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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CHLOROPHYLLS AND CAROTENOIDS

THOMAS BRAUMANN and L. HORST GRIMME

University of Bremen, Fachbereich Biologie/Chemie, 2800 Bremen 33 (F.R.G.)

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Reversed-phase high-performance liquid chromatography with octadecyl- or octylsilylated silica gel as the stationary phase provides a powerful tool in the analysis of chloroplast pigments from higher plants and green algae. Chromatographic columns packed with 10 μm chemically bonded silica gel particles allow the simultaneous separation of chlorophylls *a* and *b*, chlorophyll isomers, pheophytins *a* and *b*, α-carotene, β-carotene, lutein, violaxanthin, lutein-5,6-epoxide, antheraxanthin, neoxanthin and several minor carotenoids from a single sample within a short analysis time. The quantitative analysis requires a minimum of 1–5 pmol for carotenoids and 5–10 pmol for chlorophylls. Pigment degradation products, formed on polar stationary phases, are not found in reversed-phase high-performance liquid chromatography due to the weak hydrophobic forces on which the separation mechanism is based. The production of altered pigments however, either induced by various treatments or generated during the isolation, can be monitored as the reversed-phase system is selective enough to separate *cis*-isomers and oxidation products from their parent compounds. The reproducibility of the individual retention time for each pigment is better than ±1.5% which facilitates the identification of unknown pigments. The method is applied to the analysis of the pigment composition of *Chlorella fusca*, spinach (*Spinacia oleracea*) chloroplasts, and to the rapid determination of the ratio of chlorophyll *a* to chlorophyll *b*.

Introduction

Biochemical and physiological work on plants and plant organelles often relies on the determination of the total pigment content as a reference figure. Several methods are available to extract chlorophylls and carotenoids from leaves, chloroplasts and algae by acetone [1], methanol [2] or alkaline pyridine [3] and to estimate the pigment content of the extract spectrophotometrically by the use of specific absorption coefficients for chlorophylls [4], chlorophyll derivatives [3] or carotenoids [5]. A more differentiated need is the estimation of the quantitative molecular pattern of pigments and changes in their stoichiometry under ecological, physiological or biochemical influences.

Such detailed studies are hampered by the fact that carotenoids and chlorophylls are labile structures which are sensitive to light, heat, oxygen and chemical degradation [6–8]. Consequently, harmful influences have to be excluded during the isolation and separation of the photosynthetic pigments. An additional difficulty for the separation is the structural similarity within each group of the pigments, namely the chlorophylls with chlorophyll *a* and chlorophyll *b*, the nonpolar carotenes with α-carotene and β-carotene, and the polar xanthophylls with one or more hydroxy and/or epoxide groups at their carbon skeleton. In some cases, pigment molecules differ from each other only by

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Abbreviation: rp-HPLC, reversed-phase high-performance liquid chromatography.

the position of a functional group or a double bond. Thus, a separation technique has to meet high demands with respect to selectivity.

Among the chromatographic methods used, thin-layer chromatography (TLC) and paper chromatography have found the widest application [9,10], low-pressure column chromatography being mainly used in preparative work [11]. However, these chromatographic techniques have several limitations. It is in no case possible to separate the whole set of pigments with good resolution in a single run so that further manipulations, such as saponification to remove the chlorophylls, prefractionation through the distribution between two immiscible solvents, successive chromatography on different systems, or two-dimensional chromatography, are required [8]. These manipulations are laborious and can lead to the formation of artefacts [12,13]. The widespread use of silica gel and other polar stationary phases offers an additional source for erroneous results. These phases can react with the pigments leading to several coloured and uncoloured alteration products [7,9,13].

As a result, there are many divergent conclusions regarding the number and identity of the chloroplast pigments which constitute the in vivo set within the photosynthetic apparatus. A further confusion arises from many inconsistencies in the reported quantitative distribution of the pigments among the functional entities of the thylakoids. TLC and paper chromatography require considerable amounts of the separated pigments – in the range of 0.2–1.0 μg for carotenoids and chlorophylls, respectively – for a reliable quantification. This is often difficult to achieve for the minor pigments. A further source of mistake comes into play when the visible parts of the chromatogram are scraped or cut off and subsequently eluted. Besides the risks of overlooking minor pigments, double zoning [10] and unsharp or even overlapping zone boundaries, the recovery of the pigments rarely approaches 90–95% [9].

