Development of a Panel of Immunoassays for Monitoring DDT, Its Metabolites, and Analogues in Food and Environmental Matrices

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A panel of antisera was prepared using analogues and derivatives of metabolites of the organochlorine insecticide, *p*,*p*′-DDT as haptens. The assays developed exhibited differing cross-reactions for different DDT analogues and metabolites, and the choice of hapten for the detecting enzyme conjugate had almost as much effect on assay specificity and sensitivity as the structure of the hapten used for antibody production. Those assays developed using hapten **I**, based on esters of bis(*p*-chlorophenyl)acetic acid (DDA), typically detected DDA with greater sensitivity than *p*,*p*′-DDT or *p*,*p*′-DDE. The most sensitive assay for *p*,*p*′-DDT (lower limit of detection of 0.3 μg/L) was obtained using an immunogen based on bis(*p*-chlorophenyl)ethanol (hapten **IV**), although a significant cross-reaction with dichlorodiphenyltrichloroethane (DDD) and DDE was obtained. The most specific assay for *p*,*p*′-DDT was obtained using an immunogen (hapten **VI**) that includes all elements of the DDT structure, except that one of the *p*-chloro groups was replaced by β-alanine carboxamide for coupling to carrier proteins. Antibodies based on a similar DDE hapten (**V**) exhibited specificity for *p*,*p*′-DDE over *p*,*p*′-DDT. Greater specificity and sensitivity for dicofol were obtained by using an immunogen derived from ester hydrolysis of chlorbenzilate (hapten **II**). The assays provided methods for detection of *p*,*p*′-DDT plus *p*,*p*′-DDE either by using the antibody raised to hapten **IV** with conjugate based on hapten **Ib** or by using the assay based on hapten **V**, with treatment of samples with warm alcoholic KOH, which converted DDT to DDE. Some of the immunoassays were applied to the detection of DDT and DDE in water, soil, and selected foods.

Keywords: *DDT; DDE; organochlorine; ELISA; immunoassay; water; soil; food*

INTRODUCTION

The insecticidal properties of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) were first recognized by Muller of Switzerland in 1939, who was awarded the Nobel Prize in Medicine and Physiology for his discovery. DDT was patented as the first synthetic multipurpose insecticide the same year and applauded for its lethal effects, persistence, and low nontarget toxicity (Waterhouse, 1972). DDT proved economical and versatile for use in both agricultural and public health applications. In the 1960s, sensitive analytical techniques such as gas−liquid chromatography (de Faubert Maunder et al., 1964; Edwards, 1973) revealed the bioaccumulation of DDT in the fat of animals higher in the food chain (Brewerton, 1969). This problem of biological accumulation is aggravated by the natural conversion of DDT to the even more stable noninsecticidal product, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE). This compound has recently been associated with hormone-related cancers in humans (Kelce et al., 1995). Detection of DDT and metabolites in milk and meat for human consumption, together with the appearance of insect resistance to DDT, led to many countries restricting, and eventually abandoning, the use of DDT. Despite not being used by many countries for some years, the persistence of DDT and its metabolites in soil [including DDE and 1,1-dichloro-2,2-bis(*p*-chlorphenyl)ethane (DDD); Guenzi and Beard, 1967; Agarwal et al., 1994] means that residues remain of concern when landuse changes for potentially contaminated sites are considered. In humans, and other mammals, the major metabolite of DDT is bis(*p*-chorophenylacetic acid) (DDA). DDA itself does not accumulate but is a marker for exposure to DDT (Hassall, 1990). Some developing countries such as India and Mexico have continued to use DDT, especially for public health applications (WHO, 1969), leading to the potential for considerable buildup in the environment and hazards in foodstuffs (Mukherjee et al., 1993; Boul, 1995; Waliszewski et al., 1996). The use of dicofol [the 2-hydroxy analogue of DDT, namely, 1,1,1-trichloro-2-hydroxy-2,2-bis(*p*-chlorophenyl)ethane], an effective miticide and acaricide, has not been subject to the same restrictions, and it is widely used on horticultural crops. However, a considerable problem existed with many production batches of dicofol, especially those manufactured before the mid-late 1980s, with several of these containing a significant percentage of DDT as a contaminant (Gillespie et al., 1994). In addition, the biodegradation patterns of dicofol suggest that it too can persist as residues in foods and environmental samples (Liapis et al., 1995; Domaglaski, 1996), and dicofol has been associated with egg thinning in wild bird species

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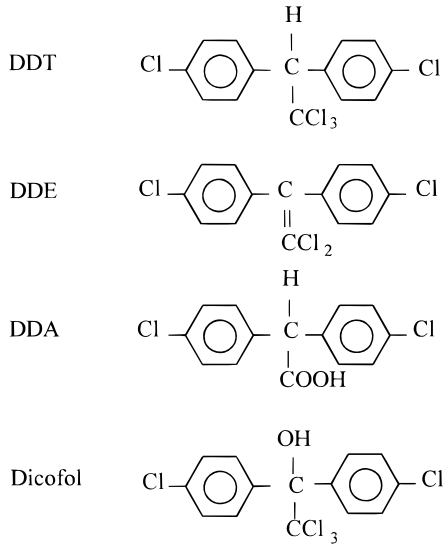

Figure 1. Chemical structures of the four target analytes.

(Schwarzbach et al., 1988; Clark et al., 1995). As a result, several food-importing countries are concerned about its continuing use (U.S. EPA, 1985).

Immunological methods for detecting insecticide residues in food and environmental samples offer several advantages over gas chromatographic methods. While maintaining comparable sensitivity, they are simple and cost-effective and can be adapted for field use (Gee et al., 1995; Lee et al., 1995). Earlier, several groups have attempted to develop antibodies for detection of DDT and metabolites by forming esters or amides of DDA (Haas and Guardia, 1968; Centero et al., 1970; Furuya and Urasawa, 1981; Banerjee, 1987); however, typically these antibodies detected DDT much more poorly than they did DDA. Burgisser et al. (1990) designed a hapten based on dicofol, which, unlike the DDA-derived haptens, retained the $-CCl_3$ moiety of DDT, and developed a competitive radioimmunoassay of low to moderate sensitivity. Banerjee et al. (1996) utilized diamino derivatives of DDT, DDE, and DDA as haptens and developed several competitive ELISAs with IC_{50} values in the range of 180−360 μg/L for standards of each of these pesticides. In the late stages of completion of this manuscript, a report was published describing the development of quite sensitive monoclonal antibodies to DDT and DDE (Abad et al., 1997), although in none of these reports was application to samples described. In the present study, we describe the development of a panel of antibodies to different DDT analogues and metabolites using a series of haptens, and their incorporation into ELISA assays. Some of these assays exhibited cross-reactions with other stable DDT metabolites, making them useful as a screening tool for determining total DDT loads, whereas others were more specific for either DDT, DDE, DDA, or dicofol. We report initial results demonstrating the application of assays for residue analysis in selected food and environmental matrices.

EXPERIMENTAL METHODS

Hapten Preparation and Conjugation. We aimed to develop immunoassays with differing specificities for *p,p′*-DDT, DDA, *p,p′*-DDE, and dicofol (Figure 1). Five classes of haptens were produced based on (1) formation of various amide derivatives of DDA (haptens **Ia**, **Ib**, and **Ic**); (2) the utilization of chlorbenzilate [2-hydroxy-2,2-bis(*p*-chlorophenyl)acetic acid

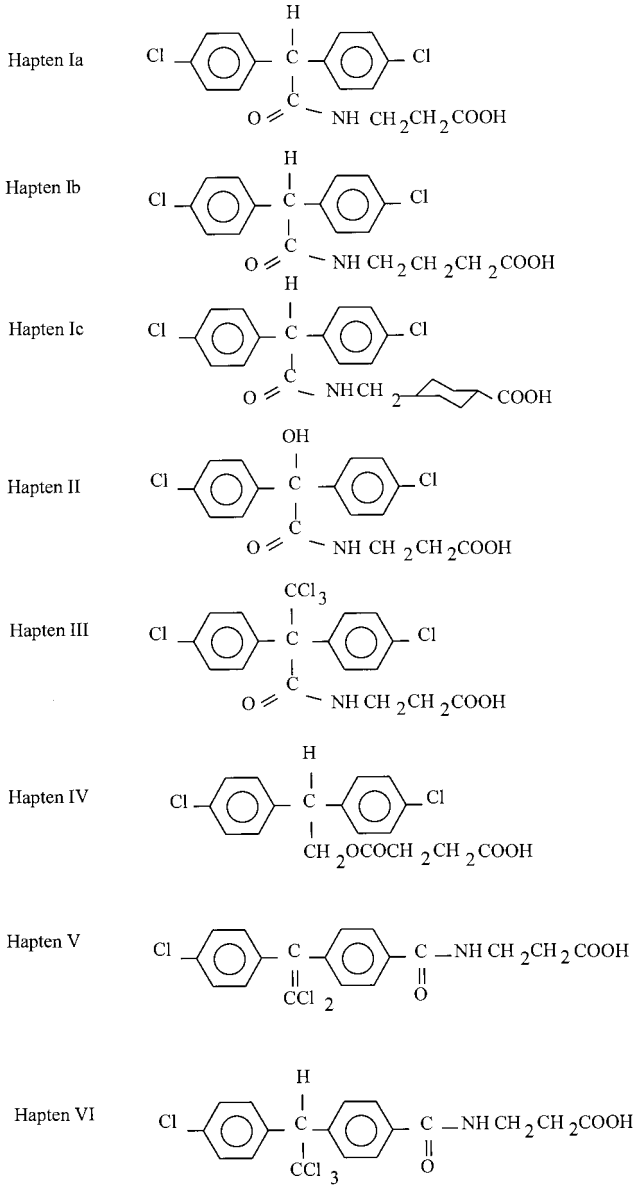

Figure 2. Chemical structures of the haptens prepared (carboxylic acid forms shown).

ethyl ester] to develop an analogue of dicofol (hapten **II**); (3) utilization of dicofol to develop a derivative that retains the 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) functionality of DDT (hapten **III**); (4) utilization of bis(*p*-chlorophenyl)ethanol (hapten **IV**); (5) a DDE derivative (hapten **V**); and (6) a DDT derivative through synthesis of an analogue bearing a substitution at one of the *p*-chloro atoms (hapten **VI**) (Figure 2).

