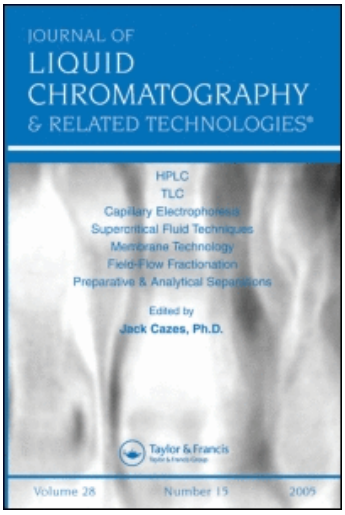

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Physico-Chemical Properties of the Most Suitable Spray Reagent for Fluorescence Enhancement in Thin-Layer Chromatography

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PHYSICO-CHEMICAL PROPERTIES OF THE MOST SUITABLE SPRAY REAGENT FOR FLUORESCENCE ENHANCEMENT IN THIN-LAYER CHROMATOGRAPHY[1]

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ABSTRACT

In a fluorescence-enhancing method by spraying with a viscous organic solvent in thin-layer chromatography(TLC), the relationship between fluorescence enhancement and the properties of spray reagents was investigated, using 5-dimethylamino-1-naphthalene-sulfondimethylamide(DNS-DMA) as a fluorescent compound. A stuitable reagent should have two properties of giving high fluorescence intensity to a fluorescent compound and good transmigration from an adsorbent to the reagent medium, i.e., having the large product of relative fluorescence quantum number(RFQN) in the reagent solution and Rf value of the fluorescnet compound in TLC using the reagent as a developing solvent.

A mixture of Triton X-100 and chloroform(2:8) showed the largest product for DNS-DMA, so that the mixture was expected to be the most suitable reagent, and DNS-amine fluorescences on a plate were enhanced about 100-fold practically. Standard curves for DNS-amines were linear in the range of 1 to 20 pmole per spot. The Triton X-100 mixture also was useful to other DNS-derivatives such as DNS-amino acids and DNS-peptides.

This selection of a suitable spray reagent was also discussed on other fluorescent compounds.

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INTRODUCTION

A method for enhancing the fluorescences of spots on thin-layer plates has been devised, based on the fact that the uniform spraying of some fluorescent spots with a nonvolatile and viscous organic solvent after development resulted in a considerable increase in the fluorescence intensities and the enhanced intensities were maintained for a long period(1). The fluorescence enhancement was not related to the light transmission through the thin-layer but to the properties of the spray reagents, i.e., polarity, viscosity and acidity. 5-Dimethylamino-1-naphthalenesulfondimethylamide(DNS-DMA) had high fluorescence intensities in nonpolar, viscous and nonacidic solvents such as liquid paraffin (2).

There remained, however, another important factor except for the above properties of reagents. The factor is the transmigration of DNS-DMA from the adsorbent to the spray reagent medium after spraying on a plate.

The present paper describes the elucidation of the fluorescence-enhancing action on a plate involving the transmigration of DNS-DMA. From these studies, a suitable spray reagent for DNS-DMA was chosen and applied for other fluorescent compounds.

MATERIALS

All chemicals were obtained from commercial suppliers and other sources. All organic solvents were of reagent grade. 5-Dimethylamino-1-naphthalenesulfonyl chloride(DNS-Cl) was purchased from Seikagaku Industries, LTD. DNS-DMA was prepared from DNS-Cl and dimethylamine by Seiler's method(3); yellow needles, m.p. 103-104°C ; mass spectrum, m/e calculated for $C_{14}H_{18}O_2N_2S$ 278.1073(M^+), found 278.1059. A silica gel TLC plate(Merck) was used, the layer thickness pre-coated on the glass plate being 0.25 mm.

DNS-amines except for DNS-DMA, DNS-ammonia and DNS-peptides were obtained from the author's method(4) and were developed on

a silica gel plate. DNS-bovine serum albumin was obtained from the Nakajima's method(5). Fluorescamine-amino acids were obtained from the Imai's method(6) and were developed on a silica gel plate.

