Journal of Chromatography, 170 (1979) 264–268
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,512

Note

Single-step separation and identification of photosynthetic pigments by high-performance liquid chromatography

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(Received September 25th, 1978)

The analysis of the pigment composition of the photosynthetic apparatus still suffers from a number of difficulties. The main problems arise from the inherent instability of the extracted pigments and their extreme sensitivity to light, heat and oxygen[1]. Further, it is difficult to separate all of the pigments in a single-step procedure owing to the diverse polar characteristics of the pigments: a total pigment extract from the photosynthetic apparatus of higher plants and green algae contains xanthophylls with relatively high polarity (due to their free hydroxyl or carbonyl groups), chlorophylls with medium polarity and phaeophytins, carotenes and their epoxides, which are of low polarity. Hitherto, the analysis of the pigment extract and the characterization of its components have relied mainly on the use of thin-layer or column chromatography[2,3] followed by spectroscopic examination of the isolated pigments.

Recently, Evans *et al.*[4] applied high-performance liquid chromatography (HPLC) to the analysis of porphyrins and chlorophyll derivatives. The separation of carotenoids[5–7] and of chlorophyll *a* and chlorophyll *b*[8] by HPLC has also been reported. Iriyama *et al.*[9] described a micro-method for the separation of a plant pigment extract on a silica gel column by HPLC and Eskins *et al.*[10] reported an HPLC preparative procedure using a C_{18} reversed-phase column. Both methods resulted in the separation of at least seven identifiable pigments, taking 75 min in the analytical procedure of Iriyama *et al.*[9] and about 4 h in the preparative procedure of Eskins *et al.*[10].

Using a self-packed C_{18} reversed-phase column and a step-gradient elution technique, we have improved the HPLC separation of a total pigment extract of the green alga *Chlorella* by decreasing the separation time and increasing the number of pigments separated. Further, the identification of each individual separated pigment during the elution was made possible by the use of a built-in spectrophotometric detector system and a stop-flow procedure.

EXPERIMENTAL

The unicellular green alga *Chlorella fusca* (strain 211-15, obtained from the algal culture collection, University of Göttingen, G.F.R.) was grown autotrophically according to the method of Grimme and Boardman[11]. Algal cells were harvested by centrifugation and washed. The pigments were extracted into acetone by shaking the

cells with glass beads of 5-mm diameter and acetone in a Vibrogen homogenizer (Bühler, Tübingen, G.F.R.). The pigments were transferred from acetone into diethyl ether in a separating funnel. The ether layer was washed thoroughly with 10% sodium chloride solution to remove trace amounts of acetone. The ether extract was then used immediately for analysis.

All reagents and solvents used were of analytical-reagent grade and were used without further purification. All operations were conducted in darkened rooms and the extracts were kept in the cold as far as possible.

For HPLC analysis an Altex Model 100A liquid chromatograph (Altex Scientific Instruments, Berkeley, Calif., U.S.A.) was used, equipped with an LC-55 UV–visible light detector and a digital scan unit (Perkin-Elmer, Norwalk, Conn., U.S.A.). The glass column (300 mm × 3 mm I.D.) was self-packed with HPLC-Sorb-Sil 60-D 10 C_{18} (Macherey, Nagel & Co. Düren, G.F.R.) according to the balanced-density method[12] and protected by a stainless-steel mantle according to Stahl (Riedel-de Haën, Hannover, G.F.R.).

The samples were introduced into the column by the stop-flow technique from a 10-μl precision syringe via a septum inlet port. Elution was performed by applying a stepwise methanol–water gradient as shown in Fig. 1. The detector was set at 440 nm, which proved adequate for the detection of most of the pigments.

The visible spectra were then recorded at the elution peak maximum after stopping the flow.

The pigments were identified by comparing their retention times with those of authentic standards and by their spectral characteristics. Authentic standards were prepared according to Hager and Meyer-Bertenrath[13].

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of an ether extract obtained from exponentially growing cells of *Chlorella fusca*. The pigments elute in four discrete groups as indicated by I, II, III and IV. The groups of pigments and the individual pigments within each group separate according to their polarities, showing that the stepwise gradient elution is adequate for minimizing the elution time without loss of resolution. The complete run takes about 40 min and results in 15 peaks. Only partial resolution was obtained with the phaeophytin(s) from β-carotene (peaks 14 and 15), but a complete separation was achieved when the separation time was extended and a slightly different gradient was used. However, the main aim of this programme was to achieve a short separation time.

