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Note

Purification of halogenated fluorescein derivatives by gel chromatography

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As part of a study of the photosensitizing properties of fluorescein derivatives, a careful purification of the commercially available substances was carried out. It is well known[1] that, besides impurities of organic carbonate, halide and volatile compounds, these derivatives are also contaminated by molecules of similar structure but showing a lower degree of halogenation.

Most of these dyes have been studied (mainly because $FlBr_4$ or other fluorescein derivatives were used in the Romanowsky-type stains[2]) by analytical-scale separation techniques[3–6]. Little use has been made of preparative purification methods, some of which have drawbacks. For example, purification by acid precipitation does not allow one to separate the halogenated derivatives of fluorescein. Among chromatographic methods, the use of a talc column[5] implies the destruction of the column after each purification and subsequent desorption of the different fractions with an appropriate solvent. This method, although highly effective, is inconvenient: elution rates are low, only relatively small amounts of dye are separated in each run and the column packing requires frequent cleaning to remove the immobilized components. Recently, a valuable method[6] for the separation of xanthene was developed using high-performance liquid chromatography (HPLC) with a reversed-phase column. This technique was used to verify the purity of commercial products, but seems to be inappropriate for the purification of large amounts of halogenated fluorescein dyes.

This paper reports a simple and rapid method for the preparative purification of commercial fluorescein derivatives on Sephadex G-25 (superfine grade). The method was derived from Parrish's work[7] on food dyes. It allows good preparative purification of fluorescein as well as of halogenated fluoresceins such as rose bengal by adjustment of the concentration of NH_3 in the eluent.

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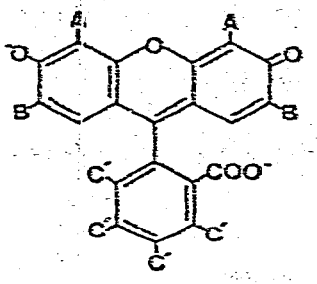
EXPERIMENTAL

Chemicals

The commercial dyes were obtained as indicated in Fig. 1. Ethanol, methanol and acetone were purchased from E. Merck (Darmstadt, G.F.R.). Double distilled water was used. Ammonia was from UCB (Brussels, Belgium).



	A	B	C'	Supplier
FlI_4Cl_4	I	I	Cl	Eastman
$FlBr_4Cl_4$	Br	Br	Cl	Merck
FlI_4	I	I	H	Merck
$FlBr_4$	Br	Br	H	Merck
$FlCl_4$	Cl	Cl	H	Hopkin & Williams
$FlBr_2(NO_2)_2$	Br	NO_2	H	Merck
$FlBr_3$	*	*	H	*
FlI_2	I	H	H	Gurr
$FlBr_2$	Br	H	H	Hopkin & Williams
2'7'$FlCl_2$	H	Cl	H	Merck
4'5'$FlCl_2$	Cl	H	H	Aldrich
Fl	H	H	H	Aldrich

Fig. 1. Structure of fluorescein and its halogenated derivatives. The names of the suppliers are given in the last column of the table. As $FlBr_3$ was obtained as a secondary product of $FlBr_4$ purification, the positions of the three bromines between the four positions (A and B) is unknown.

Methods

Preparative chromatography (10–30 mg of commercial dye per run) was carried out on Sephadex G-25 (superfine grade, Pharmacia), using an aqueous solution of ammonia as eluent. The concentration of ammonia was adjusted in order to obtain the optimum separation. The column was a Pharmacia SR 25/45 (2.5 × 30 cm). The various products eluted from the gel were detected by measuring their absorbance at 260 nm. For coloured products, the wavelength at the maximum of the visible absorption peak, λ_{max}^{abs} was measured. Products having the same λ_{max}^{abs} and the same ratio between the absorbance at 260 nm and the absorbance at λ_{max}^{abs} were pooled, partially evaporated in a rotavapor (Büchi) and lyophilized. The resulting powder was kept in the dark in closed vessels.

Desalting of the samples was performed on a shorter column (2.5 × 13 cm) packed with Sephadex G-25 and eluted with water.

The purity of the dyes was checked by thin-layer chromatography (TLC) on silica gel plates (Kieselgel 60, 20 cm high; Merck) as described by Cramer and Spears[6].

