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Short communication

Modulated anisotropy fluorescence for quantitative determination of carbaryl and benomyl

A. Navas Díaz *, F. García Sánchez, M.M. López Guerrero

Departamento de Química Analítica, Facultad de Ciencias, Universidad de Málaga, 29071 Malaga, Spain

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Abstract

Two individual components in mixtures have been resolved by frequency domain fluorescence technique by measuring the observable quantities which characterize the anisotropy decay; differential anisotropy phase and modulated anisotropy ratio (MAR), which in turn are related to the rotational correlation time. The method presented here is capable of directly resolving binaries mixtures of fluorophores on the basis of differences in their rotational diffusion rates. Our results demonstrate that modulation anisotropy ratio measurements can be used for quantitative determination of small analytes, carbaryl and benomyl, having identical or nearly identical fluorescence spectra. This methodology can be applied with good results when the fluorophores have a suitable MAR difference.
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1. Introduction

Fluorimetric measurements based on selection of excitation and emission wavelengths generally lack of selectivity because of the wide profiles of its excitation and emission spectra. However, additional fluorescence parameters can be used to resolve fluorophore mixtures and obtain the benefits of the high sensitivity of fluorescence. Selective quenching, fluorescence anisotropy and fluorescence lifetimes are examples of such parameters [1–3].

Measurements of fluorescence anisotropy or polarization are widely used in the biophysical and biomedical sciences, and more barely in analytical applications. Upon excitation with polarized light, the emission from many samples is also polarized. The extent of polarization of the emission is described in terms of the anisotropy. Depolarization of the emission can be caused by a number of phenomena, being rotational diffusion of fluorophores one common cause [3].

These diffusive motions, depend upon the viscosity of the solvent and the size and shape of the rotating molecule. That is, a large fluorescent species will rotate more slowly than a small species. Thus, for small fluorophores in solutions of low viscosity, the rate of rotational diffusion is

* Corresponding author. Tel.: +34-52-131880; fax: +34-52-131884.
E-mail address: a_navas@uma.es (A.N. Díaz).

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typically faster than the rate of emission. Under these conditions, the emission is depolarized and the anisotropy is close to zero. Because of this, polarization measurements are less used for small molecules.

On the other hand, because of molecular motion, solute–solvent interactions and collisions, an anisotropy decay as a function of time is induced. The result of this anisotropy decay is a time-dependent depolarization (i.e. rotational diffusion rate) of the resulting fluorescence, which can also be employed as a selectivity parameter [4].

Because the time scale of rotational diffusion of large molecules is comparable to the decay time of many fluorophores, the dependence of fluorescence anisotropy upon rotational motion has resulted in numerous applications in biochemical research. In contrast, analytical application to small molecules is scarce, although mixtures have been analysed successfully by using steady-state anisotropy measurements [5–10], including small molecules. Also, anisotropy dynamic fluorescence measurements have been used to multicomponent analysis but of large molecules [11].

In this study, we apply the anisotropy frequency domain fluorimetric technique to resolve mixtures of two pesticides, benomyl and carbaryl. These compounds have similar excitation-emission profiles and their steady-state anisotropy difference has been previously used to resolve the binary mixture [9]. In this work anisotropy dynamic fluorescence allows to unequivocally resolving of these spectrally overlapping constituents on the basis of the difference between the rotational diffusion rates of the species.

2. Theory

The average anisotropy ($\bar{r}$)is given by

$$\bar{r} = \sum_{\mathrm{i}} r_{\mathrm{i}} f_{\mathrm{i}} \tag{1}$$

where r_{i} indicates the anisotropies of the individual species and f_{i} corresponds to the fractional fluorescence intensities for each fluorophore.

Furthermore, following pulsed excitation, the decay of fluorescence anisotropy [$r(t)$] is given by

$$r(t) = \sum_{\mathrm{j}} r_0 \exp(-t/\theta_{\mathrm{j}}) \tag{2}$$

where r_0 is the anisotropy at $t = 0$ and θ_{j} is the rotational correlation time.

