Development of an Enzyme-Linked Immunosorbent Assay for the Analysis of the Thiocarbamate Herbicide Molinate

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The development of an enzyme-linked immunosorbent assay (ELISA) for the herbicide molinate (Ordram, *S*-ethyl hexahydroazepine-1-carbothioate) is described. By a thiol replacement reaction of thiocarbamate sulfones, several haptens were synthesized. These haptens were coupled to carrier proteins via mixed-anhydride and diazotization procedures. Antibodies raised against these antigens were screened for sensitivity and specificity for molinate. An assay is described with a limit of detectability of 3 ng/mL that is specific for molinate with some cross-reactivity (15%) to molinate sulfone. Other thiocarbamate pesticides had cross-reactivities of <1.4%. Propylene glycol, as a carrier solvent, interfered in the ELISA at concentrations of 12.5% and greater, whereas acetonitrile had no effect at 12.5% but had increased and then decreased absorbances relative to control at 5% and 25%, respectively. The half-lives of molinate in a laboratory study as measured by liquid scintillation counting and ELISA were 2.6 and 2.2 days, respectively. Paired *t* statistics on this data gave a significant correlation with $0.05 < p < 0.02$. Techniques allowing current sample preparation methods to be easily adapted for use with ELISA analysis are also described.

Immunoassays have been used extensively in clinical chemistry for the detection of hormones, peptides, proteins, drugs, and microbes. However, their use in environmental chemistry has been very limited until last year when an increasing number of academic, governmental, and industrial laboratories took note of their well-documented flexibility and advantages (Hammock and Mumma, 1980; Hammock et al., 1987). All immunoassays are physical assays based on the law of mass action but can be delivered in a bewildering variety of forms. The enzyme-linked immunosorbent assay (ELISA) is a form that has been found particularly applicable to the field of pesticide chemistry (Van Emon et al., 1986; Kelley et al., 1985; Newsome, 1986).

Molinate (Ordram, *S*-ethyl hexahydroazepine-1-carbothioate; Patchett and Batchelder, 1967; Zweig, 1972) is an excellent and selective herbicide, and the use of this or other compounds of similar properties is considered critical to profitable rice culture (Cornacchia et al., 1984). Fish kills in rice water drainage canals have led to speculation that molinate was not being dissipated as anticipated on the basis of field studies. An unusually high level of molinate in drainage canals also meant a potential for unacceptable levels of molinate in local rivers (Ross and Sava, 1986; Cornacchia et al., 1984).

An ELISA was developed that is potentially a rapid and inexpensive method for analyzing the large sample loads generated in monitoring the field, drainage canals, and rivers. Methods allowing this ELISA to be integrated with classical GLC techniques to provide very sensitive assays for comparison are also discussed.

MATERIALS AND METHODS

Chemicals. 3-Mercaptopropionic acid was purchased from Evans Chemetics, Inc., and dodecacarbonyltriiron from Morton Thiokol, Inc. (Alfa Products). The proteins used in coupling and *p*-nitrophenyl phosphate substrate were obtained from Sigma Chemical Co. Tween 20 was from Biorad, Freund's complete adjuvant was from Calbiochem, and the goat anti-rabbit IgG conjugated to alkaline phosphatase was from Miles Scientific. Molinate and the thiocarbamate compounds used in inhibition studies were gifts from Stauffer Chemical Co. Thiobencarb was a gift from Chevron Chemical Co. The other chemical reagents were supplied by Aldrich Chemical Co. Analytical TLC was performed on 0.25-mm precoated silica gel 60 F254 plastic sheets (EM Reagents). Compounds were detected by UV light (254 nm), iodine vapor, and/or 0.5% palladium chloride in diluted HCl ($PdCl_2$ reagent). Column chromatography was carried out on Baker silica gel (60–200 mesh) or Baker silica gel for flash chromatography (ca. 40-μm diameter). The solvent systems are described in the individual experiments. The purity of all compounds was supported by TLC in several solvent systems.

IR spectra were determined with a Beckman Model 4240 or an IBM IR/32 FTIR spectrometer. ^1H NMR spectra were measured with a Varian EM-390 90-MHz NMR spectrophotometer using tetramethylsilane as an internal standard. Low-resolution mass spectra (MS) and high-resolution mass spectra (HRMS) were obtained on a VG ZAB-2F with a VG 11-250 data system.

Synthesis of Haptens. Haptens were synthesized according to Figure 1. Intermediate compounds were confirmed by ^1H NMR and/or IR. Final haptens were characterized more completely.

Molinate Sulfone (***2***). To 7.62 g (40.0 mmol) of molinate (1) in 75 mL of CH_2Cl_2 was added dropwise with stirring 19.0 g (88 mmol) of *m*-chloroperoxybenzoic acid (80–90%) in 225 mL of CH_2Cl_2 under ice cooling for 1.5 h. The mixture was stirred overnight at room temperature after adding 10.3 g of anhydrous KF [dried at 100 °C (0.5 torr) for 1.5 h]. After filtration, the combined filtrate and CH_2Cl_2 wash was concentrated to provide 9.07 g (103%) of molinate sulfone as an amber oil: TLC R_f 0.29 (hexane–ethyl acetate, 4:1); ^1H NMR ($CDCl_3$) δ 1.39 (t, J = 7.3 Hz, 3 H, CH_3), 1.7 (m, 8 H, ring CH_2), 3.32 (q, J = 7.3 Hz, 2 H, CH_2SO_2), 3.51 (t, J = 5.1 Hz, 2 H, CH_2N), 3.85 (t, J = 6.0 Hz, 2 H, CH_2N).

S-2-Carboxyethyl Hexahydroazepine-1-carbothioate (***3a***). A mixture of 3.0 g (14 mmol) of **2**, 1.2 g (11 mmol) of 3-mercaptopropionic acid, and 4.4 g (44 mmol) of triethylamine in 80 mL of ethanol was stirred at room temperature for 4 h. After removal of ethanol, the residue was dissolved in ethyl acetate and the resultant mixture extracted three times with 25 mL each of 1 M NaOH solution. The combined basic extract was acidified with 1 M HCl and extracted with four 30-mL portions of ethyl acetate. The combined organic extract was dried (Na_2SO_4)

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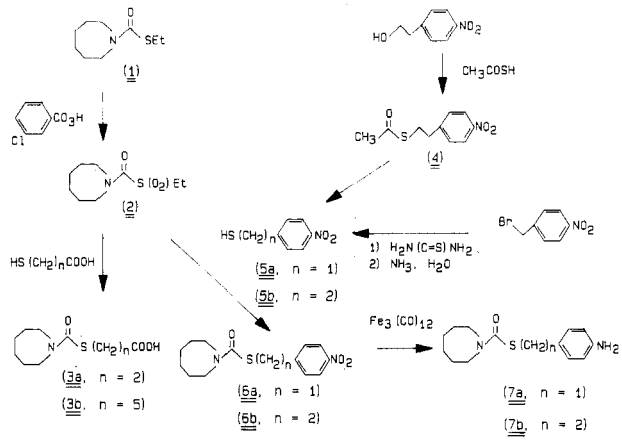


