA recoveries from immunoaffinity columns are summarized in Tables 1 and 2. Competition by chromatographic fractions was compared directly to standard

TABLE 1. Recovery of zearalenone in distilled water from immunoaffinity columns by competitive direct ELISA.

zearalenone added, ng/ml	sample ^a	recovery ^{b,c}		interwell CV, ^{d,e} %
		ng/ml	%	
1	1	0.9 ± 0.4	92	46.3
1	2	1.1 ± 0.1	110	10.8
1	3	1.2 ± 0.1	120	10.1
5	1	4.4 ± 0.5	88	10.6
5	2	3.8 ± 0.2	77	6.1
5	3	4.6 ± 0.1	92	2.6
10	1	9.3 ± 1.5	93	16.5
10	2	7.6 ± 0.2	76	3.0
10	3	11.5 ± 0.9	115	8.1
25	1	23.3 ± 2.2	93	9.6
25	2	25.0 ± 0.7	100	2.8
25	3	23.2 ± 0.5	93	2.0
50	1	49.0 ± 5.7	98	11.6
50	2	45.1 ± 1.3	90	2.9
50	3	43.9 ± 1.1	88	2.5

^aEach sample was spiked separately, passed through the immunoaffinity column, and then assayed in triplicate.

^bMean recoveries for samples containing 1, 5, 10, 25, and 50 ng/ml of zearalenone.

^cInterassay coefficient of variation (n = 3) for 1, 5, 10, 25, and 50 ng/ml was 11.6, 8.0, 16.8, 3.5, and 4.7%, respectively. Mean interassay CV was 8.9%.

^dCoefficient of variation.

^eMean interwell CV was 9.7%.

TABLE 2. Recovery of zearalenone in spiked dehydrated skim milk from immunoaffinity columns by competitive direct ELISA.

zearalenone added, ng/ml	sample ^a	recovery ^{b,c}		interwell CV, ^{d,e} %
		ng/ml	%	
1	1	1.9 ± 1.2	190	60.5
1	2	2.0 ± 1.0	200	51.0
1	3	1.7 ± 0.2	170	14.1
5	1	6.0 ± 0.5	118	8.6
5	2	6.2 ± 0.7	123	11.6
5	3	4.9 ± 0.2	98	4.8
10	1	10.6 ± 1.1	106	10.6
10	2	9.3 ± 0.6	93	6.7
10	3	12.1 ± 1.9	121	15.9
25	1	27.7 ± 2.7	111	9.6
25	2	23.2 ± 2.5	93	10.6
25	3	31.8 ± 0.7	127	2.2
50	1	56.0 ± 2.9	112	5.1
50	2	59.2 ± 1.4	118	2.4
50	3	52.3 ± 2.0	105	3.9

^aEach sample was spiked separately, passed through the immunoaffinity column, and then assayed in triplicate.

^bMean recoveries for samples containing 1, 5, 10, 25, and 50 ng/ml of zearalenone.

^cInterassay coefficient of variation (n = 3) for 1, 5, 10, 25, and 50 ng/ml was 6.7, 10.0, 11.0, 12.7, and 5%, respectively. Mean interassay CV was 9.1%.

^dCoefficient of variation.

^eMean interwell CV was 14.5%.

zearalenone competition curves in PBS. Recoveries for spiked water samples (Table 1) containing 1, 5, 10, 25, and 50 ng/ml were 107, 86, 95, 95, and 92%, respectively. The mean recovery was 95%. Recoveries for spiked milk (Table 2) at the same toxin concentrations were 187, 113, 107, 110, and 112%. The mean recovery was 126%.

No zearalenone was detected by ELISA in 12 marked samples of reconstituted dry milk. As a follow-up experiment, 5 and 10 ml milk samples were also applied to the immunoaffinity column. Eluates which were obtained reflected 5- and 10-fold concentration, respectively, thus enabling samples to be assayed with greater sensitivity (0.5-0.1 ng/ml). In spite of the concentration, ZEA was undetectable in these samples.

