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## Quantification of 2,4,5-trichlorophenoxyacetic acid by fluorescence enzyme-linked immunosorbent assay with secondary antibody

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### Abstract

This paper describes a fluorescence enzyme-linked immunosorbent assay (ELISA) for the quantification of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) with an anti-rabbit secondary antibody conjugated to peroxidase (goat anti-rabbit IgG-HRP) for sandwich antibody assay. Human gamma globulin (HGG) conjugated to 2,4,5-T was readily coated on a polystyrene microplate. Data acquisition on microtiter wells is performed by a spectrofluorimeter through a fibre optic. The standard curve was produced for 0.01–5000 ng ml<sup>-1</sup> 2,4,5-T. The minimum detectable concentration was 0.023 ng ml<sup>-1</sup> and the relative standard deviation was 6.9% for a 5 ng ml<sup>-1</sup> sample ( $n=10$ ). The ELISA procedure was selective with respect to structurally similar compounds usually found in pesticide formulations. This method was applied to 2,4,5-T previously added to apple juice. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* 2,4,5-Trichlorophenoxyacetic acid; ELISA; Fluorescence

### 1. Introduction

Analysis for pesticide residues is generally carried out for two distinct purposes, one of which is the screening analysis which is focused to detect components in a sample. In this case dynamic techniques, such as liquid and gas chromatography (LC and GC), are used. The other type of analysis is related to the detection and quantification at ng ml<sup>-1</sup> levels of a specific pesticide in a sample. In this case, an immunoassay is adequate because it is an analytical

technique based on the specific combination of antigens and antibodies that have been used for specifically detecting very small quantities of pesticides.

High levels of specificity are achieved with such immunoassays due to the specific, high-affinity, reversible binding of antigens to antibodies. In addition, a large number of samples can be detected simultaneously within a relatively short period of time (hours). Assay sensitivities have also been greatly enhanced by methods for attaching easily detected enzyme labels to antigens or antibodies [1] such as for example in enzyme-linked immunosorbent assay (ELISA) procedures [2]. In this aspect, fluorimetry based on the use of fluorogenic substrates in ELISA

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should in principle be superior to spectrophotometric detection based on the use of chromogenic substrates [3,4].

The quality of an enzyme immunoassay (EIA) depends very much on the purity of the antigen or hapten used for immunization, calibration and conjugation, the specificity of the antibody and the choice of a suitable enzyme label. The activity of enzymes is also affected by substrate concentration, pH, temperature and ionic strength. The enzyme labels most commonly used for heterogeneous EIAs are peroxidase, alkaline phosphatase and  $\beta$ -galactosidase.

A heterogeneous EIA for antigen has been demonstrated using an enzyme-labelled second antibody. In this method free antigen prevents antigen-specific antibody from binding to solid-phase antigen. Thus the amount of enzyme-labelled second antibody bound to the solid phase is inversely proportional to the amount of free antigen in the sample [5]. This method has the advantage that one label can be used for measuring any antigen for which a suitable first antiserum is available.

The herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) falls in the class of aryloxyalkylcarboxylic acids, which are intended for weed control of grass plants and are the compounds of large-scale production. 2,4,5-T is used after emergency alone or with 2,4-dichlorophenoxyacetic acid (2,4-D) for the control of shrubs and trees. It is applied on foliage, dormant shoot or basal bark spray. It is also used for girdling, injection or cut-stump treatment. It is absorbed through roots, foliage and bark [6]. The two main metabolites produced by light and water action are 2,4,5-trichlorophenol and hydroxysubstitutes of the ring chlorines [7]. Significant methods used for determination of 2,4,5-T include capillary electrophoresis [8], micellar electrokinetic chromatography with laser-induced fluorescence detection [9], polarization fluoroimmunoassay [10], photochemical-fluorimetric detection [11] and flow-injection analysis using photochemically induced fluorescence detection [12].

We propose a fluorescence ELISA for 2,4,5-T determination with data acquisition on microtiter wells by a spectrofluorometer through a fibre optic. This method gives the best detection limit and specificity.

