Development of an Enzyme-Linked Immunosorbent Assay for the Detection of the Triazole Fungicide Myclobutanil

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A competitive enzyme-linked immunosorbent assay (ELISA) system for quantitative detection of myclobutanil was developed. Polyclonal antibodies were raised in rabbits against the haptenic compound 2-(4-chlorophenyl)-2-[(1,2,4-triazol-1-yl)methyl]hexanoic acid, conjugated to conalbumin through the carboxylic acid moiety. The standard curve based on a homologous system using the above hapten conjugated to bovine serum albumin displayed a detection range from 0.3 to 200 μg/mL for myclobutanil with an IC_{50} value of 4.9 ± 0.5 μg/mL. The effects of various assay conditions, including pH, organic solvent content, sample matrix effects (surface water, soil and fruit extracts), and protein stabilizer, on the sensitivity have been evaluated. Cross-reactivities with various related fungicides, insecticides, pharmaceuticals, and intermediates containing triazole, imidazole, or pyrimidine groups were tested and found to be below 15%. The detection system appears to be a versatile device for the environmental monitoring of myclobutanil.

Keywords: *Monitoring; immunoassay; ELISA; biotechnology-based methods; fungicide; myclobutanil; analytical chemistry*

INTRODUCTION

Triazole fungicides represent a class of highly effective, broad-spectrum antifungal agents of both medicinal and agricultural applications (Scheinpflug and Kuck, 1987). These compounds are inhibitors of sterol biosynthesis. Among them, myclobutanil (**1**) is a highly effective systemic fungicide active on a wide range of pathogens (Quinn *et al.*, 1986). Myclobutanil has therefore been registered as an agricultural (Fujimoto, 1985; Fujimoto *et al.*, 1987) and industrial (Leightley *et al.*, 1992) fungicide and as a component in microbicide combinations (Savagre and Evans, 1992). As a result, it is being used on numerous plants including pome and stone fruits, cereals, and vegetables. In addition, the compound has found application as a nonseasonal antifungal agent in seed treatment and is broadly applied as a storage fungicide (Gaudet *et al.*, 1989). Although showing strong curative activity and efficacy against certain fungicide-resistant strains (Köller and Wubben, 1989), the compound displays no preventive activity (Quinn *et al.*, 1986). Moreover, emerging fungal resistance has been reported against myclobutanil (Braun and McRae, 1992). These features require more frequent and increased volume applications. The parent compound is rapidly metabolized and excreted in animals (FAO/WHO, 1992a). Nevertheless, it does not decompose under anaerobic conditions: the half-life in soil is 20–80 days, and no degradation in water occurs within 28 days at the pH range of 5–9 (FAO/WHO, 1992b). As a consequence, myclobutanil has been classified as slightly hazardous.

In contrast to their outstanding activity and extensive usage, residue analysis methods for triazole fungicides are less advanced. The procedures developed for the detection of these fungicides mainly include liquid (HPLC) and gas chromatographic (GC) methods (Cabras *et al.*, 1992; Ivie, 1980; Mendes, 1991; Reynaud *et al.*, 1991). A recent multiresidue screening method utilized GC/MS detection for monitoring myclobutanil, among numerous other pesticidal compounds (Liao *et al.*, 1991). Although the mammalian toxicity of most triazole fungicides is rather low (the LD_{50} value of myclobutanil for rats ranges from 1600 to 2290 mg/kg), monitoring these compounds is important because by affecting the biosynthetic pathway of ergosterol, they may affect nontarget organisms, as seen in the case of the closely related azole derivatives of metyrapone (Bélai *et al.*, 1988). Moreover, triazole fungicides—including myclobutanil—have been found to display mobility in soil, possibly causing leaching problems (Jamet and Eudeline, 1992).

Recent developments in the field of immunodetection of small molecules have proven that immunoassays serve as facile time- and cost-effective alternatives to instrumental analysis (Hammock and Mumma, 1980; Hammock *et al.*, 1987; Jung *et al.*, 1989; Hall *et al.*, 1990). Enzyme-linked immunosorbent assays (ELISAs) are particularly applicable in environmental monitoring, where large numbers of, primarily aqueous, samples are analyzed for toxicants and pollutants (Vanderlaan *et al.*, 1988; Hammock *et al.*, 1990).

Within the scope of the extending collaboration between our laboratories at Davis and Budapest (Hammock *et al.*, 1990; Jung *et al.*, 1991; Székács *et al.*, 1995) and in a development project sponsored by the U.S.–Hungarian Joint Fund, we aim to develop ELISA systems for the environmental monitoring of pesticides and toxins. Driven by the lack of a specific detection system for myclobutanil and to continue our previous work on development of immunodetection systems for pesticides containing a triazole moiety, one of our present objectives was to develop an ELISA system for this target analyte. This objective is especially desirable since to date no selective chromophoric or spectroscopic detection of the triazoles in foodstuff or in environmental samples has been developed.

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MATERIALS AND METHODS

Reagents. A small sample of myclobutanil (**1**) was kindly provided by Rohm and Haas Co. through Nitrokémia Ipartelepek Co. (Fúzfógyártelep, Hungary). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). Anti-rabbit immunoglobulin (raised in goats) conjugated to horseradish peroxidase was purchased from Institute "Human" for Serological Research and Production (Budapest, Hungary). Other immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and ICN ImmunoBiologicals (Lisle, IL).

The purity and structures of the haptenic intermediates were supported by melting points, analytical TLC and IR spectroscopy, and ^1H and ^{13}C NMR, as well as mass spectroscopy. Analytical TLC was performed on 0.2 mm silica gel F_{254} plates (Merck, Darmstadt, Germany), while preparative TLC was carried out on plates prepared with Kieselgel 60 GF 254 (Reanal, Budapest, Hungary) using hexane/ethyl acetate (v/v 1:2). Compounds were detected by UV light (254 nm) and/or iodine vapor staining. Melting points are uncorrected.

Instruments. Proton nuclear magnetic resonance (^1H NMR) spectra were obtained on a Bruker AW-80 (Bruker, Karlsruhe, Germany) instrument, while carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Varian EM-390 (Varian, Sunnyvale, CA) in dilute solutions in $CDCl_3$ with or without CF_3COOD and D_2O saturation and using tetramethylsilane as internal standard. Infrared spectra (IR) were obtained on a Buck 500 instrument (Buck, East Norwalk, CT). Positive and negative ion fast atom bombardment (FAB) and electron ionization (EI) mass spectra (MS) were obtained on a ZAB-HS-2F spectrometer (VG Analytical, Wythenshawe, U.K.) with 1 mA, 8 keV xenon FAB or 70 eV electron ionization. Samples were introduced as solid probes (EI) or FAB probes using 3-nitrobenzyl alcohol for low resolution or a mixture of polyethylene glycol 300 and 3-nitrobenzyl alcohol for high resolution (FAB). Enzyme-linked immunosorbent assays (ELISA) were carried out in high-capacity 96-well microplates (Nunc, Roskilde, Denmark, no. 442404) and read with a Titertek Multiskan microplate reader (Flow Laboratories, McLean, VA).