DISCUSSION

Extraction and clean-up procedures are sometimes required when zearalenone is analyzed in complex matrices such as foods and feeds. Current extraction procedures are time consuming and generally include use of organic solvents which are incompatible with immunoassay techniques and also present potential health risks to the analyst. However, rapid and reliable extraction procedures compatible with immunoassays involving organic solvents have been devised and are being currently used (4,31). Recently, monoclonal antibody-affinity chromatography columns have been utilized as preparative clean-up step for detection of aflatoxins by liquid chromatography (6). This procedure requires the availability of a high affinity antibody reactive with principal analogues and metabolites. Here, a high affinity zearalenone MAb that was cross reactive with ZEA analogues (4) was used in an immunoaffinity gel as a preparative step for ZEA detection. A simple scheme of the ZEA extraction using the MAb affinity columns is shown in Fig. 2.

ELISA recoveries in distilled water from immunoaffinity columns approached 100% (Table 1), whereas recoveries in spiked milk slightly exceeded 100% (Table 2). This might reflect presence at trace levels of ZEA or ZEA analogues in milk or from partial interference by the sample. Similarly, slightly higher ELISA ZEA recoveries have been reported in spiked corn (4,31).

The mean interwell coefficient of variation for spiked samples, 9.7 and 14.5% (6.5 and 7.6% excluding 1 ng data) in water and milk, respectively, reflects variability of antibody coating to the polystyrene solid phase and nonspecific binding of the peroxidase conjugate during the course of the assay. These values are similar to other previously found for ZEA (4,31) immunoassays, where zearalenone recoveries were carried out in spiked corn in the range of 50-500 ppb. The mean interassay coefficient of variation for spiked samples, 8.9 and 9.1% (8.2 and 9.6% excluding 1 ng data) in water and milk, respectively, reflects other sources of variation such as spiking, immunoaffinity extraction, and evaporation that may occur in the overall procedure.

Feeding studies carried out in cows administered with

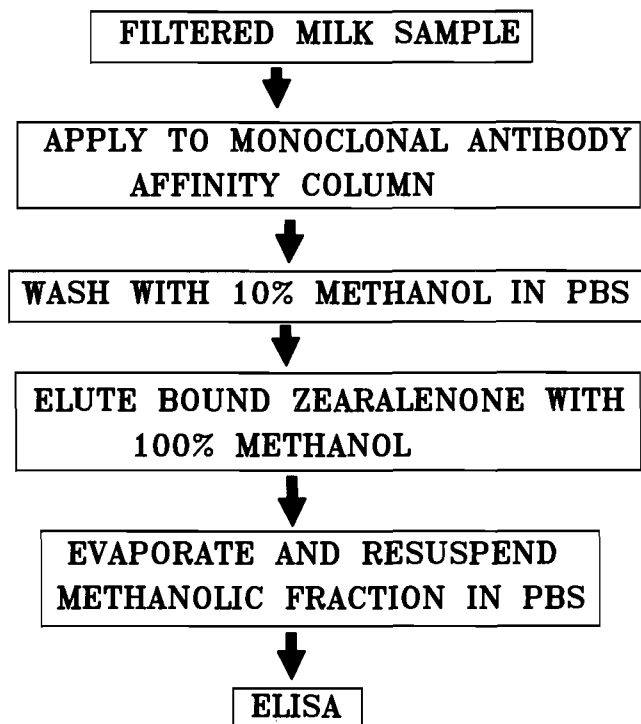


Figure 2. Tandem immunoaffinity chromatography-ELISA procedure for zearalenone detection.