## 2. Experimental

### 2.1. Reagents

2,4,5-T, MCPA (4-chloro-*o*-tolylxyacetic acid), and 2,4-DB [4-(2,4-Dichlorophenoxy)butyric acid] were provided by Riedel-de-Haen (Seelze, Germany). Dichlorprop [( $\pm$ )-2-(2,4-dichlorophenoxy)propanoic acid] was provided by Dr. Ehrenstoffer Laboratories (Augsburg, Germany). 2,4-D (2,4-dichlorophenoxyacetic acid), DCC (*N,N'*-dicyclohexylcarbodiimide), NHS (*N*-hydroxysuccinimide), HGG (human  $\gamma$ -globulin) and *p*-hydroxyphenylacetic acid were from Sigma. All other chemicals used were from Merck R.A. Goat anti-rabbit-IgG HRP conjugates were provided by Amersham International (Amdex conjugates). The protein concentration is approximately 1 mg peroxidase ml<sup>-1</sup>.

Antisera of immunized rabbits were obtained from ÕImmunotekæ Research and Production Company (Russia). The stages of preparing the immunogens and obtaining antisera are described in detail elsewhere [13]. Six mg of lyophilized antibody diluted in 1 ml of dilution buffer is used as stock solution of antibody ( $D=1$ ).

Stock standards of 2,4,5-T (5000 and 1000  $\mu\text{g ml}^{-1}$ ), MCPA, 2,4-DB, 2,4-D and dichlorprop (all 10000  $\mu\text{g ml}^{-1}$ ) were prepared in methanol and stored in the dark at 4°C. Buffer solutions were prepared from sodium phosphate (0.1 M) pH 7.5 supplemented with 0.1% Tween 20 (washing solution) and with 0.05% Tween 20 (dilution solution), carbonate (50 mM) pH 9.6 (coating buffer) and Tris-HCl (0.1 M) pH 8.5 (substrate solution).

### 2.2. Instrumentation

Fluorescent readings were accomplished in a Perkin-Elmer plate reader accessory, supplied with polystyrene well plates. A bifurcated high-grade fused silica fibre optic (Oriel, Stratford, CT, Ref. 77565) with a high transmission range 240–2200 nm was used to transfer the excitation and emission energies between the well plate and the spectrometer. An IBM-PC was used for on-line data acquisition at an integration time of 0.9 s. Data were collected and processed by fluorescence data manager (FLDM) software (Perkin-Elmer).

### 2.3. Coating of solid surfaces with HGG–2,4,5-T conjugate

2,4,5-T was conjugated to HGG according to a procedure described by Fleeker [14]. Hundred  $\mu\text{mol}$  of DCC (0.0206 g), 100  $\mu\text{mol}$  2,4,5-T (0.0256 g) and 200  $\mu\text{mol}$  of NHS (0.023 g) were dissolved in 1 ml of dimethyl formamide and agitated for 1 h at room temperature. The solution was filtered through 0.2  $\mu\text{m}$  nylon filters to remove the precipitate of dicyclohexylurea. The intermediate 2,4,5-T dihydroxy-succinimide ester (25  $\mu\text{mol}$ ) was added to 0.25  $\mu\text{mol}$  (0.0375 g) of HGG in 2 ml of 0.13 M  $\text{NaHCO}_3$ . The solution was left to stand overnight at 12°C. The resulting solution was dialysed against eight changes of water at 4°C. To estimate the amount of 2,4,5-T bound to protein, the number of free amino groups in the conjugate was determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) titration [15]. The number of amino groups substituted by the hapten in each carrier molecule (HGG) was 5.

For coating titer, Cantarero et al. [16] recommended adding about 500 ng of protein per 6.5  $\text{cm}^2$  of polystyrene, which appears to give a monolayer of protein. The most favourable coating tested was 0.5  $\mu\text{g ml}^{-1}$ .

### 2.4. ELISA procedure

The immunoassay used was a double antibody sandwich (DAS) ELISA which is described in detail elsewhere [2]. Polystyrene microtiter plates were coated by adding, to each well, 200  $\mu\text{l}$  of HGG–2,4,5-T dissolved in 50 mM carbonate buffer at pH 9.6 (0.5  $\mu\text{g ml}^{-1}$  antigen coating) and incubated for 24 h at 4°C. The plates were emptied and washed three times with washing solution. Diluted antiserum (1:1250) (400  $\mu\text{l}$  of  $D=1:50$  for a final volume of 10 ml) in phosphate buffer, pH 7.5, supplemented with 0.05% Tween 20 was preincubated for 1 h with 2,4,5-T at concentrations covering the range 0.01–5000  $\text{ng ml}^{-1}$  (this is the sufficient time for free antigen (2,4,5-T)-antibody interaction, that is to say the specific recognition of the free antigen by the antibody); 200  $\mu\text{l}$  of these preincubated mixtures was transferred to the wells of the microtiter plate and incubated for 15 min at 4°C (this is the most favourable time for competition between free antigen and immobilized antigen (HGG–2,4,5-T) for antibody

