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Selective determination of carbaryl and benomyl by fluorescence polarization

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Abstract

The potential of fluorescence polarization for fluorescence mixture resolution was assessed and compared with liquid chromatography (LC). Careful selection of excitation wavelengths and the use of an appropriate viscous medium (glycerine) allows significant differences in polarization to be obtained. For two component mixtures, information about fluorescence polarization and the total intensity of the samples is sufficient to calculate the relative contributions when the polarization of the two pure components are known. The results obtained for benomyl and carbaryl show detection limits ($K\,S_B/m$) of 11.4 ng ml^{-1} and 1.2 ng ml^{-1}, mid-range relative standard deviations of 0.8% and 0.9% n=3 and recoveries between 104 and 113% and 91 and 107%, respectively.

Keywords: Fluorescence polarization; Carbaryl; Benomyl

1. Introduction

Fluorescence spectroscopy has been applied widely in quantitative analysis because of its inherent sensitivity. Fluorescence as a quantitative tool is not limited to native fluorescent compound determination. The use of fluorescent molecules as labels, probes, and tracers has extended the applicability of fluorimetry to include determinations of species that cannot be directly determined fluorimetrically. Selectivity in fluorimetric determinations is most commonly based on excitation and emission wavelengths. However, selectivity of measurements based solely on selection of such wavelengths is generally poor because of the wide profiles of its excitation and emission spectra. To avoid these impediments, modifications such (as synchronous, derivative or variable angle spectra) or signal processing by mathematical algorithms (principal component regression, partial least square regression, rank annihilation methods, etc) have been used.

Other fluorescence parameters can also be used to resolve fluorophore mixtures and obtain the benefits of the high sensitivity of fluorescence. Selective quenching, fluorescence polarization and fluorescence lifetimes are examples of such parameters.

Polarization is a result of the photoselection of fluorophores according to their orientation relative to the direction of the polarized excitation. Rotational diffusion of fluorophores is a common cause of depolarization. Diffusive motion depends upon the viscosity of the solvent and the size and shape of the diffusing species. So, to get significant polarizations for small

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molecules, it is necessary that they have either short fluorescence lifetimes or long rotational relaxation times which require high viscosity. Fluorescence polarization measurements have been used to probe membrane structure and fluidity [1], to determinate the mobility of solutes at interfaces [2], to quantify the mean distance between donors and acceptors [3], to develop homogeneous immunoassays [4,5], to follow the aging of inorganic glass composites [6], and to investigate molecular-level interactions in supercritical fluids [7]. Polarization is a non-concentrational parameter. However, homogeneous immunoassays based on polarization measurements have been widely developed. Some previous attempts have been made to resolve binary fluorophore mixtures with overlapping spectra using fluorescence polarization measurements [8–10].

Benomyl is a systemic fungicide used for the pre-harvest treatment of fruits and vegetables, mainly to prevent botrytis. It is also used in post-harvest treatments of seed fruits to avoid rotting during storage under refrigeration [11]. Analytical methods for benomyl are mainly performed by liquid chromatography (LC) with fluorimetric [12] or ultraviolet-visible (spectrophotometric) [13–15] detection. Because of the fluorescent character of benomyl and its main degradation product carbendazim, several fluorimetric methods have been described [16–18]. Gas-liquid chromatography for determining cabendazim has been described [19–22]. Detection and identification of benzimidazole fungicides by thin layer chromatography and enzyme immunoassay have also been investigated [23,24].

Among the carbamate pesticides, carbaryl is one of the most widely used under, the trade name of Sevin, as an agricultural and forest spray. Recently, several immunoassay methods [25–27] and biosensors [28,29] have been developed for carbaryl quantification. Analytical methods for carbaryl are performed by LC [30] and combined with mass spectrometry [31]. Fluorescence detection [32], ultraviolet–visible (UV–VIS) spectrophotometric methods [33,34] and phosphorimetric methods [35] have also been investigated for carbaryl determination.

An alternative approach to analyze binary mixtures using, differences in fluorophore polarization is discussed in the present work. This approach has been applied to resolve a mixture of two pesticides, benomyl and carbaryl. These compounds have similar excitation-emission profiles and their polarization difference allows us to resolve the binary mixture.

