Fluorescent Excitation Transfer Immunoassay for the Determination of Spinosyn A in Water

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A fluorescent excitation transfer immunoassay for spinosyn A, a fermentation derived insect control agent, has been developed and applied to the analysis of tap water and wastewater effluent from manufacturing plants. Fluorescein (F) and tetramethylrhodamine (TMR) were chosen as donor and quencher, respectively, for the excitation transfer. Fluorescence quenching was observed from the binding of F-labeled antigen to TMR-labeled antibody. By employing nonlabeled antigen in a competitive immunoassay format, we reversed fluorescence quenching. The assay provides a limit of detection of 0.01 ppb and a working range of 0.05–1 ppb and allows for the rapid determination of spinosyn A in water with recovery values ranging from 96% to 120%. With the exploitation of the small size of optical fibers, fluorescence from an assay volume of 24 μL could be measured without special vessels.

Keywords: *Immunoassay; fluorescent excitation transfer; spinosyn; fiber optic; water*

INTRODUCTION

The wide use of agrochemicals has generated a need to monitor and control the level in soil and groundwater. Presently, chromatographic analyses such as GC/MS and HPLC are the most widely used monitoring methods (West, 1996; West, 1997; Yeh et al., 1997). These techniques are sensitive and accurate, but need relatively expensive instrumentation and numerous time-consuming manipulation steps such as extraction and preconcentration. Many types of immunoassays have provided complementary and/or alternative methods to these chromatographic methods recently because such cleanup steps are not required (Meulenberg et al., 1995; Brecht et al., 1995). Among immunoassays, fluorescence immunoassay methods have received considerable attention in recent years due to their rapidity and high sensitivity (Eremin and Samsonova, 1994; Lee and Durst, 1996; Matveeva et al., 1997). Homogeneous fluorescence immunoassays do not require separation of bound from free label prior to measurement, while several separation and wash steps are required in heterogeneous immunoassays. As a result, higher accuracy and shorter analysis times can be achieved in homogeneous fluorescence immunoassays due to fewer manipulations.

In this report, a homogeneous fluorescent excitation transfer immunoassay is described for spinosyn A, the predominant active ingredient of the spinosyns with the other spinosyns present at significantly lower concentrations (Thompson et al., 1996; Porteous et al., 1996). The spinosyns are a new family of macrolide pest control agents isolated from the actinomycete, *Saccharopolyspora spinosa,* and the common name of the product is spinosad (Mertz and Yao, 1990). A total of 23 naturally

Spinosyn A: R = N(CH₃)₂
Spinosyn C: R = NH₂
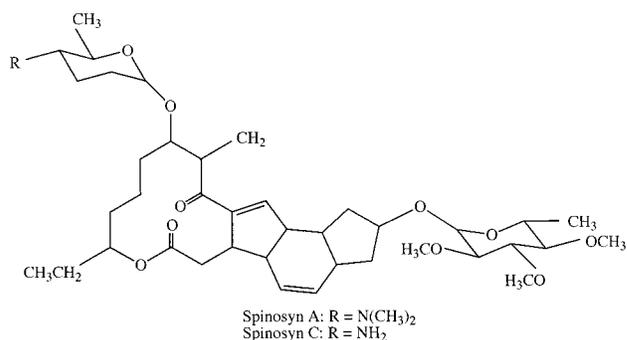
Figure 1. Structure of spinosyn.

occurring spinosyns A–Y have been isolated (Kirst et al., 1992). Figure 1 shows the structures of spinosyns A and C. Spinosyn C has a primary amine on the forosamine sugar, and this amino group was utilized to conjugate the materials described in this study. The other spinosyns vary in the substitution of one or more methyl groups on the tetracyclic core, the forosamine sugar, or the rhamnose sugar.

Fluorescent excitation transfer is a phenomenon that involves the transfer of excitation energy from one substance (donor) to another (acceptor). As a result, fluorescence quenching from the donor is observed. The two most important variables are the degree of spectral overlap between the donor and acceptor and their dipole–dipole distance. The donor's emission spectrum must overlap with the acceptor's excitation spectrum for energy transfer to occur. The most common combination is fluorescein, in its various reactive forms, and tetramethylrhodamine (TMR) (Figure 2). The other critical requirement for energy transfer is the distance between donor and acceptor. Energy transfer occurs only over distances less than 100 Å (Ullman and Khanna, 1981). In immunoassays, the dimensions of Ag–Ab complexes are usually within this critical distance, making immunoassays suitable for the excitation energy transfer

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Fluoroimmunoassay for Spinosyn A

