Journal of Fluorescence, Vol. 7, No. 4, 1997

Detection of 2,4-Dichlorophenoxyacetic Acid in Reverse Micelles AOT/*n*-Octane by Polarization and Quenching Fluoroimmunoassays

Evgenia G. Matveeva,[1,3] Valentina A. Popova,[2] and Sergei A. Eremin[2]

Received March 13, 1997; revised June 12, 1997; accepted September 26, 1997

Two fluoroimmunoassays for detection of 2,4-dichlorophenoxyacetic acid (2,4-D) in an apolar organic solvent, reverse micelles of Aerosol OT in n-octane, are proposed. Both assays are homogeneous methods, meaning that no separation of free and bound fraction of analyte and no washing steps are required. To perform the assay just add the fluorescein-labeled 2,4-D (FD) and monoclonal antibody to the sample and measure the analytical signal on the Abbott TDx Analyzer. The binding of antibodies to FD can be detected by the decrease in fluorescence intensity and also by the increase in fluorescence polarization. The sensitivity of the developed methods was strongly dependent on the micellar matrix (micellar hydration degree and surfactant concentration). The detection limits of 2,4-D in optimal reverse micellar medium are 0.10 μg/L (polarization method) and 0.12 μg/L (quenching method). These sensitivities are significantly better compared to the detection limits in an aqueous medium, 0.6 and 0.4 μg/L, respectively, using the same reagents.

KEY WORDS: Polarization; quenching; fluoroimmunoassay; 2,4-dichlorophenoxyacetic acid; reverse micelles.

INTRODUCTION

The method of polarization fluoroimmunoassay (PFIA) is widely used now for rapid determination of pesticides,[1-5] among others the widespread herbicide 2,4-dichlorophenoxyacetic acid (2,4-D).[1,3-5] PFIA is a homogeneous competition method based on detection of the difference of fluorescence polarization between a small fluorescence-labeled hapten (quick rotation of the molecule; low polarization) and its immunocomplex with specific antibody (low rotation of the large immunocomplex; high polarization).[1,6,7] In a similar manner, the quenching fluoroimmunoassay (QFIA) is based on the detection of the difference of fluorescence intensity between a fluorescence-labeled hapten and its immunocomplex.[7,8] The effect of fluorescence quenching at binding of specific antibodies to fluorescein-labeled antigens (for example, conalbumin,[9] haloperidol,[10], and fluorescein itself[11-14]) was used in investigations of the kinetic and thermodynamic binding parameters. The quenching phenomena resulting from binding of fluorescein to antifluorescein antibodies are due to the hydrophobic nature of the active site and the corresponding change in the fluorescein microenvironment to be more hydrophobic after binding.[11-14]

Reverse micellar systems of surfactants in nonpolar organic solvents are known as homogeneous organic media which are able to solubilize biologically active substances, among others antibodies,[15-18] giving optically clear solutions. The specific activity of the antibodies is retained after their solubilization in reverse micellar systems. The advantage of reverse micellar sys-

[1] A.N. Bakh Institute of Biochemistry, Russian Academy of Science, Leninsky pr., 33, Moscow 117071, Russia.
[2] Division for Chemical Enzymology, Chemistry Department, M. V. Lomonosov Moscow State University, Moscow 119899, Russia.
[3] To whom correspondence should be addressed. Fax: +7-095-9542732. e-mail: inbio@glas.apc.org.

1053-0509/97/1200-0251$12.50/0

tems is that the analyte can be added when dissolved in a nonpolar organic solvent. The possibility of PFIA of atrazine[19] and QFIA of propazine[20] in reverse micellar systems was demonstrated in our previous publications.

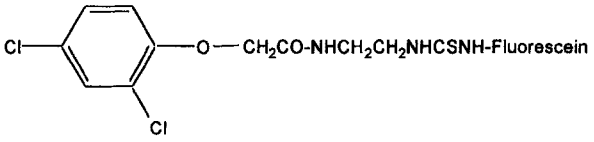
In this work, we used a reversed micellar system for the determination of 2,4-D, a hydrophobic pesticide which was different from the triazine pesticides studied before. The aim of this work was (1) to demonstrate the possibility of use the two methods, PFIA and QFIA, for the analysis of 2,4-D in reverse micellar medium, (2) to optimize the system by variation of the main properties of the micellar matrix [surfactant hydration degree W_0 (W_0 = [H_2O]/[AOT]) and surfactant concentration], and (3) to compare the detection limits of 2,4-D obtained by developed methods with those provided by PFIA and QFIA in aqueous medium using the same equipment and reagents (antibody preparation, labeled antigen).

