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Analysis of xanthene dyes by reversed-phase high-performance liquid chromatography on a polymeric column followed by characterization with a diode array detector

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SUMMARY

A high-performance liquid chromatographic method on a polymeric column was developed for the analysis of xanthene dyes. The rigid polystyrene–divinylbenzene column was connected to a photodiode array detector to verify the identity and the purity of the dyes. For eosin Y a within-day precision of 1–2% was obtained, and on a day-to-day basis the coefficient of variation was 4.2%. The purity of commercial xanthene dyes was investigated, and the results show the divergence between the actual dye contents and the dye contents indicated on the label.

INTRODUCTION

Quality control of histological dyes and stains is a recognized necessity, but often problematic owing to a lack of a stable and accurate analysis method. The purity and stability of eosin Y, a xanthene dye and anionic fraction of the Romanowsky–Giemsa (RG) stain, is of major importance as only this anionic dye in combination with the cationic azure B gives a complete staining pattern and RG effect [1]. Different methods have been described for the analysis of xanthene dyes. Many authors use paper chromatography [2–5], electrophoresis [5], thin-layer chromatography (TLC) [5–11], ion-pair high-performance liquid chromatography (HPLC) [12–17] or reversed-phase HPLC [17–22]. White and Harbin [23] recently applied the more rigid and stable polymeric column for analysis of a series of acidic dyes.

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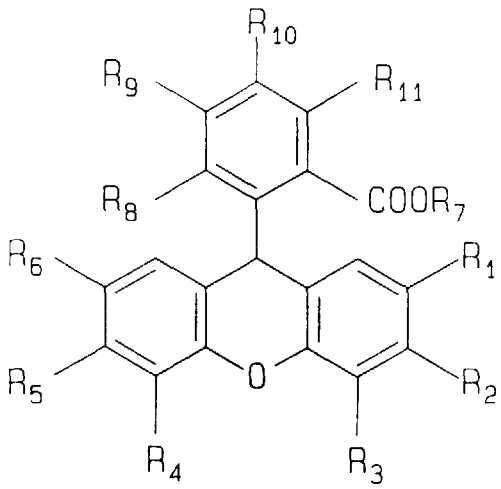
This paper describes the HPLC separation of eosin Y and halogenated homologues (Fig. 1) on a polystyrene–divinylbenzene column. This method is an extension of the previously reported HPLC method for the purity assessment of the cationic fraction of RG stain [24]. The identity of the anionic compound of the RG stain, eosin Y, is verified by comparing spectra, taken by a photodiode array detector, with spectra from the literature and of the standard if available. The purity of each peak is controlled by plotting the spectra of up- and downslope and top simultaneously, as conventionally done with a photodiode array detector [25,26].



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	R_{11}
fluorescein	H	OH	H	H	OH	H	H	H	H	H	H
4',5'-dibromofluorescein	H	OH	Br	Br	OH	H	H	H	H	H	H
eosin Y	Br	OH	Br	Br	OH	Br	H	H	H	H	H
ethyleosin	H	OH	Br	Br	OH	Br	C_2H_5	H	H	H	H
2',7'-dichlorofluorescein	Cl	OH	H	H	OH	Cl	H	H	H	H	H
4,5,6,7-tetrachlorofluorescein	H	OH	H	H	OH	H	H	Cl	Cl	Cl	Cl
4',5'-diiodofluorescein	H	OH	I	I	OH	H	H	H	H	H	H
erythrosin B	I	OH	I	I	OH	I	H	H	H	H	H
phloxine B	Br	OH	Br	Br	OH	Br	H	Cl	Cl	Cl	Cl

Fig. 1. Structures of all the xanthine homologues investigated.

EXPERIMENTAL

Chemicals and reagents

4′,5′-Dibromofluorescein, 2′,7′-dichlorofluorescein and 4′,5′-diiodofluorescein were purchased from Aldrich (Milwaukee, WI, U.S.A.). Eosin Y, rose bengal, tetramethylammonium hydroxide (TMAH, 20% in methanol) and erythrosin B came from Janssen Chimica (Beerse, Belgium), the last-named also from Merck (Darmstadt, F.R.G.). Eosin Y standard and tetrachlorofluorescein were gifts from D. Wittekind (Freiburg, F.R.G.). Ethyleosin was supplied by Sigma (St. Louis, MO, U.S.A.) and by Matheson-Coleman & Bell (Norwood, OH, U.S.A.) and phloxine B by Hopkin & Williams (Romford, U.K.). Fluorescein, absolute ethanol and phosphoric acid (85%) were obtained from UCB (Braine L'Alleud, Belgium) and acetonitrile (HPLC grade) from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Apparatus

The liquid chromatograph used was equipped with a Spectra-Physics 8700 pump, a Spectra-Physics 4270 integrator (Spectra-Physics, San Jose, CA, U.S.A.) and an N60 sample valve with a 50-μl loop (Valco Instruments, Houston, TX, U.S.A.).