200 mg/7 d, 1 g/1 d, and 1 g/2 d of ZEA estimated the presence of ZEA- α -zearalenol, and β -zearalenol (either in free or conjugated form) in milk as 0.77, 0.12, and 0.17%, respectively, of the total ZEA consumed (17). A more recent feeding study in cows administered with 25 and 100 mg/toxin during 6 d has demonstrated that presence of ZEA and analogues in milk represented less than 0.1% of total ZEA consumed (21). Our inability to detect ZEA in the market samples is consistent with these reports, because, as predicted, unusually high levels of toxin in the feed would be required for detectable carryover in milk. Further large-scale surveys of the milk supply would be necessary to verify this result.

In summary, the ZEA immunoaffinity extraction method reported here was easy to perform, rapid, and reproducible. Estimated ELISA recovery using this procedure was 95% in water within the range of 1 to 50 ng and 110% in milk within the range of 5 to 50 ng. The affinity columns (0.2 ml volume size) remained active after eluting bound toxin with methanol and after re-equilibrating in PBS were ready for reuse. Typically, 10 samples could be passed through columns with no loss of activity being observable. We believe that the tandem procedure will be valid to detect ZEA in contaminated samples containing as low as 5 ppb (or less after sample concentration), while highly contaminated samples would be assayed by direct dilution of the chromatographic extracts before the ELISA analysis. Affinity columns would be potentially useful to detect conjugated forms of zearalenols in samples previously treated with β -glucuronidase and aryl sulphatase (17,22) and also

can be used in the isolation/concentration of zearalenone in clinical samples and other agricultural commodities prior to ELISA or other conventional methods such as HPLC, GC, or MS.

ACKNOWLEDGMENTS

Juan I. Azcona is the recipient of a postdoctoral fellowship from the Ministerio de Educación y Ciencia, Spain. This work was also supported by the Research Excellence Fund from the State of Michigan and by the Michigan State University Agricultural Experiment Station.

REFERENCES

1. Association of Official Analytical Chemists. 1984. Book of Methods, 14th ed. AOAC, Washington, DC, Sec. 26.124-26.132.
2. Bennett, G. A., O. L. Shotwell, and W. F. Kwolek. 1985. Determination of α -zearalenol and zearalenone in corn by high performance liquid chromatography: collaborative study. *J. Assoc. Offic. Anal. Chem.* 68:958-961.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
4. Dixon, D. E., R. L. Warner, B. P. Ram, L. P. Hart, and J. J. Pestka. 1987. Hybridoma cell line production of a specific monoclonal antibody to the mycotoxin zearalenone and α -zearalenol. *J. Agr. Food Chem.* 35:122-126.
5. Gimeno, A. 1983. Rapid thin layer chromatographic determination of zearalenone in corn, sorghum, and wheat. *J. Assoc. Offic. Anal. Chem.* 66:565-569.
6. Groopman, J. D., and K. F. Donahue. 1988. Aflatoxin, a human carcinogen: determination in foods and biological samples by monoclonal antibody affinity chromatography. *J. Assoc. Offic. Anal. Chem.* 71:861-867.
7. Hagler, W. M., Gy. Dankó, L. Horváth, M. Palyusik, and C. J. Mirocha. 1980. Transmission of zearalenone and its metabolite into ruminant milk. *Acta Vet. Acad. Sci. Hung.* 28:209-216.
8. Hagler, W. M., C. J. Mirocha, S. V. Pathre, and J. C. Behrens. 1979. Identification of the naturally occurring isomer of zearalenol produced by *Fusarium roseum* "Gibbosum" in rice culture. *Appl. Environ. Microbiol.* 37:849-853.
9. Hart, L. P., W. E. Braselton, and T. C. Stebbins. 1982. Production of zearalenone and deoxynivalenol in commercial sweet corn. *Plant. Dis.* 66:1133-1135.
10. Hebert, G. A., P. L. Pelhman, and B. Pittman. 1973. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse and goat antisera. *Appl. Microbiol.* 37:26-36.
11. Holder, C. L., C. R. Nony, and M. C. Bowman. 1977. Trace analysis and/or zearalenol in animal chow by high pressure liquid chromatography and gas-liquid chromatography. *J. Assoc. Offic. Anal. Chem.* 60:272-278.
12. Hurd, R. N. 1977. Structure activity relationships in zearalenones. pp. 379-391. *In* J. V. Rodricks, C. V. Hesseltine, and M. A. Mehlman (eds.). *Mycotoxins in Human and Animal Health*. Pathotox Publishers, Park Forest South, IL.
13. James, L. J., L. G. McGirr, and T. K. Smith. 1982. High pressure liquid chromatography of zearalenone and zearalenols in rat urine and liver. *J. Assoc. Offic. Anal. Chem.* 65:8-13.
14. Kuiper-Goodman, T., P. M. Scott, and H. Watanabe. 1987. Risk assessment of the mycotoxin zearalenone. *Regul. Toxicol. Pharmacol.* 7:255-306.
15. Liu, M. T., B. P. Ram, L. P. Hart, and J. J. Pestka. 1985. Indirect enzyme-linked immunosorbent assay for the mycotoxin zearalenone. *Appl. Environ. Microbiol.* 50:332-336.
16. Mirocha, C. J., S. V. Pathre, and C. M. Christensen. 1977. Zearalenone. pp. 345-364. *In* J. V. Rodricks, C. V. Hesseltine and M. A. Mehlman (eds.). *Mycotoxins in Human and Animal Health*. Pathotox Publishers, Park Forest South, IL.
17. Mirocha, C. J., S. V. Pathre, and T. S. Robison. 1981. Comparative metabolism of zearalenone and transmission into bovine milk. *Food Cosmet. Toxicol.* 19:25-30.