EXPERIMENTAL

2,4-D was purchased from Aldrich. Aerosol OT, sodium bis(2-ethylhexyl)sulfosuccinate (AOT), was purchased from Serva. *n*-Octane was purchased from Reachim (Russia). Other chemicals were from Sigma. The aqueous phase was buffered with 50 m*M* Tris, pH 8.45, in all experiments. This buffer was optimal for solubilization of antibodies (Ab) and a good quantum yield of fluorescence. Reverse micellar solutions with different hydration degrees W_0 (W_0 = [H_2O]/[AOT]) were prepared by the addition of different volumes of the aqueous buffer solution to equal volumes of AOT/*n*-octane. For example, in the case of W_0 = 16.7 we kept constant in each sample the ratio of 30 µl of aqueous solution (containing or not containing antibodies or fluorescein labeled 2,4-D) per 1 ml of 100 m*M* AOT/*n*-octane.

The TDx Analyzer (Abbott Laboratories, USA) was used for all experimental work. This instrument on Photo-Check mode makes it possible to measure simultaneously fluorescence polarization and intensity. Fluorescence polarization (P) was determined by exciting the sample with vertically polarized light and measuring the intensity of both the vertically (I_v) and the horizontally (I_h) polarized components of the emitted fluorescence; $P = (I_v - I_h)/(I_v + I_h)$. The TDx Analyzer measured 10 samples in one cycle at 7 min and printed the fluorescence polarization in milliunits, mP (mP = 1000 P), and fluorescence intensity (in related units).

Fluorescein-labeled 2,4-D (FD) was synthesized from 2,4-D and fluoresceinthiocarbamyl ethylenediamine, which was prepared as described before.[3] In brief, 4 mg (20 µmol) of 2,4-D, 4 mg (40 µmol) of *N*-hydroxysuccinimide, and 16 mg (80 µmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were dissolved in 1 ml of dimethylformamide and stirred for 2 h at room temperature, then 5 mg (10 µmol) of fluoresceinthiocarbamyl ethylenediamine was added. After 1 h of stirring, a small portion (50 µl) of this reaction mixture was separated by thin-layer chromatography and developed with ethyl acetate/methanol/acetic acid (60/15/1). The major yellow band at R_f 0.9 was scraped from the plates and extracted with methanol, and the labeled product was stored at −20°C in the dark. The concentration of FD was estimated spectrophotometrically at 492 nm, assuming that the absorptivity in sodium bicarbonate buffer (50 m*M*, pH 9.0) is the same as for fluorescein ($8.78 \cdot 10^4$ l·mol^{-1}·cm^{-1}). Working FD solutions (1–10 µ*M* in 50 m*M* Tris buffer (pH 8.5) were prepared from stock FD solution in methanol ($1 \cdot 10^{-4}$ *M*). The structure of FD is given below.



The solution of FD in reversed micelles (with W_0 = 16.7) was prepared by the addition of 300 µl of FD solution (1.3 µ*M*) in 50 m*M* aqueous Tris buffer to 10 ml of 100 m*M* AOT in *n*-octane. The system became optically transparent after 3–5 s of shaking. Reverse micellar solutions of FD with different W_0 values (2.8–55.5) were prepared in the same way by the addition of different volumes of FD solutions (5–120 µl of aqueous FD solution per 1 ml of 100 m*M* AOT/*n*-octane).

Monoclonal antibodies against 2,4-D (Lot 4/E2/G2) were a generous gift from Dr. Milan Franek, Veterinary Research Institute, Brno, Czech Republic.[21] The IgG fraction from ascitic fluids was precipitated by a half-saturated aqueous ammonium sulfate solution and dialyzed against 50 m*M* Tris (pH 8.5). Protein concentration was estimated spectrophotometrically at 280 nm (Hitachi-557 spectrophotometer), assuming an absorptivity $\varepsilon = 9.3 \cdot 10^6$ M^{-1} cm^{-1}.