*1. Synthesis of DDA-Derived Haptens (Haptens **Ia**, **Ib**, and **Ic**).* Bis(2-*p*-chlorophenyl)acetic acid (0.32 mmol, 78 mg) was reacted with thionyl chloride (2 mL, caution) and refluxed at 90 °C for 1 h. Excess thionyl chloride was then removed by rotary evaporation, toluene was added to remove the thionyl chloride residue, and the solvent was removed by a second evaporation step. The product was dissolved in 2 mL of benzene and cooled to 0 °C; β-alanine (72 mg) in 2 mL of 1 M NaOH solution was added, and the mixture was allowed to react overnight. Unreacted acid chloride was removed by solvent extraction with benzene and the aqueous layer acidified, then partitioned into ethyl acetate, and washed with water and then brine. The product was dried over magnesium sulfate and filtered under vacuum. It was reconstituted in acetone and purified by flash chromatography on silica (eluted in 50% ethyl acetate/50% petroleum ether with 0.01% acetic acid). The fractions identified as containing the acid by TLC

analysis (ethyl acetate/petroleum ether, 1:1) were combined, and toluene was added to remove the acetic acid residue as an azeotrope, followed by addition of chloroform to remove residual toluene, to provide the β-alanine derivative (48% yield). The product was detected on TLC using 2% $CeSO_4$ in 2 M sulfuric acid. The β-alanine amide (0.13 mmol, 46 mg) was combined with *N*-hydroxysuccinimide (NHS; 0.3 mmol, 32 mg) and 5 mL of dry methylene chloride, at 0 °C. Dicyclohexylcarbodiimide (DCC; 0.16 mmol, 40 mg) was added, followed by (dimethylamino)pyridine (DMAP; 1 mg) and allowed to react overnight. The reaction mixture was filtered and the white precipitate discarded; the filtrate was shaken in slightly acidified water, saturated with sodium hydrogen carbonate, water, and brine, and dried over magnesium sulfate; the product was filtered off and reconstituted in chloroform for preparative TLC (in ethyl acetate/petroleum ether, 1:1) on silica gel (R_f 0.62, 80% yield, mp 95−97 °C) [hapten **Ia**, 3-[bis(4-chlorophenyl)acetylamino]propionic acid]: ^{1}H NMR (CD_3OD) of acid δ 2.33 (t, 2H, $-CH_2-COO$), 3.17 (m, 2H, $-CH_2-NHCO$), 4.78 (s, H, $-CH-$), 7.15 (4H, Ar, J = 8.21 Hz), 7.24 (4H, Ar, J = 8.3 Hz); ^{1}H NMR ($CDCl_3$) of NHS ester δ 2.55 (t, 2H, $-CH_2-COO$), 2.85 [m, 4H, $CO(CH_2)_2-CO$], 3.52 (m, 2H, $-CH_2-NH$), 4.79 (s, 1H, CH), 7.19 (d, 4H, Ar, J = 9.0 Hz), 7.33 (d, 4H, Ar, J = 9.0 Hz).

Related haptens were prepared using similar methods, and a similar molar excess of amino acid used for the spacer, with the following: (1) A γ-aminobutyric acid spacer [hapten **Ib**, 4-[bis(4-chlorophenyl)acetylamino]butanoic acid]: ^{1}H NMR ($CDCl_3$) of acid δ 1.8 (m, 2H, $-CH_2-$), 2.34 (t, 2H, $-CH_2-COO$), 3.32 (m, 2H, $-CH_2-NHCO$), 4.8 (s, H, $-CH-$), 5.93 (t, 1H, NH), 7.15 (4H, Ar, J = 8.34 Hz), 7.29 (4H, Ar, J = 8.4 Hz); ^{1}H NMR ($CDCl_3$) of NHS ester δ 2.0 (m, 2H, $-CH_2-$), 2.61 (t, 2H, $-CH_2-COO$), 2.85 (m, 4H, $CO(CH_2)_2-CO$), 3.4 (m, 2H, $-CH_2-NH$), 4.82 (s, 1H, CH), 7.17 (d, 4H, Ar, J = 9 Hz), 7.30 (d, 4H, Ar, J = 9 Hz); NHS mp 110−113 °C. (2) 4-(Aminomethyl)cyclohexylcarboxylic acid [hapten **Ic**, 4-[[bis-(4-chlorophenyl)acetylamino]methyl]cyclohexylcarboxylic acid], using the methods of Hill et al. (1993), instead of β-alanine: ^{1}H NMR ($CDCl_3$) of acid δ 0.95 (m, 2H, CH_2-ax), 1.4 (m, 3H, CH + CH_2-ax), 1.75 (m, 2H, CH_2-eq), 2.01 (m, 2H, CH_2-eq), 2.25 (m, 1H, CH-eq), 3.15 (t, 2H, $-CH_2-N$), 4.72 (s, 1H, $-CH-$), 5.60 (t, 1H, $-NH-$), 7.17 (d, 4H, Ar, J = 8.4 Hz), 7.30 (d, 4H, Ar, J = 10.5 Hz); ^{1}H NMR ($CDCl_3$) of NHS ester δ 1.0 (m, 2H, CH_2-ax), 1.53 (m, 3H, CH + CH_2-ax), 1.76 (m, 2H, CH_2-eq), 2.45 (m, 2H, CH_2-eq), 2.55 (m, 1H, CH-eq), 2.82 (s, 4H, $CO-CH_2CH_2-CO$), 3.14 (t, 2H, $-CH_2-N$), 4.81 (s, 1H, $-CH-$), 5.78 (t, 1H, $-NH-$), 7.17 (d, 4H, Ar, J = 8.46 Hz), 7.31 (d, 4H, Ar, J = 8.37 Hz); NHS mp 102−103 °C.

*2. Hydrolysis of Chlorbenzilate To Produce Hapten **II**.* Chlorbenzilate (50 mg, 0.154 mmol) was added to 2 mL of 2 M KOH in ethanol and stirred for 2 h at room temperature. After the reaction appeared to go to completion by TLC in ethyl acetate/petroleum ether (30:70), a further 10 mL of KOH solution was added and unreacted chlorbenzilate removed by extraction with dichloromethane. The aqueous layer was removed and then acidified to pH 4, and the acid product was extracted into dichloromethane and dried over magnesium sulfate. The product (40 mg, 0.135 mmol, yield 88%) in 2 mL of dichloromethane was stirred at 0 °C with DCC (33.5 mg, 0.16 mmol) and (dimethylamino)pyridine (1 mg, 0.007 mmol) for 30 min before addition of *tert*-butyl-β-alanine ethyl ester (21.5 mg, 0.15 mmol), and the mixture stirred for 4 h. The dicyclohexylurea was removed by filtration and the dichloromethane solvent removed under reduced pressure. The product was extracted into ethyl acetate and washed with 1 M sodium hydrogen carbonate, water, and brine before being dried over $MgSO_4$; the ethyl acetate was removed under reduced pressure. The product was isolated by radial chromatography using 30% ethyl acetate in petroleum ether and then hydrolyzed to the acid by dissolving in 1 mL of trifluoroacetic acid and stirring for 2 h at room temperature, to produce the acid, hapten **II** [3-[[bis(4-chlorophenyl)hydroxyacetyl]amino]propanoic acid] in 55% yield: ^{1}H NMR ($CDCl_3$) of acid δ 1.27 (t, 2H, $-CH_2-$), 4.32 (q, 2H, $-CH_2-NH-$), 5.1 (s, br, 1H, $-NH$), 7.30 (d, 4H, $-$Ar, J = 9.0 Hz), 7.35 (d, 4H, Ar, J = 6.45 Hz). The resultant carboxylic acid was converted to the NHS ester using the method described above to yield an oil: ^{1}H NMR ($CDCl_3$) δ 1.32 (t, 2H, $-CH_2-$), 3.62 (q, 2H, $-CH_2-NH-$), 2.85 [t, 4H, $-CO(CH_2)_2-CO-$], 5.1 (s, br, 1H, $-NH$), 7.33 (d, 4H, $-$Ar, J = 9.0 Hz), 7.38 (d, 4H, Ar, J = 9 Hz).

*3. Utilization of Dicofol To Produce Hapten **III**.* This synthesis was similar to that described by Burgisser et al. (1990). Dicofol (3.5 g, 9.5 mmol) was combined with 3-bromopropionitrile (11.4 g, 85 mmol) in 2.5 mmol of concentrated sulfuric acid, and the reagents were stirred at room temperature for 2 days. The mixture was extracted into ethyl acetate and washed with 1 M sodium carbonate solution and brine, before drying over magnesium sulfate. The bromo derivative product was recrystallized from hot ethanol and shown to be chromatographically pure by TLC in 10% ethyl acetate in petroleum ether. This bromo compound was converted to the nitrile by reaction of 0.6 mmol of **I** with 0.8 mmol of NaCN in 2 mL of dimethyl sulfoxide at 75 °C for 2 h (Friedman and Shechter, 1960). The mixture was washed with ethyl acetate, water, 1 M $NaHCO_3$, and then brine. The yellow residue from filtration and evaporation was taken up in chloroform and analyzed by TLC in 40% ethyl acetate in petroleum ether. The presence of the nitrile group in this intermediate was confirmed by IR spectroscopy. The nitrile was hydrolyzed to the carboxylic acid by dissolving in a 15 mol excess of concentrated HCl and then stirred at room temperature for 5 days. Two liters of water was then added, and the product was extracted into ethyl acetate; after washing in water and brine, and drying over $MgSO_4$, solvent was removed to produce an oil, hapten **III** [3-[2,2-bis(4-chlorophenyl)-3,3,3-trichloropropanoylamino]propanoic acid]: ^{1}H NMR ($CDCl_3$) of acid δ 2.51 (t, 2H, $-CH_2-CO-N-$), 2.97 (t, 2H, $-CH_2-COO-$), 7.28 (d, 4H, Ar, J = 9.0 Hz), 7.49 (d, 4H, Ar, J = 9.0 Hz); ^{1}H NMR ($CDCl_3$) of NHS ester δ 2.69 (t, 2H, $-CH_2-CO-N-$), 2.85 (m, 4H, $CO-CH_2CH_2-CO$), 3.15 (m, 2H, $-CH_2-CO-O-$), 7.29 (d, 4H, Ar, J = 7.8 Hz), 7.53 (d, 4H, Ar, J = 6.66 Hz).