The advantages of this methodology are related to the use of a fluorescence-based parameter, anisotropy or polarization, with a different dependence on analyte properties in relation to fluorescence intensity, allowing incremental selectivity parameters in the resolution of a given mixture. The use of disposable polymethacrylate cuvettes simplifies the operating procedures, and losses in sensitivity by the use of polarizers is not a problem because fluorescence is an intrinsically sensitive technique.

2. Theory

In a fluorescence polarization experiment, one excites the sample with polarized electromagnetic radiation and monitors the parallel and perpendicular components of the fluorescence. The polarization, P, is given by the ratio:

$$P = \frac{I_{\text{LI}} - I_{\perp}}{L_{\text{LI}} + I_{\perp}} \tag{1}$$

where I_{LI} and $I_{\perp}$ are the measured intensities with the excitation polarizer aligned vertically while the emission polarizer is oriented first vertically (I_{LI}) and then horizontally ($I_{\perp}$).

We will assume a mixture of two components which differ in polarization and their concentrations are in a range where fluorescence intensity is directly proportional to concentration. The polarization for each component is:

$$P = \frac{I_{\text{LIA}} - I_{\perp\text{A}}}{L_{\text{LIA}} + I_{\perp\text{A}}} \tag{2}$$

Polarizations of both fluorophores can be measured. The polarization of the pure compounds is invariant at a fixed excitation wavelength, independently of its concentrations. As has been described previously [36,37], in a defined range of wavelengths within the polarization excitation spectrum, the observed polarization or anisotropy from a sample containing more than one fluorescent species is given by

$$P = \sum_i P_i f_i \tag{3}$$

where P_i and f_i are the emission polarization of species i and its fractional contribution to the total intensity, respectively.

The polarization of a binary mixture gives an intermediate value, and this intermediate polarization value depends on the ratio of the two fluorophores in the mixture. If the total fluorescence intensities of the mixtures are constant, the fraction of each fluorophore in the mixture can be calculated, by only measuring the polarization of the mixture. The fractional contributions of each fluorophore is represented by f_A and f_B, and their values can be calculated by applying the following equations:

$$\frac{P_A - P_M}{P_M - P_B} = \frac{f_B}{f_A} \quad f_A + f_B = \text{cte} \tag{4}$$

where P_A and P_B are the polarization of the two fluorophores, P_M is the polarization of the mixture and P_A and P_B are the fraction of each fluorophore in the mixture. The equation deduction is shown clearly in Fig. 1. If there is difference in polarization the term of the first equation equals zero and this approach cannot be applied.

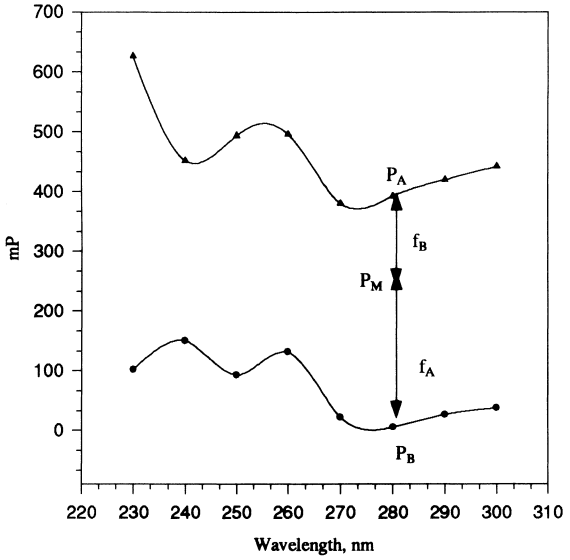

Fig. 1. Polarization spectra of two fluorophores; f_A and f_B, fractional contributions deduced from Eq. (4).

3. Experimental

3.1. Instrumentation

Two spectrofluorometers have been used to make the polarization measurements. A Perkin–Elmer LS50 spectrofluorometer (Beaconsfield, UK) equipped with film polarizers is used where the polarization accessory is operated from the PC. An Aminco 48000S spectrofluorometer (Urbana, IL) equipped with Glan-Thompson polarizers was also employed. The polarizers in the Aminco spectrofluorometer are rotated manually using wheels projecting from the top of the optical module. Both spectrofluorometers comprise two polarizing elements. One polarizer is located in the excitation beam between the excitation monochromator and the sample and the other polarizer is located in the emission beam between the sample and the emission monochromator.

