Polarization Fluoroimmunoassay of the Herbicide Dichlorprop

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A polarization fluorescence immunoassay has been developed for the screening of the herbicide dichlorprop [(±)-2-(2,4-dichlorophenoxy)propanoic acid]. The assay is based on the difference in fluorescence polarization between free and antibody-bound fluoresceinamine-labeled dichlorprop. Antibodies toward dichlorprop were produced by immunizing a rabbit with conjugates of dichlorprop to bovine serum albumin. Polarization measurements were performed by using two spectrofluorometers which use different optic configurations. Calibration curves fitted to an IC_{50} four-parameter logistic model gives chi-square 10^{-5}. The upper end of the standard curve for polarization fluorescence immunoassay is 0.01 μg/mL and the lower end 100 μg/mL. The method offers good analytical specifications and little cross-reactivity against similar compounds. The proposed method is compared with enzyme-linked immunosorbent assay using the same set of reagents. The effectiveness of the method on real samples was checked in spiked apple samples.

In the analysis of pesticide residues, immunoassay is rapidly becoming an important technique (Hock, 1989; Jung et al., 1989; Sherma, 1991). Although the development of an immunoassay method involves high technology and may be costly, once the specific antibody is available and the method has been optimized and validated, immunoassay can provide many analyses at a throughput rate and cost much improved over those of conventional approaches.

Many immunoassay methods, namely homogeneous, allow the analysis of the macromolecule bound fluorophore in the presence of the free fraction, without separation, even when both emit in the same spectral region. The analyte concentration in a sample can be monitored directly from the reaction mixture through its effects of the fluorescence properties of the labeled antigen or antibody. Thus, the assays are very rapid and simple (one incubation and no washing), and their development has been among the main objectives in fluoroimmunoassay research. However, the sensitivity of homogeneous assays is often seriously limited by interferences from samples (serum) and by the low degree of fluorescence change (quenching, enhancement, polarization, energy transfer) in an immunoreaction.

Polarization fluorescence immunoassay (PFIA) is a homogeneous one. The method basis is that haptens labeled with a fluorophore and irradiated with polarized light emit light of greater polarization if the hapten is bound to an antibody rather than free in solution. The advantages of this technique as a means of measuring analytes in serum were outlined in original publications on this subject (Dandliker et al., 1973; Spencer et al., 1973). Most of the applications of homogeneous PFIA are for analytes present in substantial concentrations, for example, drugs (Eremin et al., 1989, 1992a; Nithipatikom et al., 1989; Colbert et al., 1991). PFIA has been scarcely applied to the determination of pesticide contaminants. A metabolite of the carbamate fungicide benomyl, namely 2-aminobenzimidazole, has been the subject of the first PFIA of a pesticide, reporting a sensitivity of subnanogram levels (Lukens et al., 1975). More recently is also described a PFIA for food contamination by 2,4-dichlorophenoxyacetic acid (Eremin et al., 1992b).

Commercial instruments dedicated for PFIA measurements are available for automated drug monitoring. Users are fully dependent on the supplier for assay procedures and cannot develop their own methods. However, a dedicated polarization fluorometer it is not necessary because a standard and versatile fluorometer provided with two polarizers is sufficient to perform polarization measurements.

Dichlorprop is a herbicide of translocation, its residues are a problem of potential health hazards. Methods of dichlorprop quantitation include gas chromatography (GC) (Ahmed et al., 1989; Blessington et al., 1989; Steinwandter, 1989) and high-performance liquid chromatography (HPLC) (Vaughan, 1981; Bogus et al., 1990; Schuessler, 1990). From the development of pesticide-specific antibodies, immunochemical procedures offer advantages over GC and HPLC methods (Hammock et al., 1980; Van Emon et al., 1985; Thurman et al., 1990). A test kit based on immunoassay technology for dichlorprop residue detection in water, soil, and food is commercially available (Millipore). This is a heterogeneous method, and a physical separation between the liquid phase and the solid phase is necessary.