A reverse micellar solution of Ab with hydration degree W_0 = [H_2O]/[AOT] = 16.7 was prepared by the addition of 300 µl of Ab solution (40 µ*M*) in 50 m*M* aqueous Tris buffer to 10 ml of 100 m*M* AOT in *n*-octane; the final concentration of Ab was 1.17 µ*M*. The system became optically transparent after 0.1–1 min of shaking. Antibody solutions in reverse micelles with different Ab concentrations were prepared by step-to-step dilution (1:2) of the initial micellar Ab solution with

reverse micellar solution without Ab with the same hydration degree. For reverse micelles with hydration degree W_0 = 16.7 this solution was prepared by the addition of 300 μl of 50 m*M* aqueous Tris buffer to 10 ml of 100 m*M* AOT in *n*-octane. Reverse micellar solutions of Ab with different W_0 values (2.8–55.5) were prepared in the same way by the addition of different volumes of Ab solutions (5–120 μl of aqueous Ab solution per 1 ml of 100 m*M* AOT/*n*-octane).

Antibody Dilution Curve. Two-tenths milliliter of FD in reverse micelles was mixed with 0.8 ml of Ab solution in reverse micelles (both mixed components were of the same hydration degree); a series of the mixtures was obtained using Ab solutions of different concentrations. After 10–30 min of incubation at room temperature the mixtures were measured on the Abbott TDx Analyzer in the Photo-Check mode. Dilution curves in the cases of other hydration degrees were obtained similarly using Ab and FD solutions in reversed micelles of the desired W_0.

Calibration Curve. The initial mixture of 2,4-D and FD solutions in reverse micelles was prepared by the addition of 10–20 μl of the stock 2,4-D solution in methanol (0.46 mg/ml) to 1.5 ml of FD-containing micellar solution. The standard solutions of the mixtures of 2,4-D and FD in reverse micelles were prepared by serial dilution of the initial solution of 2,4-D in reverse micelles with the same FD-containing micellar solution without 2,4-D. Then 100 μl of Ab reverse micellar solution was added to 1 ml of each of the standard solutions of the mixture of 2,4-D and FD solution in reverse micelles; the hydration degree of both solutions was the same. After a short incubation (15–30 min) at room temperature, the fluorescence intensity and polarization of the resulting mixtures were measured on the Abbott TDx Analyzer in the Photo-Check mode. A calibration curve in water was obtained in a similar manner using FD- and Ab-containing aqueous solutions instead of the reverse micellar ones.

RESULTS AND DISCUSSION

The antigen–antibody interaction in the reverse micellar system of Aerosol OT in *n*-octane has been investigated in our previous research. Homogeneous fluoroimmunoassays based on the fluorescence quenching measurement (QFIA) for atrazine[19] and on the fluorescence polarization (PFIA) for propazine[20] in apolar organic media were developed. The main advantage of these two new methods is that the analyte can be directly analyzed in nonpolar organic solvents. In this paper the same phenomena were investigated for the more polar pesticide 2,4-D, which cannot be directly analyzed by the gas chromatographic (GC) method and must first be at derivatized to ether.

The sensitivity of the immunoassay is one of the crucial parameters for pesticide detection. Therefore, the main purpose of assay optimization was to find the minimal detection limit of analyte in the reverse micellar system. The sensitivity of the PFIA and QFIA to 2,4-D was estimated according to IUPAC recommendations by the "3-σ method"[22] from calibration curves as follows. The standard deviation of the response (fluorescence polarization or intensity) for a zero 2,4-D concentration ("zero response") was calculated (10 replicates). Then the detection limit (DL) was determined from the calibration curve as the response at zero concentration minus the triple standard deviation (for PFIA; polarization decreases with increasing [2,4-D]) or plus the triple standard deviation (for QFIA; fluorescence intensity increases with increasing [2,4-D]).

The 50 m*M* Tris buffer (pH 8.5) was used in all experiments, because this basic buffer system is optimal for solubilization of Ab in reverse micelle AOT/*n* octane and a good quantum yield of FD fluorescence. It is clear that the concentration of fluorescein-labeled analyte must be as low as possible (depending on the equipment) to reach the lowest DL in competitive immunoassay. The background fluorescence intensity for 50 m*M* Tris buffer (pH 8.5) was 200–400 relative units. For this reason, the fluorescence of a fluorescein labeled hapten which can be correctly detected by the TDx Analyzer must be about 2000–3000 relative units. The concentration of FD corresponding to this level was approximately 5–8 n*M*, and this value was chosen as the minimal FD concentration.

