Journal of Chromatography, 129 (1976) 277–285

CHROM. 9305

THIN-LAYER CHROMATOGRAPHY OF SOME CATIONIC DYES COMMONLY USED IN HISTOLOGY

P. N. MARSHALL
Department of Haematology, R.P.M.S., Hammersmith Hospital, London W12 OHS (Great Britain)
(First received January 26th, 1976; revised manuscript received April 12th, 1976)

SUMMARY

A number of cationic dyes commonly used in histology may be conveniently and effectively analysed using the thin-layer chromatographic system of Marshall and Lewis. Commercial samples of Acridine Orange, Crystal Violet, Janus Green B, Methyl Violet, Neutral Red, Pyronin B, Pyronin Y(G), Safranin, Victoria Blue B and Victoria Blue 4R have been analysed with this system. All have been found to be complex mixtures of coloured components. Chromatographic data on these components are presented.

INTRODUCTION

The cationic dyes Acridine Orange, Crystal Violet, Janus Green B, Methyl Violet, Neutral Red, Pyronin B, Pyronin Y(G), Safranin, Victoria Blue B and Victoria Blue 4R are commonly used in histology[1]. With the current awareness of problems caused by batch variations in biological dyes in general, a rapid and highly effective chromatographic system for these dyes would be helpful for identification, analysis and quality control. Many published systems have been paper chromatographic ones[2–12]. These are slow and often of low resolution. Most published thin-layer chromatographic (TLC) systems for these dyes[6,7,13–21], although rapid, are also of low resolution, or where highly effective, are only applicable to one or two of the named dyes.

In the author's hands the most rapid and effective means of analysis of all these dyes is the TLC system previously developed by Marshall and Lewis[22] for Romanowsky-type blood stains. In this paper TLC data obtained with this system are reported.

EXPERIMENTAL

Practical details were identical to those already described[22]. The effectiveness of the system may be judged by comparison of the numbers of components separated from batches of dye presented in the following tables with those previously reported for the same samples using other thin-layer systems[6]. Using Marshall and Lewis'

system, and applying dyes as narrow bands, most components are separated after the solvent front has travelled 10 cm. Separated bands are narrow, clearly defined and totally free from tailing. Bands which have R_F differences of only 0.02 require increased development. In these cases the front should be allowed to travel *ca.* 18 cm. The bands are then completely separated since during this increased development they remain narrow and sharply defined.

RESULTS AND DISCUSSION

Acridine Orange (CI 46005) (Table I)

All batches contained the same major component, presumably the nominal dye. A total of seventeen components were identified in the seven samples examined, but individual samples contained only seven to ten of these. Several authors have

TABLE I

TLC DATA ON COMMERCIAL SAMPLES OF ACRIDINE ORANGE

m Indicates a major component, t a trace one and i one present in an intermediate amount.

Supplier and batch No.	*Component*																
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
B.D.H., 2050450	t		t		t				t		m	t	i	i		t	t
G. T. Gurr, 422		t		t		t	t	t		i	m		i			t	
G. T. Gurr, 17316		t		t		t	t	t		i	m		i			t	
R. A. Lamb, 0700		i		t			t	t		i	m		i		t	t	
R. A. Lamb, 0770		i		t			t	t		i	m		i		t	t	
R. A. Lamb, 1109		t		t			t	t		i	m		i	t		t	
Supplier unknown, sample many years old		i		t			t	t		i	m		i				

Data on components

No.	*Mean R_F*	*Colour*	*Fluorescence colour in short-wave UV light*
1	immobile	none	pink
2	0.05	pink	yellow
3	0.07	none	blue
4	0.10	none	yellow
5	0.13	none	pink
6	0.16	yellow	blue
7	0.19	none	pink
8	0.22	none	yellow
9	0.25	none	pink
10	0.28	orange	green
11	0.31	orange	yellow
12	0.34	none	blue
13	0.37	yellow	green
14	0.40	yellow	blue
15	0.43	pink	yellow
16	0.46	none	blue
17	0.49	none	cream

detected impurities in Acridine Orange by TLC[6,7,15,21] although others have noted its homogeneity[16].

Crystal Violet (CI 42555) and Methyl Violet (CI 42535) (Table II)

These dyes are mixtures of hexa-, penta- and tetramethyl pararosanilines together with lower homologues. The three batches of Crystal Violet contained the same two major components and one present in an intermediate amount. These components of R_F 0.24, 0.27 and 0.31 are possibly the hexa-, penta- and tetramethyl derivatives, respectively. Only four components were identified in the samples examined, individual samples contained three or all of these. Several authors[6,15,17] have previously analysed this dye by TLC.

