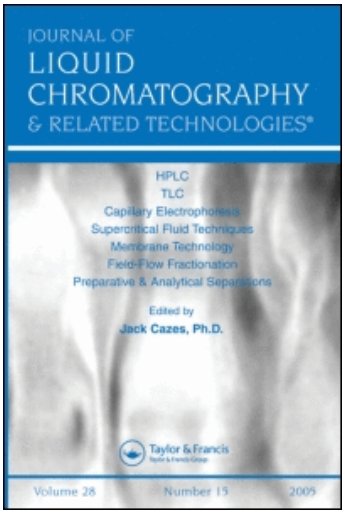

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THE PURIFICATION OF XANTHENE DYES BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reliable method for the separation of fluorescein dyes from their impurities was developed using high performance liquid chromatography and involved a μBondapak C_{18} reverse phase column and mixtures of methanol and ammonium acetate buffer. This technique was used to verify the purity of commercial products as well as to aid in the development of an empirical theory related to retention of halogenated fluorescein dyes by reverse phase columns.

INTRODUCTION

Commercial preparations of halogenated fluorescein dyes exhibit varying degrees of purity. From this fact was borne the need for a rapid reliable technique to separate the dyes from impurities and to monitor the quality of the commercial dyes (1, 2). Open column and thin layer chromatography do not possess the separative capacity for this task (3). Reverse phase high performance liquid chromatography (HPLC) does provide the necessary capability.

MATERIALS AND METHODS

The dyes used in this study were a series of chemical compounds based on the structure of fluorescein (Fig. 1), and contained different degrees of halogenation of the ring systems. The Hilton-Davis Chemical Company provided fluorescein; 3', 4', 5', 6'-tetrachlorofluoran; 2, 4, 5, 7-tetrabromofluorescein (eosin yellowish); 2, 4, 5, 7-tetraiodofluorescein (erythrosin B); 2, 4, 5, 7-tetrabromo-3', 4', 5', 6'-tetrachlorofluorescein (phloxin B); 2, 4, 5, 7-tetraiodo-3', 4', 5', 6'-tetrachlorofluorescein (rose bengal); and 2, 4, 5, 7, 3', 4', 5', 6'-octabromofluorescein. 4, 5-Diiodofluorescein and 4, 5-dibromofluorescein were ob-

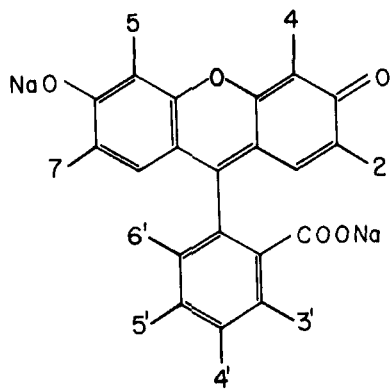

Figure 1

The basic structure for fluorescein and all dyes that are related by structure. Each available position is numbered.

tained from Eastman Chemical Company and 2, 7-dichlorofluorescein was obtained from Gallard-Schlessinger.

Tetrachlorofluoran was converted to tetrachlorofluorescein by addition to 0.1N NaOH before dilution with distilled water. Each dye was dissolved in distilled water, passed through a sample clarification kit equipped with a 0.45 μ filter, and introduced onto a Waters μBondapak C_{18} reverse phase column (3.9mm ID x 30cm) via a Waters U-6K injector. A mobile phase consisting of variable amounts of methanol (Burdick and Jackson) and ammonium acetate buffer (0.02M, pH = 3.5) was used at a flow of 2.0 ml/min.

Other equipment for the study included 2 Waters M6000A pumps, a Waters Model 660 Solvent Programmer, a Waters uv-visible Model 440 fixed wavelength detector, a Perkin-Elmer uv-visible Model 55 variable wavelength detector, and a Houston Ommniscribe recorder.

RESULTS AND DISCUSSION

The series of xanthene dyes was monitored for purity using HPLC. Excellent separations were obtained for each dye by varying the methanol concentration in the methanol-ammonium acetate elution solution. The range of methanol concentrations varied from a minimum of 46% methanol used for fluorescein to a maximum of 68% used for 3',4',5',6'-tetrachlorofluorescein.