The detector system consisted of a diode array spectrophotometer (Model 1040A, Hewlett-Packard, Winnersh, U.K.) interfaced to a microcomputer (Model 85, Hewlett-Packard) and a plotter (Model 7470A, Hewlett-Packard). Programs were available to monitor the flow-cell absorbance at different UV and visible wavelengths, to capture spectra when components were detected and to evaluate and plot all the chromatographic and spectral data.

Chromatographic conditions

The liquid chromatographic column used was a 10-μm Hamilton PRP-1, 25 cm$\times$0.41 cm I.D. (Hamilton, Bonaduz, Switzerland).

The mobile phase was water–acetonitrile (78:22, v/v) containing 0.142 *M* TMAH or water–acetonitrile (85:15, v/v) containing 0.154 *M* TMAH. To both eluents sufficient phosphoric acid was added to adjust the pH of the aqueous component to 7.0.

The flow-rate was 1.0 ml/min and the temperature ambient.

Analytes

The compounds were dissolved in absolute ethanol and diluted with the aqueous component of the mobile phase.

Calibration

An external calibration procedure was used. The primary reference standard, eosin Y (MW 643.9), was dissolved in absolute ethanol to a concentra-

tion of ca. 0.78 m*M*. The absorption of a 1:25 dilution in ethanol was determined at 530 nm, and the exact concentration of eosin Y in both solutions was calculated using a molar absorption coefficient of 113 000 l/mol/cm. A working solution was prepared by diluting the stock solution ten-fold with absolute ethanol. From this solution, a series of dilutions in the aqueous component of the eluent was made, to give a final concentration range from $7.0 \cdot 10^{-3}$ to $2.6 \cdot 10^{-2}$ m*M*. A 50-μl portion of each solution was injected on the column. The peak area or height of the eosin Y peak was measured by electronic integration. A standard curve was constructed by plotting peak heights or areas versus the corresponding eosin Y concentrations.

RESULTS AND DISCUSSION

The aim of this study was to develop a rigid and stable system for quality assessment of the anionic fraction of the RG stain, namely eosin Y. As the cationic compound of the RG stain, azure B, could already be analysed on the polymeric column, and the latter provides a robust stationary phase, it was logical to develop a method based on the same concept. After optimization of the eluent, calibration curves for eosin Y were obtained, the linearity was investigated, and coefficients of variation (C.V.) were calculated. The purity of different commercial halogenated homologues of eosin Y was examined.

Calibration curves, linearity and coefficients of variation

Over the concentration range from $7.0 \cdot 10^{-3}$ to $2.6 \cdot 10^{-2}$ m*M*, linear relationships were found between peak heights or peak areas and concentrations, with correlation coefficients of up to 0.999. Both the peak area and the peak height yielded comparable results. The within-day precision, calculated after repeated injections ($n=10$) of the same sample (three samples with different concentrations covering the concentration range of the calibration curves) varied between 1 and 2%. The C.V. on the slope of the calibration graphs on a day-to-day basis was 4.2% ($n=8$). The average equation of the calibration curve, based on peak-height measurements, was $y=0.1368x-0.0151$.

Identity and purity control of commercial xanthene dyes

To resolve all early eluting impurities, the weaker eluent (water–acetonitrile, 85:15, v/v) was used. All compounds were injected and eluted by both eluent systems.

The identities of the xanthene dyes were investigated by recording continuous spectra of eluting peaks and comparing them with spectra from the literature [27], or with spectra of a reference standard. In Fig. 2 the spectra of the eosin Y standard and commercial eosin Y are plotted. Now from this, it can be concluded that the peak eluting with a capacity factor (k') of 4.58 (eluent water–acetonitrile, 78:22, v/v) is eosin Y.