Multiexponential anisotropy decay can also occur for a mixture of independently rotating fluorophores. For a mixture of two fluorophores the anisotropy from the mixture $r(t)$ is an intensity-weighted average of the contribution from each fluorophore.

$$r(t) = r_1(t) f_1(t) + r_2(t) f_2(t) \tag{3}$$

where $r_1(t)$ and $r_2(t)$ are the anisotropy decays and $f_1(t)$ and $f_2(t)$ are the fractional time-dependent intensities for each fluorophore; $f_1(t) = I_1(t)/I_1(t) + I_2(t)$, similarly for $f_2(t)$, being $I_1(t)$ and $I_2(t)$ the time-dependent fluorescences which, in turn, are proportional to concentrations of components 1 and 2, respectively. Additionally, the fractional time-dependent intensities can be determined by the decay times of each fluorophore.

In the frequency domain, there are two observable quantities, which characterize the anisotropy decay [4] and which are used for the experimental procedures and the data treatment. These are the phase shift Δ_ω, at the modulation frequency ω, between the perpendicular ($\phi_\perp$) and the parallel ($\phi_\parallel$) components of the emission,

$$\Delta_\omega = (\phi_\perp) - (\phi_\parallel) \tag{4}$$

and the ratio of the modulated amplitudes of the parallel ($m_\parallel$) and the perpendicular ($m_\perp$) components of the modulated emission,

$$\Lambda_\omega = (m_\parallel)/(m_\perp) \tag{5}$$

At present, almost all fluorescence domain anisotropy decays are measured by the differential method; it is to say, by rotation of the emission polarizer, and direct measurement of the phase difference (Δ_ω) and/or modulated anisotropy ratio (MAR) (Λ_ω).

For a mixture of two fluorophores each having different rotational correlation times, the phase difference $\Delta_{\omega\mathrm{M}}$ or modulated anisotropy $\Lambda_{\omega\mathrm{M}}$, at fixed wavelength and frequency, similarly to the Eq. (3) for measurements in the time-domain, is an intensity-weighed average of the contribution from each fluorophore:

$$\Lambda_{\omega M} = \Lambda_{\omega 1} f_1(t) + \Lambda_{\omega 2} f_2(t) \quad (6)$$

being $\Lambda_{\omega 1}$ and $\Lambda_{\omega 2}$ the modulated anisotropy and $f_1(t)$ and $f_2(t)$ the fractional time-dependent intensities for each fluorophore. Keeping $f_1(t) + f_2(t) = \text{constant}$, and combining this expression with Eq. (6) it is deduced:

$$f_2(t)/f_1(t) = \Lambda_{\omega 1} - \Lambda_{\omega M}/\Lambda_{\omega M} - \Lambda_{\omega 2} \quad (7)$$

Analogous expression exists for the phase difference (Δ_ω).

Since $\Delta_{\omega 1}$ and $\Delta_{\omega 2}$, or $\Lambda_{\omega 1}$ and $\Lambda_{\omega 2}$ of the pure compounds are invariant at a fixed frequency (whenever the concentration of pure compounds) and all the experimental conditions are constant, the changes in $\Delta_{\omega M}$ or $\Lambda_{\omega M}$ occur as a result of concentration changes in the system. Therefore, simply by determining Δ_ω and/or $\Lambda_{\omega,}$ and $f_1(t)$ and $f_2(t)$ at fixed wavelength and modulation frequency (Fig. 1), and without need of additional knowledge of the system, quantitative determination of one analyte in presence of the other can be calculated by using the expression in Eq. (7).

3. Experimental

3.1. Chemicals

Carbaryl (1-naphtyl-*N*-methylcarbamate) (M.W., 202.2) was provided by Riedel de Haen and benomyl (methyl1-(butylcarbamoyl)benzimidazol-2-yl carbamate) (M.W., 290.3) by Dr S. Ehrenstofer. Both pesticides stock solutions were prepared in methanol. Methanol, glycerine and ethyleneglycol were obtained from Merck. Mixtures of carbaryl and benomyl in glycerine and in ethyleneglycol were prepared by appropriately diluting stock solutions of each pesticide.