Fig. 2 shows the HPLC traces of 4,5-diiodofluorescein (retention time = 13.5 min) and its impurities monitored at 4 wavelengths: 536nm, 520nm, 515nm, and 488nm. The absorbance maximum (λ_{max}) for 4,5-diiodofluorescein is 536 nm (Fig. 3). If each impurity is assumed to have the same λ_{max} and extinction coefficient (ε), the sample is shown to be no more than 80% pure. This is an invalid assumption as evidenced by the variation in peak heights for the impurities observed at the other wavelengths. Therefore, the sample may be much less pure than 80%, but in the absence of isolation and characterization of the λ_{max} and ε for

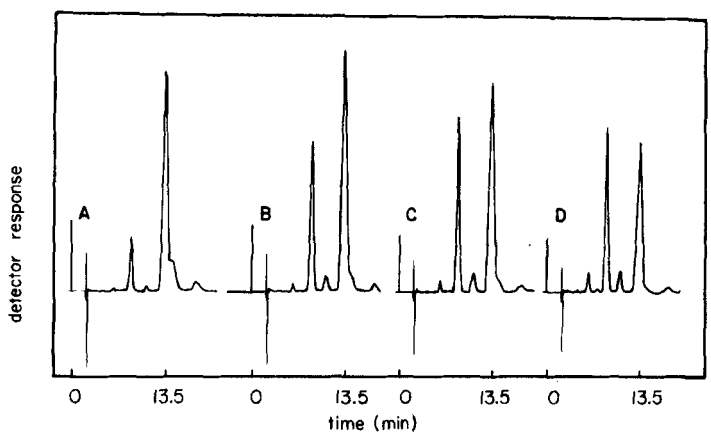

Figure 2

Commercially available diiodofluorescein chromatographed on reverse phase and detected at 4 specific wavelengths: curve A, 536nm; curve B, 520nm; curve C, 515nm; curve D, 488nm.

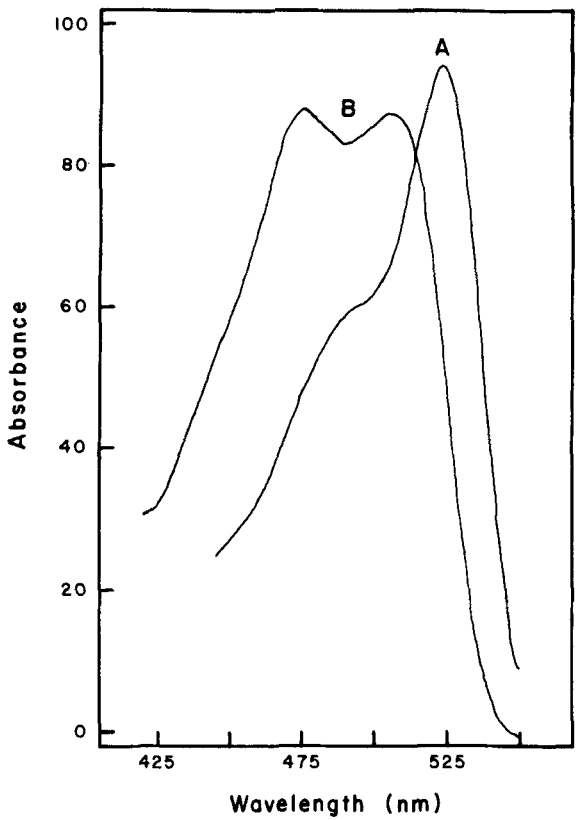

Figure 3

Absorbance maxima for 4, 5-diiodofluorescein (A) and one impurity (B) isolated from commercial diiodofluorescein.

each impurity, no more precise evaluation is possible. Corresponding data for the other xanthene dyes was also obtained.

