Journal of Chromatography, 135 (1977) 217–220
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 9870

Note

High-performance liquid chromatography of plant pigments

KENNETH ESKINS, CHARLES R. SCHOLFIELD and HERBERT J. DUTTON
*Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture**, *Peoria, Ill. 61604 (U.S.A.)*
(First received May 31st, 1976; revised manuscript received December 7th, 1976)

A number of papers on the analytical and preparative separation of plant pigments have been published. Methods investigated include column[1], paper[2], countercurrent distribution[3], and thin-layer chromatography[4]. No one of these, however, is entirely suitable for both analysis and preparative work on a wide range of plant pigments. We report preliminary results on a liquid chromatographic method which combines simplicity, sensitivity and reproducibility with little or no degradation. The sources of pigments used in this study, spinach and the brown sea diatom, *Nitzschia closterium*, represent well-characterized and diverse systems of the chlorophyll and carotenoid pigments.

EQUIPMENT AND METHODS

Pigments

Pigment mixtures were obtained by extraction of fresh spinach or cultures of the brown sea diatom, *Nitzschia closterium* f. *minutissima* (Bloomington 642). The diatoms were grown in an artificial seawater medium[5] which was maintained at 18° within a cylindrical arrangement of 12 Gro-lux fluorescent lamps. The culture was magnetically stirred and aerated with a mixture of 5% carbon dioxide in air. Samples were centrifuged and immediately extracted for chromatography.

Liquid chromatography

High-performance liquid chromatography (HPLC) was run on a Waters Assoc. ALC-202 instrument with two 61 cm × 7 mm I.D. stainless-steel columns in series packed with 37–75 μm Bondapak C_{18}–Porasil B. The UV detector was modified with a kit to measure absorbance at 440 nm. Runs were made at 28° or 18° with a solvent flow-rate of 2.5 or 4 ml/min. Pump pressure varied from 600 to 250 p.s.i. depending on the solvent used.

Reagents

All reagents and solvents used were ACS grade chemicals and were used

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

without purification. All extractions and operations were conducted in darkened rooms at room temperature.

Pigment extracts

Nitzschia closterium. One liter of heavy culture (5×10^6 cells per ml) was centrifuged (2–3 ml wet packed cells) and extracted with 15 ml volumes of acetone by mixing with sand and grinding in a mortar. The grinding and extracting were repeated until no more color could be eluted. The acetone extract was reduced in volume under vacuum to approximately 10 ml, then redissolved in ether (100 ml). The ether solution was extracted six times with 50-ml portions of 10% sodium chloride to remove colorless lipids and acetone solvent. The ether layer was dried over sodium sulfate then reduced to dryness under vacuum. The residue was taken up in 6 ml of methanol. Several drops of acetone were added to completely dissolve the residue.

Spinach. Six medium leaves stripped of main stems and veins (16.4 g fresh weight) were blended with 100 ml of acetone. After filtration the extract was treated as described above for *Nitzschia closterium.*

Identification of pigments

Pigments were characterized by comparison of their visible spectra with literature values. In addition, the pigments were paper chromatographed using a system of 30% chloroform in petroleum ether on Whatman 3MM chromatography paper[2].

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram for the pigments of *Nitzschia closterium.* The good resolution of all major pigments was obtained with aqueous methanol by a solvent program of 80, 90, 95, 97.5 and 100% methanol followed by 10, 50 and

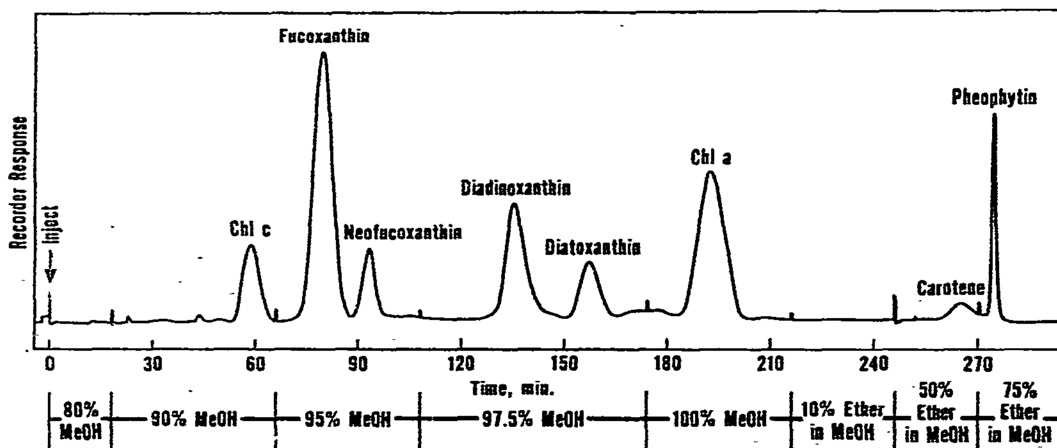

Fig. 1. HPLC of *Nitzschia closterium* pigments run on a Waters Assoc. ALC-202 with two 61 cm × 7 mm I.D. stainless-steel columns packed with 37–75 μm Bondapak C_{18}–Porasil B. Solvent flow-rate 2.5 ml/min at 28°.

