Quantitation of Bioresmethrin, a Synthetic Pyrethroid Grain Protectant, by Enzyme Immunoassay

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An enzyme immunoassay was developed for the synthetic pyrethroid, bioresmethrin, by use of a novel approach for synthesis of the pyrethroid–protein hapten conjugate for antibody preparation. Bioresmethrin was hydrolyzed at the ester linkage, and following protection of the chrysanthemic acid group, the 2-methylprop-1-ene substituent was oxidatively cleaved. The newly formed and unprotected acid group was reesterified to the other bioresmethrin hydrolysis product [[2-(phenylmethyl)-4-furyl]-methanol], and following substitution of the protecting group, the hapten was coupled to either protein for antibody production or peroxidase for use in the immunoassay. The most sensitive assay employed an antibody prepared to a derivative with a 4-carbon spacer arm between bioresmethrin and carrier protein, but used a bioresmethrin–enzyme reporter prepared using a 4-(aminomethyl)cyclohexane-carboxylic acid spacer arm (limit of detection 2 ppb in buffer, 50 ppb in whole wheat or barley grain). Good correlations between HPLC and ELISA determinations of bioresmethrin in whole or ground barley grain were obtained. The sensitivity of the assay was slightly lower in ground grain or flour milling fractions due to interference from coextractives in methanol extracts. Apart from resmethrin, of which bioresmethrin is the 1*R*,3*R*-*trans*-isomer, the assay did not detect a variety of other pyrethroids in commercial use.

INTRODUCTION

Synthetic pyrethroids are finding an increasing place in agriculture, because they are of lower toxicity to man and the environment than most other insecticide groups (Davies, 1985), such as organophosphates, carbamates, and organochlorines. Several members of the group are designed to decompose over a period of months after application (Hevre, 1985). They are effective against many domestic insects and stored commodity pests and, because of their lower human toxicity, are approved for direct application to a number of stored products, including harvested cereal grains. In grain protection practice, synthetic pyrethroids are not typically used on their own, but rather in admixture with an organophosphate, since the spectrum of insect pests of concern in most countries is too broad to enable a single compound to be used (Desmarchelier et al., 1981; Snelson, 1987). As a result of their use on foodstuff commodities, it is important for grain trading and for human health to develop simple methods for monitoring synthetic pyrethroid residues (Papadopoulous-Mourkidow, 1983). Analysis of synthetic pyrethroids can be more challenging than that of organophosphates, because a number of pyrethroids lack a suitable element for use of element-specific detection following fractionation by gas chromatography (Sharp et al., 1988). In addition, their application rates and their residue levels are generally lower than those of other insecticides (Desmarchelier et al., 1981).

High-performance liquid chromatography has been used by several groups, but since detection is less specific and the methods usually less sensitive, significant sample cleanup and concentration are required in certain matrices (Gunew, 1978; Bottomley and Baker, 1984). Another approach, gas chromatography–mass spectrometry, can offer the necessary selectivity (Mestres et al., 1979), but equipment is expensive and sample throughput rather limited. Because of these concerns, a number of enzyme-linked immunosorbent assay (ELISA) methods have been developed for detection of synthetic pyrethroids. Pyrethroids detected by immunoassay include separate assays for (*S*)-bioallethrin (Wing et al., 1978), permethrin/1*R*-phenothrin (Stanker et al., 1989; Skerritt et al., 1992a), cypermethrin (Wraith et al., 1986; B. Hock, Technical University of Munich, Freising, Germany, 1992, unpublished), and deltamethrin (Demoute et al., 1987). These have been applied to a limited number of matrices including water (Wengatz et al., 1991), meat (Stanker et al., 1989), and black tea (Wraith, unpublished). In addition, we have recently modified and simplified an assay for permethrin and phenothrin in grain and grain milling fractions (Skerritt et al., 1992a).

The major synthetic pyrethroid used on Australian domestic and export grain is bioresmethrin (BRM; Figure 1A), the 1*R*,3*R*-*trans*-isomer of resmethrin (Bengston et al., 1980, 1983), which is also used widely in domestic insect control. It differs from the two major classes of pyrethroid in not being an ester of either phenoxybenzyl alcohol or α-cyanophenoxybenzyl alcohol, but rather an ester of [2-(phenylmethyl)-4-furyl]methanol. The presence of the furan ring and the chrysanthemic acid moiety render the molecule more susceptible to photolytic and hydrolytic breakdown after application than compounds with halogenated acid moieties and *m*-phenoxybenzyl and similar alcohols. Environmentally, this is a desirable attribute, but it complicated the chemical approaches to hapten synthesis. In this paper, we report the development of a sensitive and specific immunoassay for the detection of BRM and describe its application to wheat and barley grain matrices.

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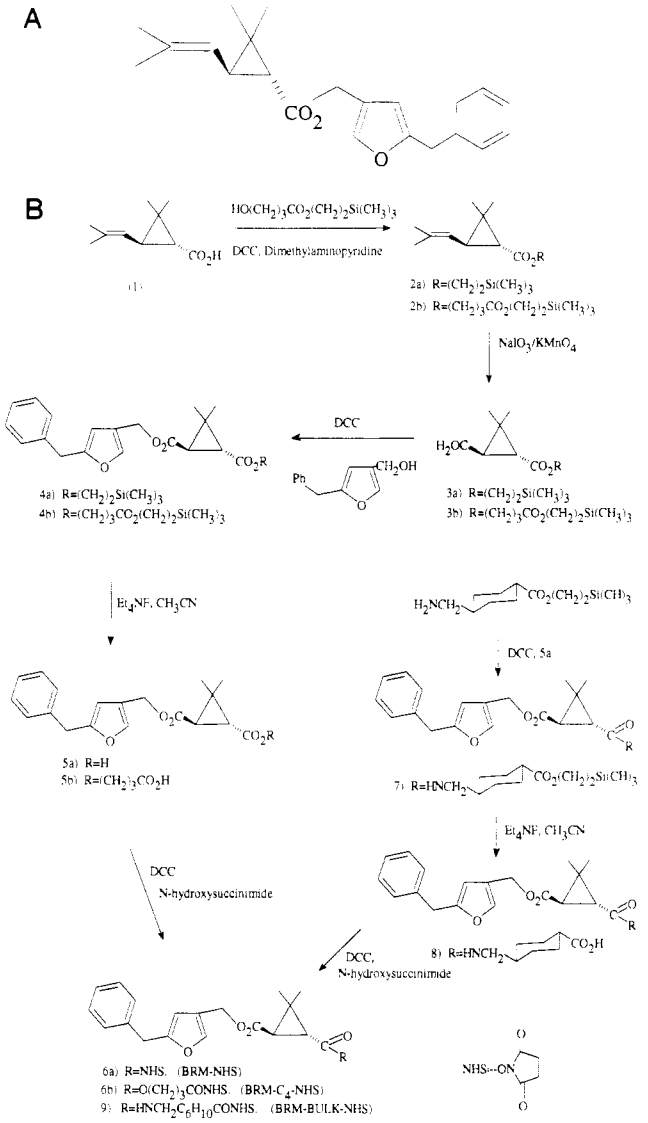

Figure 1. (A) Structure of bioresmethrin. (B) Synthesis of bioresmethrin–protein conjugates.