For the isomers neoxanthin (peak 3) and neoxanthin neo A (peak 2) and for the isomers chlorophyll *a* (peak 13) and chlorophyll *a'* (peak 12) the resolution of the reversed-phase column is remarkably high under the conditions described. The xanthophylls were also separated satisfactorily. In particular, antheraxanthin and neoxanthin, which differ chemically by the presence of only one additional hydroxyl group in neoxanthin, were separated clearly (for spectra see Fig. 2a and b); previous work failed to obtain a distinct separation of the two pigments[9,10].

Table I summarizes the data obtained for the identification of the separated pigments from the *Chlorella* extract. All absorption maxima were obtained from spectra that were actually recorded during the separation procedure. Even pigments in

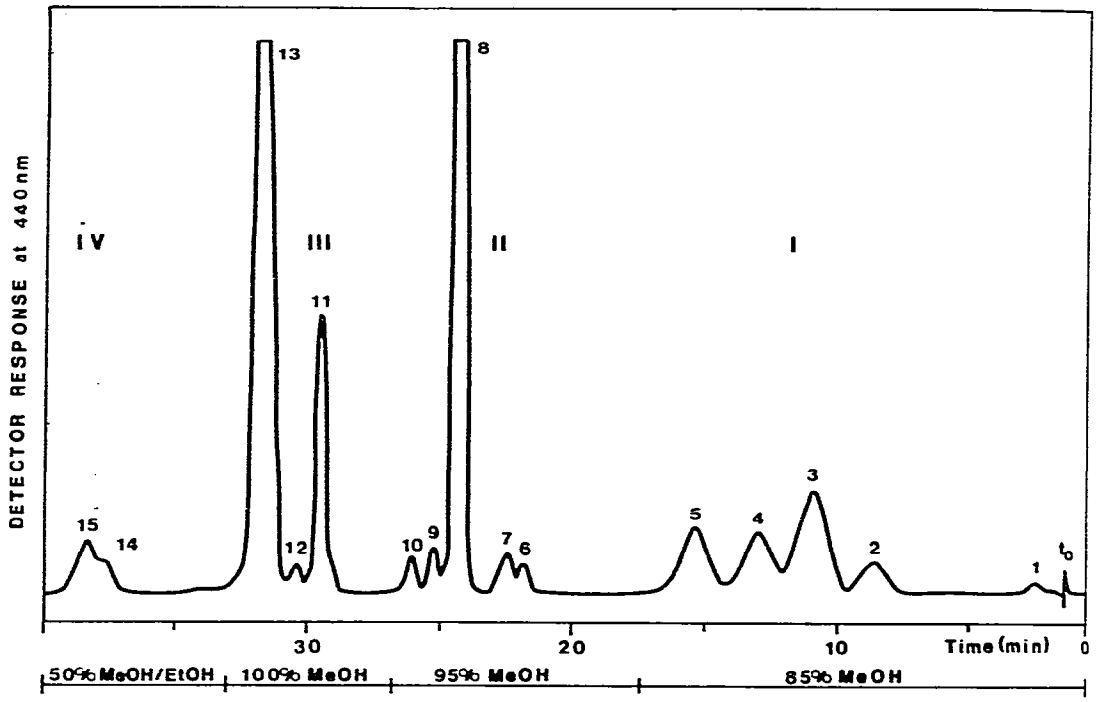

Fig. 1. Elution chromatogram obtained by HPLC of a pigment extract from *Chlorella fusca* on a C_{18} reversed-phase column and with the stepwise gradient elution indicated. Flow-rate 2.0 ml/min (baseline corrected).