Figure 1. Route of synthesis for haptens of molinate: **1**, molinate (*S*-ethyl hexahydroazepine-1-carbothioate); **2**, molinate sulfone; **3a**, *S*-2-carboxyethyl hexahydroazepine-1-carbothioate; **3b**, *S*-5-carboxypentyl hexahydroazepine-1-carbothioate; **4**, 2-(*p*-nitrophenyl)ethyl thioacetate; **5a**, *p*-nitrobenzyl mercaptan; **5b**, 2-(*p*-nitrophenyl)ethanethiol; **6a**, *S*-(*p*-nitrobenzyl) hexahydroazepine-1-carbothioate; **6b**, *S*-2-(*p*-nitrophenyl)ethyl hexahydroazepine-1-carbothioate; **7a**, *S*-(*p*-aminobenzyl) hexahydroazepine-1-carbothioate; **7b**, *S*-2-(*p*-aminophenyl)ethyl hexahydroazepine-1-carbothioate.

and concentrated. The resulting syrup was subjected to silica gel column chromatography using benzene–ethyl acetate (1:1) as eluant to obtain 1.8 g (71%) of transparent liquid **3a**: IR (film) 3185–3020, 2650–2545, 1705, 1630, 1605, 1410 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.6 (br m, 8 H, ring CH_2), 2.71 (t, J = 6.3 Hz, 2 H, CH_2), 3.11 (t, J = 7.0 Hz, 2 H, CH_2), 3.5 (br m, 4 H, CH_2N); MS (70 eV) m/z (relative intensity) 231 (9, M^+), 158 (3, M – CH_2CH_2COOH), 126 (100, M – SCH_2CH_2COOH), 105 (9, SCH_2CH_2COOH), 98 (19, M – $COSCH_2CH_2COOH$), 97 (7, C_5H_7NO), 55 (55, C_2HNO); HRMS for $C_{10}H_{17}NO_3S$, calcd m/z 231.0929, found 231.0947.

6-Mercaptohexanoic Acid. The acid was prepared according to Minisci (1959) from 2.00 g (1.03 mmol) of 6-bromohexanoic acid, 1.62 g (10.3 mmol) of $Na_2S_2O_3$, 2 mL of water, and 3 M NaOH (sufficient to attain pH 7) under reflux for 1 h. The mixture was then acidified to pH 1 with concentrated HCl and boiled for 3 h. After cooling, the acid mixture was extracted with CH_2Cl_2 (3 × 25 mL), which on removal provided the acid as a colorless oil, 1.51 g (10.2 mmol, 99%), showing only two trace impurities on TLC: R_f 0.48 (THF–ethyl acetate–hexane, 2:13:35); ^1H NMR ($CDCl_3$) δ 1.31 (t, J = 7.8 Hz, 1 H, SH), 1.5 (m, 6 H, CH_2), 2.32 (t, J = 6.8 Hz, 2 H, CH_2COOH), 2.5 (m, 2 H, CH_2S), 10.4 (br m, 1 H, OH).

S-5-Carboxypentyl Hexahydroazepine-1-carbothioate (***3b***). To a stirred solution of 2.30 g (10.5 mmol) of **2** and 40.4 g (40.0 mmol) of triethylamine in 40 mL of dry ethanol was added 1.44 g (9.71 mmol) of 6-mercaptohexanoic acid at room temperature. After 12 h, the solvent was removed (rotovap) and the residual oil taken up in 75 mL of ether. The ethereal solution was washed with 1 N HCl and then extracted with 1 N NaOH. The basic layer was acidified to pH 1 with 6 N HCl and extracted with $CHCl_3$ (3 × 20 mL). Concentration of the combined $CHCl_3$ layers gave a yellow oil that was subjected to flash chromatography on 150 g of silica gel, being eluted with hexane–ethyl acetate (2:1). Combination of the fractions showing only one component on TLC [R_f 0.39 (THF–ethyl acetate–hexane, 2:13:35)] provided 1.00 g (35%) of **3b** as a white solid: mp 58.0–59.0 °C; IR (KBr) 1712 (vs, C=O), 1641 (vs, C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.6 (m, 14 H, CH_2), 2.32 (t, J = 6.6 Hz, 2 H, CH_2COOH), 2.85 (t, J = 6.8 Hz, 2 H, CH_2S), 3.4 (m, 4 H, CH_2N), 9.6 (br, 1 H, OH); MS (70 eV) m/z (relative intensity) 273 (11, M^+), 126 [98, M – $S(CH_2)_5COOH$], 98 [29, M – $COS(CH_2)_5COOH$], 97 (17, C_5H_7NO), 83 (53, C_4H_5NO), 69 (45, C_3H_3NO), 55 (100, C_2HNO).

p-Nitrobenzyl Mercaptan (***5a***). A solution of 10.8 g (50.0 mmol) of *p*-nitrobenzyl bromide and 5.0 g (67 mmol) of thiourea in 25 mL of water was heated under reflux for 2 h and cooled in ice, and the salt was collected and dried (13.1 g, 83%). The isothiouronium salt was decomposed by boiling several times with concentrated ammonia and hexane. Concentration of the combined hexane extracts provided 6.33 g (75%) of the mercaptan **5a**, mp 52–54 °C [lit. (Saville, 1960) mp 51–52 °C].

S-(p-Nitrobenzyl) Hexahydroazepine-1-carbothioate (***6a***). To 2.19 g (10.0 mmol) of **2** and 3.33 g (33.3 mmol) of triethylamine in 50 mL of absolute ethanol under N_2 was added 1.77 g (10.5 mmol) of *p*-nitrobenzyl mercaptan (**5a**), while noting a 6 °C temperature increase. The mixture was stirred overnight and concentrated at reduced pressure. The residue was taken up in 100 mL of ethyl acetate and washed successively with 1 M NaOH (2 × 25 mL), 1 M HCl (2 × 25 mL), and saturated NaCl (3 × 25 mL). Removal of the solvent at reduced pressure provided **6a** as an amber, viscous oil that finally solidified: TLC R_f 0.67 (THF–ethyl acetate–hexane, 2:13:35); ^1H NMR ($CDCl_3$) δ 1.6 (m, 8 H, ring CH_2), 3.38 (t, J = 5 Hz, 2 H, CH_2N), 3.50 (t, J = 5 Hz, 2 H, CH_2N), 4.15 (s, 2 H, CH_2S), 7.45 (d, J = 8.7 Hz, 2 H, aromatic), 8.08 (d, J = 8.7 Hz, 2 H, aromatic).

