An Enzyme Immunoassay for the Environmental Monitoring of the Herbicide Bromacil†

Hassan K. M. Bekheit,‡ Anne D. Lucas, Ferenc Szurdoki,§ Shirley J. Gee, and Bruce D. Hammock*

Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616

Competitive enzyme-linked immunosorbent assays (ELISAs) were devised for the environmental monitoring of the herbicide bromacil. The polyclonal antibodies used in this work were raised against two haptens. The bromacil molecule was derivatized at the N-1- and 6-methyl-positions to obtain these haptens with carboxyalkyl [$(CH_2)_nCO_2H$] spacer arms. The antibodies have been examined in several immunoassay formats. Two additional haptens were also synthesized and used for the preparation of coating antigens and enzyme tracers. Some of the heterologous indirect ELISAs in a coating antigen format showed promising sensitivities and, with only a few exceptions, slight cross-reactivities with a series of bromacil metabolites and related compounds. The best sensitivity (IC_{50} = 0.25 ppb) and specificity were achieved with a system using antibodies derived from the hapten bearing the handle at the 6-methyl group (n = 1) and coating antigen synthesized from hapten with the bridging group at the N-1-position (n = 5). Further investigations were performed with this ELISA. Changing the pH value in the range 5–8.5 did not influence the sensitivity of the optimized assay. Human urine, however, exercised a strong effect on sensitivity, which varied from sample to sample. Organic solvents also affected assay sensitivity; nevertheless, IC_{50}s remained below 11 ppb with solvent concentrations up to 12.5%. Water samples spiked with bromacil were analyzed by ELISA. The results showed excellent correlation to spiked amounts at levels of 0.1–160 ppb. Soil samples fortified with bromacil were extracted with 1% aqueous NaOH, and then the obtained solutions were simply diluted with the assay buffer and analyzed by ELISA. Recoveries in the concentration range 0.04–20 ppm were between 92.5 and 102.5%. The direct ELISAs in enzyme tracer formats did not perform better than the heterologous coating antigen assays. However, use of this format in homologous assays dramatically improved the sensitivity from poor inhibition to IC_{50}s of 3–10 ppb.

INTRODUCTION

Bromacil [5-bromo-6-methyl-3-(1-methylpropyl)-2,4-(1*H*,3*H*)-pyrimidinedione (1, Table I)] is the active ingredient of Hyvar, a major herbicide used on citrus and pineapple as a selective weed killer as well as on noncrop land for general vegetation control (Gardiner, 1975; Worthing and Hance, 1991). Considerable interest has been focused on the environmental monitoring of this compound as well as the study its transport processes in environmental elements (Alva and Singh, 1990; U.S. Environmental Protection Agency, 1990; Worthing and Hance, 1991; Allender, 1991; Jaynes, 1991; Reddy et al., 1992) because of its wide application and recent concerns about health hazards (Garrett et al., 1986; Call et al., 1987; Parent et al., 1990). Most chromatographic residue analyses for bromacil at trace levels require laborious cleanup procedures or expensive instrumentation (Gardiner, 1975; Bennett and de Beer, 1984; Putzien, 1987; Goewie and Hogendoorn, 1987; Fröhlich and Meier, 1989; Lipschitz et al., 1989; Wylie and Oguchi, 1990; Stan and Heil, 1991; Tuinstra et al., 1991; Foster et al., 1991). An immunochemical determination could provide a simpler approach for detection of this pesticide at trace levels (Hammock and Mumma, 1980; Gee et al., 1988; Schlaeppi et al., 1989; Goodrow et al., 1990; Li et al., 1991; Forlani et al., 1992). Recently, we reported syntheses of haptens and conjugates and the preliminary results of an immunoassay for bromacil (Szurdoki et al., 1992). In the present study, several ELISAs were used to compare the sensitivity and selectivity of antisera from rabbits immunized with different bromacil derived conjugates. In addition, the polyclonal antibodies were examined in several immunoassay formats. The method of numbering of the compounds in Tables I and II and throughout this work conforms to that used by Szurdoki et al. (1992).

* Author to whom correspondence should be addressed.

† This work was supported in part by the California Department of Food and Agriculture, NIEHS Superfund Grant ES04699-01, the University of California, Systemwide Toxic Substance Program (Ecotox), U.S. EPA Cooperative Agreement CR-814709-01-0, EPA Center for Ecological Health Research CR819047-01-0, and NIEHS Center for Health Effects of Agrochemicals 1P30 ES05707. H.K.M.B. was supported by U.S. AID. B.D.H. is a Burroughs Wellcome Toxicology Scholar.

‡ Permanent address: Plant Protection Research Institute, A.R.C., Dokki Giza, Nadi El-Syd, Egypt.

§ Permanent address: Plant Protection Institute, Hungarian Academy of Sciences, P. O. Box 102, H-1525 Budapest, Hungary.

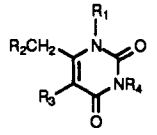
MATERIALS AND METHODS

Reagents. Solvents and chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI). 3,3′,5,5′-Tetramethylbenzidine (TMB) and immunochemicals were purchased from Sigma Chemicals Co. (St. Louis, MO) if not otherwise stated. Horseradish peroxidase (HRP) was obtained from Boehringer Mannheim (Indianapolis, IN). Alkaline phosphatase (AP) and goat anti-rabbit IgG were obtained from Miles Laboratory (Elkhart, IN). Samples of bromacil, terbacil, and their metabolites were supplied by E. I. du Pont de Nemours & Co. (Wilmington, DE). A Sybron/Barnstead Nanopure II water purification system provided water (16.7 megohm/cm) for water spiking experiments and for the preparation of aqueous buffers.

Apparatus. ELISA experiments were carried out in 96-well microplates (Nunc 442402) and read with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode

0021-8561/93/1441-2220$04.00/0

Table I. Structure of Bromacil, Terbacil, and Related Compounds: Cross-Reactivities of ELISAs Including Anti-Bromacil Antibodies (Format 1)[a]



