# Hapten Synthesis for the Development of a Competitive Inhibition Enzyme-Immunoassay for Thiram

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An enzyme-linked immunosorbent assay (ELISA) was developed for the fungicide thiram. Two types of haptens were synthesized. The first type exhibits the two symmetrical *N*-alkyl dithiocarbamate patterns of thiram with a spacer arm linked to one of the *N*-methyl terminal group. The second type exhibits one of the two symmetrical *N*-alkyl dithiocarbamate patterns of thiram with a variable-length spacer arm linked to one sulfur atom. Polyclonal antibodies suitable for thiram detection were obtained from immunization with an hapten of the first type, while haptens of the second type were used as coating antigens to develop a competitive ELISA against thiram. The $IC_{50}$ value for thiram was estimated to be 0.24 $\mu$g/mL, with a detection limit of 0.03 $\mu$g/mL. The assay seems to be thiram-specific since no or little cross-reaction with other dithiocarbamates were observed.

**Keywords:** *Thiram; dithiocarbamates; hapten; ELISA*

## INTRODUCTION

Dithiocarbamates (DTCs), including thiram, are wide-spectrum nonsystemic fungicides that have widely been used in agriculture and horticulture over several decades. They also have important uses in inorganic analysis, in rubber industry and medicine (Thorn and Ludwig, 1962). These fungicides are classified into two main groups: dimethyldithiocarbamates and ethylenebis(dithiocarbamates) (EBDCs). All, except thiram, are salts or complexes including a metal ion. Dimethyldithiocarbamates include thiram (Figure 1), ferbam, and ziram, while EBDCs, include maneb, mancozeb, nabam, and zineb (Figure 1). Propylenebis(dithiocarbamates) (propineb) form a third, smaller group. In agriculture, some dithiocarbamates are formulated as seed treatments, and all have been used, in some way, for disease control on growing crops. The range of crops treated with dithiocarbamates is very wide and includes cereals, potatoes, and other field crops, soft, top fruit, and tropical fruit (Smart, 1989).

Dithiocarbamates are considered to be not very toxic; however, there is some concerns over chronic exposure to these fungicides since they are suspected to be carcinogens (Engst and Schnark, 1974; Woodrow and Seiber, 1995). Furthermore, they have been a focus of attention for many years because of the hazard associated with ethylene thiourea (ETU), a metabolite and decomposition product of EBDCs which has shown teratogenic, goitrogenic, and carcinogenic properties in animal experiments (Lentza-Rizos, 1990). So maximum residue limits (MRL) for dithiocarbamates in foodstuffs were defined. These MRL were generally expressed as $CS_2$ equivalent, so they involved all the DTCs. In Codex

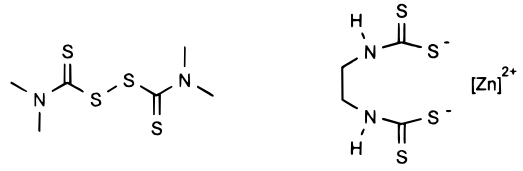


**Figure 1.** Structures of two fungicidal dithiocarbamates.

Alimentarius, the MRL for dithiocarbamates range from 10 mg/kg on lettuce to 0.05 mg/kg on milks or eggs.

Although many methods for determination of dithiocarbamates residues have been described in the literature, the official and most commonly used method is that of Keppel (1969). In this method, the DTCs are hydrolyzed with hot mineral acid in the presence of $SnCl_2$ to form carbon disulfide ($CS_2$) which is then determined spectrophotometrically as cupric complexes of *N*,*N*′-bis(2-hydroxylethyl) dithiocarbamic acid. Nevertheless the analysis of DTCs as $CS_2$ provides only a measure of total DTCs and thus does not allow discrimination of individual dithiocarbamates. Results of these analyses may also include $CS_2$ from other sources and from natural plant constituents (for example, in cabbages) in the sample (Hill, 1992). Moreover, collaborative study did not fully confirm this procedure, as somewhat low results were obtained with some DTCs and some erratic results with others (Keppel, 1971). Another type of procedure being increasingly used is sensitive determination of $CS_2$ produced during hydrolysis by means of headspace gas chromatography (Committee for Analytical Methods for Residues of Pesticides and Veterinary Products in Foodstuffs of the Ministry of Agriculture, Fisheries and Food, 1981; Hill, 1992). Studies have also been carried out on modification of the Keppel method in order to improve its reproducibility (Vuik et al., 1992; Bohrer et al., 1999). Otherwise, the distinction between residues of indi-

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vidual dithiocarbamates or their metabolites has been the aim of many workers. For example a specific high performance liquid chromatography (HPLC) method for determination of thiram, salts of alkylenebis(dithiocarbamic acids), and *N*,*N*′-dimethyl(dithiocarbamic acid) has been reported by Gustafsson and Thompson (1981). Approaches to specific determination of thiram (Irth et al., 1986, 1990; Baumann et al., 1991) and EBDCs (Miles and Zhou, 1991; Irth et al., 1990) have been examined in a number of laboratories. Validation of HPLC method in which the EBDCs can be separated from thiram has been reported by Jongen et al. (1991). More recently Lo et al. (1996) have developed an HPLC and atomic absorption method to distinguish propineb, zineb, maneb, and mancozeb fungicides. However none of the above methods can be performed directly on foodstuff. All of them need an initial extraction step and require sophisticated equipment such as HPLC or gas chromatography (GC), available in only well-equipped centralized laboratories. They are more labor-intensive, time-consuming, and expensive than the reference method. Furthermore the complete identification of residues of the complex DTCs still remains impossible.

The problem of monitoring the residual level of pesticides in foodstuffs need the development of simple, rapid, and sensitive procedures adequate for handling a large number of samples. In this way, immunoassay has been proved to fulfill such analytical requirement, provided that appropriate antibodies are available (Kaufman and Clower, 1995; Ferguson et al., 1993). Immunoassay may offer either an alternative to the traditional method or the potential for an initial rapid screening method to reduce the number of samples requiring chemical analysis. So immunoassays have been developed for some pesticides. In the case of dithiocarbamates, there is no report of an immunoassay development.