TABLE II

TLC DATA ON COMMERCIAL SAMPLES OF CRYSTAL VIOLET AND METHYL VIOLET

m Indicates a major component, t a trace one and i one present in an intermediate amount. Suppliers' suffixes for Methyl Violet samples are given in parentheses.

Supplier and batch No.	*Component*																
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
Crystal Violet																	
B.D.H., 2105200									t	m	m	i					
Difco, 1881									t	m	m	i					
R. A. Lamb, 3518										m	m	i					
Methyl Violet																	
B.D.H., 2089030 (6B)	i	t	t	t	t	t				i	m	m	m	t	t		t
Difco, 0123 (2B)	i	i	t	i	t	t					m	m	m	i	t	t	
R. A. Lamb, 1009 (2B)	t	t	t	t	t	t				i	m	m	m	i	t	t	
R. A. Lamb, 2572 (6B)	i	i	t	i	t	t					m	m	m	i	t	t	
Merck, 424030 (KB rein)	i	t	t	t	t	t	t	i		m	m	m	m	m			

Data on components

No.	*Mean R_F*	*Colour*	*Fluorescence colour in short-wave UV light*
1	immobile	blue	none
2	0.03	turquoise	none
3	0.06	lilac	none
4	0.08	blue	none
5	0.10	lilac	none
6	0.13	lilac	none
7	0.16	magenta	none
8	0.19	magenta	none
9	0.21	blue	none
10	0.24	violet	none
11	0.27	violet	none
12	0.31	lilac	none
13	0.37	lilac	none
14	0.45	lilac	none
15	0.50	lilac	none
16	0.53	lilac	none
17	0.64	blue	none

Three major components were common to all samples of Methyl Violet, in a single sample two additional major components were present. The three common major components are possibly the penta-, tetra- and trimethyl pararosanilines, although it has been suggested that Methyl Violet is principally a mixture of the hexa-, penta- and tetramethyl derivatives[23]. The compounds of high R_F may include the di- and monomethyl pararosanilines and pararosaniline itself. A total of sixteen components were identified in five samples, individual ones contained twelve to thirteen of these. Using paper chromatography Gasparič and Matrka[4] separated several components from Methyl Violet.

Janus Green B (CI 11050) (Table III)

Most samples of this dye contained the same two major components, although many other components were present in smaller proportions. A total of eleven components were identified, individual samples contained nine to eleven of these. Horobin and Murgatroyd[6] detected a coloured impurity in a sample of this dye by TLC but a sample examined by Marshall and Horobin[15] was apparently homogeneous.

TABLE III

TLC DATA ON COMMERCIAL SAMPLES OF JANUS GREEN B

m Indicates a major component, t a trace one and i one present in an intermediate amount.

Supplier and batch No. or purchase date	*Component*										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
B.D.H., 1853810	i	i	i	m	m	t	i	i	i	i	i
B.D.H., 1955350	t	t	i	m			i	t	i	t	t
Difco, 0372	t	t	i	m	i	t	i	i	i	i	t
E. Gurr, July '72	i	i		m	m	i	i	t	i	t	t
G. T. Gurr, 16791	i	i		m	m	i	i	t	i	t	t
R. A. Lamb, 0385	i	i	i	m	m	t	i	i	i	i	i

Data on components

No.	*Mean R_F*	*Colour*	*Fluorescence colour in short-wave UV light*
1	0.02	grey	none
2	0.04	blue	none
3	0.07	pink	none
4	0.12	blue-green	none
5	0.17	blue-green	none
6	0.20	blue	none
7	0.24	blue	none
8	0.27	mauve	pink
9	0.30	blue	none
10	0.33	mauve	yellow
11	0.44	mauve	pink

Neutral Red (CI 50040) (Table IV)

All samples of this dye contained a common major component, presumably the nominal dye. One contained an additional major component. A total of eight components were identified in the four samples, four to eight of these being present in individ-

TABLE IV

TLC DATA ON COMMERCIAL SAMPLES OF NEUTRAL RED

m Indicates a major component, t a trace one and i one present in an intermediate amount.

Supplier and batch No.	*Component*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
B.D.H., 2051130		t	t		m	i	t	t
G. T. Gurr, 15841		t	t		m	i	t	
R. A. Lamb, 0622		t			m	i	t	
R. A. Lamb, 3675	t	t	m	t	m	i	i	i

Data on components

No.	*Mean R_F*	*Colour*	*Fluorescence colour in short-wave UV light*
1	0.02	none	cream
2	0.07	none	orange
3	0.10	pink	red
4	0.13	none	cream
5	0.40	red	scarlet
6	0.47	pink	yellow
7	0.57	none	yellow
8	0.63	none	yellow

ual samples. Using TLC, Horobin and Murgatroyd[6] reported that a sample of Neutral Red was homogeneous, whilst Wittekind[20] identified several components in a number of samples. With this dye Wittekind's TLC system appears as effective as that used here.