*4. Utilization of Bis(p-chlorophenyl)ethanol To Produce Hapten **IV**.* 2,2′-Bis(4-chlorophenyl)ethanol (100 mg, 0.37 mmol) was reacted with succinic anhydride (375 mg, 3.74 mmol) in 5 mL of dry pyridine with 5 mg of DMAP for 16 h at room temperature. After addition of 20 mL of water, the water/pyridine mixture was removed by rotary evaporation and then the residue rinsed with toluene and solvent removed by evaporation. The residue was then dissolved in ethyl acetate and washed with 1 M HCl, water, and brine, before evaporation and drying over $MgSO_4$ to produce the hemisuccinate in 92% yield. This acid was then activated with NHS, and the product, hapten **IV** [mono bis(2,2-(4-chlorophenyl)ethyl ester of butanedioate] was isolated using radial chromatography in ethyl acetate/petroleum ether (40:60) to provide the NHS ester in 78% yield: ^{1}H NMR ($CDCl_3$) of acid δ 2.58 (dd, 4H, $-CO_2-CH_2-CH_2-CO_2-$), 4.31 (t, 1H, $-CH$), 4.58 (d, 2H, $-O-CH_2-$), 7.12 (d, 4H, Ar, J = 8.46 Hz), 7.27 (d, 4H, Ar, J = 8.52 Hz); ^{1}H NMR ($CDCl_3$) of NHS ester δ 2.67 (t, 2H, $-CH_2-$), 2.83 (m, 6H, 3 × $-CH_2$), 4.38 (t, 1H, $-CH-$), 4.60 (d, 2H, CH_2-OCO-), 7.13 (d, 4H, Ar, J = 8.31 Hz), 7.28 (d, 4H, Ar, J = 8.52 Hz); NHS mp 98−100 °C.

*5. Synthesis of Analogues of DDE (Hapten **V**) and DDT (Hapten **VI**), Coupled through the Ring.* Initially, 2-(*p*-chlorophenyl)-2-(*p*-tolyl)-1,1,1-trichloroethane was synthesized according to the method of Chattaway and Muir (1934). A *p*-tolyltrichloromethylcarbinol (Dinesman, 1905) was first synthesized by slow addition of 0.1 mol of toluene to a vigorously shaken solution of chloral hydrate (0.3 mol) in 35 mL of concentrated sulfuric acid. The mixture was shaken for 2 h, and the emulsion so formed was poured into excess ice−water. The oil that separated was washed with water and steam-distilled to yield a colorless oil [bp 155−157 °C, 13.5 mmHg (lit. 155 °C)], which solidified to give a solid with mp 60−61 °C (lit. 63 °C) in 25% yield. The carbinol was dissolved in a 1.1 molar excess of chlorobenzene and an equal volume of concentrated sulfuric acid added with vigorous shaking. An emulsion formed, which was poured into ice−water. An oil separated and was washed with warm (40 °C) water until the

wash was no longer acidic. The oil was then dissolved in boiling ethanol and cooled, and after 2−3 days, 2-(*p*-chlorophenyl)-2-(*p*-tolyl)-1,1,1-trichloroethane crystallized, mp 79−80 °C (lit. 81 °C). The tolyl (methyl) group was oxidized to form the carboxylic acid (Haskelberg and Lavie, 1949) by adding 2.7 g of the *p*-tolyl compound to a mixture of 7.7 g of potassium dichromate and 15 mL of water. Concentrated sulfuric acid (17 mL) was added with stirring. This caused the mixture to boil, and after 60 min of sitting, a product precipitated. The reaction mix was diluted with 100 mL of water, and the precipitate was washed with 30 mL of 5% sulfuric acid in water. The precipitate was dissolved in 20 mL of 5% NaOH in water and then reprecipitated by addition of excess dilute sulfuric acid before being recrystallized from 50% ethanol. The product had an mp of 92 °C (lit. 94−96 °C), and its identity was confirmed by TLC analysis in benzene/dioxane/acetic acid (200:6:3) (lit. R_f 0.33; Kapoor et al., 1973). For the production of the DDE analogue [hapten **V**, 3-[4-[1-(4-chlorophenyl)-2,2-dichloroethen-1-yl]benzoylamino]propanoic acid], it was then dehydrohalogenated by treatment for 2 h at 60 °C in 10% KOH in ethanol. This step was not performed in the synthesis of the DDT analogue [hapten **VI**, 3-[4-[1-(4-chlorophenyl)-2,2,2-trichloroethyl]benzoylamino]propanoic acid].

Subsequently, identical manipulations were performed in the syntheses of haptens **V** and **VI**. Eighty milligrams (0.24 mmol) of the acid was dissolved in 2 mL of thionyl chloride and refluxed at 90 °C for 1 h. The thionyl chloride was removed by rotary evaporation, toluene was added to remove the thionyl chloride residue, and the azeotrope was removed by evaporation. The yield was 78%, and the product was used without further purification. All of the acid chloride was dissolved in 2 mL of benzene, and then 72 mg of β-alanine in 2 mL of 1 M NaOH solution was added and the mixture allowed to react overnight. The product was obtained by solvent extraction, benzene was removed, the aqueous layer was acidified, and ethyl acetate was added followed by washing with water and then brine; the product was dried using magnesium sulfate and filtered under vacuum. It was reconstituted in acetone and purified by flash chromatography on silica (eluted in 20% acetone/chloroform/0.01% acetic acid). The fractions containing the acid by TLC analysis [ethyl acetate/chloroform (1:1), R_f 0.13 for hapten **V** synthesis and 0.15 for hapten **VI** synthesis] were combined, and toluene was added to remove the acetic acid residue by evaporation of the azeotrope, followed by addition of ethanol to remove residual toluene as an azeotrope. The NHS esters were formed as follows. The β-alanine amide (0.19 mmol, 75 mg) was combined with 0.4 mmol (49 mg) of NHS and 5 mL of dry methylene chloride, at 0 °C. DCC (0.23 mmol, 51 mg) was added, followed by DMAP (1.2 mg, 0.01 mmol) and allowed to react overnight. The reaction mixture was filtered and the precipitate discarded; the filtrate was evaporated and reconstituted in ethyl acetate. The mixture was shaken in slightly acidified water, saturated with sodium hydrogen carbonate, water, and brine, and dried over magnesium sulfate; the product was filtered off and reconstituted in chloroform for preparative TLC, performed in ethyl acetate/chloroform (1:1) (R_f 0.46 for hapten **V** synthesis and 0.51 for hapten **VI** synthesis).

The hapten **V** acid had an mp of 134−136 °C: ^{1}H NMR ($CDCl_3$) δ 2.72 (t, 2H, CH_2), 3.72 (m, 2H, CH_2), 6.81 (t, 1H, NH), 7.20 (d, 2H, ArH, J = 10.2 Hz), 7.32 (d, 2H, ArH, J = 8.8 Hz), 7.34 (d, 2H, ArH, J = 8.4 Hz), 7.74 (d, 2H, ArH, J = 8.0 Hz). The hapten **V** NHS ester had an mp of 150−152 °C: ^{1}H NMR ($CDCl_3$; confirmed the product) δ 2.89 (t, 2H, CH_2), 3.90 (m, 2H, CH_2), 7.0 (t, H, NH), 7.21 (d, 2H, ArH, J = 10 Hz), 7.34 (d, 2H, ArH, J = 10 Hz), 7.36 (d, 2H, ArH, J = 10 Hz), 7.82 (d, 2H, Ar, J = 8 Hz).

The hapten **VI** acid had an mp of 103−106 °C: ^{1}H NMR (acetone-d_6) δ 2.64 (t, 2H, $-CH_2-COOH$), 3.62 (m, 2H, $-CH_2-NHCO$), 5.47 (s, 1H, −CH−), 7.43 (d, 2H, Ar, J = 7.8 Hz), 7.57 (d, 2H, Ar, J = 6.3 Hz), 7.83 (d, 2H, Ar, J = 6.6 Hz), 7.89 (d, 2H, Ar, J = 9.0 Hz). The hapten **VI** NHS ester had an mp of 131−133 °C: ^{1}H NMR ($CDCl_3$) δ 2.90 (m, 2H, CH_2), 3.90 (m, 2H, CH_2), 5.12 (s, 1H, −CH), 6.98 (t, 1H, −NH), 7.31 (d, 2H, ArH, J = 8 Hz), 7.51 (d, 2H, ArH, J = 8 Hz), 7.65 (d, 2H, ArH, J = 8 Hz), 7.81 (d, 2H, ArH, J = 8.1 Hz).

The NHS esters of each hapten were then coupled to ovalbumin (OA), keyhole limpet hemocyanin (KLH), and horseradish peroxidase (HRP) in 0.1 M potassium phosphate buffer, pH 9.1 (McAdam et al., 1992), using 40-, 30-, and 13-fold molar excess of haptens, respectively.

Antibody Production. The OA and KLH conjugates of each immunogen (1 mg/mL) were emulsified with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO) and injected half-subcutaneously, half-intramuscularly into New Zealand White rabbits. Following two booster injections, each 0.5 mg/mL, of immunogen in Freund's incomplete adjuvant (Sigma) 4 weeks apart, blood was collected from the marginal ear vein 8−10 days after the last injection and clotted to form serum. Boosting and bleeding were continued monthly; IgG fractions from sera were purified by Protein G−Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography (Akerstrom et al., 1985)

Pesticide Standards. For the development of enzyme immunoassays and specificity studies, the following analytical grade standards were obtained from either Riedel-de-Haen, Seelze, Germany, or the Australian Government Analytical Laboratory, Sydney: bis(4-chlorophenyl)acetic acid (DDA), *p*,*p*′-DDD, *o*,*p*′-DDT, *o*,*p*′-DDE, and 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (*p*,*p*′-DDT). 2,2-Bis(4-chlorophenyl)-1,1-dichloroethylene (*p*,*p*′-DDE) and the analogue 2,2-bis(4-chlorophenyl)ethanol (DDT-ethanol) were obtained from Aldrich. The following analytical grade compounds were obtained from ChemService (West Chester, PA) for the cross-reaction study: fluometuron, metobromuron, chlorbromuron, linuron, metoxuron, monolinuron, diuron, neburon, bensulfuron-methyl, lindane, heptachlor, aldrin, dieldrin, endrin, methoxychlor, chlorbenzilate, phenothrin. Cypermethrin stock was prepared from a 75% pure technical grade product (ICI, Australia). Cross-reactivity (% ×) is calculated as the concentration of analyte that causes a reduction of 50% in the assay color relative to a pesticide-free control (IC_{50}), expressed as a percentage of the IC_{50} of the cross-reaction compound. Pesticide stocks were prepared by dissolving in methanol at 1 mg/mL; assay standards were prepared by diluting the stock solutions in disposable borosilicate glass tubes and used within 30 min to avoid pesticide loss through adhesion to glass surfaces.