In summary, the conventional chromatographic techniques have inherent limitations which render more difficult any conclusions drawn from the elaborated qualitative and quantitative pigment composition presented until now.

Since the introduction of reversed-phase high-performance liquid chromatography (rp-HPLC) during the last years, a rapidly growing amount of separation problems in biochemistry have been solved [15,16]. This technique relies on the hydrophobic interaction between solute, mobile phase and stationary phase. The latter normally consists of totally porous silica gel with a particle size of 5–10 μm. 'Reversed-phase' indicates that a non-polar hydrocarbonaceous layer is chemically bonded onto the surface of the silica gel by silylation of the free hydroxyl groups with an alkyl trichlorosilane (usually octadecyl- or octyltrichlorosilane). The nature of the retention mechanism in rp-HPLC is a matter of controversy [16,17]. As a rule of thumb, the pigments elute from the column in the order of an ascending partition coefficient, xanthophylls are eluted first before the chlorophylls, and carotenes and pheophytins are eluted last. Because of the small particle size and the weak surface energies of bonded phases, separations are rapid and highly efficient also for complex mixtures.

Up to now only few reports exist which describe the separation of plant pigments by HPLC, and which are moreover limited to the analysis of chlorophylls [18–20] or carotenoids [21–24], respectively. As far as they deal with a total extract of chloroplasts [25,26], the results are unsatisfactory with respect to resolution and separation time. Stransky [27] described the separation and quantification of pigments from spinach chloroplasts by HPLC, but his system is based on the reactive silica gel as the stationary phase, and is furthermore not applicable to the analysis of minor chlorophylls and carotenoids. Recently, we have presented preliminary results for the qualitative separation of photosynthetic pigments from the green alga *Chlorella fusca* using octadecyl silica and a step-wise gradient of methanol/water as the mobile phase [28]. Although being a considerable improvement, the binary solvent system failed to separate the lipophilic carotenes from the pheophytins. Secondly, some minor peaks consisted of a mixture of xanthophyll epoxides and *cis*-isomers so that an unequivocal identification has not been possible.

The present paper describes a chromatographic system which exploits fully the versatility and separation power of rp-HPLC, and which is applicable to a wide variety of problems concerning the qualitative and quantitative analysis of chlorophylls and carotenoids.

Materials and Methods

Pigment extraction

Chloroplasts of spinach (*Spinacia. oleracea*) were prepared by a standard procedure [29] and resuspended in 80% acetone (v/v) at room temperature. The extract was centrifuged at 5 000 rev./min for 5 min (Labofuge III, Heraeus-Christ, Osterode, F.R.G.) to remove cell debris. Pigments were transferred to diethyl ether by adding an equal volume of ether to the acetone extract in a separating funnel and by shaking with twice the volume of 10% KCl (w/v) to improve pigment migration to the ether layer. The ether layer was washed thoroughly with distilled water to remove the remaining acetone and used immediately for analysis. If necessary, the ether extract was concentrated by blowing a stream of pure nitrogen over the surface of the extract.

The green alga *Chlorella fusca* (strain 211-15, algal culture collection, University of Göttingen, F.R.G.) was grown autotrophically according to Grimme and Boardman [30]. The cells were harvested by centrifugation, resuspended in ethanol and homogenized with glass beads of 0.5 mm diameter in a Vibrogen homogenizer (Bühler, Tübingen, F.R.G.) at 0–4°C for 4 × 15 s. We found in preliminary experiments that ethanol is slightly more effective in extracting the carotenoids from *Chlorella* cells than 80% acetone; the chlorophylls on the other hand are equally well extracted. The extract was filtered, the cell residues were washed with 96% ethanol (v/v) to remove the remaining pigments. The pigments were transferred to diethyl ether as described above.

All operations were conducted in darkened rooms and the extracts were kept in the cold as far as possible. Reagents and solvents were of analytical grade and used without further purification. Diethyl ether was routinely checked for peroxides by a commercial test kit (Merck, Darmstadt, F.R.G.).