Polarization Fluoroimmunoassay of 2,4-D in Reverse Micellar Medium

The fluorescence polarization of the labeled hapten FD in reverse micellar medium increases in the presence of specific antibodies (Fig. 1a), as well as in aqueous medium. The increase in polarization in the reverse micellar system is direct confirmation of the antigen–antibody binding. The antibody dilution curve in aqueous medium is nearly the same, but the polarization of FD not bound to Ab (at [Ab] = 0) is higher in reverse micellar solutions (40–100 mP, depending on the hydration degree of reverse micelles and surfactant concentration) than that in aqueous medium (27–30 mP). This difference is due to the more hydrophobic environment of FD in micellar medium compared to that in water. The po-

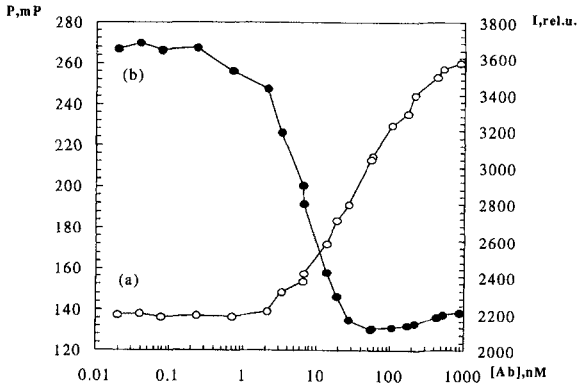


Fig. 1. The antibody dilution curve in reverse micelles of AOT/*n*-octane (W_0 = 16.7, [AOT] = 100 m*M*, [FD] = 7.8 n*M*): (a) FD fluorescence polarization (open circles); (b) FD fluorescence intensity (filled circles).

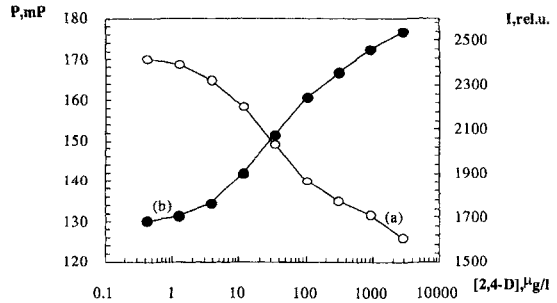


Fig. 2. PFIA (a; open circles) and QFIA (b; filled circles) calibration curves for 2,4-D in reverse micelles of AOT/*n*-octane (W_0 = 16.7, [AOT] = 100 m*M*, [FD] = 4.7 n*M*, [Ab] = 13 n*M*).

Table I. Effect of Surfactant Hydration Degree W_0 on the Detection Limits (DL) of 2,4-D in Reverse Micelles of AOT/*n*-Octane ([AOT] = 100 m*M*) by Polarization (PFIA) and Quenching (QFIA) Fluoroimmunoassays

W_0	DL of PFIA (μg/L)	DL of QFIA (μg/L)
2.8	*[a]	*
4.2	200	*
8.3	3.60	1.93
12.1	1.36	0.40
16.7	1.26	1.03
33.3	1.44	0.63
41.7	0.23	0.28
55.5	0.37	0.14

[a] Not detectable.

larization of FD varies from 27 to 350 mP in water and from 40–100 to 250 mP in reverse micelles of AOT/*n*-octane. As a result, the sensitivity of the PFIA in the organic solvent could decrease, because the range of polarization change is wider in water than in reverse micelles.

The antipropazine antibody dilution curves using fluorescein-labeled propazine in the same reverse micellar system[19] look similar to those presented in Fig. 1a. This confirms the strong antigen–antibody interaction in the reverse micellar system for any type of analyte and the possibility of developing a direct immunoassay in organic solvents.