Hapten Synthesis and Verification. *2-(4-Chlorophenyl)-2-[(1,2,4-triazol-1-yl)methyl]hexanoic amide* (**2**) was prepared by the partial hydrolysis of myclobutanil (**1**) under phase transfer conditions (Cacchi *et al.*, 1980). To a solution of myclobutanil (1.45 g, 5 mmol) dissolved in 2 mL of dichloromethane was added 2.5 mL of aqueous hydrogen peroxide solution (30 vol %), tetrabutylammonium hydrogen sulfate (0.34 g, 1 mmol), and 1.9 mL of a 20% aqueous solution of sodium hydroxide. The reaction mixture was stirred at room temperature for 24 h and then diluted with 5 mL of dichloromethane; the organic layer was separated and washed with 5 × 2 mL of brine. The precipitated white solid was collected to give the crude product (1.15 g). Thin layer chromatography using a hexane/ethyl acetate (1:2) solution as eluent indicated that the mixture still contained unreacted myclobutanil. Therefore, the product was purified using preparative TLC with the above eluent to give **2** (0.74 g, 2.4 mmol) with a yield of 48.2%: mp 210 °C. TLC R_f = 0.33 (hexane/ethyl acetate 1:2); IR (KBr, cm^{-1}) 3400–3100 (ν_{NH_2}), 1716 ($\nu_{C=O}$), 1040 (ν_{Cl}, *para*-aromatic), 825 (γ_{C-H}, aromatic); ^1H NMR ($CDCl_3$–CF_3COOD) δ 0.95 (3H, CH_3, t), 1.43 (4H, CH_2, m), 2.00 (2H, CH_2, t), 4.83 (2H, CH_2, m), 7.04, 7.30 (4H, aromatic, dd), 8.41 (1H, triazole CH, s), 9.05 (1H, triazole CH, s); ^{13}C NMR ($CDCl_3$) δ 13 (CH_3), 22, 25, 32 (3 CH_2), 39 (C), 53 (NCH_2), 127 (4 aromatic C), 132 (aromatic C), 138 (aromatic CCl), 142, 152 (2 triazole CH), 174 (CONH); FAB-MS, m/z 307.02 $[M + H]^+$; EI-MS, m/z 306 $[M]^+$. Anal. Found: C, 58.4; H, 6.7; Cl, 11.2%. Calcd for $C_{15}H_{19}ClN_4O$: C, 58.73; H, 6.24; Cl, 11.56%.

In another process using the traditional method of hydrolysis of nitriles in acidic media (Sperber *et al.*, 1948), myclobutanil was hydrolyzed to amide **2** in quantitative yield, when a concentrated sulfuric acid (20 mL) solution of myclobutanil (1 g, 3.5 mmol) was maintained at room temperature for 3 weeks. The mixture was carefully neutralized by adding it dropwise to a solution of 30 g of sodium hydroxide in 1300 mL of water, cooled in an ice–water bath. The solid precipitate was filtered off, washed extensively with water, and dried to give 1.03 g (3.3 mmol) of the product (at a yield of 96.8%) that was proven to be identical to **2**.

2-(4-Chlorophenyl)-2-[(1,2,4-triazol-1-yl)methyl]hexanoic acid (**3**) was prepared by the nitrosation (Olah and Olah, 1965) of **2**. To a solution of the amide **2** (0.736 g, 2.4 mmol) dissolved in 0.96 mL of concentrated sulfuric acid and heated to 70 °C was added a solution of sodium nitrite (0.21 g, 3.12 mmol) in 0.3 mL of water. The reaction mixture was stirred at 105 °C for 3 h. Upon cooling to room temperature, the mixture was alkalized with a 50% aqueous sodium hydroxide solution and extracted with 3 × 2.5 mL of dichloromethane to remove impurities. Then, the aqueous phase was acidified with concentrated sulfuric acid, and the white solid precipitate was filtered out, washed extensively with water and with a small amount of hexane, and dried to give **3** (0.44 g, 1.4 mmol) at a yield of 59.1%: mp 179 °C: TLC R_f = 0.05 (hexane/ethyl acetate 1:2); IR (KBr, cm^{-1}) 3500–2500 (ν_{OH}), 1680 ($\nu_{C=O}$), 1035 (ν_{Cl}, *para*-aromatic), 825 (γ_{C-H}, aromatic); ^1H NMR ($CDCl_3$–CF_3COOD) δ 0.92 (3H, CH_3, t), 1.39 (4H, CH_2, m), 2.05 (2H, CH_2, t), 4.89 (2H, CH_2, m), 7.05, 7.31 (4H, aromatic, dd), 8.41 (1H, triazole CH, s), 9.20 (1H, triazole CH, s), 12.5 (1H, exchanged COOH proton in CF_3COOD); ^{13}C NMR ($CDCl_3$) δ 13 (CH_3), 22, 25, 32 (3 CH_2), 39 (C), 54 (NCH_2), 125 (4 aromatic C), 130 (aromatic C), 138 (aromatic CCl), 143, 150 (2 triazole CH), 173 (COOH); FAB-MS, m/z 308.21 $[M + H]^+$; EI-MS, m/z 307 $[M]^+$. Anal. Found: C, 58.6; H, 6.1; Cl, 11.3%. Calcd for $C_{15}H_{18}ClN_3O_2$: C, 58.54; H, 5.89; Cl, 11.52%.

Hapten Conjugation and Verification. The hapten (**3**) was conjugated to the carrier proteins conalbumin (CONA) and bovine serum albumin (BSA) through amide bonds by the active ester method (Tijssen, 1985). Thus, 100 mg (0.32 mmol) of **3** and 24 mg (0.35 mmol) of *N*-hydroxysuccinimide were dissolved in 7 mL of dry tetrahydrofuran, 82 mg (0.40 mmol) of dicyclohexylcarbodiimide (DCC) was added, the mixture was stirred at room temperature for 2 h, and the solid precipitate was filtered out. Two portions (3.5 mL) of the filtrate were added to two batches of 160 mg of CONA or BSA dissolved in a mixture of 17.5 mL of water and 1 mL of tetrahydrofuran. The solutions were stirred at 4 °C for 24 h. The products were dialyzed against water at 4 °C for 1 week, lyophilized, and stored at −20 °C.