Chromatography

The liquid chromatograph consisted of a Series 2/2 reciprocating pump (Perkin-Elmer, Norwalk, CT, U.S.A.), a model LC-55 variable wavelength UV/VIS-detector (Perkin-Elmer, Norwalk, CT, U.S.A.) set at 445 nm and a model Servogor S pen recorder (Metrawatt, Nürnberg, F.R.G.). Injections were performed by a model 7105 sample valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 175-µl loop. The sample volume was between 2–10 µl and was introduced into the valve by means of a precision syringe. The chromatograph was connected to a model Sigma-10 integrating unit (Perkin-Elmer, Norwalk, CT, U.S.A.) which performed the quantitative evaluation of the chromatograms.

Two different columns were used during the experiments: a 10-µm Lichrosorb-RP 8 column (25 cm × 4.6 mm internal diameter) (Merck, Darmstadt, F.R.G.) and a steel-protected glass column (30 cm × 3.0 mm internal diameter) (Riedel-de Haen, Hannover, F.R.G.), self-packed according to the balanced-density method [28] with 10 µm Sil 60-RP 18 (Riedel-de Haen, Hannover, F.R.G.). The solvents were degassed prior to use with an ultrasonic bath under low pressure. The mobile-phase composition and other chromatographic properties are indicated in the legends of the figures.

Identification of the pigments

The pigments were identified by comparing their retention times with those of authentic standards and by their spectral characteristics. Xanthophyll-standards were prepared according to Hager and Meyer-Bertenrath [31], α-carotene and β-carotene were of the highest purity available (type V and type IV, respectively, from Sigma, München, F.R.G.). Spectra of the eluted pigments were recorded with a model DW-2 spectrophotometer (Aminco, Silver Spring, MD, U.S.A.). Antheraxanthin and lutein-5,6-epoxide were additionally identified by their hypsochromic shift after treatment with HCl [28], and trihydroxy-α-carotene (identical with loroxanthin and pyrenoxanthin [30]) by the acetylation of the hydroxyl groups [29] and subsequent separation of the resulting acetates on the RP 18 column.

Quantitative analysis

Known amounts of pure pigment in the range of 20–2 000 pmol were injected and the area under the peak was measured by the integrating unit described above. The following extinction coefficients were

TABLE I

EXTINCTION COEFFICIENTS OF CHLOROPHYLLS AND CAROTENOIDS AT WAVELENGTH MAXIMUM

Pigment	$\epsilon^{1\%}_{1\,\mathrm{cm}}$	Solvent	Ref.
Neoxanthin	2 270	Ethanol	31
Trihydroxy-α-carotene	2 540	Ethanol	34
Violaxanthin	2 500	Ethanol	31
Lutein-5,6-epoxide	2 400	Ethanol	31
Antheraxanthin	2 350	Ethanol	31
Lutein	2 540	Ethanol	31
Chlorophyll *b*	535	Acetone	35
Chlorophyll *a*	926	Acetone	35
α-Carotene	2 525	Hexane	*
β-Carotene	2 212	Hexane	*

* Given by the supplier for the specific lot used.

used to calculate the amount of pigments injected (Table I).

Six to ten measurements for each of four different concentrations were performed; the resulting calibration curves were linear over the whole concentration range with a linear correlation coefficient of 0.991–0.998. The S.D. of the mean for each concentration was better than ±3.0%.

Results

Fig. 1A shows the separation of a total extract from *C. fusca* on a self-packed RP 18 column, routinely used for the analytical work. Complete separation of all pigments was obtained within 30 min, using a ternary mobile phase together with a linear gradient.

Table II shows the identification data for the chlorophylls and carotenoids.