					Ab 2365 cAg 3eo		Ab 2369 cAg 3eo		Ab 2370 cAg 3eo		Ab 2005 cAg 2cb	
compd	R_1	R_2	R_3	R_4	IC_{50}, ppb	CR, %	IC_{50}, ppb	CR, %	IC_{50}, ppb	CR, %	IC_{50}, ppb	CR, %
1	H	H	Br	$CH(CH_3)C_2H_5$	1.56	100	4.77	100	1.64	100	0.247	100
6	H	OH	Br	$CH(CH_3)C_2H_5$	37.6	4.14	19.8	24.1	31.7	5.24	7.54	3.28
7	H	H	Br	$CH(CH_3)CH(OH)CH_3$	654	0.34	131	3.64	158	1.04	828	0.030
8	H	H	Br	$CH(CH_3)CH_2CH_2OH$	729	0.21	558	0.85	515	0.32	164	0.15
9	H	H	H	$CH(CH_3)C_2H_5$	490	0.32	561	0.85	502	0.33	37.3	0.66
4	H	H	Cl	$C(CH_3)_3$	0.414	377	2.40	199	13.1	12.5	7.58	3.26
11	H	OH	Cl	$C(CH_3)_3$	29.0	5.38	14.2	33.6	743	0.22	63.0	0.39
2a	$(CH_2)_5CO_2C_2H_5$	H	Br	$CH(CH_3)C_2H_5$	23.8	6.55	41.3	11.6	31.8	5.16	952	0.026
2b	$(CH_2)_5CO_2H$	H	Br	$CH(CH_3)C_2H_5$	7.38	21.1	0.572	827	0.577	284	56.4	0.44
2c	$(CH_2)_5$CONH–PROT	H	Br	$CH(CH_3)C_2H_5$	NT	NT	NT	NT	NT	NT	NT	NT
2d	CH_3	H	Br	$CH(CH_3)C_2H_5$	1.25	125	0.761	627	0.196	837	414	0.060
2e	$(CH_2)_3CH_3$	H	Br	$CH(CH_3)C_2H_5$	68.5	2.28	52.3	9.12	44.0	3.73	8020	0.003
2f	$CH_2CO_2CH_3$	H	Br	$CH(CH_3)C_2H_5$	2.23	70.0	4.24	113	3.55	46.2	NT	NT
2g	CH_2CO_2H	H	Br	$CH(CH_3)C_2H_5$	NT	NT	NT	NT	NT	NT	NT	NT
2h	CH_2CONH–PROT	H	Br	$CH(CH_3)C_2H_5$	NT	NT	NT	NT	NT	NT	NT	NT
3a	H	Br	Br	$CH(CH_3)C_2H_5$	8.20	1.99	55.0	8.67	33.0	4.96	3.38	7.31
3b	H	$CH(CO_2C_2H_5)_2$	Br	$CH(CH_3)C_2H_5$	528	0.30	1250	0.38	948	0.17	NT	NT
3c	H	$CH(CO_2H)_2$	Br	$CH(CH_3)C_2H_5$	NT	NT	NT	NT	NT	NT	56.4	0.44
3d	H	CH_2CO_2H	Br	$CH(CH_3)C_2H_5$	NT	NT	NT	NT	NT	NT	12.2	2.03
3e	H	CH_2CONH–PROT	Br	$CH(CH_3)C_2H_5$	NT	NT	NT	NT	NT	NT	NT	NT
12a	H	H	Br	$CH(CH_3)CH_2CH_2O_2C(CH_2)_3CO_2H$	NT	NT	NT	NT	NT	NT	NT	NT
12b	H	H	Br	$CH(CH_3)CH_2CH_2O_2C(CH_2)_3$CONH–PROT	NT	NT	NT	NT	NT	NT	NT	NT

[a] IC_{50} values were calculated on the basis of a 10-point standard curve (four well replicates at each concentration). The ELISA system utilizing Ab 2005 and cAg **2cb** showed no cross-reactivity (IC_{50} > 10 000 ppb) for a series of related heterocyclic compounds [e.g., 2,4(1*H*,3*H*)-pyrimidinedione, 5-bromo-2,4(1*H*,3*H*)-pyrimidinedione, 5-methyl-2,4(1*H*,3*H*)-pyrimidinedione, 5,6-diamino-1,3-dimethyl-2,4(1*H*,3*H*)-pyrimidinedione, thymidine, uridine, caffeine, 1-methyluric acid]. NT indicates not tested. Antibody dilutions used were 1/2000, 1/4000, 1/4000, and 1/4000 for Ab 2365, 2369, 2370, and 2005, respectively. Coating antigen concentrations used were 5, 5, 5, and 1.25 μg/mL for Ab 2365, 2369, 2370, and 2005, respectively.

(405–650 nm). The software package Softmax (Molecular Devices) was used for fitting the 12-point sigmoidal standard curves based on a four-parameter logistic method described by Rodbard (1981) and for interpolation of unknown sample values. Melting points uncorrected were taken on a Thomas-Hoover capillary apparatus. UV spectra were recorded on a DU-6 spectrometer (Beckman Instruments, Inc., Palo Alto, CA). Infrared spectra were measured on an IR/32 FTIR spectrophotometer (IBM Instruments, Inc.); wavenumber (cm^{-1}) values are given. ^1H and ^{13}C NMR spectra were obtained with a QE-300 spectrometer (General Electric, 300 MHz for ^1H and 75 MHz for ^{13}C nuclei). Chemical shifts (δ) are given relative to tetramethylsilane as internal reference. Electron impact mass spectra were determined on a Trio 2 (VG Masslab, Altrincham, U.K.) apparatus at 70 eV; data are reported as *m/z* (relative intensity).

Synthesis of Haptens. The homogeneity and the structure of the compounds were verified by TLC and spectral investigations. We previously described strategies for syntheses of haptens **2b** and **3d** (Table I) (Szurdoki et al., 1992). Substances **2f** and **2g** (Table I) were obtained by methods very similar to the syntheses of related compounds **2a** and **2b** reported earlier (Szurdoki et al., 1992). Bromacil (**1**) was alkylated with methyl bromoacetate in the presence of NaH. The resulting ester (**2f**) was then hydrolyzed by NaOH in wet THF to the corresponding acid (**2g**). Spectral and physical characteristics are as follows.

Methyl [5-Bromo-6-methyl-3-(1-methylpropyl)tetrahydro-2,4-dioxopyrimidin-1-yl]acetate (**2f**). The compound was obtained as a colorless oil: IR (NaCl) 1753, 1705, 1657, 1612, 1432, 767 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.83 (t, *J* = 7.5 Hz, 3 H), 1.43 (d, *J* = 7 Hz, 3 H), 1.77 (m, 1 H), 2.05 (m, 1 H), 2.44 (s, 3 H), 3.80 (s, 3 H), 4.69 (s, 2 H), 4.95 (m, 1 H); MS (EI) 277 (100), 279 (99), 303 (4), 305 (2), 317 (4), 319 (2), 332 (35, M^+), 334 (32, M + 2).

[5-Bromo-6-methyl-3-(1-methylpropyl)tetrahydro-2,4-dioxopyrimidin-1-yl]acetic Acid (**2g**). The acid was a crystalline product: mp 118–119 °C; IR (KBr) 3500–2500, 1714, 1626, 1454, 1186, 766 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.82 (t, *J* = 7.5 Hz, 3 H), 1.43 (d, *J* = 7 Hz, 3 H), 1.77 (m, 1 H), 2.04 (m, 1 H), 2.46 (s, 3 H), 4.72 (s, 2 H), 4.95 (m, 1 H), 7.85 (b, 1 H); ^{13}C NMR ($CDCl_3$ δ 11.12, 17.32, 20.39, 25.85, 46.87, 54.01, 99.80, 148.83, 150.95, 159.05, 172.00; MS (EI) 263 (100), 265 (95), 289 (3), 291 (3), 301 (2), 303 (4), 305 (2), 318 (4, M^+), 320 (3, M + 2).

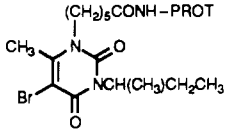
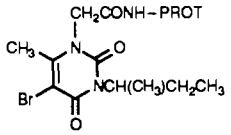
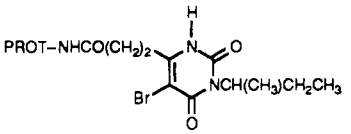
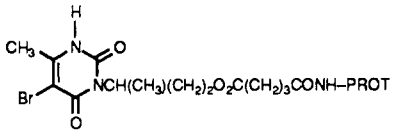
3-(5-Bromo-6-methyltetrahydro-2,4-dioxopyrimidin-3-yl)-3-methyl-1-propyl Hydrogen Pentanedioate (**12a**, Table I). Compound 8 (33.3 mg, 120 μmol) was partly dissolved in 2.5 mL of warm dry dichloromethane. Dry pyridine (9.5 mg, 144 μmol), glutaric anhydride (97%, 16.9 mg, 144 μmol), and 4-(dimethylamino)pyridine (1.5 mg, 12 μmol) were added to this solution at room temperature. The reaction mixture was stirred at ambient temperature under nitrogen for a day. HCl (1 N, 5 mL) was added, and the product was extracted with ether (4 × 5 mL), dried, evaporated, and purified by preparative TLC (EtOAc/acetic acid 100:1) to provide 31 mg (66%) of crystalline product: mp 124–126 °C; IR (KBr) 3400–2500, 1736, 1711, 1658, 1631, 1431, 769 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.48 (d, *J* = 7 Hz, 3 H), 1.85–2.10 (m, 4 H), 2.25–2.67 (m, 4 H), 2.33 (s, 3 H), 3.98 (m, 1 H), 4.16 (m, 1 H), 5.12 (m, 1 H), 10.3–11.4 (b, 2 H); MS (EI) 42 (100), 187 (30), 204 (52), 205 (68), 206 (58), 207 (66), 243 (24), 245 (25), 258 (58), 260 (57), 311 (2.4), 390 (1.5 M^+), 392 (1.4, M + 2).