In this paper a homogeneous polarization fluorescence immunoassay for the pesticide dichlorprop [(±)-2-(2,4-dichlorophenoxy)propanoic acid] is described. The specific antibody for dichlorprop has been developed by immunizing a rabbit. The hapten has been labeled with fluoresceinamine. Competition between pesticide hapten and fluorescent labeled hapten for antibody binding sites allows the construction of a standard curve which describes the decrease in polarization by increasing concentrations of pesticide.

EXPERIMENTAL METHODS

Instrumentation. Spectrofluorometer Perkin-Elmer LS-50 equipped with a 9.9-W pulsed xenon lamp source and using an automatic interchangeable wheel with film polarizers was used. The instrumental configuration is conventional, arranged in L-format, i.e., fluorescence emitted at 90° to the direction of excitation is detected with a Hamamatsu R928 photomultiplier tube detector.

Spectrofluorometer Aminco SLM-48000S equipped with a 450-W xenon lamp source and provided with Glan-Thompson polarizers arranged in T-format was also used. There are two channels with two distinct Hamamatsu R928 photomultipliers receiving the emission polarization from the sample holder; one receives the parallel and the other the perpendicular polarized

0021-8561/93/1441-2215$04.00/0

light. An IBM PC-AT microcomputer was used for on-line data acquisition and for data analysis. Steady-state excitation and emission spectra were obtained in the "10 average" mode, with all of the slits set at 2-nm band-pass. Fluorescence intensity and polarization measurements were made in the "25 average" mode and reported as the average of five measurements. The excitation monochromator slits were set to 4-nm band-pass and the band-pass filter was used for emission wavelength selection. Fluorescence polarization was printed out in "millipolarization" units (mP).

The polarization (P) of the fluorescence was calculated as

$$P = (I_V - CI_H)/(I_{VV} + CI_{VH})$$

The subscripts to the fluorescence intensity values (I) refer to orientations of the excitation and emission polarizers, in that order (V, vertical; H, horizontal). The correction factor C ($C = I_{HV}/I_{HH}$) is required for the L-format and was experimentally determined along with I_{VV} and I_{VH}.

Readings of fluorescence ELISA were accomplished in a Perkin-Elmer plate reader accessory, supplied with polystyrene well plates. A bifurcated high-grade fused silica fiber optic (Oriel, Stratford, CT, ref 77565), with a light transmission range of 240–2200 nm was used to transfer the excitation and emission energies between the well plate and the spectrometer. An IBM-PC computer was used for on-line data acquisition at an integration time of 10 s. Data were collected and processed by FLDM software (Perkin-Elmer).

Reagents and Solvents. Dichlorprop [(±)-2-(2,4-dichlorophenoxy)propanoic acid], triclopyr [[(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid], MCPA [[(4-chloro-*o*-tolyl)oxy]acetic acid], and bentazone [3-isopropyl-(1*H*)-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide] were provided by Dr. Ehrenstoffer Laboratories (Augsburg, Germany). 2,4,5-T [(2,4,5-trichlorophenoxy)acetic acid], *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), bovine serum albumin (BSA), human gammaglobulin (HGG), goat anti-rabbit IgG peroxidase conjugate, β-phenylethylamine, homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid), and fluoresceinamine (isomer II) were from Sigma. Ioxynil (4-hydroxy-3,5-diiodobenzonitrile) was obtained from Riedel-de Haën (Seelze, Germany). HPTLC silica gel 60 (F_{254}) plates and silica gel 60 for column chromatography were obtained from Merck. All other chemicals used were Merck R.A.

Stock standards of dichlorprop (4.2×10^{-3} M) were prepared in ethanol and stored in the dark at 4 °C. Buffer solutions were prepared from phosphate (pH 7.5, 0.1 M) and borax (pH 9.0, 0.1 M).

Preparation of Fluoresceinamine-Labeled Dichlorprop. To a magnetically stirred suspension of fluoresceinamine (200 mg, 0.002 mol) in 5 mL of dry ethanol was added 124 mg (0.002 mol) of DCC and 71 mg (0.001 mol) of dichlorprop. The reaction was carried out at room temperature for 24 h and the precipitated dicyclohexylurea filtered through 0.2-μm nylon filter. The solution was concentrated at 2 mL under vacuum at 35 °C on a rotary evaporator. The mixture was checked by TLC using chloroform/ethanol (9:1) as mobile phase. By comparing with standards an intense band at R_f 0.5 was identified as corresponding to fluoresceinamine-labeled dichlorprop. Separation was achieved by column chromatography using the same mobile phase. The chloroform/ethanol solution was dried under vacuum at 35 °C, and the yellow precipitate formed was recrystallized in ethanol/ether (50:50).