MATERIALS AND METHODS

Preparation of Bioresmethrin Haptens. The bioresmethrin–protein conjugates prepared for immunization and use in the assay were coupled through the cyclopropane end of the pyrethroid moiety (Figure 1B). Three different spacer arm (bridge) strategies were employed: (1) no spacer arm—direct esterification to protein; (2) a linear 4-carbon spacer (4-hydroxybutanoic acid), derived from succinic acid; and (3) a bulky spacer, based on 4-(aminomethyl)cyclohexanecarboxylic acid (Okano et al., 1972). Since earlier work on the development of antibodies to permethrin and cypermethrin had demonstrated that improved sensitivity was obtained when esters of the phenoxybenzyl group (originally esterified to chrysanthemic acid) were coupled to protein or enzyme for use in the ELISA (Stanker et al., 1989; Skerritt et al., 1992a), the corresponding benzylfuran derivatives were also prepared. Column chromatography was performed using silica gel 60 (70–230 mesh, Merck, Darmstadt, Germany). The identities of chemical intermediates in each synthesis were confirmed by proton and ^{13}C NMR spectroscopy on a 200-MHz (Gemini 200) instrument. Infrared spectroscopy (Hitachi 279-30 spectrometer) and melting point determinations were also performed.

Preparation of 2-(Trimethylsilyl)ethyl 4-Hydroxybutanoate. Succinic anhydride (2 g, 20 mmol) was treated with 2-(trimethylsilyl)ethanol (2.0 g, 17 mmol) in dichloromethane (20 mL) and pyridine (2 mL) by stirring overnight at room temperature. The solution was diluted with dichloromethane (20 mL), then washed (1 M HCl, water, brine), and dried over $MgSO_4$. Filtration, followed by concentration, gave 2-(trimethylsilyl)ethyl succinate as an oil (2.98 g, 81%). This was reduced using ethyl chloroformate and sodium borohydrate (Ishizumi et al., 1968), then chromatography on silica (2% methanol in chloroform), to give the 2-(trimethylsilyl)ethyl 4-hydroxybutanoate as an oil (860 mg, 31%) (Mei et al., 1991).

(1R-trans)-2,2-Dimethyl-3-(2-methylprop-1-enyl)cyclopropane-1-carboxylic Acid, 2-(Trimethylsilyl)ethyl Ester (**2a**), *and (1R-trans)-2,2-Dimethyl-3-(2-methylprop-1-enyl)cyclopropane-1-carboxylic Acid, 4-[2-(Trimethylsilyl)ethoxy]-4-oxobutyl Ester* (**2b**). To the acid [(1*R-trans*)-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropane-1-carboxylic acid (1)] (620 mg, 3.3 mmol) in dichloromethane (15 mL) containing oxalyl chloride (300 μL, 3.4 mmol), precooled in an ice bath, was added DMF (50 μL), and the solution was stirred for 2 h at room temperature. 2-(Trimethylsilyl)ethyl 4-hydroxybutanoate (610 mg, 3.0 mmol) and 1 mL of pyridine were added to the mixture, and stirring was continued overnight. The solution was concentrated, the residue partitioned between ethyl ether and water, and the organic layer washed (water, 1 M HCl, sodium carbonate, water, brine) and dried over $MgSO_4$. Following filtration and concentration, the residue was chromatographed on silica (50% chloroform–50% petroleum ether) to yield **2a** as an oil (270 mg, 29% yield): ^1H NMR δ 4.89, d, *J* 7.8 Hz, =C*H*; 4.16, t, *J* 8.1 Hz, OCH_2; 2.04, dd *J* 5.2 Hz, H2; 1.71, s, 2 × CH_3; 1.35, d, C*H*CO; 1.26, 1.13, s, 2 × CH_3; 0.99, t, CH_2Si; 0.04 s, Si$(CH_3)_3$; ^{13}C NMR δ 172.7, CO; 135.3, $(CH_3)_2C$=; 121.2, =CH; 62.3, CH_2O; 34.9, 32.5, $(CH_3)_2C$=; 28.4, *C*(CH_3); 25.6, *C*HCO; 22.2, 20.4, 2 × CH_3; 18.5, *C*HC; 17.5, CH_2Si; −1.5, $(CH_3)_3Si$.

Further elution of the column gave **2b** as an oil (550 mg, 52%): ^1H NMR δ 4.87, dd, *J* 8.0 Hz, *J* 1.3 Hz, =CH; 4.17, t *J* 8.4, OCH_2; 4.09, t, *J* 6.4 HZ, OCH_2; 2.37, t, *J* 7.5 Hz, CH_2CO; 1.96, m, CH, CCH_2C; 1.69, s, 2 × CH_3; 1.36, d, *J* 5.4, CHCO; 1.25, 1.12, s, 2 × CH_3; 0.97, t, CH_2Si; 0.03, s, 3 × CH_3; ^{13}C NMR δ 173.0, 172.4, CO; 135.5, =C; 121.1, =CH; 63.2, 62.7, 2 × OCH_2; 34.7, 32.7, 2 × CH_3; 31.0, CH_2CO; 28.6, *C*$(CH_3)_2$; 27.6, *C*HCO; 24.2, CCH_2C; 22.2, 20.4, 2 × CH_3; 18.5, CH; 19.4, CH_2Si; 0.5, Si$(CH_3)_3$.

The compound **2a** was also prepared as above using 2-(trimethylsilyl)ethanol (350 mg, 3.0 mmol) to yield 640 mg (80%) after column chromatography on silica.

(1R-trans)-2,2-Dimethylcyclopropane-1,3-dicarboxylic Acid, 2-(Trimethylsilyl)ethyl Monoester (**3a**). The alkene **2a** (540 mg, 2.0 mmol) in *tert*-butyl alcohol (7 mL) was added to a mixture of pyridine (2.5 mL) and oxidant (25 mL of 0.1 M KIO_3, 1.7 mM $KMnO_4$), and the mixture was stirred (4 h). The mixture was then acidified to pH 2 with 1 M HCl and extracted twice with ethyl acetate. The organic layers were combined and then washed (0.1 M $Na_2S_2O_5$, water, brine), dried ($MgSO_4$), and concentrated. The residue was chromatographed on silica (methanol–chloroform, 5:95) to give an oil which crystallized on standing at 0 °C (390 mg, 75%): mp 65–67 °C (heptane, −10 °C); ^1H NMR δ 4.19, t, *J* 8.5 Hz, OCH_2; 2.23, s, 2 × C*H*CO; 1.33, 1.32, s, 2 × CH_3; 1.01, t, CH_2Si; 0.05, s, Si$(CH_3)_3$; ^{13}C NMR δ 176.7, COOH; 170.2, COO; 63.2, CH_2O; 34.4, 33.1, 2 × COCH; 31.2, *C*$(CH_3)_2$; 20.5, 20.5, 2 × CH_3; 17.4, CH_2Si; −1.5, Si$(CH_3)_3$.

(1R-trans)-2,2-Dimethylcyclopropane-1,3-dicarboxylic Acid, 4-[2-(Trimethylsilyl)ethoxy]-4-oxobutyl Monoester (**3b**). The alkene **2b** (170 mg, 0.5 mmol) was treated as for **2a**, which gave an oil (**3a**, 160 mg, 93%) after chromatography on silica (0.5% acetic acid in chloroform): ^1H NMR δ 4.16 m, 2 × $COCH_3$, 2.39, t, *J* 7.4 Hz, CH_2CO; 2.25, s, 2 × C*H*CO; 1.98, m, CCH_2C; 1.34, 1.31, s, 2 × CH_3; 0.99, t, *J* 8.6 Hz, CH_2Si; 0.04, s, Si$(CH_3)_3$; ^{13}C NMR δ 176.2, COOH; 173.0, 170.0, 2 × CO; 63.9, 62.8, 2 × OCH_2, 34;1, 33.3, 2 × CHCO; 31.7, *C*$(CH_3)_2$; 30.9, CH_2CO; 24.1, CCH_2C; 20.5, 20.4, 2 × CH_3; 17.3, CH_2Si; 0.5 Si$(CH_3)_3$.