Method

Apparatus

The fluorescence spectra were recorded with a Hitachi Model MPF-4 spectrofluorimeter. The fluorescence intensity of a spot was measured with an Ozumer Model SD-92 spectrophotodensitometer, the excitation wavelength being 365 nm and the whole emission being measured. The mass spectrum of DNS-DMA was measured with a JEOL Model JMS-OISG-2 mass spectrometer. The dielectric constants of the solvents were measured with a Shibayama Model SS-208 dielectric constant meter. The uniformly-spraying equipment consisted of a sprayer with N_2 gas pressure regulator, a belt conveyer to carry a plate and a plastic spray cabinet with a ventilation duct(1).

Spraying Procedure with a Fluorescence-enhancing Reagent

After development and standing for an hour, the plate was sprayed with an appropriate reagent and then allowed to stand for a futher hour in the dark. The fluorescence intensity of the spot was then measured.

Relative Fluorescence Quantum Number

The relative fluorescence quantum number(RFQN) of the whole fluorescence was taken as the ratio(area shown in a specific solution)/(area shown in a n-hexane solution) under the compensated fluorescence spectrum, which was measured at an excitation wavelength of 365 nm, a temperature of 20°C and a slit width of 10 nm. The RFQN of DNS-DMA in n-hexane solution was assigned a value of 1.0.

RESULT

Selection of Fluorescence-enhancing Reagent

It was found in the previous paper that DNS-DMA had high fluorescence intensities in nonpolar, viscous and nonacidic solvents

such as liquid paraffin. There was, however, a question about transmigration of DNS-DMA from an adsorbent on a plate to a sprayed reagent medium. The principle of fluorescence enhancement on a plate by spraying should be elucidated accordingly.

If a thin layer containing a fluorescent spot is exposed to a light source, the relation(I) holds, where F = total fluorescence intensity, quanta per second; I = intensity of exciting light, quanta per second; Ca_1 = concentration of DNS-DMA in an adsorbent; εa = molecular extinction coefficient in the adsorbent; ϕa = quantum efficiency(yield) of fluorescence in the adsorbent; da = optical depth of the adsorbent.

$$F_o = I \cdot \phi a \cdot 2.303 \cdot \varepsilon a \cdot Ca_1 \cdot da \qquad (I)$$

After spraying, the transmigration of DNS-DMA occurs from the adsorbent to the spray reagent, so that the relation(II) holds, where Cr = concentration of DNS-DMA in the reagent; dr = optical depth of the reagent medium on a plate; Ca_2 = remaining concentration of DNS-DMA in the adsorbent.

$$F = Fa + Fr$$
$$= I \cdot \phi a \cdot 2.303 \cdot \varepsilon a \cdot Ca_2 \cdot da + I \cdot \phi r \cdot 2.303 \cdot \varepsilon r \cdot Cr \cdot dr \qquad (II)$$

Consequently, the fluorescence enhancement is given by the relation(III).

$$\frac{F}{F_o} = \frac{I \cdot \phi a \cdot 2.303 \cdot \varepsilon a \cdot Ca_2 \cdot da + I \cdot \phi r \cdot 2.303 \cdot \varepsilon r \cdot Cr \cdot dr}{I \cdot \phi a \cdot 2.303 \cdot \varepsilon a \cdot Ca_1 \cdot da} \qquad (III)$$

As regards the amount of DNS-DMA, there is the relation(IV) before and after spraying, where Va = volume of the adsorbent containing DNS-DMA; Vr = volume of the reagent containing DNS-DMA. If an equilibrium state is maintained between the reagent and the adsorbent, the relation(V) holds, where K = distribution coefficient.

$$Ca_1 \cdot Va = Cr \cdot Vr + Ca_2 \cdot Va \qquad (IV)$$

$$K = \frac{Ca_2}{Cr} \qquad (V)$$

K value must be inversely proportional to the transmigration. If the area of a spot is the same before and after spraying, the relation(IV) is converted to $Ca_1 \cdot da = Cr \cdot dr + Ca_2 \cdot da$.

