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A Quenching Fluoroimmunoassay for Analysis of the Pesticide Propazine in an Apolar Organic Solvent, Reverse Micelles of AOT in *n*-Octane: Effect of the Micellar Matrix and Labeled Antigen Structure

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A simple way of directly observing antigen–antibody binding in a reverse micellar system, *n*-octane containing reverse micelles of aerosol OT (AOT), using the hydrophobic pesticide propazine as antigen, is described. We observed two processes during fluorescein-labeled propazine (FP)–antibody (Ab) interaction in reverse micelles: (1) quenching of the fluorescence of FP after mixing of Ab and FP (due immune complex formation) and (2) restoration of FP fluorescence after addition of excess propazine to the immune complex formed. We found that the quenching efficiency depends on both the properties of the reverse micellar system (surfactant concentration, hydration degree W_0 = [water]/[surfactant]) and the structure of the labeled antigen. A quenching fluoroimmunoassay of propazine both in apolar organic solvents and in water is developed. The method is homogeneous. The quenching time is 10–30 min, and the detection limit of propazine is 100 n*M* (20 μg/L) in organic solvent and 10 n*M* (2 μg/L) in water. Propazine can be added to the reverse micellar system when dissolved in AOT/octane, or in an octane/chloroform mixture, or in chloroform. This makes possible the use of the analysis directly for pesticide extracts in nonpolar organic solvents.

KEY WORDS: Propazine; pesticide; immunoassay; polyclonal antibodies; reverse micelles; aerosol OT; fluorescence quenching.

INTRODUCTION

Recently much attention has been paid to the study of biologically active substances in reverse micellar systems, which are apolar organic solvents containing small water droplets surrounded by a monolayer of hydrated surfactant molecules. The main advantage of such a system is the possibility of using both hydrophilic and hydrophobic reagents at the same time in one system. It is known that the entrapment of proteins or any other biologically active substance into reverse micelles leads to protein-sized, optically clear assemblies of surfactants in nonpolar organic solvents. The catalytic properties of many enzymes entrapped in the water pool of reverse micelles have been studied in detail (see, for example, Refs. 1 and 2). However, there have been only a few studies devoted to a very interesting and important biointeraction: antigen–antibody binding in reverse mi-

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[4] Abbreviations used: Ab, polyclonal antibodies against propazine; AOT, aerosol OT, sodium bis(2-ethylhexyl)sulfosuccinate; DL, detection limit; ELISA, enzyme-linked immunosorbent assay; Fab, fragment antigen binding; FP, propazine labeled with fluorescein; QFIA, quenching fluoroimmunoassay; W_0, surfactant hydration degree (W_0 = [H_2O]/[surfactant]).

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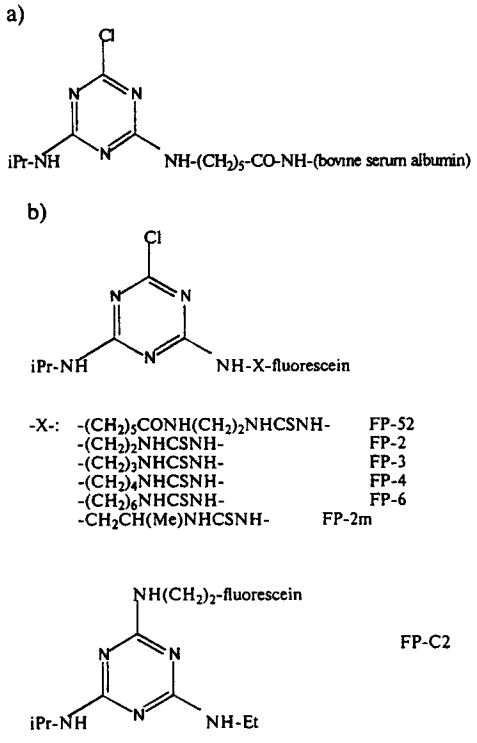

Fig. 1. Structures of the conjugates used: (a) atrazine–bovine serum albumin, used for rabbit immunization; (b) propazine–fluorescein conjugates (FP).