S-Aminobenzyl Hexahydroazepine-1-carbothioate (***7a***). In a N_2 atmosphere and with vigorous stirring, 3.0 g (5.7 mmol) of dodecacarbonyltriiron (95% containing 5–10% methanol), 1.25 mL of anhydrous methanol, 1.47 g (5.00 mmol) of **6a**, and 50 mL of dry benzene were heated under reflux for 10 h. The mixture was filtered and the filtrate washed with 1 N HCl (2 × 20 mL) and then made basic to pH 10 with 3 N NaOH. This solution was extracted with $CHCl_3$ (4 × 25 mL); the extracts were combined and concentrated to provide a red-brown, viscous oil that, on TLC, was found to contain five trace-amount impurities.

The 1.07 g of oil was chromatographed on 30 g of silica gel and eluted with hexane–ethyl acetate (3:1) to obtain 0.709 g of oil still containing one trace impurity. A second chromatogram on 30 g of silica gel, eluting with hexane–$CHCl_3$ (2:1), resolved the mixture and yielded fractions containing only one spot on TLC, R_f 0.25 (THF–ethyl acetate–hexane, 2:13:35). Concentration of these fractions at 25 °C (0.1 torr) provided 0.658 g (50%) of **7a** as an amber oil: IR (neat) 3445 (m, N—H), 3360 (s, N—H), 1620 (vs, C=O), 1285 (s, C—N) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.6 (m, 8 H, ring CH_2), 3.4 (m, 6 H, CH_2N and NH_2), 4.02 (s, 2 H, CH_2S), 6.51 (d, J = 8.1 Hz, 2 H, aromatic), 7.04 (d, J = 8.1 Hz, 2 H, aromatic); MS (70 eV) m/z (relative intensity) 266 (2, M + 2), 265 (5, M + 1), 264 (30, M^+), 158 (5, M – $CH_2C_6H_4NH_2$), 138 (3, $SCH_2C_6H_5NH_2$), 126 (8, M – $SCH_2C_6H_4NH_2$), 106 (100, $CH_2C_6H_4NH_2$), 98 (7, $C_6H_{12}N$).

S-2-(p-Nitrophenyl)ethyl Thioacetate (***4***). To an ice-cooled solution of 23.9 g (91.2 mmol) of triphenylphosphine in 300 mL of THF was added dropwise over 40 min a solution of 18.8 mL (92.6 mmol) of 97% diisopropyl azodicarboxylate in 30 mL of THF. Stirring was continued for 100 min. Then, a mixture of 7.6 g (45 mmol) of 4-nitrophenethyl alcohol and 6.9 mL (91 mmol) of thioacetic acid in 100 mL of THF was added dropwise to this suspension with stirring and ice-cooling over 70 min. Stirring was continued at 0 °C for 1 h, and then the mixture remained overnight at room temperature. After removal of

THF at reduced pressure, the residue was extracted 8X with 50-mL portions of hexane. The combined extract on cooling provided 10.8 g of crude solid 4. Recrystallization from hexane gave 7.6 g (75%) of 4 sufficiently pure for subsequent synthesis: TLC R_f 0.32 (hexane–ethyl acetate, 4:1); ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3 H, CH_3CO), 3.1 (br m, 4 H, CH_2), 7.36 (d, J = 8.5 Hz, 2 H, aromatic), 8.14 (d, J = 8.5 Hz, 2 H, aromatic).

2-(p-Nitrophenyl)ethanethiol (***5b***). To 3.6 g (16 mmol) of thioester **4** in 25 mL of methanol was added dropwise with stirring 25 mL of 1 M NaOH solution under a stream of N_2 at room temperature for 30 min. Stirring was continued for 20 min. After removal of methanol at 35 °C, the residue was extracted four times with 35-mL portions of ethyl acetate, and the combined extract was dried (Na_2SO_4) and concentrated. The resulting solid was extracted with six 30-mL portions of hot hexane, and after cooling, the organic phase was pooled and concentrated to obtain 1.0 g (34%) of crude thiol **5b**: TLC R_f 0.36 (hexane–ethyl acetate, 4:1); ^{1}H NMR ($CDCl_3$) δ 1.38 (t, J = 7.0 Hz, 1 H, SH), 2.9 (m, 4 H, CH_2), 7.33 (d, J = 9.0 Hz, 2 H, aromatic), 8.13 (d, J = 9.0 Hz, 2 H, aromatic).

S-2-(p-Nitrophenyl)ethyl Hexahydroazepine-1-carbothioate (***6b***). A mixture of 1.6 g (8.7 mmol) of thiol **5b**, 1.8 g (8.2 mmol) of **2**, and 2.7 g (27 mmol) of triethylamine in 50 mL of ethanol was stirred at room temperature for 3 h and allowed to stand overnight at room temperature. After removal of ethanol, the residue was dissolved in ethyl acetate, washed with two 25-mL portions of 1 M NaOH solution followed by 1 M HCl, dried (Na_2SO_4), and concentrated. The syrup was dissolved in 75 mL of hot hexane, filtered to remove insoluble material, and concentrated. The residue was chromatographed on silica gel with hexane–ethyl acetate (4:1) as eluant to obtain 0.96 g (38%) of carbothioate **6b**: TLC R_f 0.22 (hexane–ethyl acetate, 4:1); ^{1}H NMR ($CDCl_3$) δ 1.6 (br m, 8 H, ring CH_2), 3.1 (m, 4 H, CH_2), 3.5 (br m, 4 H, CH_2N), 7.35 (d, J = 9.0 Hz, 2 H, aromatic), 8.11 (d, J = 9.0 Hz, 2 H, aromatic).

S-2-(p-Aminophenyl)ethyl Hexahydroazepine-1-carbothioate (***7b***). A mixture of 738 mg (2.3. mmol) of carbothioate **6b**, 1.37 g (2.39 mmol) of dodecacarbonyltriiron (95%, containing 5–10% methanol), and 0.89 mL of methanol in 25 mL of benzene was refluxed for 10 h. After the mixture was filtered in vacuo and the residue was washed with benzene, the combined filtrate and wash was extracted three times with 30-mL portions of 1 M HCl. The combined acid solution was made strongly basic to litmus with 3 M NaOH and extracted with three 50-mL portions of ethyl acetate. The combined organic extract was dried (Na_2SO_4) and concentrated to obtain 366 mg (55%) of **7b**. Carbothioate **7b** was detected as a single spot on TLC: R_f 0.05 (hexane–ethyl acetate, 4:1), 0.37 (benzene–ethyl acetate, 1:1); IR (Nujol) 3460, 3365, 1640, 1610, 1520, 1480, 1400 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.7 (br m, 8 H, ring CH_2), 2.8 (m, 2 H, CH_2), 3.1 (m, 2 H, CH_2), 3.3 (br s, 2 H, NH_2, disappears on addition of D_2O), 3.5 (br m, 4 H, CH_2N), 6.58 (d, J = 8.5 Hz, 2 H, aromatic), 7.00 (d, J = 8.5 Hz, 2 H, aromatic); MS (70 eV) m/z (relative intensity) 278 (0.3, M^+), 150 (1, $SCH_2CH_2C_6H_4NH_2$), 120 (9, $CH_2CH_2C_6H_4NH_2$), 119 (100, $CH_2{=}CHC_6H_4NH_2$), 106 (16, $CH_2C_6H_4NH_2$); HRMS for $C_{15}H_{22}N_2OS$, calcd m/z 278.1453, found 278.1457.