J. Agric. Food Chem., Vol. 47, No. 7, 1999 **2767**
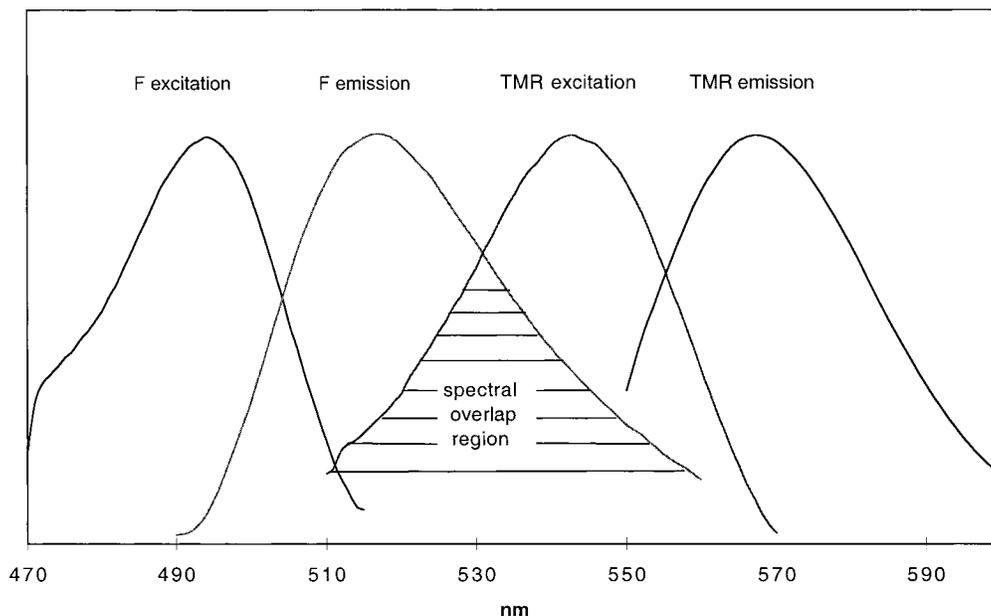
Figure 2. Excitation and emission spectra of fluorescein (F) and tetramethylrhodamine (TMR). The area between F emission and TMR excitation is defined as the spectral overlap and determines the efficiency of energy transfer.

assay format. Immunoassays using energy transfer have been reported for morphine (Ullman et al., 1976), IgG (Barnard and Walt, 1991; Calvin et al., 1986), atrazine (Agayn and Walt, 1993), thyroxine (Ozinskas et al., 1993), and human serum albumin (Youn et al., 1995).

For this work, we labeled the antibody (Ab) with TMR and the antigen (Ag) with fluorescein (F). When TMR-labeled Ab (TMR–Ab) binds to F-labeled Ag (F–Ag), the complexation should result in fluorescence quenching. For the fluorescein–rhodamine dye pair, R_0 (distance at which 50% transfer efficiency occurs) has been reported to be 58 Å (Khanna, 1988). Therefore, efficient energy transfer between F and TMR can take place in the Ag–Ab complex. When sample Ag is introduced into solution, competition for the Ab binding sites occurs between the nonlabeled sample Ag and labeled Ag, and the amount of fluorescence quenching will be reduced in proportion to the concentration of nonlabeled Ag.

The antibodies are generally the most expensive component of the immunoassay, since antibody preparation is time-consuming and is not always successful. Many fluorometers are available for fluoroimmunoassays; however, fluorometers accepting less than 100 μL sample vessels are not commercially available. The small size and flexibility of optical fibers make them suitable for this niche. These features of optical fibers were exploited previously in our laboratory in the format of controlled-release polymer systems (Barnard and Walt, 1991). In this study, a total assay volume of 24 μL was illuminated by using a fiber optic fluorometer without special vessels. By reducing the total analysis volume, one can minimize inefficient antibody use.

MATERIALS AND METHODS

Materials. Spinosyn A (CAS Registry No. 131929-60-7), spinosyn C (CAS Registry No. 131929-62-9), and polyclonal antibody to spinosad were provided by DowAgroSciences (Indianapolis, IN). Tetramethylrhodamine protein labeling kit and fluorescein isothiocyanate (FITC) were obtained from Molecular Probes, Inc. (Eugene, OR). Protein A affinity adsorbent was purchased from Bioprocessing Inc. (Princeton, NJ). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydro-