Table 1 lists the dyes, the k' values, λ_{max}, the optimum solvent system, and maximum purity of each

Table 1

Dye	Solvent[1]	k'	λ_{max}(nm)[2]	Maximum % Purity
Fluorescein	46/54	7.14	478	99
4,5-Diiodofluorescein	60/40	7.01	536	80
4,5-Dibromofluorescein	52/48	21.44	517	80
2,7-Dichlorofluorescein	56/44	9.42	490	95
Erythrosin B	62/38	4.77	536	95
Phloxin B	64/36	5.41	545	95
Rose Bengal	66/34	5.67	555	85
3',4',5',6'-Tetrachloro-fluorescein	68/32	5.41	495	80
Octabromofluorescein	63/37	7.81	545	95
Eosin Yellowish	58/42	5.79	525	90

[1]Ratio of Methanol to Buffer in the solvent system.

[2]The λ_{max} was determined spectrophotometrically using 50% Methanol and 50% Buffer as the solvent.

dye based on the assumption of equal absorbance for each component at that wavelength.

With this knowledge, the necessity for further purification was obvious. Using optimum separation conditions, the presence of each dye, its purity, and some insight concerning the necessary scale-up for preparative HPLC, may be obtained.

A comparison of the retention times of the selected xanthene dyes within a single solvent system is shown in Table 2. Comparison of retention time to the type of halogen substituents, the degree of halogenation, and the location of the halogen substituents allowed an empirical theory to be developed to explain the retention observed.

Table 2

Dye	Functional Groups Lower Ring	Upper Ring	k'[1]
Fluorescein	-	-	1.31
Eosin Yellowish	-	4 Br	3.23
2,7-Dichlorofluorescein	-	2 Cl	4.26
4,5-Dibromofluorescein	-	2 Br	4.38
Erythrosin B	-	4 I	5.67
4,5-Diiodofluorescein	-	2 I	6.12
Phloxin B	4 Cl	4 Br	8.48
Octabromofluorescein	4 Br	4 Br	9.38
3',4',5',6'-Tetrachlorofluorescein	4 Cl	-	11.69
Rose Bengal	4 Cl	4 I	11.95

[1]The k' for each compound calculated in a solvent system consisting of 62% Methanol and 38% Buffer.

A comparison of the retention times of all of the other xanthenes to fluorescein (no halogens) indicated that the presence of any halogen on the molecule caused retention of the compound to be extended. Lower ring halogenation apparently had a greater effect on retention than halogenation of the upper rings. The 4 xanthenes containing halogen on the lower ring exhibited longer retention times. Further, rose bengal, containing 4 chlorine atoms on the lower ring, exhibited a retention time twice that of erythrosin B, which was identical save the lack of the chlorine atoms. Similarly, phloxin B had a retention time twice that of eosin yellowish.

Closer inspection indicated that a relationship based on specific halogens to retention times may be

made in the dyes exhibiting upper ring halogenation only. Considering the 3 dihalogenated xanthenes: diiodofluorescein, dichlorofluorescein, and dibromofluorescein, the elution order followed the order of increasing size. This relationship held for the two xanthenes with tetrahalogenated upper rings, eosin yellowish (4Br) and erythrosin B (4I), that were studied.

Detectable impurities have been found to elute both before and after the target peak of a dihalogenated xanthene. Theoretically, the elution order of xanthenes would be dependant upon the degree of halogenation. Thus, xanthenes exhibiting fewer than two halogens would elute prior to the dihalogenated xanthene and those exhibiting more than two halogens would elute after the dihalogenated xanthene. Therefore, the xanthene impurities in the diiodofluorescein shown in Fig. 2 would be fluorescein, iodofluorescein, triiodofluorescein, and/or tetraiodofluorescein. Other impurities would not be xanthenes.

CONCLUSION

A method for separation, purification, and quality control of xanthene dyes was developed. This HPLC technique proved to be a simple, accurate, and rapid method of monitoring the xanthenes that were routinely used in the experiments. A theory relating halogenation

of the xanthene ring to retention time in reverse phase HPLC was also developed.

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REFERENCES

(1) Chudy, J., Crosby, N. T., and Patel, I., "Separation of Synthetic Food Dyes Using High Performance Liquid Chromatography", J. Chromatog. 154: 306 (1978).

(2) McKone, H. T., "Identification of FD&C Dyes by Visible Spectroscopy", J. Chem. Educ., 54: 376 (1977).

(3) McKone, H. T. and Nelson, G. J., "Separation and Identification of Some FD&C by TLC", J. Chem. Educ., 53: 722 (1973).