Development of an Immunoassay (ELISA) for the Quantification of Thiram in Lettuce

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Two competitive immunoassays, a laboratory assay based on microwell plates and a field test based on the use of polystyrene tubes, have been developed for the quantification of thiram in lettuces. Concerning the laboratory assay, the calibration curve for thiram had a linear range of 11 to 90 ng/mL and a detection limit of 5 ng/mL. Precision of the assay presented coefficient of variation values <9% and the recovery of thiram from lettuce averaged 89% across the range of the immunoassay method using 30 min extraction with water/acetone (50:50, v/v). The tube-based method was developed in order that an extract of lettuce, containing thiram at the MRL (8 ppm), would be found on the linear part of the standard curve. The calibration curve for thiram has a linear range of 100 to 800 ng/mL (1.39 to 11.1 ppm in lettuce) and a detection limit of 40 ng/mL.

Keywords: *Thiram; dithiocarbamates; ELISA; lettuce*

INTRODUCTION

Thiram (tetramethylthiuram disulfide) [bis(dimethylthiocarbamyl)disulfide] is a nonsystemic dithiocarbamate fungicide. Thiram is widely used as protectant on foliage and fruits (apples, pears, strawberries, grapes, lettuce leafy, and fruiting vegetables) to control a variety of fungal diseases (rust, scab, *Anthracnosis*, *Botrytis*, *Fusarium*, and *Monilia*). It is also commonly applied for seed treatment on maize, cotton, cereals, vegetables, and ornamentals.

The maximum residue level (MRL) refer to total dithiocarbamates determined as carbon disulfide (CS_2) evolved during acid digestion and are expressed as mg of CS_2/kg. According to the European Community MRLs for dithiocarbamate residues in France range from 0.05 to 5 ppm upon the fruits or vegetables (*1*). These residues included ethylene(bisdithiocarbamates), methylene(bisdithiocarbamates), propineb, and thiram.

The most commonly used procedures, for determining thiram residues, are based on the CS_2 liberated by an acid hydrolysis of the foodstuffs. Then CS_2 is quantified by various methods such as spectrophotometric (*2*) or gas chromatographic (GC) headspace (*3*) procedures. These methods, based on degradation of dithiocarbamates, are not able to discriminate between different dithiocarbamates. Another disadvantage is that reproducibility is greatly influenced by the sample preparation (*4*), and precision and reproducibility are affected by time and temperature of heating.

Several quantifications of dithiocarbamates without degradation have also been reported. Determination of residue of thiram in foodstuffs can be performed with the classical photometric method after reaction of thiram with a transition metal such as copper. In the official method (*5*, *6*), thiram is extracted from samples with $CHCl_3$ and treated with copper. The complex, which was formed, is then measured at 440 nm. Modifications of this method have been described. Verma et al. (*7*) used tetraacetonitrilecopper (I) perchlorate ($CuClO_4$, $4CH_3CN$) for the spectrophotometric determination of thiram in grains with a limit of detection (LOD) of 0.4 μg/mL. Malik et al. (*8*) converted thiram into copper dimethyldithiocarbamate complex, which was dissolved in water by cetyltrimethylammonium bromide, and the complex formed was then measured at 430 nm. This method was applied to detect thiram in grain and in vegetables with a LOD of 0.44 μg/mL. Several high-pressure liquid chromatography (HPLC) methods have been also reported. Gustafsson and Thompson (*9*) developed a procedure in which thiram was extracted with chloroform, cleaned-up on a silica gel column, and analyzed with the HPLC method with UV detection at 272 nm, the LOD in water solution was 0.01 ppm. Brandsteterova et al. (*10*) analyzed dithiocarbamates by normal and reverse-phase chromatography and applied this method to detect thiram in maize and strawberries, the LODs were 0.089 and 0.08 mg/kg, respectively. Irth et al. (*11*) developed a selective reversed phase liquid chromatography (LC) method that allows detection of thiram among the other dithiocarbamates. The method is based on an LC separation with a postcolumn derivatization system using a solid-state reactor filled with metallic copper; the LOD was 3 ng for thiram. Bauman et al. (*12*) found that this method had some drawbacks such as instability of the copper column and zero recovery of thiram in spiked extracts at a concentration lower than 0.2 ppm. They optimized a new LC technique that presented a LOD for thiram in apple extracts at 0.01 ppm.