Hapten–Protein Conjugates. The preparations of conjugates **2c** and **3e** (Tables I and II) by the mixed anhydride method were described previously (Szurdoki et al., 1992).

Conjugates of Hapten 2g (**2h**). The procedure of coupling acid **2g** (14.5 mg, 45 μmol dissolved in DMF, divided into five aliquots) to BSA, CONA, KLH, OVA, and THY (15 mg of each) to obtain protein conjugates **2hb**, **2hc**, **2hk**, **2ho**, and **2ht** (Tables I and II) was essentially identical with those reported earlier for coating antigens **2c** and **3e** (Szurdoki et al., 1992).

Conjugates of Hapten 12a (**12b**). Acid (**12a**) (9.9 mg, 25 μmol), monosodium salt of (±)-1-hydroxy-2,5-dioxo-3-pyrrolidinesulfonic acid (*N*-hydroxysulfosuccinimide, **13**) (Aldrich, 97%, 9.0 mg, 40 μmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (5.8 mg, 30 μmol) were added to dry DMF (2 mL).

Table II. Composition of Protein Conjugates

structure		carrier protein	conjugate
2c		BSA	**2cb**
		CONA	**2cc**
		KLH	**2ck**
		OVA	**2co**
2h		BSA	**2hb**
		CONA	**2hc**
		KLH	**2hk**
		OVA	**2ho**
		THY	**2ht**
3e		BSA	**3eb**
		KLH	**3ek**
		OVA	**3eo**
12b		BSA	**12bb**
		CONA	**12bc**
		KLH	**12bk**
		OVA	**12bo**

The mixture was stirred under nitrogen overnight, divided into four equal aliquots, and then added dropwise to the stirred protein solutions [12 mg each of BSA, CONA, KLH, and OVA in 3 mL of PBS (phosphate-buffered saline)] at 4 °C. The reaction mixtures (**12bb**, **12bc**, **12bk**, and **12bo**, Tables I and II) were stirred at 4 °C for 0.5 h and at room temperature overnight and then dialyzed exhaustively against PBS.

Hapten–Enzyme Conjugate. The enzyme tracer was prepared as follows: Hapten **2b** (5.0 mg, 13 μmol), *N*-hydroxysuccinimide (1.7 mg, 15 μmol), and 1,3-dicyclohexylcarbodiimide (6.2 mg, 30 μmol) were dissolved in 130 μL of dry DMF and stirred for 4 h at room temperature. The mixture was centrifuged; then the supernatant was added slowly to a stirred solution of HRP (2 mg of enzyme of 1490 units/mg activity, 24 839 nkat, 0.05 μmol) in 3 mL of 0.13 M sodium bicarbonate at 4 °C. The reaction mixture was stirred at 4 °C for 12 h and subsequently dialyzed exhaustively against 0.1 M sodium borate buffer (pH 7.8).

Production of Polyclonal Anti-Bromacil Antibodies. Anti-bromacil antisera were obtained following the protocol reported earlier (Szurdoki et al., 1992). Briefly, three New Zealand white rabbits (2–4 kg) were used for each immunizing antigen. The antigen solutions (3 × 100 μg dissolved in physiological NaCl solution) were emulsified with Freund's complete adjuvant (1:2 volume ratio) and injected intradermally on the back for 2 weeks (one injection a week). After 1 week, the animal was boosted with an additional 100 μg of antigen in physiological NaCl solution and Freund's incomplete adjuvant (1:2 volume ratio) and bled 10 days later. If the resulting antibody displayed sufficiently high titer, boosting and bleeding continued as above for 10 days. The serum was isolated by centrifugation and stored at −20 °C. The results of antibody characterization are obtained from sera of terminal bleeds.

ELISA Procedures. *Indirect ELISA with Coating Antigen (Format 1).* The method was similar to that previously described briefly by Szurdoki et al. (1992). Each well of a 96-well microplate was coated with 100 μL of the appropriate coating antigen concentration in 0.1 M carbonate–bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Different concentrations of bromacil or other potential inhibitors in PBSTA (phosphate-buffered saline plus Tween 20, azide) were mixed with the appropriate dilution of the anti-bromacil antibody and incubated at room temperature overnight in a separate, uncoated microtiter plate. The coated plates were washed with PBSTA. The inhibitor/anti-bromacil antibody mixture was added to the coated plate (100 μL/well). The plates were incubated at room temperature for 2 h and then washed thoroughly. Goat anti-rabbit IgG conjugated to AP diluted in PBSTA buffer (1/2500) was added (50 μL/well), and the plates were incubated at room temperature for 2 h. After the plates were washed, phosphatase activity was measured by adding 100 μL/well of *p*-nitrophenyl phosphate (1 mg/mL) in 10% diethanolamine buffer (pH 9.8). The plates were incubated for 30 min at room temperature, and then absorbance at 405–650 nm was measured. The dose–response curves were analyzed to calculate IC_{50} values, i.e., parts per billion (micrograms per liter) concentrations giving 50% inhibition.

Direct ELISA Using Enzyme Conjugates (Format 2). Format 2A. The plates were coated with antibody diluted (1:8000) in carbonate–bicarbonate buffer (pH 9.6, 100 μL/well), incubated overnight at 4 °C, and finally washed with PBSTA. The enzyme tracer, prepared from HRP above, mixed with the different concentrations of bromacil in PBSTA was then added to the antibody-coated plates (100 μL/well) and incubated at room temperature for 25 min. The plates were washed, and then 100 μL/well of TMB stock solution (a mixture of 200 μL of 6 mg/mL TMB solution in DMSO, 50 μL of 1% aqueous H_2O_2 solution, and 12.5 mL of 0.1 M sodium acetate buffer, pH 5.5) was added. After incubation for 20 min at room temperature, the reaction was stopped with 4 M H_2SO_4 (50 μL/well) and the optical density (OD) at 450–595 nm determined.

Formats 2B and 2C. The plates were coated with protein A (1 μg/mL) or goat anti-rabbit IgG (1:2000), respectively, at 4 °C, washed, and recoated with the anti-bromacil Ab. The remaining procedure was identical with that of format 2A.

Analysis of Water Samples. Well and deionized water samples spiked in a blind fashion with bromacil were analyzed by ELISA (format 1, Ab 2005, cAg **2cb**).

Analysis of Soil Samples. Twenty-five grams of soil (Yolo silt loam with 1.7% organic matter) was extracted by stirring with 100 mL of 1% NaOH for 0.5–1 h (Pease, 1966). The turbid mixture was allowed to settle, and 20 mL of the supernatant was removed. This basic aqueous extract was diluted (1:10) with the assay buffer and then analyzed by ELISA (format 1, Ab 2005, cAg **2cb**).