Synthesis of Haptens. *Synthesis of Dichlorprop Methyl Ester.* Concentrated H_2SO_4 was added dropwise to a solution of dichlorprop (700 mg) in 3 mL of methanol; the methyl ester formed was extracted into ethyl ether. The solution was evaporated under vacuum. The mixture was checked by TLC using chloroform as mobile phase and HPTLC silica gel 60 plate with fluorescence indicator. By comparison with dichlorprop standard a band at R_f 0.32 was identified as dichlorprop methyl ester (yield about 100%).

Synthesis of Dichlorprop β-Phenylethylamide. Dichlorprop (700 mg) was treated with DCC (620 mg) in 6 mL of methanol. After the mixture was stirred for 30 min at room temperature, the solution was filtered through 0.2-μm nylon filter. Excess of β-phenylethylamine was added to a methanolic solution containing redissolved precipitate. The reaction was carried at 4°C overnight, and the precipitate was removed by centrifugation. The supernatant was evaporated under vacuum at 35 °C. The compound appeared to be highly pure when analyzed by TLC in several systems, and its structure was confirmed by ^{1}H NMR.

Preparation of Immunogen. Unmodified dichlorprop was used as the hapten and was conjugated to BSA according to a procedure described by Fleeker (1987). Equimolar amounts of dichlorprop (470 mg), NHS (220 mg), and DCC (390 mg) were dissolved in 25 mL of dioxane. After incubation at room temperature for 18 h, the solution was filtered through 0.2-μm nylon filter and the solution evaporated to dryness under vacuum at 35 °C. A white powder of dichlorprop dihydroxysuccinimide ester was obtained. This product had a decomposition point of 118.6 °C. Structure confirmation was done by ^{1}H NMR (CCl_4) showing δ 1.84 (d, J = 6.9 Hz, 3H CH_3), 4.9 (q, J = 6.9 Hz, 1H, CH), 6.94 (d, J = 8.8 Hz, 1H aromatic), and 7.2 (q, J = 6.3 Hz, 1H aromatic).

The intermediate dichlorprop dihydroxysuccinimide ester (28 mg) was added to 100 mg of BSA in 3 mL of pH 9.0 borate buffer solution and agitated gently for 1 h. The resulting solution was dialyzed against four changes of water at 4 °C. The conjugate was then lyophilized and weighed into vials for storage under N_2 at −40 °C.

Determination of free amino groups in the conjugate was performed by titration with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Habeeb, 1966). The conjugate was identified by electrophoretic analysis, using polyacrylamide gel and sodium dodecyl sulfate (PAGE–SDS) (Laemmli, 1970).

The same procedure was used to conjugate human gammaglobulin (HGG) with dichlorprop for use as coating antigen in the ELISA well plate.

Immunization. A New Zealand white female rabbit was immunized with 300 mg of the lyophilized antigen–BSA conjugate in 7 mM phosphate buffer, pH 7.5, in the first injection, and 500 mg in the following immunizations. This suspension was emulsified with an equal amount of Freund's complete adjuvant (Chard, 1989). A total volume of 4 mL of antigen emulsion was injected at multiple subcutaneous sites. Booster injections were then given at 2-week intervals by using the same antigen emulsified in Freund's incomplete adjuvant. Blood was obtained from the rabbit ear vein 8 weeks after the first injection, and the serum was separated by centrifugation and stored at −20 °C.

Antibody Dilution Curve. To several antibody solutions covering the dilution range between 1:10 and 1:10000 was added 300 μL of 10^{-5} M ethanolic solution dichlorprop–fluoresceinamine (final concentration 10^{-6} M), and the final volume was adjusted to 3 mL with phosphate buffer solution (pH 7.5, 0.1 M). After a 30-min incubation at 25 °C, polarization readings were made.