binding sites). One column of the plate received no 2,4,5-T and no antiserum, to determine nonspecific binding of the secondary antibody-labelled horseradish peroxidase enzyme in the following step. Another column received no 2,4,5-T to determine the maximum fluorescent reading. The plates were emptied and washed as described above. The immobilized antibodies were detected by labelled anti-Ig antibodies. Goat anti-rabbit-horseradish peroxidase conjugate (1:300) was added (200  $\mu\text{l/well}$ ) to the plates. The plates were incubated for 1 h at 4°C, emptied and washed as before.

### 2.5. Substrate

A substrate solution containing 3  $\text{mg ml}^{-1}$  *p*-hydroxyphenylacetic acid,  $\text{H}_2\text{O}_2$  ( $7.35 \times 10^{-5}$  M) and Tris–HCl (0.1 M) pH 8.5 buffer was prepared; 200  $\mu\text{l}$  of this solution was added to each well. Fluorescence was allowed to develop for exactly 1 h and was measured as a 320 nm wavelength excitation (slit 5 nm) and 420 nm wavelength emission (slit 10 nm). Readings were corrected for nonspecific binding of the enzyme-labelled antibody. Fluorescent values of standards ( $F$ ) were divided by the maximum fluorescent value ( $F_0$ ) representing those wells in which binding of antibody to the coating conjugate was not challenged with free 2,4,5-T in solution. The  $F/F_0$  values were plotted against the logarithm of 2,4,5-T concentration to construct a standard curve.

## 3. Results and discussion

### 3.1. Standard curves

To construct a calibration curve aliquots of 2,4,5-T covering the range 0.01–5000  $\text{ng ml}^{-1}$  were preincubated with antiserum (1:1250) for 1 h at 4°C, and then incubated for 15 min with previously coated HGG–2,4,5-T, as described in ELISA procedure. Free 2,4,5-T was then quantified by its competition with the coated 2,4,5-T for binding to anti-2,4,5-T (antibody). In the sandwich antibody assay, the antibody bound to the 2,4,5-T–HGG is detected by goat anti-rabbit-IgG HRP which reacts with fluorescent substrates. In this method, HRP is conjugated to a secondary antibody and its concentration on the wells is inversely proportional to 2,4,5-T concentration.

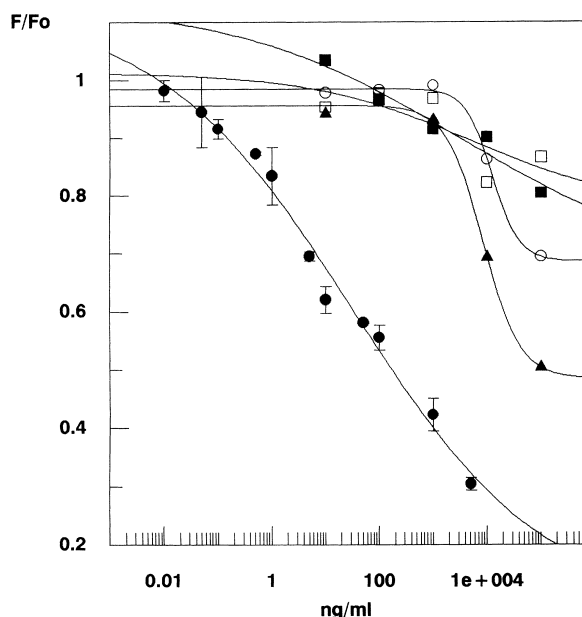


Fig. 1. ELISA standard curve for 2,4,5-T and displacement curves for crossreactant: (●) 2,4,5-T calibration; (▲) 2,4 DB; (○) 2,4-D; (□) dichlorprop; (■) MCPA.

A logarithmic plot of fluorescent intensity against concentration ( $\text{ng ml}^{-1}$ ) of the standard 2,4,5-T (Fig. 1(●)) displays a typical calibration graph fitted to an  $IC_{50}$  four parameter logistic model. A dynamic range covering standard concentrations of 2,4,5-T between 0.023 and 5000  $\text{ng ml}^{-1}$  is achieved.