(1R-trans)-2,2-Dimethylcyclopropane-1,3-dicarboxylic Acid, [2-(Phenylmethyl)-4-furyl]methyl Ester, 2-(Trimethylsilyl)-ethyl Ester (**4a**). To a mixture of **3a** (260 mg, 1.0 mmol) and [2-(phenylmethyl)-4-furyl]methanol (300 mg, 1.6 mmol) in dichloromethane (10 mL) at 0 °C were added 1,3-dicyclohexylcarbodiimide (DCC, 330 mg, 1.6 mmol) and 4-(dimethylamino)-pyridine (20 mg). The mixture was stirred overnight, while warming to room temperature, and then filtered, and the filtrate was concentrated. The ester **4a** was obtained after chromatography on silica (ethyl acetate–petroleum ether, 4:1) as an oil (340 mg, 79%): ^1H NMR δ 7.29, m, 6H, ArH, HC=; 6.04, s, HC=C;

4.93, d, J 2.4 Hz, CCH_2O; 4.18 m, OCH_2; 3.94, s, $ArCH_2$; 2.22, 2.21, s, CHCO; 1.28, 1.27, s, $C(CH_3)_2$; 1.00, m, CH_3Si; 0.04, s, $Si(CH_3)_3$; ^{13}C NMR δ 170.5, 170.4, 2 × CO_2; 155.6; C5; 140.5, C2; 137.7, Ph (C1); 128.7, 128.5 Ph (C2,6 and C3,5); 126.6 Ph (C4); 120.9, C3; 107.3, C4; 63.2, OCH_2CH_2; 58.3, CCH_2O; 34.5, $ArCH_2$; 33.9, 33.4, 2 × CHCO; 30.5, $C(CH_3)_2$; 20.4, 2 × CH_3; 17.4, CH_2Si; −1.5, $Si(CH_3)_3$.

(1R-trans)-2,2-Dimethylcyclopropane-1,3-dicarboxylic Acid, [2-(Phenylmethyl)-4-furyl]methyl Ester, 4-[2-(Trimethylsilyl)-ethoxy]-4-oxobutyl Ester (**4b**). The acid **3b** (440 mg, 1.3 mmol) was treated as for **3a**. The ester **3b** was obtained after chromatography on silica (chloroform–petroleum ether, 4:1) as an oil (430 mg, 65%): ^1H NMR δ 7.29, m, 6H, ArH, HC=; 6.04, s, HC=; 4.93, d, J 2.2 Hz, CCH_2O; 4.12, m, 4H, 2 × OCH_2; 3.95, s, $ArCH_2$; 2.39, t, J 7.3 Hz, CH_2CO; 2.22, s, 2H, 2 × $CHCO_2$; 1.98, m, CCH_2C; 1.27, s, 2 × CH_3; 0.99, t, CH_2Si; 0.05, s, $Si(CH_3)_3$; ^{13}C NMR δ 172.0, CO; 170.2, 2 × CO; 155.6, C5; 140.5, C1; 137.6, Ph (C1); 128.7, 128.5, Ph (C2,6 and C3,5); 126.6, Ph (C4); 120.9, C3; 107.2, C4; 63.8, 62.8, 2 × OCH_2; 58.3, CCH_2O; 34.5, $ArCH_2$; 33.6, 2 × CCO; 30.9, CCH_2CO; 29.7, $C(CH_3)_2$; 24.1, CCH_2CH; 20.4, $C(CH_3)_2$; 17.3, CH_2Si; 1.5, $Si(CH_3)_3$.

(1R-trans)-2,2-Dimethylcyclopropane-1,3-dicarboxylic Acid, [2-(Phenylmethyl)-4-furyl]methyl Monoester (**5a**). To the ester **4a** (330 mg, 0.77 mmol) in acetonitrile (10 mL) was added tetraethylammonium fluoride (300 mg, 2.0 mmol), and the solution was stirred overnight at 20 °C. The solution was concentrated, the residue was dissolved in ethyl acetate and then washed (1 M HCl, water, brine), dried ($MgSO_4$), and after chromatography on silica (35% ethyl acetate–0.5% acetate acid–64.5% petroleum ether), the residue was concentrated to give **5a** an oil (154 mg, 61%): ^1H NMR δ 7.29, m, ArH, HC=C; 6.04, s, HC=C; 4.94, d, $J_{H,H}$ 2.2 Hz, CCH_2O; 3.95, s, $ArCH_2$; 2.26, 2.25, s, 2 × CHC; 1.32, 1.29, s, $C(CH_3)_2$; ^{13}C NMR δ 170.0, 2 × CO; 155.7, C5; 140.5, C2; 137.6, Ph (C1); 128.7, 128.6, Ph (C2,6 and C3,5); 126.6, Ph (C4); 120.9, C3; 107.2, C4; 58.4, CH_2O; 34.5, $ArCH_2$; 34.1, 3, 3.7, 2 × CHCO; 31.3, $C(CH_3)_2$; 20.5, 20.4, $(CH_3)_2C$.

(1R-trans)-4-[[[3-[[[2-(Phenylmethyl)-4-furyl]methoxy]carbonyl]-2,2-dimethyl-1-cyclopropyl]carbonyl]oxy]butanoic Acid (**5b**). The ester **4b** (420 mg, 0.82 mmol) was treated as for **4a** to give the acid **5b** as an oil (265 mg, 80%), which was pure by proton NMR analysis: ^1H NMR δ 7.29, m, 6H, ArH, HC=C; 6.04, s, HC=C; 4.94, d, J 2.3, CCH_2O; 4.14, t, J 7.0 Hz, CH_2O; 3.94, s, $ArCH_2$; 2.47, t, J 7.1 Hz, CH_2CO, 2.23, s, 2 × CHCO; 1.99, m, CCH_2C; 1.28, 1.27, s, $C(CH_3)_2$; ^{13}C NMR δ 178.7, COOH; 170.3, 2 × CO; 155.7, C5; 140.5, C2; 137.6, Ph (C1); 128.7, 128.5, Ph (C2,6 and C3,5); 126.6, Ph (C4); 120.9, C3; 107.2, C4; 63.6, CH_2CH_2O; 58.4, CCH_2O; 34.6, $ArCH_2$; 33.6, 33.5; 2 × CHCO; 30.6, CH_2CO, 29.7, $C(CH_3)_2$; 23.8, $CH_2CH_2CH_2$; 20.4, $C(CH_3)_2$.

(1R-trans)-2,2-Dimethylcyclopropane-1,3-dicarboxylic Acid, [2-(Phenylmethyl)-4-furyl]methyl Ester, 2,5-Dioxo-1-pyrrolidinyl Ester (**6a**). To the acid **5a** (150 mg, 0.65 mmol) in dichloromethane (10 mL), cooled in an ice bath, was added *N*-hydroxysuccinimide (100 mg, 0.9 mmol) followed by dicyclohexylcarbodiimide (DCC, 165 mg, 0.8 mmol), and the mixture was stirred overnight, allowing it to warm to 20 °C. Filtration, concentration of the filtrate, and chromatography of the residue (35% ethyl acetate–65% petroleum ether) gave **6a** as an oil (190 mg, 98%): ^1H NMR δ 7.28, m, ArH, HC=C; 6.05, s, HC=C; 4.95, s, CCH_2O; 3.95, s, $ArCH_2$; 2.83, s, $COCH_2CH_2CO$; 2.49, d, J 5.5 Hz, COCH; 2.35, d, COCH; 1.34, 1.33, s, $C(CH_3)_2$; ^{13}C NMR δ 169.1, 169.0, 2 × CO_2; 165.8, 2 × CON; 155.8, C5; 140.7, C2; 137.6, Ph (C1); 128.7, 128.5, Ph (C2,6 and C3,5); 126.6, Ph (C4); 120.6, C3; 107.3, C4; 58.7, CH_2O; 34.9 $ArCH_2$; 34.5, CHCON; 32.4, CHCO; 30.0 $C(CH_3)_2$; 25.6, 2 × CH_2CO; 20.4, 20.1, CH_3.