TABLE I

IDENTIFICATION DATA FOR PIGMENTS OF *NITZCHIA CLOSTERIUM*

Pigment	*Retention time (min)*	*Visible spectra*				*Paper chromatography*	
		Max. reported	*Solvent*	*Ref.*	*Max. found*	*R_F reported*	*R_F found*
Chlorophyll c	58.5	445, 580, 625	Ethanol	2	445, 582, 625	0.00	0.00
Fucoxanthin	79.5	425, 450, 478	Hexane	8	425, 449, 478	0.28	0.27
Neofucoxanthin (A, B)	93.2	447	Ethanol	2	446	0.08	0.06
Diadinoxanthin	135.2	448, 478	Ethanol	2	448, 477	0.46	0.43
Diatoxanthin	157.2	453, 481	Ethanol	2	451, 481	0.55	0.59
Chlorophyll a	192.5	428, 660	Ether	9	428, 660	0.27	0.29
Carotenes	265.0	425, 451, 482	Pet. ether	8	425, 449, 476	0.93	0.96
Pheophytin	274.5	409, 667	Ether	10	408, 667	0.92	0.96

75% ether in methanol. The column temperature was maintained at 28°. A programmed elution was necessary because straight elution using 95% methanol failed to resolve the first three peaks, while greater concentrations of water greatly extended elution time. The retention times and identifying data for each of the major peaks are given in Table I.

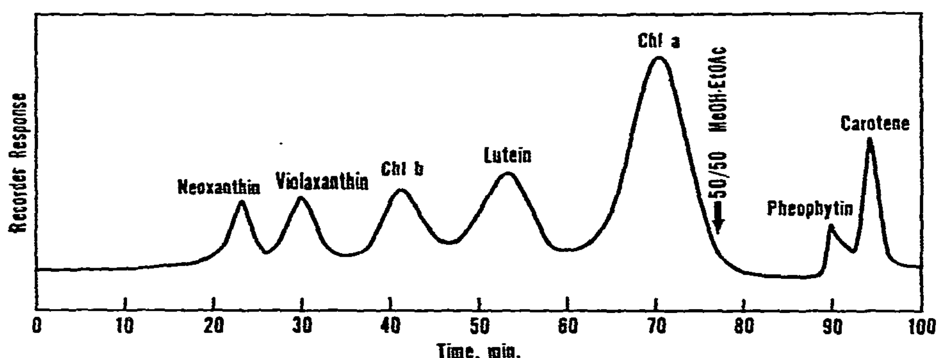

Fig. 2. HPLC of spinach pigments. Solvent flow-rate, 4.0 ml/min at 18°. Other conditions, see Fig. 1.

TABLE II

IDENTIFICATION DATA FOR PIGMENTS OF SPINACH

Pigment	*Retention time (min)*	*Visible spectra*			*Paper chromatography*	
		Max. reported[6]	*Solvent*	*Max. found*	*R_F reported*	*R_F found*
Neoxanthin	23	414, 436, 466	Ethanol	414, 437, 466	0.05	0.05
Violaxanthin	30	421, 440, 470	Ethanol	421, 443, 469	0.48	0.48
Chlorophyll b	41	645, 598, 455	Acetone	455, 595, 644	0.10	0.12
Lutein	53	422, 445, 476	Ethanol	423, 445, 474	0.73	0.70
Chlorophyll a	72	*		*	*	*
Pheophytin	92	*		*	*	*
Carotenes	96	*		*	*	*

* Reported in Table I.

Fig. 2 shows a chromatogram for spinach extract eluted with 98% methanol then 50% ethyl acetate in methanol. Good resolution of the pigments is also obtained. It was necessary, however, to lower the column temperature to 18° before a satisfactory separation of chlorophyll b (Chl b) and lutein was accomplished. Identifying data for the spinach pigments are given in Table II.

CONCLUSIONS

Of all prior methods of pigment separation, column chromatography represented the best combination of virtues. With HPLC, these good features are improved by the use of a standard re-useable column and reproducible retention times. The ability to separate complex mixtures under reproducible conditions without undue exposure to air or light represents a definite improvement in pigment chromatography. The reverse-phase partition chromatography used here is an extension of earlier solvent partition separations by countercurrent distribution using similar hydrocarbon–alcohol systems[6,7]. In our present system, a preparative column was used because we were interested in obtaining larger quantities of plant pigment for additional experiment. Due to the use of this larger column, the separation times were long (4½ h) especially when a solvent program was used. It was possible, however, to use smaller quantities on an analytical column and to reduce the total elution time to less than 1 h.

In summary, we feel that the separation of plant pigment by HPLC is a remarkably versatile system and that the difficulties presently encountered can be overcome. We intend to pursue these studies using a variety of column materials and solvents to improve separation of the pigments of algae and higher plants.

REFERENCES

1 H. H. Strain, *Annu. Priestley Lect.*, 32 (1958).
2 S. W. Jeffrey, *Biochem. J.*, 80 (1961) 336.
3 C. R. Lancaster, E. B. Lancaster and H. J. Dutton, *J. Amer. Oil Chem. Soc.*, 27 (1950) 386.
4 M. F. Bacon, *J. Chromatogr.*, 17 (1965) 322.
5 J. E. Mann and J. Myers, *J. Phycol.*, 4 (1968) 349.
6 M. C. Burnett, R. L. Lohmar and H. J. Dutton, *J. Agr. Food Chem.*, 6 (1958) 374.
7 A. L. Curl, *J. Agr. Food Chem.*, 1 (1953) 456.
8 T. W. Goodwin, in K. Paech and M. V. Tracey (Editors), *Modern Methods of Plant Analysis, Vol. III, Carotenoids*, Springer, Heidelberg, 1955, p. 272.
9 H. H. Strain, M. R. Thomas and J. J. Katz, *Biochim. Biophys. Acta*, 75 (1963) 306.
10 F. C. Pennington, H. H. Strain, W. A. Svec and J. J. Katz, *J. Am. Chem. Soc.*, 86 (1964) 1418.