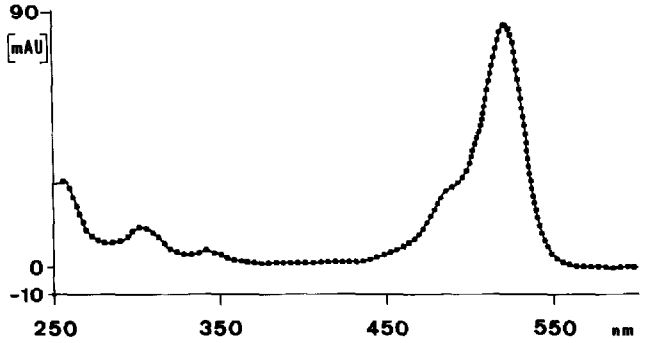


Fig. 2. Plots of spectra of eosin Y standard and commercial eosin Y taken at the top of the peak when eluting from the PRP-1 column (25 cm×0.41 cm I D.) with the mobile phase water–acetonitrile (85 15, v/v) containing 0.154 *M* TMAH (pH 7.0) at a flow-rate of 1.0 ml/min. The two spectra overlap completely and show a λ_{max}=521 nm. (—) Spectrum of eosin Y standard; (■) spectrum of commercial eosin Y.

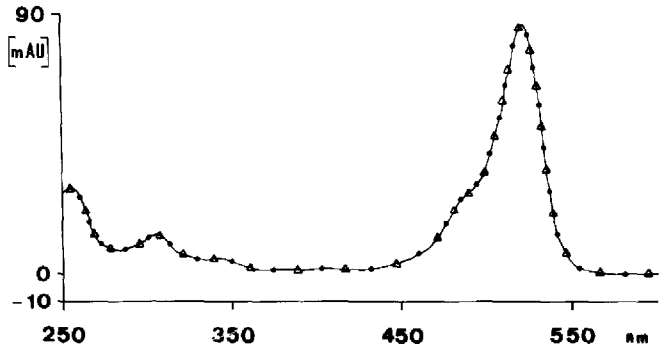


Fig. 3. Plots of the spectra of commercial eosin Y taken at up- and downslope and top Chromatographic conditions: column, same as in Fig. 2; mobile phase, water–acetonitrile (78.22, v/v) containing 0.142 *M* TMAH (pH 7.0); flow-rate, 1.0 ml/min. Complete overlapping of the spectra proves the purity of the peak. (△) Spectrum at upslope, (—) spectrum at top; (●) spectrum at downslope.

The purity of each peak was controlled by plotting the spectra, taken at the up- and downslope and top, simultaneously. Complete overlapping of the spectra confirms the purity. This is shown in Fig. 3 for commercial eosin Y.

Table I lists the capacity factors (k') of each compound in both eluent systems and the impurities detected. The observed λ_{max} and the amount of impurity in each dye is also indicated.

The labels on the different dye containers mentioned their contents, but our results indicated that the label indication on dye contents and the actual amount present did not match. Other coloured substances are almost always present and can interfere in staining procedures. This is a factor that can additionally

TABLE I

CAPACITY FACTORS IN BOTH ELUENTS, AMOUNT OF COLOURED IMPURITIES PRESENT IN EACH DYE, AND ABSORPTION MAXIMUM OF COMPOUNDS ANALYSED

Compound	Capacity factor[a]		Amount of dye present, eluent 85 15 (%)	λ_{max} (nm)	Dye contents indicated on container label (%)
	Eluent 78.22	Eluent 85 15			
Fluorescein	0.77	2.3	100	493	-
4′,5′-Dibromofluorescein	0.72	1 06	0.39	-[b]	95
	1.14	2.26	29.10	499	
	1.75	3.46	44.04	507	
	4.56	4.68	1.70	-[b]	
	8.44	5 80	2.54	-[b]	
	9.00	11 24	9.29	509	
	11.33	36 97	9.55	523	
		39.48	0.54	-[b]	
		39.83	0.53	-[b]	
		43.07	2.32	-[b]	
Eosin Y (commercial)	0.76	4.09	0 60	-[b]	83
	1.76	13.17	18 60	515	
	4.58	42.04	80.80	521	
Eosin Y (standard)	4.45	3.25	0.58	-[b]	-
		41.52	99.42	521	
Ethyleosin (Sigma)	1 71	12.4	3.96	-[b]	95
	4.59	40.14	96.04	521	
Ethyleosin (Matheson)	1 71	11.8	5.65	-[b]	-
	4.57	40.32	94.36	521	
2′,7′-Dichlorofluorescein	0.80	1.93	1.07	-[b]	95
		2.82	2.96	499	
		3.82	1.51	505	
		5.02	94.46	507	
Tetrachlorofluorescein	0.66	2 90	0.13	-[b]	-
	1 22	4 60	0.09	-[b]	
	2 21	7.61	5.81	513	
		8.48	18.00	511	
		17.88	75.97	519	
4′,5′-Diiodofluorescein	0.72	1.66	0 98	-[b]	-
	1.00	2.31	0.52	-[b]	
	1.44	3.58	7.54	501	
	2.00	6.52	61 46	511	
	3 55	10.42	1.83	-[b]	
		15.20	1 86	-[b]	
		34 56	25.81	521	
Erythrosin B	0.93	-	0.37[c]	-[b]	-
	1.20		0.83[c]	-[b]	
	3.37		1.32[c]	-[b]	
	4.57		0.65[c]	-[b]	
	13.23		96.84[c]	529	
Phloxine B	0.73	-	6.95[c]	507	-
	1.56		9.46[c]	515	
	4.16		81.94[c]	521	
	7.49		0.07[c]	-[b]	
	10.92		1 59[c]	515	