Standard Curves. Antiserum to give a 1:200 final dilution was incubated for 30 min at room temperature with 300 μL of ethanolic solution of 10^{-5} M dichlorprop–fluoresceinamine (final concentration 10^{-6} M) and 300 μL of ethanolic solution dichlorprop covering standard concentrations between 0.01 and 100 μg/mL and adjusted to a volume of 3 mL with phosphate buffer solution (pH 7.5, 0.1 M). Using the Perkin-Elmer LS-50 with L-format configuration, the excitation monochromator was placed at 490 nm and the emission monochromator at 520 nm. The readings in the SLM 48000S were performed using T-format configuration, excitation monochromator set at 490 nm, excitation slits 16/4 nm, and cutoff emission filter of 550 nm.

ELISA. Microtiter plates were coated by adding to each well 200 μL of HGG–dichlorprop dissolved in 50 mM carbonate buffer, pH 9.6 (10 μg/mL of antigen coating) and incubated overnight at 4 °C. The plates were emptied and washed three times with washing solution (0.1 M phosphate buffer, pH 7.5 supplemented with 0.1% Tween 20. Unoccupied sites on the polystyrene well surface were blocked by adding 200 μL of 0.2% (w/v) gelatin solution in phosphate buffer and incubated for 20 min at 4 °C. The plates were emptied and washed as described above. Diluted antiserum (1:3000) in phosphate buffer, pH 7.5, supplemented with 0.05% Tween 20 was preincubated for 1 h with dichlorprop methyl ester standard. Aliquots of the preincubated mixture were transferred to the wells of the microtiter plate (200 μL/well) and incubated for 15 min at 4 °C. One column of the plate received no dichlorprop and no antiserum to determine nonspecific binding of the secondary antibody-labeled horseradish peroxidase enzyme

in the following step. Another column received no dichlorprop to determine the average maximum fluorescence reading. The plates were washed as before. Goat anti-rabbit horseradish peroxidase conjugate diluted in phosphate buffer (1:500) was added (200 μL/well) to the plates. The plates were incubated for 1 h at 4 °C, emptied and washed.

Substrate. Sixty microliters of a 2 mg/mL solution of homovanillic acid, 60 μL of 0.1% hydrogen peroxide solution, and 100 μL of 0.1 M Tris buffer, pH 8.5, were added to the plate. Fluorescence was allowed to developed for 5 h and measured at 315-nm wavelength excitation and 425-nm wavelength emission (Guilbault et al., 1968). Readings were corrected for nonspecific binding of the antibody-labeled enzyme.

Fluorescence values of the standards (F) were divided by the maximum fluorescence value (F_0) representing those wells in which binding of antibody to the coating conjugate was not challenged with free dichlorprop in solution. The F/F_0 values were plotted against the logarithm of dichlorprop concentration to construct a standard curve.

Apple Extraction Procedure. Apples (250 g) were chopped in a food chopper, and 15 g was transferred to a blender cup; 25 mL of acetone containing 0.3 mL of orthophosphoric acid was added, and the contents were blended for 5 min. The homogenate was filtered through a glass fiber filter of medium porosity. The filtrate was made up to 50 mL with acetone, and aliquots were fortified with a methanolic solution of dichlorprop for use in the polarization fluoroimmunoassay.

RESULTS AND DISCUSSION

Excitation of a fluorophore with polarized light causes the resultant fluorescence emission to be partially polarized. The emission is depolarized by a number of phenomena as rotational diffusion. For small molecules such as dichlorprop–fluoresceinamine, the fluorescence depolarization is essentially complete. Complexation by a high molecular weight molecule such as the antibody slows tumbling of the resultant complex, and the emission remains partially polarized. Thus, complexed and uncomplexed fluorescent molecules can be distinguished by the polarization of the emitted light.