RESULTS AND DISCUSSION

Synthesis. Hapten **2g** and its protein conjugates **2h** were obtained by routine methods (Tables I and II; Szurdoki et al., 1992). Metabolite 8 was selectively derivatized at the hydroxyl group of the side chain by glutaric anhydride to give hapten **12a**. No significant *N*-acylation occurred under identical, mildly basic conditions (Scriven, 1983) with the equimolar mixture of bromacil and ethanol in a control experiment. The hapten **12a** was conjugated by a special method to obtain coating

Table III. Sensitivities of ELISA Systems Using Coating Antigens 12b and Anti-Bromacil Antibodies (Format 1)[a]

	IC_{50} with indicated antiserum, ppb		
coating Ag	Ab 2365	Ab 2369	Ab 2370
12bb	222	14.0	212
12bc	235	13.8	147
12bo	50	5.38	8.33

[a] The assay was conducted as described under Materials and Methods with coating antigen concentration of 5 μg/mL and the antibody dilution at 1/2000 for all three systems. IC_{50} was calculated from a 10-point standard curve with four well replicates at each concentration. The average coefficient of variance for the well replicates was 8.25%, range 3–14%.

antigens **12b**. The technique of combination of water-soluble carbodiimides and *N*-hydroxysulfosuccinimide (**13**), developed by Staros et al. (1986), greatly enhanced the carbodiimide-mediated coupling reactions of proteins with water-soluble compounds (e.g., peptides) in aqueous solution. The additive **13**, an auxiliary nucleophile (Bodanszky, 1984), traps sensitive, highly activated intermediates by forming active esters *in situ* (Staros et al., 1986). Compound **13** was also utilized for obtaining various protein modification reagents, mostly water-soluble, protein cross-linking agents (Staros, 1982; Ludwig and Jay, 1985; Anjaneyulu and Staros, 1987). The conjugation of proteins with organic compounds, insoluble in water, is a common problem during the development of immunoassays. The use of a mixed organic–aqueous solvent system (Goodrow et al., 1990) alone does not always give satisfactory results. The conversion of a hapten with a carboxylic acid functional group to an acylating agent with higher water solubility (Mei, 1991) may result in improved coupling efficiency. In our studies, bromacil derivative **12a** was condensed with compound **13** in DMF. The solution of the active ester was then added to aqueous protein solutions to produce conjugates. Besides the coupling reaction reported here, we are currently using this method with highly lipophilic substrates.

Antisera Characterization and Sensitivity. Titers of all antisera were tested using different coating antigens. ELISA results showed high titers on homologous as well as heterologous systems with all antigens.

Format 1. Inhibition studies for bromacil were performed for each of the coating antigen and serum combinations giving a significant titer (>1:1000). Homologous ELISAs (i.e., employing the same hapten for both coating and immunization) showed invariably that bromacil did not inhibit the binding of antibodies to antigen-coated wells even at concentrations as high as 1 ppm. With the heterologous systems (i.e., involving a coating hapten which is different from that used for immunization) the IC_{50} values varied widely depending on the antibody–coating antigen combinations (Table I) used. Only one of the haptens, acid **12a**, has handle attachment at the isobutyl side chain (R_4, Table I). It is interesting that antibody 2005 did not recognize the coating antigens (**12bb**, **12bc**, and **12bo**) prepared from hapten **12a**. However, antibodies 2365, 2369, and 2370 that were less discriminating against changes in the structure of the aliphatic substituent at the N-3-position (cf. Table I) did recognize conjugates of **12a**. The resulting assays gave fair, although less satisfying, results (Table III) with these reagents than the best systems reported below (Table I). (No efforts were made to determine cross-reactivities in this case.) None of the antisera recognized coating antigens (**2h**) derived from hapten **2g**; thus, no further studies were performed with these reagents. This failure may be due to the very short spacer arm in hapten **2g**. The most promising optimized

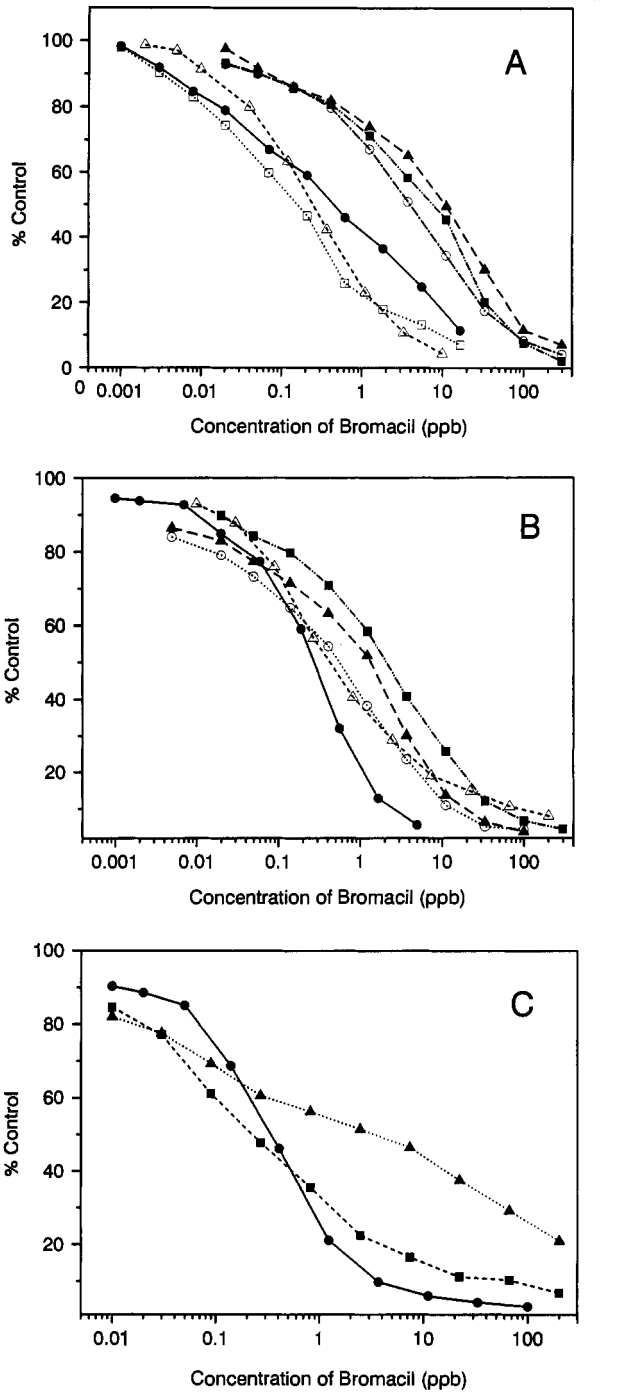


Figure 1. (A) Standard curves obtained with antibodies raised against immunizing antigen **2ck**, sera 2365, 2369, and 2370 combined with coating antigen **3eb**, as well as with antibodies raised against immunizing antigen **3ek**, sera 1960, 2005, and 2007 combined with coating antigen **2cb**. Antibody/coating antigen combinations (Ab/cAg) were 1960/**2cb** (●), 2005/**2cb** (△), 2007/**2cb** (□), 2365/**3eb** (▲), 2369/**3eb** (■), and 2370/**3eb** (○). (B) Standard curves obtained with antibodies raised against immunizing antigen **2ck**, sera 2365, 2369, and 2370 combined with coating antigen **3eo**, as well as with antibodies raised against immunizing antigen **3ek**, sera 2005 and 2007 combined with coating antigen **2co**. Antibody/coating antigen combinations (Ab/cAg) were 2005/**2co** (●), 2007/**2co** (△), 2365/**3eo** (○), 2369/**3eo** (▲), and 2370/**3eo** (■). (C) Standard curves obtained with antibodies raised against immunizing antigen **3ek**, sera 1960, 2005, and 2007 combined with coating antigen **2cc**. Antibody/coating antigen combinations (Ab/cAg) were 2005/**2cc** (●), 2007/**2cc** (■), and 1960/**2cc** (▲).