Immunization and Serum Collection. Three female, 3-month-old New Zealand white rabbits were immunized intradermally with the immunogen **4b** (CONA conjugate). After the initial immunization with 0.1 mg of the immunogen in phosphate-buffered saline (PBS) and emulsified in Freund's complete adjuvant (1:1 v/v), injections of 0.15 mg of the immunogen in PBS and Freund's incomplete adjuvant (1:1 v/v) were given: the first three booster injections were at 3-week intervals and the subsequent ones at 1-month intervals. The rabbits were bled 1 week after each immunization, and after coagulation of the blood at 4 °C overnight, the serum was centrifuged at 2400*g* for 15 min. To demonstrate no related humoral immune response prior to immunization, preimmune sera were also collected from each rabbit.

Antibody Purification. The immunoglobulin fraction of the serum was collected by sodium sulfate precipitation. Thus, 2.03 g of sodium sulfate was added to a 10 mL batch of the crude serum under continuous stirring, and upon 1 h of stirring and 1 h of sedimentation, the white fluffy precipitate of the immunoglobulin fraction was centrifuged at 2400*g* for 15 min. The supernatant was decanted, and the precipitate was dissolved in 0.015 M phosphate buffer (pH 8.0) and extensively dialyzed (48 h) against the above buffer.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA determinations were performed in 96-well microplates following the basic solid phase immunoassay principle of Voller (1976). BSA conjugates in 0.1 M carbonate–bicarbonate buffer (pH 9.6) were used as plate coating antigens. After washing, sample or standard solutions and the antiserum diluted in PBS containing 0.05% Tween 20 and 1% gelatin (pH 7.4) (PBSTG) were dispensed into the wells and incubated. After another wash, bound antibodies were exposed to anti-rabbit IgG (1:2500 dilution in PBST) conjugated to horseradish peroxidase (HRP) and enzymatic activity was measured using 0.01 M hydrogen peroxide as a substrate and 3 mM *o*-phenylenediamine (OPD) as a chromophore in 0.5 M citrate buffer (pH

5.0). Analyte concentrations were measured indirectly by competition with the coating antigen for antibody binding sites. For standard curves, stock solutions of different triazole derivatives in methanol or in acetonitrile (10 mg/mL) were diluted from 1 mg/mL to 10 ng/mL in 1:4 dilution steps. Standard curves were calculated from the raw data using a four-parameter (sigmoidal) equation (Rodbard, 1981). The detection limit was defined as the lowest concentration of hapten showing a reduction of 3 standard deviations from the mean blank standard absorbance.

In the equilibrium test (Zettner, 1973), the antibody and hapten solutions were added to the coated plate without preincubation followed by incubation either at 4 °C for 4 h, at room temperature for 2 h, or at 37 °C for 1 h. Sequential tests (Zettner and Duly, 1974) were done by preincubating antibody and hapten in glass tubes at 4 °C and transferring aliquots to coated plates. The amount of specific antibody bound was detected as above.

Assay Optimization. *pH Effect.* The influence of pH on assay performance was studied by varying pH values between 4.4 and 9.4 in 0.5 pH increments with no analyte present or in the presence of 5 μg/mL myclobutanil. Assays were carried out in triplicates; standards were run in the usual buffer (0.2 M PBS, pH 7.5).

Solvent Effects. To test assay tolerance to various commonly used organic solvents (methanol, ethanol, acetone, acetonitrile, DMSO), the solvents were added to the assay buffer PBSTG (pH 7.4) at five concentration levels (starting with 1% and with an increment of 2.5). The assays were performed in the absence of the analyte or in the presence of 5 μg/mL myclobutanil. Assays were carried out in triplicates.

Sample Preparation for Matrix Effect Studies. *Water Samples.* Tap water and water from Putah Creek, Davis, CA, were used for matrix effect studies. The water from Putah Creek is very high in organic matter and is taken from a stagnant oxbow lake. To remove floating contaminants, the creek water sample was filtered through a Whatman No. 1 paper filter. After the pH of the water samples had been set to 7.4, the samples were spiked with myclobutanil to final concentrations of 167 and 70 μg/mL. The latter solution was serially diluted with the same unspiked sample. The concentration of myclobutanil was detected by standard competition ELISA.

Soil Samples. Three soil types (Yolo silt loam, 33% sand, 49% silt, 18% clay, organic carbon content, 1.05%; Sacramento clay, 68% sand, 30% silt, 2% clay, organic carbon content, 2.14%; Placer county Aiken complex, 64% sand, 29% silt, 7% clay, organic carbon content, 5.61%) were used for matrix effect studies. Soil extraction was carried out according to the recommended FAO/WHO procedure allowing 87–95% recoveries (FAO/WHO, 1992b), in a single-step methanol extraction without further cleanup steps. Briefly, 20 g of soil was shaken in a flask overnight at room temperature in 50 mL of methanol. The slurry was then centrifuged for 30 min at 13000*g*, decanted, and filtered through a Whatman No. 1 paper filter. The filtrate was evaporated and the residue reconstituted in 200 μL of methanol and diluted to 10 mL with PBS. These aqueous extracts were spiked with myclobutanil (167 and 70 μg/mL), diluted further with the same unspiked extract, and used for ELISA determination.

Produce Samples. Fruit extracts were prepared by the routinely used FAO/WHO procedure (FAO/WHO, 1992b) using apples and grapes, the two fruits on which myclobutanil is used to the greatest extent. Organically grown fresh produce samples were weighed (100 g) and homogenized with 20 mL of PBS in a Waring 7012 (New Hartford, CT) blender. The suspension was then centrifuged for 30 min at 13000*g*, decanted, and filtered through a Whatman No. 1 paper filter (affording 72 and 93 mL of filtrate for apple and grape, respectively). After the pH was adjusted to 7.4, the filtrates were spiked with myclobutanil (167 and 70 μg/mL), diluted further with the same unspiked extract, and used for ELISA determination. The determined concentration values of myclobutanil in different spiked matrices (using the standard curve obtained in buffer) along with statistical descriptors are shown in Table 1.

Determination of Cross-Reactivities. Cross-reactivities (CR) were calculated in relationship to myclobutanil. The data were obtained from standard curves of the tested compounds, listed in Table 2, and calculated as percentage ratio values based on the IC_{50} values of myclobutanil and that of the given compound.