Con't. on p. 627

REFERENCES

1. Adekeye, D. 1979. Resistance of *Staphylococcus aureus* isolates of man and other animals to five antibiotics commonly used in Nigeria. *Nigerian Med. J.* 9:195-197.
 2. Adesiyun, A. A., J. O. Adekeye, J. U. Umoh, and M. Nadarajah. 1983. Studies on well water and possible health risks in Katsina, Nigeria. *J. Hyg. (Camb.)* 90:199-205.
 3. Adetosoye, A. I. 1980. *Escherichia coli* and diarrhea in kids, lambs, and piglets. *Bull. Anim. Hlth. Prod. Afr.* 28:300-306.
 4. Adetosoye, A. I. 1980. Serological grouping of haemolytic *Escherichia coli* isolated from diarrhoeic piglets. *Bull. Anim. Hlth. Prod. Afr.* 28:190-193.
 5. Adetosoye, A. T., H. T. Willinge, and M. M. Awad. 1976. Enterotoxigenicity of haemolytic *Escherichia coli* isolated from diarrhoeic piglets. *J. Nigerian Vet. Med. Assoc.* 5:32-34.
 6. Alabi, D. A., and A. A. Adesiyun. 1986. Studies on microbial quality of filtered water in households of a university community in Nigeria. *J. Hyg. (Camb.)* 96:239-248.
 7. Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turk. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Tech. Bull. Reg. Techn.* 36:493-496.
 8. Bonita, A. G., and A. B. Steven. 1980. Survey of commercially available cheese for enterotoxigenic *Escherichia coli*. *J. Food Prot.* 43:395-398.
 9. Cowan, S. T., and K. J. Steel. 1974. Identification of medical bacteria. Cambridge University Press, London.
 10. DeBoy, M. J., I. K. Wachsmuth, and B. R. Davis. 1980. Antibiotic resistance of enterotoxigenic and non-enterotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* 12:264-270.
 11. DeBoy, M. J., I. K. Wachsmuth, and B. R. Davis. 1980. Haemolytic activity of enterotoxigenic and non-enterotoxigenic strains of *Escherichia coli*. *J. Clin. Microbiol.* 12:193-198.
 12. Falade, S., and M. O. Oluwadiya. 1975. Serologic grouping and in vitro drug resistance of 13 *Escherichia coli* isolates from cattle and pigs. *J. Nigerian Vet. Med. Assoc.* 4:117-118.
 13. Habiballa, N., and A. Jubcir. 1978. Isolation of haemolytic *Escherichia coli* from cases of bovine abortion. *Trop. Anim. Hlth. Prod.* 10:214.
 14. Igene, J. O. 1983. Total plate count and coliform density for retail "suya" (Tsire). *Trop. Vet.* 1:85-91.
 15. Jackson, G. 1981. A survey of antibiotic resistance of *Escherichia coli* isolated from farm animals in Great Britain from 1971-1977. *Vet. Rec.* 108:325-328.
 16. Jones, G. A., D. C. Gibson, and K. J. Chang. 1967. Coliform bacteria in Canadian pasteurized dairy products. *Can. J. Pub. Hlth.* 58:257-264.
 17. Kornacki, J. L., and E. H. Marth. 1982. Fate of non-pathogenic and enteropathogenic *Escherichia coli* during the manufacture of Colby cheese. *J. Food Prot.* 45:310-316.