The determination of the molar extinction coefficients of the purified dyes in alkaline solution 10^{-2} *M* NaOH was carried out in siliconed tubes in order to avoid adsorption of the charged dye on the tube walls which introduced large dilution errors.

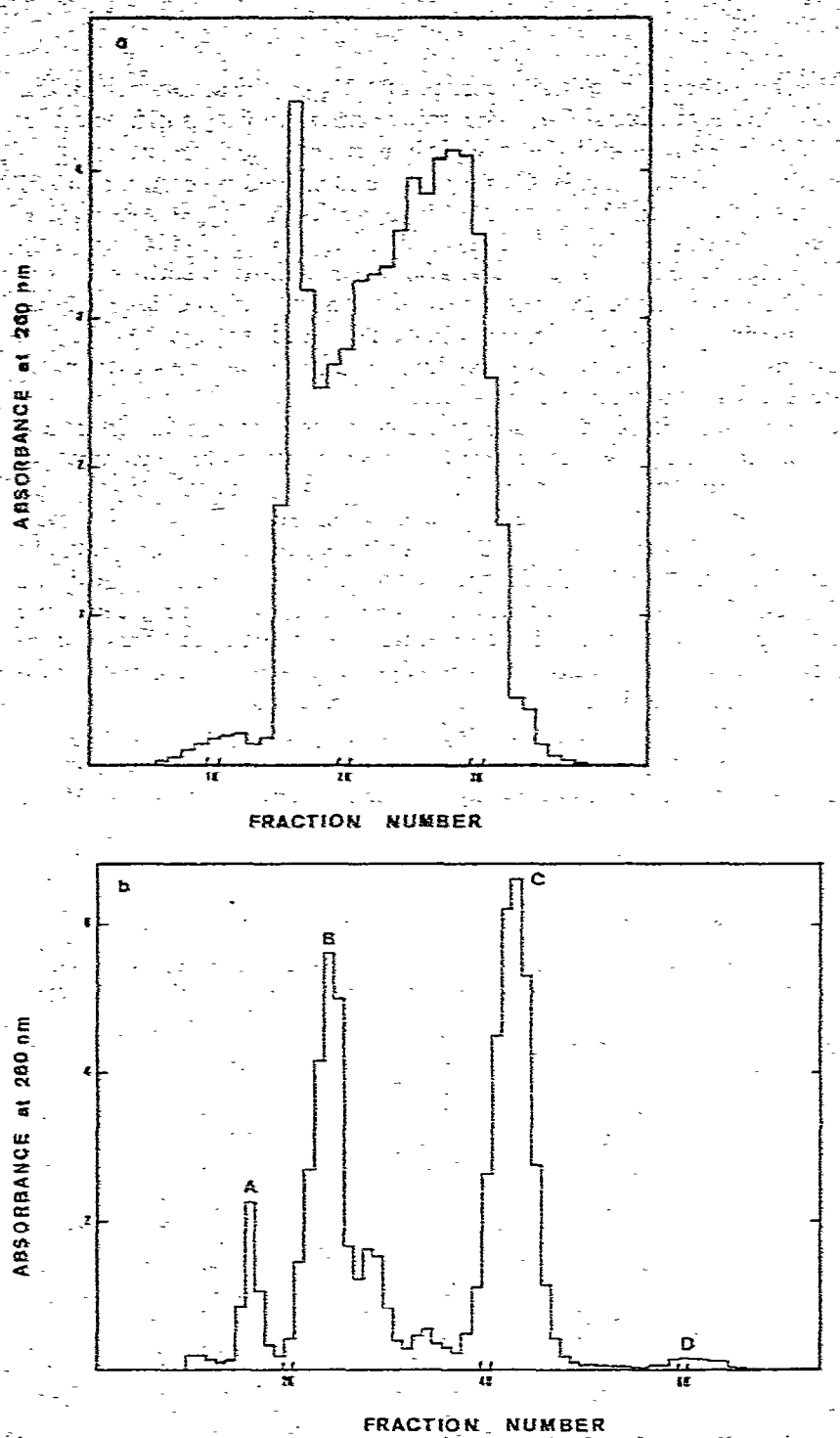


Fig. 2. Elution profile of the FII_2 purification by gel chromatography on Sephadex G-25 superfine eluted with water (a) and an aqueous solution of ammonia ($5 \cdot 10^{-2}$ *M*) (b). For the other experimental conditions, see text.

