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Development of an Enzyme-Linked Immunosorbent Assay for Triadimefon in Foods

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Triadimefon (1-(4-chlorophenoxy)-3,3-dimethyl-1(1H-1,2,4-triazol-1-yl)-2-butanone) is a fungicide registered for use in the United States on several crops including pineapple, apples, grapes and pears. One method of analysis has been described (Specht, 1977) which involves extraction with acetone, partitioning into dichloromethane, purification on Florisil and determination by gas-chromatography with a nitrogen selective detector. Another procedure developed for grape juice and wine employed an XAD-2 resin for isolation of triadimefon and determination by capillary column gas chromatography (Nickless et al., 1981).

Recently, immunochemical methods such as enzyme linked immunosorbent assay (ELISA) have been applied to the analysis of foods for a variety of compounds including the mycotoxin ochratoxin A (Michael et al., 1983), the growth regulator diflubenzuron (Wie and Hammock, 1982) and the herbicide diclofop-methyl (Schwalbe et al., 1984). These procedures are capable of achieving the lower limit of detection required for residue analysis without recourse to the lengthy cleanup steps required in conventional chemical methods. The present study was initiated to investigate the applicability of ELISA to the analysis of triadimefon in foods and compare it to a gas chromatographic procedure.

MATERIALS AND METHODS

Triadimefon (95.3%) was supplied by Chemagro, Kansas City, MO. A stock solution was prepared and diluted in methanol to give a 50 $\mu g\ mL^{-1}$ spiking solution. Other dilutions were made to give 40, 80, 160, 320 and 640 $ng\ mL^{-1}$ for determination of the calibration curve. Bovine serum albumin (RIA grade), human serum albumin, ovalbumin, anti-rabbit IgG paroxidase conjugate, o-phenylenediamine dihydrochloride and Tween 20 were obtained from Sigma Chemical Co., St. Louis, MO. Freund's complete adjuvant was purchased from Difco Laboratories, Detroit, MI. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI.

Phosphate buffered saline (PBS) contained 2.42 g NaH_2PO_4 and 8.26 g NaCl, adjusted to pH 7.2 with NaOH before making to 1 L with distilled water. PBS-Tween washing solution contained 0.1% Tween 20 in PBS. Antiserum and IgG were diluted with 0.1% bovine serum albumin in PBS. Substrate for the peroxidase conjugate consisted of O-phenylenediamine

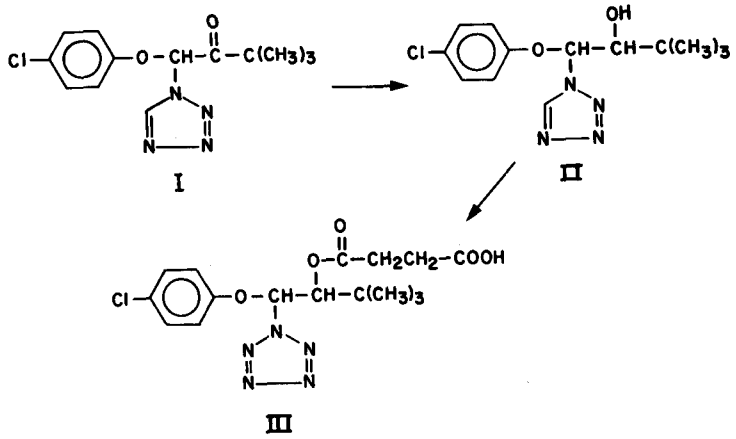

Figure 1. Route for the synthesis of succinyl triadimenol from triadimefon.

dihydrochloride (70 mg) and 40 µL of 30% H_2O_2 in 100 mL of citrate-phosphate buffer, pH 5.0.

Four New Zealand white rabbits were immunized by intradermal injection of 0.5 mL each of an emulsion of immunogen in 1:1 saline: Freund's complete adjuvant (1 mg mL^{-1}). Booster injections were given at monthly intervals and blood was collected 1 week after each injection by bleeding from the marginal ear vein. The immunogen was prepared by coupling human serum albumin with succinyl triadimenol (III), prepared by the route shown in Fig. 1.