In the present paper, we describe the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of thiram using anti-thiram polyclonal antibodies. In this aim, different haptens, belonging to two families, were synthesized and used in order to obtain antibodies. They were also screened for their capacity to improve ELISA sensitivity in heterologous competitive ELISA.

## MATERIALS AND METHODS

Bovine serum albumin (BSA), ovalbumin (Ova), and goat anti-rabbit IgG were obtained from SIGMA. Other chemicals were from Aldrich. Thin-layer chromatography and column chromatography were carried out using Merck silica gel 60. The structure of haptens was confirmed by $^1$H and $^{13}$C NMR spectroscopy (Brucker DRX 400 or JEOL FX 100) using tetramethylsilane as internal standard.

**Hapten Synthesis.** *Disodium 4-[Carbodithioato(methyl)-amino]butanoate* ***3***. To a water solution (50 mL) of sodium hydroxide (1.2 g, 30 mmol) and 4-(methyl-amino)butanoic acid chlohydrate salt (1.53 g, 10 mmol) was added dropwise, at room temperature, carbon disulfide (0.91 g, 12 mmol). After 1 h, the mixture was warmed to 40 °C for 2 h. Then, the mixture was extracted with acetone (2 × 20 mL), and the acetone phase was discarded. The product, **3**, precipitated as a white powder. Yield: 2 g (84%). $^1$H NMR (99.54 MHz, $D_2O$): $\delta$ 1.8−2.05 (m, 2H), 2.2 (t, 2H, $J$ = 7 Hz), 3.45 (s, 3H), 4.05 (t, 2H, $J$ = 7 Hz). $^{13}$C NMR (25.05 MHz, $CDCl_3$): $\delta$ 25.4 (C−$CH_2$−C), 36.5 ($CH_2$−$CO_2^-$), 44.8 (N−$CH_3$), 58.1 ($CH_2$−N), 184.2 (*C*=O), 209, 0 (*C*=S).

*5,11-Dimethyl-6,10-dithioxo-7,9-dithia-5,11-diazadodecanoic Acid, Hapten* ***1***. A mixture of disodium 4-[carbodithioato(methyl)amino]butanoate **3** (0.78 g, 3.3 mmol), and sodium dimethyldithiocarbamate (1.17 g, 8 mmol) in 20 mL of methanol/dichloromethane (15:5) was stirred for 20 h at room temperature and concentrated in vacuo. The residue was dissolved into water (20 mL), acidified (pH 3) with 3 M HCl, and extracted with dichloromethane (2 × 15 mL). The organic phase was dried with $MgSO_4$, filtered, and concentrated in vacuo. The product, **1,** ($R_f$: 0.2) was purified by flash chromatography (ethyl acetate/hexane, 1:2). Yield: 0.3 g (27%) amorphous powder (mp: 105−106 °C, dec). $^1$H NMR (400.13 MHz, $CDCl_3$): $\delta$ 2.0 (p, 2H, $J$ = 7 Hz), 2.45 (t, 2H, $J$ = 7 Hz), 3.75, 4.1 (2t, 2H, $J$ = 7 Hz), 3.32, 3.36, 3.50, 3.55 (4s, 9H), 5.33 (s, 2H,S−C$H_2$−S), 10.7 (s, 1H). $^{13}$C NMR (100.62 MHz, $CDCl_3$): $\delta$ 21.3, 22.0 (C−$CH_2$−C), 30.5, 30.8 ($CH_2$−$CO_2$H), 39.6, 43.6 (N−$CH_3$), 41.5, 45.5 (($CH_3$)$_2$N−), 46.2 (S−$CH_2$−S), 53.3, 55.8 (N−$CH_2$−), 178.1, 178.6 (*C*=O), 195.0, 195.8 (($CH_3$)N−*C*=S), 195.13 (($CH_3$)$_2$N−*C*=S). Anal. Calcd for $C_{10}H_{18}N_2O_2S_4$: C, 36.78; H, 5.56. Found: C, 36.62; H, 5.51. Two symmetrical products were also obtained ($R_f$ = 0.9, $C_7H_{14}N_2S_4$; $R_f$ =0.05, $C_{13}H_{22}N_2O_4S_4$).

*{[(Dialkylamino)carbothioyl]sulfanyl}alkanoic Acid* ***2***. General Procedure. To a water solution (25 mL) of sodium hydroxide (1.2 g, 30 mmol) and 40% aqueous solution of dimethylamine (2.25 g, 20 mmol) was added dropwise, at room temperature, carbon disulfide (1.9 g, 25 mmol). After 30 min, a mixture of 6-bromohexanoic acid (3.9 g, 25 mmol), sodium hydroxide (0.8 g, 20 mmol), and water (15 mL) was added. The mixture was stirred for 20 h, acidified (pH 3) with 3 M HCl, and extracted using dichloromethane (2 × 20 mL). The organic phase was dried with $MgSO_4$, filtered, and concentrated in vacuo. The crude product was dissolved into dichloromethane and precipitated by hexane at 0 °C to give **2c** (3.2 g, 13.6 mmol, 68% yield), a white powder (mp: 85−86 °C, dec).

The structure of haptens **2** was fully ascertained using $^1$H and $^{13}$C NMR spectroscopy and by comparison with reported data.

*2-{[(Dimethylamino)carbothioyl]sulfanyl}ethanoic Acid* ***2a***. (Garraway, 1962). White powder (mp: 145−146 °C, dec). $^1$H NMR (99.54 MHz, $CDCl_3$): $\delta$ 3.4, 3.5 (2s, 6H), 4.15 (s, 2H), 11.2 (s, 1H). $^{13}$C NMR (25.05 MHz, $CD_3OD/CDCl_3$): $\delta$ 39.2 ($CH_2$), 41.4, 45.5 (N($CH_3$)$_2$), 170.5 (*C*=O), 195.2 (*C*=S).