chloride (EDC) was obtained from Pierce (Rockford, IL). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Instrumentation. A fiber optic fluorometer, described previously (Luo and Walt, 1989), was assembled from the following components: a 75 W Oriel (Stratford, CT) Model 6263 xenon arc lamp, a Spex (Edison, NJ) Model 1680 double spectrometer, a UniBlitz (Rochester, NY) shutter, an AMP Inc. (Harrisburg, PA) fiber optic coupler, a Pacific Instruments (Concord, CA) Model 126 photon counting photometer, and a Pacific Instruments 315 ORF photomultiplier tube. The optical fiber was 550 μm in diameter and 30 cm in length, with a numerical aperture of 0.22 and a protective jacket coating (SpecTran Specialty Optics Co., Avon, CT). Both ends of the fiber were polished, washed with acetone, and used without further treatment. A LabVIEW software system (version 3.1, National Instruments, Austin, TX) was used to collect data and control the movements of the stepping motors in the excitation and emission spectrometers.

Purification of Ab. Anti-spinosad Ab was raised in rabbits using spinosad cross-linked to KLH (keyhole limpet hemocyanin) and purified from whole serum by passing through a protein-A column following the manufacturer's recommendation of loading with phosphate-buffered saline (PBS), pH 7.4, and eluting with 0.1 M glycine/HCl, pH 3.0. Further purification was achieved by immunoaffinity chromatography with KLH immunosorbent prepared from CNBr-activated Sepharose 4B and KLH. For the preparation of KLH immunosorbent, CNBr-activated Sepharose 4B (4 mL) was washed with cold 0.1 M sodium bicarbonate (pH 9.0) and treated with 20 mg of KLH in 4 mL of 0.1 M sodium bicarbonate at 4 °C (Cuatrecasas, 1970). After 12–16 h, the gel was thoroughly washed until KLH was no longer in the eluent.

Labeling of Ab. The amine-reactive reagent, tetramethylrhodamine-succinimidyl ester (TMR-SE), reacted with lysine amine residues in the Ab following the manufacturer's instructions. Two hundred microliters of antibody solution (1 mg/mL) was mixed with 20 μL of freshly prepared 1 M sodium bicarbonate solution (pH 8.3). TMR-SE (500 μg) was dissolved in 50 μL of dimethyl sulfoxide (DMSO), and 2.9 μL of this dye solution was added to the tube containing the antibody while stirring. The solution was stirred at room temperature for 1–1.5 h, with protection from light. Freshly prepared 1.5 M hydroxylamine (pH 8.5) was then added to the solution to a final concentration of 0.15 M. This step eliminates any dye molecules that were noncovalently attached to the protein or

were attached to tyrosine or histidine residues by labile bonds. After 15 min of incubation, TMR–Ab was purified from unreacted dye by a spin column with P-6 gel (Bio-Rad, Hercules, CA). By using an absorbance method, we determined the concentration of the purified TMR–Ab and the degree of TMR in TMR–Ab. The absorbance of TMR–Ab was measured both at 280 nm (OD_{280}) and at the wavelength for maximum absorbance of TMR (OD_{555}). The following equations were applied for the determination.

$$\text{TMR–Ab concentration (mg/mL)} = (OD_{280} - 0.3OD_{555})/1.4 \quad (1)$$

$$\text{TMR per Ab} = (OD_{555}/\epsilon)[\text{molecular weight of antibody}/(\text{mg/mL of TMR–Ab})] \quad (2)$$

Where 1.4 is the absorbance of most antibodies at 1 mg/mL and ϵ is the molar extinction coefficient ($cm^{-1} M^{-1}$) of TMR. Texas Red (TR) was conjugated to the spinosyn antibody using a similar procedure.

Labeling of Ag. The amino group on spinosyn C was used to conjugate the amine-reactive fluorescein dye, fluorescein isothiocyanate (FITC). Spinosyn C (0.5 μmole) was dissolved in 2.5 mL of 0.1 M sodium bicarbonate (pH 9.0). FITC in DMSO (10 mg/mL) was added to the spinosyn C solution at 10 molar excess. The reaction was monitored by thin-layer chromatography on silica by using a solvent system of chloroform/methanol/acetic acid (21:3:1). F–Ag was purified by a preparative TLC method using the same solvent system. B-phycoerythrin (BPE) was conjugated to spinosyn using EDC following the manufacturer's instructions. One milligram of spinosyn C was dissolved in 250 μL of 0.1 M MES (2-(*N*-morpholino)ethane sulfonic acid) buffer (pH 4.7) and added to a solution of 1 mg of BPE in 100 μL of MES buffer. Fifty microliters of EDC solution (2.6 μmol) in deionized water was added to BPE-spinosyn C solution, and the solution was stirred for 2 h at room temperature. BPE-spinosyn C was purified by a spin column.