celles.[3–10] Antigen–antibody binding in reverse micelles was demonstrated for antibodies against thyroxin in reverse micelles of aerosol OT [AOT;[4] sodium bis(2-ethylhexyl)sulfosuccinate] in *n*-octane[5] and for dansylated synthetic human myelin basic protein peptide with Fab from monoclonal antibodies against myelin basic protein in reverse micelles of AOT in isooctane.[6] Recently we studied the quenching of fluorescein-labeled atrazine[9] and polarization of fluorescein-labeled propazine in the presence of antitriazine antibodies[10] in the reverse micellar system AOT/*n*-octane with varying surfactant concentrations and surfactant hydration degrees. The reverse micellar system AOT/hexane was used in competitive ELISA for the detection of parathion dissolved in hexane without prior solvent removal.[11]

Due to the growing need for environmental monitoring and food control of pesticide contamination, several enzyme immunoassays[12,13] and polarization immunoassays[14] for triazine pesticides have been developed; these methods are carried out in aqueous solutions.

In this work we present a fluorescence quenching study of the antigen–antibody interaction in a reverse micellar system and the development of a quenching fluoroimmunoassay for propazine. We have chosen a well-known reverse micellar system, *n*-octane containing reverse micelles of AOT, and propazine as one of the hydrophobic triazine pesticides.

EXPERIMENTAL

Materials

Propazine (2-chloro-4,6-diisopropylamino-1,3,5-triazine) was purchased from Sigma. AOT, purchased from Serva, was not further purified. *n*-Octane was purchased from Reachim (Russia). Other chemicals were of analytical grade.

Polyclonal antisera against triazines were obtained after immunization of rabbits with an atrazine–bovine serum albumin conjugate (the structure of the conjugate is given in Fig. 1a) as described in Ref. 9. Antibodies were precipitated with a saturated aqueous ammonium sulfate solution, dissolved in 50 m*M* Tris buffer, and dialyzed against 50 m*M* Tris buffer (pH 8.7).

Conjugate FP-C2 was kindly donated by Abion (Germany). Other fluorescein–propazine conjugates were obtained using carbodiimide binding of the carboxylic propazine derivatives and amine derivatives of fluorescein, as described in Ref. 10.

Methods

Micellar solutions were prepared by solubilization of aqueous solution of antibodies (0.5–1.5 mg/ml) in 1–20 ml of 0.025–0.3 *M* AOT in *n*-octane (Ab-containing micellar solution). The system became optically transparent after 0.1–1 min of shaking. One hundred microliters of stock FP solution in 0.1 *M* AOT/octane was added to 1 ml of Ab-containing micellar solution, and the fluorescence intensity was measured using a TDx analyzer (Abbott Laboratories, USA) at 37°C. The total time for the measurement of 10 samples was about 7 min. An excess of free propazine was made by the addition of 5–20 μl of stock propazine solution in chloroform (2.4 mg/ml) to 1–2 ml of a micellar mixture of Ab and FP. A reverse micellar solution containing Ab and an excess of propazine, but not FA, showed no detectable fluorescence.

The aqueous phase was 0.1 *M* Tris, pH 8.7, in all experiments. This buffer was optimal for the solubilization and activity of antibodies and for a good quantum yield of FP fluorescence in reverse micelles of AOT/octane/water.