A Merck-Hitachi liquid chromatograph (Darmstadt, Germany) was used. This chromatograph consists of an L-6200 pump, an AS-4000 autosampler, a D-6000 interface and F-1080 fluorescence detector. The compounds were analysed using a Lichrosper 100RP-8 analytical column (25 cm×4.6 mm; 10 μm particle size) from Merck. The injected volume was 5 μl for both standard and sample solutions; the flow rate was 1 ml min^{-1}. Mobile phase composition was acetonitrile/methanol/water (70:20:10).

3.2. Chemicals and solutions

Carbaryl (1-Naphthyl-N-methylcarbamate) were provided by Riedel de Haen and benomyl [methyl 1-(butylcarbamoyl)benzimidazol-2-yl carbamate by Dr. S. Ehrenstofer. Both pesticide stock solutions were prepared in methanol. Methanol and glycerine were obtained from Merck. Mixtures of benomyl and carbaryl in glycerine were prepared by appropriately diluting stock solutions of each pesticide and shaking to obtain a homogeneous solutions. The measurements were carried out in quartz cuvettes.

3.3. Procedure

The appropriate volumes of glycerine, methanol, benomyl and carbaryl solutions were placed in a quartz

cuvette to obtain a final 50% (v:v) glycerine proportion. The cuvettes were covered by film and shaken. The polarization of the pure compounds was measured in the same way using a pure solution of each compound. From Eq. (4) fractional contributions of each compound can be calculated and its value plotted against concentration. The standard calibration graph is then used as a reference to calculate the sample concentration.

4. Results and discussion

Rotational diffusion of fluorophores is a dominant cause of fluorescence depolarization. The rotational correlation time depends directly on the viscosity of the solution. To minimize the depolarization of the molecules in the solution and so to obtain good measurements of polarization a highly viscous medium was employed. The high viscosity of glycerine minimizes the depolarization of fluorescence due to molecular rotation during the lifetime of the excited state.

Polarization varies with the excitation wavelength. To observe the true polarization spectrum the solution must be sufficiently diluted so that energy transfer or reabsorption of fluorescence does not occur. Generally, the polarization is independent of the emission wavelength so only excitation polarization spectra are reported (Fig. 2). The polarization spectra show a high polarization difference between benomyl and carbaryl through all the studied wavelengths. To develop this approach and obtain good results a suitable polarization difference between the two compounds must be measured. The selected excitation wavelength to carry out the benomyl and carbaryl assay was 280 nm. This wavelength corresponds to the maximum excitation wavelength for both pesticides and gives a good polarization difference (P_{benomyl}=0.386 and P_{carbaryl}=0.043). The emission wavelength was fixed at 440 nm which corresponds to the maximum emission wavelength for the two pesticides.

The viscosity of the medium is responsible for the molecular rotation during the lifetime of the excited state and hence of the polarization measurements. When the solution viscosity decreases, the fluorescence depolarization of the molecules increases. Then a change in the solution viscosity produces a variation in the polarization of the fluorophores. To observe which is the polarization change, a reduction of glycerine proportion in the solution has been made (Fig. 3A). A reduction of glycerine proportion implicates a smaller solution viscosity and a polarization decrease of benomyl. Thus, when the viscosity of the solution decreases, the depolarization increases and the polarization difference becomes smaller.