ELISA Methods. All steps were performed at room temperature (18−23 °C). Microwell plates (Maxisorp, Nunc, Roskilde, Denmark) were coated for 16 h with antibody (100 μL per well) after dilution to 10 μg/mL in 50 mM sodium carbonate buffer, pH 9.6. Microwells were washed three times in 50 mM sodium phosphate/0.9% NaCl, pH 7.2 (PBS)/0.05% Tween 20 (PBS-T), and then nonspecific antibody binding was blocked with 150 μL per well of 1% bovine serum albumin (BSA) in PBS for 1 h. The assay was performed by the addition of 100 μL/well organochlorine standard in methanol or methanol sample extract [after 1:10 dilution in 0.1% *Teleostean* fish skin gelatin (FG, Sigma) in PBS] and 100 μL/well enzyme conjugate (diluted in 0.5% FG/PBS, providing a final oncentration of 0.3% FG) and incubation for 1 h. Other sample and conjugate diluents were also assessed (see Results and Discussion). For water analysis, the standards were diluted in distilled water to below 1% methanol concentration and the samples added directly to the microwells. The conjugate concentrations used were the lowest concentrations that provided an optical density (OD) in the assay of 0.7−1.2 and are listed in Table 1. After washing the microwells five times with distilled water, color was developed by the addition of 150 μL/well 3,3′,5′-tetramethylbenzidine (Sigma)/peroxide-based substrate (Hill et al., 1991) for 30 min. Color development was stopped by addition of 50 μL/well 1.25 M sulfuric acid, and OD values were read at 450 nm using a microplate reader, interfaced with a personal computer and data fitted to a four-parameter logistic plot using SOFTmax software (Molecular Devices, Menlo Park, CA).

Analysis of Food and Environmental Samples. The extent of matrix interference was initially determined by extracting a pesticide-free matrix, preparing standards with

Table 1. Detection of DDT, DDE, DDA, and Dicofol by Antibody/Enzyme−Hapten Combinations[a]

	Antiserum to hapten:	Ia	II	II	IV	V	VI
Enzyme-labeled hapten:	Carrier protein:	OA	KLH	OA	KLH	KLH	OA
Ia (conjugate, ng/mL)		*6*	*20*	*75*	*400*	*450*	*1600*
	p,p'-DDT	30	1000	1000	50	*	13
	p,p'-DDE	65	60	90	40	60	130
	DDA	**2**	12	50	30	1000	>1000
	dicofol	30	20	20	25	nt	500
Ib (conjugate, ng/mL)		*10*	*3.5*	*3*	*7*	*>4000*	>
	p,p'-DDT	22	*	640	**2**	*	nt
	p,p'-DDE	65	*	112	**3.5**	*	nt
	DDA	50	*	75	20	*	nt
	dicofol	520	*	25	**7**	1000	nt
Ic (conjugate, ng/mL)		*5*	*20*	*4000*	*20*	*>4000*	>
	p,p'-DDT	8	700	*	15	nt	nt
	p,p'-DDE	30	515	*	10	nt	nt
	DDA	5.5	600	*	300	nt	nt
	dicofol	3	40	*	110	nt	nt
II (conjugate, ng/mL)		*30*	*7*	*35*	*1000*	*>4200*	>
	p,p'-DDT	25	*	*	5	nt	nt
	p,p'-DDE	30	800	100	5	nt	nt
	DDA	1.5	600	70	4	nt	nt
	dicofol	15	100	10	17	nt	nt
III (conjugate, ng/mL)		*10*	*10*	*400*	*2200*	*>4000*	>
	p,p'-DDT	350	710	*	*	*	nt
	p,p'-DDE	700	140	4	*	*	nt
	DDA	5	66	190	*	*	nt
	dicofol	210	**13**	20	*	*	nt
IV (conjugate, ng/mL)		*125*	*550*	*>5000*	*30*	*>5000*	>
	p,p'-DDT	200	*	*	20	*	nt
	p,p'-DDE	190	*	*	34	*	nt
	DDA	5	*	*	*	*	nt
	dicofol	400	*	*	*	*	nt
V (conjugate, ng/mL)		*4000*	*2400*	*>4900*	*610*	*10*	>
	p,p'-DDT	*	9	*	*	130	nt
	p,p'-DDE	*	*	*	*	**9**	nt
	DDA	*	5.5	*	*	*	nt
	dicofol	nt	*	*	*	*	nt
VI (conjugate, ng/mL)		*160*	*400*	*>1600*	*16*	*1.5*	*16*
	p,p'-DDT	100	400	*	5	9	8
	p,p'-DDE	6	400	*	15	**3**	300
	DDA	*	100	*	210	*	>1000
	dicofol	*	0.01	*	90	*	350

[a] Data shown are concentrations (in μg/L) providing 50% inhibition of antibody binding; conjugate concentrations are shown in italics. > denotes control OD <0.5; * denotes <50% inhibition at 1000 μg/L; nt, not tested.

known concentrations of pesticide, and comparing the standard curve to a similar one produced in the particular extractant only. These experiments were followed by spike and recovery studies.

River Water. Initial experiments demonstrated that river water did not interfere with the DDT + DDE assay or the DDE assay (see Results and Discussion) and could be analyzed directly without the need for cleanup procedures. Both distilled and river waters (from Lane Cove River, NSW, Australia) were spiked to 50, 25, 12.5, 6.25, 3.1, 1.6, and 0.8 μg/L from 1 mg/mL *p,p'*-DDE and *p,p'*-DDT stock solutions. All spikes were sealed and left at room temperature overnight and analyzed the following day with respect to DDE and DDT standard curves prepared freshly in distilled water. A set of spikes was also prepared using both *p,p'*-DDT and *p,p'*-DDE (50:50) to form a combined total of 50, 25, 12.5, 6.25, 3.1, 1.6, and 0.8 μg/L in the sample. These spikes were analyzed separately by the DDT + DDE and DDE assays, using the degree of cross-reaction with the nontarget pesticide (Table 1) to calculate the total recoveries obtained.

Soil, Tomato Puree, and Apple Puree. The soil (gray loam, Canberra, Australia) was sieved using a 2 mm sieve and contained 12% moisture. Twenty grams of either soil or tomato or apple puree (obtained from a local supermarket) or dried sultana grapes (after dipping in a K_2CO_3/ethyl oleate emulsion in water before drying, obtained from CSIRO Hor-

ticulture Research Laboratory, Merbein, Australia) was extracted by shaking at 100 cycles/min in a reciprocal shaker for either 1 or 16 h in 100 mL of pure methanol [or methanol/water (90:10) in the case of soil]. The extracts were allowed to settle for 15 min and then analyzed in the indicated assays after a 1:10 dilution of sample extracts in 0.5% FG/PBS.

Milk. Fresh, homogenized, and pasteurized full-cream milk was obtained for matrix and spike and recovery studies from a commercial supplier. For overnight incubation of spikes, amber glass bottles were used for storage and the lids covered with aluminum foil before sealing. Milk had a significant effect on the DDE immunoassay (see Results and Discussion) and the following methods, derived from the instrumental analysis literature, for potentially removing interference were examined:

1. Partitioning into N,N′-Dimethylformamide (DMF; de Faubert Maunder, 1964). Forty milliliters of milk was extracted with 80 mL of acetone and 80 mL of hexane in a Waring blender for 2 min. The hexane layer was removed and dried with 2 g of sodium sulfate. Forty milliliters of DMF was added and partitioned; this layer was collected for immunoassay after 1:10 dilution.

2. Sulfuric Acid Treatment (Waliszewski et al., 1982). Twenty milliliters of concentrated sulfuric acid was added to 25 mL of milk, mixed gently, and allowed to cool to room temperature. Forty milliliters of petroleum ether was added and mixed for a further 3 min before 2 mL of the petroleum ether layer was removed, evaporated, and resuspended in 625 μL of methanol for analysis.

3. Alcohol and Alkali (Single Drying; Tuinstra et al., 1980). Ten milliliters of milk was extracted in 10 mL of petroleum ether and 20 mL of saturated potassium hydroxide in ethanol and left overnight at room temperature. After 5 mL of distilled water was added, the petroleum ether layer was removed and dried with sodium sulfate. A 2 mL extract was evaporated to dryness and resuspended in 1 mL of methanol for analysis.

4. Alcohol and Alkali (Double Drying). Fifty milliliters of milk was extracted with an equal volume of petroleum ether and left overnight. The petroleum ether layer was removed, evaporated, and resuspended in alcoholic potassium hydroxide. After heating to 70 °C for 30 min, and cooling to room temperature, 30 mL of petroleum ether and 20 mL of distilled water was added. The petroleum ether layer was collected and dried with 2 g of sodium sulfate and a 2 mL extract evaporated and then resuspended in 1 mL of methanol.

5A,B. Selective Solvent Extraction (Prapamontol and Stevenson, 1991). Four milliliters of milk was extracted in 20 mL of a mixture of ethyl acetate/methanol/acetone (2:4:4), vortex mixed for 2 min, and then either incubated at room temperature overnight (A, overnight extraction method) or vortex mixed for 1 min and ultrasonicated for 10 min (B, rapid extraction method). In both methods the extracts were centrifuged at 2000 rpm for 15 min, and the upper phase was collected for pesticide analysis.

6. Solid-Phase Extraction Using Florisil or Silica (Sapp, 1989). Ten milliliters of milk was extracted in 5 mL of hexane and mixed, and 1 mL of the hexane layer was collected to be run through Florisil or silica Sep-Pak Vac 6 cc (1 g) (Waters, Milford, MA) prerinsed with 10 mL of 1% diethyl ether in hexane. The eluate was collected, and 2 mL was evaporated and resuspended in 2 mL of methanol for analysis.

7. Addition of Tween 20. Tween 20 (1−10% final concentration) was added to milk and heated to 60 °C for 30 min.

All milk extracts were diluted 1:10 into 0.5% FG/PBS before analysis by immunoassay. Methods 5A and 5B were of greatest use (see Results and Discussion).