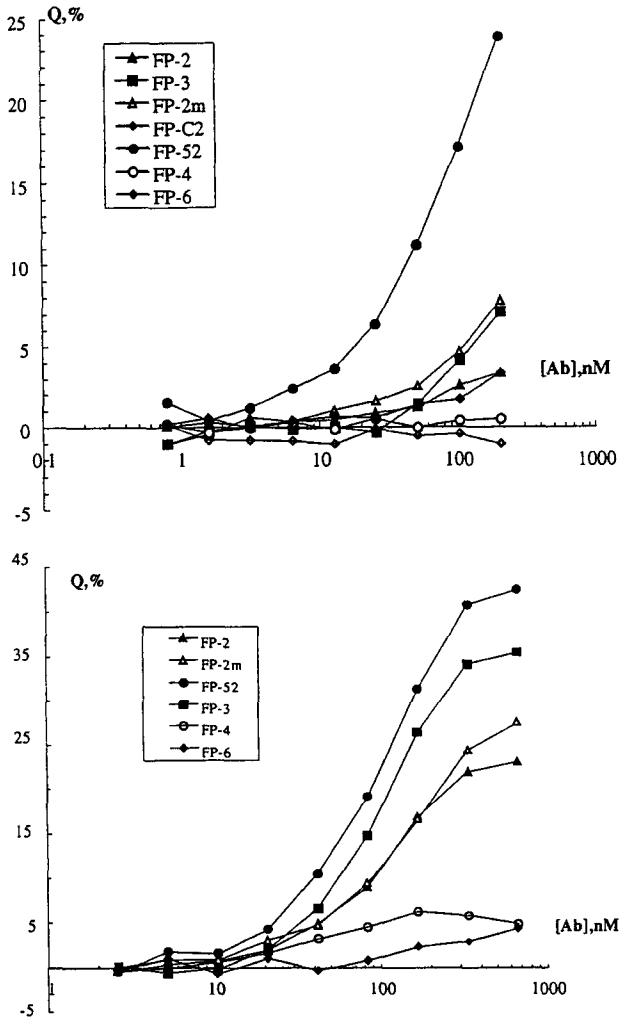

Fig. 2. Quenching of FP fluorescence in the presence of Ab: effect of FP structure (a) in reverse micelles of AOT/octane (W_0 = 16.7, [AOT] = 100 m*M*, [FP] = 8 n*M*) and (b) in water (50 m*M* Tris, pH 8.7; [FP] = 8 n*M*).

A calibration curve was obtained as follows. Standard solutions of propazine in reverse micelles were obtained by serial dilution of the stock propazine solution in 100 m*M* AOT/octane. Then 100 µl of FP in AOT/octane/water and 100 µl of Ab in AOT/octane/water were added to 0.8 ml of each propazine-containing sample. The resulting concentrations of the components were (FP) 5–7 n*M*, (Ab) 370 n*M*, and (propazine) 0 to 40 m*M*, W_0 = 16. After 10 min–2 h of incubation (the effect of the incubation time was negligible), the fluorescence intensity was measured. A propazine calibration curve in water was obtained in a similar manner using water instead of reverse micellar solution. The sensitivity of the assay to propazine was estimated as follows. The standard deviation of the response (fluorescence intensity) for zero propazine concentration was calculated (20 replicates). The propazine detection limit (DL) was calculated from the calibration curve as the concentration corresponding to the response value equal to three times the standard deviation.

RESULTS AND DISCUSSION

We found that FP fluorescence in a reverse micellar system is quenched after the addition of antibodies against propazine. The initial fluorescence level of FP is restored after the addition of excess free propazine, which competes for binding sites on the antibody. A similar reversible quenching effect was observed for interaction of the fluorescein-labeled atrazine and antiatrazine antibodies in the same reverse micellar system.[9]

Fluorescein quenching by binding to antifluorescein antibodies is probably due to the hydrophobic nature of the active site and the corresponding change in the fluorescein microenvironment to being more hydrophobic after binding.[15–19] With fluorescein quenching by binding to antifluorescein antibodies, the quenching effect can be up to 100%. We propose that, in our case, if the antigen is not fluorescein itself, but fluorescein bound to a small antigen molecule, the cause of this quenching could be the same (hydrophobic nature of the active site), but fluorescein is located more remotely from the active site, so the quenching effect is less.

We expressed the quenching effect in the following form: quenched fluorescence, $I_0 - I$, as a percentage of the initial fluorescence, I_0:

$$Q(\%) = 100\% * (I_0 - I)/I_0$$

where I is the fluorescence intensity of the mixture Ab + FP, and I_0 is the fluorescence intensity of the same mixture after the addition of an excess of free propazine. In this way, we consider only specific quenching caused by competitive Ab–propazine interaction. It turned out that the quenching efficiency depends on the FP structure and on the properties of the reverse micellar system (surfactant concentration and hydration degree).