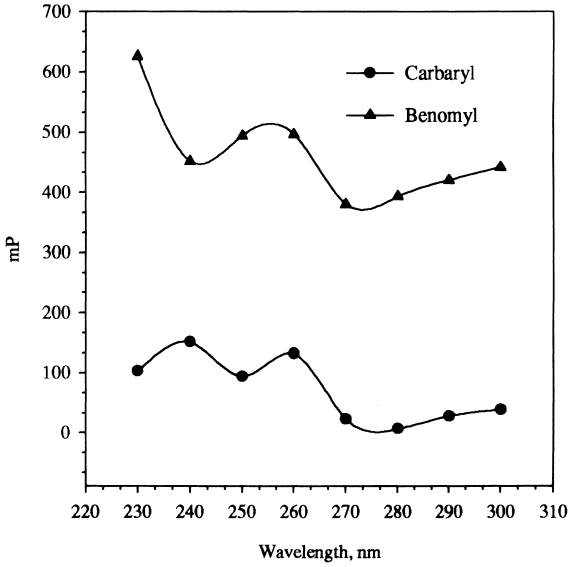

Fig. 2. Polarization spectra of benomyl and carbaryl.

Another parameter that has to be studied is the temperature. The rotational correlation time is also governed by the temperature. In consequence, the polarization can be affected by temperature changes. A polarization decrease of benomyl can be observed in Fig. 3B due to an increase of temperature which produces a greater depolarization. Room temperature was selected to develop the assay because the polarization decrease with temperature is not dramatic and good polarization differences remain over the studied range of studied temperatures.

The polarization was measured in two different spectrofluorometers, one equipped with film polarizers and another equipped with Glan-Thompson polarizers. The two instruments give similar results (Table 1). The subsequent polarizations were measured in the spectrofluorometer with film polarizers for several reasons: the measurements were faster because the polarization accessory is operated from the PC and so polarization was measured automatically.

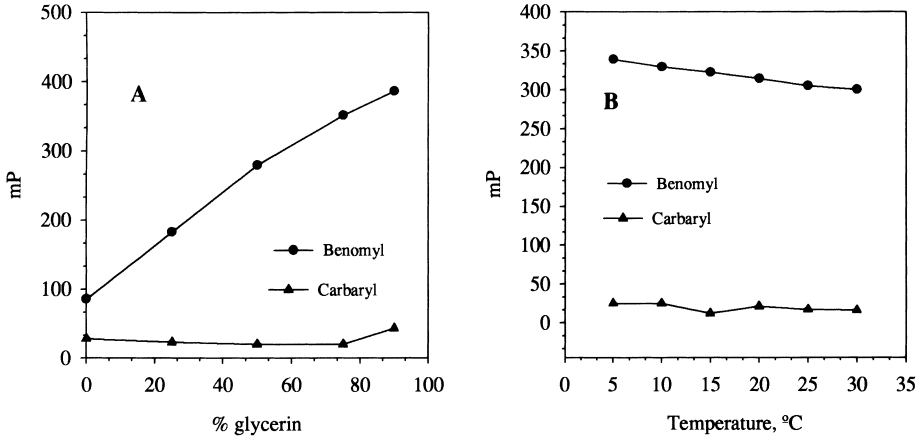

Fig. 3. Dependence of benomyl and carbaryl polarization against (A) glycerine ratio and (B) temperature.

Besides, a lower relative standard deviation (RSD) was obtained.

In general an increase in viscosity promotes enhancement of rotational relaxation times and polarization. Regarding the structure and configuration, an increase in viscosity promotes more or less an increase in polarization. Thus, a compound having some chromophores with free rotation in a non-viscous medium will show more dependence on the viscosity. In the case of benomyl and carbaryl, the more branched configuration of benomyl gives some support to the different polarization behaviour.

A good polarization difference was observed between benomyl and carbaryl up to 50% glycerine (Fig. 3A). Three different proportions of glycerine were been selected (90, 75 and 50%) to develop the pesticides determination and the results obtained have been compared. Equal analytical parameters are found when 90 and 75% glycerine is employed. However, a better detection limit (DL), defined by IUPAC as $K S_B/m$ where K is a numerical factor, S_B is the blank signal and m is the slope of the calibration graph, and RSD and a lower linear range are found by using 50% glycerine (Table 1). A high glycerine proportion gives a plot of fluorescence intensity pesticide concentrations with a smaller slope than when a lower proportion of glycerine is used. Besides, in 90% and 75% glycerine, benomyl concentrations 37 ng ml^{-1} give the same fluorescence intensities; there is no linear range below this concentration. The linear range