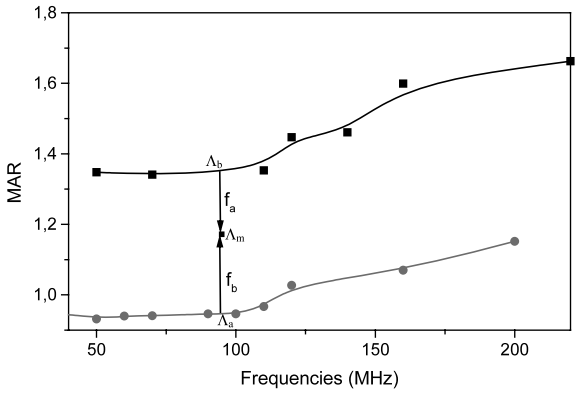

Fig. 1. Modulation ratio for 1×10^{-4} M carbaryl (below) and 1×10^{-4} M benomyl (up) in 50% (v:v) glycerine:water.

3.2. Apparatus and measurements

Fluorescence measurements were made with an Aminco SLM 48000S spectrofluorimeter equipped with a 450 W Xenon lamp source, Hamamatsu R928 photomultiplier tube detector, Pockel cell electro-optic modulator and Glan–Thompson polarizers which are rotated manually using wheels projecting from the top of the optical module.

The excitation monochromator was set at 290 nm. For selection of the emission wavelength a cut-off filter (320 nm) was placed in the sample emission receiving channel and the start channel received the reference solution. The measurements (sample and reference) were made in quartz cuvettes. The temperature was fixed to 20 °C with a thermostat.

Modulation ratio measurements were made using multifrequency modulated excitation beams. Multifrequency measurements of MAR were made at several modulations between 30 and 250 MHz. Glycogen was used as a scatter reference with a lifetime of $\tau = 0.0$ ns. Quantitative determination based on modulation ratio measurements was obtained at a fixed modulation frequency of 110 MHz at the '150 average' mode in which each measurements is the average of 150 samplings carried out automatically by the instrument circuitry. Eleven replicate measurements were taken for each sample.

3.3. Procedure

The appropriate volumes of carbaryl or benomyl, glycerine and water to give a final glycerine:water 50:50 (v:v) dilution were placed in a calibrate flask. Similarly, carbaryl or benomyl solutions were prepared in ethyleneglycol:water 50:50 (v:v). The mixtures of carbaryl and benomyl were prepared directly in cuvettes, which were covered

by parafilm and shaken. The MAR of pure compounds and mixtures was then measured.

4. Results and discussion

The selected excitation and emission wavelengths to carry out the carbaryl, benomyl and their mixtures determination were 290 and 320 nm, respectively [9]. These values correspond to the maximum excitation and emission wavelengths for both pesticides.

Rotational diffusion of a fluorophore is a dominant cause of fluorescence depolarisation. The extent of the rate of rotational diffusion is described in terms of the rotational correlation time, which, in turn depends directly on the viscosity of the solution. The rotational correlation time of the fluorophore (θ) is given by:

$$\theta = \eta V / RT$$

where θ is the correlation time of fluorophore, η is the viscosity, T is the temperature in Kelvin, R is the gas constant, and V is the volume of the rotating unit. The correlation time is related to the rotational diffusion coefficient by $\theta = (6D)^{-1}$.

By supposing that the carbaryl and benomyl are spherical fluorophores, which display a single rotational correlation time, values for this parameter were obtained by applying the previous expression. The results obtained were a rotational correlation time of 0.4 ns for carbaryl and 0.6 ns for benomyl in 50% glycerine ($\eta = 6.05$ cP). The rotational diffusion coefficient was 0.4 and 0.3 ns^{-1} for carbaryl and benomyl, respectively. Similarly, in 90% ethyleneglycol ($\eta = 19.9$ cP) a single rotational correlation time of 1.5 ns for carbaryl and 2.1 ns for benomyl and rotational diffusion coefficient of 0.1 ns^{-1} for carbaryl and 0.08 ns^{-1} for benomyl were obtained. These results, as expected, show that for carbaryl and benomyl the rotational correlation time, and hence polarization, is higher for the high molecular weight compound and it increases with viscosity.