[a]Capacity factors on the same line for both eluents do not necessarily reflect the same compound. Impurities are resolved with the weaker eluent (85 15) and inserted in the list.
[b]Amount of substance too small to determine λ_{max} exactly.
[c]Eluent 78 22.

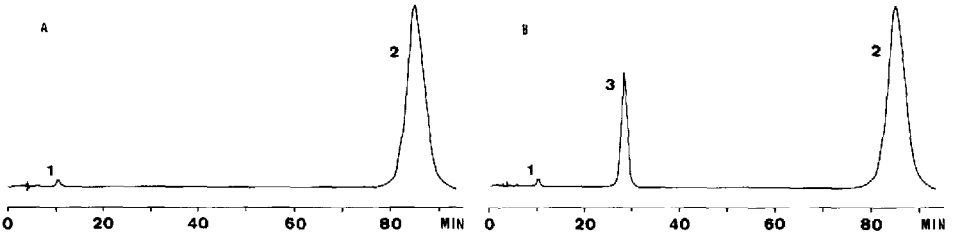


Fig. 4 (A) Chromatogram of eosin Y reference standard. (B) Chromatogram of commercial eosin Y. Chromatographic conditions as in Fig. 2. Peaks. 1 and 3 = impurities; 2-eosin Y.

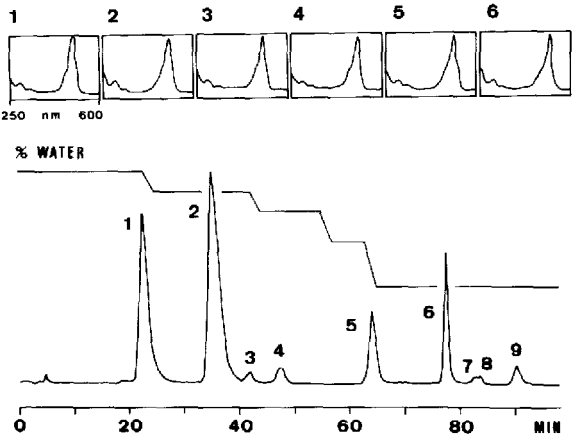


Fig. 5. Chromatogram of 4',5'-dibromofluorescein with plots of the spectra of the six main peaks present. The purity of each peak was checked by plotting the spectra of up- and downslope and top simultaneously (not shown). Chromatographic conditions: stepwise gradient elution was performed from 89:11 (v/v) to 78·22 (v/v) water–acetonitrile as shown on the figure. Peaks: 1, 3, 4, 5, 7, 8 and 9 = impurities; 2 = 4',5'-dibromofluorescein; 6 = eosin Y.

complicate investigations concerning staining mechanisms [28]. When elucidating the mechanisms in staining it is crucial to use pure dyes. This will be possible in the future as the analytical method is now being expanded to a preparative scale. The impurity of commercial dyes is illustrated in Fig. 4A and B, which shows chromatograms of eosin Y standard and commercial eosin Y. Fig. 5 shows the chromatogram of 4',5'-dibromofluorescein. The product is so impure that it requires stepwise gradient elution from 89:11 to 78:22 (v/v) water–acetonitrile to resolve all the impurities in an acceptable time. The purity of the six main peaks is controlled by superposing the spectra of up- and downslope and top.

CONCLUSIONS

This HPLC method is rigid, stable and precise, and enables the identification of xanthene homologues and quantification of eosin Y as there is a refer-

ence standard for this dye available. The method is now being expanded to a preparative scale, so that the purification of impure commercial dyes will become possible.

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