RESULTS AND DISCUSSION

Fig. 2a and 2b show the effect of the presence of NH_3 on the separation of the various constituents of the commercial FII_2. The improvement in FII_2 purification by using the optimal NH_3 concentration ($5 \cdot 10^{-2}$ *M*) is illustrated. The different halogenated derivatives present in fractions A, B, C and D were identified by comparison of their λ_{max}^{abs} with published data[9]. In this particular case, peaks A ($\lambda_{max}^{abs} = 490$ nm), B ($\lambda_{max}^{abs} = 497$–$498$ nm), C ($\lambda_{max}^{abs} = 506$–$507$ nm) and D ($\lambda_{max}^{abs} = 515$–$516$ nm) correspond to FI, FII, FII_2 and FII_3, respectively. The order of elution is the opposite of that expected on the basis of the exclusion principle and the molecular weights. This shows that gel chromatography of fluorescein derivatives is mainly based upon adsorption interactions, in agreement with the known affinity of the dextran gel matrix for aromatic and pseudo-aromatic substances[10,11].

The expected enhancement of the adsorption with increasing salt concentration is observed for all the dyes. However, a limiting value of the NH_3 concentration must be considered since adsorption of aromatics leads to longer purification times. The optimal concentrations of NH_3 for each dye are presented in Table I. The best separations were obtained with Sephadex G-25 superfine since elution was difficult from the less swollen G-10 and G-15 and adsorption was too greatly reduced with the more swollen G-50 gel. This method was applied to all the dyes, using the same experimental set up and adjusting the NH_3 concentration to the specific case considered. The number of coloured fractions was always equal to the number of coloured spots observed in previous TLC assays (column 2 of Table I).

Some commercial dyes contained coloured impurities which did not migrate and could perturb the chromatography. A pre-run on a short column (a few cm) of the same gel eluted with pure water was generally sufficient to eliminate the undesired compounds.

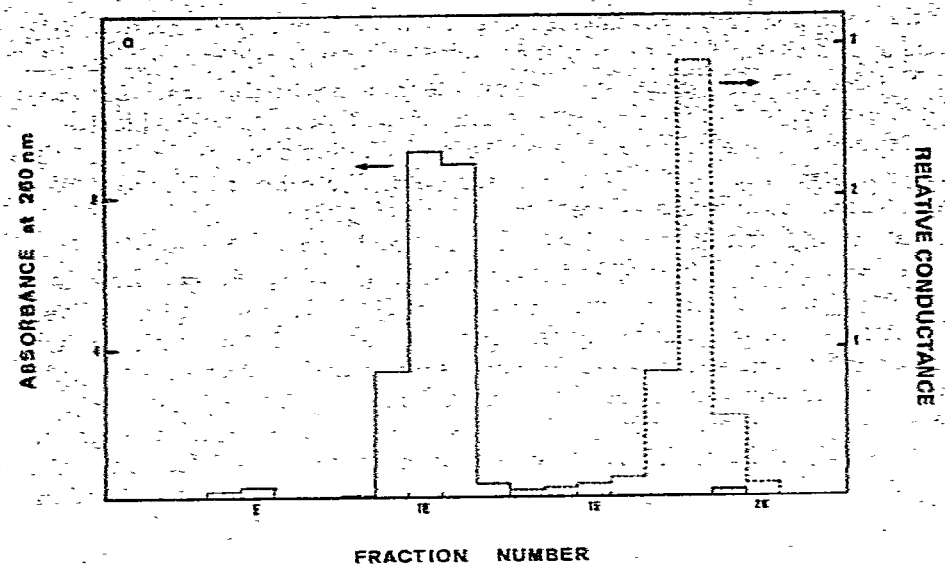
TABLE I

EXPERIMENTAL RESULTS OBTAINED DURING PURIFICATION OF THE DYES

Shown are the number of coloured spots observed by TLC prior to purification, the optimal NH_3 concentrations for separation of the different components, and the absorption characteristics of the purified dyes.