(1R-trans)-2,2-Dimethylcyclopropane-1,3-dicarboxylic Acid, [2-(Phenylmethyl)-4-furyl]methyl Ester, 4-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-4-oxobutyl Ester (**6b**). The acid **5b** (265 mg, 0.65 mmol) was treated as for **5a** to give **6b** as an oil (190 mg, 98%) after chromatography (35% ethyl acetate–65% petroleum ether): ^1H NMR δ 7.29, m, ArH, HC=C; 6.05, d, J 1.0 Hz, HC=C; 4.94, d, J 1.8 Hz, CCH_2O; 4.18, t, J 6.2 Hz, CH_2O; 3.94, s, $ArCH_3$; 2.83, s, $COCH_2CH_2CO$; 2.74, t, J 7.6 Hz, CH_2; 2.24, s, 2 × CHCO; 2.09, m, $CH_2CH_2CH_2$; 1.28, s, $C(CH_3)_2$; ^{13}C NMR δ 170.2, 2 × CO; 168.9, CONCO; 167.9, CO; 155.6, C5; 140.5, C2; 137.7 Ph (C1); 128.7, 128.5, Ph (C2,6 and C3.5); 126.6, Ph (C4); 120.9, C3; 107.2, C4; 63.0, CH_2CH_2O; 34.5, $ArCH_2$; 33.7, 33.5, 2 × CHCO; 30.7, $C(CH_3)_2$; 27.8, CH_2CH_2O; 25.6, $COCH_2CH_2CO$; 23.8, $CH_2CH_2CH_2$; 20.4, $C(CH_3)_2$.

(1R-trans)-3-[[[[4-[[2-(Trimethylsilyl)ethoxy]carbonyl]-cyclohexyl]methyl]amino]carbonyl]-2,2-dimethylcyclopropane-1-carboxylic Acid, [2-(Phenylmethyl)-4-furyl]methyl Ester (**7**). DCC (210 mg, 1.0 mmol) was added to the acid **5a** (250 mg, 0.75 mmol) in dichloromethane (10 mL), and the mixture was stirred for 20 min at 0 °C. [2-(Trimethylsilyl)ethyl] *trans*-4-(aminomethyl)cyclohexanecarboxylate (250 mg, 1.0 mmol; Okano et al., 1972) was then added to this mixture, and it was stirred overnight at room temperature. Following filtration, concentration, and chromatography on silica (30% ethyl acetate–70% petroleum ether), the product **7** (265 mg, 61%) crystallized on standing: mp 78–79 ° petroleum ether); ^1H NMR δ 7.28, m, 6H, ArH, HC=C; 6.03, s, HC=C; 5.72, b, NH; 4.92, d, J 2.5 Hz, CCH_2O; 4.14, t, J 8.4 Hz, OCH_2; 3.94, s, $ArCH_2$; 3.12, m, NCH_2; 2.30–1.00, m, 10H, cyclohexyl; 1.26, 124, s, $C(CH_3)_2$; 0.97 m, CH_2Si; 0.44, s, $Si(CH_3)_3$; ^{13}C NMR δ 176.0, CON; 171.1, 168.7, 2 × CO; 155.6; C5; 140.4, C2; 137.7, Ph (C1); 128.7, 128.5 Ph (C2,6 and C3,5); 126.6, Ph (C4); 121.0, C3; 107.2, C4; 62.5, CH_2O; 58.3, CCH_2O; 45.7, CH_2N; 43.4, $CHCH_2N$; 37.5, $CHCO_2$ (cyclohexyl); 35.7, CHCON; 34.5, $ArCH_2$; 32.3, $CHCO_2$; 29.8, 2 × CH_2CHCO; 29.8, $C(CH_3)_2$; 28.4, 2 × CH_2CHCH_2; 20.5, 20.2, $C(CH_3)_2$; 17.3, CH_2Si; −1.4, $Si(CH_2)_3$.

trans-4-[[[[(1R-trans)-3-[[[2-(Phenylmethyl)-4-furyl]methoxy]-carbonyl]-2,2-dimethyl-1-cyclopropyl]carbonyl]amino]methyl]-cyclohexane-1-carboxylic Acid (**8**). The ester **7** (265 mg, 0.47 mmol) was treated with tetraethylammonium fluoride (150 mg, 1.0 mmol) as for **4a**, to give **8** as an oil (230 mg, 97%), which solidified as standing: ^1H NMR δ 7.29, m, 6H, ArH, HC=C; 6.04, s, HC=C 5.72, b, t, NH; 4.92, J 2.2 Hz, CCH_2O; 3.94, s, $ArCH_2$; 3.12, m, CH_2N; 2.30–1.70, m, 8H, cyclohexyl, 2 × CHCO; 1.60–0.95, m, 4H, cyclohexyl; 1.26, 1.24, s, $C(CH_3)_2$; ^{13}C NMR δ 180.9, COOH; 171.1, CO; 168.8, CON; 155.6, C5; 140.4, C2; 137.7, Ph (C1); 128.7, 128.5, Ph (C2,6 and C3,5); 126.6, Ph (C4); 120.9, C3; 107.2, C4; 58.3, CH_2O; 45.6, CH_2N; 42.8, $CHCH_2N$; 37.4, CHCOOH; 35.7, CHCON; 34.5, $ArCH_2$; 32.3, CH_2COO; 29.9, $C(CH_3)_2$; 29.7, 2 × CH_2CHCH_2N; 28.7, 2 × CH_2CHCO; 20.5, 20.2, $C(CH_3)_2$.

(3R-trans)-3-[[[[trans-4-[[(2,5-Dioxo-1-pyrrolidinyl)oxy]carbonyl]cyclohexyl]methyl]amino]carbonyl]-2,2-dimethylcyclopropane-1-carboxylic Acid, [2-(Phenylmethyl)-4-furyl]methyl Ester (**9**). The acid **8** (95 mg, 0.2 mmol) was treated as for **5b** with NHS (35 mg, 0.3 mmol) and DCC (70 mg, 0.35 mmol) to give **6c** as an oil (93 mg, 81%) after chromatography on silica (60% ethyl acetate–40% petroleum ether): ^1H NMR δ 7.29, m, ArH_2, HC=C; 6.04, HC=C; 5.74, b, NH; 4.92, d, $J_{H,H}$ 2.5 Hz, CCH_2O; 3.94, $ArCH_2$; 3.15, d, d, J 6.6 Hz, J 6.3 Hz, CH_2N; 2.83, s, $COCH_2CH_2CO$; 2.3–1.05, m, 10H, cyclohexyl; 1.26, 1.25, s, $C(CH_3)_2$; ^{13}C NMR δ 171.1, CO_2; 170.6, CON; 169.2, 2 × $NCOCH_2$; 168.8, CONH; 155.5, C5; 140.3, C2; 137.6, Ph (C1); 128.7, 128.5, Ph (C2,6 and C3,5); 126.5, Ph (C4); 120.9, C3; 107.2, C4; 58.2, CH_2O; 45.4, NCH_2; 40.5, NCH_2CH; 37.2, CHCON (cyclohexyl); 35.7, CHCON; 34.4, $ArCH_2$; 32.2, CHCOO, 29.8, $C(CH_3)_2$; 29.3, 2 × CH_2CHCO; 28.1, 2 × CH_2CH; 25.5, 2 × $COCH_2$; 20.4, 20.2, CH_3.