Lipophilic adsorbents which retain the pigments according to their partition coefficients, are usually not very selective for carotenes and xanthophylls that differ only in the arrangement of the double bonds. The RP 18 stationary phase however, used in this study, can clearly separate α-carotene from β-carotene and lutein-5,6-epoxide from antheraxanthin. A second striking feature is the appearance of a chlorophyll *a* isomer running between chlorophyll *b* and chlorophyll *a*. On the contrary, we were not able to detect any zeaxanthin which probably elutes

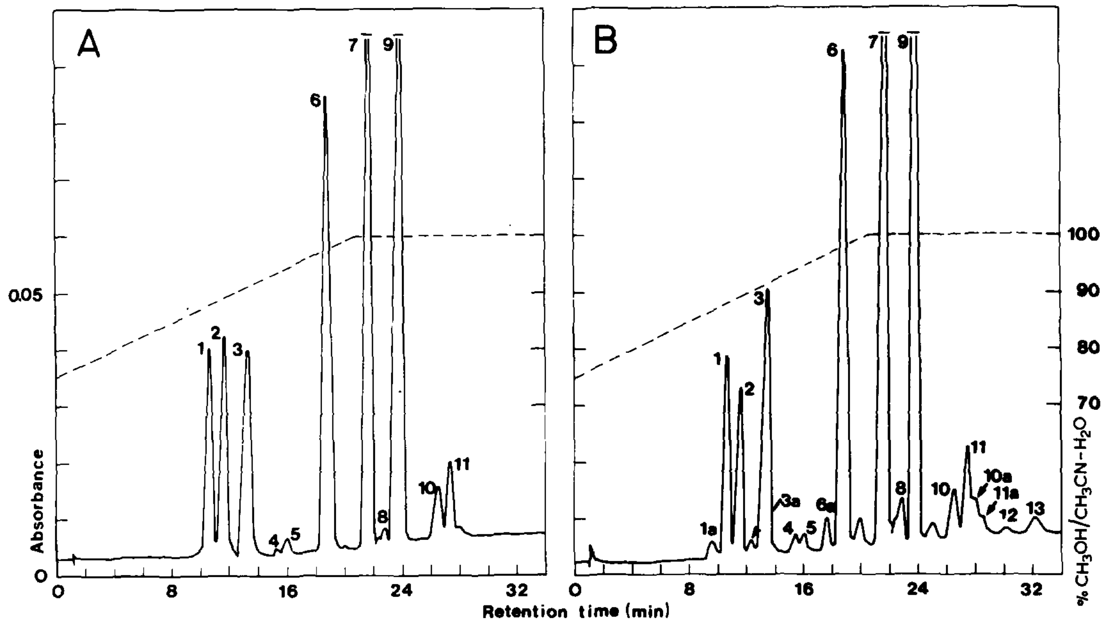

Fig. 1. Chromatogram of a total pigment extract from *C. fusca*. (A) Extract immediately injected after preparation. (B) Extract aged for 3 h and injected. Column: 10 μm RP-18; eluent: linear gradient of methanol/acetonitrile (25 : 75, v/v)-water as indicated by the dotted line; flow rate: 1.5 ml/min; sample volume: 5 μl. The peaks are numbered according to Table II.

TABLE II

IDENTIFICATION DATA FOR THE SEPARATED CHLOROPHYLLS AND CAROTENOIDS FROM THE GREEN ALGA *CHLORELLA FUSCA*

The spectra were recorded in methanol/acetonitrile (25 : 75, v/v) and different amounts of water according to the elution conditions indicated in Fig. 1. III/II indicates the relation between the absorbance of the long-wavelength maximum (III) and the absorbance at wavelength maximum (II, underlined). Acetylation of trihydroxy-α-carotene yielded five acetates indicating three accessible hydroxyl groups. HCl treatment of antheraxanthin and lutein-5,6-epoxide yielded the corresponding 5,8-epoxides (maxima in ethanol are 402, 449 and 339, 422, 452, respectively).

Peak No.	Pigment	Maxima found (nm)	Maxima reported (nm)	III/II found	III/II reported	Ref.
1	Neoxanthin	414, 438, 467	415, 438, 467 *	89	89	36
2	Trihydroxy-α-carotene	445, 471	445, 470 *	44	33	37
3	Violaxanthin	418, 441, 471	418, 441, 471 *	94	97	36
4	Lutein-5,6-epoxide	416, 441, 470	416, 440, 469 *	83	79	31
5	Antheraxanthin	423, 445, 473	421, 443, 473 *	45	50	36
6	Lutein	422, 447, 475	422, 446, 475 *	62	60	36
7	Chlorophyll *b*	460, 648	453, 648 **	–	–	38
8	Chlorophyll *a* isomer	431, 662	– , –	–	–	–
9	Chlorophyll *a*	431, 664	430, 662 **	–	–	38
10	α-Carotene	423, 446, 474	423, 444, 473 *	50	65	36
11	β-Carotene	426, 453, 479	– , 452, 478 *	25	27	

* In ethanol.
** In diethyl ether.

together with lutein (peak 6).

