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THIN-LAYER CHROMATOGRAPHIC SEPARATION AND SUBSEQUENT DETERMINATION OF SOME WATER-SOLUBLE DYESTUFFS

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SUMMARY

Separation of the water-soluble dyes, Naphthol Yellow S, Tartrazine, Ponceau 3R, Amaranth, Rhodamine B, Erythrosine B, Indigo Carmine and Patent Blue V by thin-layer chromatography on silica gel G is described. Plates were developed in the single ascending mode using ethanol–*n*-butanol–water (9:2:1, v/v) as solvent. Determinations of the separated dyes by (1) elution and absorption spectrophotometry, (2) densitometry and (3) a spectral reflectance method are examined and discussed.

INTRODUCTION

Water-soluble dyes, especially of the Food, Drugs and Cosmetics (F D & C) class, are widely used as colouring agents. Because the quantity of dye used is likely to be low (usually $\leqslant 0.2\%$, w/w) and the finished product complex, isolation and quantitative measurement of the colouring substance is not necessarily straightforward. Preliminary isolation from a complex mixture such as a foodstuff is usually accomplished by one of four separation steps[1], namely, (a) extraction into an organic solvent, (b) adsorption on wool and subsequent removal with ammonia, (c) adsorption on polyamide powder followed by chemical desorption or (d) chromatographic separation on ion-exchange papers or columns by selective elution. The initial crude separation is followed by a clean-up step to remove residual contaminants and to separate dyes if a mixture of the latter is encountered. Thin-layer chromatography (TLC) is one of the best methods for such purposes even although difficulties may arise in subsequent quantitative measurements.

In the work presented here a method is described for the separation of some widely used water-soluble dyes by TLC on silica gel. Determination of the separated dyes is examined using (a) an elution method with spectrophotometric finish, (b) an *in situ* densitometric method and (c) a recently discussed[2] spectral reflectance method.

EXPERIMENTAL

Reagents

The dyes (see Table I for the list), obtained from Hopkin & Williams, Chad-

TABLE I

PHYSICAL PROPERTIES RELEVANT TO THE SEPARATION AND QUANTITATIVE ANALYSIS OF SOME DYES

$\nu_{max.}$ in soln. the wavelength of maximum absorption in solution and ϵ = the molar absorptivity were determined in ethanol–water (4:1, v/v).

Dye	*Designation No.*	R_F	$\nu_{max.}$ *in soln. (nm)*	$\nu_{max.}$ *on silica gel G (nm)*	*Chromascan filter*	ϵ *(l mol^{-1} cm^{-1})*	*Recovery** *(%)*
Naphthol Yellow S (CI 10316; F D & C Yellow No. 2)	I	0.65	426	435	GB490	21,000	93
Tartrazine (CI 19140; F D & C Yellow No. 5)	II	0.20	423	420	GB490	23,000	88
Ponceau 3R (CI 16155)	III	0.33	499	495	G520	18,000	90
Amaranth (CI 16185; F D & C Red No. 2)	IV	0.10	522	526	G520	20,000	89
Rhodamine B (CI 45170)	V	0.45	552	550	G520	96,000	84
Erythrosine B (CI 45430; F D & C Red No. 3)	VI	0.95	522	530	G520	68,000	91
Indigo Carmine (CI 73015; F D & C Blue No. 2)	VII	0.00	603	600	Y575	15,000	77
Patent Blue V (CI 42051)	VIII	0.75	634	635	Y575	198,000	92

* For each dye adsorbed on dried silica gel G by immersion in 10 ml of ethanol–water (4:1, v/v) eluting solvent.

well Heath, Great Britain, were recrystallised from ethanol–water (4:1, v/v) and the purity of each checked by a single chromatographic development on silica gel G.

Commonly available solvents and other chemical substances, unless otherwise stated, were of analytical-reagent grade and supplied by Fisons Labs., Loughborough, Great Britain: all were used as received.

Silica gel G, Kieselguhr G, aluminium oxide G adsorbents and pre-coated silica gel 60 plates were obtained from E. Merck, Darmstadt, G.F.R.

Chromatographic separation

For chromatography, nominal 0.25-mm layers of adsorbent were prepared on glass, dried at 100–105° for 30 min, washed by a single development in ethanol and re-activated at 100–105° for 30 min. Dyes were applied as spots in an ethanol–water (4:1, v/v) mixture and the plates developed (single ascending mode) in a tank saturated in the vapour of the developing solvent at 25°. After a 10-cm solvent run the plates were removed and dried at 100–105° for 1 h before quantitative measurement.

Dyestuff determination

For elution, a dye spot was removed with supporting adsorbent and immersed in 10 ml of the eluting solvent in a stoppered centrifuge tube which was then shaken for 5 min and finally centrifuged. The absorbance of the dye transferred to the eluting solvent was measured against a blank consisting of eluting solvent equilibrated with "clean" thin-layer material. A Hitachi–Perkin-Elmer 139 spectrophotometer was used for absorbance measurement.