Triadimefon (I) was reduced to triadimenol (II) by a procedure similar to that described by Rouchaud et al., (1982). A suspension of sodium borohydride (400 mg) in ethanol (12 mL) was added to a stirred solution of triadimefon (767 mg) in ethanol (15 mL). After reaction at room temperature overnight, conc. HCl (5.8 mL) was added and the alcohol removed on a rotary evaporator. The residue was taken up in dichloromethane washed with water, and the solvent removed to yield a syrup (II) which was refluxed in pyridine (10 mL) with succinic anhydride (520 mg) for 24 h. Most of the pyridine was removed on a rotary evaporator and the residue dissolved in dichloromethane. The solution was washed with 1 N HCl, then water, dried, and the solvent removed to yield a clear light brown syrup (479 mg). Thin-layer chromatography on silica with

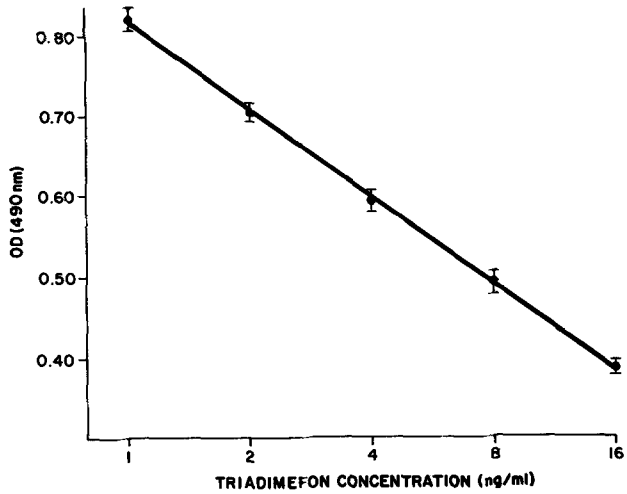

Figure 2. Standard curve for the determination of triadimefon concentration. Values are the means of quadruplicate determinations and vertical bars represent the standard deviations.

acetonitrile as the mobile phase showed a single spot which remained at the origin. Electron impact mass spectrometry gave a molecular ion for the succinate (m/z 395) and a fragment for (M-chlorophenoxy) (m/z 268). Succinyl triadimenol was coupled to human serum albumin by adding a solution of 1-[3-(dimethylamino)propyl]-3-ethyl-carbodiimide hydrochloride (95 mg) and triadimenol succinate (1.69 mg) in 0.01 M phosphate buffer, pH 7.2 (2 mL) to a stirred solution of human serum albumin (14 mg) in 1.0 mL of the buffer. After reaction for 20 h at room temperature, the protein was dialyzed for 3 days at 4° against frequent changes of distilled water, then lyophilized.

Assays were carried out using 96 well microtiter plates ("Cook Microtiter", Fisher Scientific, Ottawa, Ont.) previously sensitized with succinyl triadimefon-ovalbumin conjugate. The conjugate was prepared by the mixed anhydride method as described by Wie and Hammock (1982). Plates were sensitized by incubation overnight at 4° with 200 µL/well of a solution consisting of 0.2 µg mL^{-1} conjugate and 10 µg mL^{-1} ovalbumin in 0.05 M carbonate-bicarbonate buffer, pH 9.6. After sensitization, plates were washed 3 times with PBS-Tween and twice with water, then stored in polystyrene bags in a freezer.

Recovery studies were carried out on various commodities which were spiked with a methanol solution of triadimefon prior to extraction. Samples (10 g) of apple, pear or pineapple were extracted by homogenizing in a Sorvall Omni-Mixer with methanol (40 mL), filtered through Whatman no. 1 paper on a Buchner funnel, and brought to 50 mL with methanol. An aliquot of the extract (25 µL) was added to a 1:4000 dilution of antiserum (1.0 mL) and pre-equilibrated for 30 min at room temperature. Samples of grape were extracted in a similar manner, substituting ethyl acetate for methanol. Aliquots (25 µL) of the ethyl acetate extract were taken to dryness in a 12 x 75 mm tube and the residue dissolved in methanol (25 µL) prior to the addition of diluted antiserum.

After pre-equilibration, 200 µL of antiserum was added to the sensitized plate which was incubated at 4° for 1 h. The wells were emptied, washed 4 times with PBS-Tween, then filled with a 1:1000 dilution of anti-rabbit IgG conjugate (225 µL). After 30 min at room temperature the wells were again emptied, washed, and the adsorbed conjugate measured by reaction for 15 min with 225 µL of O-phenylenediamine/H_2O_2 substrate. The enzyme reaction was terminated by the addition of 2.5 M H_2SO_4 (50 µL) and the optical density of the wells read at 490 nm or a Dynatech MR 600 plate reader.

Samples were quantitated by comparison of the optical density to that obtained from a semi-log plot of optical density against concentration for standards carried through the immunoassay procedure simultaneously. Sample and standard assays were carried out in quadruplicate.