Fluorescence Excitation Transfer Measurements. The assay was performed on the fiber optic fluorometer. Fluorescence measurements were made after preincubating TMR–Ab, F–Ag, and Spinosyn A, mixed in order, for 20–30 min at room temperature in PBS (pH 8.0). The fluorescence intensities were read at 520 nm with 490 nm excitation.

RESULTS AND DISCUSSION

Fluorescence Excitation Transfer Assays from F–Ag and TMR–Ab. The fluorescence quenching by energy transfer was demonstrated by adding donor-labeled spinosyn (F–Ag) to quencher-labeled antibody (TMR–Ab). Apparently, the fluorescein-labeled spinosyn has less affinity for the antibody than nonlabeled Ag. Fifty molar excess of Ag–F relative to Ab solution was required for optimum response. When F–Ag was added to mixtures of premixed TMR–Ab and spinosyn A, it did not give a sensitive response. Therefore, the components must be mixed in the order TMR–Ab, F–Ag, and spinosyn A. Figure 3 shows the effect of spinosyn A on the fluorescence intensity of a premixed F–Ag and TMR–Ab solution. The fluorescence data were recorded as arbitrary units and transformed as in eq 3, where F is the fluorescence at a given concentration of spinosyn A, F_0 is the fluorescence at [spinosyn A] = 0, F_∞ is the fluorescence in the presence of excess of spinosyn A, and $\%B/B_0$ is the percentage of bound to unbound spinosyn.

$$\%B/B_0 = (F - F_0)/(F_\infty - F_0) \quad (3)$$

In this application, about 20 nM TMR–Ab was used and the dye/Ab ratio was 6.8. Labeled Ab with an average
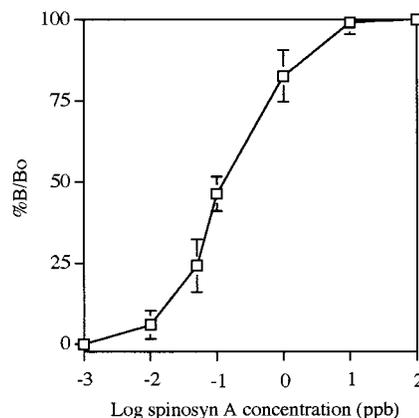
Figure 3. Fluorescence intensity versus antigen (spinosyn A) concentration with fluorescein-labeled spinosyn and tetramethylrhodamine-labeled antibody. Each point represents the mean of three measurements with error bars indicating ±1 sd.

number of TMR bound to Ab between 3 and 10 gave good fluorescence quenching results in this assay. TMR-labeled Ab did not contribute to the background. As expected, additions of nonlabeled Ag to the solutions reduced the fluorescence quenching. As the incubation time increased, more nonlabeled Ag occupied Ab binding sites and fluorescence quenching was reversed. A 20–30 min incubation time was found to give the most sensitive response curve. As shown in Figure 3, 0.05 ppb of spinosyn A could be detected easily in this assay with a dynamic range of 0.05–10 ppb. The limit of detection was calculated as three times the standard deviation of mean B_0 value and was 0.01 ppb spinosyn A in this study. With this low detection limit, environmental samples can be diluted before the assay to avoid the matrix effects commonly encountered in immunoassays when field water samples are analyzed. Even though a dynamic range of 0.05–10 ppb was obtained here, it was often observed that the fluorescence intensity sometimes was less than the 1 ppb value when the concentration of spinosyn A was over 1 ppb. Therefore, only a dynamic concentration range of 0.05–1 ppb could be used from this dose–response curve, and higher concentrations of spinosyn A must be diluted to meet this range. To avoid this cumbersome dilution, we sought a different excitation transfer assay format to obtain a response curve with a dynamic range over 1 ppb of spinosyn A. We selected BPE as the donor and TR as the acceptor. In this case, the multiple NH_2 residues on BPE allow various amounts of spinosyn to be conjugated, unlike the 1:1 conjugation of FITC to spinosyn. There is significant overlap between the BPE emission spectrum and the TR excitation spectrum (Kronick and Grossman, 1983). It was expected that energy transfer would occur when these fluorophores were brought into proximity by the antibody–antigen binding. Although the extent of incorporation of spinosyn into BPE was not determined, conjugates with varying incorporation ratios were prepared by varying the amount of spinosyn C during the conjugation procedure. These BPE–spinosyns with varying incorporation ratios gave dose–response curves with different dynamic ranges (data not shown). This assay format had a higher detection limit (over 1 ppb of spinosyn A) and higher dynamic ranges (from 1 ppb to 100 ppm) than the F–Ag/TMR–Ab format (Figure 4). By measuring samples concurrently using these two assay formats, one can analyze samples