The complete separation capacity of rp-HPLC is demonstrated in Fig. 1B where an extract of *C. fusca* was allowed to stand in an ice-bath for 3 h without a cover. Additional to the pigments found in a fresh extract (Fig. 1A), a variety of artefacts appear which are produced through the influence of light and oxygen. The absorption spectra of these compounds verify that some carotenoids had undergone *cis-trans* isomerization: the long-wavelength maxima show a hypsochromic shift, the III/II-ratios drop drastically, and the so-called *cis*-peak [6] appears between 320 and 340 nm. Davies [5] has pointed out that the difference between the long-wavelength maximum of an all-*trans* carotenoid and the *cis*-peak of the isomer is practically a constant of 142 ± 2 nm, if the molecule contains 11 double bonds. This statement is confirmed in Table III where the spectral characteristics of some of the artificial isomers are compared with those of their parent compounds.

After chlorophyll *a*, another chlorophyll *a* derivative appears which can be ascribed to the classical chlorophyll *a* isomer of Strain and Manning [39], a slightly more lipophilic compound with the same absorption spectrum as chlorophyll *a*. Peak 12 represents pheophytin *b* (maxima: 414, 435, 522, 598, 652 nm), peak 13 consists of pheophytin *a* (maxima: 409, 507, 536, 608, 664 nm). The absorption maxima agree very well with those reported by Goedheer [38]. We found pheophytin *a* sometimes also in fresh extracts from *C. fusca* but only in trace amounts.

With respect to the reproducibility of the separation capacity described above, the rp-HLPC is superior over the conventional chromatographic techniques. The S.D. of the retention times approaches ±1.0%, if a conditioning phase between two separations is given to allow reequilibration between stationary and initial mobile phase. Table IV depicts the small experimental error in determining the capacity factor k', which represents the analogue to the R_f value in TLC and paper chromatography.

TABLE III

IDENTIFICATION DATA OF SOME CAROTENOIDS OCCURRING IN AN EXTRACT OF *CHLORELLA FUSCA*, AGED FOR 3 h

The spectra were recorded in methanol/acetonitrile (25 : 75, v/v) and different amounts of water according to the elution conditions indicated in Fig. 1. III/II indicates the relation between the absorbance of the long-wavelength maximum (III) and the absorbance at wavelength maximum (II, underlined); *cis*, wavelength maximum of the *cis*-peak in the region of 325–340 nm.

Peak No.	Pigment	Maxima found (nm)	III/II	*cis*-II	III-*cis*
1	Neoxanthin	414, $\underline{438}$, 467	89	–	–
1a	*cis*-Neoxanthin	326, 408, $\underline{432}$, 459	35	62	141
3	Violaxanthin	418, $\underline{441}$, 471	94	–	–
3a	*cis*-Violaxanthin	329, 413, $\underline{437}$, 464	42	80	142
6	Lutein	422, $\underline{447}$, 475	62	–	–
6a	*cis*-Lutein	332, 420, $\underline{442}$, 469	34	27	143
10	α-Carotene	423, $\underline{446}$, 474	50	–	–
10a	*cis*-α-Carotene	332, 422, $\underline{445}$, 471	5	30	142
11	β-Carotene	426, $\underline{453}$, 479	25	–	–
11a	*cis*-β-Carotene	338, – , $\underline{452}$, 477	6	25	141

The calibration curves of the three carotenoids α-carotene, β-carotene and trihydroxy-α-carotene are given in Fig. 2. The calibration factors are of course strongly dependent on the measuring wavelength, the specific integrator used and the technical design of the detector so that the whole set of calibration curves have to be performed once in every laboratory. However, some important features can be drawn from Fig. 2. The analytical method covers a concentration range of 5–500 pmol and can be extended to 300 nmol with only a minor loss in resolution (data not shown). Consequently, 4–10 ng are sufficient to detect and to quantify the pigments within a small experimental error. On the other hand, the same separation system can be used for semi-preparative work without overloading the stationary phase if an unknown pigment has to be isolated and identified.