In application of the reflectance method the dye spot was removed with supporting adsorbent and additional clean adsorbent was added to make the total weight of the latter up to 200 mg on a balance. The weight-adjusted powder was thoroughly mixed and a reflectance measurement made using a Pye-Unicam SP500-2 spectrophotometer with reflectance attachment having its sample holder modified to take smaller samples[2]. "Clean" adsorbent was used as reference substance.

For densitometric scanning of spots directly on the chromatogram a Chromoscan densitometer (Joyce Loebl, Gateshead, Great Britain) was used.

RESULTS AND DISCUSSION

Chromatography

Silica gel G was considered to be generally preferable to Kieselguhr G and aluminium oxide G. Kieselguhr is not satisfactory for reflectance measurements because of its high light absorption[2] and alumina produces colour changes in some dyes after development and drying of the plates. The latter can react with dyes which are acid–base indicators and hence pH responsive, *e.g.* VIII (Table I).

With silica gel G the solvents, water, methanol, ethanol, acetone, *n*-butanol, ethyl acetate, chloroform and benzene were each tried separately as developing solvent. Then miscible binary and ternary mixtures of these solvents each covering a range of compositions were examined. From the many combinations of pure solvents explored it was concluded that ethanol–*n*-butanol–water (9:2:1, v/v) ternary mixture was the most satisfactory. It does not give rise to any tailing or fronting with these dyes and furthermore the resolved dye spots remain compact throughout develop-

ment if the initial spot is small ($\leqslant$ 5 mm diameter). However, when used with silica gel 60 the results are less satisfactory for separation of these dyes.

The R_F values of the dyes developed separately on silica gel G with this solvent system are given in Table I. (Somewhat different R_F values were sometimes obtained when dye mixtures were chromatographed.) From the R_F values, it is seen that good separations of all these dyes should be possible and this has been confirmed in practice. The present and previous studies do not cover the identical list in Table I but where dyes are common to each a comparison can be made. Thus, none of the 7 solvent systems examined by Griffiths[3] using paper chromatography separate all of the dyes I to III (V was excluded in her study) and in the TLC method of Mathew *et al.*[4] II is not separated from VII in mixtures containing II, IV, VI and VII. None of the 5 developing solvent systems examined by Chiang[5] separate III, IV, V and VI completely from each other. However, the TLC method of Chiang and Lin[6] separates I and II and that of Davídek and Davídkova[7] II, IV and VI. Thus the proposed new separation method increases the scope of TLC dye separations in a convenient way (development of a chromatogram takes about 2 h).

Quantitative analysis

From direct visual observation the TLC separation enables the number of dyes present in a mixture to be readily established. Comparison of R_F values with reference substances and spectroscopy will usually enable each to be identified. For quantitative analysis an elution method with spectrophotometric finish, a densitometric method and a reflectance method were examined and compared. Amounts of each dye in the range 0 to about 35 μg were studied by each of these methods. In Table I data relating to the conditions applicable to individual dye determinations are set out. Thus it is seen that the wavelengths of maximum absorbance in solution and on silica gel G powder differ slightly as is generally observed to be the case[8].

Four different solvents, namely, water, ethanol, methanol and ethanol–water (4:1, v/v) were tested for elution purposes. Of these, the last mentioned was most effective and therefore applied for quantitative spectrophotometry. Under the conditions employed for elution, transfer into the solvent was not quantitative. However, for each dye, a constant fraction, given in Table I, was eluted over the weight range studied in the present work. The Lambert–Beer relation was found to hold for absorbance [measured at the wavelength of maximum absorbance (Table I)] plotted against weight for each dye over the complete range studied.

Application of the densitometric method showed that in most cases the effective weight range suitable for quantitative purposes depends on the dye. The relationship between densitometric reading and sample weight is such that above a certain weight the instrument response becomes relatively insensitive (see, for example, Fig. 1).

For the weight range studied measurements by the reflectance method gave rectilinear plots of $-\log R_x$ (the "absorbance" scale reading of the instrument) against dye weight with three exceptions (R_x is the ratio of light reflected from the sample to that from the reference surface). The exceptions were those dyes (V, VI and VIII) having large molar absorbtivities: for each of these, however, the plot of $F(R_x) = (1 - R_x)^2/2R_x$ has the expected form[2,9]. Typical calibration curves for reflectance measurements on Patent Blue V are presented in Fig. 1. The $-\log R_x$ calibrations are more satisfactory in practice for low weights on the substrate but, for larger amounts, particularly of V, VI and VIII, the $F(R_x)$ plots will be preferable.