RESULTS AND DISCUSSION

Hapten Synthesis and Conjugation. The structures of the haptenic compounds are shown in Figure 1. The nitrile group appeared to be unusually resistant to chemical hydrolysis, probably due to steric hindrance. Similarly, it appeared to be resistant even to catalytic hydrogenation. The hydrolysis using hydrogen peroxide and phase-transfer catalytic conditions resulted—although at a moderate yield—in the corresponding amide, and the acidic hydrolysis, using an exceedingly long reaction time, proceeded only to the amide stage. This amide, however, could be readily hydrolyzed by the nitrosation method used for unreactive amides (Olah and Olah, 1965; Challis and Challis, 1970).

The hapten (**3**) was conjugated to proteins (CONA, BSA) by the active ester method (Tijssen, 1985). A molar ratio of over 100 hapten molecules per mole of protein was used in the conjugation reactions for all conjugates based on the results of Jung *et al.* (1991). The quantity of the hapten coupled to the protein was determined by the back-titration of the free amino groups available on the protein before and after conjugation according to the method of Habeeb (1966) and was found to be 0.19 and 0.23 mmol of hapten/g of protein for the CONA and BSA conjugates, respectively.

Antibody Characterization. The titer, defined as the antiserum dilution that binds 50% of the antigen, was determined after the third immunization. Useful antiserum titers were obtained 6–8 months after the first immunization and resulted in average dilutions of 1000–1500. Although the antibody titer for all rabbits increased with repeated injections, a significant variance in the immune response was observed among the animals. Two of three rabbits immunized produced antibodies of significant affinity toward the hapten–homologous coating antigen, **4b**.

Because the titer of the crude antiserum appeared to be low, purification of the immunoglobulin fraction seemed to be desirable. As seen by the two-dimensional titration curves in Figure 2, the purification resulted in an approximately 2-fold concentration of the antibody; moreover, it provided better reproducibility and lower standard deviation of the determinations. The sensitivity of the resulting assay with the antibody, however, was not affected by purification of the antiserum.

The final working dilution of the antiserum was chosen from the titer curves (at 70% of the total activity). Thus, dilutions of 1:500 and 1:2000 were used for the crude and purified antibodies, respectively. For the anti-rabbit IgG conjugated to HRP, a dilution factor of 1:1000 was used.

Antibody Affinity and Inhibition. For inhibition tests in the chosen ELISA format, it was necessary to optimize parameters that can influence the antibody flexibility and binding to the hapten. The influence of the following factors was tested in assay buffer: pH (4.9–9.4); incubation temperatures of 4 °C, room temperature, and 37 °C; amount of the coating antigen (1–20 μg/mL); as well as the addition of 0.05% Tween 20 and gelatin.

Inhibition by myclobutanil appeared to be in the micromolar range, which is close to the sensitivity of similar immunoassays reported (Newsome, 1986; For-

Table 1. Statistical[a] and Regression[b] Analysis of Determination of Spiked Concentrations of Myclobutanil in Various Matrices

	detected concn (μg/mL)		
spiked concn (μg/mL)	tap water	creek water	water samples[c]
55.6	44.4 ± 2.3	61.9 ± 8.3	53.2 ± 8.8
18.6	12.2 ± 0.7	18.5 ± 1.2	14.2 ± 3.0
6.17	6.34 ± 0.35	6.94 ± 0.21	6.64 ± 0.42
2.06	2.47 ± 0.30	2.22 ± 0.12	2.35 ± 0.24
0.686	0.910 ± 0.116	0.881 ± 0.226	0.895 ± 0.180
0.229	0.660 ± 0.039	0.314 ± 0.072	0.487 ± 0.182
0.0762	0.236 ± 0.037	0.308 ± 0.082	0.272 ± 0.103
residual variance	$(s_r^2) = 0.009$	variance within groups	$(s_e^2) = 0.057$

	detected concn (μg/mL)			
spiked concn (μg/mL)	loam soil	clay soil	forest soil	soil[c]
55.6	26.76 ± 5.1	37.5 ± 2.4	36.9 ± 5.3	35.0 ± 5.7
18.6	12.7 ± 1.6	11.8 ± 2.2	10.1 ± 0.3	11.3 ± 1.7
6.17	5.48 ± 0.11	5.49 ± 0.40	6.16 ± 0.08	5.71 ± 0.40
2.06	1.97 ± 0.07	2.76 ± 0.11	2.81 ± 0.03	2.51 ± 0.39
0.686	0.917 ± 0.041	0.909 ± 0.021	0.862 ± 0.253	0.896 ± 0.150
0.229	0.465 ± 0.074	0.442 ± 0.015	0.259 ± 0.046	0.389 ± 0.086
0.0762	0.272 ± 0.029	0.299 ± 0.023	0.285 ± 0.097	0.286 ± 0.029
residual variance	$(s_r^2) = 0.475$		variance within groups	$(s_e^2) = 0.105$

	detected concn (μg/mL)		
spiked concn (μg/mL)	apple juice	grape juice	juice samples[c]
55.6	46.8 ± 2.3	nd[d]	46.8 ± 2.3
18.6	12.7 ± 1.3	24.9 ± 3.1	18.8 ± 3.6
6.17	7.99 ± 0.37	8.93 ± 0.71	8.46 ± 0.74
2.06	3.98 ± 0.04	4.29 ± 0.15	4.14 ± 0.19
0.686	1.13 ± 0.07	1.70 ± 0.03	1.42 ± 0.23
0.229	0.234 ± 0.017	0.191 ± 0.012	0.213 ± 0.026
0.0762	0.271 ± 0.058	0.275 ± 0.010	0.273 ± 0.041
residual variance	$(s_r^2) = 2.43$	variance within groups	$(s_e^2) = 0.221$

[a] Concentration values were calculated from assay signals in the given matrix using the four-parameter (sigmoid) standard curve obtained in buffer. Calculated values are expressed as average values ± the standard error of the mean. [b] Regression equations were calculated for the average value within each group between concentrations of 0.229 and 6.17 μg/mL. [c] Average values within groups for each concentration. [d] nd, not detected.