 18. Kwaga, J. K. P., and A. A. Adesiyun. 1984. Antibigrams of *Staphylococcus aureus* isolated from some ready-to-eat products. *J. Food Prot.* 47:865-867.
 19. Lechevallier, M. W., S. C. Cameron, and G. A. Mefetter. 1983. New medium for improved recovery of coliform bacteria from drinking water. *Appl. Environ. Microbiol.* 45:484-492.
 20. Linton, A. H. 1977. Antibiotic resistance. The present situation reviewed. *Vet. Rec.* 100:354-360.
 21. Ojo, M. O., and L. D. Ahanihu. 1974. Minimum inhibition concentration of furazolidone for *Escherichia coli* and *Salmonella* strains isolated from Nigeria. *Vet. Rec.* 94:174.
 22. Ojo, M. O., and C. Moomaw. 1972. *Escherichia coli* of pigs, O. K. groups, and antibiotic resistance - Preliminary Report. *Nigerian Vet. J.* 1:54.
 23. Ray, J., and M. L. Speck. 1978. Plating procedure for the enumeration of coliforms in dairy products. *Appl. Environ. Microbiol.* 35:820-822.
 24. Rosenberg, M. L., J. P. Koplun, I. K. Wachmuth, J. G. Wells, E. T. Gangarosa, R. L. Guerrant, and D. A. Sack. 1977. Epidemic diarrhea at Crater lake from enterotoxigenic *Escherichia coli*, A large water-borne outbreak. *Ann. Int. Med.* 86:714-718.
 25. Sack, B. R., D. A. Sack, I. J. Mehlman, F. Orskov, and I. Orskov. 1977. Enterotoxigenicity of *E. coli* isolated from food. *J. Inf. Dis.* 135:313-317.
 26. Schroeder, S. A., J. R. Caldwell, I. M. Vernon, P. C. White, S. I. Granger, and J. V. Bennet. 1968. A water-borne outbreak of gastroenteritis in adults associated with *Escherichia coli*. *Lancet* 737-740.
 27. Sherwood, D., D. R. Snodgrass, and G. H. K. Lawson. 1983. Prevalence of enterotoxigenic *Escherichia coli* in calves in Scotland and Northern England. *Vet. Rec.* 113:208-212.
 28. Umoh, V. J., A. A. Adesiyun, and N. E. Gomwalk. 1986. Enumeration of staphylococci isolated from Nigerian fermented milk products using Baird-Parker agar (BPA) as a selective medium. *Nigerian J. Microbiol.* (in press).
 29. Umoh, V. J., A. A. Adesiyun, and N. E. Gomwalk. 1988. Enterotoxin production by staphylococci isolated from Nigerian fermented milk products. *J. Food Prot.* 51:534-537.
 30. Wachmuth, I. K. 1980. *Escherichia coli* typing and pathogenicity. *Clin. Microbiol. (Newsletter)* 2:4-7.
 31. Wolf, H. W. 1972. The coliform count as a measure of water quality. In R. Mitchell (ed) *Water Pollution Microbiology*. Wiley Inter-Science, a division of John Wiley and Sons Inc., New York.
-
- Pestka et al., cont.** from p. 580
18. National Toxicological Program. 1982. Carcinogenesis bioassay of zearalenone in F 344/N rats and B6C3F1 mice. National Toxicology Program, Technical report series No. 235, Department of Health and Human Services, Research Triangle Park, NC.