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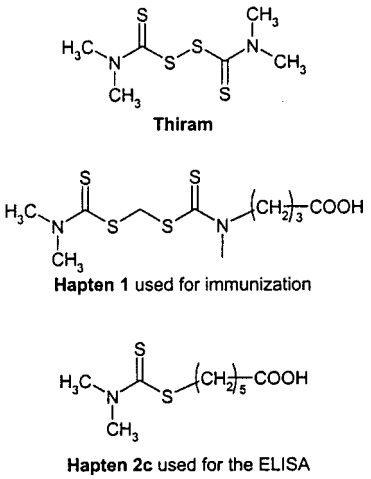

Figure 1. Structures of the thiram and the haptens used in the assay.

Methods, which were described above, involving HPLC or GC are time-consuming, tedious, and require specialized equipment and experimental skill to obtain good results. The wide use of thiram requires convenient, fast, and economic methods for its determination.

Immunoassays such as the enzyme linked immunosorbent assay (ELISA) have proved to fulfill to these requirements. Over the past few years, many immunoassays have been developed for detecting numerous pesticides in foodstuffs (*13*−*23*).

Concerning thiram analysis, Gueguen et al. (*24*, *25*) have produced polyclonal antibodies that were used to develop an indirect competitive ELISA format. The most sensitive dose response curve was performed with a serum from rabbit immunized with hapten **1** and using hapten **2c** as coating antigen (Figure 1). The competitive ELISA, reported by Gueguen et al. (*25*), presented little or no cross-reaction with other dithiocarbamates. In this study, the anti-thiram serum produced by Gueguen et al. (*25*) was used to develop and validate a selective analytical method to allow thiram determination in lettuce with an easy-to-use sample treatment.

MATERIAL AND METHODS

Chemicals. Buffer salts were of reagent grade and obtained from Aldrich Chemical Co. Alkaline phosphatase conjugated affinipure goat anti-rabbit IgG (H + L) was obtained from Jackson Immunoresearch, the *p*-nitrophenyl phosphate substrate tablets and the Fish Gelatin (G-7765) from Sigma Chemical Co.

The anti-thiram serum was produced by Gueguen et al. (*25*) and was used without purification.

ELISA. *Laboratory (Microwell) Assay.* Microtitration plates (FALCON 3915, Pro-Bind Assay Plate) were coated with 100 μL of the Ova-hapten **2c** (0.6 μg/mL) conjugate dissolved in carbonate buffer pH 9.6 (Na_2CO_3, 15 mM; $NaHCO_3$, 35 mM) which was equivalent to 0.06 μg of Ova-hapten **2c** coating conjugate per well. The plates were incubated overnight at 4 °C. Unbound antigen was removed by washing three times with phosphate buffered saline (PBS: NaCl, 0.137 M; KCl, 0.0027 M; KH_2PO_4, 0.0015 M; Na_2HPO_4, 0.008 M, pH 7.4) supplemented with 0.05% Tween 20 (PBS-T). Unspecified binding sites were blocked by adding 200 μL of 0.5% (w/v) fish gelatin solution in PBS (PBS-G) and incubated for 2 h at 25 °C. After washing, 50 μL/well of thiram and 50 μL/well of anti-thiram serum were added to the wells and incubated for 1.5 h under stirring. The competitors, dissolved in acetone, were diluted in water, and the serum was diluted (1/30 000) in 2× PBS-G. The final concentration of acetone in the well was 3%. After incubation at 25 °C, the plates were washed as previously described. Goat anti-rabbit IgG-alkaline phosphatase conjugate diluted in PBS-G (1/5 000) was added and incubated at 25 °C for 1.5 h. After washing the plates, 100 μL of the *p*-nitrophenyl phosphate substrate (1 mg/mL) in diethanolamine buffer (diethanolamine 1 M; $MgCl_2$, 5×10^{-7} M; pH = 9.8) was added, and color was allowed to develop for 1 h. Absorbance of each well was measured at 405 nm with a microtiter plate scanner (Multiscan MCC 340 MK, Flow Laboratories).