Pyronin B (CI 45010) and Pyronin Y(G) (CI 45005) (Table V)

The single sample of Pyronin B contained four major components, one of which was present as a major component of one sample of Pyronin Y(G). Other authors[6,13,14] have detected coloured impurities in samples of Pyronin B using TLC. Several major components were identified in the samples of Pyronin Y(G) examined, although in only two samples were these the same. In the five samples thirty components were identified, four to thirteen of these being present in individual samples. Several authors have previously analysed this dye by TLC[6,13–15,18], finding in many cases that several components were present in samples from various sources.

Safranin (CI 50240) (Table VI)

All samples of this dye contained the same two major components, possibly dimethyl- and trimethylphenosafranin. A total of thirteen components were identified in the eight samples, five to eleven being present in individual samples. The heterogeneity of this dye has been reported previously by workers using TLC[6,15].

Victoria Blue B (CI 44045) and Victoria Blue 4R (CI 42563) (Table VII)

Two major components were common to the samples of Victoria Blue B; in one sample an additional major component was present. Twenty-three components were separated in the three samples examined, fifteen to nineteen being present in

TABLE V

TLC DATA ON COMMERCIAL SAMPLES OF PYRONIN B AND PYRONIN Y(G)

m Indicates a major component, t a trace one and i one present in an intermediate amount.

Supplier and batch No.	*Component*																														
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*33*
Pyronin B																																	
Difco, 0551													m				m		t									m		m			
Pyronin Y(G)																																	
B.D.H., 418688/530810						m				t					t				t			t		t			t						
B.D.H., 1911210						m					t															t							i
Difco, 2126					m	i						t		t						t													i
Hopkin & Williams, 27293	t		t					t					t					i			i		m		i		i		m		m	m	i
Merck, 432204		m		i			t		t				m			t																	

Data on components

No.	*Mean R_F*	*Colour*	*Fluorescence colour in short-wave UV light*	*No.*	*Mean R_F*	*Colour*	*Fluorescence colour in short-wave UV light*
1	0.02	none	cream	18	0.36	pink	yellow
2	0.04	pink	gold	19	0.38	mauve	none
3	0.06	none	cream	20	0.40	mauve	yellow
4	0.08	salmon-pink	green	21	0.42	yellow	green
5	0.10	mauve	none	22	0.44	yellow	blue
6	0.12	pink	gold	23	0.46	orange	yellow
7	0.14	yellow	blue	24	0.48	mauve	none
8	0.16	pink	cream	25	0.50	pink	yellow
9	0.18	pink	grey	26	0.52	none	blue
10	0.20	pink	cream-blue	27	0.54	pink	yellow
11	0.22	pink	gold	28	0.56	yellow	blue
12	0.24	pink	yellow	29	0.58	pink	orange
13	0.26	pink	orange	30	0.60	orange	yellow
14	0.28	pink	cream-blue	31	0.65	orange	yellow
15	0.30	salmon-pink	green	32	0.81	yellow	green
16	0.32	pink	cream-blue	33	0.86	none	blue
17	0.34	yellow	green				

TABLE VI

TLC DATA ON COMMERCIAL SAMPLES OF SAFRANIN

m Indicates a major component, t a trace one and i one present in an intermediate amount.

Supplier and batch No.	*Component*												
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
B.D.H., 2121070		t	t			t		m	i	m	i	t	t
Comak, no batch no.								m	i	m	t	i	
Difco, 1548		t	t			t		m	i	m	i	t	t
G. T. Gurr, 16634	t	t	t	t		i	i	m	i	m	i	i	
G. T. Gurr, 18941	t	t	t	t				m	i	m	i	t	
G. T. Gurr, 20486	t	t	t	t				m	i	m	i	t	
Hopkin & Williams, 814	t	t	t	t	t			m	i	m	i	t	
R. A. Lamb, 3609								m	i	m	t	i	

Data on components

No.	*Mean R_F*	*Colour*	*Fluorescence colour in short-wave UV light*
1	0.04	none	yellow
2	0.07	blue	pink
3	0.11	blue	none
4	0.16	none	yellow
5	0.19	none	pink
6	0.22	purple	red
7	0.25	pink	gold
8	0.33	red	gold
9	0.38	pink	yellow
10	0.44	red	gold
11	0.50	pink	yellow
12	0.54	purple	pink
13	0.60	none	yellow

individual samples. Using TLC previous authors[6] found that a sample of Victoria Blue B was apparently homogeneous. Both samples of Victoria Blue 4R showed three major components in common, but in one sample two additional major components were identified. Twenty-two components were identified in the samples, twenty to twenty-one being present in individual samples. By TLC a single sample of this dye was found to be homogeneous[15].