*4-{[(Dimethylamino)carbothioyl]sulfanyl}butanoic Acid* ***2b***. (Garraway, 1962). White powder (mp: 92−93 °C, dec). $^1$H NMR (99.54 MHz, $CDCl_3$): $\delta$ 2.05 (p, 2H, $J$ = 7 Hz), 2.5 (t, 2H, $J$ = 7 Hz, C$H_2$−$CO_2$H), 3.35 (t, 2H, $J$ = 7 Hz), 3.4 (s, 3H), 3.55 (s, 3H). $^{13}$C NMR (25.05 MHz, $CDCl_3$): $\delta$ 23.9 ($CH_2$), 32.9 ($CH_2$−$CO_2$H), 36.3 ($CH_2$−S), 41.4, 45.2 (N($CH_3$)$_2$), 178.8 (*C*=O), 196.5 (*C*=S).

*6-{[(Dimethylamino)carbothioyl]sulfanyl}hexanoic Acid* ***2c***. (Garraway, 1962). White powder (mp: 85−86 °C, dec). $^1$H NMR (99.54 MHz, $CDCl_3$): $\delta$ 1.2−1.9 (m, 6H), 2.35 (t, 2H, $J$ = 7 Hz, C$H_2$−$CO_2$H), 3.25 (t, 2H, $J$ = 7 Hz, S−C$H_2$−), 3.32 (s, 3H), 3.50 (s, 3H). $^{13}$C NMR (25.05 MHz, $CDCl_3$): $\delta$ 24.2, 28.3, 28.4, 33.9 ($CH_2$−$CO_2$H), 37.2 (S−$CH_2$−), 180 (*C*=O), 197.3 (*C*=S).

*11-{[(Dimethylamino)carbothioyl]sulfanyl}undecanoic Acid* ***2d***. White powder (mp: 90−92 °C, dec). $^1$H NMR (99.54 MHz, $CDCl_3$): $\delta$ 1.1−2 (m, 16H), 2.4 (t, 2H, $J$ = 7 Hz), 3.3 (t, 2H, $J$ = 7 Hz), 3.5 (s, 6H), 10.2 (s, 1H). $^{13}$C NMR (25.05 MHz, $CDCl_3$): $\delta$ 24.6, 28.5, 28.9 (2C), 29.1 (2C), 29.3, 32.8, 34.1 ($CH_2$−$CO_2$H), 37.6 ($CH_2$−S), 41.4, 45.3 (2 −$CH_3$), 180.4 (*C*=O), 197.5 (*C*=S).

*2-{[(Diethylamino)carbothioyl]sulfanyl}ethanoic Acid* ***2e***. White powder (mp: 88−90 °C, dec). $^1$H NMR (99.54 MHz, $CDCl_3$): $\delta$ 1.35 (t, 6H, $J$ = 7 Hz), 3.95 (m, 4H), 4.26 (s, 2H), 11.9 (s, 1H). $^{13}$C NMR (25.05 MHz, $CDCl_3$): $\delta$ 11.1, 12.2 (2 −$CH_3$), 38.3 ($CH_2$−$CO_2$H), 46.9, 49.9 (2 −$CH_2$), 173.8 (*C*=O), 192.8 (*C*=S).

**Preparation and Characterization of Hapten-Protein Conjugates.** All haptens used in this study contained a free carboxylic group suitable to react with amine groups of proteins. Hapten−protein conjugations were carried out by the *N*-hydroxysuccinimide (NHS) active ester method according to Langone and Van Vunakis (1975).

The carboxylic acid hapten (50 mg) was dissolved in 1.0 mL of dimethylformamide (DMF) with 70 mg of NHS and 60 mg

of dicyclohexylcarbodiimide (DCC). After being stirred for 30 min at room temperature, the precipitated dicyclohexylurea was removed by centrifugation and the DMF supernatant was added to protein solutions. Proteins, 200 mg of BSA to prepare the immunoconjugate or 200 mg of Ova for the coating antigen, were dissolved in 10 mL of 0.1 M bicarbonate sodium solution. The reaction mixtures were stirred gently at 4 °C for 3 h to complete the conjugation and then dialyzed exhaustively against phosphate buffered saline (pH 7.4), (PBS: 1.5 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 2.7 mM KCl, 137 mM NaCl, and 0.02% $NaN_3$) and then lyophilized. All conjugates easily dissolved in distilled water at 1.0 mg/mL for measurement of hapten substitution or ELISA plate coating with the exception of conjugate Ova-**2d**.

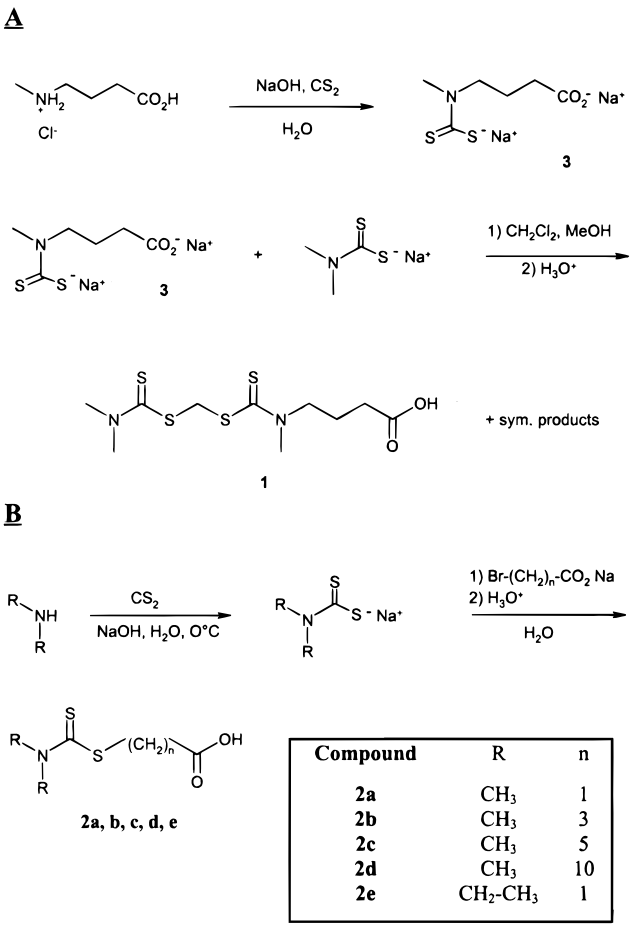
Protein concentration in conjugate solutions was measured according to the method of Bradford (1976). The level of hapten substitution was estimated by measuring the loss of free amino groups on the protein (Queffelec et al., 1998) using the orthophthaldialdehyde (OPDA) method (Svedas et al., 1980).