On the other hand, if the thin-layer chromatography of DNS-DMA is conducted using a spray reagent as a developing solvent, there is the relation(VI) between distribution coefficient K and Rf value, where Ar = section area of mobile phase; Aa = section area of stationary phase.

$$Rf = \frac{Ar}{Ar + KAa} \qquad (VI)$$

If an amount of the sprayed reagent is the same as that of developing solvent on an adsorbent in TLC, the relation(VI) is also converted to

$$Rf = \frac{dr}{dr + Kda} .$$

Although there are several conditions , the fluorescence enhancement(III) may be converted to the relation(VII) by substitution of equation(IV), (V) and (VI) into (III).

Subsequently, the fluorescence enhancement is expressed as the relation(VIII) containing RFQN and Rf, because RFQN is proportional to the product of fluorescence quantum efficiency and molecular extinction coefficient.

$$\frac{F}{F_o} = \left[\frac{\phi r \cdot \varepsilon r}{\phi a \cdot \varepsilon a} - 1\right] Rf + 1 \qquad (VII)$$

$$= \frac{RFQNr \cdot Rf}{RFQNa} + 1 - Rf \qquad (VIII)$$

In the relation(VIII), since the RFQNa, which is RFQN of DNS-DMA in the adsorbent, is constant and also the quantity of 1-Rf in the second term is negligibly small, i.e., less than 1, the fluorescence enhancement is almost proportional to the product of RFQN in the reagent and the Rf value.

Relationship between RFQN and Rf Value

Fig.1 shows the relationship between RFQN and Rf value of DNS-DMA for various organic solvents and viscous mixtures. The product of RFQN and Rf value, i.e., indicating the magnitude of fluorescence enhancement, is high in viscous mixtures such as No.6 which is a mixture of liquid paraffin, chloroform and monoethanolamine(1:6:1), No.15 which is a mixture of triethe-

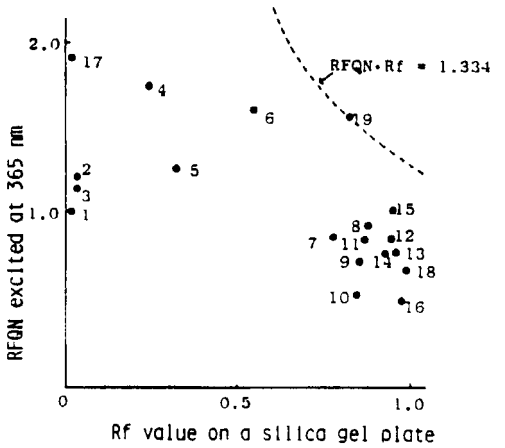

FIGURE 1

Relationship between RFQN and Rf value of DNS-DMA in various reagents

RFQN in n-hexane is expressed as 1.0. The curve in the figure represents a contour line on which the product of RFQN and Rf value is 1.334. Number, 1 = n-hexane; 2 = benzene; 3 = toluene; 4 = chloroform; 5 = 1,2-dichloroethane; 6 = liquid paraffin + chloroform + monoethanolamine(1:6:1); 7 = 1-butanol; 8 = ethyl acetate; 9 = ethanol; 10 = methanol; 11 = methyl cellosolve; 12 = methyl cellosolve + glycerol(9:1); 13 = acetone; 14 = acetonitrile; 15 = triethanolamine + isopropanol(2:8); 16 = ethanolamine; 17 = liquid paraffin + n-hexane; (2:1); 18 = glycerol + ethanol(1:1); 19 = Triton X-100 + chloroform(2:8).

nolamine and isopropanol(2:8), and No.19 which is a mixture of Triton X-100(a detergent) and chloroform(2:8). The mixture of Triton X-100 and chloroform among these mixtures was expected to be the most suitable reagent because of its large product of RFQN and Rf value.