ELISA systems with IC_{50} values lower than 5 ppb are listed in Table I (see also Figure 1). The best assay consisting

Table IV. Comparison of Homologous ELISA Systems Using Anti-Bromacil Antibodies Immobilized on the Walls of the Wells and Enzyme Tracers[a]

Ab	format	IC_{50}, ppb	absorbance max	absorbance min	slope	r
2365	2A	3.11	0.658	0.032	0.759	0.999
	2B	6.67	0.596	0.059	0.791	0.999
	2C	26.9	0.482	0.148	0.690	0.965
2369	2A	6.87	0.452	0.009	0.756	0.993
	2B	4.21	0.188	0.031	0.881	0.990
	2C	13.2	0.654	0.063	0.562	0.983
2370	2A	3.11	0.658	0.032	0.759	0.999
	2B	3.34	0.373	0.006	0.541	0.976
	2C	7.26	0.617	0.084	0.470	0.953

[a] All formats utilized hapten **2b** conjugated to HRP under conditions described under Materials and Methods. Data shown are generated from a 10-point standard curve with four well replicates at each concentration. IC_{50}, absorbance maximal and minimal values, slope, and *r* were determined from the four-parameter fit. Dilutions of antibodies and enzyme tracers were 1/8000 and 1/20000, respectively.

of antibody (Ab 2005, 1:4000 dilution) derived from the conjugate **3ek** and coating antigen **2cb** (1.25 μg/mL) resulted in the highest sensitivity (IC_{50} = 0.25 ppb) and specificity (Table I; Szurdoki et al., 1992). Generally, the antibodies (e.g., Ab 2005) raised against the conjugate **3ek** (derivatization at 6-methyl group) showed higher affinity for bromacil than those (e.g., Abs 2365, 2369, and 2370) derived from **2ck** (handle attachment at the N-1-position). This difference was due to the coupling position of the bromacil hapten in the immunogen which strongly affects the sensitivity of the ELISA (Wie and Hammock, 1984; Harrison et al., 1991; Li et al., 1991; Szurdoki et al., 1992).

Format 2. After checkerboard titration (Gee et al., 1988; Schneider and Hammock, 1992) of the antibodies 2365, 2369, and 2370, the inhibition studies were performed with working dilutions of 1:20000 for the enzyme tracer (HRP conjugated with hapten **2b**) and 1:8000 for the specific antibodies. The results listed in Table IV showed that the enzyme tracer format (2A, 2B, and 2C) generally increased the sensitivity about 1000-fold as compared to the coating antigen format when homologous systems were used. However, there was no improvement of the assay sensitivity for the heterologous systems (data not shown). Using protein A or goat anti-rabbit IgG trapping (formats 2B and 2C) did not give better results. Enzyme tracers obtained from haptens **2g** and **3d** failed to give any significant color development. The negative results were again probably due to the rather short handles in these haptens which may hamper the association of the antibody and the sterically crowded bromacil moiety on the enzyme tracer.

Cross-Reactivity Studies for Assays in Format 1. Structures of immunizing haptens **2b** and **3d** were designed to minimize the affinity of sera for terbacil (a related herbicide, **4**; Table I) and for the decomposition products of bromacil effected by light (**5**) and metabolic processes (**6–9**, Table I) (Gardiner, 1975; Szurdoki et al., 1992). Cross-reactivities (CRs) of systems including anti-bromacil Abs 2365, 2369, and 2370, combined with cAg **3eo**, and of Ab 2005 with cAg **2cb** for terbacil, metabolites of bromacil and terbacil, and several related compounds were determined (Table I). Ratios of IC_{50} values [CR (%) = 100 × $^{\text{bromacil}}IC_{50}/^{\text{analyte}}IC_{50}$] of the same system for different compounds were calculated to characterize the relative affinity of the analytes. Data obtained show that substitution of the 5-bromo atom for hydrogen (metabolite **9**) or introduction of a hydroxy substituent at the 6-methyl group (metabolite **6**) or at the 3-(1-methylpropyl) side chain (metabolites **7** and **8**) generally resulted in weak affinity (most CRs are <5%; Table I). Ab 2365 recognized terbacil (**4**, IC_{50} = 0.41 ppb, CR = 377%) but only weakly recognized compound **11** (Table I), the major metabolite of terbacil. Thus, this antiserum appears to be suitable for the quantitative analysis of terbacil in environmental samples. *N*-Alkyl derivatives (**2a,b,d–f**, Table I) cross-reacted with antibodies raised against the *N*-substituted hapten (**2b**). The *N*-methylbromacil (**2d**) gave particularly high CR values. Thus, these ELISAs (e.g., Ab 2370 with cAg **3eo**, IC_{50} = 0.2 ppb, CR = 837%) are potentially useful for the sensitive and selective detection of bromacil in the derivatized form (**2d**) after extraction of this pesticide with aqueous base and methylation of the salt formed. In general, antibody 2005, raised against **3ek** conjugate, showed lower cross-reactivities than those derived from **2ck**. These results confirm our preliminary results on the system comprising Ab 2005 and cAg **2cb** as an assay potentially useful for the selective environmental monitoring of bromacil (Szurdoki et al., 1992). It is also worth noting that this ELISA had no cross-reactivity with a series of related heterocyclic compounds (e.g., caffeine, 1-methyluric acid; see Table I), some of which are possible components of samples of biological origin (e.g., urine).

Solvent and Matrix Effects. The effect of several solvents, human urine, and pH on the baseline response of the best ELISA system (format 1, Ab 2005 with cAg **2cb**) was investigated by combining antibody and bromacil in a buffer containing various amounts of solvent or urine. The results (Table V) demonstrate that all organic solvents studied significantly influence assay sensitivity, although IC_{50}s remained below 10 ppb in solvent concentrations of up to 12.5%. Fluctuation of the slope and curve fit as well as subtle changes in minimal and maximal absorbances also occurred with addition of organic solvents. The IC_{50} values increased 2–110-fold depending on solvent, which may create difficulties when bromacil is measured in samples obtained using organic solvents (e.g., extracts of soil samples). However, volatile extraction solvents can usually be removed completely by evaporation, the analyte trapped by a small amount of a nonvolatile, water-miscible solvent, and the solution taken up in buffer prior to analysis. Alternatively, bromacil, having a slightly acidic amide proton, can be extracted into dilute sodium hydroxide solution from soil (Pease, 1966, 1968). We found that, after buffering, this basic aqueous solution can be directly analyzed by ELISA (see below), thus overcoming any problem associated with organic solvents.

A strong matrix effect was observed when bromacil standard curves were measured employing various dilutions of human urine even at 1.6% concentration level (Figure 2). The effect of urine on the assay performance varied greatly from sample to sample. Thus, a quantitative ELISA for bromacil at a high sensitivity in urine, a typical example of rather comples matrices, would require some sample preparation.