Quantitative recoveries of a number of pigments were studied by injecting a known amount of pigment, and by collecting the eluted pigment. A spectroscopic determination proved that essentially no pigment was retained on the column; however, some colourless compounds can accumulate

TABLE IV

RETENTION TIMES OF SOME CAROTENOIDS AND CHLOROPHYLLS (IN s) DURING SIX SUCCESSIVE SEPARATIONS UNDER IDENTICAL CONDITIONS

Chromatography was performed as in Fig. 1, except a flow rate of 1.7 ml/min. The capacity factor k' is given by $k' = (t_R - t_0)/t_R$, where t_R and t_0 are the retention times of a retained and an unretained solute in a given system, respectively.

Pigment	Separation						Mean ± S.D.	k' ± S.D.
	1	2	3	4	5	6		
Neoxanthin	473	465	463	474	464	472	468.5 ± 5.0	3.46 ± 0.05
Violaxanthin	656	644	639	653	640	651	647.2 ± 7.1	5.16 ± 0.07
Chlorophyll *b*	1 246	1 238	1 230	1 243	1 225	1 237	1 236.5 ± 7.9	10.78 ± 0.08
Chlorophyll *a*	1 393	1 386	1 378	1 390	1 386	1 387	1 387.7 ± 5.1	12.21 ± 0.05
β-Carotene	1 625	1 602	1 614	1 610	1 600	1 616	1 611.2 ± 9.3	14.34 ± 0.09

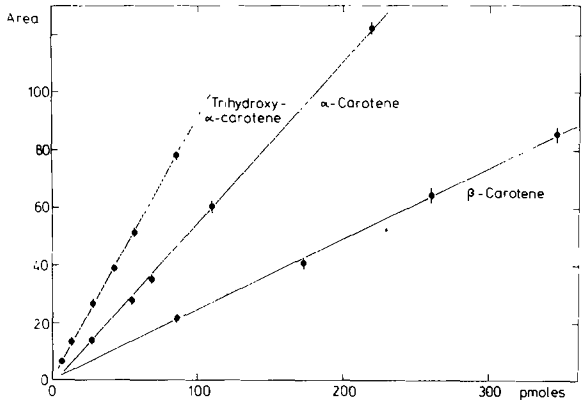

Fig. 2. Calibration curves of three selected carotenoids α-carotene, β-carotene, and trihydroxy-α-carotene. The bars represent the S.D. of the mean, calculated from six to ten independent measurements. The area is given in arbitrary units as produced by the integrating unit.

during several successive separations causing a higher pressure drop and a loss in resolution. This can be circumvented if the column is washed overnight with pure acetonitrile or methanol. Table V shows the concentration of chlorophylls and carotenoids of *C. fusca*, analysed by rp-HPLC. This is to demonstrate that the sensitivity and accurateness of the method now permit quantitative determinations of photosynthetic pigments to be made.

An important criterion for the quality of a chromatographic technique is its ability to avoid pigment destruction during the separation.. In rp-HPLC, the retention mechanism relies on the hydrophobic interaction between the nonpolar hydrocarbonaceous layer, solute and mobile phase. As a consequence, only weak hydrophobic forces comparable to the partitioning between two immiscible solvents are working, so it is expected that these weak forces are far less destructive than ionic and polar forces in conventional chromatography. The validity of this assumption is illustrated in Fig. 3. A total extract from spinach chloroplasts was separated into a xanthophyll fraction and a chlorophyll fraction on a low-pressure silica gel column, and the xanthophyll fraction was rechromatographed on our rp-HPLC system. Besides the in vivo xanthophylls, a variety of alteration products appears, among them another neoxanthin isomer (neoxanthin Neo A

TABLE V

THE PIGMENT COMPOSITION OF AUTOTROPHICALLY GROWN *CHLORELLA FUSCA*

C. fusca was grown as described in the text.

Pigment	μg/g dry weight		
	Found	Reported	Ref.
α-Carotene	140	220	40
β-Carotene	360	440	40
Lutein	2 300	1 910	40
Antheraxanthin	traces	–	–
Lutein-5,6-epoxide	50	–	–
Violaxanthin	900	500	40
Trihydroxy-α-carotene	860	–	–
Neoxanthin	770	370	40
Chlorophyll *a*	18 920	19 000	41
Chlorophyll *a* isomer	590	–	–
Chlorophyll *b*	7 340	6 870	41