Dilution Curves. Antibody dilution curves involve the incubation of a fixed amount of dichlorprop labeled with various amounts of antibody and the determination of the proportion of dichlorprop labeled that is bound compared to unbound. It also can be used to determine the optimum antiserum dilution to be used in the calibration curve construction. An incubation time of 30 min was selected because polarization measurements increased over 30 min and then were stabilized for 1 h. Polarization readings were made (λ_{exc} = 490 nm, λ_{em} = 520 nm, $slit_{exc}$ = 5 nm, $slit_{em}$ = 7.5 nm) and plotted against the serum dilution. Also, a dilution curve using preimmune serum was obtained. Figure 1 displays the curves obtained with immune and preimmune sera. From these results it can be deduced that effective production of antibody against dichlorprop is accomplished after immunization. The semilogarithmic plot for immune serum fitted to an IC_{50} four-parameter logistic function shows a reduced chi-square of 9×10^{-5}. Also, 50% of antibody bound to the labeled dichlorprop is obtained at 1:400 antibody dilution (IC_{50} = 407) and a slope factor of 1.432.

Standard Curves. *PFIA.* The calibration curve involves the incubation of fixed amounts of antibody and antigen labeled with different analyte concentrations. Plots of polarization signal against analyte concentration in logarithmic scale give a sigmoidal curve. The dynamic range of the assay is the step of maximum slope. The standard curves were constructed from antigen labeled concentration of 10^{-6} M and a 1:400 final antiserum dilution in buffered, pH 7.5, solution. Two sets of measurements with different apparatus were made, using L-format

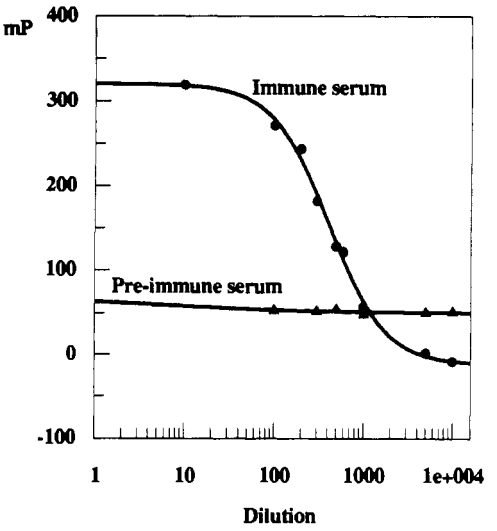


Figure 1. Antibody dilution curve. Instrumental parameters (L-format) λ_{exc} = 490 nm, λ_{em} = 520 nm, $slit_{exc}$ = 5 nm, $slit_{em}$ = 7.5 nm.

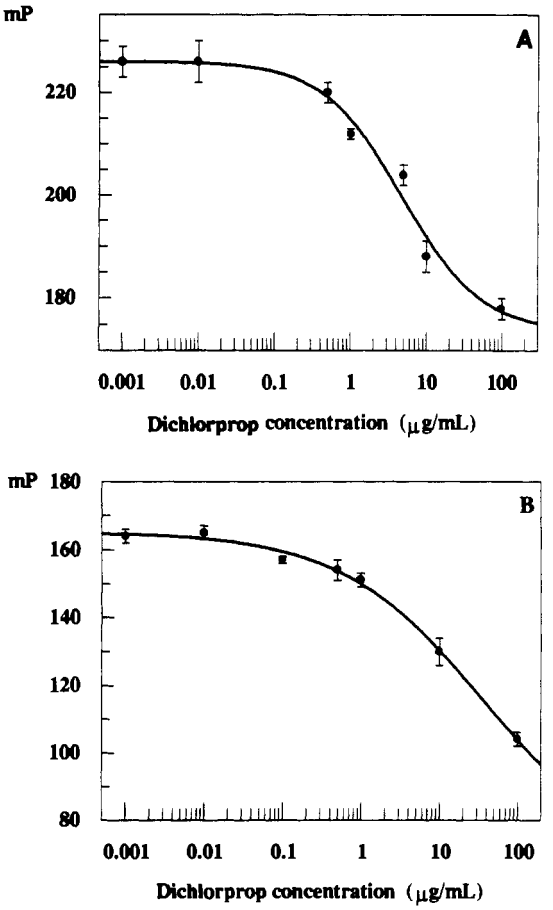


Figure 2. PFIA standard calibration curve for dichlorprop. (A) Spectrofluorometer configuration in T-format, excitation monochromator set at 490 nm, excitation slits 16/4 nm and cutoff emission filter of 550 nm. (B) Spectrofluorometer configuration in L-format, excitation monochromator placed at 490 nm, and emission monochromator at 520 nm.

configuration and T-format configuration, respectively (Lakowicz, 1983).