Curves were normalized by expressing experimental absorbance values y as $(y/A_{max}) \times 100$. Standard curve data were obtained by plotting $(y/A_{max}) \times 100$ against logarithm of analyte concentration and fitted to a four-parameter logistic equation of Rodbard by Table Curve 2D software from Jandel Scientific (Erkrath, Germany). The general form of the four-parameter equation is

$$Y = \frac{(A - D)}{(1 - (X/C)^B)} + D$$

where X = concentration of thiram (ng/mL); Y = absorbance at 405 nm; A = absorbance at zero concentration; B = the curve slope at the inflection point; C = the center of the transition; and D = absorbance at infinite concentration.

Tube-Based Assay. All volumes were measured with graduated transfer pipets of 1 mL. Microtitration tubes (MAXISORP 7 × 11 mm, Nunc) were coated with 1 mL of the Ova-hapten **2c** (0.200 μg/mL) conjugate dissolved in carbonate buffer and were incubated overnight at 4 °C. After the tubes were washed three times with PBS-T, the unbound sites were blocked by adding 2 mL of PBS-G and incubated for 1 h at 25 °C. After washing, standard solution or sample (500 μL) and anti-thiram serum (500 μL) were added per tube, and the sample was vigorously shaken and incubated for 1 h. The competitors, dissolved in acetone, were diluted in water and the serum was diluted (1/5 000) in 2× PBS-G. After incubation, the tubes were washed as previously described. Goat anti-rabbit IgG-alkaline phosphatase conjugate diluted in 1× PBS-G (1 mL, 1/5 000) was added and incubated at 25 °C for 1 h. After washing the tubes, 1 mL of the *p*-nitrophenyl phosphate substrate (1 mg/mL) in diethanolamine buffer was added and color was allowed to develop for 1 h. The solution was transferred into a spectrophotometer cuvette and the absorbance was measured at 405 nm with a spectrophotometer (UV 160A Shimadzu).

The measured absorption (A) data were normalized as B/Bo according to the following formula

$$\% B/Bo = (A - A\infty)/(A_o - A\infty)$$

where A = measured absorbance value of the sample or standard; A_o = absorbance value in the absence of analyte; and $A\infty$ = measured absorption of a thiram saturated solution.

Lettuce Extraction. Cabbage lettuces were from biological agriculture to ensure that the vegetable was pesticide free. This was confirmed by the method of Keppel: thiram was hydrolyzed to carbon disulfide which is subsequently determined spectrophotometrically as cupric complexes (*26*). MRL expressed in terms of CS_2 must be multiplied by a molecular weight factor of 1.58 to obtain thiram concentration in mg/kg. In this work, all concentrations were expressed in thiram concentration and not in terms of CS_2.

Spiking was performed as follows: 30 g of nonchopped fresh lettuce leaves were spiked by adding aliquots of standard solutions of thirame in acetone (0.7−27 ppm). Lettuces were exposed to the thiram for 30 min prior to extraction. Lettuce leaves were then placed into a 200-mL flask followed by the addition of 100 mL (microwells plates: laboratory assay) or 50 mL (tube-based assay) of acetone/water (50/50 v/v). The flask was incubated for 30 min with four vigorous shakes (1 min each), and then the sample was filtered through a Whatman #1 filter paper. The filtrate was then diluted in water to obtain an acetone concentration of 6% in the sample.

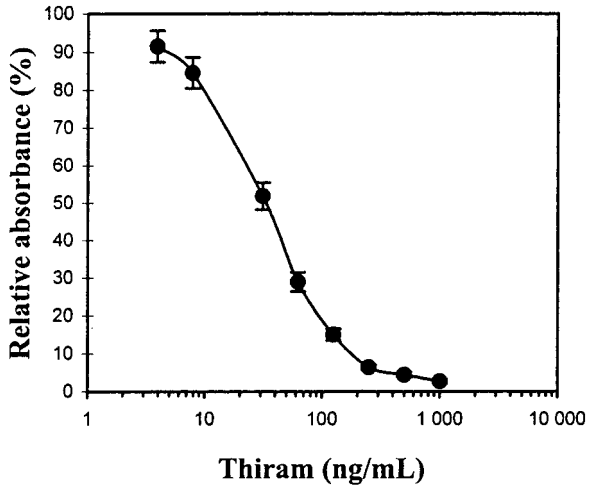

Figure 2. Dose response curve for thiram. The assay was performed in microwell plates. Each point represents the mean of 76 determinations. Vertical bars indicate ± SD about the mean.