**Polyclonal Antibodies Production.** Three-month-old rabbits were immunized subcutaneously. Each rabbit received 2 mL of an emulsion (1:1) containing 0.5 mg/mL of BSA−hapten conjugate in PBS and complete Freund's adjuvant. The rabbits were boosted three times within 2-week intervals using the same amount of antigen emulsified in incomplete Freund's adjuvant for the first boost and in PBS alone for the subsequent ones. Blood was collected from the marginal ear vein 10 days after the last injection. Coagulum was allowed to form at 4 °C overnight. The serum was recovered by centrifugation at 10 000 *g* for 5 min and stored at −20 °C.

**Immunoassays.** *Hapten-Immobilized Immunoassay.* The titer of rabbit sera used in the competitive assays was determined according to this format. Microwell plates (Maxisorp, Nunc) were coated with 100 $\mu$L of Ova−hapten conjugate dissolved at 10 $\mu$g/mL in coating buffer (50 mM carbonate−bicarbonate buffer, pH 9.6) by overnight incubation at 4 °C. Plates were washed twice with PBS supplemented with 0.05% Tween 20 (PBS-T), and blocked with 300 $\mu$L/well 0.5% (w/v) casein in PBS-T for 2 h at room temperature. Serial dilutions of 100 $\mu$L/well of antiserum in PBS-T were added to the wells and incubated for 1.5 h at room temperature. After the plates were washed three times with PBS-T, a goat anti-rabbit IgG−alkaline phosphatase conjugated (Jackson Immuno Research) diluted 1:5000 (100 $\mu$L/well) in PBS-T-0.5% casein was added. Plates were incubated for 1.5 h at room temperature, and then after three washes in PBS-T and one in reaction buffer (Diethanolamine, 1 M; $MgCl_2$, $5 \times 10^{-7}$ M; pH 9.8), 100 $\mu$L of 1 mg/mL *p*-nitrophenyl phosphate in reaction buffer was added. After 1.5 h at 37 °C, the absorbance was measured at 405 nm using a microplate reader (Multiscan MCC 340, Flow Laboratories).

*Competitive Indirect ELISA.* A competitive ELISA, using different coating antigen, was used for assessing the specificity of the antibodies to free thiram and their cross-reactivity with related structural compounds and pesticides. To microwell plates coated with selected concentration of Ova−hapten conjugates in coating buffer and then blocked with PBS-T-0.5% casein as described above, 50 $\mu$L/well of serial dilutions of the analyte in PBS-T containing 6% acetone was added, immediately followed by 50 $\mu$L of antiserum at appropriate dilution in PBS-T. In this way, a competition was established between immobilized hapten and free analyte for the antibody binding sites. Plates were incubated at room temperature for 1.5 h, and after being washed six times, a 1:5 000 dilution of goat anti-rabbit IgG−alkaline phosphatase conjugated was added. After 1.5 h at room temperature and six washes, color was developed as described above. All incubation steps were carried out under stirring.

The results are expressed in percent inhibition as follows: % inhibition = % $B/B_o = 100[(A_{405}^{(x)} - A_{405}^{(0)})/(A_{405}^{(ctl)} - A_{405}^{(0)})]$, where $A_{405}^{(x)}$ = OD value in the presence of competitor, $A_{405}^{(ctl)}$ = OD value in the absence of competitor, and $A_{405}^{(0)}$ = OD value when the antibodies are omitted.




| Compound | R | n |
|---|---|---|
| **2a** | $CH_3$ | 1 |
| **2b** | $CH_3$ | 3 |
| **2c** | $CH_3$ | 5 |
| **2d** | $CH_3$ | 10 |
| **2e** | $CH_2$-$CH_3$ | 1 |

**Figure 2.** Synthesis and structures of haptens for conjugation to carrier proteins: (A) hapten **1** (immunizing hapten), (B) hapten **2** (coating hapten).

## RESULTS AND DISCUSSION

**Synthesis of Thiram Haptens.** Whereas immunoassay specificity and sensitivity are determined primarily by the antibody produced in response to the immunogen, hapten design and synthesis are critical steps in the development of immunoassays to low-molecular-weight analytes (Harrison et al., 1991; Goodrow et al., 1995; Goodrow and Hammock, 1998).

The simplicity of the thiram structure, which results from the oxidation of two *N*,*N*′-dimethyldithiocarbamic acids, leaves a few choices for the placement of a spacer arm. The first idea was to synthesize a derivative of one of each symmetrical *N*,*N*′-dithiocarbamate pattern with the attachment of the spacer arm through a thioester linkage (hapten **2**, Figure 2B). The route to dithiocarbamate esters of type **2** is well-known (Nachmias, 1952; Reid, 1962; Thorn et al., 1962). The method most commonly used involves the alkylation of a dithiocarbamate salt with an alkyl halide (see Figure 2B). In this work, the sodium salt of *N*,*N*′-dimethyldithiocarbamic acid was reacted with various $\omega$-bromoalkanoic acids.