Figure 2 shows a typical calibration graph as successfully fitted to an IC_{50} four-parameter logistic, for T-format (Figure 2A) and L-format configurations (Figure 2B) with chi-square of 3×10^{-6} and 3.7×10^{-6}, respectively. A dynamic range covering standard concentration of dichlorprop between 0.01 and 100 μg/mL is deduced. The

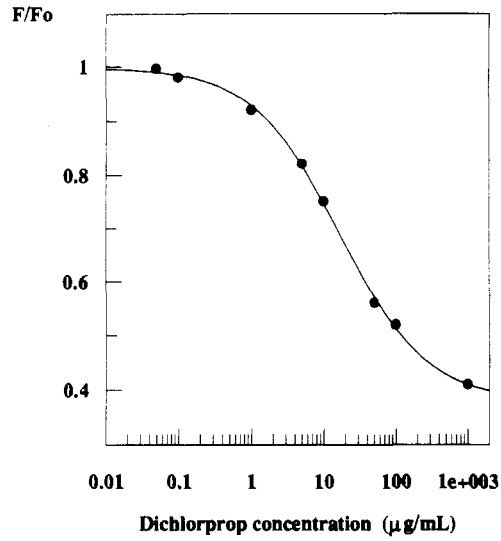


Figure 3. ELISA standard calibration curve for dichlorprop methyl ester.

minimum detectable concentrations at the 95% confidence level was 0.1 and 0.20 μg/mL for measurements in the T-format and L-format, respectively. Analytical sensitivities, defined as $s_A = \sigma_{n-1}/m$, were 0.014 and 0.13 μg/mL, respectively.

The precision of the method was assessed measuring three replicates between runs of standard dichlorprop at seven concentration levels and is displayed in Figure 2 as a bar plot. Standard deviations are in the range between 0.001 and 0.007 mP for measurements in T-format and between 0.002 and 0.006 mP for L-format. The average standard deviations were 0.002 and 0.003 mP, respectively. Ten separate samples, each containing 10 μg/mL, gave relative standard deviations of 15% and 13% in the L and T configurations, respectively.

ELISA. To construct a calibration curve, aliquots of dichlorprop methyl ester covering the range 0.01–1000 μg/mL were preincubated with antiserum (1:3000) during 1 h at 4°C and then incubated for 15 min with previously coated HGG–dichlorprop, as described in the ELISA procedure. Plot of fluorescence readings against log concentration of the standard dichlorprop methyl ester (Figure 3) displays a typical calibration graph fitted to an IC_{50} four-parameter logistic. A dynamic range covering standard concentration of dichlorprop methyl ester between 0.05 and 1000 μg/mL is deduced. The minimum concentration detectable at the 95% confidence level was 0.06 μg/mL, and analytical sensitivity was 0.01 μg/mL.

The precision of the method was determined by measuring the fluorescence intensity of 10 separate samples, each containing 10 μg/mL of dichlorprop methyl ester, and gave a relative standard deviation of 9.37% ($P = 0.05$).

Specificity of the Assay. To characterize the specificity of the assay, several pesticides having structures closely similar to that of the dichlorprop (2,4,5-trichlorophenoxyacetic acid, triclorpyr, dichlorprop methyl ester, and dichlorprop β-phenylethylamide) or usually found in formulations (Worthing, 1983) (ioxynil, bentazone, MCPA) were tested using L-format configuration.

The concentration of compound required to produce 50% inhibition was determined in each case. The cross-reactivity of a compound was determined as [(concentration of dichlorprop at the assay midpoint)/(concentration of structurally related compound at the assay midpoint)] × 100 = % cross-reactivity. Figure 4 gives formulas, IC_{50} values, and percent cross-reactivity for the compounds studied. From these results it can be deduced that bentazone and ioxynil do not affect the analytical method to determine dichlorprop. The methyl ester and amide derivatives display better affinity for the antiserum than the pure hapten (unmodified dichlorprop), especially the methyl ester. For this reason we used the methyl ester derivative of dichlorprop to develop the ELISA instead of dichlorprop.