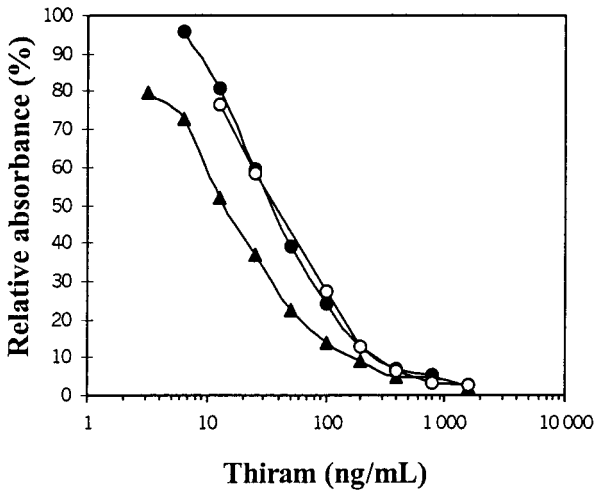

Figure 3. Dose response curve for thiram diluted in different solutions: -●- standard prepared in water/acetone (94:6, v/v); -○- standard prepared in water/water−acetone lettuce extract (88:12, v/v); -▲- standard prepared in water/acetone lettuce extract (94:6, v/v).

This sample was then analyzed by ELISA without other cleanup procedure as described above.

Statistical Validation. Statistical data were validated in compliance with the criteria from Caporal-Gautier et al. (*27*).

Linearity. To assess the linearity of the method, measurements were carried out for five different concentrations of thiram; each measurement was made in triplicate. Statistical tests were performed on the whole set of data at a significance level of 1%.

Reproducibility Study. To determine the intra-assay and inter-assay variation of the enzyme immunoassay method, lettuces were spiked at three different levels (0.83; 1.67; 3.33 ppm), and then extracted and analyzed six times on the same day, and this was repeated at three different days.

Recovery Study. Lettuces were spiked with various amount of thiram: 0.83, 1.67, 2.5, 3.33, 5, 6.67 ppm, each measurement was made in triplicate. This study was used to ascertain the accuracy of the immunoassay and the efficiency of the thiram extraction technique.

RESULTS AND DISCUSSION

Plate-Based Method. *Dose Response Curve and Sensitivity*. The sensitivity of the assay depends on both the coating antigen amount and antibody concentration. The best combination of reagents must yield to a competitive curve with adequate signal and with the lowest IC_{50} (value concentration of thiram required for 50% inhibition of the absorbance of the positive control). In a previous work (*25*), we had developed a competitive ELISA for thiram which presented an IC_{50} of 240 ng/mL and an LOD of 30 ng/mL. The conditions of this competitive ELISA were modified to improve its sensitivity. The optimal ELISA for thiram detection was performed with 60 ng/well of coating antigen Ovahapten **2c** and a serum dilution of 1/30 000. Figure 2 represents the mean standard curve for the thiram calibrators collected over 76 runs during a six-month period. The IC_{50} value was 34 ng/mL. The linear working range of the assay system was established between concentrations producing 20% and 80% inhibition (11−90 ng/mL). The LOD, defined as the lowest detectable analyte concentration based on 90% *B*/*B*o concentration (*28*), was 5 ng/mL.

Solvent Tolerance. Changes (A_{max}, IC_{50}) were recorded to determine the effect of the solvent on the calibration curve. Acetone, acetonitrile, and methanol were added to the assay buffer at four levels: 3, 5, 10, and 20% final concentrations. Acetonitrile, at 10% concentration, inhibited antibody binding by 70% and thus was unacceptable for assay purposes. Methanol concentrations (3−20%) did not reduce ELISA control values, but the standard curve was shifted to the right what increased IC_{50} value. Concerning acetone, the assay supported concentrations of solvent below 10% without modification of the values of IC_{50} and A_{max}. On the basis of these results, a 3% final concentration of acetone was routinely used for subsequent work.