Effect of FP Structure

To observe antigen–antibody interaction in the reverse micellar system, we chose a number of fluorescein-labeled propazine conjugates. The structures of FP conjugates used are presented in Fig. 1b. Quenching by binding of different FP with Ab in the reverse micellar system is demonstrated in Fig. 2a. We see that effective

Table I. Effect of FP Structure on Quenching of FP by Binding with Ab

Medium	Quenching effectivity		
	Strong	Moderate	Low
Reverse micelles	FP-52	FP-2m	FP-2
		FP-3	FP-4
			FP-6
			FP-C2
Water	FP-52	FP-2	FP-4
	FP-3	FP-2m	FP-6
			FP-C2

quenching (25%) takes place only for one conjugate, FP-52; the use of two other conjugates, FP-3 and FP-2m, results in noticeable quenching (up to 7–8%). Other conjugates used do not give marked quenching. Fluorescence quenching for different FP conjugates in an aqueous medium is presented in Fig. 2b. We observed effective quenching for FP-52 (up to 42%) and FP-3 (35%), moderate quenching for FP-2 and FP-2m (up to 20–25%), and low quenching for FP-4 and FP-6.

The quenching efficacy (Figs. 2a and b) in water and reverse micelles using conjugates of different structure is presented in Table I. The optimal FP structure both in reverse micellar medium and in water is that of FP-52 (similar to the structure of antigen used for antiserum production). There also appears to be an interesting spacer length optimum with FP-3. However, this optimum is clear only in water (Fig. 2b); in reverse micelles (Fig. 2a) FP-2m has properties similar to those of FP-3.

Effect of Micellar Hydration Degree

We varied the hydration degree of these reverse micellar systems (W_0) by three methods.

(1) We diluted the Ab-containing reverse micellar solution with an "empty" reverse micellar solution with the same hydration degree; this procedure was repeated several times at different hydration degrees to obtain Ab dilution curves.

(2) We measured the quenching of a series of reverse micellar solutions containing the same amount of Ab and FP; the hydration degree being varied by adding different amounts of Tris buffer to the samples.

(3) We measured the quenching of a series of the samples with the same FP concentration but different amounts of aqueous Ab solution added; Q was calculated for each sample

Quenching Ab dilution curves measured for three W_0 values (38.9, 22.2, and 11.1) according to method 1 are given in Fig. 3a. Quenching values measured according to method 2 are given in Figs. 3b (for FP-2) and 3c (for FP-2m), and those according to method 3 in Fig. 3d (for FP-52). Using method 3, two curves were obtained from one experiment. The open circles in Fig. 3d are quenching data with different Ab concentrations ([Ab] increases proportional to W_0 increase), and the filled circles represent the same data recalculated per one Ab concentration (Ab = 400 nM) assuming that the response (Q) is proportional to the Ab concentration (i.e., filled circles are open circles divided per [Ab]/400 nM). These two curves have one joint point at W_0 17 ([Ab] = 400 nM).

In none of the experiments with systems of low W_0 (from 1.5 to 10) did we observe reversible quenching. At $W_0 > 10$ (see Figs. 3a–d) we observed a slight increase in quenching with an increase in W_0.

Effect of Surfactant Concentration

The most effective quenching by Ab is observed for the conjugate FP-52, so we used this conjugate to study the effect of AOT concentration on quenching in reverse micelles. Quenching curves for reverse micellar systems of different surfactant concentrations are presented in Fig. 4. The action of Ab is expressed more strongly (the same quenching effect is reached with lower Ab concentrations) at low AOT concentrations. These results are in agreement with our polarization study of the effect of AOT concentration on antipropazine Ab dilution curves in the same reverse micellar system:[10] the best response was obtained using the minimal AOT concentration of 25 mM. It is evident that if we use very high surfactant concentrations, at fixed W_0 values and total concentrations of FP and Ab, we have a higher percentage of water, and the reagents (labeled antigen and Ab) become more diluted per aqueous phase (water pool of the reverse micelle).

Propazine Calibration Curves

Propazine calibration curves (with FP-52) measured on the basis of the quenching effect (quenching fluoroimmunoassay; QFIA) are given in Fig. 5 (a, reverse micelles; b, water). We see that the minimal propazine concentration which can be detected by QFIA using FP-52 is about 100 nM (20 μg/L) in the reverse micellar system and about 10 nM (2 μg/L) in the aqueous solution. Thus, the sensitivity in reverse micellar media is lower than that in aqueous medium, but the advantage