To improve the target molecule recognition, a second approach involved the synthesis of an hapten bearing *N*,*N*′-dithiocarbamate pattern (hapten **1**, Figure 2A). The effect of the length of a spacer arm on the sensitivity and specificity of the antibodies produced against pesticides have been well documented by Harrison et al. (1991). A spacer arm that is neither too long nor too short maximizes the exposure of the analyte for antibody production and increase the assay sensitivity.

Consequently we linked a three carbon alkyl spacer arm ($(CH_2)_3$) with a carboxylic group to one of the *N*-methyl terminal group. The position of such a spacer arm should give a minimum alteration of the structure, geometry, and electronic nature of the hapten compared to the target analyte.

The disulfide ($n = 2$) derivative was made by thioacylation of the corresponding dithiocarbamic acid **3**. Unfortunately this compound was not stable enough to give the BSA conjugate. Abstraction of a sulfur atom from the sulfide linkage ($n = 1$) gave a mixture of compounds which could not be separated by liquid chromatography.

Regarding these difficulties, it was decided to introduce a methylene group to join the two dithiocarbamic moieties. Methylenebis(*N*,*N*′-dimethyldithiocarbamate) was described by Nakai et al. (1974). This compound, which is very useful for aldehyde synthesis, was easily prepared by reacting sodium *N*,*N*′-dimethyldithiocarbamate with methylene chloride. Using the same procedure and the appropriate sodium salts, we got a mixture of symmetrical compounds and the expected hapten **1** (Figure 2A) which was purified by liquid chromatography. Compound **1** was very stable and could be coupled to BSA or Ova.

All haptens were coupled to BSA or Ova using the DCC−NHS method (Langone and Van Vunakis, 1975). Hapten protein conjugation was verified by measuring the number of free amino group on the protein by OPDA reaction. Hapten densities were between 33 and 41 for BSA conjugates (33, 39, 41, 39 with **1**, **2a**, **2b**, **2e**, respectively) and 5 and 15 for Ova conjugates (7, 10, 12, 5, 5, 15 with **1**, **2a**, **2b**, **2c**, **2d**, **2e**, respectively).

**Production and Titration of Antibodies to Thiram.** To evaluate the suitability of the synthesized hapten **2** to raise anti-thiram antibodies, three rabbits were immunized with BSA−hapten **2b** conjugates and three other with BSA−hapten **2d** conjugates. After four injections, the titer (serum dilution that gave 3 times the background absorbance) of antisera was estimated by indirect ELISA using the respective homologous Ova−hapten as a coating antigen. The individual animals showed little differences in their antisera ranging from $1{:}2.5 \times 10^4$ at the lowest titer to $1{:}5.1 \times 10^5$ at the highest titer (data not shown). These antisera were subsequently tested for their ability to recognize free thiram by competitive indirect ELISA. Unfortunately, the raised antisera failed to recognize thiram in a competitive ELISA test. The sera screening indicated the recognition of conjugated and free immunizing hapten without significant recognition of the target analyte (data not shown). The use of these sera for thiram detection should be not possible, so they were no longer studied.

Three other rabbits were immunized with BSA−hapten **1** conjugate, and their sera were tested as above. The individual animals showed significant differences in their antisera ranging from $1{:}6.4 \times 10^4$ at the lowest titer to $1{:}5.1 \times 10^5$ at the highest titer (data not shown). The anti-thiram serum giving the best titer was subsequently tested for its ability to recognize free thiram by competitive indirect ELISA.

Conditions for the competition test were optimized in preliminary experiments with various concentrations of plate-coating antigen and anti-thiram serum. The criteria were the concentrations giving an absorbance around 1 unit of OD in absence of analyte (maximum absorbance) and an inhibition curve with the lowest $IC_{50}$ (concentration of thiram inhibiting 50% of the maximum absorbance). Thiram solutions were prepared before each assay by diluting in PBS-T a freshly prepared acetonic solution of thiram. The final acetone concentration in the wells was 3%.

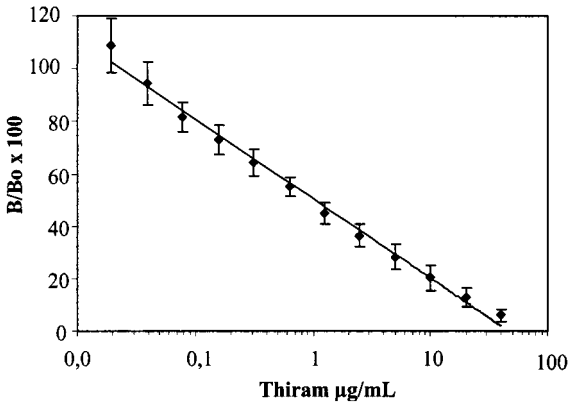



**Figure 3.** ELISA inhibition curve for thiram using anti-BSA−hapten **1** serum (1:100 000) and Ova−hapten **1** (20 ng/well) as a coating conjugate. The points represent the mean and the standard deviation for 23 determinations. The absorbance at 405 nm were converted to percent $B/B_o$ value.

The optimal competitive ELISA was performed with 0.2 $\mu$g/mL of coating antigen and a serum dilution of 1:100 000. Figure 3 shows thiram standard curve inhibition obtained by the competitive ELISA. The $IC_{50}$ value for thiram was estimated to be 0.96 $\mu$g/mL. The linear range of the assay was extended from 0.04 to 2 $\mu$g/mL. Competition between thiram and Ova−hapten **1** conjugate shows that antibodies produced against hapten **1** present a good recognition of the target compound despite the introduction of methylene group between the two sulfur atoms to stabilizes the hapten molecule.