Fluorescence Enhancement on a Plate

The practical fluorescence enhancement of DNS-DMA on a plate was shown in Fig.2 by spraying with viscous mixtures of No.6, No.15, No.17 and No.19. The fluorescence enhancements by spraying with them increased almost with the magnitude of the products. No.19 mixture afforded the highest fluorescence enhan-

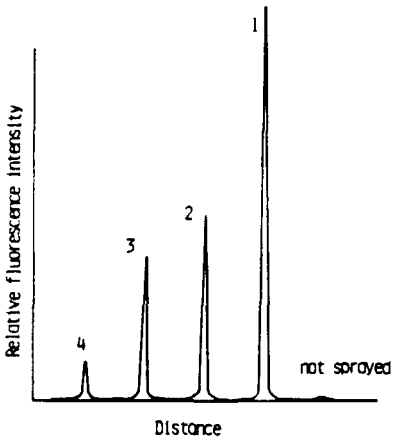

FIGURE 2

Fluorescence densitometry of 0.4 nmole per spot of DNS-DMA on a silica gel plate sprayed with various reagents
Peak, 1 = Triton X-100 + chloroform(2:8); 2 = liquid paraffin + chloroform + monoethanolamine(1:6:1); 3 = triethanolamine + isopropanol(2:8); 4 = liquid paraffin + n-hexane(2:1).

cement, 98.8-fold, and its fluorescence intensity was stable for 24 hours or more.

No.17, which is a mixture of liquid paraffin and n-hexane, gives the highest fluorescence intensity of DNS-DMA in its solution but did not afford high fluorescence enhancement on a plate practically.

Fluorescence Enhancement of DNS-amines

The fluorescence intensities of 0.4 nmole per spot of DNS-amines, including DNS-DMA(=DNS-dimethylamine), were enhanced in the range of 97.0-fold to 107.0-fold by spraying with the Triton X-100 mixture as shown in Table 1. The standard curves for DNS-amines were linear in the range of 1 to 20 pmole per spot on a silica gel plate.

Fluorescence Enhancement of Other Fluorescent Compound

The Triton X-100 mixture was applied for other DNS-derivatives although the most suitable spray reagents for them were not investigated precisely. The maximum fluorescence enhance-

TABLE 1

Maximum Fluorescence Enhancements of DNS-derivatives on a Silica Gel Plate Produced by Spraying with a Mixture of Triton X-100 and Chloroform(2:8)

Compound	Maximum fluorescence enhancement(fold)	Compound	Maximum fluorescence enhancement(fold)
DNS-amine (0.4 nmole/spot)		DNS-amino acid (0.4 nmole/spot)	
Monomethyamine	100.5	Phenylalanine	63.7
Dimethylamine	98.8	Proline	62.1
Monoethylamine	107.0	Hydroxyproline	43.2
Diethylamine	97.0	Serine	93.6
Mono n-butylamine	105.0	Threonine	50.9
DNS-ammonia (0.4 nmole/spot)	96.7	Tryptophan	132.2
DNS-amino acid (0.4 nmole/spot)		Valine	76.1
Alanine	111.1	Norvaline	85.3
Asparagine	49.4	DNS-peptide (0.4 nmole/spot)	
Aspartic acid	82.9	Diglycine	93.0
Cysteine	33.3	Triglycine	92.0
Cystine	66.8	Tetraglycine	92.0
Glutamic acid	44.3	Pentaglycine	92.0
Glycine	99.1	Hexaglycine	88.7
Leucine	44.3	Glutathione	13.0
Isoleucine	54.2	Oxytocin	6.1
Norleucine	30.9	DNS-bovine serum albumin (20 μg/spot)	1.4
Methionine	63.3		

ments of DNS-amino acids, DNS-peptides and DNS-protein on a silica gel plate are shown in Table 1.

The fluorescence intensities of DNS-amino acids were enhanced 30.9-fold to 132.2-fold, so that DNS-amino acids were determined in the range of 1 to 20 pmole per spot on a silica gel plate. DNS-ammonia was also determined in the same range. The fluorescence intensities of DNS-glycine peptides of diglycine to hexaglycine were enhanced in the range of 88.7-fold to 93.0-fold, but that of DNS-bovine serum albumin was enhanced only 1.4-fold.