Matrix Effect. Matrix effect was studied by comparing the standard curves in competitive ELISA for a set of standards concentrations of thiram prepared by dilution in water/acetone (94:6, v/v) or in water/acetone lettuce extract (94:6, v/v). Standard curves prepared in an acetone lettuce extract and diluted appropriately to obtain a final acetone concentration of 3% in the wells exhibited a matrix effect (Figure 3). The A_{max} were generally decreased, and the results were not reproducible. The most frequently phenomenon observed was a shift to the left of the curve obtained with thiram diluted in an acetone lettuce extract (Figure 3). Interfering substances from lettuce were co-extracted and altered the specificity of the assay. To eliminate this matrix effect, instead of diluting the samples, lettuce was extracted with water/acetone (50:50, v/v). When thiram was diluted in this new extract [water/water−acetone lettuce extract (88:12, v/v)], we observed that the superimposition of the standard curves suggested the absence of matrix effect that allowed the analysis of the extracts with direct reference to a calibration curve prepared in acetone.

Precision. The precision of the assays was studied by determining the intra-assay repeatability and inter-assay reproducibility. Reproducibility and repeatability were determined by analysis of variance (ANOVA) according to Caporal-Gaultier et al. (*27*). Lettuces were spiked with thiram at three levels of concentration: 0.83, 1.66, and 3.33 ppm and extracted as described under the materials section. The precision of the combined extraction and immunoassay analysis of thiram spiked lettuces, which were assayed six times over 3 days, are represented in Table 1. Precision should usually be <20%, and repeatability should not vary by more than 15%. In the present work, coefficients of

Table 1. Precision of Thiram Measurement

	sample[a]		
	1	2	3
replicates	6	6	6
days	3	3	3
mean (ppm)	0.70	1.38	3.09
repeatability CV (%)	7.5	5.6	4
reproducibility CV (%)	8.7	5.6	8.0

[a] Thiram spiked lettuce (sample 1 spiked at 0.83 ppm, sample 2 at 1.67 ppm, and sample 3 at 3.33 ppm) assayed in six singlicates each, over 3 days.

Table 2. Accuracy of Thiram ELISA

	thiram recovered				
amount of added thiram (ppm)	mean (ppm)	*N*	SD	recovery (%)	CV (%)
0.83	0.72	5	0.07	86.7	9.7
1.67	1.43	5	0.05	85.6	3.5
2.5	2.22	5	0.14	88.8	6.3
3.33	3.14	5	0.16	94.3	5.1
5	4.58	5	0.19	91.6	4.1
6.67	6.09	5	0.32	91.3	5.2

variation (CVs) of repeatability and reproducibility were lower than 8 and 9% respectively. The total CV (n = 18) was lower than 9% at all tested concentrations.

Spike and Recovery of Thiram in Lettuce. To determine the efficiency of the acetone extraction procedure and the accuracy of the assay, lettuces were spiked at levels between 0.83 to 6.67 ppm and then analyzed; data are shown in Table 2. The equation from spiked and recovery was thiram (recovered, ppm) = 0.93 × thiram (spiked, ppm) − 0.0716 (r = 0.99). The recovery ranged from 85 to 94%. The recoveries were statistically analyzed: no significant differences at the 1% level were observed, and the slope (0.93) and the intercept (−0.00716) were not significantly different from 1 and 0, respectively. Added amounts of thiram were recovered accurately across the range of the assay. The average recovery assay was 89% with a confidence interval of 2%. These data demonstrate that good recoveries were observed for the assay.

Tube-Based Method. ***Dose Response Curve in Tubes.*** Concurrently, we developed an ELISA in tubes with the aim that it will be easily applicable by nonqualified technicians. The conditions were defined so that an extract of lettuce, containing thiram at the MRL (8 ppm of thiram corresponding to 5 ppm of CS_2) would be found on the linear part of the standard curve. The ELISA in tubes was performed with 200 ng/tube of coating antigen and an antibody dilution of 1/5 000. Standard curve (Figure 4) was obtained by plotting B/B_0 × 100 versus the logarithm of thiram concentration; the IC_{50} value was 108 ng/mL and the linear range of the assay system was extended from 100 to 800 ng/mL. The LOD was 40 ng/mL. In this assay according to the extraction procedure and the dilution of the extract, lettuce spiked with thiram at the MRL should correspond in detecting thiram at 0.57 μg/mL. As expected, this value was on the linear part of the standard curve, but this theoretical result shall be corrected by extraction recovery and possible matrix effect. The assay described above can be used to detect thiram in lettuce.