Table 1
Analytical parameters for benomyl and carbaryl determination

	Benomyl				Carbaryl			
	Linear range, ng ml^{-1}	Regression coefficient (r)[a]	DL ng ml^{-1}	RSD (n=3), (%)	Linear range (ng ml^{-1})	Regression coefficient (r)[a]	DL ng ml^{-1}	RSD (n=3) (%)
90% glycerine[b]	1.4–10.7	0.993	167	1.6	0.2–1.4	0.992	20.5	1.6
90% glycerine[c]	1.4–10.7	0.990	199	2.8	0.2–1.4	0.990	24.5	3.2
75% glycerine[c]	1.4–10.7	0.992	197	1.1	0.2–1.4	0.992	24.0	1.3
50% glycerine[c]	0.4–3.0	0.9992	11.4	0.8	0.04–0.3	0.9992	1.2	0.9
Method [8]	0.5–3.0	0.998	330	8.2	0.04–0.3	0.997	51	9.7
LC	3.0–10.0	0.997	552	2.7	0.6–4.0	0.991	217	2.0

[a] n=6.
[b] Film polarizers.
[c] Glan-Thompson polarizers.

Table 2
Recovery assay of synthetic mixtures

	Polarization[a]					Polarization[b]						LC			
	Taken (ppm)	Found (ppm)	Polarization	Recovery (%)	$\bar{R} \pm \sigma_{n-1}$	Taken (ppm)	Found (ppm)	$I_{\amalg}$	$I_{\perp}$	Recovery (%)	$\bar{R} \pm \sigma_{n-1}$	Taken (ppm)	Found (ppm)	Recovery (%)	$\bar{R} \pm \sigma_{n-1}$
Benomyl	0.684	0.713	0.084	104.2		1.11	1.22	135.9	183.7	109.9		3.5	4.57	130.5	
	0.684	0.699	0.083	102.2	106.0±5.0	1.11	1.08	131.9	181.9	97.3	105.1±6.8	3.5	4.64	132.5	128.5±5.4
	0.684	0.766	0.088	112.0		1.11	1.20	137.9	187.8	108.1		3.5	4.28	122.3	
	1.32	1.54	0.145	116.0		1.57	1.60	155.2	202.4	101.9		5.5	6.27	114.0	
	1.32	1.39	0.134	105.3	113.1±6.0	1.57	1.63	157.5	205.3	103.8	99.7±5.4	5.5	6.16	112.0	114.5±2.8
	1.32	1.56	0.147	118.1		1.57	1.47	153.9	205.1	93.6		5.5	6.47	117.6	
	2.72	2.88	0.244	105.8		2.59	2.55	196.6	237.8	98.5		9.0	9.80	108.8	
	2.72	2.85	0.242	104.7	104.7±1.1	2.59	2.43	201.4	250.2	98.8	97.6±3.4	9.0	9.77	108.5	108.5±0.3
	2.72	2.82	0.240	103.6		2.59	2.60	207.4	254.3	100.4		9.0	9.74	108.2	
Carbaryl	0.042	0.046	0.248	109.5		0.048	0.072	124.9	155.7	151.1		0.75	0.66	88.0	
	0.042	0.050	0.245	119.0	107.1±13	0.048	0.057	121.7	148.4	118.3	137.1±17	0.75	0.69	92.0	87.1±5.4
	0.042	0.039	0.253	92.9		0.048	0.068	119.5	148.6	141.9		0.75	0.61	81.3	
	0.211	0.188	0.145	89.1		0.213	0.166	169.8	228.0	77.8		1.5	1.37	91.3	
	0.211	0.203	0.134	96.2	91.0±4.5	0.213	0.234	172.4	247.1	110.1	95.5±16.4	1.5	1.27	84.7	92.7±8.7
	0.211	0.185	0.147	87.7		0.213	0.209	176.7	246.1	98.5		1.5	1.53	102.0	
	0.276	0.272	0.084	98.5		0.259	0.278	190.2	277.4	107.4		3.5	3.62	103.4	
	0.276	0.274	0.083	99.3	98.2±1.3	0.259	0.227	192.5	268.0	87.7	100.8±11	3.5	3.63	103.7	104.1±0.9
	0.276	0.267	0.088	96.7		0.259	0.278	181.0	267.1	107.4		3.5	3.68	105.1	

[a] Methodology described in this work.
[b] Methodology described previously [8].