On the other hand, the maximum fluorescence enhancements of fluorescent compounds produced by spraying with some various spray reagents are shown in Table 2. They were useful for vitamin A derivatives, fluorescamine-amino acids, ochratoxin A

TABLE 2

Maximum Fluorescence Enhancements of Fluorescent Compounds on a Silica Gel Plate Produced by Spraying with Various Reagents

Spray reagent
1 = glycerol + ethanol(1:2); 2 = liquid paraffin + n-hexane(2:1); 3 = liquid paraffin + benzene(2:1); 4 = triethanolamine + isopropanol(2:8); 5 = liquid paraffin + chloroform + monoethanolamine(1:6:1); 6 = Triton X-100 + chloroform(2:8)

Compound	Spray reagent	Maximum fluorescence enhancement(fold)
DNS-dimethylamine	1	8.0
(0.4 nmole/spot)	2	10.0
	4	34.0
	5	40.0
	6	98.8
DNS-alanine		
(0.4 nmole/spot)	2	10.0
	4	32.0
	5	40.0
	6	111.1
Fluorescamine-alanine		
(0.17 nmole/spot)	1	4.0
	4	2.0
	5	16.0
Vitamin A acetate and palmitate		
(8.5 U/spot)	1	13.0
	2	15.0
	4	23.0
	5	59.0
	6	59.0
Thiochrome(from VB_1)		
(20.9 ng/spot)	4	4.0
	5	3.6
	6	4.0
Benzo(a)pyrene		
(80 ng/spot)	1	2.0
	2	35.0
	3	38.0
	4	1.0
	5	30.0
Ochratoxin A		
(40 ng/spot)	1	10.0
	2	4.0
	4	15.0
	5	30.0

and benzo(a)pyrene. Vitamin A palmitate and acetate fluorescences were enhanced 59-fold by the Triton X-100 mixture. Fluorescamine-amino acids and ochratoxin A fluorescences were enhanced 16-fold and 30-fold by a mixture of liquid paraffin, chloroform and monoethanolamine(1:6:1), respectively. Benzo-(a)pyrene fluorescence was enhanced 38-fold by a mixture of liquid paraffin and benzene(2:1).

DISCUSSION

The fluorescence enhancement on a plate is almost proportional to the product of the RFQN and the Rf value of a fluorescent compound using the spray reagent as a developing solvent, and a suitable reagent can be chosen on this basis. Fig.2 showed the fact that this relationship was held practically on a plate, but some constituents of the reagent may evaporate faster than others, changing the composition, and this effect must be considered in selecting a suitable reagent. For example, No.15 mixture, which has a larger product than No.6 mixture for DNS-DMA, gave a little smaller fluorescence enhancement on a plate than that with No.6. This might be caused by the above reason.

The use of No.15 mixture had already been reported by Seiler (7) and the mixture seems to have mainly good transmigration property as shown in Fig.1.

On the other hand, a mixture of liquid paraffin and n-hexane afforded large RFQN for DNS-DMA but small Rf value, so that the fluorescence enhancement on a plate would not be so large as expected in the previous paper(2).

Triton X-100, detergent, itself had large RFQN and Rf value for DNS-DMA, i.e., being a suitable reagent. The Triton X-100 mixture, which was diluted by chloroform to spray smoothly, was suitable not only for DNS-amines but also for other DNS-derivatives. However, the fluorescence enhancement seems to be affected by the structure of a fluorescent compound, so that the difference of fluorescence enhancement for DNS-amino acids would

be observed and also very small fluorescence enhancement would be shown in DNS-bovine serum albumin.

The preliminary application of the method was indicated in Table 2 by using viscous organic mixtures for some fluorescent compounds. Some compound fluorescences were enhanced effectively. These fluorescence enhancements were also observed not only on a silica gel plate but also on a polyamide or cellulose plate. This method must, therefore, be effective for the fluorescence enhancements of many fluorescent compounds if a suitable reagent will be selected on the basis of the product of the RFQN and Rf value using the reagent as a developing solvent for the fluorescent compound.

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