 19. Pestka, J. J., Y. K. Li, and F. S. Chu. 1982. Reactivity of aflatoxin B_{2a} antibody with aflatoxin B₁-modified DNA and related metabolites. *Appl. Environ. Microbiol.* 44:1159-1165.
 20. Pestka, J. J., M-T. Liu, B. K. Knudson, and M. G. Hogberg. 1985. Immunization of swine for production of antibody against zearalenone. *J. Food Prot.* 48:953-957.
 21. Renz, V., and G. Terplan. 1988. Ein enzymimmunologisches Verfahren zum Nachweis des östrogen wirksamen Mykotoxins Zearalenon und seiner Metaboliten in der Milch. Proceedings of the 29th Conference in Food Hygiene, Garmish-Partenkirchen, FRG, pp. 324-327.
 22. Scott, P. M., and G. A. Lawrence. 1988. Liquid chromatographic determination of zearalenone and α - and β -zearalenols in milk. *J. Assoc. Offic. Anal. Chem.* 71:1176-1179.
 23. Scott, P. M., T. Panalaks, S. Kanhere, and W. F. Miles. 1978. Determination of zearalenone in cornflakes and other corn based foods by thin layer chromatography, high pressure liquid chromatography and gas-liquid chromatography. *J. Assoc. Offic. Anal. Chem.* 61:593-600.
 24. Shortwell, O. L., M. L. Goulden, G. A. Bennett, R. D. Plattner, and C. V. Hesselstine. 1977. Survey of 1975 wheat and soybeans for zearalenone, aflatoxin and ochratoxin. *J. Assoc. Offic. Anal. Chem.* 60:778-783.
 25. Swanson, S. P., R. A. Corley, D. G. White, and W. B. Buck. 1984. Rapid thin layer chromatographic method for determination of zearalenone and zearalenol in grains and animal feeds. *J. Assoc. Offic. Anal. Chem.* 65:580-582.
 26. Thouvenot, D. R., and R. F. Morfin. 1979. Quantitation of zearalenone by gas-liquid chromatography on capillary glass columns. *J. Chromatogr.* 170:165-170.
 27. Thouvenot, D. R., and R. F. Morfin. 1983. Radioimmunoassay for zearalenone and zearalenol in human serum: production, properties, and use of porcine antibodies. *Appl. Environ. Microbiol.* 45:16-23.
 28. Trenholm, H. L., R. M. Warner, and D. W. Fitzpatrick. 1984. Rapid, sensitive liquid chromatographic method for determination of zearalenone and α - and β -zearalenol in wheat. *J. Assoc. Offic. Anal. Chem.* 67:968-972.
 29. Ueno, Y., and F. Tashiro. 1981. α -Zearalenol, a major hepatic metabolite in rats of zearalenone, an estrogenic mycotoxin of *Fusarium* species. *J. Biochem. (Tokyo)* 89:563-571.
 30. Warner, R. L., and J. J. Pestka. 1987. ELISA survey of retail grain-based food products for zearalenone and aflatoxin B₁. *J. Food Prot.* 50:502-504.
 31. Warner, R., B. P. Ram, L. P. Hart, and J. J. Pestka. 1986. Screening for zearalenone in corn by competitive direct enzyme-linked immunosorbent assay. *J. Agr. Food Chem.* 34:714-717.