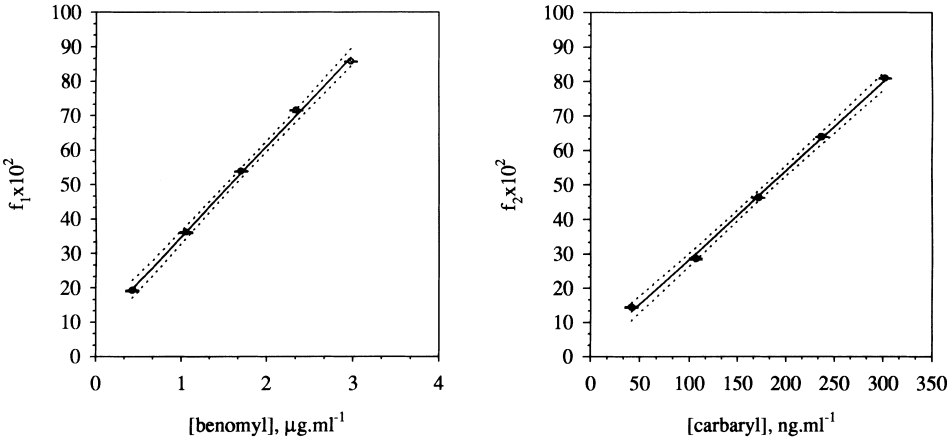

Fig. 4. Calibration plots for benomyl and carbaryl determination.

is greater in 50% glycerine and at low concentrations of benomyl the fluorescence intensity decreases as the benomyl concentration decreases. Thus, the calibration graph for benomyl and carbaryl were established 50% glycerine. The mixtures were prepared and the polarizations were measured. These polarization values were analysed in Eq. (4) and the fractional contributions were calculated. The calibration graphs were obtained by plotting the fractional contributions against the concentration of each pesticide (Fig. 4). The precision of the method was assessed by measuring three replicates of each mixture at five concentration levels, as shown in Fig. 4.

This approach has been compared with previous methodology based on polarization to resolve binary mixtures [8]. To apply this previous method, the compounds must have different polarizations. This approach makes it possible to observe polarization that is directly proportional to the concentration of one component of the mixture and independent of the concentration of the other component. Benomyl and carbaryl assay has been developed using this methodology. The methodologies have been compared and the results obtained are shown in Table 1. To apply the methodology described in this paper, polarization values are measured. To apply the methodology described in previous work the components of the emission which are parallel and perpendicular to the plane of polarization of the excitation radiation ($I_{\amalg}$ and $I_{\perp}$) are measured. The detection limits obtained for benomyl and carbaryl with the described methodology in this paper are better than those obtained by the other approach. As well, an important reduction of the RSD was achieved by the approach described here.

Validation of these methods by LC for benomyl and carbaryl was carried out. The mobile phase and flow rate were optimized for good resolution of benomyl and carbaryl with a RP8 reversed phase column. The retention times for benomyl and carbaryl were 2.79 and 3.25 min, respectively. The main analytical parameters, viz. linear range, DL and RSD, are related in Table 1.

To test the accuracy of the approach based on polarization measurements we assayed ‘unknown’ samples and a recovery assay in drinking water for both the pesticides was made. The recovery assay was applied to the three methodologies. The results are summarized in Table 2, where it can be seen that good recoveries and RSDs were obtained in every case for the polarization methodology described in this paper.

5. Conclusions

An approach for use of polarization measurements to resolve binary mixtures of fluorophores with overlapping spectra has been developed. A non-concentrational parameter has been useful for a quantification of the two compounds. This methodology can be applied with good results when the fluorophores have a suitable polarization difference. This approach has been used for benomyl and carbaryl

determination and compared with previous methodology based on the measurement of the components of the emission which are parallel and perpendicular to the plane of polarization of the excitation radiation. A comparative study of both methodologies, for these pesticides, shows better DLS and RSDs for the methodology described in this paper. The instrumentation is not expensive, the procedure is quick and the measurements are easy to obtain.

Acknowledgements

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