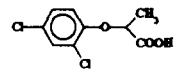
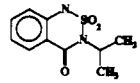
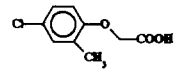
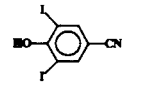
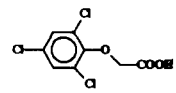
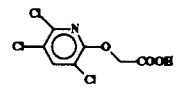
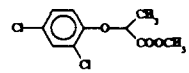
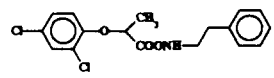
Formula	Name	IC_{50} (μg/mL)	CR %
	Dichlorprop	1.84	100
	Bentazone	>4000	0
	MCPA	32.27	5.7
	Ioxynil	62.35	3.0
	2,4,5-T	>4000	0
	Triclopyr	>4000	0
	Dichlorprop methyl ester	0.24	753.8
	Dichlorprop phenethyl amide	5.13	35.9

Figure 4. Cross-reactivity and IC_{50} of the dichlorprop PFIA and related compounds.

Table I. Recovery of Dichlorprop from Apple Samples Determined by PFIA

fortification		found	
concn, μg/mL of sample	concn, μg/g of apple	concn, μg/mL of sample	concn, μg/g of apple
10	0.22	11.71 ± 1.36[a]	0.26 ± 0.03[a]
1	0.022	1.25 ± 0.15	0.028 ± 0.003

[a] Standard deviation for three determinations.

Application. Extracts of apple samples were spiked at the 1.0 and 10.0 μg/mL levels and analyzed by PFIA. The results obtained, after the samples were submitted to the PFIA method, are summarized in Table I.

Comparison of PFIA and ELISA. The results obtained with the polarization fluoroimmunoassay proposed in this work were compared with those obtained with the ELISA developed with the same antiserum. Preliminary assays using dichlorprop as hapten show that the method failed, probably because of a higher affinity for the antiserum of the dichlorprop coated to the well plate than the standard dichlorprop. However, using as standard the methyl ester of dichlorprop, the fluorescent ELISA gives analytical specifications similar to those of the polarization assay (Table I). Figure 3 displays the standard calibration plot of dichlorprop methyl ester, which shows a shape similar to that of the polarization standard curve.

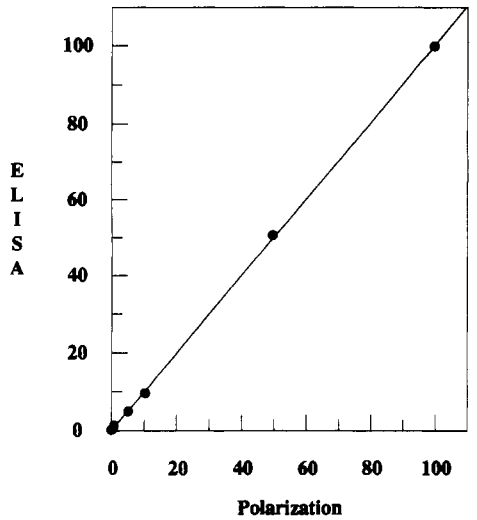


Figure 5. Correlation between PFIA and ELISA methods.

The correlation between PFIA and ELISA was 0.9999 (r); the slope of the regression line was 1.0047 and the intercept −0.0902. Thus, both methods correlated well and no systematic errors are detected (Figure 5).

Conclusion. A polarization fluoroimmunoassay for the herbicide dichlorprop has been developed. The used non-isotopically labeled hapten used is stable, and its synthesis and measurement are simple. Specificity of the antiserum employed shows induced cross-reactivity against similar compounds. The assay is sufficiently sensitive, precise, and accurate to be useful for the rapid assay of dichlorprop residue levels in agricultural or environmental samples. The total time for an assay is about 1 h. The instrumental simplicity of measurements allows the methodology to be used in routine analyses. The fluorescence polarization method compares favorably with ELISA method.

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Received for review August 2, 1993. Accepted August 6, 1993.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1993.