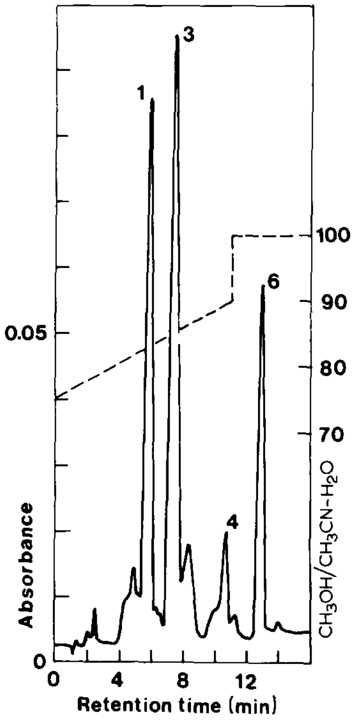

Fig. 3. Chromatogram of the xantophyll fraction of spinach chloroplasts. The xanthophylls were eluted from a low-pressure silica gel column with 100% methanol after removing the chlorophylls and part of lutein (peak No. 6) with hexane/*n*-propanol (88 : 12, v/v) and injected onto a 10 μm RP 8 column. Eluent, linear gradient of methanol/acetonitrile (25 : 72, v/v)-water as indicated by the dotted line; flow rate, 1.4 ml/min; sample volume, 20 μl. The peaks are numbered according to Table II.

according to Ref. 43, maxima at 417, 439, 469 nm) and several unidentified pigments. The chlorophyll fraction obtained from the silica gel column also contains several artificially produced chlorophyll isomers (data not shown).

It has to be stressed that not only reactive stationary phases but also prolonged contact of the pigments with air or saponification with hot alkaline methanol do alter the sensitive molecular structure of chlorophylls and carotenoids. In rp-HPLC, oxygen is excluded from the separation system through the experimental set-up. The only carrier for oxygen is the mobile phase, but this source is easily handled by degassing the solvents prior to use. Saponification can be omitted as the presence of chlorophylls does not disturb the complete resolution of the carotenoids.

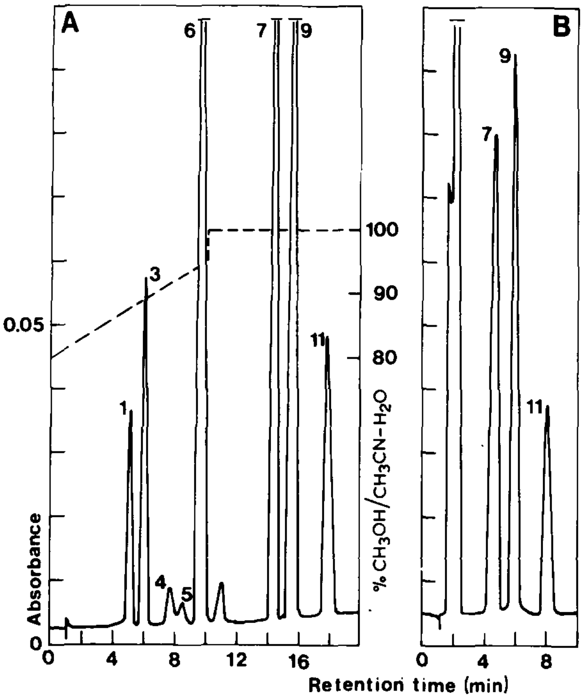

Fig. 4. Chromatogram of a total pigment extract from spinach chloroplasts. (A) Complete resolution with a 10-μm RP 8 column and the indicated gradient of methanol/acetonitrile (25 : 75, v/v)-water. Flow rate, 1.7 ml/min; sample volume, 5 μl. The peaks are numbered as before. (B) Isocratic run on a 10-μm RP 18 column with 100% methanol/acetonitrile (25 : 75, v/v) and the complete resolution of chlorophyll *b*, chlorophyll *a* and β-carotene. Flow rate, 1.5 ml/min; sample volume, 2 μl.