RESULTS AND DISCUSSION

Preparation of Assay Standards. Although the solubility of DDT and its related compounds in aqueous solutions is low (Bowman et al., 1960; Worthing, 1987), use of solvents such as methanol or dimethyl sulfoxide at 10−20% in the preparation of DDT standards did not result in significant improvements in sensitivity compared with dilution of standards in purified water from a concentrated stock in methanol. Diluents containing 1% BSA and/or 0.05% Tween 20 lowered the sensitivities of several of the assays, although use of BSA typically increased color development. For example, for detection of DDE with the assay utilizing antibody to hapten **IV** and hapten **Ib**−HRP (Figure 3A), there was little change in assay sensitivity (IC_{50} = 5 μg/L in all cases) when 1% BSA was substituted for 0.3% FG, but the color development was doubled. The color development remained elevated when the BSA concentration was reduced to 0.3% (data not shown). In contrast, for detection of DDE with the assay utilizing antibody to hapten **V** and hapten **V**−HRP (Figure 3B), the color only slightly increased with BSA but the assay sensitivity was poorer, changing from an IC_{50} = 8−10 μg/L to IC_{50} = 40−50 μg/L. The effects of BSA were unlikely to be due to binding of DDE or DDE−HRP by BSA and not FG since different phenonema were observed with different assays for the same analyte. Therefore, 0.5% FG/PBS was used routinely as the diluent for enzyme conjugate and for methanol extracts of food or soil matrices, unless otherwise specified.

Assay Selection. Each antiserum was screened against the panel of eight enzyme-labeled haptens to assess its detection sensitivity for DDT, DDE, DDA, and dicofol (Table 1). Consistent with previous immunoassays for pesticides developed in our laboratories, competitive, solid-phase antibody ELISAs were shown to be more sensitive than immobilized antigen formats; therefore, only results obtained using direct competitive assays are discussed (Hill et al., 1993). To select the most suitable combination of antibody and hapten-labeled enzyme for assaying the target analytes (DDT, DDE, DDA, and dicofol), each combination was assessed for its relative sensitivity, cross-reactivity, and performance at low enzyme-labeled hapten concentrations. Although two antisera were prepared to each hapten (except for haptens **Ib** and **Ic**, which were only used to prepare detection conjugates), only results with the antiserum that exhibited superior detection sensitivity for the target compound(s) are shown. Because this was from the haptens coupled to OA and KLH in an equal number of cases, neither protein carrier appeared to be clearly superior.

As expected, antibodies prepared using the hapten derived from DDA (with a β-alanine spacer arm) usually exhibited selectivity for DDA, with 50% inhibition values for DDA as low as 2 μg/L, although it was clear that the both the structure of the hapten used in the detecting enzyme conjugate and the particular spacer (for the DDA haptens) influenced both the sensitivity and the specificity of the assay. Use of the same β-alanine spacer for the enzyme conjugate actually provided a more sensitive assay for DDA than the assays using either a γ-aminobutyric acid spacer or the aminocyclohexane carboxylic acid ("bulky") spacer. The result with the bulky spacer arm contrasts with results obtained for the pyrethroid, bioresmethrin (Hill et al., 1993), and the carbamate insecticide, aldicarb (Brady, 1989), and the common finding that spacer heterology can increase immunoassay sensitivity (Harrison et al., 1990). It is possible that the failure of spacer heterology to increase assay sensitivity arises because the two chlorinated aromatic rings in the DDA hapten structure are sufficiently large to comprise most of the epitope

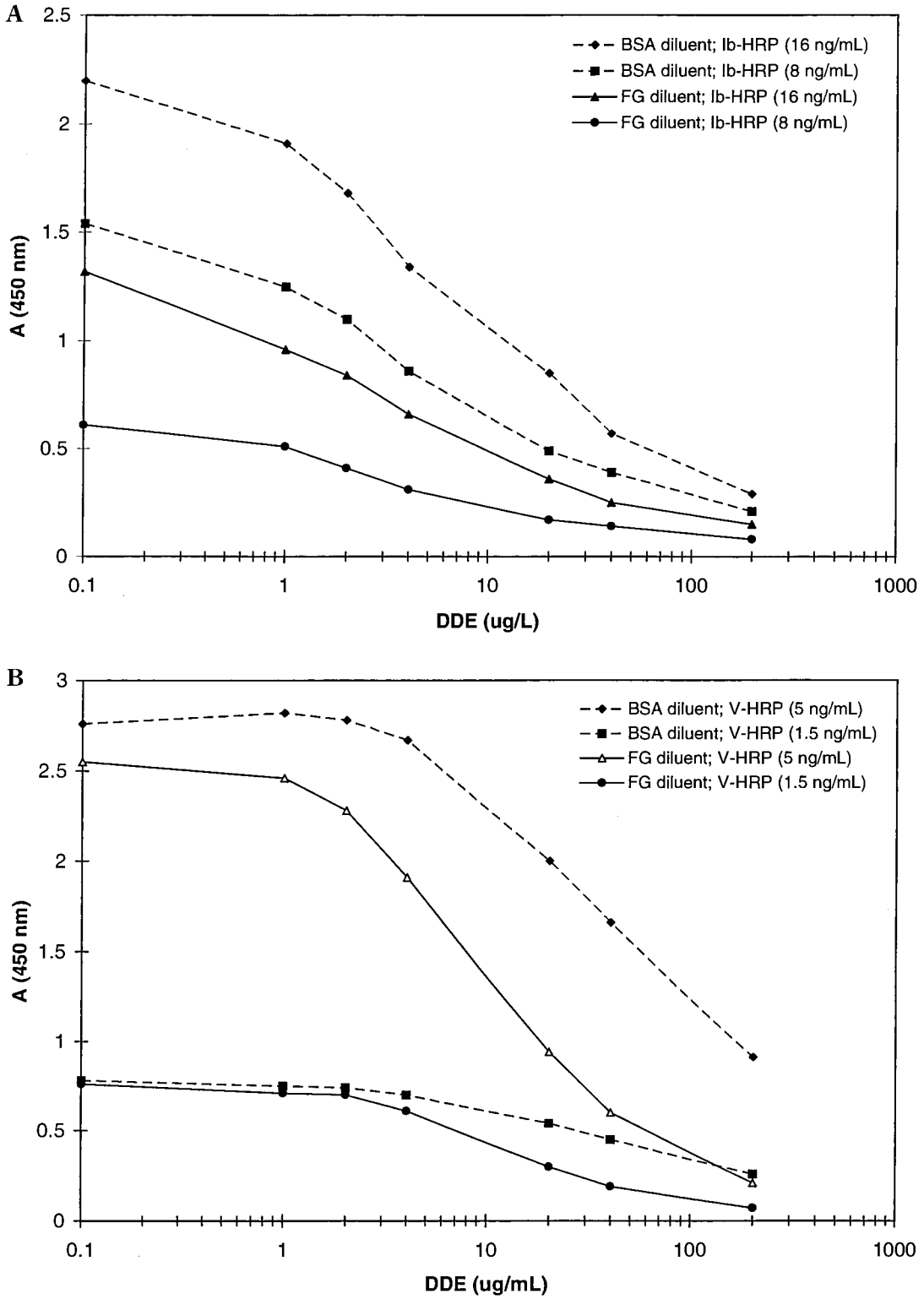

Figure 3. Comparison of assay performance for DDE standards diluted in 1% BSA/PBS/10% methanol and 0.3% FG/PBS/10% methanol, for assays (A) using antibody to hapten **V** and hapten **Ib**−HRP and (B) using antibody to hapten **V** and hapten **V**−HRP. The experiment was repeated three times in duplicate with similar results.

recognized by the antibody. However, assay specificity was affected, with the assay using the bulky spacer in the conjugate cross-reacting more equally with DDT and dicofol. Heterology in the hapten itself also greatly affected the specificity of the assay. Use of hapten **II**−HRP (identical to the immunogen hapten **Ia** except for an −OH group on carbon 1) gave similar sensitivity for DDA to the assay using hapten **Ia**, although cross-reaction with DDT, DDE, and dicofol was slightly greater. Even though their structures were different, when hapten **III**−HRP or hapten **IV**−HRP was used, the assay was virtually specific for DDA with little cross-reaction with DDT, DDE, or dicofol. Remarkably, with hapten **VI**−HRP, which differs from haptens **I**−**IV** by being coupled through one of the aromatic rings, the antibody to the DDA−β-alanine hapten failed to recognize DDA but recognized DDE with high sensitivity and DDT with moderate sensitivity.

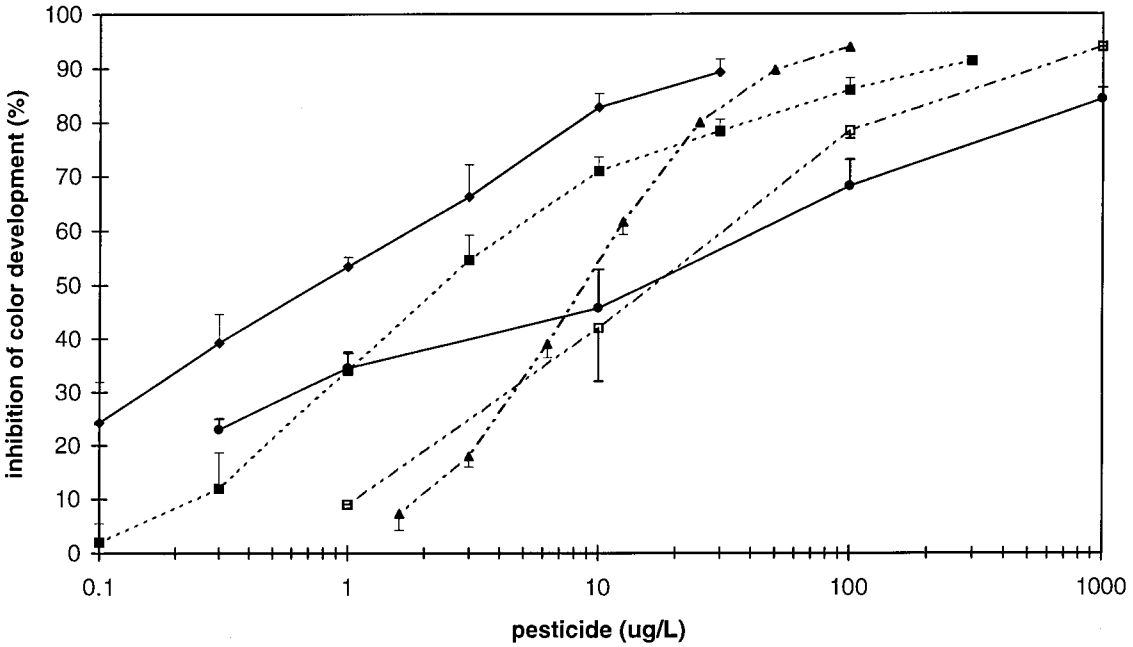

Figure 4. Standard pesticide response curves of the four selected assays for their target analytes: (◆) DDA; (■) DDT + DDE (for DDT); (□) DDT; (▲) DDE and (●) dicofol, showing standard deviations of two to four experiments.