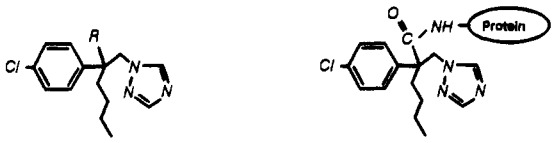
Table 2. Cross-Reactivity of the Polyclonal Antibodies Raised against 4a with Various Compounds

compound	cross-reactivity[a]	compound	cross-reactivity[a]
myclobutanil (**1**)	100	fluconazol (**17**)	nd
2-(4-chlorophenyl)-2-[(1,2,4-triazol-1-yl)methyl]-hexanoic amide (**2**)	354[b]	2,4-difluoro-α-[1-(1,2,4-triazolyl)acetophenone (**18**)	5.0
2-(4-chlorophenyl)-2-[(1,2,4-triazol-1-yl)methyl] hexanoic acid (**3**)	204	prochlorase (**19**)	7.9
hexaconazole (**5**)	14.5	imazalyl (**20**)	1.6
penconazole (**6**)	12.1	fenarimol (**21**)	nd
tetraconazole (**7**)	11.0	amitrole (**22**)	nd
propiconazole (**8**)	10.1	1,2,4-triazole	nd
imazalyl (**9**)	6.0	3-amino-5-mercapto-1,2,4-triazole	nd
triadimefon (**10**)	nd[c]	1*H*-1,2,4-triazole-3-thiol	nd
triadimenol (**11**)	nd	4-methyl-1,2,4-triazole-3-thiol	nd
bitertanol (**12**)	nd	(1,2,4-triazol-2-yl)acetic acid	nd
diclobutrazol (**13**)	(18.7)[d]	4-amino-1,2,4-triazole	nd
4-chloro-α-(1-imidazolyl)-α,α-dimethylaceto-phenone (**14**)	(30.9)	3-acetamido-1*H*-1,2,4-triazole	nd
α-[1-(1,2,4-triazol)yl]-α,α-dimethylacetophenone (**15**)	(19.9)	3-amino-1,2,4-triazole-5-carboxylic acid hemihydrate	nd
α-(1-imidazolyl)-α,α-dimethylacetophenone (**16**)	2.0	imidazole	nd
		L-histidine	(4.1)
		L-glutamic acid	nd

[a] Cross-reactivities are defined as the percentage ratio of the IC_{50} value (the concentration that inhibits the assay by 50%) of myclobutanil and of the given compound. IC_{50} values were calculated from a four-parameter fit and were not determined above 1 mg/mL concentration. [b] IC_{50} is above solubility limit. [c] Values in parentheses indicate percent inhibition at 1 mg/mL concentration. [d] nd, not detected.

lani *et al.*, 1992). Figure 3 illustrates the competitive inhibition tests with the purified antiserum under optimized conditions. The curve represents a mean of at least three replicates with a coefficient of variation of less than 7%, and the minimum detectable concentration was 0.2 μg/mL. The IC_{50} values of myclobutanil (**1**) and intermediates **2** and **3** were found to be 4.9, 1.4, and 2.4 μg/mL, respectively.

The sensitivity of the detection was found to be highly dependent on the amount of the sensitizing (coating) antigen applied (data not shown). Optimal sensitivity was obtained using plates coated with a low (5 μg/mL) concentration of the BSA conjugate (**4b**). Due to the relatively low affinity of the antibodies, several variations in assay conditions were tried. An excess of antibodies and a slight excess of the sensitizing (coating) antigen resulted in improved assay performance. In general, this procedure reduces the optimum theoretical sensitivity, but in this case it gave a better signal and improved the accuracy of the assay. Therefore, parallel experiments were carried out by applying 1 and 10 μg/mL of the sensitizing antigen, but the signal dramatically decreased in the former, while IC_{50} values for myclobutanil were significantly higher in the latter case.



1 R: CN

2 R: $CONH_2$

3 R: COOH

4a Protein: CONA

4b Protein: BSA

Figure 1. Structures of myclobutanil (**1**), the haptenic compounds (**2**, **3**), and the protein conjugates (**4**).

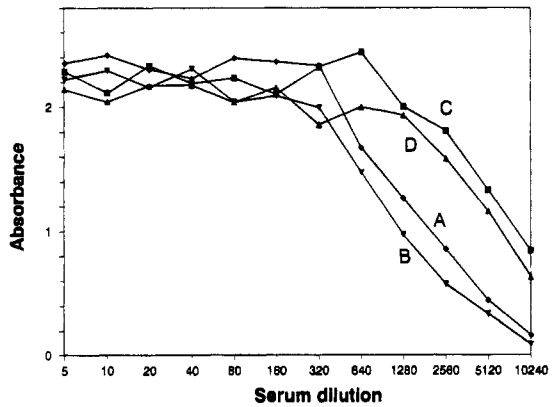


Figure 2. Two-dimensional titer of the antibodies raised against **4a**. Crude serum with the enzyme-labeled antibody was diluted 1:1000 (A) and 1:500 (B). Purified immunoglobulin fraction with the enzyme-labeled antibody was diluted 1:1000 (C) and 1:500 (D). The plate was coated with 5 μg/mL (500 ng/well) of **4b**. The determinations were carried out in triplicates.

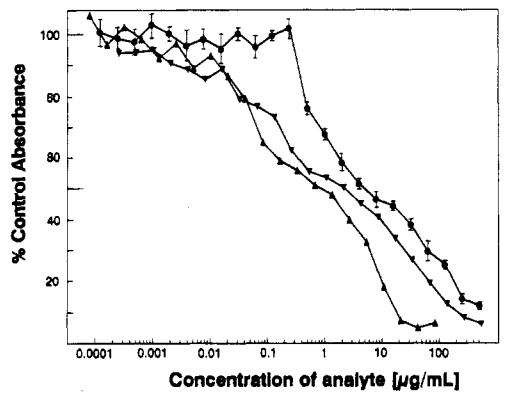


Figure 3. Competitive inhibition of the serum by myclobutanil (●), as well as haptenic derivatives **2** (▲) and **3** (▼) under optimized conditions, and the assay buffer containing 0.1% gelatin. ELISA was performed in a sequential test system using 5 μg/mL **4b** as coating antigen. Absorbance values are corrected for nonspecific binding and are the mean of at least three replicates carried out on the same day. Raw inhibition curves are plotted without curve fit. Standard deviations are shown for myclobutanil.

As expected, improved IC_{50} values were observed with increased serum dilutions, while the signal/noise ratio, in contrast, declined. A working dilution of 1:4000 for the purified antiserum appeared to be optimal, because the sensitivity of the detection was not significantly improved beyond this dilution factor. The detection of myclobutanil from spiked samples also indicated a detection limit (the minimal concentration of the analyte at which the signal produced exceeds 3 times the background) of 0.2–0.5 μg/mL.