Preparation of Hapten–Protein Conjugates. Molinate haptens **3a** and **3b** were conjugated to proteins by the mixed-anhydride method using isobutyl chloroformate (Wie and Hammock, 1982, 1984). Haptens **7a** and **7b** were conjugated to proteins by a diazotization method (Nisonoff, 1967) with an average loading of 333 mol of hapten/mol of protein. Proteins used were bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), conalbumin (CONA), ovalbumin (OA), thyroglobulin (THY), and fibrinogen (FIB). An estimate of the protein concentration was made by the Lowry protein assay (Lowry et al., 1951).

Immunization of Rabbits. Female New Zealand white rabbits (2–4 kg) each received 100 μg (protein equivalents) of a hapten–protein conjugate in 0.1 M potassium phosphate buffer (pH 7.4) mixed 1:1 with Freund's complete adjuvant intradermally in 5–10 spots on the back 3 days in a row. After 4 weeks each animal was boosted with an additional 100 μg and serum collected after 10 days. Blood was collected from the ear vein into tubes and allowed to clot. The clot was reamed and the blood centrifuged at 800g for 10 min. Serum was carefully drawn off by pipet and NaN_3 added in a final concentration of 0.02%. The serum was then aliquoted and stored at −20 °C. Subsequent boosting was done from at least 3 days from the last bleed. At least two animals each were immunized with either compound **3a** coupled to KLH (**3a**–KLH) or **7b** coupled to THY (**7b**–THY).

Direct Sandwich ELISA. Ninety-six-well titer plates (Nunc, Vangard International) were coated with appropriate coating antigen at 2 μg/mL in 0.5 M carbonate buffer at pH 9.8 at 0.2 mL/well and incubated in a moist environment at 4 °C overnight. On the same day that plates were coated, standards or samples were prepared by incubating molinate (1–2000 ng/mL) or the sample with antisera diluted 1/5000 with phosphate-buffered saline containing 0.05% Tween 20 and 0.02% NaN_3 (PBS–Tween) overnight at room temperature. The following day, the plates were washed with PBS–Tween three times. The samples and standards were added to the wells at 0.2 mL/well. After a 2-h incubation at room temperature, the plates were again washed three times and goat anti-rabbit IgG conjugated to alkaline phosphatase (Miles) diluted 1/2500 with PBS-Tween was added to each well and incubated for 2 h at room temperature. After a further three washings, 0.2 mL of a 1 mg/mL solution of *p*-nitrophenyl phosphate in 10% diethanolamine buffer was added and incubated at room temperature for 15–30 min and read in a plate reader (Titertek Multiskan, Flow Laboratories) at 405 nm.

The plate reader was interfaced (RS 232C) with an IBM personal computer (Figure 2, top), and data were collected with the PC-EIA program (Dorian Software Co., Wheaton, MA). The absorbance values and other relevant information collected by the PC-EIA program were stored in data files on floppy disks (Figure 2, top). An intermediate-step program called TRANSFER.BAS, between PC-EIA and LOTUS 1-2-3, was written. (The program and documentation are given in the supplementary material.) TRANSFER.BAS used data from the files created by PC-EIA, rearranged the data in a user-specified manner (Figure 2, bottom), and saved the data in a file. When the LOTUS 1-2-3 program was run, this file was loaded and made into a normal LOTUS worksheet file by using the /FILE IMPORT command. The spreadsheet program, LOTUS 1-2-3, was then used to display the absorbance values into an easily reviewed form, to perform simple calculations on the values, and to graph the results.

Preparation of the Standard Curves. Molinate (1 mg/mL in acetonitrile) was added to tubes containing PBS–Tween to give a concentration of 4000 ng/mL. This stock was serially diluted to 2 ng/mL. Antisera diluted 1/2500 in PBS–Tween were added to each tube so that the final concentration of antisera was 1/5000. The final concentration of standards then was 1–2000 ng/mL.

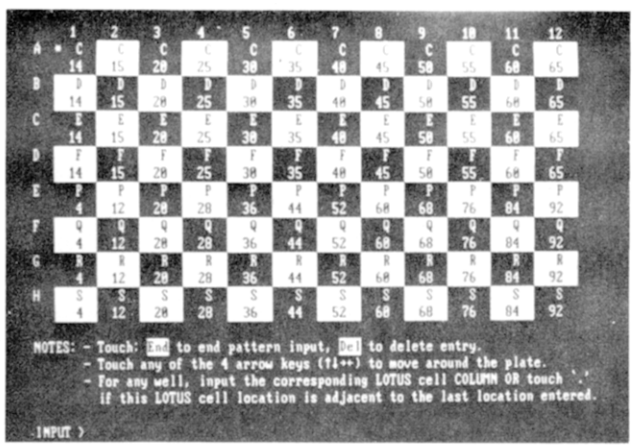


Figure 2. (Top) Data flow pattern. Absorbances are read at 405 nm in a Titertek Multiskan. Absorbances are collected via software program through an RS 232C interface. Once data are stored on a floppy disk, they may be manipulated by an in-house program (TRANSFER.BAS) and calculations and graphics completed with LOTUS 1-2-3. (Bottom) TRANSFER program pattern. The pattern is laid out as a typical 96-well plate. User specifies a location in the LOTUS spreadsheet for the absorbance from any given well.

Screening Procedure. Standard curves for molinate were developed for each of the coating antigen and serum combinations showing good titer (i.e., good color development in a reasonable amount of time). Those experiments in which inhibition was seen over a wide range for the standard were then optimized for further study. The coating antigen and serum concentrations were optimized by a checkerboard titration. Once optimized, the combinations were tested for specificity for molinate and other thiocarbamates and for utility with samples.

Determining IC_{50}s. With the optimized system for molinate (2 μg of **7b**–CONA/mL as coating and a 1/5000 dilution of antibody from a rabbit immunized with **3a**–KLH), several concentrations of a number of thiocarbamates were tested for their ability to inhibit the assay. The concentration of thiocarbamate that could inhibit the assay by 50% was denoted IC_{50}. When appropriate, the percent inhibition at the highest concentration tested also was noted.

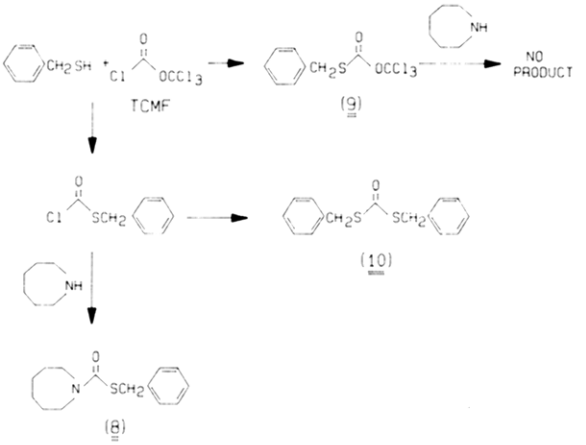


Figure 3. Reactions of trichloromethyl chloroformate (TCMF) with phenylmethanethiol followed by hexamethylenimine yielding the expected thiocarbamate **8**. Side reactions to **9** and **10** limited the utility of this procedure.