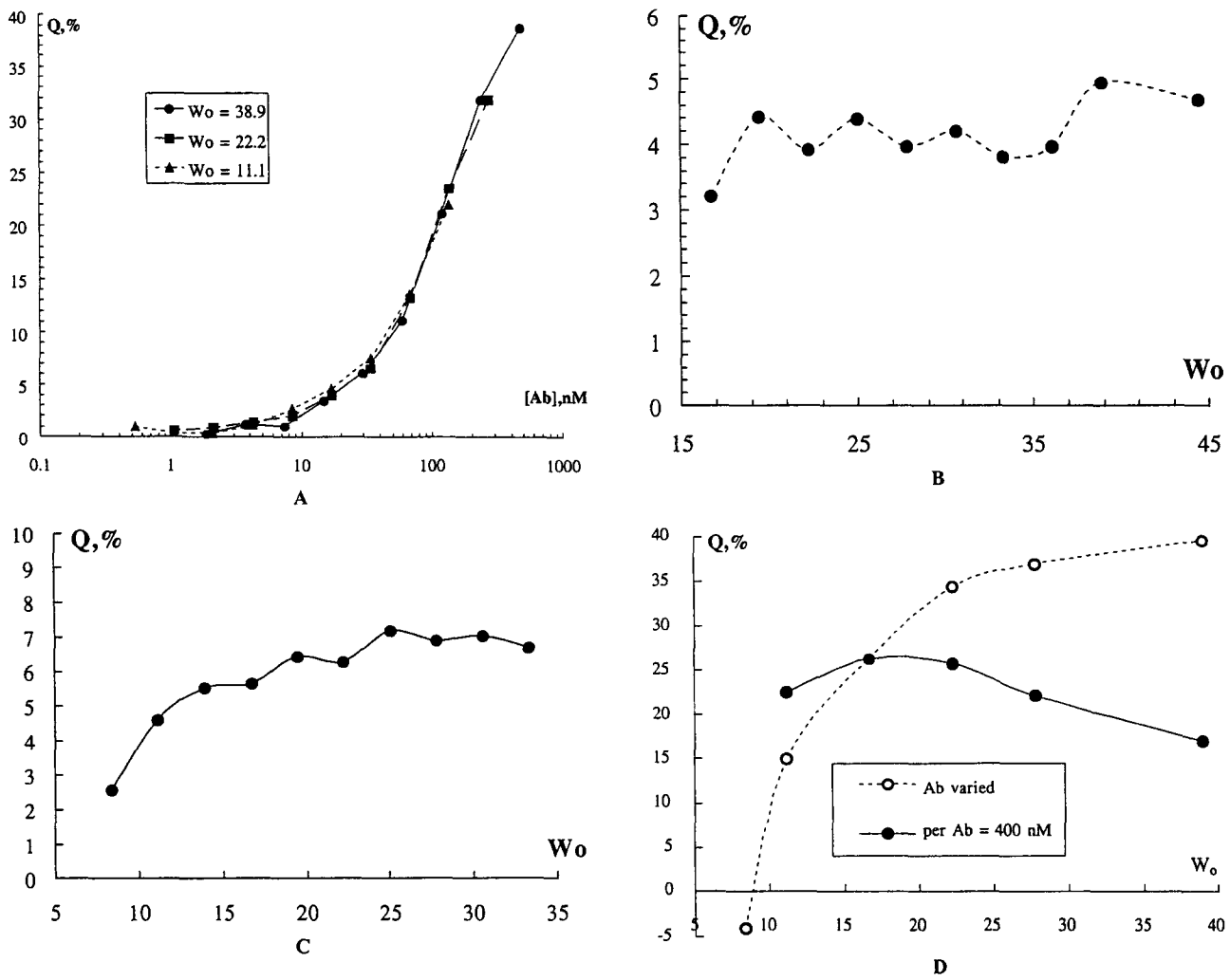

Fig. 3. FP fluorescence quenching effect Q in reverse micelles AOT/octane/water: effect of hydration degree (W_0). (a) W_0 variation by method 1 (FP = FP-52, 8 nM; time of Ab–FP-52 contact, 1 h; time of contact with the excess of added free propazine, 40 min). (b) W_0 variation by method 2 (FP = FP-2, 8 nM; [Ab] = 390 nM). (c) W_0 variation by method 2 (FP = FP-2m, 8 nM; [Ab] = 200 nM). (d) W_0 variation by method 3 (FP = FP-52, 8 nM).