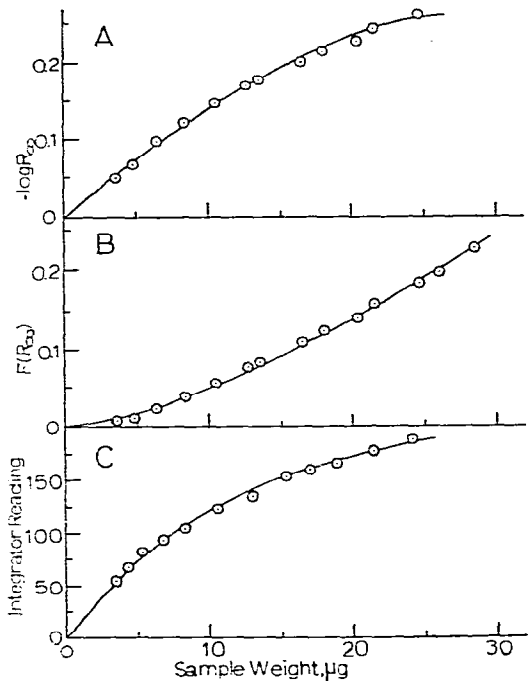

Fig. 1. Calibration curves for reflectance (A and B) and densitometric (C) determinations of Patent Blue V on silica gel G.

The upper weight limit on all quantitative methods is imposed by the adsorbent capacity in TLC separations. No upper limits were observed for determinations by the presently applied elution and reflectance methods. The lower limit for these two methods is governed by molar absorptivity, quantity of diluent present and instrument response characteristics. The upper limits for the densitometric method, where known, are $\geqslant$34, $\geqslant$30, $\geqslant$33, 21, 16, 6, 35, and 20 for dyes I to VIII in that order.

Some collected results for quantitative determinations of dyes separated from mixtures are presented in Table II. The values have mostly been chosen either near the lower limits or within the most favourable part of the weight range. The determinations within the range of 2 to 5 μg were carried out following separations of all 8 dyes from a mixture; those involving larger amounts were performed from mixtures of 3 or 4 dyes only, because of chromatographic loading limitations on the separations. The elution and reflectance methods applied to the low sample weights give discrepancies usually of 5 to 10% between expected and determined values. The large potential error arises essentially from the weak instrumental signals in this weight region. The discrepancy is somewhat more marked for VI and VII; the former is due to photosensitivity of the dye and the latter to a low elution constant and a low molar absorptivity (Table I). On the other hand the densitometric method gives consistently reliable values (usually within 5% of that expected) at these low amounts of dyes. All three methods give results within 5% of the nominal value for quantities greater than about 8 to 10 μg. The only exception is VI determined by densitometry; the upper limit of the useful measurement range is 6 μg.

Amaranth in a commercially available blackcurrant flavoured cordial was separated by a solvent extraction method[10,11] and purified by TLC as described in

TABLE II

SOME RESULTS FOR THE DETERMINATION OF DYES SEPARATED FROM MIXTURES BY TLC ON SILICA GEL G

Each value is the mean of 15 measurements.

Method	*Naphthol Yellow S*		*Tartrazine*		*Ponceau 3R*		*Amaranth*	
	Applied (μg)	*Recovered (μg)*	*Applied (μg)*	*Recovered (μg)*	*Applied (μg)*	*Recovered (μg)*	*Applied (μg)*	*Recovered (μg)*
Elution	4.3	4.6	3.9	4.1	4.5	4.1	4.4	4.0
	25.5	25.8	24.0	23.0	25.4	26.3	23.1	22.9
Reflectance	4.3	4.6	3.9	4.5	4.5	5.0	4.3	4.8
	25.5	26.0	24.0	23.3	21.2	20.4	23.4	22.8
Densitometry	4.3	4.3	3.9	3.8	4.5	4.7	4.9	4.8
	21.0	20.7	21.0	21.5	21.2	20.7	20.3	20.0
	Rhodamine B		*Erythrosine B*		*Indigo Carmine*		*Patent Blue V*	
	Applied (μg)	*Recovered (μg)*	*Applied (μg)*	*Recovered (μg)*	*Applied (μg)*	*Recovered (μg)*	*Applied (μg)*	*Recovered (μg)*
Elution	2.6	2.4	5.4	4.7	4.0	3.3	4.6	4.9
	22.5	22.9	27.1	26.3	20.6	19.7	20.2	20.6
Reflectance	2.6	2.4	2.7	2.3	4.0	5.0	3.6	3.4
	22.5	23.8	22.8	22.2	21.1	20.5	21.5	22.3
Densitometry	2.6	2.7	3.0	3.1	3.8	4.0	3.5	3.4
	16.4	17.0	6.1	7.1	21.6	21.9	13.0	12.4

this paper. Determination of the dye content by each of the three methods described gave results in good agreement with each other. Addition of known amounts of pure Amaranth to the cordial gave values consistent with those expected for total content.

To conclude, the proposed TLC separation conveniently enables two or more of the eight water-soluble dyes to be resolved. As was found in the previous study[2] there is a need for replicate separations and determinations in order to obtain a reliable measure of the quantity of dye present in a sample. The densitometric scanner used was found to operate best for dye contents at which the elution-spectrophotometric and reflectance methods are less satisfactory and to be inferior to these methods at higher loadings on separated spots.

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