Extraction Study. A field water sample was spiked with [^{14}C]molinate (0.006 μCi/mL, 10.4 μCi/μmol, specific activity) and divided into several 1-mL aliquots. Aliquots were extracted with the specified solvent or solvent combination three times by vortexing vigorously for 45 s. Organic phases were pooled and evaporated onto a bed of 25 μL of propylene glycol, and the propylene glycol was counted. The aqueous phase was counted after the final extraction.

Laboratory Half-Life Study. Eight hundred milliliters of water from a local rice field was placed in a 1-L beaker. Fifteen microcuries of [^{14}C]molinate (1.75 mCi/mmol) and enough unlabeled molinate were added to give a final concentration of about 2000 ng/mL. Beakers were placed on a roof in direct sunlight, without aeration. Samples were taken for several days and analyzed by liquid scintillation counting (LSC) and ELISA. TLC of the remaining water showed only one radioactive spot, indicating that the molinate dissipated primarily through evaporation rather than breakdown.

RESULTS

Synthesis. In general, thiocarbamates are prepared by (A) reaction of isocyanate with thiol, (B) reaction of amine with chlorothioformate, or (C) thiol replacement of thiocarbamate sulfones. As haptens used in this study are N,N-disubstituted thiocarbamates, they could not be synthesized by method A. By method B, such compounds are usually prepared by reaction of phosgene with 3-mercaptopropionic acid or 2-(*p*-nitrophenyl)ethanethiol (**5b**) followed by reaction with hexamethylenimine. However, since it is difficult to work with phosgene, a synthetic procedure using a derivative of phosgene, trichloromethyl chloroformate (TCMF), was examined. TCMF has many of the same chemical properties as phosgene and can be handled more easily and conveniently. Thus, the synthesis of *S*-benzyl hexahydroazepine-1-carbothioate (**8**; Figure 3) was investigated. The reaction of TCMF with hexamethylenimine followed by addition of phenylmethanethiol did not yield the proposed product (**8**). When TCMF was reacted with the thiol at room temperature and refluxed and the resulting solution reacted with hexamethylenimine at 0 °C, thiocarbamate **8** was obtained in 36.8% yield with byproducts **9** and **10** as shown in Figure 3. Compound **8**:

Table I. Some Representative Antibody Activities

coating antigen	absorbance (405 nm)[a] rabbit 245, 3a–KLH	rabbit 717, 7b–THY
3a–KLH	0.806	1.134
3a–BSA	0.157	0.311
3a–OA	0.179	0.276
3a–CONA	0.509	1.196
3a–THY	0.541	1.241
3a–FIB	0.536	0.552
7b–BSA	0.158	0.592
7b–OA	0.100	0.497
7b–CONA	0.375	0.913
7b–THY	0.153	0.642
7b–FIB	0.132	0.543

[a] Absorbances were measured in a checkerboard pattern with several coating antigen concentrations and several antibody dilutions. For simplicity only data from a coating antigen concentration of 2 μg/mL and an antibody dilution of 1/2000 are shown. The antibody was from the bleed after the third boost. Absorbance was measured after a 60-min incubation with substrate.

IR (film) 1640, 1610, 1590, 1500, 1470, 1455, 1405 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.6 (m, 8 H, ring CH_2), 3.5 (m, 4 H, CH_2N), 4.12 (s, 2 H, CH_2), 7.3 (m, 5 H, aromatic). Compound **9**: IR (film) 1760, 1645, 1610, 1590, 1500, 1455 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.20 (s, 2 H, CH_2), 7.28 (s, 5 H, aromatic). Compound **10**: IR (film) 1755, 1605, 1590, 1500, 1455 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.12 (s, 4 H, CH_2), 7.30 (s, 10 H, aromatic). Since acid chloride formation was undesirable, haptens **3a**, **3b**, **6a**, and **6b** were prepared by reaction of molinate sulfone and the corresponding thiol (method C) in the presence of triethylamine (Figure 1; DeBaun et al., 1978).

Molinate sulfone (**2**) was prepared by *m*-chloroperoxybenzoic acid oxidation of molinate (**1**). *p*-Nitrobenzyl mercaptan (**5a**) was prepared from the bromide and thiourea, the isothiouronium salt being decomposed in ammonia. 2-(*p*-Nitrophenyl)ethanethiol (**5b**) was obtained in low yield by alkaline hydrolysis of 2-(*p*-nitrophenyl)ethyl thioacetate (**4**), which was prepared from *p*-nitrophenethyl alcohol by slight modification in a Mitsunobu procedure described by Volante (Volante, 1981; Prestwich et al., 1984). Thiocarbamates **7a** and **7b** were prepared from the respective thiocarbamates **6a** and **6b** by reduction of nitroaryls by dodecacarbonyltriiron–methanol (Landesberg et al., 1971). Thiocarbamates **3a**, **3b** and **7a**, **7b** appeared highly pure when analyzed by TLC in several systems, and their structures were confirmed by IR, ^1H NMR, MS, and HRMS.

ELISA Development. Using a checkerboard titration, the coating antigens were each tested against the serum collected. Absorbances of representative sera are shown in Table I. An antisera is a collection of antibodies with varying specificities and affinities. Antibodies of interest were selected through the choice of coating antigen and must have bound more readily to the analyte than the coating antigen. A coating antigen, then, will not usually contain the same protein or coupling linkage as was used for the immunizing antigen. This concept is exemplified in Table I where antiserum from the rabbit immunized with the hapten coupled to KLH showed high absorbances against the coating antigen containing KLH as the carrier protein. Additionally when the same hapten was used for immunization (**3a**–KLH) as for coating (**3a**–CONA), the absorbances were higher (0.509) than if a different hapten was used to coat (**7b**-CONA, 0.375). In those cases where the absorbances were high when a different protein and a different hapten were used to coat compared to that used for immunization, specificity for molinate was indicated.