Tube Immunoassay Validation. Statistical residual analysis, according to Caporal-Gauthier et al. (*27*), indicated that the slope was significant and validated the adjustment to a linear model at the 1% level. To evaluate the matrix effect, standards were prepared in water/acetone (94:6, v/v) and in water/water−acetone lettuce extract (88:12, v/v) and used to obtain competitive curves for thiram (the final acetone concentration in the sample was 6%). Matrix effect was assessed by estimating relative recovery that ranged from 95 to 85% for thiram concentration of 400 to 100 ng/mL, respectively. Matrix effect was more important for the low concentrations of thiram.

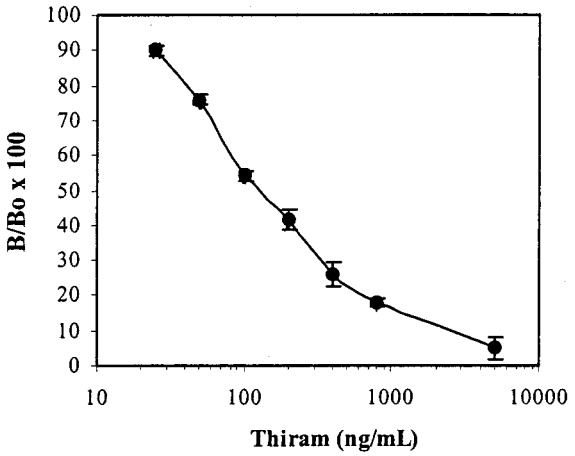

Figure 4. Dose response curve for thiram. The assay was performed in tubes. Each point represents the mean of eight replicates. Vertical bars indicate ± SD about the mean.

Table 3. Relation between the Factor K and the Concentration of Thiram in Lettuce[a]

K	16	30	40	50	58	66	73	79	85	90	96
thiram (ppm)	5	6	7	8	9	10	11	12	13	14	15

[a] K is calculated according to the formulas: $K = 100(A_2 - A_x)/(A_2 - A_1)$, with A_x absorbance of T_x A_1 and A_2 absorbance of T_1 and T_2, respectively.

Study of the combined extraction/ELISA at the following levels 4, 8, and 16 ppm, repeated six times, gave recoveries of 64, 84, and 88%, respectively. Statistical analysis indicated that the recovery variances calculated for each concentration were lower than those calculated for the total assay. It was so inaccurate to calculate a recovery average.

The precision of the method (extraction/ELISA) was evaluated with six samples of lettuce spiked with thiram at 8 ppm and repeated three times. CVs of repeatability and reproducibility were 10 and 23%, respectively.

These results allowed us to calculate a corrective factor to determine concentrations of thiram of unknown lettuce samples. This method is useful for a semiquantitative assay, with two classes results (<MRL or >MRL).

Tube Screening Assay. The knowledge of recovery allowed the development of a screening protocol based on a visual comparison of color intensity. The unknown sample (T_x) was compared to two standard tubes containing thiram concentrations corresponding to 1/2 MRL (T_1) and 2 × MRL (T_2), values that have been corrected by a precision factor: 180 and 1000 ng/mL, respectively. When intensity of unknown sample was included between the two standards, the concentration of the extract could be evaluated by measuring absorbance at 405 nm and correlated to a concentration in mg of thiram/kg of lettuce (ppm) according to Table 3.

Comparison and Validation of the Two Procedures. Samples were analyzed in a blind test to validate the data generated by the two ELISA formats. A series of 27 lettuces were spiked with thiram, extracted, and

Table 4. Results Generated by the "Blind Test"

	tube assay						plate assay	
spiked thiram (ppm)	A_1	A_2	A_x	K	calc thiram (ppm)	recovery (%)	calc thiram (ppm)	recovery (%)
1.7	0.65	1.20	1.93		<4		1.3	85
1.7	0.65	1.20	1.88		<4		1.2	80
1.7	0.65	1.20	1.93		<4		1.3	85
3.3	0.65	1.20	1.54		<4		2.8	96
3.3	0.65	1.20	1.72		<4		2.8	96
3.3	0.65	1.20	1.76		<4		2.7	92
6	0.50	0.92	0.78	33	6.3	105	5.8	109
6	0.50	0.92	0.85	18	5	83	6.0	112
6	0.50	0.92	0.80	30	6	100	5.5	103
6	0.61	1.22	1.07	24	5.5	92	5.4	101
6	0.61	1.22	0.96	42	7.2	120	5.7	107
6	0.61	1.22	0.96	43	7.3	122	5.8	109
8	0.50	0.92	0.70	53	8.4	105	7.6	107
8	0.50	0.92	0.71	49	8	100	7.0	98
8	0.50	0.92	0.71	50	8	100	7.3	102
8	0.61	1.22	0.95	44	7.4	93	7.9	111
8	0.61	1.22	0.98	39	7	88	7.3	102
8	0.61	1.22	0.98	39	7	88	7.5	106
12	0.61	1.22	0.72	81	12.3	103	11.7	109
12	0.61	1.22	0.71	83	12.5	104	11.3	106
12	0.61	1.22	0.65	93	14.5	121	11.2	104
20	0.61	1.22	0.43		>16		19.1	107
20	0.61	1.22	0.44		>16		19.4	109
20	0.61	1.22	0.44		>16		19.3	108
27	0.61	1.22	0.38		>16		22.3	93
27	0.61	1.22	0.44		>16		23.0	96
27	0.61	1.22	0.40		>16		24.0	100
mean						101		101
confidence interval						6		3.26