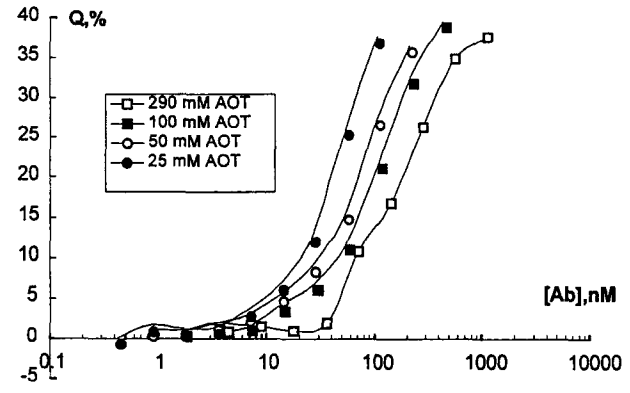

Fig. 4. Quenching efficiency by FA-52 and Ab interaction in AOT/n-octane/water as a function of surfactant (AOT) concentration (W_0 = 38; [FP] = nM).

of QFIA in reverse micelles is that the analyte can be added when dissolved in a nonpolar organic solvent, for example, chloroform, toluene, hydrocarbons, etc.

CONCLUSIONS

The antigen–antibody interaction between fluorescein-labeled propazine and antipropazine antibodies was studied by the fluorescence quenching method in nonpolar solvent (reverse micellar system n-octane/AOT). We observed quenching of the labeled hapten fluorescence after addition of the antibodies (immune complex formation) and restoration of the labeled hapten fluores-

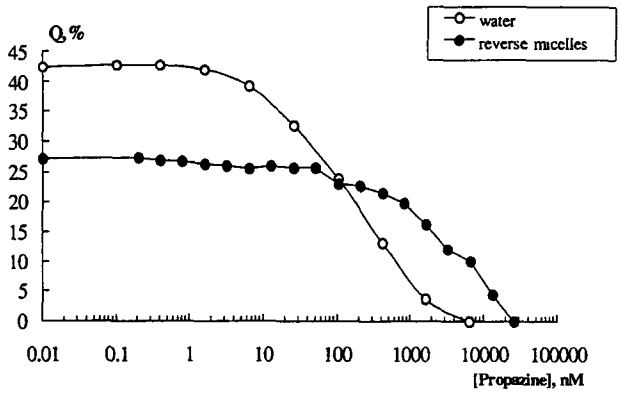

Fig. 5. Propazine QFIA calibration curve ([FP-52] = 8 n*M*; [Ab] = 0.37 m*M*; contact time of free propazine, FP-52, and Ab, 1 h) (a) in reverse micelles AOT/*n*-octane ([AOT] = 100 m*M*; W_0 = 16) and (b) in aqueous medium (50 m*M* Tris, pH 8.7).

cence after addition of an excess of free propazine to the immune complex formed. A similar quenching effect takes place in aqueous medium. We found that the quenching efficiency depends on the properties of the reverse micellar system (surfactant concentration and hydration degree) and, in both micellar and aqueous media, on the structure of the spacer between fluorescein and propazine in labeled propazine. Optimal conditions for effective quenching of the FP fluorescence by the antibodies against propazine in reverse micelles of AOT in *n*-octane are a low surfactant concentration ([AOT] = 0.1 *M* or lower) and a hydration degree allowing large reverse micelles (W_0, 15–20 or more) capable of retaining solubilized antibodies. The optimal FP structure both in reverse micellar medium and in water is that of FP-52 (similar to the structure of the antigen used for antiserum production).

Analysis of the mechanism of this quenching was not the aim of this work, but we propose that the mechanism of this quenching is similar to that of the quenching of fluorescein by monoclonal antibodies against fluorescein, studied in detail in aqueous medium.[15–19]

The presented quenching method, QFIA, could be used for propazine determination both in apolar organic solvents and in an aqueous medium. The sensitivity of QFIA in the present reversed micellar system to propazine is 100 n*M* (20 μg/L). The sensitivity of QFIA in water is 10 n*M* (2 μg/L) propazine. In the case of the reverse micellar system, propazine can be added when dissolved in a nonpolar organic solvent, for example, in AOT/octane, in an octane/chloroform mixture, or in chloroform. This makes possible its use for analysis directly in pesticide extracts in nonpolar organic solvents.

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