Conjugation of Haptens to Protein and Enzymes. Each of the products **6a**, **6b**, and **9** was reacted with two protein carriers [ovalbumin (OA) and either chicken IgG, IgY (**6b**), or keyhole limpet hemocyanin, KLH (**6a**, **9**)] as well as horseradish peroxidase (HRP) using methods described earlier (McAdam et al., 1992). In addition, [2-(phenylmethyl)-4-furyl]methanol was derivatized using succinic anhydride in pyridine (Skerritt et al., 1992a), and the activated ester was prepared using NHS/DCC, before coupling to OA, IgY, and HRP. Conjugates of **6b** had a 4-carbon spacer arm and are designated BRM-C4-, those of **6a** had no spacer arm and are designated BRM-0-, and those of **9**, with a 4-(aminomethyl)cyclohexanecarboxylate spacer, are designated BRM-AMCC-. The [2-(phenylmethyl)-4-furyl]methyl conjugates containing a 4-carbon acid spacer arm are designated BF-C4-.

Analysis of Conjugates. Conjugates were analyzed for protein content using a modified dye-binding assay (Sharma and Tjohn, 1988), and the degree of hapten substitution was measured by determination of the loss of amino groups on the protein using reaction with trinitrobenzenesulfonate (Plapp et al., 1971). Conjugates had the following hapten substitution ratios: (1) BRM-C4-OA, 7.8 mol/mol of OA; BRM-C4-IgY, 19 mol/mol of

IgY; BRM-C4-HRP, 1.5; (2) BRM-0-OA, 9.5; BRM-0-KLH, 8.5; BRM-0-HRP, 2.5; (3) BRM-AMCC-OA, 9.0; BRM-AMCC-KLH, 4.5; BRM-AMCC-HRP, 2.5; (4) BF-C4-OA, 9.4; BF-C4-KLH, 8.5; BF-C4-HRP, 1.2.

Antibody Production. Rabbits were immunized with each of the BRM-OA derivatives, the BRM-IgY derivative, and both BRM-KLH derivatives using the intradermal–intramuscular route (McAdam et al., 1992). Rabbit IgG antibodies were purified by protein A–agarose affinity chromatography (Goding, 1978). After dialysis against PBS, and concentration to 1–8 mg/mL, antibodies were stored at −20 °C.

Bioresmethrin Assays. *Sample Preparation.* Whole wheat and barley grain samples were obtained from commercially-treated bulk storages 2–18 months after treatment. Pesticide was extracted by standing 10 or 20 g of whole grain in 25 or 50 mL of methanol for 45–48 h in stoppered 100-mL flasks at 20 °C. The flasks were shaken at 20 rpm for 15 min twice during the extraction period. This method has been shown earlier to be optimal for quantitative extraction of both organophosphates and pyrethroids from grain (Sharp et al., 1988). Ground grain was extracted using a high-frequency homogenizer (Ystral, Germany) for 1 min at 15 000 rpm.

Assay for BRM. Two immunoassay formats were examined (Hill et al., 1992). In the most useful assay format, the appropriate antibody was diluted (to 10 μg/mL unless otherwise indicated) with 50 mM sodium carbonate buffer, pH 9.6, and 100 μL was added to each well of a 96-well plate and incubated at 20 °C for 1 h. After washing all wells three times with 250 μL of PBS–0.05% (v/v) Tween 20 to remove unbound antibody, 150 μL of blocking solution (1% bovine serum albumin in 50 mM sodium phosphate buffer, pH 7.2, containing 0.9% NaCl) was added and incubated for 1 h at 20 °C. Standards, methanol (for controls and blanks), and methanol grain extracts were diluted either 1/2.5, 1/3.3, 1.5, or 1/10 with diluent buffer [blocking solution plus 0.05% (w/v) Tween 20]. These dilution protocols yielded final methanol concentrations in the microwell of 20%, 15%, 10%, and 5%, respectively. After removal of blocking solution, 100 μL of diluted methanol was added to control wells and diluted methanol grain extract or diluted BRM standard added appropriately to separate wells in triplicate, and then 100 μL of the indicated BRM-HRP conjugate was added in diluent buffer to each well. Well contents were mixed by gentle agitation of the microplate for 30 s. After 60-min incubation at 20 °C, the plate was washed four times with purified water, and then 120 μL of hydrogen peroxide substrate–3,3′,5,5′-tetramethylbenzidine chromogen (Hill et al., 1991) was added and incubated 30 min at 20 °C. Stopping reagent (40 μL of 1.25 M sulfuric acid per well) was added and absorbance measured at 450 nm.

In the other assay format, BRM–protein conjugates were immobilized (typically at 150 ng/100 μL of sodium carbonate, pH 9.6) onto the microwell by incubation overnight at 20 °C. Where antibodies were prepared to KLH or IgY conjugates of BRM, OA conjugates were used to coat the microwells, while antibodies to OA conjugates were screened against immobilized KLH or IgY conjugates. Following antigen coating, wells were washed three times with PBS–Tween and then blocked. Subsequent steps followed the protocol described above, except that the assay mixture consisted of 50 μL of BRM-specific antibody and 50 μL of test sample or standard, and after 60-min incubation of this mixture, plates were washed three times with PBS–Tween, and 100 μL of peroxidase-labeled swine anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) diluted 1/400 in 1% BSA–PBS–Tween were added and incubated 30 min at 20 °C.

RESULTS AND DISCUSSION

Approaches to Bioresmethrin Hapten Synthesis. Bioresmethrin differs from many of the other major pyrethroids in having a [(phenylmethyl)furyl]methyl rather than a phenoxybenzyl moiety esterified to chrysanthemic acid. While this gave the molecule high knockdown insecticidal potency, the greater susceptibility of the furan moiety to degrade enabled the compound to be used in domestic situations and directly applied to commodities such as stored grain. However, the same susceptibility complicated the synthesis of the BRM–protein conjugate (required for development of an antibody response to BRM), as several of the attempted chemical routes led to degradation of the BRM hapten.

Table I. Effects of Spacer Arm Chemistry on Detection of Bioresmethrin Using Different Immobilized Antibody Formats and Peroxidase-Labeled Bioresmethrin Derivatives[a]

conjugate carrier	antibody					
	BRM-0-Prt		BRM-C4-Prt		BRM-AMCC-Prt	
	OA	KLH	OA	IgY	OA	KLH
BRM-0-HRP	2000	900	>2500	>2500	2500	>2500
BRM-C4-HRP	1500	800	>2500	2000	2000	2500
BRM-AMCC-HRP	1500	1000	100	25	>2500	1000
BF-C4-HRP	1000	300	>2500	>2500	>2500	>2500

[a] Data shown are concentrations in parts per billion (final, in the microwell) of bioresmethrin providing 50% inhibition of antibody binding. Data are shown for six different antibodies, prepared using each of three spacer arm chemistries and using two proteins for coupling.