The hapten structure for hapten **II** was derived by formation of an amide from chlorbenzilate, thus retaining the key functional groups of dicofol. Most of the assays with the different detection conjugates were selective for dicofol, with the exception of the assay using hapten **V**−HRP, which required very high conjugate concentrations. Unlike the DDA assay, detection of dicofol (and other organochlorines) using the hapten **II**−KLH antibody/hapten **II**−HRP conjugate was of low sensitivity. The most sensitive and specific assay for dicofol used the hapten **II**−KLH antibody and hapten **VI**−HRP conjugate, in which dicofol had an IC_{50} of 0.01 μg/mL, and detection of DDA, DDT, and DDE was 10 000−40 000-fold less sensitive. However, this assay was not used routinely as it had some disadvantages in that it required relatively high concentrations of conjugate (Table 1), and a maximal inhibition of only 65% by free dicofol was obtained, even at concentrations 10 000-fold higher than the IC_{50}. The latter result is possibly due to two populations of antibodies of differing affinities in the polyclonal antiserum to this hapten. The preferred assay for analysis of dicofol used either a hapten with a hydrogen atom replacing the −OH group on the central carbon (hapten **II**) in the immunogen conjugate or one with a trichloromethane group in the detection conjugate (hapten **III**). This assay combination was not as specific or sensitive, but it did not have the same serious limitations. Furthermore, the selected assay gave a steeper pesticide concentration−response curve, with a 20% change in inhibition for a 10-fold change in pesticide concentration (Figure 4). In general, the antisera from the second rabbit (two rabbits were used per group) that had been immunized with the same hapten (but coupled to the alternate protein carrier) exhibited similar specificity, differing only in sensitivity for the target compound. However, the antiserum from the hapten **II**−OA-immunized rabbit had quite different specificity. Dicofol could be detected with high sensitivity in the homogeneous assay (using hapten **II**−HRP); in addition, the assay using hapten **III**, which was selective for dicofol with the hapten **II**−KLH antiserum was selective for DDE with the hapten **II**−OA antiserum.

Hapten **III** produced a relatively poor antibody response, although it provided useful HRP conjugates in several of the assays. Only two conjugates gave significant (OD > 0.2) color development at 2 μg/mL, and in no case did DDT, DDE, DDA, or dicofol at 1000 μg/mL produce >10% inhibition. This result was initially thought to be surprising, given that it was the same hapten used by Burgisser et al. (1990) to develop a radioimmunoassay with moderate sensitivity for DDT and that it retained the $-CCl_3$ group as well as both of the 4-chloro groups of DDT. However, hapten **2** of Abad et al. (1997), which had a similar but not identical structure, also failed to generate polyclonal or monoclonal antibodies that were displaceable by DDT.

Hapten **IV** differed from haptens **I**−**III** by having a carbon−carbon bond between the first two atoms in the spacer; the carbon atom linked to the central carbon is thus tetrahedrally substituted rather than being in a planar amide bond. In this way hapten **IV** resembles the DDT structure. This hapten was successfully used to generate antibodies and develop assays using several conjugates with high sensitivities for DDT, including haptens **Ib**−HRP (most sensitive assay), **Ic**−HRP, and **VI**−HRP. However, in each case the assay also detected DDE with similar sensitivity. Hapten **V** retained the dichloroethylene group of DDE, and one of the 4-chloro groups on the aromatic rings was substituted by a spacer. This hapten produced antibodies and assays with high selectivity for DDE. The homologous assay was quite sensitive (IC_{50} for DDE of 9 μg/L); whereas the assay using the corresponding DDT analogue hapten (hapten **IV**) was slightly more sensitive for DDE, it was not favored since this assay cross-reacted to a much greater extent with DDT. Clearly, the antisera only poorly recognized conjugates derived from the haptens that were substituted through the central carbon atom, because the only other assay that displayed significant

Table 2. Cross-Reaction of Isomers and Metabolites of DDT and Related Compounds in Selected Assays[a]

	assay, antibody/conjugate									
	DDA assay, **Ia/Ia**		DDT assay, **VI/VI**		DDT + DDE assay, **IV/Ib**		DDE assay, **V/V**		dicofol assay, **II/III**	
compound	IC_{50}	% ×	IC_{50}	% ×	IC_{50}	% ×	IC_{50}	% ×	IC_{50}	% ×
p,p′-DDT	45	2	**13**	**100**	**2**	**100**	130	7	500	0.6
p,p′-DDD	7	14	30	40	0.3	700	0.9	900	30	10
p,p′-DDE	65	2	400	3	3	70	**9**	**100**	25	12
o,p′-DDT	350	0.3	70	20	60	3	–	–	–	–
o,p′-DDE	70	1.4	200	6	3	70	3	300	–	–
DDA	**0.8**	**100**	–	–	40	5	–	–	100	3
2,2′-bis(4-chlorophenyl)ethanol	0.6	135	–	–	0.3	700	580	2	8	40
N(1,1′)-bis(4-chlorophenyl)-2,2,2-(trichloroethyl)acetamide	–	–	–	–	–	–	–	–	–	–
dicofol	14	7	200	6	8	25	–	–	**3**	**100**
chlorbenzilate	1.0	70	–	–	6	30	–	–	2	150
methoxychlor	–	–	–	–	–	–	–	–	–	–
thiobencarb	–	–	13	100	90	2	80	0.4	–	–
lindane	–	–	–	–	–	–	–	–	–	–
HCH	–	–	–	–	–	–	–	–	–	–

[a] – denotes <20% inhibition at 1000 μg/L: aldrin, endrin, endosulfan, dieldrin, heptachlor, linuron, neburon, chlorbromuron, metobromuron, diuron, monolinuron, chlortoluron, metoxuron, fluometuron, cypermethrin, phenothrin, 2,4-dichlorophenoxyacetic acid.

DDE displacement utilized hapten **Ia**–HRP, although at high concentrations.

Hapten **VI** retained the trichloroethane structure of DDT, but with substitution through one of the aromatic rings. The only assay that performed at acceptably low HRP conjugate concentrations utilized hapten **VI**; it detected DDT almost 40 times more sensitively than it did DDE or dicofol and 200 times more sensitively than it did DDA. Intriguingly, whereas hapten **V** antisera could be combined with hapten **VI**–HRP to form a sensitive assay, the antisera to hapten **VI** did not recognize hapten **V**–HRP. The reasons for this are unclear. In common with the antisera to hapten **V**, the antiserum very poorly recognized HRP conjugates derived from the haptens that were substituted through the central carbon atom, including hapten **III**–HRP, even though it retained the $C-CCl_3$ structure. Abad et al. (1997) obtained a similar result; none of the monoclonal antibodies they generated to 4-{4-[1-(4-chlorophenyl)-2,2,2-trichloroethyl]phenyl}butanoic acid (hapten **5** of their study) detected conjugates based on other haptens that had been derivatized at the central carbon, although they functioned well in a homogeneous ELISA. In selecting a format, selectivity for the target analyte and a low enzyme concentration requirement were important considerations. On the basis of these results, five assays were selected (boxed results, Table 1) for further characterization, using the following combinations: (1) DDA assay, antibody raised to hapten **Ia**–OA with hapten **Ia**–HRP; (2) DDT + DDE assay, antibody to hapten **IV**–KLH and hapten **Ib**–HRP; (3) DDT assay, antibody to hapten **VI**–KLH and hapten **VI**–HRP; (4) DDE assay, antibody to hapten **V**–KLH and hapten **V**–HRP; and (5) dicofol assay, antibody to hapten **II**–OA and hapten **III**–HRP.

Specificities of Selected Assays. The specificities of the five selected assays were evaluated using a fuller range of DDT analogues, structurally related compounds, and other agrochemicals (Table 2). The DDA assay was 50–100 times more sensitive to free DDA than to *p,p′*-DDT, *p,p′*-DDE, and their *o,p′* analogues. Dicofol was detected slightly more sensitively. *p,p′*-DDD, which lacks one chlorine substituent on the carbon atom beta to the aromatic rings (carbon 1) and is thus less hindered, exhibited greater cross-reaction, whereas 2,2′-bis(4-chlorophenyl)ethanol, which shares high homology to the hapten (which is an ester at carbon 1, rather than a free acid), was detected with slightly greater sensitivity than DDA itself. Chlorbenzilate shares the ester group at carbon 1 and was detected with sensitivity similar to that for DDA. The DDT assay, using antisera to hapten **VI**, cross-reacted strongly with *p,p′*-DDT and *o,p′*-DDT (both of which contain the $-CCl_3$ moiety) and *p,p′*-DDD (which has a $-CHCl_2$ group). Substitution at carbon 2 with an –OH group or a double bond between carbons 1 and 2 decreased recognition by the antibody. The *p*-chloro group appeared to be very important in hapten recognition in all of the assays, because methoxychlor, which is identical to DDT except for two $-OCH_3$ groups, was not detected by any of the assays. Intriguingly, this assay recognized thiobencarb, a major rice herbicide, with sensitivity similar to that for DDT. This herbicide shares some degree of isosterism to *p,p′*-DDT, because it has a *p*-chlorophenyl group linked to a tetrahedrally substituted carbon atom. There is not a second aromatic ring, but the other half of the molecule is also electron-rich. Other *p*-chloro herbicides such as the phenoxyacetic acids and the substituted ureas were not detected, presumably because the aromatic group is linked to an oxygen or a nitrogen atom rather than a carbon atom.