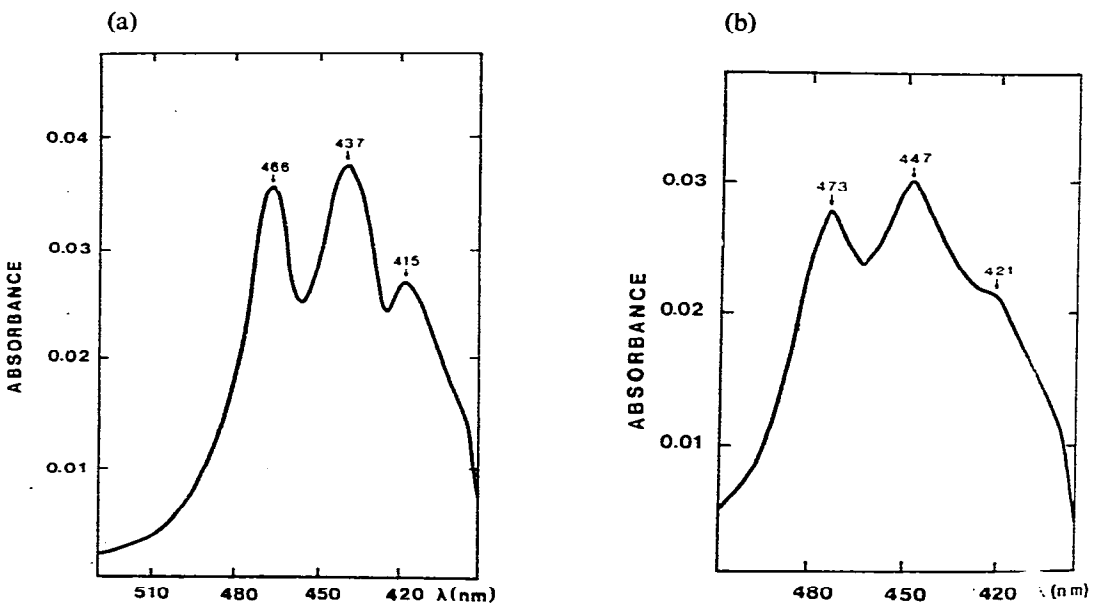

Fig. 2. Absorption spectra of (a) neoxanthin and (b) antheraxanthin (peaks 3 and 4, respectively, in Fig. 1), recorded during elution by the stop-flow method on reaching maximum absorption at 440 nm.

small amounts can be detected and spectroscopically characterized as shown in Fig. 2a and b. Absorption spectra were compared with those derived from authentic standards and with already published spectra (for references see Table I).

With this procedure it is also possible to determine pigment concentrations by introducing into the column a known amount of a pure pigment and comparing the areas under the corresponding peaks. The accuracy of this method has been demonstrated to be satisfactorily[9].

BLE I

NTIFICATION DATA FOR PIGMENTS OF *CHLORELLA FUSCA*

Pigment	*Visible spectra*		*Solvent*	*Reference*	*III/II found (%)*	*III/II reported (%)*
	Maxima found	*Maxima reported*				
Unidentified	—	—	—	—	—	—
Neoxanthin neo A	417, 439, 469	418, 440, 470	Ethanol	14	77	62 (hexane)
Neoxanthin	415, 437, 466	415, 438, 467	Ethanol	14	82	89 (hexane)
Antheraxanthin	421, 447, 473	421, 443, 473	Ethanol	15	60	50 (hexane)
Violaxanthin	417, 440, 470	417, 441, 471	Ethanol	14	88	97 (hexane)
Violeoxanthin	414, 436, 465	(415), 436, 464	Ethanol	1	75	—
Unidentified	—	—	—	—	—	—
Lutein	421, 445, 473	422, 446, 475	Ethanol	14	63	60 (hexane)
Lutein 5,6-epoxide	(417), 440, 469	416, 440, 469	Ethanol	14	73	79 (hexane)
Unidentified	—	—	—	—	—	—
Chlorophyll *b*	468, 654	453, 648	Diethyl ether	16	—	—
Chlorophyll *a'*	428	428	Diethyl ether	16	—	—
Chlorophyll *a*	432, 667	430, 662	Diethyl ether	16	—	—
Pheophytin(s)	(Identified by authentic standards)				—	—
β-Carotene	426, 451, 478	425, 450, 478	Ethanol	15	33	27 (hexane)

HPLC is a sensitive and reproducible method for the separation and qualitative and quantitative determination of photosynthetic pigments. With the *Chlorella* pigment extract examined here a separation of more than ten different pigments was achieved with good resolution in short elution times by the application of the stepwise gradient technique on C_{18} reversed-phase columns. An advantage of HPLC over other chromatographic methods for the sepatation of readily oxidizable or photolabile pigments is that the pigments can be identified by using a spectrophotometric detector system during analysis.

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