Stanker et al. (1989) produced phenothrin carboxy haptens by ozonolysis of the 2-methylpropenyl bond in the chrysanthemic acid moiety. However, application of this reaction to bioresmethrin caused opening of the furan ring. Oxidative cleavage of the bond with $KMnO_4$ had a similar effect. In the approach we employed (Figure 1), we overcame problems with furan ring opening by first hydrolyzing the central ester, cleaving the bond on chrysanthemic acid to produce an acid. With the use of protecting groups, it is actually the latter acid that is reesterified to the [2-(phenylmethyl)-4-furyl]methanol.

(Trimethylsilyl)ethyl ester protecting groups which are removable under neutral conditions were required, as initial attempts to produce a 4-hydroxybutanoic acid spacer arm using a *tert*-butyl ester protecting group showed that it could not be removed without simultaneous decomposition of the furan. The protected spacer arm, 2-(trimethylsilyl)ethyl 4-hydroxybutanoate (a) was prepared by reduction using sodium borohydride of 2-(trimethylsilyl)ethyl succinate (Ishizumi et al., 1968). The yield of the reduction was poor (45%), but the recovered starting material could readily be recycled. The same spacer arm has recently been reported (Mei et al., 1991), where it was prepared in three steps.

An alternative approach would have been to used an electrophilic substitution reaction on the furan, such as Friedel–Crafts acylation, which would have preferentially substituted at the 2-position on the furan (Olah, 1964). However, this approach was not favored because the BRM would have been derivatized at the center of the molecule, possibly necessitating use of long spacer arms. In addition, with our aim of developing a BRM-specific antibody, it was considered desirable to couple the hapten through the chrysanthemic acid end of the molecule, thus presenting the [(phenylmethyl)furyl]methyl moiety to the immune system.

Use of BRM Conjugates with Different Spacer Arms. Initial studies were conducted using antisera raised to BRM-C4–protein conjugates (Table I). The results of the assay using immobilized antigen indicated that while the antibodies recognized the BRM-C4 conjugated to the protein that had not been used for immunization, inhibition by free BRM was weak across a range of antibody and antigen concentrations. The binding was to the BRM-C4 moiety, because little color was seen when the appropriate carrier protein alone was coated. Inhibition of antibody binding by BRM was rather weak, with 50% B_0 (i.e., 50% of the color obtained in the absence of competing

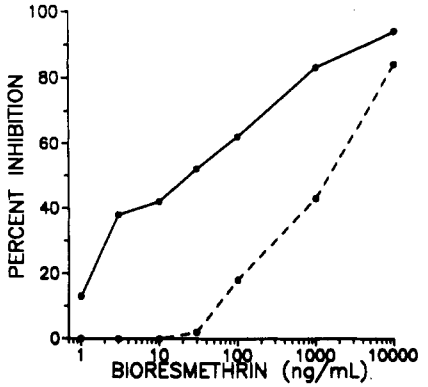

Figure 2. Standard curves for analysis of bioresmethrin using antiserum from rabbit TRI to BRM-C4-OA and BRM-C4-HRP (dashed lines) or BRM-AMCC-HRP (solid lines). Similar results were obtained with another rabbit immunized with BRM-C4-OA. Data are expressed as percentage inhibition of absorbance obtained for controls performed in the presence of 10% methanol but in the absence of competing pesticide. In each case, this was equivalent to A_{450} = 0.8–1.2. Data shown are means of standard curves on 4–5 separate days; the standard deviation of the concentrations causing 50% inhibition was less than 20% of the mean.

BRM) of about 4000 ppb (ng/mL) BRM. Use of immobilized antibody and BRM-C4-HRP also provided an assay of low sensitivity, with a 50% B_0 of 2000 ppb and a limit of detection (10% B_0) of 50 ppb (Figure 2). Since levels of BRM in grain are typically 0.1–2 ppm, a methanol extract prepared using 25 mL/10 g of grain, diluted 5-fold with aqueous buffer before addition to the microwell and then 2-fold by addition of conjugate solution, would contain only one-twenty-fifth this amount, namely, 4–80 ppb. Therefore, an assay that could be used without requirement for concentration of extracts should display 50% inhibition of antibody binding (50 B_0) of no more than 30–40 ppb.

Earlier work on the development of immunoassays to the carbamate insecticide, aldicarb (Brady et al., 1989), showed that use of a different spacer arm in the detecting pesticide–protein complex from that used in the immunogen can improve assay sensitivity and specificity, by removing cross-reaction with the spacer arm used in the immunogen. In order to maximize the reaction with the benzylfuran moiety (to enable specificity for bioresmethrin among the commonly-used grain protectant pyrethroids) and to minimize the risk of hapten folding (preventing the antibody–antigen reaction), we prepared the activated hapten **5a**, in which BRM (minus the vinyl chrysanthemic acid side arm) is coupled directly to protein. However, these derivatives were ineffective in providing a more sensitive assay when used either as microwell-bound antigens or as peroxidase-labeled reporter molecules. Antibodies raised to these zero spacer arm derivatives gave little color with a range of immobilized BRM–protein derivatives at serum dilutions above 1/500, and at lower dilutions they exhibited very weak inhibition by free BRM. The two antisera were also studied in the format involving immobilized antibody. Good ELISA color development was noted with each of four peroxidase conjugates at low concentrations (5–200 ng/mL, on a peroxidase basis), but inhibition by free BRM was less than desirable.

The most sensitive of the four assays using the zero spacer arm BRM antibodies was obtained using a conjugate of (5-benzyl-3-furyl)methanol with HRP (containing the C4 spacer arm) and gave a 50% B_0 value of 250 ppb; this is probably because most of the antibody reaction was to this part of the molecule, with the chrysanthemic acid moiety acting as a spacer arm itself. This finding contrasts with the properties of the monoclonal antibody-based assay for permethrin/phenothrin (Stanker et al., 1989; Skerritt et al., 1992a), in which the point of hapten–protein conjugation in the immunogen was similar, but acceptable assay sensitivity was only obtained when the phenoxybenzyl moiety rather than the whole pyrethroid was coupled to either solid phase-bound antigen or peroxidase for use in the assay.

A series of BRM–protein conjugates in which BRM was coupled through a bulky (aminomethyl)cyclohexanecarboxylic acid group was next prepared. These offer the same number of carbon atoms between the cyclopropane ring of BRM and the carrier protein, but have a bulky group and involve an amide link to this ring, rather than an ester. While these conjugates were immunogenic, evoking high-titer antisera when screened by an indirect ELISA, inhibition of antibody binding by free BRM was poor. Similarly, in the immobilized antibody format, recognition of the different BRM–peroxidase conjugates was good (ELISA color development of >1.0 at conjugate dilutions between 5 and 200 ng/mL), but sensitivity for free BRM was low.

Vastly differing results were obtained with the BRM-C4 antisera and the different BRM–spacer arm–protein complexes. In the immmobilized antigen assay, assay sensitivity was still rather low, with 50% B_0 values of about 1000 ppb BRM. In the immobilized antibody assay, suitably high sensitivity for analysis of BRM in grain (50% B_0 of about 25 ppb) was obtained using either of the two antisera to BRM-C4-IgY, with the peroxidase conjugate prepared using the bulky spacer arm, BRM-AMCC-HRP (Figure 2). The much greater sensitivity of this assay compared with when BRM-HRP conjugates based on other spacer arms were used probably is based on the lack of AMCC spacer arm recognition and the effect of this bulky spacer arm in lowering the affinity of the antibody–conjugate interaction relative to binding of free BRM by the antibody. This assay was used exclusively in subsequent work.