The typical calibration curve of PFIA for 2,4-D is given in Fig. 2a. The DL values defined from such curves, at a starting hydration degree of 16.7, depend on both FD and Ab concentrations. Our possibilities of varying the FD concentration were limited to the range 5–50 n*M*, due to the features of the equipment used. The range of correct fluorescence polarization and intensity measurements using the TDx Analyzer is 2000–60,000 relative units of fluorescence intensity. The optimal Ab concentration for PFIA in the reverse micellar system was 13 n*M*, which gives approximately 70% binding of FD at the minimal concentration (5 n*M*). These values ([FD] = 5 n*M*, [Ab] = 13 n*M*) were fixed and used in further experiments.

Effect of the Micellar Matrix on the Fluorescence Polarization

Hydration degree is the ratio of water to surfactant concentration (W_0 = [H_2O]/[AOT]). This parameter is the most important characteristic of the reverse micellar system; the W_0 value defines the size of the reverse micelle. The PFIA calibration curves for 2,4-D were obtained for different W_0 values in the range 2.8–55.5. The calculated DLs corresponding to each W_0 are presented in Table I. The best DL values are found to be at the maximum possible W_0 = 55.5. The W_0 could not be higher than 55.5 because the system became turbid and separated into a two-phase water–organic mixture. At low W_0, the polarization values do not depend on the concentration of 2,4-D, but depend dramatically on the time of the contact of the reagents. The detection of 2,4-D in such a system is not possible. The size of the water pool of the reverse micelle at a low W_0 is too small to enclose the Ab molecule. As a result, the micelles do not prevent Ab inactivation by the organic solvent. The highest possible hydration degree (W_0 = 55.5) of AOT in *n*-octane was chosen for the next PFIA experiments on 2,4-D.

The effect of AOT concentration on DL in reverse micelles was studied at optimal (maximal) W_0 = 55.5. The concentration of AOT was screened in the range 5–100 m*M*. The DL values depend on the AOT concen-

Table II. Effect of AOT Concentration on the Detection Limits (DL) of 2,4-D in Reverse Micelles of AOT/*n*-Octane (W_0 = 55.5) by Polarization (PFIA) and Quenching (QFIA) Fluoroimmunoassays

[AOT] (m*M*)	DL of PFIA (μg/L)	DL of QFIA (μg/L)
100	0.37	0.14
50	0.10	0.12
20	0.10	0.27
10	0.27	0.29
5	1–3	1–3

Table III. Detection Limits (DL) of 2,4-D in Reverse Micelles of AOT/*n*-Octane (W_0 = 55.5, [AOT] = 50 m*M*) and in Water by Polarization (PFIA) and Quenching (QFIA) Fluoroimmunoassays ([FD] = 5 n*M*, [Ab] = 13 n*M*)

Reaction medium	DL of PFIA (μg/L)	DL of QFIA (μg/L)
Water	0.63	0.43
Reverse micelles of AOT/*n*-octane	0.10	0.12

tration at the same hydration degree (Table II), but the optimal range of AOT concentrations is rather wide (20–50 m*M*). The AOT concentration effect[19] for antipropazine Ab dilution curves was investigated in the range from 25 to 290 m*M* and the best response was obtained using a minimal AOT concentration of 25 m*M*. Thus, AOT at concentrations of 20–50 m*M* could be used in the immunoassay in the reverse micellar system.

Quenching Fluoroimmunoassay of 2,4-D in Reverse Micellar Medium

The Abbott TDx Analyzer in the Photo-Check mode presents the possibility of simultaneous measurement of both fluorescence polarization and intensity in the same experiment. It gives the additional possibility of detection of the analytical signal. Moreover, any fluorimeter could be used for QFIA in a reverse micellar system for pesticides. As shown in Fig. 1b, the FD fluorescence intensity in the reverse micellar system is quenched after the addition of antibodies against 2,4-D. This quenching effect does not take place in the presence of excess free 2,4-D, which competes with FD in the process of immune complex formation. A typical Ab dilution curve (see Fig. 1b) and QFIA calibration curve (Fig. 2b) in reverse micelles look similar to those obtained by PFIA (Figs. 1a and 2a), but with an inverted slope. The best QFIA results were obtained using [FD] 5 n*M* and [Ab] 13 n*M*, as in the PFIA study. Quenching efficiency also depends on the properties of the reverse micellar system (surfactant concentration and hydration degree).