For determination of triadimefon by gas chromatography an aliquot of methanol extract (25 mL) was added to 40 mL of water in a separatory funnel and, after the addition of 5% Na_2SO_4 (5 mL) was extracted with dichloromethane (3 x 10 mL). The extracts were combined, dried with anhydrous Na_2SO_4 and the solvent removed on a rotary evaporator. The residue was dissolved in toluene (1 mL) and added to 2 g of 100-200 µm silica (activity I) packed in toluene in an 8 mm id x 20 cm glass chromatographic column. The flask was rinsed twice with 1 mL aliquots of toluene, then the column eluted with 10 mL of toluene followed by 10 mL of 50% ethyl acetate in toluene. The latter fraction was evaporated to dryness and taken up in toluene for determination by gas chromatography on a Varian 1400 fitted with an N-P detector and 0.25 µ DB-5 capillary column (0.25 mm x 10 m; J and W Scientific, Inc. Rancho Cordova, CA). The helium carrier gas velocity was 33 cm sec^{-1}, while flows of nitrogen make-up gas, air, and hydrogen to the detector were 30, 175, and 4.5 mL min^{-1} respectively. A cold-trap injection technique was used and the column heated from 110° to 190° as rapidly as possible. Under these conditions 0.64 ng of triadimefon produced a peak of 50% full scale deflection with a retention time of 8 min.

RESULTS AND DISCUSSION

Useful antiserum titers were obtained within 6 months of the initial immunization. Optimum sensitivity was obtained using plates coated with a low (0.2 µg mL^{-1}) concentration of ovalbumin-triadimenol conjugate.

Unconjugated ovalbumin was added to the sensitizing protein to obtain a uniform coating of the wells and thus attain a coefficient of variation among replicates of approximately 3%. A typical competitive inhibition curve is shown in Fig. 2. A 50% inhibition of binding of antibody to the coated plate was observed in the presence of 2.4 ng mL^{-1} of triadimefon. Triadimenol, a metabolite of triadimefon (Rouchard et al., 1981, 1982) was slightly more active than triadimefon, 2.1 ng mL^{-1} resulting in 50% inhibition. Thus, both compounds are determined in a single analysis.

Another metabolite, 4-chlorophenol did not exhibit significant inhibition at 500 ng mL^{-1}, nor did 1,2,4-triazole or the fungicides iprodione or imazalil.

The recovery of triadimefon from various commodities as measured by ELISA and by gas chromatography is given in Table I. It was necessary to use ethyl acetate to extract grapes for the ELISA procedure, since methanol resulted in coextractives which gave erroneously low values. Methanol gave good recoveries at 0.5 ppm and above for other commodities, and is preferable to ethyl acetate since it avoids an evaporation step.

Low recoveries obtained at 0.1 ppm were not improved by the use of ethyl acetate as an extractant. With the exception of pineapple where immunoassay results are slightly lower, recoveries obtained by ELISA correlate well with those measured by gas chromatography. The reproducibility of both methods was also similar, a coefficient of variation of 5.1% being observed by ELISA with 6 samples of pear spiked at 0.5 ppm while a value of 4.6% was found by gas chromatography.

Table 1. Recoveries of triadimefon added to various commodities.

Triadimefon added (ppm)	Triadimefon recovered[1] (%)							
	Apple		Pear		Pineapple		Grape	
	ELISA	GLC	ELISA	GLC	ELISA	GLC	ELISA	GLC
0.1	62	-[2]	48	-	60	-	56	-
0.5	112	96	91	91	77	90	94	80
1.0	101	90	89	98	74	95	95	93
2.0	90	90	85	103	76	86	81	96

[1]Values are the means of duplicate determinations.

[2]Not analyzed.

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REFERENCES

Morgan MRA, McNerney R, Chan HWS (1983) Enzyme-linked immunosorbent assay of ochratoxin A in barley. J Assoc Off Anal Chem 66:1481-1484

Nickless G, Spitzer T (1981) Determination of triadimefon in grape juice and wine using capillary gas chromatography. J Chromatog 208:409-413

Rouchaud J, Moons C, Meyer JA (1981) The products of metabolism of [^{14}C] triadimefon in the grain and straw of ripe barley. Bull Environ Contam Toxicol 27:543-550

Rouchaud J, Moons C, Meyer JA (1982) Metabolism of [^{14}C] triadimefon in barley shoots. Pestic Sci 13:169-176

Schwalbe M, Dorn E, Beyermann K (1984) Enzyme immunoassay and fluoroimmunoassay for the herbicide diclofop-methyl. J Agric Food Chem 32:734-741

Specht W (1977) Gas chromatographic method for determining residues of the fungicides fuberidazole, fluotrimazole and triadimefon in plants and soil. Pflanzenschutz-Nachr 30:55-71

Wie SI, Hammock BD (1982) Development of enzyme-linked immunosorbent assays for residue analysis of diflubenzuron and BAY SIR 8514. J Agric Food Chem 30:949-957

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