The effect of pH on the optimized assay system of Ab 2005 and cAg **2cb** was investigated according to the method described by Harrison et al. (1989). The results indicated that there was no remarkable influence on the assay sensitivity in the pH range 5–8.5. However, the effect of pH did become significant at higher antibody concentrations (1:2000, data not shown). These results appear to be consistent with the weak acidity of bromacil (pK_a = 9.1; Alva and Singh, 1990). Thus, we assume that in the investigated pH range the major effect is exerted through the influence on the antibodies rather than on the analyte.

Table V. Influence of Selected Solvents on the ELISA Systems Using Anti-Bromacil Antibodies (Serum 2005) and Coating Antigen 2cb[a]

solvent concn,	methanol			acetonitrile			2-propanol			acetone		
% v/v	IC_{50}, ppb	slope	r	IC_{50}, ppb	slope	r	IC_{50}, ppb	slope	r	IC_{50}, ppb	slope	r
50	NT	NT	NT	NT	NT	NT	6.62	2.12	0.636	6.02	1.10	0.892
25	41.3	0.538	0.993	8.58	2.00	0.623	NT	NT	NT	4.55	1.52	0.950
12.5	3.22	0.854	0.991	7.11	0.651	0.994	9.24	0.556	0.992	10.7	0.336	0.983
6.25	2.27	0.901	0.995	3.02	0.731	0.997	3.02	0.531	0.979	3.98	0.744	0.998
3.12	1.28	0.779	0.998	2.39	0.874	0.995	1.40	0.776	0.997	2.11	0.724	0.998
1.56	1.22	0.815	0.998	2.07	1.28	0.986	0.83	0.885	0.993	1.88	0.853	0.999
0	0.39	0.821	0.996	0.40	0.801	0.997	0.40	0.851	0.996	0.38	0.819	0.996

[a] IC_{50} was determined from a 10-point standard curve, with four well replicates at each concentration. The average coefficient of variance for the well replicates was 8.25%, range 3–14%. IC_{50}, slope, and r were determined from the four-parameter fit. NT indicates not tested.

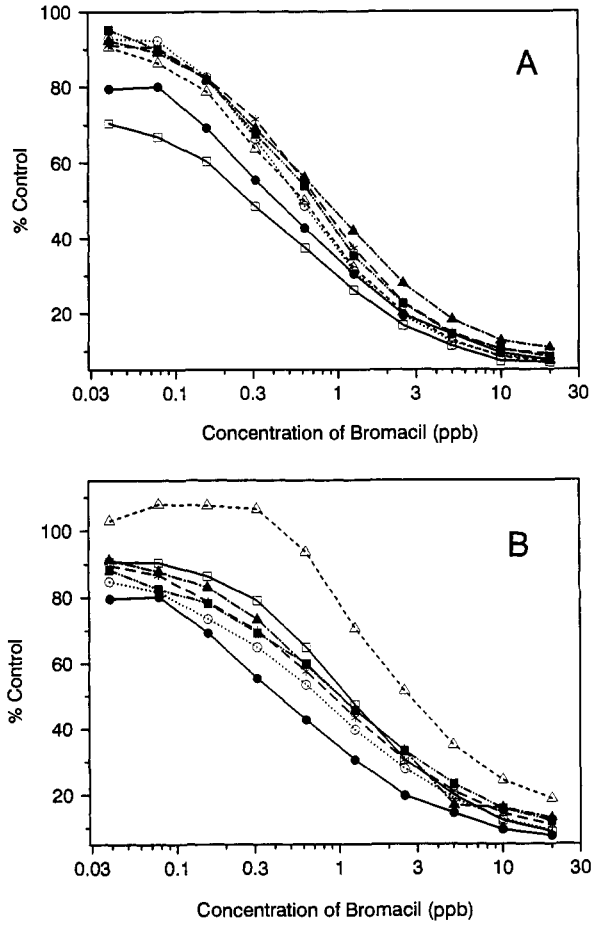


Figure 2. (A) Effect of various concentrations of a urine sample (1) on the ELISA including Ab 2005 and cAg **2cb**. The urine concentrations used were 50 (□), 25 (△), 12.5 (○), 6.25 (*), 3.12 (■), and 1.56% (v/v) (▲) and buffer (●). (B) Effect of various concentrations of a urine sample (2) on the ELISA including Ab 2005 and cAg **2cb**. The urine concentrations used were 50 (□), 25 (△), 12.5 (○), and 1.56% (v/v) (▲) and buffer (●).

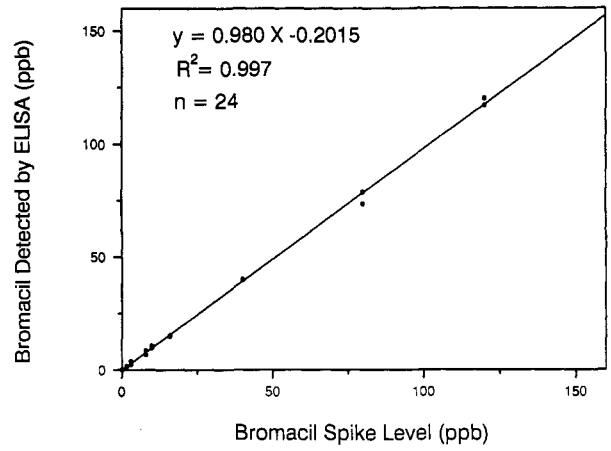


Figure 3. Results of ELISA system including Ab 2005 and cAg **2cb** on water samples spiked with bromacil. The low-concentration region of this graph illustrates results for a well-water sample, obtained in the Sacramento area, spiked with bromacil (two of each: 0.1, 0.3, 3, and 10 ppb). The remainder of the points depict results for bromacil spiked into deionized water. The regression equation and the statistical parameters are not significantly different for the two parts of the graph.

Assay Validation. Since the combination of Ab 2005 with cAg **2cb** (format 1) showed the lowest IC_{50} value, the greatest linear range of inhibition, the highest specificity, the best reproducibility, and no sensitivity to the change of pH, this was the system of choice for assay validation.

Two sets of water samples, spiked with bromacil concentrations ranging from 0 to 160 ppb, were analyzed in blind fashion by ELISA. Well water was used for the low-concentration region, while the rest of the samples were prepared from deionized water. The linear regression analysis of ELISA results showed excellent correlation ($Y = 0.9789X - 0.0213$, $R^2 = 0.9979$, $n = 24$; Figure 3). The results were not significantly different for the samples made of well and deionized water. All recoveries were over 95% of the spiked values. These results demonstrate that the polyclonal assay is suitable for quantitative detection of bromacil at trace level in real water samples without sample preparation. This ELISA seems to be promising for drinking water and well-water contamination studies where very low detection limits are required, and it compares favorably with several recent chromatographic methods (Putzien, 1987; Goewie and Hogendoorn, 1987) in terms of simplicity, sensitivity, and specificity.

Conventional residue analysis of bromacil in soil are usually based on the extraction of the analyte into dilute alkaline solutions and on further complicated extraction/cleanup procedures (Pease, 1966, 1968; Jolliffe et al., 1967; U.S. Food and Drug Administration, 1990; Worthing and Hance, 1991). In our studies, soil samples were extracted with 1% aqueous sodium hydroxide solution according to the method of Pease (1966, 1968), and then the basic solutions were diluted (at least 1:10) with the assay buffer and analyzed by ELISA. Recoveries of soil spiked from 0.04 to 20 ppm ranged from 92.5 to 102.5% (Table VI), thus verifying that the simple dilution and buffering of the extracts was sufficient to minimize the matrix effect of the particular soil type used (a Yolo silt loam with 1.7% organic matter). This single extraction is much simpler than the usual tedious sample preparation methods (Pease, 1966, 1968; Jolliffe et al., 1967). The investigated lowest concentration (0.04 ppm) in this study is identical with the detection limit of the literature method (Pease, 1966) based on a 25-g sample in both cases.