Although the sensitivity of ELISA systems against various pesticides often ranges in the low parts per billion level (Marco *et al.*, 1993; Bekheit *et al.*, 1993; Schneider *et al.*, 1994), triazoles are known to be of low natural immunogeneity. In our hands, an aminotriazole herbicide, amitrole, was found to be a poor immune epitope (Jung *et al.*, 1991; Székács *et al.*, 1995). ELISA systems have been developed for the detection of triazole fungicides with more complex chemical structures, i.e. triadimefon (Newsome, 1986), tetraconazole, and penconazole (Forlani *et al.*, 1992), but the assays did not display cross-reactivity with 1,2,4-triazole. The latter assay developed against the BSA conjugate of 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propanol (DTP) also displayed IC_{50} values in the high parts per billion–low parts per million range. Moreover, the estimated detection limit of a GC/MS method for the determination of myclobutanil in various crop samples has been found to be 2 μg/mL (0.05 μg/g) (Liao *et al.*, 1991).

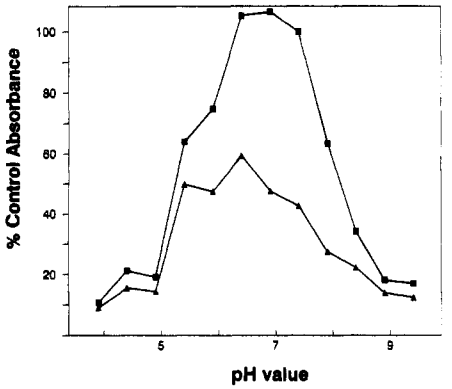


Figure 4. pH dependence of the interaction between the serum and the coating antigen **4b** (BSA conjugate) in the presence of no analyte (■) and in the presence of 5 μg/mL myclobutanil (▲). Absorbances are expressed as percent of the control absorbance at pH 7.4. The data are means of three replicates with standard deviations less than 20%.

pH Effect. The influence of the pH on assay performance was studied with no analyte present or at an analyte concentration causing 50% inhibition at pH 7.4 (5 μg/mL myclobutanil). Since the triazole ring can behave as a base only in relatively acidic conditions (pK_a = 2.19), the pH probably effects the antibody more than the analyte. The assay seemed to better tolerate basic than acidic conditions. As seen in Figure 4, the absorbance displayed a plateau between pH 6.4 and 7.4, above and below which values the signal intensity rapidly deteriorated. In addition to color development, the pH also effected inhibition: myclobutanil was less able to inhibit the assay. Due to the pH influence, standard inhibition curves could not be recorded at pH values below 5.4 and above 7.9.

Solvent Effects. Organic solvents frequently used for extraction were tested for their influence on assay performance. Figure 5 shows the effect of methanol, ethanol, acetone, and DMSO on the assay signal. All of these solvents displayed significant reduction of color development at concentrations over 8%. Methanol and ethanol were tolerated best, while acetone appeared to show a strong inhibitory effect on the assay. In contrast, DMSO caused a more intense color development at concentrations below 5% but was detrimental to color formation at higher levels. The effects of the above solvents were similar in the presence of 5 μg/mL myclobutanil, as well. Nevertheless, they had a strong influence on the inhibition by myclobutanil: 2.5% acetone content caused a 43% decrease in sensitivity, while methanol and ethanol appeared to be better tolerated. Apparently, the signal activation caused by DMSO with no analyte present was not seen in the presence of 5 μg/mL myclobutanil.

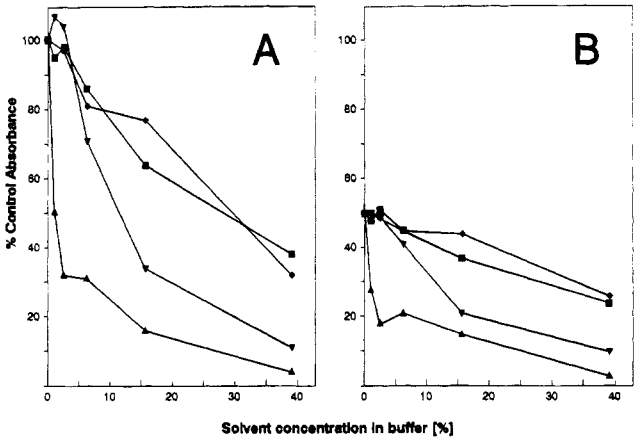


Figure 5. Effect of various solvents, e.g. methanol (■), ethanol (◆), acetone (▲) and DMSO (▼) on the interaction between the serum and the coating antigen **4b** (BSA conjugate) in the presence of no analyte (A) and in the presence of 5 μg/mL myclobutanil (B). Data points represent the average of three replicates with variation coefficients below 15%.

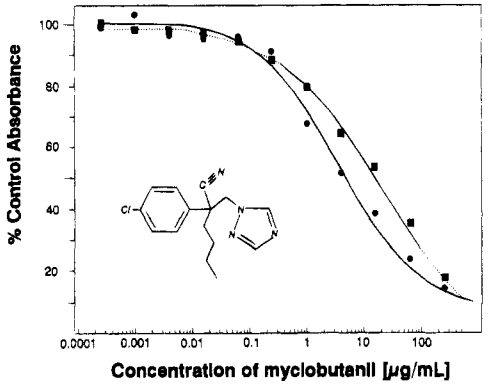


Figure 6. Standard curves of myclobutanil in PBST buffer (■) and with PBST buffer with 0.1% gelatin (●). Standard curves were obtained by a four-parameter curve fit. The addition of gelatin provided a steeper standard curve and decreased nonspecific binding which, in turn, allowed for longer incubation time and better signal/noise ratio. The data are corrected for background and nonspecific binding and are the average of triplicate determinations.

In contrast to other immunoassays in which the organic solvent content of the media affects assay sensitivity rather than assay signal (Marco *et al.*, 1993; Schneider *et al.*, 1994), our findings indicate unusually strong solvent sensitivity of the assay signal, while the apparent IC_{50} value appeared to be less affected.

Effect of Additives. On the basis of the previous observations of Pagani and co-workers (Forlani *et al.*, 1992), the effects of a buffer protein stabilizer were also investigated. Assay sensitivity to myclobutanil was favorably affected by the addition of gelatin to the assay buffer. This additive resulted in a steeper standard curve with an apparent 3-fold decrease in the IC_{50} value from about 15 to 4.9 ± 0.5 μg/mL. Optimal effect was seen at 0.1−1% gelatin content. Figure 6 shows standard curves in the presence and absence of gelatin in the assay buffer.