Fig. 2 shows the shape of modulation ratio versus percentage of glycerine (A) and ethyleneglycol (B) for carbaryl and benomyl at two modulation frequencies, 40 and 110 MHz. It is

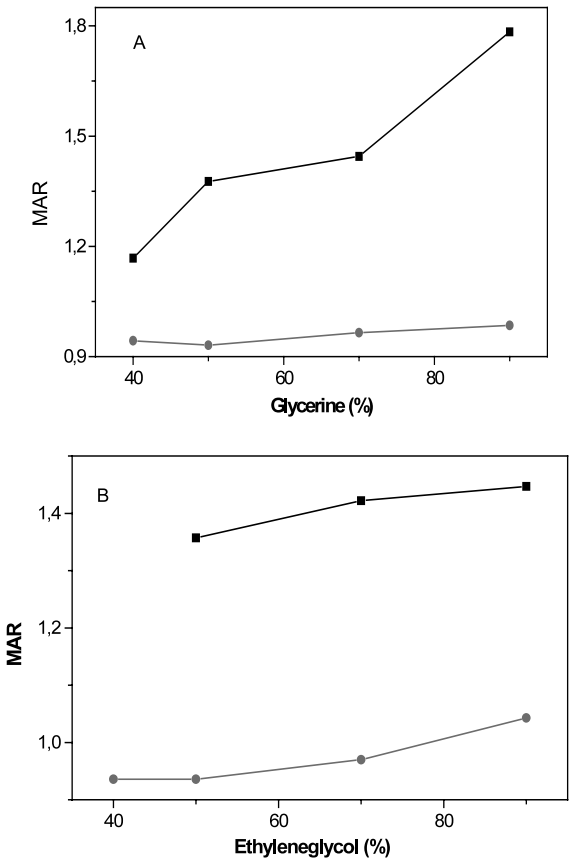

Fig. 2. (A) Modulation ratio versus percentage of glycerine, 1×10^{-4} M carbaryl (below) and 1×10^{-4} M benomyl (up). (B) Modulation ratio versus percentage of ethyleneglycol, 1×10^{-4} M carbaryl (below) and 1×10^{-4} M benomyl (up).

observed that an increase in viscosity causes an increase in the modulation ratio and in the difference of this parameter for both pesticides.

Regarding precision and selectivity criteria of an analysis a great difference in MAR values for the analytes is preferable. This was the criterion followed to optimise the instrumental parameters. The higher MAR difference between benomyl and carbaryl was observed both above 50% glycerine and for 90% ethyleneglycol. Subsequent experiments were realized with 50% glycerine, higher glycerine proportion gives higher difficulties for accurate volumes measurement, and 90% ethyleneglycol.

To select the best frequency of modulation to determine the analytes in mixtures, the MAR for

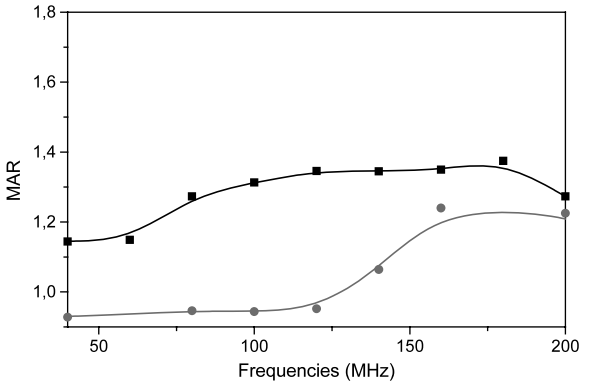

Fig. 3. Modulation ratio for 1×10^{-4} M carbaryl (below) and 1×10^{-4} M benomyl (up) in 90% (v:v) ethyleneglycol.

1×10^{-4} M carbaryl and 1×10^{-4} M benomyl in 50% glycerine (Fig. 1) and in 90% ethyleneglycol (Fig. 3) were obtained as a in function of modulation frequency. An increase of frequency produced an increase of modulation ratio and the highest difference in modulation ratio was achieved at 110 MHz for 50% glycerine and 90% ethyleneglycol. This frequency was selected for quantitative determination.