Thus, of the 12 antibody–conjugate combinations (Table I), only one yielded an assay of sufficient sensitivity. The corresponding assays using immobilized antigen were of low sensitivity. In addition, antisera raised to the various conjugates of BRM with ovalbumin gave less sensitive assays than those using either IgY or KLH as carrier proteins. This was not a general phenonemon with other pesticide haptens; for example, the most sensitive antisera to organophosphates such as chlorpyrifos and pirimiphos-methyl arose from OA conjugates (Skerritt et al., 1992b). However, in keeping with most, but not all, pesticide immunoassay systems, the assay format involving immobilized antibody gave more sensitive detection than those utilizing immobilized BRM–protein conjugates.

Solvent and Matrix Effects. A number of solvents has been shown to extract BRM and other synthetic pyrethroids efficiently from whole or ground cereal grain. These include both polar solvents, such as methanol or ethanol, acetone, and acetone–methanol, and acetonitrile, as well as nonpolar solvents, such as hexane and petroleum ether (Desmarchelier, 1980; Okadu et al., 1983; Sharp et al., 1988; Chamberlain, 1990). Direct dilution and analysis of grain extracts by immunoassay usually requires use of a water-miscible extractant. Methanol, acetone, and acetonitrile were first tested for effects on ELISA absorbance values in the absence of grain extract. Using the standard antibody coating concentration (0.1 mL of 10 μg/mL), these solvents had markedly different effects on antibody–antigen interactions. Acetone inhibited anti-

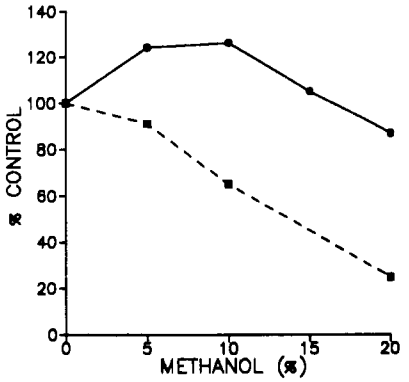

Figure 3. Effects of differing concentrations of methanol on binding of BRM-AMCC-HRP to antibodies to BRM-C4-OA, coated at either 1 μg/well (●) or 0.025 μg/well (■). Data shown are the percentage of control OD values obtained in the absence of free BRM for two experiments in which OD values differed by less than 15% of the mean.

Table II. Matrix Effects in Immunoassay for Bioresmethrin Using Immobilized Antibody to BRM-C4-Chicken IgG and BRM-AMCC-HRP[a]

matrix	final concentration (%) of methanol solvent: 5	10	15	20
	50% B_0 Value (ng/mL), Solvent Only			
mean	27	21	21	10
range	20–40	17–35	15–25	6–12
	Relative Sensitivity[b]			
whole wheat (48-h extraction)	1.10 ± 0.04	1.00 ± 0.07	1.00 ± 0.05	0.91 ± 0.05
ground wheat (homogenized)	0.81 ± 0.13	0.46 ± 0.28	nt[c]	0.23 ± 0.06
whole barley (48-h extraction)	nt	1.10 ± 0.05	nt	0.83 ± 0.06
ground barley (homogenized)	nt	0.46 ± 0.13	nt	0.29 ± 0.05

[a] Data shown are means of 2–5 determinations and are means ± standard deviations. [b] Calculated as 50% B_0 for BRM (methanol solvent only) divided by 50% B_0 (for BRM in the particular grain extract). [c] nt, not tested.

body binding markedly (about 50% at 10% solvent) and thus could not be considered for direct dilution in to the assay buffer. Acetonitrile inhibited antibody binding 25% at 10% solvent concentration, but by 59% at 15% solvent. In contrast, methanol at 5–10% enhanced antibody binding, while at 15–20% methanol, there was little net effect on antibody binding (Figure 3). The antibody coating density also played a role; at 40-fold lower antibody coating, the ELISA absorbance was reduced at each methanol concentration. On the basis of these results, methanol was used as the primary assay solvent, together with microwells coated with 1 μg of antibody.

The potential sensitivity of the assay was increased in two ways as the methanol concentration increased from 5% to 20%—the requirement to dilute an extract of grain less before assay directly increased the potential sensitivity, and it was noted that the inhibition of antibody binding at a given concentration of bioresmethrin increased at higher concentrations of methanol (Table II). Standard curves for bioresmethrin prepared in methanol or in the methanol extracts of whole grain were superimposable, indicating the absence of matrix effects in whole grain (Table II). Therefore, methanol extracts of whole wheat or barley could be analyzed with direct reference to a calibration curve prepared in methanol. In contrast, matrix effects were seen with methanol extracts of ground grain. When ground wheat extracts were diluted 1/20 for the assay (i.e., final methanol concentration of 5%), the

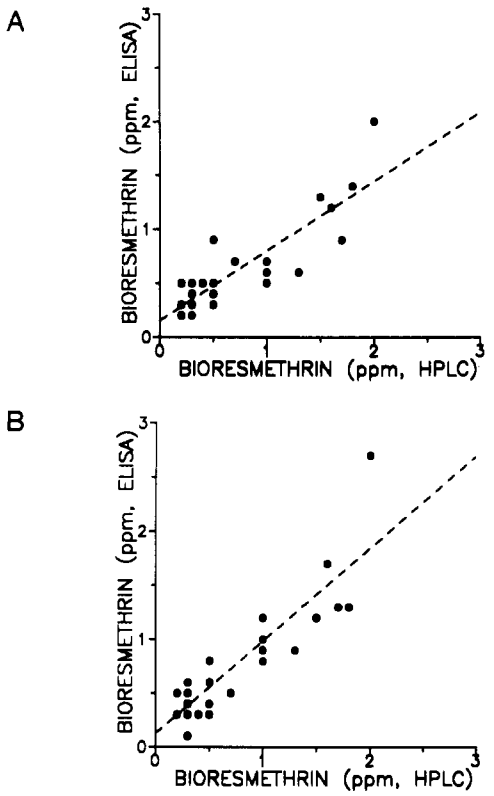

Figure 4. Relationship between pesticide determined by HPLC and ELISA for barley: (A) (Whole grain) $n = 34$, $r = 0.879$, $P < 0.001$; ELISA value (ppm) = 0.65 × HPLC value (ppm) + 0.15. (B) (Ground grain) $n = 34$, $r = 0.895$, $P < 0.001$; ELISA value (ppm) = 0.86 × HPLC value (ppm) + 0.12.

shift in the standard curve was minor, but at a 1/10 dilution the loss in sensitivity for wheat or barley was about 2-fold, and at a 1/5 dilution, 4–5-fold (Table II). Thus ground grain extracts were best analyzed at a 1/10 dilution, where 50% B_0 was 40–50 ppb (1–1.25 ppm in the grain).

Assay Specificity. A range of concentrations of other pyrethroids and other grain protectants was screened for detection in the assay. Resmethrin [[2-(phenylmethyl)-4-furyl]methyl (+)-*cis,trans*-chrysanthemate] was detected with approximately 50% the sensitivity of bioresmethrin, indicating that the assay probably has selectivity for the (+)-*trans*-isomer (bioresmethrin). A wide variety of other synthetic pyrethroids [allethrin, bioallethrin, (*S*)-bioallethrin, cyhalothrin, cypermethrin, deltamethrin, fenvalerate, permethrin, and tetramethrin] exhibited little or no binding, as all had 50% B_0 values greater than 5000 ppb. Chrysanthemic acid also was not recognized by the antibody at concentrations below 2000 ppb, while the alcohol of the BRM ester, (5-benzyl-3-furyl)methanol, was recognized rather poorly (50% B_0 of 1500 ppb). Thus the entire molecule is required for recognition by the antibody. Finally, a variety of other grain protectants such as chlorpyrifos-methyl, fenitrothion, pirimiphos-methyl, and carbaryl were not detected by the assay at concentrations up to 5000 ppb.