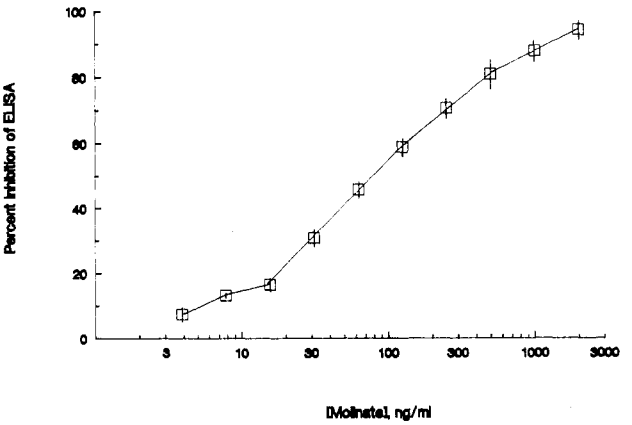


Figure 4. Standard curve for molinate. Each point represents the mean ± SD for three separate experiments run in quadruplicate. The linear range extends from 15 to 500 ng/mL, and the limit of detectability is 3 ng/mL. Plates were coated with 2 μg/mL **7b**–CONA. First antibody was diluted 1/5000 from rabbit 245 immunized with **3a**–KLH and bled on 6/11/85. The curve represented is a point to point connection. For actual calculation using this data, a curve-fitting program was used.

Haptens coupled to BSA or OA were not used as coating antigens as the resulting assays commonly showed low absorbance values. Assays utilizing haptens coupled to CONA, THY, or FIB had routinely higher absorbance values. Table I also points out that there were exceptions for each of the stated examples. Individual responses can vary, and so immunization of several rabbits for each immunizing antigen was warranted.

The combinations with specificity for molinate were further tested in the competition assay to generate a standard curve for molinate for an assessment of sensitivity. Those combinations then showing a good standard curve with linearity over a broad range and reproducibility were optimized for coating antigen concentration and antibody dilution. The standard curve shown in Figure 4 was made with **7b**–CONA as the coating antigen (2 μg/mL) and a 1/5000 dilution of serum from rabbit 245 immunized with **3a**–KLH and bled on 6/11/85. These conditions were used for all further experiments reported here.

Integration of the ELISA with the Classical Analysis Method. The classical method for the analysis of molinate from environmental matrices involves extraction with toluene or dichloromethane–ether, concentration, and analysis by gas chromatography [referenced in Seiber et al. (1986)]. Use of ELISA as another analytical technique for measuring molinate in these same sample extracts is not possible without modification since organic solvents can disrupt the antigen–antibody reaction. However, some organic cosolvent may be necessary to maintain the solubility of the analyte in aqueous solution under experimental conditions for ELISA. Polar, water-miscible solvents such as propylene glycol, acetonitrile, or dimethyl sulfoxide are frequently used in this capacity in immunoassay. In order to integrate the use of ELISA into the classical analysis method, we needed to find an extraction solvent with higher volatility than toluene to determine recoveries after a solvent exchange of this extraction solvent with a more ELISA-compatible solvent such as propylene glycol or acetonitrile and to test the effect of the ELISA-compatible solvents on this particular assay.

Table II shows that dichloromethane alone was the best extraction solvent of those tried under these extraction conditions. Spiked field water samples at three concentrations (25, 100, 500 ppb) extracted with dichloromethane

Table II. Recovery of Molinate from Water after Extraction by Several Solvents and Solvent Exchange into Propylene Glycol

solvent	% recovery by LSC[a]
toluene	89.6 ± 1.3
hexane	78.7 ± 1.4
dichloromethane	95.1 ± 0.9
ethyl acetate	90.5 ± 3.4
petroleum ether	87.7 ± 4.7
pentane–dichloromethane (0.5:9.5)	94.7 ± 0.1
pentane–dichloromethane (1:9)	92.8 ± 1.3
hexane–ethyl acetate (9.5:0.5)	80.8 ± 0.4
hexane–ethyl acetate (9:1)	80.7 ± 2.4
hexane–ethyl acetate (8:2)	84.2 ± 3.1
hexane–ethyl acetate (7:3)	88.2 ± 5.0

[a] Water samples spiked with [^{14}C]molinate (1 mL of 320 ng/mL and 40 000 dpm/mL) were extracted three times with equal volumes of solvent or solvent mixture. Pooled organic phases were evaporated onto a bed of propylene glycol (25 μL) and the propylene glycol was counted. Data are the mean ±SD for n = 3 replicates.

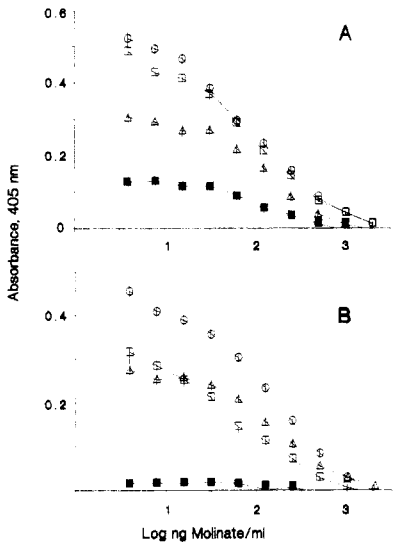


Figure 5. Effect of propylene glycol (A) or acetonitrile (B) on the molinate standard curve. Experimental conditions are the same as in the legend for Figure 4. A: no propylene glycol (□); 5% (○); 12.5% (△); 25% (■) propylene glycol added. B: no acetonitrile (□); 5% (○); 12.5% (△); 25% (■) acetonitrile added. Values are the mean ± SD of three replicates.

followed by exchange of this solvent with propylene glycol gave recoveries of 100, 95, and 107%, respectively.

Figure 5 shows the effect of propylene glycol and acetonitrile on the molinate standard curve. Propylene glycol at 5% did not appreciably affect the standard curve. At 12.5% and greater the sensitivity was significantly decreased (Student's t-test, $p < 0.05$). Acetonitrile, however, had no effect at 12.5%. Surprisingly, 5% acetonitrile actually increased the absorbances in the assay, a phenomenon previously observed with some but not all immunoassays in this laboratory.

IC_{50} for Several Thiocarbamates. To characterize the specificity of the assay, several thiocarbamates and other pesticides were tested in the ELISA system. The molar concentration of compound required to produce 50% inhibition was determined for each compound. Table III gives the IC_{50}s for these compounds. Hapten **3a** was used to raise the antibody used here; hence, the percent cross-reactivity was greater than molinate for **3a** and its close structural analogue **3b**. Other closely related compounds, haptens **7a**, **7b** and molinate sulfone (**2**), had a significant amount of cross-reactivity. This assay system did not detect the hexamethylenimine ring alone (no inhibition at 5×10^{-4} M).

Other thiocarbamates cross-reacted 1% or less. All thiocarbamates giving a measurable IC_{50} had curves parallel to the one for molinate, indicating cross-reactivity rather than interference. Thiobencarb, also a thiocarbamate herbicide used in rice fields, did not cross-react as 5×10^{-4} M (the solubility limit of this compound in the assay). Other pesticides commonly used in rice fields (methyl parathion and carbaryl) were not detected by this assay at concentrations up to 5×10^{-4} M.

Limit of Detectability and Precision of Assay. We defined two assay limits. One was a practical limit of quantitation, that is the lowest concentration observed on the linear portion of the standard curve. The second is the more classically defined limit of detection, that is the lowest concentration that can be detected as two times over background. The assay had a practical limit of detectability in buffer of 15 ng/mL. The classical limit of detectability was determined to be 3 ng/mL. From the standard curve shown in Figure 4 it can be seen that 3 ng/mL would not be on the linear portion of the standard curve so absorbances derived from samples containing this low a concentration would not be accurately quantitated without further concentration of the sample, preparation of very detailed standard curves, or use of mathematical systems to extend the linear portion of the standard curve.