Fluoroimmunoassay for Spinosyn A

J. Agric. Food Chem., Vol. 47, No. 7, 1999 **2769**
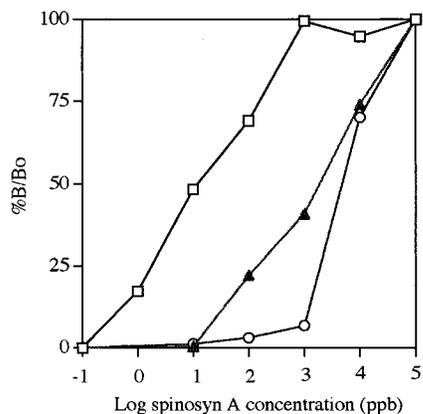
Figure 4. Dose−response curves of BPE spinosyns with various concentrations of TR-Ab: 34 nM TR-Ab (□), 94 nM (▲), 188 nM (○).
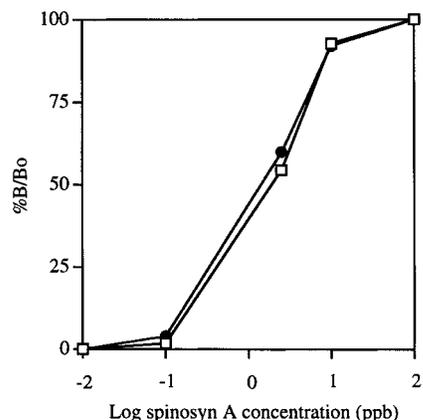
Figure 5. Effect of lyophilization of F−Ag and TMR−Ab mixture on dose−response curve. (●) was obtained from F−Ag and TMR−Ab in solution and (□) was from reconstitution of lyophilized F−Ag and TMR−Ab mixture.

with unknown concentrations of spinosyn A without interruption.

Lyophilization of the Components. A simpler way to perform the assay was sought for the convenience of potential end users to reduce error-prone steps. TMR−Ab and F−Ag were premixed and lyophilized. The lyophilized components were then reconstituted to the original volume by adding high-purity water. The rest of the steps were the same as for TMR−Ab and F−Ag in solution, and the standard curve obtained by this lyophilization method was compared to the result obtained by using an aliquot of premixed TMR−Ab and F−Ag solution (Figure 5). The similarity of the response curves demonstrates that binding of F−Ag to TMR−Ab was not affected by lyophilization and allows the method to be adapted to an easy-use commercial kit.

Detection of Spinosyn A in Fortified Water Samples. To evaluate the potential environmental applications of this assay, we spiked two types of water, tap (pH 6.0) and plant wastewaters (pH 5.4), with three concentrations of spinosyn A covering the established working range of the dose−response curve. Plant wastewater samples, previously analyzed and known to have no spinosyns, were supplied by DowAgroSciences. Each fortified sample was first diluted 10 times with PBS to circumvent matrix effects and analyzed three times in triplicate by the assay format using F−Ag and TMR−Ab. The results are presented in Table 1. The mean recovery data for two waters at three levels of fortifica-

Table 1. Recovery of Spinosyn A from Spiked Water Samples

	n	spinosyn A added (ppb)	mean ±sd (ppb)	recovery (%)
tap water	9	0.5	0.48 ± 0.07	96
	9	1	0.98 ± 0.15	98
	9	5	5.0 ± 0.47	100
plant effluent	9	0.5	0.60 ± 0.14	120
	9	1	0.96 ± 0.12	96
	9	5	5.9 ± 1.1	118

tion were 105% (from 96% to 120%). These data proved that acceptable recoveries can be obtained by this assay. However, it is expected that, because of the structural similarity with other spinosyns (minor components of spinosad and their degradation products), there will be some cross-reactivity in real environmental water samples.

Conclusions. We have devised a fluorescent excitation transfer immunoassay for spinosyn A. A fluorescein (donor)-labeled antigen and a TMR (quencher)-labeled antibody were employed. We observed fluorescence quenching of F-spinosyn after mixing with TMR−Ab and restoration of fluorescence after adding excess spinosyn A to the reaction solution. The fluoroimmunoassay described herein does not require sample cleanup for the water samples tested. Samples were diluted 10-fold with PBS before testing to avoid sample matrix effects. Application of this immunoassay for the determination of spinosyn A in fortified real environmental water samples was satisfactory, since acceptable recoveries and precision were obtained.

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