The DDT + DDE assay detected 2,2′-bis(4-chlorophenyl)ethanol and *p,p′*-DDD with higher sensitivity than it did DDT or DDE. In the former case, this is probably because the hapten used for antibody production (**IV**) is based on this compound. DDD is also more similar to the hapten at carbon 1 in that it is less spatially hindered. The assay cross-reacted 25–30% with dicofol and chlorbenzilate (apparently the –OH substitution on C2 did not markedly hinder antibody binding) and 2–5% with thiobencarb, *o,p′*-DDT, and DDA. The DDE assay, using antibodies to immunogen prepared with hapten **V**, showed preferential recognition of the DDE and DDD compounds and was 10–80 times less sensitive for the *p,p′*-DDT parent compound. Thiobencarb, a herbicide used in rice cultivation, was detected slightly by the DDT assay (2.2% cross-reaction) and only at very high levels (>1000 μg/L) by the DDE, DDA, and dicofol assays (0.4, <0.1, and <0.3% cross-reaction, respectively). Finally, the dicofol assay, using a hapten derived from chlorbenzilate, actually detected this

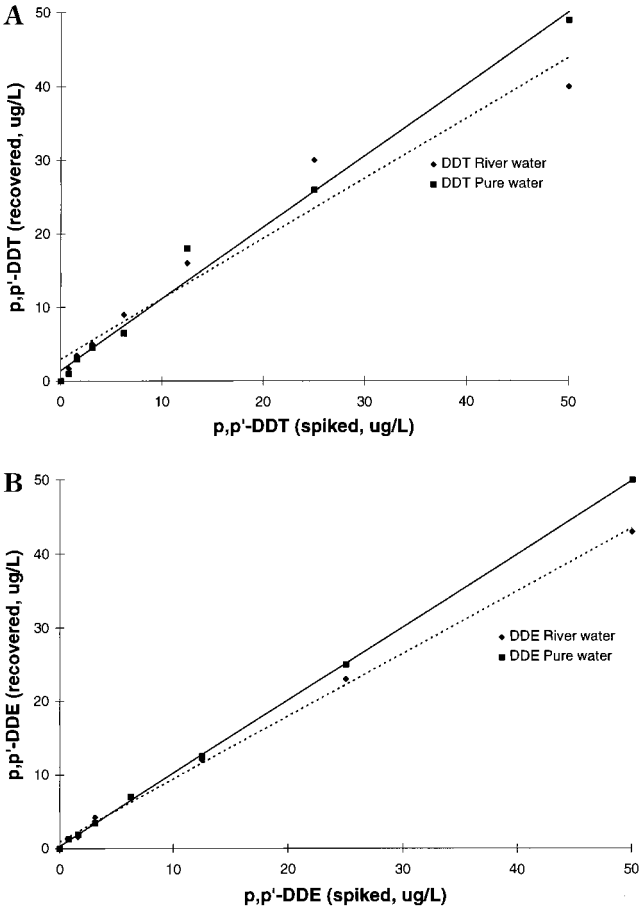

Figure 5. (A) Recovery of *p,p′*-DDE spikes prepared in river water [◆, dashed line, DDE (recovered) = 0.85 (spiked) + 1.2 μg/L, r = 0.99] and distilled water [■, solid line, DDE (recovered) = 0.99 (spiked) + 0.4 μg/L, r = 0.99] as measured by the DDE assay. (B) Recovery of *p,p′*-DDT spikes prepared in river water [◆, dashed line, DDT (recovered) = 0.80 + 3.7 μg/L, r = 0.97] and distilled water [■, solid line, DDT (recovered) = 0.96 + 1.8 μg/L, r = 0.99].

compound slightly better than it did dicofol. The hapten is an amide, and the assay detected 2,2′-bis(4-chlorophenyl)ethanol rather well (40% cross-reaction), whereas an approximately 10% cross-reaction was noted with DDE and DDD. DDT and DDA were detected only at high concentrations. Methoxychlor is an analogue of DDT with *O*-methyl groups in the place of the chlorine substituents on the aromatic rings. It was not detected by any of the assays, suggesting that the chlorine substituents play a key role in influencing antibody specificity. None of the assays detected (at 1000 μg/L) lindane or HCH, cyclodiene organochlorines (endrin, aldrin, endosulfan, dieldrin, heptachlor), or linuron, monolinuron, diuron, chlortoluron, chlorbromuron, metoxurin, fluormeturon, 2,4-D, cypermethrin, and phenothrin.

Recovery of DDE and DDT Residues from River Water. The performance of the DDE and DDT + DDE assays was assessed in spike and recovery studies. Using the DDT + DDE assay, analysis of DDT spikes in purified and river water showed recoveries of between 98 and 145% and between 80 and 161%, respectively, for samples spiked with DDT between 3 and 50 μg/L (Figure 5A). Over the same pesticide concentration range, the DDE assay also gave good recoveries of DDE spikes in purified and river water of between 100 and 113% and between 86 and 135%, respectively (Figure 5B). These high recoveries indicated there was not a significant loss of pesticide by adhesion to glass. Spikes in river water were prepared using a 50:50 mixture of DDT and DDE (at seven levels between 1.6 and 60 μg/L total DDT + DDE) and the mixtures analyzed using the DDT + DDE assay. Calculated recoveries, based on this assay having a 67% cross-reaction with DDE relative to DDT, showed good correlations between the level spiked and the recoveries, averaging between 71 and 93% (Figure 6). Similarly, the recovery for these DDT + DDE spiked samples analyzed by the DDE assay (based on this assay having a cross-reaction with *p,p′*-DDT of 3%) showed recoveries between 88 and 128%. In all cases the lowest spiked concentration gave slight overestimates, with the higher spiked concentrations being much closer to 100% recovered.

Analysis of Food and Soil Samples. The assays were next applied to the analysis of soil and selected foods (tomato puree, milk custard) in the DDT + DDE and DDE assays and to dried sultana grapes in the dicofol assay (because dicofol is registered for use in viticultural crops in many countries). In these assays, methanol extracts of the sample matrix were diluted 1:10 before analysis. Using the DDT + DDE assay, initial examinantion of pesticide standard curves produced in pesticide-free extracts of each sample matrix showed that none of the extracts affected the color developed in the absence of added pesticide standard. Both of the soil extracts decreased the sensitivity of the assay, altering the IC_{50} from 13 μg/L to 15 μg/L (soil) and 20 μg/L (custard) DDT for the 1 h extracts and to 20 μg/L (soil) and 50 μg/L (custard) DDT for the 16 h extracts. This suggests that the longer extraction period either extracted a larger proportion of interfering substances or allowed binding of the pesticide to components in the sample matrix. The recoveries of DDT spiked at 0.01−10 mg/kg in each of these matrices, using the DDT + DDE assay and determined with reference to standards prepared in methanol, are shown in Figure 6A. Slightly higher recoveries were noted for the two food samples when 16 h of extraction was used, probably due to more complete extraction of the residue, although no false positive results were observed. However, in soil, the apparent recovery increased from 109 to 189%. DDE spikes into soil, tomato puree, and custard were also analyzed with respect to DDE standards prepared in methanol, using both the DDT + DDE assay and the DDE assay (Figure 6B). Pesticide-free extracts of soil and tomato did not significantly alter the sensitivity of the DDE assay, but custard extract decreased the sensitivity of this assay 3-fold. In contrast to the DDT + DDE assay, quantitative recoveries were obtained using soil, but low (25−65%) recoveries were obtained for tomato puree and custard. This would suggest that sample cleanup strategies (or, alternatively, analysis of samples with respect to standards prepared in residue-free matrix extracts) may be required if the DDE assay were to be used for quantitative analysis rather than screening.

This latter concept was explored using the DDT + DDE and dicofol assays applied to the analysis of dried sultana grapes. These comprise a complex matrix comprising grape and grape skin components, oil, and traces of alkali. Again the curves for pesticide standards prepared in methanol and in a methanol extract of the fruit differed. Using the DDT assay, the absorbance value in the absence of pesticide decreased from

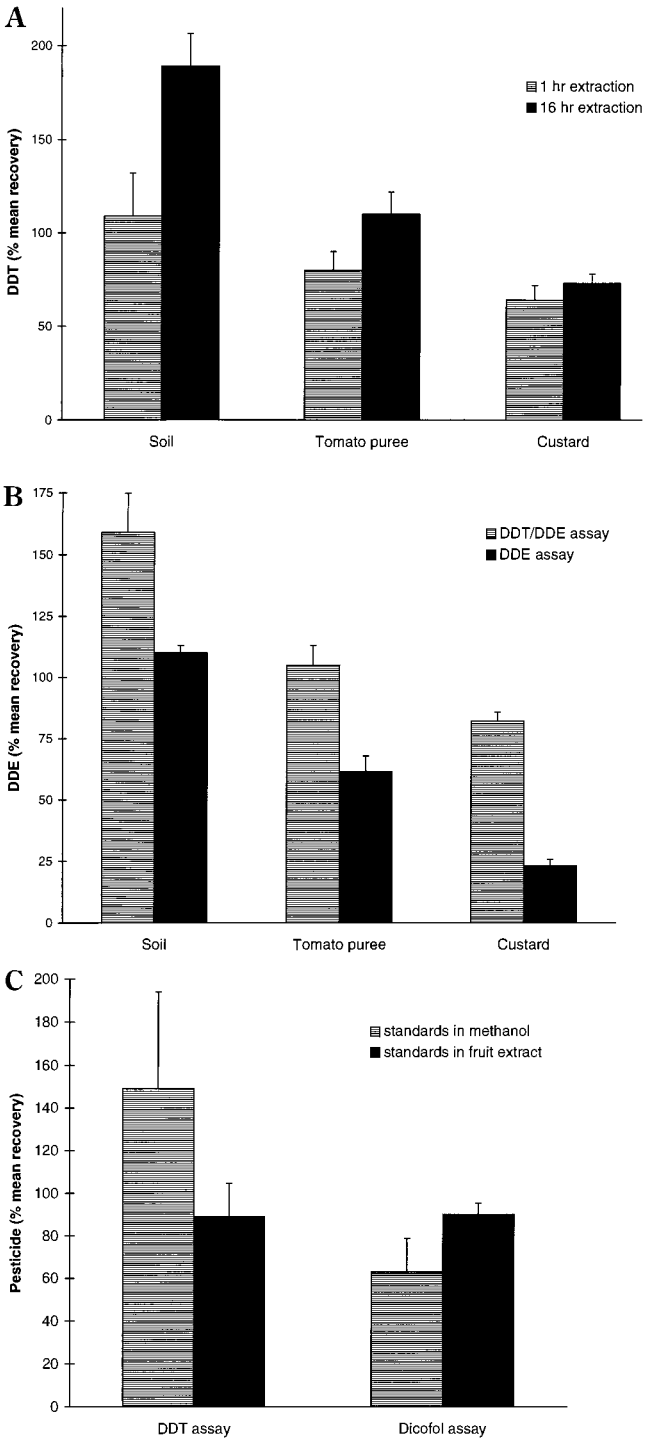

Figure 6. (A) Recoveries of DDT spiked at six concentrations (0.01−10 mg/kg) in soil, tomato puree, and custard, extracted for either 1 or 16 h, and analyzed using the DDT + DDE assay with reference to DDT standards prepared in methanol. (B) Recoveries of DDE spiked at six concentrations (0.01−10 mg/kg) in soil, tomato puree, and custard, extracted for 16 h, and analyzed using either the DDT + DDE assay or the DDE assay with reference to DDE standards prepared in methanol. (C) Recoveries of DDT and dicofol spiked at five concentrations (0.2−5 mg/kg) in dried sultana grapes, extracted for 16 h, and analyzed using the DDT + DDE and dicofol assay, respectively, with reference to standards prepared in either methanol or methanol extract of dried sultana grapes. Data shown are means of two to three experiments.