Sample Matrix Effects. To study possible matrix effects from various samples including surface water, soil extracts, and fruit extracts and to estimate analyte recoveries, spiked samples were tested for assay performance. Water samples were used undiluted and without purification except for a filtration step for creek water. Since the pH of the water samples (8.2 and 9.1 for tap and creek water, respectively) was slightly alkaline, a pH adjustment was required. The water

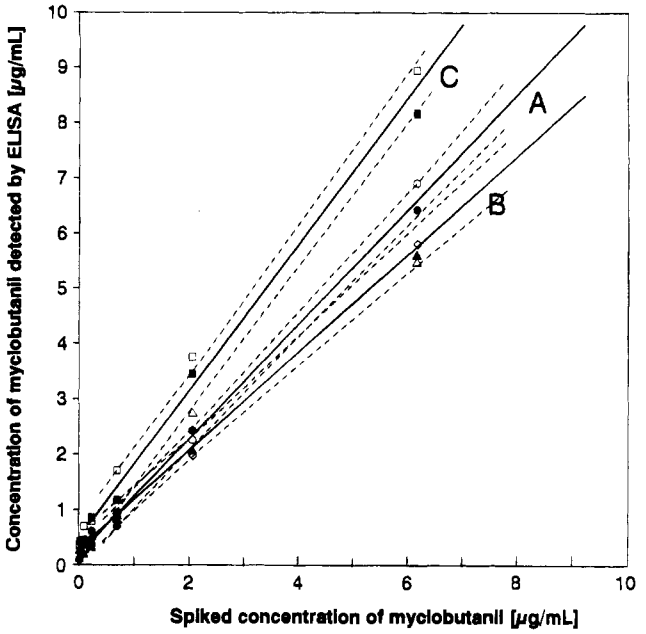


Figure 7. Correlation between the spike level of myclobutanil in different matrices and the concentration detected by ELISA. Matrices included water samples (A), e.g. tap water (●) and creek water (○), aqueous soil extracts (B) from loam (▲), clay (△) and forest (◇) soil, as well as fruit extracts (C) of apple (■) and grape (□). Dotted lines indicate 95% confidence belt for each line.

samples were then spiked above (333 μg/mL) and below (140 μg/mL) the solubility limit of myclobutanil (142 μg/mL at 25 °C) (FAO/WHO, 1992b), and serial dilution was carried out, resulting in a final concentration range between 25 ng/mL and 167 μg/mL.

To test possible matrix effects in soil, three different soil types were used, ranging from low to extremely high organic matter content. Yolo silt loam, a well-drained soil, was collected from a Sacramento Valley field; Sacramento clay, a poorly drained, fine-textured soil, was from the flood plain of the Sacramento river; and Aiken Complex, a forest red soil rich in plant roots and leaf parts, was from the Tahoe National Forest. All soil samples were collected from the upper 15 cm of soil, air-dried, passed through a 2 mm sieve, and homogenized prior to extraction. Because the required limit of determination for chromatographic methods in soil is 10 μg/kg (FAO/WHO, 1992b), soil extracts had to be concentrated prior to ELISA determination. For this purpose a 5-fold concentration step was carried out. According to previous supervised trials (FAO/WHO, 1992b), myclobutanil residue levels are 0.01−0.46 and 0.01−1.52 mg/kg in apple and grape, respectively. Consequently, similar concentration of the fruit extracts was also necessary. Because the fruit extracts were rather acidic (pH 3.8 and 4.0 for apple and grape, respectively), the pH had to be adjusted to 7.4 prior to spiking with myclobutanil.

Table 1 lists spiked and estimated myclobutanil concentrations in various matrices with statistics on each matrix and within matrix groups. Concentration values were estimated in each sample using a four-parameter calibration curve obtained in measurements in buffer.

Comparison of correlations between spiked levels of myclobutanil and the corresponding concentrations detected by the present ELISA system (Figure 7) gave three slightly different correlation lines described below:

water

$$c_{\text{detected}} = 1.040c_{\text{spiked}} + 0.214$$

$$n = 16 \quad r = 0.994 \quad F = 1100 \quad s = 0.294$$

soil

$$c_{\text{detected}} = 0.882c_{\text{spiked}} + 0.358$$

$$n = 24 \quad r = 0.985 \quad F = 738 \quad s = 0.373$$

fruit

$$c_{\text{detected}} = 1.332c_{\text{spiked}} + 0.511$$

$$n = 16 \quad r = 0.977 \quad F = 299 \quad s = 0.733$$

In these equations, c means concentration, n represents the number of data points used to derive the equation, r is the correlation coefficient, F is the F statistic for significance of the regression of the average values at 95% confidence level, and s is the standard deviation from regression.

As seen from the 95% confidence belts (depicted in Figure 7), the regression line for soil samples was statistically different from that for water samples only above 4.7 μg/mL, while the regression line for fruit extracts was statistically different from that for water at the 95% confidence level. Regression equations and regression statistics are shown in Table 1. Standard deviations were best for water samples and worst for fruit extract samples. Residual variance values (s_r^2) for soil and fruit extract samples were greater than errors within groups (s_e^2). The intercept was greater than zero for all three lines, but when forced through the origin (zero), regression slopes were 1.085, 0.957, and 1.440 for water, soil extract, and fruit extract samples.

Water samples provided optimal assay performance, while myclobutanil content was slightly underestimated in soil extracts and overestimated in fruit extracts as calibrated in buffer. Nevertheless, the correlation coefficient was over 0.97 even for fruit extracts. It is important to emphasize that these regression equations were obtained using a calibration curve with mycobutanil in buffer. Obviously, myclobutanil residue analysis using standard curves obtained in the actual matrix (free from the analyte) provides better estimates. Assay sensitivity (IC_{50}) appeared to be virtually unaffected by these matrices.

Cross-Reactivities by Related Compounds. As expected, the haptenic analogues—regardless of whether crude or purified antiserum was used—exceeded myclobutanil in their inhibitory potencies. The amide derivative (**2**)—being closer in structure to the immunizing antigen (**4**) than myclobutanil—showed similar concentration dependence but a 3.5-fold lower IC_{50} value. The IC_{50} value of the haptenic compound with a carboxyl moiety (**3**) appeared to be over 2 times lower than that of myclobutanil.

Although partial inhibition at high concentrations was shown, no significant cross-reactivity with the triazole fungicides studied (the structures seen in Figure 8) was found. The commercial triazole fungicides hexaconazole, penconazole, tetraconazole, and propiconazole displayed cross-reactivities below 15%. These results are in agreement with the similarly low cross-reactivity (0.2%) of the anti-tetraconazole antibodies (Forlani *et al.*, 1992) with myclobutanil (Székács and Pagani, unpublished results). A triazole fungicide used in humans, fluconazol (**17**) did not cross-react at all, while its pharmaceutical intermediate (**18**) closer in chemical structure (containing only one triazole ring) displayed a low (5%) cross-reactivity.