The minimum detectable concentration (MDC) is defined for a four-parameter logistic model as [17] “the lowest concentration which results in an expected response less than the one-sided  $I$  level ( $I=0.05$ ) lower confidence limit at zero concentration”. The MDC is 0.023  $\text{ng ml}^{-1}$  and was calculated by substituting the average response minus the relative standard deviation of three samples containing 0.01  $\text{ng ml}^{-1}$  in each in the calibration equations.

The precision of the method was determined by measuring the fluorescent intensity of 10 separate samples, each containing 5  $\text{ng/ml}$  2,4,5-T, giving a relative standard deviation of 6.9%.

### 3.2. Specificity of the assay

To characterize the specificity of the assay several pesticides with structures very similar to that of

the 2,4,5-T (2,4-D, 2,4-DB, MCPA, dichlorprop) or usually found in formulations [6] (2,4-D) were tested.

Analytical interference due to the limited selectivity of antibodies is commonly referred to as crossreactivity. Crossreactivity in immunoassay is defined as the ratio of competitive binding between two or more structurally similar ligands with the antibody binding sites. The crossreactivity was calculated by using the 50% displacement method. In this method, the doses of 2,4,5-T and structurally related compounds necessary to displace 50% of the bound labelled tracer are compared. Displacement curves were prepared by incubation of various doses of crossreactant with a constant amount of antibody, followed by separation of bound and unbound fractions, and quantification of the crossreactant at each dose. After fitting the curves by the four-parameter logistic model the doses of crossreactant that give 50% displacement were determined and the crossreactivity (CR) of a compound was determined as [18]

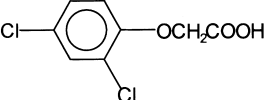
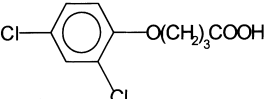
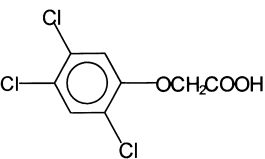
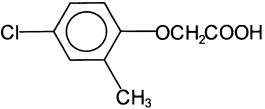
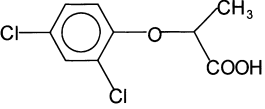
$$\%CR_{50\%} = \frac{2,4,5-T_{50\%}}{CR_{50\%}} \times 100,$$

where 2,4,5-T<sub>50%</sub> is the concentration of 2,4,5-T at the assay midpoint ( $F/F_0=0.652$ ), and  $CR_{50\%}$  is the concentration of crossreactant at the assay midpoint ( $F/F_0=0.652$ )

Table 1 gives formulae and crossreactivity percentages for the components studied by the ELISA. From these results it can be deduced that MCPA, dichlorprop and 2,4-D do not affect the method of determining 2,4,5-T. Displacement curves for 2,4,5-T and crossreactant are illustrated in Fig. 1. At 50% displacement, 2,4-DB displays affinity for the antiserum, and it has an  $I_{50}$  value (concentration giving 50% inhibition of maximum response) of 13  $\mu\text{g ml}^{-1}$ . The  $I_{50}$  value for 2,4,5-T is 5  $\text{ng ml}^{-1}$ .

We can compare specificity in two different methods (fluorescence ELISA and polarization fluoroimmunoassay) using the same polyclonal antibody. A comparison of the results of the estimation of the antibody specificity in the automated polarization fluoroimmunoassay without prepreparation of 2,4,5-T [10] and the ELISA shown in Table 1, established that the ELISA shows more specificity than the polarization fluoroimmunoassay.

Table 1  
Crossreactivity of 2,4,5-T and related compounds

Formula	Compound	%CR <sub>50%</sub> <sup>a,b</sup>	%CR <sub>50%</sub> <sup>a,c</sup>
	2,4-D	0	1.4
	2,4-DB	0.0374	3.3
	2,4,5-T	100	100
	MCPA	0	0.6
	Dichlorprop	0	

<sup>a</sup>Crossreactivity estimated at 50% displacement of signal.

<sup>b</sup>Fluorescence ELISA.

<sup>c</sup>Polarization fluoroimmunoassay.