Dye	*TLC analysis*	$[NH_3]$ *(M)*	λ_{max}^{abs} *(nm)*	ε_{max} *(cm^2 $mmole^{-1}$)*
FII_4Cl_4	5	$5 \cdot 10^{-4}$	548.5	99,800
$FIBr_2Cl_2$	5	$5 \cdot 10^{-3}$	538.5	103,500
FII_4	2	$2 \cdot 10^{-3}$	526.5	90,000
$FIBr_4$	4	$5 \cdot 10^{-2}$	517	111,500
$FICl_4$	1	$5 \cdot 10^{-2}$	510	94,000
$FIBr_2(NO_3)_2$	3	$5 \cdot 10^{-2}$	517	105,000
$FIBr_3$	*	$5 \cdot 10^{-2}$	510	100,000
FII_2	4	$5 \cdot 10^{-2}$	506.5	95,000
$FIBr_2$	4	$5 \cdot 10^{-2}$	504	98,000
2'7'$FICl_2$	2	$5 \cdot 10^{-2}$	502.5	97,000
4'5'$FICl_2$	2	$5 \cdot 10^{-2}$	502.5	96,000
FI	1	$5 \cdot 10^{-2}$	490	90,000

* $FIBr_3$ is a secondary product of the purification of $FIBr_4$.



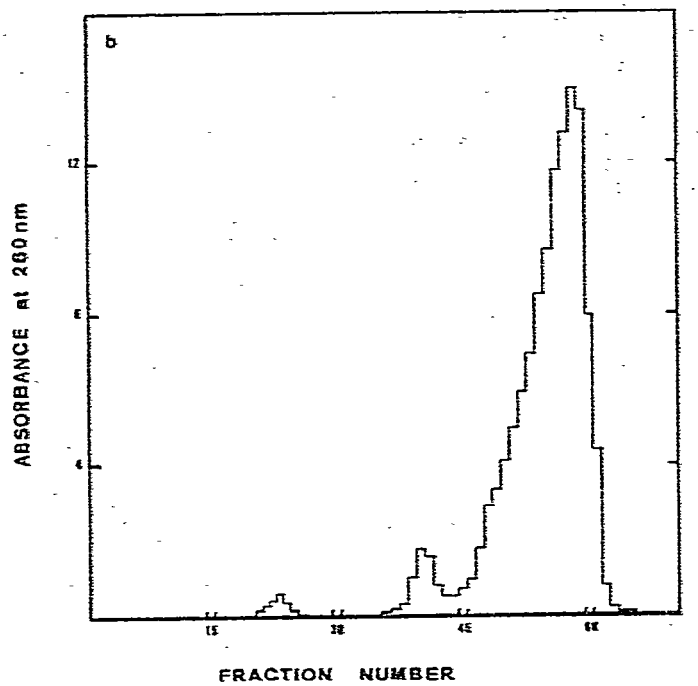


Fig. 3. Elution profile of the $FICl_4$ purification by gel chromatography on Sephadex G-25 superfine: a, desalting chromatography; b, purification chromatography. For the experimental conditions, see text.

When the solubility of the commercial dye was low (acidic form) the following procedure was applied. The dye was transformed to its sodium salt by reaction with an excess of NaOH. After lyophilization, the powder was desalted by chromatography on a 13 cm high column of G-25 superfine (elution with pure water) as illustrated in Fig. 3a. The measurement of the absorbance and conductance of the fractions indicated the good separation which had been achieved. The coloured fractions were pooled, concentrated and purified as described above (Fig. 3b).

The values of λ_{max}^{abs} and of the molar extinction coefficients of the purified dyes are collected in Table I. The values of λ_{max}^{abs} agree with previously published data[9] and the high values of ε_{max} are indicative of the purity of the dyes. Moreover, TLC analysis of the purified products always gave only one spot. So, this simple and rapid method of purification of halogenated fluorescein derivatives by gel chromatography on Sephadex G-25 superfine eluted with aqueous ammonia solution gives very good separations. Although NH_3 can be replaced by other substances, it has some advantages: in addition to its volatility, the basic pH allows direct measurement of the λ_{max}^{abs} of the coloured form of the eluted dye (dianionic form) and subsequently its identification.

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