The affinity of the serum toward certain imidazole and triazole analogues of metyrapone (Bélai *et al.*, 1988) revealed an interesting detail concerning the specificity of the present polyclonal antibodies toward the triazole

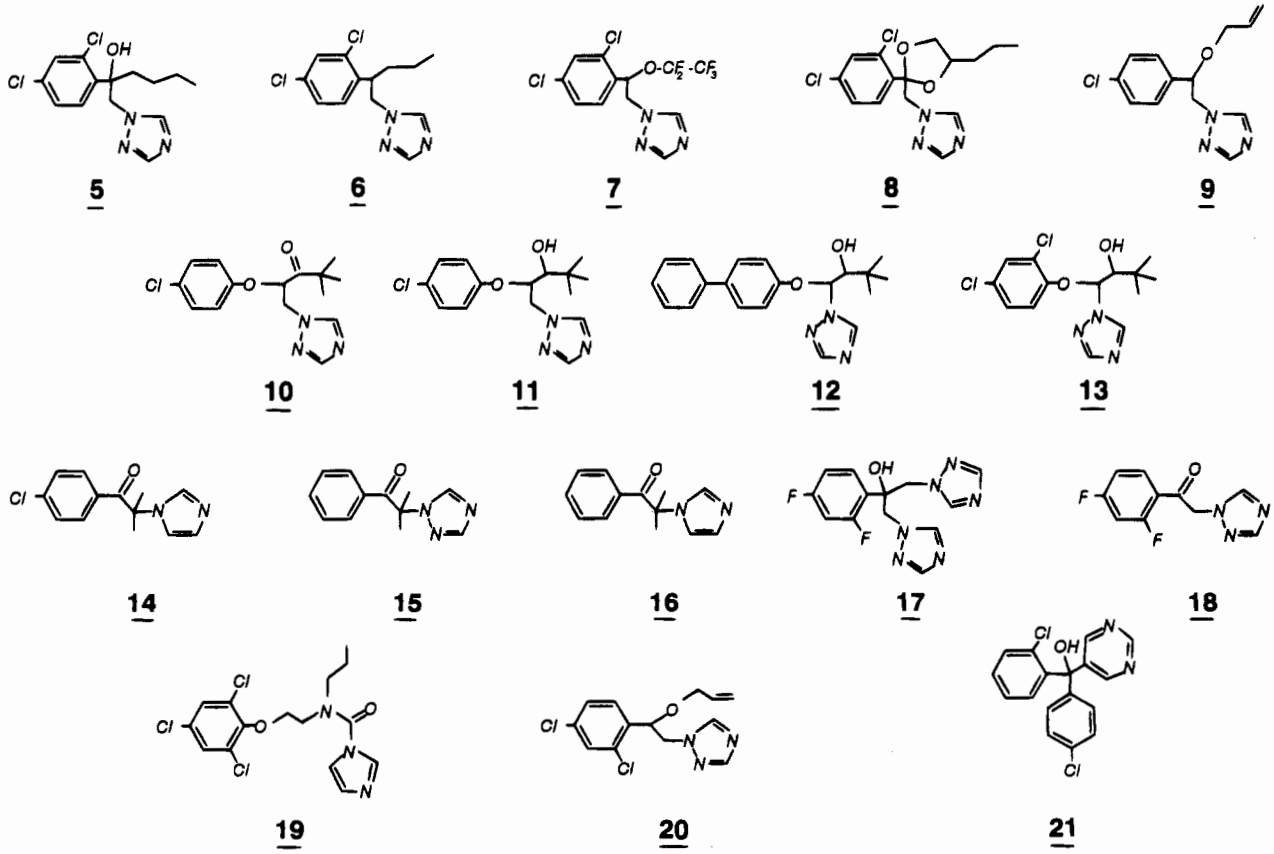


Figure 8. Structures of the azole fungicides, pharmaceuticals, and intermediates tested for cross-reactivity.

ring, because a significant increase in binding to the appropriate analogue with a triazole ring (**15**) was observed compared to the one containing an imidazole moiety (**16**). This effect seemed to exceed sensitivity toward the *p*-chlorophenyl moiety (**14**).

No cross-reactivity was detected for the structurally related small single-ring triazole derivatives in Table 2. These results conclusively show that—even though a certain range of antibody specificity for the triazole ring has been shown—the triazole moiety is not large enough or characteristic in electronic properties to be solely recognized by B-lymphocytes. These results are in accordance with the earlier results on ELISA systems against triadimefon and triadimenol (Newsome, 1986) and against tetraconazole and penconazole (Forlani *et al.*, 1992), as well as against aminotriazoles (Jung *et al.*, 1991; Székács *et al.*, 1995) for which no cross-reaction with triazole has been found.

Conclusion. An ELISA method has been developed and optimized for the detection of myclobutanil. Although the assay is of relatively low sensitivity, the detection limit appears to be similar to that reported in a GC/MS method (Liao *et al.*, 1991), although somewhat higher than that reported for an ELISA to detect related triazole fungicides, tetraconazole, penconazole, and intermediate DTP (Forlani *et al.*, 1992).

Assay optimization studies revealed the sensitivity of the antiserum to pH and organic cosolvents, while the application of a protein stabilizer, gelatin, resulted in a 3-fold increase in assay sensitivity.

The structures of different triazole compounds that bind the antibody provided information about antibody specificity. From those data, at least the obvious conclusions can be drawn that (a) the binding to the antibody follows amply different structure–activity relationships from the biological activity on sterol demethylase (Fujimoto *et al.*, 1988) and (b) none of the compounds studied—except the haptens that are not present in environmental samples—would significantly interfere with the detection of myclobutanil.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent immunoassay; IC_{50}, 50% inhibitory concentration; HPLC, high-performance liquid chromatography; GC, gas chromatography; LD_{50}, 50% lethal dose; TLC, thin layer chromatography; IR, infrared spectroscopy; NMR, nuclear magnetic resonance spectroscopy; FAB-MS, fast atom bombardment mass spectroscopy; EI-MS, electron ionization mass spectroscopy; CONA, conalbumin; BSA, bovine serum albumin; DCC, dicyclohexylcarbodiimide; PBS, phosphate-buffered saline; Tween 20, polyoxyethylene sorbitan monolaurate; PBSTG, PBS with 0.05% Tween and 1% gelatin; PBST, PBS with 0.05% Tween; IgG, immunoglobulin G; HRP, horseradish peroxidase; OPD, *o*-phenylenediamine; DMSO, dimethyl sulfoxide; CR, cross-reactivity; DTP, 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propanol.

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Registry No. Supplied by the Author: Myclobutanil, 88671-89-0; 4-amino-1,2,4-triazole, 584-13-4; imidazole, 288-32-4; L-histidine, 71-00-1; L-glutamic acid, 56-86-0.

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