We also determined whether the matrix of interest, water, interfered with the molinate ELISA assay. Standard curves run in distilled, creek, or rice field water were parallel to that run in PBS but were shifted to the right, giving higher limits of quantitation. When the salt and Tween 20 concentrations of these field water samples were normalized by addition of 10× concentrated PBS–Tween, the curves were indistinguishable from that of the standard. All samples were subsequently buffered by the addition of 10× concentrated PBS–Tween.

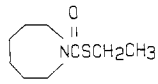
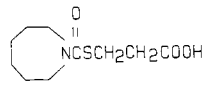
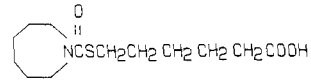
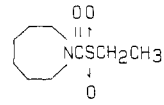
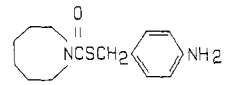
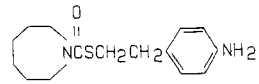
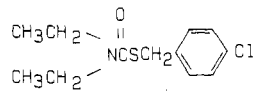
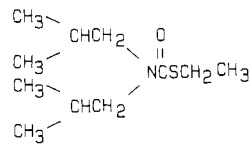
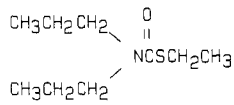
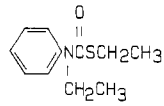
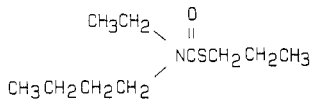
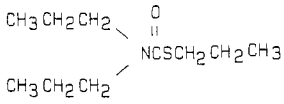
To assess within run variability, nine field-collected samples were each run in quadruplicate on the same day. The mean percent coefficient of variance (CV) for the sample concentrations was 7.8%. Three of these nine samples were run in quadruplicate on four separate days. The average percent CV for the sample concentrations was 9.4%. The percent CV for absorbance values between wells for any given sample or standard was less than 4%. Absorbances greater than 2 times the standard deviation of the four replicates were rejected.

Laboratory Half-Life Study. The results of the laboratory half-life study are shown in Table IV. The half-life values determined by LSC and ELISA for rice field water were 2.6 ± 0.32 and 2.2 ± 0.12 days, respectively. These values are within range of the 2.5–10-day half-life values reported following field application of molinate (Crosby, 1983; Cornacchia et al., 1984; Ross and Sava, 1986). A paired t statistic showed the data to be correlated with the t statistic lying between $0.05 < p < 0.02$.

DISCUSSION

The synthetic procedures used for the haptens investigated were straightforward. Initial synthetic attempts utilizing TCMF as in Figure 3 were more cumbersome and provided low yields of thiocarbamate **8**. Thus, the replacement reactions (Figure 1) were used. These utilized less hazardous starting materials and provided products in reasonable yields and high purities. Once a sulfone intermediate such as **2** is prepared from a readily available thiocarbamate pesticide, a multitude of haptens with desirable functional groups for attachment to proteins can be prepared.

Table III. Inhibition of the Molinate ELISA by Some Thiocarbamates and Related Compounds[a]

compound	structure	I_{50}, M × 10^{-7}	% cross-reactivity	% inhibn at 5 × 10^{-4} M[b]
molinate (Ordram)		5.21 ± 3.80	100	
3a		3.85 ± 2.42	135	
3b		1.64 ± 0.20	318	
molinate sulfone		35.8 ± 34.7	15	
7a		18.8 ± 1.40	28	
7b		14.9 (13.1, 8.4)[c]	35	
hexamethylenimine			0	11.8 (0, 23.7)
thiobencarb (Bolero)			0	29.9 ± 8.8
butylate (Sutan)			0	28.9 ± 5.1
EPTC (Eptam)		491 ± 113	1.1	
cycloate (Roneet)		697 ± 146	0.8	
pebulate (Tillam)		381 ± 232	1.4	
vernolate (Vernam)		539 ± 192	1.0	

[a] Polystyrene 96-well microtiter plates were coated with 2 μg/mL **7b**–CONA. A 1/5000 dilution of serum from rabbit 245 was incubated with several concentrations of the compounds listed. Molar I_{50} values were calculated using Finney's probit analysis. All data are the mean ± SD of three experiments unless otherwise noted. A number of other compounds tested (chemical intermediates and pesticides used in rice culture) gave no inhibition at the highest concentration tested (5 × 10^{-4} M). [b] Highest concentration tested for all compounds. [c] Mean of two experiments, individual values in parentheses.

This paper demonstrates that ELISAs can be used in conjunction with classical techniques for pesticide residue analysis. The limit of detectability of 3 ng/mL is comparable to that obtained by other investigators using ELISA for pesticide quantitation (Van Emon et al., 1986; Wie and Hammock, 1984; Schwalbe et al., 1984; Warner et al., 1986; Huber and Hock, 1985). The assay is specific for molinate over other thiocarbamate herbicides and some other herbicides and insecticides; however, there is some cross-reactivity with molinate sulfone. With spiked samples the ELISA appears precise, simple, and fast. The within-run and between-run variability was less than 10%.

When samples contain ppm levels of molinate, analyses can be conducted without prior extraction or cleanup so that many samples can be analyzed simultaneously. The methodology involving extraction and solvent exchange for samples containing low ppb levels of molinate allows molinate to be presented to the antibody in an acceptable form. This concept of solvent exchange should be widely applicable as immunoassays for other lipophilic materials are integrated into classical cleanup systems.

ABBREVIATIONS USED

BSA, bovine serum albumin; CONA, conalbumin; ELISA, enzyme-linked immunosorbent assay; FIB, fibrinogen; HRMS, high-resolution mass spectrum; IC_{50}, the

Table IV. Comparison of ELISA and LSC Methods for Measuring Molinate (ppm) in Water: Laboratory Half-Life Study[a]

day	ELISA	LSC	day	ELISA	LSC
0	1.34	1.71	2.0	1.7	1.07
0.04	1.28	1.80	2.5	1.21	1.20
0.17	2.82	1.82	3.0	1.1	1.0
0.33	2.2	1.69	3.5	1.18	0.51
0.5	2.19	1.85	4.0	1.05	0.81
1.0	1.85	1.35	4.5	0.64	0.86
1.5	1.95	1.41			

[a] Rice field water was spiked with [^{14}C]molinate and enough unlabeled molinate to yield 2000 ng/mL and about 2000 cpm/mL. Beakers were placed in the sun without aeration, and 1 mL was sampled at each time point. Each value is the mean ± SD of three sample determinations.

concentration inhibiting 50% of an ELISA absorbance value; IR, infrared spectrometry; KLH, keyhole limpet hemocyanin; LSC, liquid scintillation counting; MS, low-resolution mass spectrum; ^{1}H NMR, nuclear magnetic resonance; OA, ovalbumin; PBS, phosphate-buffered saline; TCMF, trichloromethyl chloroformate; THF, tetrahydrofuran; THY, thyroglobulin; TLC, thin-layer chromatography.