**Assay Development.** The sensitivity of any inhibition ELISA is affected by the strength of interaction between the antibodies and the coating antigen. So heterologous assays are well-known procedures to improve immunoassays sensitivity (Harrison et al., 1991, Lee et al., 1998). As reviewed by Goodrow et al. (1995), suitable heterology can be accomplished at different levels by hapten (use of partial structure or change of key determinants), site, and spacer modification (composition, length, and conjugation chemistry). Therefore, the series of hapten **2**, sharing with the immunizing hapten one of each symmetrical *N*-alkyl dithiocarbamate pattern of thiram, with varying length spacer arm, were used as heterologous haptens to improve the sensitivity of thiram determination performed by competitive ELISA with the serum produced against hapten **1**.

The five conjugates Ova−hapten **2** were tested for recognition by anti-hapten **1** serum in hapten-immobilized immunoassay (Figure 4). All conjugates were recognized by the antiserum, but the responses differed according to the hapten. Ova−haptens **2c** and **2b** ($R = CH_3$, $n = 3$ or 5) were the best recognized, showing a response comparable to those of homologous conjugate (Ova−hapten **1**), the response with Ova−hapten **2a** ($R = CH_3$, $n = 1$) was weaker. With the conjugates Ova−haptens **2d** ($R = CH_3$, $n = 10$) and **2e** ($R = CH_2{-}CH_3$, $n = 1$) the responses were markedly lower than with the three other conjugates. These differences were not correlated with hapten densities on conjugates. The

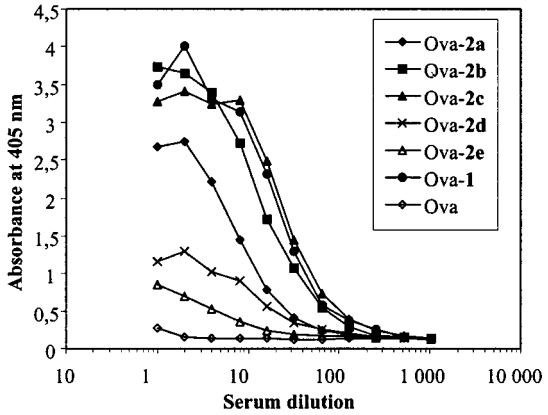



**Figure 4.** Antibody binding of homologous and heterologous hapten conjugates. ELISA was used to evaluate the binding of antibodies raised against BSA−hapten **1** to unconjugated Ova or conjugates Ova **1** and Ova **2a**−**e**. Concentrations of all coating antigens was 1 $\mu$g/mL (100 ng/well expressed as dry weight).

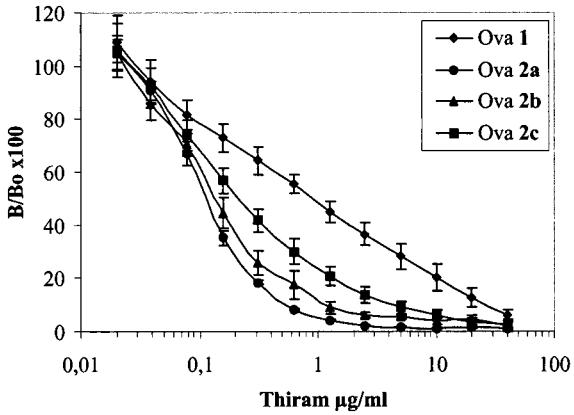



**Figure 5.** Standard curves for thiram obtained under optimized assay conditions using conjugates Ova **1** and **2a**−**c**. Each point represented the mean ± standard deviation from 5 (**2a**) to 15 determinations. The absorbance at 405 nm were converted to percent $B/B_o$ value.

**Table 1: Relationship between the $IC_{50}$ Value for Homologous and Heterologous Haptens in Competitive ELISA Using Ova 1 as a Coating Antigen**

| hapten | $IC_{50}$ for hapten $\mu$g/mL | $IC_{50}$ for hapten $\mu$M |
|---|---|---|
| 1 | 0.043 | 0.132 |
| 2a | 173 | 966 |
| 2b | 49 | 236 |
| 2c | 3 | 12 |
| 2d | 1.3 | 4 |
| 2e | >500 | >2400 |

serum anti-hapten **1** appeared to be sensitive to the *N*-alkyl substitution (hapten **2e**) but also to the length of the spacer arm (haptens **2a**−**d**).

To assess that the conjugated protein was not implicated in the response, the recognition of free haptens was also conducted in competitive ELISA. The results are expressed as $IC_{50}$ of different haptens and compared to those of hapten **1** and thiram (Table 1). As in the hapten-immobilized immunoassay, the anti-hapten **1** serum recognized the four haptens with a methyl substitution (**2a**−**d**), showing an affinity increasing with the length of the spacer arm. Hapten **2e** with an *N*-alkyl substitution had too little affinity and was not able to compete in the tested range. The relative binding of antibodies to these haptens was similar regardless of whether they are conjugated or free, with the exception of hapten **2d** which seems to be the best recognized as it showed the lower $IC_{50}$ value. The low titer of the conjugate Ova−hapten **2d** in hapten-immobilized immunoassay may be related to a change of conformation during conjugation or to a lack of solubility of this compound.

Although all heterologous haptens presented a reduced antibody affinity relative to the reference analyte and the immunizing hapten, only **2a**−**c**, which showed good recognition by the serum as conjugates, were tested as coating antigens to perform competitive ELISA for thiram determination. After selecting optimized conditions, sensitive assays were obtained with the three haptens. Figure 5 shows mean standard curves representing data from 5 to 15 curves obtained using Ova−haptens **1** and **2a**−**c** as coating antigens. These curves were established with thiram concentrations ranging from 0.02 to 40 $\mu$g/mL. As expected, the $IC_{50}$ value of the homologous assay (0.96 $\mu$g/mL) was improved by the heterologous ones (0.12, 0.15, 0.24 $\mu$g/mL with **2a**−**c**, respectively). Additionally, the most sensitive assay resulted from the heterologous hapten which has the lowest affinity (hapten **2a**) relative to the analyte, providing a 8-fold improvement assay sensitivity over the homologous one. However, the use of this hapten over hapten **2c** led to only a 2-fold sensitivity enhancement while high immunoreagent concentrations were required. A heterology approach resulted in steeper slope standard curves with 4−8-fold decreases in $IC_{50}$ value and narrower linear detection zones from 0.02 to 3.125, 0.625, and 1.25 $\mu$g/mL using heterologous hapten **2a**−**c**, respectively; whereas the linear detection zone of homologous assay extended from 0.04 to 40 $\mu$g/mL. The limit of detection (LOD, also called the least-detectable dose) calculated as the concentration to the zero dose absorbance minus three standard deviations was very closed to the analyte concentration giving 10% inhibition of the maximum absorbance (Hennion and Barcelo, 1998). The LOD values determined according to the second method were found around 0.03 $\mu$g/mL for the three heterologous assays. A higher value of 0.045 $\mu$g/mL was calculated from the homologous curve. The LOD for each assay was on the linear portion of the standard curves. So absorbances derived from samples containing such a low concentration would be accurately quantified.