### 3.3. Application

Recovery experiments (to describe the accuracy of the ELISA and to evaluate of matrix effect) were carried out with apple juice samples spiked at 500, 50, 5 and 0.5 ng ml<sup>-1</sup> with 2,4,5-T. The samples were prepared in the following way: 100 µl of ethanolic solution containing 50, 5, 0.5 and 0.05 µg ml<sup>-1</sup> of 2,4,5-T were added to 3 ml of apple juice and agitated. Diluted antiserum (1:1250) in phosphate buffer, pH 7.5, was added. The final concentrations of 2,4,5-T were 500, 50, 5 and 0.5 ng ml<sup>-1</sup> because the final volume of each sample was 10 ml.

Recovery results for 2,4,5-T in the spiked apple juice samples are given in Table 2.

Table 2  
Recovery of 2,4,5-T from spiked apple juice

Spike concentration in sample (ng ml <sup>-1</sup> )	Found concentration (ng ml <sup>-1</sup> )	RSD (%)	Recovery (%)
500	550	10.1 <sup>a</sup>	109.9
50	51.3	6.7 <sup>a</sup>	102.5
5	4.75	11.8 <sup>b</sup>	95.0
0.5	0.47	15.6 <sup>b</sup>	93.9

<sup>a</sup>n=3.

<sup>b</sup>n=8.

### 3.4. Conclusions

A fluorescence ELISA for the quantification of 2,4,5-T over a concentration range 0–5000 ng ml<sup>-1</sup> has been developed. The assay is very sensitive, due to

significantly enhanced activity of polymeric conjugates (Ab 2°). ELISA with fluorescent detection gives a good MDC ( $0.023 \text{ ng ml}^{-1}$ ), precision and accuracy. The high specificity of the antibody and the assay was demonstrated. The antiserum employed only shows induced crossreactivity against 2,4-DB. A fluorescence ELISA shows more specificity than a polarization fluoroimmunoassay.

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### References

- [1] E.T. Maggio, Enzyme Immunoassay, CRC Press, Boca Raton, FL, 1980.
- [2] M.F. Clark, R.M. Lister, M. Bar-Joseph, Methods Enzymol. 118 (1986) 742.
- [3] Z. Huang, N.A. Olson, W. You, R.P. Haugland, J. Immunol. Methods 149 (1992) 261.
- [4] G.G. Guilbault, Practical Fluorescence, Dekker, New York, p. 683.
- [5] E. Gnemmi, M.J. O'Sullivan, G. Chieriegatti, M. Simmons, A. Simmonds, J.W. Bridges, V. Marks, in: S.B. Pal (Ed.), Enzyme-linked Immunoassay of Hormones and Drugs, Walter de Gruyter, Berlin, 1978, p. 29.
- [6] The Pesticide Manual, 7th ed., Br. Crop Protect. Council, Suffolk, UK, 1983.
- [7] Metabolic Maps of Pesticides, Hiroyasu Aizawa, Yokohama, Japan, 1982.
- [8] Y. Mechref, Z. El Rassi, Anal. Chem. 68 (1996) 1771.
- [9] M. Jung, W.C. Brumley, J. Chromatogr. A 717 (1995) 299.
- [10] S.A. Eremin, A.M. Egorov, O.A. Melnichenko, A.A. Tumanov, J. Anal. Chem. 50 (1995).
- [11] S.A. Eremin, B. Laassis, J.J. Aaron, Talanta 43 (1996) 295.
- [12] L.F. García, S.A. Eremin, J.J. Aaron, Anal. Lett. 29 (1996) 1447.
- [13] S.A. Eremin, O.A. Mel'nichenko, A.A. Tumanov, N.V. Sorokina, E.V. Molokova, A.M. Egorov, Vopr. Med. Khim. 40 (1994) 57.
- [14] J.J. Fleeker, Assoc. Anal. Chem. 70 (1987) 874.
- [15] A.F.S.A. Habeed, Anal. Biochem. 14 (1966) 328.
- [16] L.A. Cantarero, J.E. Butler, J.W. Osborne, Anal. Biochem. 105 (1980) 357.
- [17] M.A. O'Connell, B.A. Balanger, P.D. Haaland, Chemom. Intell. Lab. Syst. 20 (1993) 97.
- [18] J.T. Miller, R. Valdes Jr., J. Clin. Immunoassay 15 (1992) 97.