Registry No. 1, 2212-67-1; 2, 54404-54-5; **3a**, 114583-21-0; **3b**, 114583-22-1; **4**, 114583-24-3; **5a**, 26798-33-4; **5b**, 100949-14-2; **6a**, 81712-33-6; **6b**, 114583-25-4; **7a**, 114583-23-2; **7b**, 114583-26-5; EPTC, 759-94-4; hexamethylenimine, 111-49-9; thiobencarb, 28249-77-6; butylate, 2008-41-5; cycloate, 1134-23-2; pebulate, 1114-71-2; vernolate, 1929-77-7; *m*-chloroperoxybenzoic acid, 937-14-4; 3-mercaptopropionic acid, 107-96-0; 6-mercaptohexanoic acid, 17689-17-7; 6-bromohexanoic acid, 4224-70-8; *p*-nitrobenzyl bromide, 100-11-8; thiourea, 62-56-6; dodecacarbonyltriiron, 17685-52-8; diisopropyl azodicarboxylate, 2446-83-5; 4-nitrophenethyl alcohol, 100-27-6.

Supplementary Material Available: Program and documentation for TRANSFER.BAS written in BASIC using data from the files created by PC-EIA (Dorian Software) and rearranging the data in a user-specified manner for import into LOTUS 1-2-3 (Lotus Development Corp.) (17 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

Cornacchia, J. W.; Cohen, D. B.; Bowes, G. W.; Schnagl, R. J.; Montoya, B. L. *Rice Herbicides, Molinate (Ordram) and Thiobencarb (Bolero): A Water Quality Assessment*; California State Water Resources: Sacramento, CA, 1984.

Crosby, D. G. "The Fate of Herbicides in California Rice Culture". In *IUPAC Pesticide Chemistry, Human Welfare and the Environment*; Miyamoto, J., et al., Eds.; Pergamon: Oxford, 1983; pp 339–346.

DeBaun, J. R.; Bova, D. L.; Tseng, C. K.; Menn, J. J. "Metabolism of [*ring*-^{14}C]Ordram (Molinate) in the Rat. 2. Urinary Metabolite Identification". *J. Agric. Food Chem.* **1978**, *26*, 1098–1104.

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

Hammock, B. D.; Gee, S. J.; Cheung, P. Y. K.; Miyamoto, T.; Goodrow, M. H.; Van Emon, J.; Seiber, J. N. "Utility of Immunoassay in Pesticide Trace Analysis". In *Pesticide Science and Biotechnology*; Greenhalgh, R., Roberts, T. R., Eds.; Blackwell Scientific: Ottawa, 1987; pp 309–316.

Huber, S. J.; Hock, B. "A Solid-Phase Enzyme Immunoassay for Quantitative Determination of the Herbicide Terbutryn". *Z. Pflanzenkrankh. Pflanzenschutz* **1985**, *92*, 147–156.

Kelley, M. M.; Zahnow, E. W.; Petersen, C.; Toy, S. T. "Chlorsulfuron Determination in Soil Extracts by Enzyme Immunoassay". *J. Agric. Food Chem.* **1985**, *33*, 962–965.

Landesberg, J. M.; Katz, L.; Olsen, C. "Reduction of Nitroaryls by Dodecacarbonyltriiron-Methanol". *J. Org. Chem.* **1972**, *37*, 930–936.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. "Protein Measurement with the Folin Phenol Reagent". *J. Biol. Chem.* **1951**, *193*, 265–275.

Minisci, F. "Perossidi e Sali di Diazonio. Nota IV. Solfocian e Ditio-Derivati da l-Ossi-Idroperossidi". *Gazz. Chim. Ital.* **1959**, *89*, 2428–2437.

Newsome, W. H. "Development of an Enzyme-Linked Immunosorbent Assay for Triadimefon in Foods". *Bull. Environ. Contam. Toxicol.* **1986**, *36*, 9–14.

Nisonoff, A. "Coupling of Diazonium Compounds to Proteins". In *Methods in Immunology and Immunochemistry*; Williams, C. A., Chase, M. W., Eds.; Academic: New York, 1967; Vol. 1, pp 120–126.

Patchett, G. G.; Batchelder, G. H. "Ordram". In *Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives*; Zweig, G., Ed.; Academic: New York, 1967; Vol. V, pp 469–472.

Prestwich, G. D.; Eng, W.-S.; Roe, R. M.; Hammock, B. D. "Synthesis and Bioassay of Isoprenoid 3-Alkylthio-1,1,1-trifluoro-2-propanones: Potent, Selective Inhibitors of Juvenile Hormone Esterase". *Arch. Biochem. Biophys.* **1984**, *228*, 639–645.

Ross, L. J.; Sava, R. J. "Fate of Thiobencarb and Molinate in Rice Fields". *J. Environ. Qual.* **1986**, *15*, 220–225.

Saville, B. "Reaction of Thiols with Tetra-alkylthiuram Disulphides and Related Compounds". *J. Chem. Soc.* **1960**, 1730–1734.

Schwalbe, M.; Dorn, E.; Beyermann, K. "Enzyme Immunoassay and Fluoroimmunoassay for the Herbicide Diclofop-Methyl". *J. Agric. Food Chem.* **1984**, *32*, 734–741.

Seiber, J. N.; McChesney, M. M.; Sanders, P. F.; Woodrow, J. W. "Models for Assessing the Volatilization of Herbicides Applied to Flooded Rice Fields". *Chemosphere* **1986**, *15*, 127–138.

Van Emon, J.; Hammock, B. D.; Seiber, J. N. "Enzyme-Linked Immunosorbent Assay for Paraquat and Its Application to Exposure Analysis". *Anal. Chem.* **1986**, *58*, 1866–1873.

Volante, R. P. "A New, Highly Efficient Method for the Conversion of Alcohols to Thiolesters and Thiols". *Tetrahedron Lett.* **1981**, *22*, 3119–3122.

Warner, R.; Ram, B. P.; Hart, L. P.; Pestka, J. J. "Screening for Zearalenone in Corn by Competitive Direct Enzyme-Linked Immunosorbent Assay". *J. Agric. Food Chem.* **1986**, *34*, 714–717.

Wie, S. I.; Hammock, B. D. "Development of Enzyme-Linked Immunosorbent Assays for Residue Analysis of Diflubenzuron and BAY SIR 8514". *J. Agric. Food Chem.* **1982**, *30*, 949–957.

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.

Zweig, G.; Sherma, J. "Ordram". In *Analytical Methods for Pesticides and Plant Growth Regulators*; Zweig, G., Ed.; Academic: New York, 1972; Vol. VI, pp 668–670.

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