Effect of the Micellar Matrix on the Fluorescence Intensity

The effect of the hydration degree W_0 on the DL of 2,4-D studied by QFIA is presented in Table I. This effect is similar to that obtained by PFIA; the best DL values were achieved with the maximal W_0 used (W_0 = 55.5). In the case of a low W_0 (small micelles), the reverse micellar system cannot be used for 2,4-D detection. This conclusion is in agreement with the results obtained in antigen–antibody interactions in the reverse micellar system AOT/*n*-octane for fluorescein-labeled atrazine[20] and propazine:[23] the fluorescence quenching of fluorescein-labeled hapten by antibodies was effective only at a high hydration degree ($W_0 > 10$–15).

The effect of surfactant concentration on quenching (studied by W_0 = 55.5) is identical to that on polarization fluorescence: the best DL values are obtained at optimal AOT concentrations (see Table II). However, the optimal AOT concentrations for the DL of 2,4-D by QFIA are somewhat higher (50–100 m*M*) than those by PFIA (20–50 m*M*). This concentration of AOT is also somewhat higher than the AOT concentration optimal for QFIA of labeled atrazine—20 m*M*[20] (AOT in Ref. 20 was varied from 20 to 300 m*M*)—and for QFIA of labeled propazine—25 m*M*[22] (AOT in Ref. 23 was varied from 25 to 290 m*M*). Detailed analysis of surfactant concentration effects was not the aim of this work, but our conclusion is that, for both QFIA and PFIA, there is an optimal range of surfactant concentrations. If the surfactant concentration is higher, at fixed W_0 and total concentrations of FD and Ab, the percentage of water in reversed micellers will also be higher. Therefore, the reagents (FD and Ab) became more diluted in the aqueous phase (water pool of the reverse micelle). If the surfactant concentration is lower than 25–50 m*M*, its amount will be, probably, insufficient to construct the Ab-containing reverse micelle.

Analytical Parameters of PFIA and QFIA for 2,4-D in the Reverse Micellar System

The detection limit was chosen as the main characteristic to study the effect of the micellar matrix on the sensitivity of both methods, PFIA and QFIA, in the reverse micellar system AOT/*n*-octane. The DL values under optimal conditions in reverse micelles and in aqueous solution are summarized in Table III. The sensitivity of both developed immunoassays of 2,4-D in re-

verse micelles is ca. five times better than that in aqueous solution.

Precision was studied by adding two amounts of 2,4-D to the samples, to obtain a final concentration of 10 and 100 μg/L in the reverse micellar solution (W_0 = 55.5, [AOT] = 50 m*M*). The relative standard deviations (n = 10) were 6.3 and 5.1% (PFIA) and 7.2 and 4.3% (QFIA), respectively.

Recovery was studied by the addition of different amounts of 2,4-D dissolved in 5 m*M* AOT/*n*-octane (10–20 μl) to 2 ml of reverse micellar medium (W_0 = 55.5, [AOT] = 50 m*M*); the final concentrations of 2,4-D in reverse micelles were 10, 50, and 100 μg/L (each in triplicate). The recoveries obtained by developed PFIA and QFIA varied from 90 to 112% (10 and 100 μg/L) and from 82 to 118% (50 μg/L).

CONCLUSIONS

The reverse micellar system Aerosol OT/*n*-octane was used for 2,4-D detection by two homogeneous immunoassays, PFIA and QFIA. The sensitivities of both PFIA and QFIA depend on the characteristics of the reverse micellar system (surfactant concentration and hydration degree W_0). Optimal conditions for PFIA and QFIA of 2,4-D in reverse micelles of AOT in *n*-octane were an [AOT] of 50 m*M* and a hydration degree high enough to obtain a large size of reverse micelles (W_0 = 40–55) which were capable of retaining solubilized antibodies. The same effects of reverse micelles of AOT/*n*-octane were observed for detection of the herbicide propazine by PFIA[19] and QFIA[23] and atrazine by QFIA.[20]

The two immunoassays can be used for 2,4-D detection both in apolar organic solvents and in aqueous media. The minimum detection limit of both PFIA and QFIA in the presented reverse micellar system for 2,4-D is about 0.1 μg/L. This DL is several times lower than that in aqueous medium using the same reagents and equipment (Table III). The PFIA method is a little more sensitive and independent of endogenous fluorescein compounds in real water samples than QFIA. However, special instrument like the Abbott TDx Analyzer polarization fluorimeter is needed to carry out the PFIA method. The QFIA method can be performed using any simple and sensitive fluorimeter. At the end, in the case of PFIA or QFIA in a reverse micellar system, hapten can be added when dissolved in a nonpolar organic solvent. This makes it possible to simplify the sampling process and to analyze directly the pesticide extracts in nonpolar organic solvents.