In conclusion, extensive studies with various assay formats and, in particular, in a coating antigen format with numerous ELISAs based on four haptens led us to

Table VI. Amounts of Bromacil Recovered from Soil Samples after Extraction with 1% NaOH[a]

spike level, ppm	amount recovered		recovery, %
	ppm	SD	
20	20.44	4.32	100.2
4	4.02	1.03	100.5
0.4	0.410	0.40	102.5
0.04	0.034	0.03	92.5

[a] The ELISA system used for analysis comprises serum 2005 and coating antigen **2cb**. Recovered amounts were determined in 1 extract per level with 12 replicates for each concentration.

a system highly sensitive (IC_{50} = 0.25 ppb) and selective for bromacil. Organic solvents and human urine exercised strong matrix effects on this optimized immunoassay. However, the ELISA was successfully applied to the quantitative detection of trace amounts of bromacil in water and in soil. The water samples were directly analyzed, while the soil was only extracted with aqueous base prior to the test. The most expensive and time-consuming part of conventional pesticide residue analyses is usually the sample cleanup (Wie and Hammock, 1984). Thus, our simple assays requiring no cleanup procedure seem to be candidate methods for cost- and labor-effective environmental monitoring of bromacil.

ABBREVIATIONS USED

AP, alkaline phosphatase; BSA, bovine serum albumin; CONA, conalbumin; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EI, electron impact; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IC_{50}, analyte concentration required for 50% inhibition; IR, infrared spectroscopy; KLH, keyhole limpet hemocyanin; MS, mass spectrometry; *N*-hydroxysulfosuccinimide, (±)-1-hydroxy-2,5-dioxo-3-pyrrolidinesulfonic acid; NMR, nuclear magnetic resonance spectroscopy; OD, optical density; OVA, ovalbumin; PBS, 0.2 M phosphate buffer + 0.8% NaCl (pH 7.5); PBSTA, 0.2 M phsophate buffer + 0.8% NaCl + 0.02% NaN_3 + 0.05% Tween 20 (pH 7.5); THF, tetrahydrofuran; THY, thyroglobulin; TLC, thin-layer chromatography; TMB, 3,3′,5,5′-tetramethylbenzidine.

ACKNOWLEDGMENT

We thank A. D. Jones, M.-P. Marco, and P. Schneider (University of California, Davis, CA) for helpful discussions. We greatly appreciate K. S. Goh (California Department of Pesticide Regulation, Sacramento, CA) for offering well-water samples spiked with bromacil. F. E. Henze and E. W. Zahnow (E. I. du Pont de Nemours & Co., Wilmington, DE) generously provided samples of bromacil, terbacil, and the metabolites of these herbicides. H.K.M.B. thanks Profs. E. A. Gomaa, M. Ashour, and G. Moawad (Plant Protection Research Institute, A.R.C., Dokki Giza, Nadi El-Syd, Egypt) for encouragement and advice.

LITERATURE CITED

Allender, W. J. Movement of Bromacil and Hexazinone in a Municipal Site. *Bull. Environ. Contam. Toxicol.* **1991**, *46*, 284–291.

Alva, A. K.; Singh, M. Sorption of Bromacil, Diuron, Norflurazon, and Simazine at Various Horizons in Two Soils. *Bull. Environ. Contam. Toxicol.* **1990**, *45*, 365–374.

Anjaneyulu, P. S. R.; Staros, J. V. Reactions of N-Hydroxysulfosuccinimide Active Esters. *Int. J. Pept. Protein Res.* **1987**, *30*, 117–124.

Bennett, P. H.; de Beer, P. R. A Rapid Quantitative Bioassay for the Determination of Biologically Available Bromacil in Soils. *Pestic. Sci.* **1984**, *15*, 425–430.

Bodanszky, M. *Principles of Peptide Synthesis (Reactivity and Structure, Vol. 16)*; Hafner, K., Len, J.-M., Rees, C. W., Schleyer, P. R., Trost, B. M., Zahradník, R., Eds.; Springer-Verlag: New York, 1984; pp 44–49.

Call, D. J.; Brooke, L. T.; Kent, R. J.; Knuth, M. L.; Poirier, S. H.; Hout, J. M.; Lima, A. R. Bromacil and Diuron Herbicides: Toxicity, Uptake, and Elimination in Freshwater Fish. *Arch. Environ. Contam. Toxicol.* **1987**, *16*, 607–613.

Forlani, F.; Arnoldi, A.; Pagani, S. Development of an Enzyme-Linked Immunosorbent Assay for Triazole Fungicides. *J. Agric. Food Chem.* **1992**, *40*, 328–331.

Foster, G. D.; Foreman, W. T.; Gates, P. M. Performance of the Goulden Large-Sample Extractor in Multiclass Pesticide Isolation and Preconcentration from Stream Water. *J. Agric. Food Chem.* **1991**, *39*, 1618–1622.

Fröhlich, D.; Meier, W. HPLC Determination of Triazines in Water Samples in the ppt-Range by On-Column Trace Enrichment. *J. High Resolut. Chromatogr.* **1989**, *12*, 340–342.

Gardiner, J. A. Substituted Uracil Herbicides. In *Herbicides. Chemistry, Degradation, and Mode of Action*; Kearney, P. C., Kaufman, D. D., Eds.; Dekker: New York, 1975; pp 293–322.

Garrett, N. E.; Stack, H. F.; Waters, M. D. Evaluation of the Genetic Activity Profiles of 65 Pesticides. *Mutat. Res.* **1986**, *168*, 301–325.

Gee, S. J.; Miyamoto, T.; Goodrow, M. H.; Buster, D.; Hammock, B. D. Development of an Enzyme-Linked Immunosorbent Assay for the Analysis of the Thiocarbamate Herbicide Molinate. *J. Agric. Food Chem.* **1988**, *36*, 863–870.

Goewie, C. E.; Hogendoorn, E. A. *Analysis* of Bromacil, Diuron and 3,4-Dichloroaniline in Contaminated Well Water, Using a High-Performance Liquid Chromatographic Column-Switching Procedure *J. Chromatogr.* **1987**, *410*, 211–216.

Goodrow, M. H.; Harrison, R. O.; Hammock, B. D. Hapten Synthesis, Antibody Development, and Competitive Inhibition Enzyme Immunoassay for *s*-Triazine Herbicides. *J. Agric. Food Chem.* **1990**, *38*, 990–996.

Hammock, B. D.; Mumma, R. O. Potential of Immunochemical Technology for Pesticide Analysis. In *Recent Advances in Pesticide Analytical Methodology*; Harvey, J., Jr., Zweig, G., Eds.; ACS Symposium Series 136; American Chemical Society: Washington, DC, 1980; pp 321–352.

Harrison, R. O.; Braun, A. L.; Gee, S. J.; O'Brien, D. J.; Hammock, B. D. Evaluation of an Enzyme-Linked Immunosorbent Assay (ELISA) for the Direct Analysis of Molinate (Ordram) in Rice Field Water. *Food Agric. Immunol.* **1989**, *1*, 37–51.