[a] Recovery takes account of the matrix effect and percent of recovery. [b] Recovery takes account of average recovery of 89%.

then analyzed in ELISA using the tubes and plates format assays. Results are represented in Table 4.

Statistical analysis of these results indicated that the tube-based procedure was accurate from 6 to 12 ppm, values corresponding to 0.75 MRL and 1.5 MRL, since a mean recovery of 101% was found at a confidence interval of 6%. This result was attempted since standards were corrected with the precision factor calculated with the initial procedure. CV of repeatability was 10%, the same as the validation procedure. The limit of repeatability was evaluated at 3 ppm, indicating that this method is particularly interesting for a semiquantitative detection in lettuce.

Statistical analysis of the plate assay indicated that the procedure was accurate, since a mean recovery of 101% was found at a confidence interval of 3%. CV of repeatability was 2%, and the limit of repeatability was calculated to be 0.5 ppm. This assay could be used for a quantitative detection of thiram in lettuce.

CONCLUSION

This paper describes the development and evaluation of an ELISA for the quantification of thiram residues in lettuce. Two enzyme immunoassays, a laboratory assay using microwells plates and a tube-based assay using polystyrene tubes, have been developed. These assays are sufficiently sensitive to detect thiram in lettuce at the MRL level. Extraction of thiram from lettuce was performed with a simple sample treatment: the lettuce samples need only a simple extraction by shaking in water/acetone (50:50, v/v) followed by a dilution of the extract for the immunoassays. The ELISA has a IC_{50} of 34 ng/mL and a LOD of 5 ng/mL for the laboratory test and a IC_{50} of 108 ng/mL and a LOD of 40 ng/mL for the tube-based method. The data proved that good recoveries were obtained for both formats.

According to the validation procedure, the proposed tube-based method is interesting for a preliminary semiquantitative screening of lettuce samples. The laboratory test exhibits good precision and accuracy in the analysis of lettuce samples with an average recovery of 89%. The competitive ELISA reported in this study presented little or no cross-reaction with other dithiocarbamates (*25*). This specificity was not observed using the methods based on the measurement of CS_2 that cannot differentiate dithiocarbamates. In addition both ELISA methods allow detection of several samples in the same experiment. The time-consuming step is not the ELISA itself but the extraction procedure. In our laboratory, the average analysis time for 15−20 samples is 6.5 h. It is less time-consuming than the official method of Keppel. So the assays presented in this study may be very useful tools for regular monitoring of lettuce for thiram contamination.

This ELISA is the first reported for thiram detection in foodstuffs. This method would be validated by correlation with an official method using fields lettuces, and this could be done in interlaboratory assay. The actual results presented in this paper allow this ELISA to be considered as a promising analytical alternative for the quantitative measurements of thiram.

ABBREVIATIONS USED

A_{max}, absorbance in the absence of competiting analyte; ANOVA, analysis of variance; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high performance liquid chromatography; IC_{50}, concentration of analyte giving 50% inhibition of the maximum absorbance; LC,

liquid chromatography; LOD, limit of detection; MRL, maximum residue level; Ova-hapten **2c,** ovalbumine conjugated to hapten **2c**; PBS, phosphate buffered saline; PBS-G, PBS containing 0.5% fish gelatin and 0.05% Tween 20; PBS-T; PBS containing 0.05% Tween 20; SD, standard deviation; UV, ultraviolet.

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