Precision of the Assay. The intra-assay repeatability and between-assay reproducibility of the assay was studied at zero, 11, 33, and 100 ppb BRM. For 8 determinations performed in the same assay, the coefficients of variation (cv) of absorbance values were 7%, 7%, 7%, and 11%, respectively, whereas for assays performed on three consecutive days, cv values between 2% and 6% were obtained.

Performance of the Assay with Wheat and Barley Samples. Barley (Figure 4) and wheat (Figure 5) samples

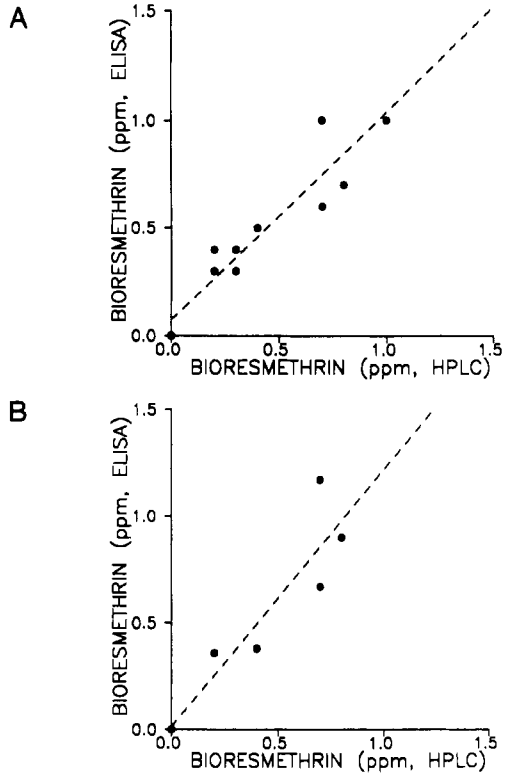

Figure 5. Relationship between pesticide determined by HPLC and ELISA for wheat: (A) (Whole grain) $n = 15$, $r = 0.941$, $P < 0.001$; ELISA value (ppm) = 0.96 × HPLC value (ppm) + 0.07. (B) (Ground grain) $n = 8$, $r = 0.939$, $P < 0.005$; ELISA value (ppm) = 1.20 × HPLC value (ppm) + 0.01.

containing residues incurred 2–6 months before analysis were studied in separate experiments, and the ELISA data obtained were compared with HPLC analyses. Good correlations between HPLC and ELISA data were seen with whole wheat (Figure 5A) and ground barley samples (Figure 4B). An initial comparison of extraction methods and use of a final 1/5 or final 1/10 dilution of the methanolic grain extract was made using 34 barley samples. Data were as follows: For the 1/5 dilution: whole grain: r = 0.842, ELISA value = 0.44 × HPLC value + 0.14; ground grain: r = 0.894, ELISA value = 0.79 × HPLC value + 0.09. For the 1/10 dilution: whole grain: r = 0.878, ELISA, value = 0.65 × HPLC value + 0.15; ground grain: r = 0.895, ELISA value = 0.86 × HPLC value + 0.12. Therefore, linear regression of ELISA and HPLC data for each combination of dilution and extraction technique gave highly significant correlation coefficients, but for barley, the ELISA method gave underestimates with whole grain extracted for 48 h. Recoveries were somewhat better for ground grain extracted by rapid homogenization. For whole and ground grain, the samples diluted 1/10 (final) gave better correlations with HPLC data. The latter finding was also made with wheat; in addition, the presence of methanol at 20% caused intermittent precipitation of the serum albumin used in the sample diluent. Therefore, although the sensitivity of the assay for methanol standards and for extracts of ground grain was somewhat higher when the final methanol concentration was 20%, samples were routinely assayed at a final dilution of 1/10 (i.e., 10% methanol in the assay incubation).

In contrast to the results obtained with barley, good recoveries were found for ELISA analyses of both whole (Figure 5A) and ground (Figure 5B) wheat grain. In practice, the greater sensitivity of the assay for extracts of whole wheat grain would suggest that analysis of whole grain is preferred. The difference between wheat and barley probably arises from the morphological difference between the grains; barley is hulled, while wheat has a naked caryopsis. Possibly grinding of the barley grain is necessary to extract pyrethroid that may have penetrated the barley pericarp. Differences in pesticide recovery between whole and ground barley have not been noted for the organophosphates, which are more polar (Edward and Skerritt, unpublished results).

Attempts To Remove Matrix Interference with Ground Grain. Alternative extraction or simple cleanup methods were investigated in order to attempt to avoid the inconvenience of requiring standards to be prepared in a pesticide-free methanol extract of the matrix under study, in situations where ground grain, milling fractions, or baked goods were to be analyzed. In initial studies, methanol extracts of ground wheat grain were applied at 1 mL/min to Sep-pak devices (Millipore, Bedford, MA) containing either Florisil, basic alumina, or acidic alumina, in an attempt to adsorb lipid or protein coextractives (Chamberlain, 1990) that may have interfered with the immunoassay. The extracts were then diluted 1/10 (final) for analysis. However, while the alumina "cleanup" clarified the turbid grain extracts, none of the treatments improved the sensitivity of detection of BRM to that found in extracts of whole grain or in methanol standards: 50% B_0 values of BRM were as follows: methanol standards, 25 ppb; homogenized ground grain, 47 ppb; Florisil, 51 ppb; basic alumina, 42 ppb; acidic alumina, 45 ppb; and neutral alumina, 50 ppb. The inability of basic alumina to clean up grain components that interfere with immunoassay of BRM contrasts with the earlier success with another pyrethroid, permethrin, in grain matrices (Skerritt et al., 1992a).

An alternative approach investigated involved extraction in a volatile solvent that does not extract protein from ground grain, with subsequent evaporation before redissolving the residue in methanol. However, evaporated and methanol-redissolved extracts of grain prepared using acetone, hexane, or petroleum ether still exhibited a matrix effect (50% B_0 values of about 50, 50, and 80 ppb, respectively), and in the case of the latter two solvents, components were extracted from the ground grain that gave false-positive results in the immunoassay. The acetone extracts could be cleaned up before drying using Florisil to provide a 50% B_0 of 25 ppb. However, the requirement for two cleanup steps meant that direct analysis of methanol extracts was the simplest and cheapest approach to analysis of BRM residues in ground grain; thus it was used in these studies, and in subsequent analyses of baked goods for bioresmethrin (Skerritt, Hill, and Edward, unpublished results).

GENERAL DISCUSSION

We have developed a sensitive immunoassay, detecting bioresmethrin and resmethrin only, and applied it to the analysis of grain samples containing incurred residues. The specificity of the assay for these pesiticides arises from the site of conjugation of the immunogens, distal from the benzylfuran moiety. Only one antibody/conjugate combination gave sufficient sensitivity for direct analysis of bioresmethrin in grain. BRM is applied to grain at severalfold lower concentrations than many other grain protectants such as the organophosphates (Snelson, 1987), so a correspondingly more sensitive assay was required. The suitability of methanol as an extractant for BRM in grain is an advantage, as simultaneous analysis of organophosphates in the same extract by ELISA is possible (Skerritt et al., 1992).

While the matrix under study in the current work was grain, the high knockdown potency but low mammalian toxicity of bioresmethrin and resmethrin has led to their use in domestic sprays and for surface treatments of food stores and restaurants. The current test method could be readily modified for urban exposure monitoring.

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