TABLE VII

TLC DATA ON COMMERCIAL SAMPLES OF VICTORIA BLUE B AND VICTORIA BLUE 4R

m Indicates a major component, t a trace one and i one present in an intermediate amount.

Supplier and batch No.	*Component*																															
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*
Victoria Blue B																																
B.D.H., 1878440		t	t			t		t				t	m	i		m	t		i	t		t		t						t		t
Difco, 0292		t	i			i		t				t	m	t		m	t		i	t		t		i	t					t		t
R. A. Lamb, 0503			t	i	t	t	t	t		t	t	t	m	i		m	t		m			t	t			t				t		t
Victoria Blue 4R																																
Difco, 0373			i	i	t		i		t	i	i	i	m	i	m		t	m			t	t		t			i	t	i		t	
R. A. Lamb, 1819	t		i	i	t	i			t	i	i	t	m	m	m		t	m			i	i		i			m	t	i		t	

Data on components

No.	*Mean R_F*	*Colour*	*Fluorescence colour in short-wave UV light*	*No.*	*Mean R_F*	*Colour*	*Fluorescence colour in short-wave UV light*
1	0.01	blue	none	17	0.33	mauve	none
2	0.03	blue	none	18	0.36	mauve	none
3	0.05	blue	none	19	0.38	blue	none
4	0.07	blue	none	20	0.41	blue	none
5	0.09	blue	none	21	0.43	blue	none
6	0.11	blue	none	22	0.45	blue	none
7	0.13	blue	none	23	0.47	blue	none
8	0.15	blue	none	24	0.50	mauve	none
9	0.17	blue	none	25	0.53	none	cream
10	0.19	turquoise	none	26	0.61	blue	none
11	0.21	blue	none	27	0.64	mauve	none
12	0.23	blue	none	28	0.67	blue	none
13	0.25	blue	none	29	0.70	blue	none
14	0.27	blue	none	30	0.73	grey	none
15	0.29	mauve	none	31	0.76	blue	none
16	0.31	blue	none	32	0.79	yellow	blue

REFERENCES

1 R. D. Lillie, *H. J. Conn's Biological Stains*, Williams and Wilkins, Baltimore, Md., 8th ed., 1969.
2 J. S. Bellin and M. E. Ronayne, *J. Chromatogr.*, 24 (1966) 131.
3 J. Ciglar, J. Kolšek and M. Perpar, *Chem.-Ztg.*, 86 (1962) 41.
4 J. Gasparič and M. Matrka, *Collect. Czech. Chem. Commun.*, 24 (1959) 1943.
5 V. S. Gupta, S. C. Kraft and J. S. Samuelson, *J. Chromatogr.*, 26 (1967) 158.
6 R. W. Horobin and L. B. Murgatroyd, *Histochemie*, 11 (1967) 141.
7 F. H. Kasten, *Int. Rev. Cytol.*, 21 (1967) 141.
8 E. G. Kiel and G. H. A. Kuypers, *Tex*, 22 (1963) 533.
9 S. Nagai, *Exp. Cell Res.*, 27 (1962) 14.
10 J. E. Scott, *Histochemie*, 9 (1967) 30.
11 J. Šrámek, *J. Chromatogr.*, 15 (1964) 57.
12 S. I. Rosenthal, H. Puchtler and F. Sweat, *Arch. Pathol.*, 80 (1965) 190.
13 H. L. Dobres and W. A. Moats, *Stain Technol.*, 43 (1968) 27.
14 R. W. Horobin and L. B. Murgatroyd, *Stain Technol.*, 44 (1969) 297.
15 P. N. Marshall and R. W. Horobin, *Histochemie*, 36 (1973) 303.
16 A. Niederwieser and M. Brenner, *Experientia*, 21 (1965) 105.
17 M. B. Naff and A. S. Naff, *J. Chem. Educ.*, 40 (1963) 534.
18 A. Stier and W. Specht, *Naturwissenschaften*, 50 (1963) 549.
19 R. Takeshita, N. Itoh and Y. Sakagami, *J. Chromatogr.*, 57 (1971) 437.
20 D. Wittekind, *Acta Histochem., Suppl.*, XIII (1973) 305.
21 V. Zanker, *Acta Histochem., Suppl.*, XIII (1973) 281.
22 P. N. Marshall and S. M. Lewis, *Stain Technol.*, 49 (1974) 235.
23 *The Colour Index*, Society of Dyers and Colourists, Bradford, 2nd ed., 1956.