Upon inspection, the difference between the two data sets is narrow but enough for the quantitative determination of both compounds. Table 1 show results of quantitative determination for various mixtures of benomyl/carbaryl in glycerine. Table 2 shows additional results in ethyleneglycol. Measuring five replicates of each mixture assessed the precision of the method. As observed in Table 1 increasing the percentage of carbaryl and decreasing the amount of benomyl induced a raise in the values of R.S.D. The R.S.D. for carbaryl and benomyl range of between 1.2 and 17.5% (Table 2).

From these results it can be concluded that different mixtures of benomyl and carbaryl can be resolved with good accuracies. The values of percentage recovery for both compounds are near to 100% in both media: glycerine and ethyleneglycol. However, in glycerine the values

Table 1
Mixtures of carbaryl/benomyl in glycerine:water 50% (v:v)

Carbaryl taken %	Benomyl taken %	Carbaryl found %	Recovery carbaryl %	R.S.D.%	Benomyl found %	Recovery benomyl %	R.S.D.%
10.0	90.0	18.6	108.0	8.0	81.4	108.0	8.0
40.0	60.0	39.5	98.7	5.7	60.5	102.0	5.7
50.0	50.0	52.9	105.8	2.8	47.7	95.4	2.6
60.0	40.0	60.5	100.8	5.7	40.8	100.8	1.8
90.0	10.0	89.2	99.1	1.7	10.8	90.4	1.7

Table 2
Mixtures of benomyl/carbaryl in ethyleneglycol:water 90% (v:v)

Carbaryl taken %	Benomyl taken %	Carbaryl found %	Recovery carbaryl %	R.S.D.%	Benomyl found %	Recovery benomyl %	R.S.D.%
10.0	90.0	15.5	155.0	7.9	84.5	93.8	7.9
40.0	60.0	36.4	91.0	1.2	63.7	106.0	1.2
50.0	50.0	45.4	90.8	6.7	54.6	109.2	6.7
60.0	40.0	63.3	105.5	1.9	36.7	91.7	1.9
90.0	10.0	88.6	98.4	17.5	11.4	114.0	7.4

of recovery percentage are smaller than the values in ethyleneglycol.

5. Conclusion

A method has been presented which is capable of directly resolving binarys mixtures of fluorophores on the basis of differences in their rotational diffusion rates without the need to know the lifetimes of the individual species and the quantum yield of fluorophores. Our results for benomyl and carbaryl mixtures demonstrate that modulation anisotropy ratio measurements can be used for quantitative determination of small analytes mixtures. This methodology can be applied with good results when the fluorophores have a suitable MAR difference.

References

[1] F.J. Knorr, J.M. Harris, Anal. Chem. 53 (1981) 272.
[2] T.T. Ndou, I.M. Warner, Chem. Rev. 57 (1985) 463A.
[3] J.R. Lakowicz, in: J.R. Lakowicz (Ed.), Topics in Fluorescence Spectroscopy, vol. 1–6, Kluwer Academic/Plenum Publishers, New York, 1991, p. 2000.
[4] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Kluwer Academic, 1999.
[5] P.M. Roemelt, A.J. Lapen, W.R. Seitz, Anal. Chem. 52 (1980) 771.
[6] E.A. Bozhevol'nov, V.I. Gribkov, L.P. Tropina, O.A. Fakeeva, J. Anal. Chem. 32 (1978) 1594.
[7] F.V. Bright, L.B. McGown, Anal. Chem. 58 (1986) 1424.
[8] F. García Sánchez, A. Navas Díaz, M.C. Torijas, Quim. Anal. 20 (2001) 113.
[9] F. García Sánchez, A. Navas Díaz, M.C. Torijas, Anal. Chim. Acta 414 (2000) 25.
[10] Y. Yan, M.L. Myrick, Anal. Chim. Acta 441 (2001) 87.
[11] F.V. Bright, Appl. Spectrosc. 42 (1988) 1245.