1.57 (methanol) to 1.27 (fruit extract), although the assay IC_{50} values were similar (17 and 16 μg/L, respectively). Using the dicofol assay, the absorbance value in the absence of pesticide also decreased [from 0.97 (methanol) to 0.80 (fruit extract)], and the assay IC_{50} value increased from 17 μg/L in methanol standards to 36 μg/L in fruit extract. Recoveries for a series of DDT spikes were analyzed using the DDT + DDE assay and dicofol spikes with respect to the dicofol assay, and results were obtained by reference to the two different types of standard curve. Because of the different effects of the dried grape matrix on the two assays, analysis with respect to a methanol standard curve (Figure 6C) provided significant overestimates for DDT and underestimates for dicofol, whereas in both cases, recoveries close to 100% were provided when results were analyzed with reference to standards prepared in a pesticide-free extract of the matrix. These results suggest that if analyzed pesticide-free reference samples are available, by reference to a standard curve prepared in an extract of the sample matrix, it is possible to obtained accurate recovery data without the need for sample cleanup.

Milk had a severe effect on the DDE assay, both completely eliminating inhibition by DDE color development when analyzed without dilution and reducing sensitivity by 10-fold when diluted to 1% (Figure 7). As milk is composed of approximately 4% fat and DDE is known to be highly fat soluble (Worthing, 1987), several cleanup methods that had been reported for gas chromatographic analysis were assessed (see Experimental Methods), initially by evaluating the effect of a sham cleanup procedure on pesticide-free matrix. Loss of inhibition sensitivity by pesticide standards was noted for many of the treatments; for example, standards prepared in milk after Florisil treatment provided only 30% inhibition at 0.1 mg/L DDE. One treatment, the alcohol and alkali (double drying) procedure, gave superimposable curves for DDE standards prepared in methanol and pesticide-free milk extract (Figure 7). Unfortunately, subsequent spiking experiments showed that pesticide was removed by the cleanup procedure together with the interfering fat. Milk spiked with 0.01, 0.1, and 1 mg/L DDE and processed through the alcohol and alkali (double drying) cleanup procedure gave pesticide recoveries in the range of 6−23%; thus, the method was of little practical use. The "selective solvent cleanup" method did not provide a pesticide standard curve that was superimposable with the methanol curve (Figure 7) but enabled good pesticide recovery from milk samples when samples were read with reference to pesticide standards prepared in pesticide-free milk that had been processed in the same manner. For *p,p′*-DDE spikes of 5, 1, 0.5, 0.2, and 0.1 mg/L, the method gave mean recoveries (two experiments) of 75, 112, 210, 125, and 110%, respectively. A rapid extraction format, which did not use an overnight extraction step, simplified the procedure and also showed good (but lower) recoveries at the same spike levels (60, 85, 60, 75, and 80%). The sample required a 1 in 50 dilution during the cleanup process and was analyzed with respect to a less sensitive milk extract curve (Figure 7); hence, the lower limit of detection of this method is 0.1 mg/L in the sample.

CONCLUSIONS

A number of competitive immunoassays have been developed for the sensitive analysis of *p,p′*-DDT, *p,p′*-DDE, DDA, and the miticide dicofol using a series of DDT metabolites and analogues as the basis for hapten design and synthesis. From a panel of possible antibod-

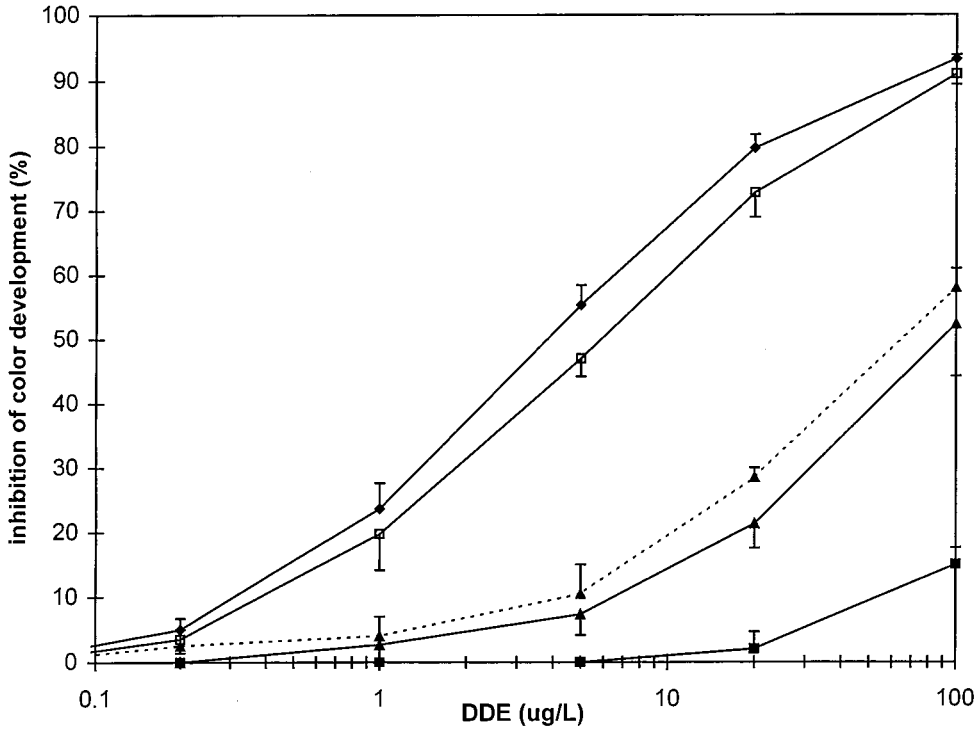

Figure 7. Effect of cleanup processes on the determination of DDE residues in milk using the DDE assay showing pesticide response curves prepared in the following: milk-free methanol (◆); milk without cleanup (■); milk after alcohol and double-drying cleanup (□); and milk after selective solvent cleanup (▲) by the overnight (solid line) or rapid (dashed line) method. Standard deviations from three to four experiments are indicated, with the error bars on the rapid selective solvent curve showing the range of data from two experiments.

ies and enzyme-labeled hapten combinations, five assays were selected and evaluated for application to food and environmental matrices. Other immunoassays have been developed for DDA (Haas and Guardia, 1968; Centero et al., 1970; Furuya and Urasawa, 1981), although much of this work predated the development of sensitive nonisotopic methods, such as the ELISA. In the current study, DDA-derived immunogens produced highly sensitive assays for DDA. The antibodies were usually most sensitive for DDA when used in combination with enzyme conjugates that also utilized DDA-derived haptens. The slightly longer spacer arm of hapten **Ib**−HRP gave the lowest limit of detection. By utilizing chlorbenzilate, an analogue of dicofol, very specific and sensitive antibodies to dicofol cross-reacting with chlorobenzilate were raised. The DDT + DDE assay, based on antibodies raised to a bis(*p*-chlorophenyl)ethanol derivative, could detect as little as 0.3 μg/L DDT in water, with significant detection of the DDE metabolite (67%), allowing for the first time the ability to directly detect trace levels of these organochlorines in water without sample concentration, at levels relevant to environmental analytical programs (Pham et al., 1993). An immunoassay described recently by Banerjee et al. (1996) had broad specificity, but is approximately 10 times less sensitive for DDT than the DDT + DDE assay described in this paper. The DDT + DDE assay described herein is also 10 times more sensitive (and the DDT assay twice as sensitive) as the competitive solid-phase radioimmunoassay described by Burgisser et al. (1990) using haptens based on dicofol derivatives. In the late stages of preparation of this paper, Abad et al. (1997) described the development of monoclonal antibodies of similar detection sensitivity to those described in the present paper. Typically, the major determinant of assay specificity is the hapten used for antibody production (Gee et al., 1995). Whereas we have earlier observed that the choice of hapten for the enzyme label used in the development of panels of antibodies for structurally related haptens (Edward et al., 1993; Lee et al., 1995, 1998) can affect assay specificity, in the present study the hapten in the detecting conjugate had remarkable effects on specificity.

Selected assays were applied to different sample matrices. Organochlorine residues in river water and extracts of two foods (tomato puree and dried sultana grapes) could be quantitatively analyzed directly without the need for extract concentration or sample cleanup. Since the 1996/1997 harvest, assays based on some of these antisera have been routinely use for residue screening by the Australian dried fruit industry; full details of their performance and validation will be described elsewhere (J. H. Skerritt and T. Phongkham, unpublished results). With the introduction of a simple alkali dehydrohalogenation step, which converts DDT residues in a sample extract to DDE, we have adapted the DDE assay for the large-scale quantitative analysis of total DDT plus DDE residues in cotton cropping soils (Shivaramiah, Kennedy, and Skerritt, 1998). Milk (and milk-based custard) proved a difficult matrix for analysis of organochlorine residues due to its fat content and the high fat solubility of these compounds, with procedures that remove the interfering fat often removing the pesticide strongly associated with it. A simple method involving treating the milk with a selection of solvents gave a less sensitive assay than the standard format, but allowed samples to be analyzed against a standard curve prepared in pesticide-free milk. The method had a lower limit of detection of 0.1 mg/mL DDE in milk; a further modification such as a concentration step would be required to increase the detection sensitivity.

ABBREVIATIONS USED

BSA, bovine serum albumin; DCC, dicyclohexylcarbodiimide; DDA, bis(*p*-chlorophenyl)acetic acid; DDD, dichlorodiphenyltrichloroethane; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane or 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DMAP, (dimethylamino)pyridine; FG, *Teleostean* fish skin gelatin; KLH, keyhole limpet hemocyanin; NHS, *N*-hydroxysuccinimide; OA, ovalbumin. Note: Where not specified, DDT and DDE refer to the *p*,*p*′ isomers.

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