Harrison, R. O.; Goodrow, M. H.; Hammock, B. D. Competitive Inhibition ELISA for the *s*-Triazine Herbicides: Assay Optimization and Antibody Characterization. *J. Agric. Food Chem.* **1991**, *39*, 122–128.

Jaynes, D. B. Field Study of Bromacil Transport under Continuous-Flood Irrigation. *Soil Sci. Soc. Am. J.* **1991**, *55*, 658–664.

Jolliffe, V. A.; Day, B. E.; Jordan, L. S.; Mann, J. D. Method for Determining Bromacil in Soils and Plant Tissues. *J. Agric. Food Chem.* **1967**, *15*, 174–177.

Li, Q. X.; Hammock, B. D.; Seiber, J. N. Development of an Enzyme-Linked Immunosorbent Assay for the Herbicide Bentazon. *J. Agric. Food Chem.* **1991**, *39*, 1537–1544.

Lipschitz, C.; Irth, H.; De Jong, G. J.; Brinkman, U. A. T.; Frei, R. W. Trace Enrichment of Pyrimidine Nucleobases, 5-Fluorouracil and Bromacil on a Silver-Loaded Thiol Stationary Phase with On-Line Reversed Phase High-Performance Liquid Chromatography. *J. Chromatogr.* **1989**, *471*, 321–334.

Ludwig, R.; Jay, F. A. Reversible Chemical Cross-Linking of the Light-Harvesting Polypeptides of *Rhodopseudomonas viridis*. *Eur. J. Biochem.* **1985**, *151*, 83–87.

Mei, J. V.; Yin, C-M.; Carpino, L. A.; Ferguson, B. S. Hapten Synthesis and Development of Immunoassays for Methoprene. *J. Agric. Food Chem.* **1991**, *39*, 2083–2090.

Parent, D.; Hourmant, N.; Guedes, Y.; Riche, C.; Thouvenot, D. Evaluation of the Hepatotoxicity and Hematotoxicity of Plant

Protection Agents in Rat Cells in Culture. *Meded. Fac. Landbouwwet., Rijksuniv. Gent.* **1990**, *55*, 1369–1375.
Pease, H. L. Determination of Bromacil Residues. *J. Agric. Food Chem.* **1966**, *14*, 94–96.
Pease, H. L. Determination of Terbacil Residues Using Microcolumetric Gas Chromatography. *J. Agric. Food Chem.* **1968**, *16*, 54–56.
Putzien, J. Enrichment and Determination of Pesticides in Drinking Water. *Vom Wasser* **1987**, *68*, 33–41.
Reddy, K. N.; Singh, M.; Alva, A. K. Sorption and Leaching of Bromacil and Simazine in Florida Flatwoods Soils. *Bull. Environ. Contam. Toxicol.* **1992**, *48*, 662–670.
Rodbard, D. Mathematics and Statistics of Ligand Assay. An Illustrated Guide. In *Ligand Assay*; Langan, J., Clapp, J. J., Eds.; Masson Publishing: New York, 1981; pp 45–99.
Schlaeppi, J.-M.; Fory, W.; Ramsteiner, K. Hydroxyatrazine and Atrazine Determination in Soil and Water by Enzyme-Linked Immunosorbent Assay Using Specific Monoclonal Antibodies. *J. Agric. Food Chem.* **1989**, *37*, 1532–1538.
Schneider, P.; Hammock, B. D. Influence of the ELISA Format and the Hapten Enzyme-Conjugate on the Sensitivity of an Immunoassay for *s*-Triazine Herbicides Using Monoclonal Antibodies. *J. Agric. Food Chem.* **1992**, *40*, 525–530.
Scriven, E. F. V. 4-Dialkylaminopyridines: Super Acylation and Alkylation Catalysts. *Chem. Soc. Rev.* **1983**, *12*, 129–161.
Stan, H. J.; Heil, S. Two-Dimensional Capillary Gas Chromatography with three Selective Detectors as a Valuable Tool in Residue Analysis—State-of-the Art. *Fresenius' J. Anal. Chem.* **1991**, *339*, 34–39.
Staros, J. V. *N*-Hydroxysulfosuccinimide Active Esters: Bis-(*N*-Hydroxysulfosuccinimide) Esters of Two Dicarboxylic Acids are Hydrophilic, Membrane-Impermeant, Protein Cross-Linkers. *Biochemistry* **1982**, *21*, 3950–3955.
Staros, J. V.; Wright, R. W.; Swingle, D. M. Enhancement by *N*-Hydroxysulfosuccinimide of Water-Soluble Carbodiimide-Mediated Coupling Reactions. *Anal. Biochem.* **1986**, *156*, 220–222.
Szurdoki, F.; Bekheit, H. K. M.; Marco, M. P.; Goodrow, M. H.; Hammock, B. D. Synthesis of Haptens and Conjugates for an Enzyme Immunoassay for Analysis of the Herbicide Bromacil. *J. Agric. Food Chem.* **1992**, *40*, 1459–1465.
Tuinstra, L. G. M. T.; Roos, A. H.; Matser, A. M.; Traag, W. A.; Van Rhijn, J. A. Development of a Multi-Residue/Multi-Matrix Method for Pesticide Analysis in Agricultural Products. *Fresenius' J. Anal. Chem.* **1991**, *339*, 384–386.
U.S. Environmental Protection Agency. *National Pesticide Survey, Survey Analytes. Fall 1990*; U.S. EPA Office of Water, Office of Pesticides and Toxic Substances: Washington, DC, 1990.
U.S. Food and Drug Administration. *FDA Pesticide Analytical Manual*; U.S. FDA: Rockville, MD, 1990; Vol. II.
Wie, S. I.; Hammock, B. D. Comparison of Coating and Immunizing Antigen Structure on the Sensitivity and Specificity of Immunoassays for Benzoylphenylurea Insecticides. *J. Agric. Food Chem.* **1984**, *32*, 1294–1301.
Worthing, C. R., Hance, R. J., Eds. *The Pesticide Manual. A World Compendium*, 9th ed.; The British Crop Protection Council: Farnham, Surrey, U.K., 1991.
Wylie, P. L.; Oguchi, R. Pesticide Analysis by Gas Chromatography with Novel Atomic Emission Detector. *J. Chromatogr.* **1990**, *517*, 131–42.

Received for review January 11, 1993. Accepted July 30, 1993.[⊕]

Registry No. Supplied by the Author: 1, 314-40-9; **2d**, 7692-45-7; 4, 5902-51-2; 5, 15018-56-1; 6, 22663-43-0; 7, 22663-44-1; 8, 22663-47-4; 9, 6589-36-2; 11, 25546-02-5; 5-bromo-2,4(1*H*,3*H*)-pyrimidinedione, 51-20-7; caffeine, 58-08-2; 1,3-dicyclohexylcarbodiimide, 538-75-0; 5,6-diamino-1,3-dimethyl-2,4(1*H*,3*H*)-pyrimidinedione, 5440-00-6; (±)-1-hydroxy-2,5-dioxo-3-pyrrolidinesulfonic acid, monosodium salt, 106627-54-7; *N*-hydroxysuccinimide, 6066-82-6; 5-methyl-2,4(1*H*,3*H*)-pyrimidinedione (thymine), 65-71-4; 1-methyluric acid, 708-79-2; 2,4(1*H*,3*H*)-pyrimidinedione (uracil), 66-22-8; 3,3′,5,5′-tetramethylbenzidine, 54827-17-7; thymidine, 50-89-5; uridine, 58-96-8.

[⊕] Abstract published in *Advance ACS Abstracts*, October 1, 1993.