These results confirmed the suitability of the hapten heterology approach to improve the sensitivity of the pesticide immunoassays. Furthermore, the prediction of the sensitivity of heterologous assays could be achieved by preliminary screening of heterologous hapten cross reactivity. A rational approach for enzyme tracer selection, based on cross reactivity study of a variety of monuron−, diuron−, and linuron−hapten have already been reported by Schneider et al. (1994). Haptens, recognized about 100 times less than the "best" hapten (homologous one), were found to be useful as enzyme tracers. In our experiments, the difference between $IC_{50}$ values of the most efficient heterologous hapten and the best-recognized hapten was much greater than 7300 times (966/0.132 $\mu$M; $IC_{50}$ **2a**/$IC_{50}$ **1**). However this variation was reduced to 240 times (966/4 $\mu$M) when $IC_{50}$ values of the heterologous hapten and the reference analyte were compared.

**Specificity.** Specificity of the anti-thiram serum in competitive ELISA was investigated using as a coating antigen the heterologous **2c** conjugate giving the highest

**Table 2: Inhibition of Heterologous Competitive ELISA Using 2c Hapten by Some Dithiocarbamates and Related Compounds[a]**

| Compounds | R | n | M | $IC_{50}$[b] µg/mL | $IC_{50}$[b] µM | % Cross reactivity[c] |
|---|---|---|---|---|---|---|
| Thiram | Me | 2 | | 0.24±0.05 | 1 | 100 |
| Tetramethylthiurammonosulfide | Me | 1 | | 0.58±0.02 | 2.8 | 36 |
| Tetraethylthiuram disulfide | Et | 2 | | 11.5±0.4 | 38.9 | 2.6 |
| Dimethyldithiocarbamic acid sodium salt | Me | | $Na^+$ | 0.65±0.09 | 4.5 | 22 |
| Dimethyldithiocarbamic acid zinc salt | Me | | $Zn^{2+}$ | 1.75±0.28 | 5.7 | 17.5 |
| Diethyldithiocarbamic acid sodium salt | Et | | $Na^+$ | 183±26 | 1070 | <0.1 |
| **3** | | 3 | | >500 | | ND[d] |
| N,N,N',N'-tetramethylthiourea | | | | >500 | | ND |
| Nabam | | | $Na^+$ | >500 | | ND |
| Zineb | | | $Zn^{2+}$ | >500 | | ND |
| Maneb | | | $Mn^{2+}$ | >500 | | ND |
| Ethylenethiourea | | | | 363±18 | 3550 | <0.03 |
| Molinate | | | | 310±35 | 1660 | <0.06 |
| Hapten **1** | | 3 | | $92.10^{-5}$±$4.10^{-5}$ | 0.0028 | >35000 |
| Hapten **2a** | Me | 1 | | 7.1±0.8 | 39.7 | 2.5 |
| **2b** | Me | 3 | | 3.1±0.4 | 15 | 6.7 |
| **2c** | Me | 5 | | 0.12±0.01 | 0.51 | 196 |
| **2d** | Me | 10 | | $7.10^{-3}$±$8.10^{-4}$ | 0.023 | 4348 |
| **2e** | Et | 1 | | 58±7 | 280 | 0.36 |

[a] ELISA test were performed under optimized conditions: 80 ng/well of the coating antigen Ova-**2c** and a 1:20 000 dilution of the anti-hapten **1** serum. [b] The $IC_{50}$ value of different compounds are the average ± SD (standard deviation) of 2−5 determinations with the exception of thiram $IC_{50}$ which was the mean ± SD of 30 determinations. [c] Percentage of cross reactivity calculated according to the formula: %CR = ($IC_{50}$ of thiram/$IC_{50}$ of other compound) × 100. [d] Not detectable.

sensitivity along with a minimal immunoreagent requirement. Under optimum conditions, the specificity was evaluated by obtaining competitive curves with several related structural compounds and pesticides as competitors. $IC_{50}$ value and relative cross reactivity (CR) for each compound are summarized in Table 2.As expected, the immunizing hapten exceeded thiram in its inhibitory potency. However, the replacement of one of the symmetrical pattern of the thiram structure by an alkyl chain of 5 and 10 carbons led surprisingly to a better recognition by the serum (see CR of **2c** and **2d**). This effect should be ascribed to closer structural characteristic of the haptenic compounds with the immunizing hapten, stabilized by the presence of a middle methylene group, than the reference analyte. These results suggested that the thiram molecule, taken as a whole, was not involved in the immunological interaction. Just one of the two symmetrical pattern should play an important role in the analyte antibody interaction. The length of their handle had great influence on the cross reactivity of haptenic compounds of the series **2**. Thus, haptens with a spacer length chain of 1 and 3 carbons showed CRs of 2.5 and 6.7%, respectively. This effect may arise from the highly polar amide carbonyl group and its close proximity to the antibody binding pocket in the case of the shorter handled hapten. The size of these haptenic compounds modulated by the length of their handle should be also an important factor in their recognition by the serum. This hypothesis was supported by the similarity of the sizes of **2d** and **2c** with those of the immunizing hapten and the reference analyte, respectively.