ACKNOWLEDGMENTS

The authors greatly appreciate the financial support of this work by INTAS Grant 93-2223 and by Russian Foundation of Fundamental Research Grant 95-03-08039a.

REFERENCES

1. S. A. Eremin (1995) in J. O. Nelson, A. E. Karu, and R. B. Wong (Eds.), *Immunoanalysis of Agrochemicals*, ACS Symposium Series 586, American Chemical Society, Washington, DC, pp. 223–234.
2. F. Garcia-Sanchez, A. Navas, F. Alonso, and J. Lovillo (1993) *J. Agr. Food Chem.* **41**, 2215–2219.
3. S. A. Eremin, I. M. Lunskaya, and A. M. Egorov (1993) *Bioorg. Khim.* (Russ.) **19**, 836–843.
4. Yu. V. Lukin, I. M. Dokuchaev, I. M. Polyak, and S. A. Eremin (1994) *Anal. Lett.* **27**, 2973–2982.
5. S. A. Eremin, A. M. Egorov, O. A. Melnichenko, and A. A. Tumanov (1995) *J. Anal. Chem.* **50**, 198–201 [translated from Russian].
6. M. C. Gutierrez, A. Gomez-Hens, and D. Perez-Bendito (1989) *Talanta* **36**, 1187–1201.
7. R. M. Nakamura (1992) in R. M. Nakamura, Y. Kasahara, and G. A. Rechnitz (Eds.), *Immunochemical Assays and Biosensors Technology for the 1990s*, American Society for Microbiology, Washington, DC, pp. 205–227.
8. P. R. Droupadi, T. Nanavaty, C. Smith, D. D. Johnson, M. Adamczyk, and D. S. Linthicum (1995) *J. Fluoresc.* **5**, 273–277.
9. R. P. Tengerdy (1967) *J. Immunol.* **99**, 126–132.
10. C. Mandal, C. Harrington, M. Adamczyk, and D. S. Linthicum (1992) *Biochem. Pharmacol.* **43**, 855–863.
11. D. Lopatin and E. W. Voss, Jr. (1971) *Biochemistry* **10**, 208–213.
12. D. M. Kranz, J. H. Herron, and E. W. Voss, Jr. (1982) *J. Biol. Chem.* **257**, 6987–6995.
13. J. H. Herron, D. M. Kranz, D. M. Jameson, and E. W. Voss, Jr. (1986) *Biochemistry* **25**, 4602–4609.
14. W. D. Bedzyk, C. A. Swindlehurst, and E. W. Voss, Jr. (1992) *Biochim. Biophys. Acta* **1119**, 27–34.
15. A. N. Eryomin, M. I. Savenkova, and D. I. Metelitsa (1986) *Bioorg. Khim.* (Russ.) **12**, 606–612.
16. A. V. Kabanov, M. M. Khrutskaya, S. A. Eremin, N. L. Klyachko, and A. V. Levashov (1989) *Anal. Biochem.* **181**, 145–148.
17. N. P. Groome, M. Vacher, Cl. Nicot, and M. Waks (1990) *Biochem. Int.* **21**, 1–7.
18. J. M. Francis and D. H. Craston (1994) *Analyst* **119**, 1801–1805.
19. E. G. Matveeva, J. V. Samsonova, and S. A. Eremin (1996) *Bioorg. Khim.* (Russ.) **22**, 931–937.
20. E. G. Matveeva, N. S. Melik-Nubarov, P. Miethe, and A. V. Levashov (1996) *Anal. Biochem.* **234**, 13–18.
21. M. Franek, V. Kolar, M. Granatova, and Z. Nevorankova (1994) *J. Agr. Food. Chem.* **42**, 1369–1374.
22. G. L. Long and J. D. Winefordner (1983) *Anal. Chem.* **55**, 712A–724A.
23. E. G. Matveeva, J. V. Samsonova, and S. A. Eremin (1997) *J. Fluoresc.* **7**, 211–216.