A simple change of the *N*-methyl group by an ethyl one (diethyl dithiocarbamate over dimethyl dithiocarbamate, and tetraethyl dithiocarbamate over tetramethyl dithiocarbamate) markedly reduced the recognition, indicating the importance of this moiety in the immunological interaction. That is not surprising because this structure in the immunizing hapten was the portion most distal from the location of the spacer arm, a position which maximizes the exposure of this determinant to the immune system. However, no CR was detected with the tetramethylthiourea. So the minimal requirement for cross reactivity comprises a larger structure, including probably $CS_2$ moiety. It is especially interesting to note the lack of recognition of **3** by the serum. This reinforced the assumption that this structure, which corresponds, in the immunogen, to the part bound to the carrier protein, did not play a significant role in the antibody selectivity.

The tetramethyl thiuram monosulfide cross-reacted strongly with the antiserum. On the contrary, the

sodium and zinc (ziram) salts of dimethyl dithiocarbamic acid displayed CRs at 22 and 17.5%, respectively. The low CR values of these compounds were likely due to the presence of a charged sulfur atom which may interfere with binding of antibody, but ferbam was not tested.

Although partial inhibition was observed at very high concentrations, no significant CR was found with the ethylenebis(dithiocarbamates) and their main metabolite, the ethylene thiourea. Molinate, the only thiocarbamate tested, displayed an almost negligible CR.

The specificity pattern of the serum differed when using homologous and heterologous haptens. Thus, **2c** and **2d,** which were better recognized than thiram in the competitive assay using hapten **2c** as a coating antigen, showed $IC_{50}$ values similar or slightly higher than those of the thiram in the homologous assay. The relative binding of the other tested compounds were similar in both assay types (data not shown). The sensitivity of the assay depends on the antibody affinity to the analyte in relation to the antibody affinity to the coating antigen. The use of an antigen coating made with a different hapten may lead to better sensitivity and consequently to better distinction between some competitors by the serum. A different subgroup of antibodies able to produce a different assay sensitivity could also be selected through the choice of a different ELISA antigen. Lee et al. (1998) has reported a similar behavior in the analysis of synthetic pyrethroids. For example, the $IC_{50}$ values for isomerized deltamethrin, cypermethrin, and cyhalothrin found at 4, 4, and 5 $\mu$g/L using a first tracer were changed to 120, >1000, and 48 $\mu$g/L, respectively, using another tracer. On the contrary, the specificity of a monoclonal antibody raised against the insecticide chlorpyrifos did not change significantly, using different coating haptens (Manclus et al., 1996).

## CONCLUSION

To develop an immunoassay for thiram detection, six haptens belonging to two families were synthesized, conjugated to proteins, and used for production of polyclonal antibodies.

Haptens of type **2** showing one of two symmetrical *N*-alkyl dithiocarbamate patterns of thiram with variable-length spacer arms linked to one sulfur atom were easily synthesized by alkylation of the sodium salt of *N*,*N*′-dimethyldithiocarbamic acid with various $\omega$-bromoalkanoic acids. Some of these haptens led to the production of antisera with good titer; unfortunately these antisera presented little or no reaction with thiram and were not usable for thiram detection. Nevertheless these haptens were used as coating antigens for the development of the competition ELISA.

On the other hand, hapten of type **1** showing the two symmetrical *N*-alkyl dithiocarbamate patterns of thiram with a spacer arm linked to one of the *N*-methyl terminal group was more difficult to obtain. Different approaches to synthesize a thiram molecule with a spacer arm had led to an unstable compound that was not usable for protein conjugation. The solution was to introduce a methylene group to join the two dithiocarbamic moieties (Gueguen et al., 1998). The obtained compound was very stable and could be coupled to protein. Antisera against this hapten showed a good titer and were usable for thiram detection.

The best results in thiram detection were obtained in competitive ELISA using one of the anti-hapten **1** sera and one of the type **2** haptens as coating antigens (hapten **2c**). This assay allows the detection of free thiram dissolved in buffer with 3% acetone, in a range from 0.02 to 40 $\mu$g/mL, with an $IC_{50}$ of 0.24 $\mu$g/mL and a LOD of 0.03 $\mu$g/mL. This limit of detection was not very low but would be sufficient for thiram detection in vegetables in the range of the MRL (5 ppm as $CS_2$ equivalent on lettuce in France). Compared to other detection methods, the immunodetection of thiram is not as sensible but offers the advantage of a specific detection because the competitive ELISA present little or no cross reaction with other dithiocarbamates. This is not true for Keppel's method or headspace gas chromatography which measure the $CS_2$ released by DTCs without distinction between them and cannot discriminate the thiram. Another advantage would be the possibility to analyze simultaneously several samples in a reduced time, without sophisticated equipment.

This work will be continued by the development of an immunoassay for thiram detection on vegetables. This will require the development of an extraction method allowing the recovery of thiram from vegetable without interference of the matrix, while keeping the idea of an assay as easy as possible.

## ABBREVIATIONS USED

BSA, bovine serum albumin; BSA−hapten, BSA conjugated to hapten; $^{13}C$ NMR, carbon-13 nuclear magnetic resonance; CR, cross reactivity; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DTC, dithiocarbamate; EBDC, ethylenebis(dithiocarbamate); ELISA, enzyme-linked immunosorbent assay; ETU, ethylene thiourea; GC, gas chromatography; $^1H$ NMR, proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; $IC_{50}$, value of the concentration inhibiting 50% of the maximum ELISA absorbance value; LOD, limit of detection; MRL, maximum residue limits; NHS, *N*-hydroxysuccinimide; OD, optical density; OPDA, orthophthaldialdehyde; Ova, ovalbumin; Ova−hapten, Ova conjugated to hapten; PBS, phosphate buffered saline; PBS-T, PBS plus 0.05% Tween 20.

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