Fig. 4A shows the application of rp-HPLC on a frequent separation problem, namely the routine analysis of a plant extract. As freshly prepared spinach chloroplasts do not contain detectable amounts of pheophytins and only trace amounts of α-carotene, octyl silica can be used instead of octadecyl silica. Octyl silica shortens the retention time to about a half with only a minor loss in resolution. However, the prerequisite for the successful exploitation of this time-saving procedure is the establishment of a preparation procedure which does not lead to the formation of artefacts, because the altered pigments could be masked due to the lower separation capacity of the adsorbent. If this is guaranteed, the whole analysis including the quantitative evaluation can be obtained within 20 min.

A second important application of rp-HPLC is demonstrated in Fig. 4B. Starting the elution isocratically with 100% acetonitrile/methanol (75 : 25, v/v), the xanthophylls elute together in one broad peak leaving behind the chlorophylls and β-carotene as well-resolved zones. The spectroscopic determination of the chlorophylls from a total plant extract is an inexhaustive source for errors. Therefore, it is recommended [9] to separate the chlorophylls and to quantify each of them individually. Chromatography on octadecyl silica successfully resolves the chlorophylls within 10 min and without any danger of producing artefacts.

Discussion

The instability of the chlorophylls and carotenoids on polar adsorbents precludes use of such stationary phases for the quantitative determination of these plant pigments. Principally, better suited are reversed-phase systems where the pigments are separated according to their lipophilicity. Here, only weak hydrophobic interactions come into play ensuring the intactness of the labile structures during the separations. However, the main objection against reversed-phase systems is their low selectivity with respect to structurally related compounds [11]. The stationary phase used in this work offers a new approach by using a microparticulate silica gel on which surface a hydrocarbonaceous layer is chemically bonded. The retention mechanism on hydrocarbon silica is often described as an analogy to the conventional partition systems, e.g. lipid-coated silica gel in TLC. However, this is not the case as it is demonstrated in Fig. 1A. All carotenoids are clearly separated, although some of the pigments have nearly the same partition coefficient [44]. As we have shown previously with structurally related herbicides [17], the retention behaviour in rp-HPLC is very sensitive to small variations of the physicochemical properties of the structures concerned. This is due to the characteristics of the chemically bonded phase which does not act as a true liquid phase, but shows a quasi adsorptive property. The binding process [16] depends to a great extent on Van der Waals and electrostatic contributions which result from the interaction between the mobile phase and the solute. The driving force for retention in rp-HPLC is therefore not the favourable interaction of the solute with the surface of the adsorbent, but the effect of the solvent in forcing the solute to the hydrocarbon ligands. Consequently, the composition of the mobile phase plays a great role in polar-group selectivity and nonpolar-group selectivity [45].

This appreciation has led us to the use of a ternary mobile phase consisting of water and two organic modifiers, namely methanol and acetonitrile, for the precise control of the eluent selectivity to isomers and solutes with different functionalities. The success of this approach is shown in Fig. 1B. Besides the in vivo pigments, also the isomers and oxidation products are separated from an 'aged' extract. rp-HPLC is therefore not only suitable for the detection of trace amounts of structurally very similar pigments but also a powerful tool in monitoring deviations from the in vivo set of pigments, whether induced experimentally or produced during the isolation procedure.

The only restriction lies in the inability of the method to separate zeaxanthin from lutein. Sometimes, we found a shoulder in front of the lutein peak but we were not able to reproduce this appearance. Since zeaxanthin is found in varying amounts due to its participation in the xanthophyll cycle [46], and its presence in *Chlorella* is furthermore dependent on the nutritional conditions of the alga [40], it seems likely that our rp-HPLC system is principally able to separate also zeaxanthin from lutein by small variations in the mobile-phase composition.

Since rp-HPLC needs only a few pmoles of pigment for a quantitative determination (Fig. 2), the analysis of trace amounts, e.g. in partial structures of the photosynthetic apparatus, is now open to study. The experimental error in determining the quantitative distribution of the pigments is well below ±5%, a great improvement if compared with the conventional chromatographic techniques. We expect that rp-HPLC will lead to a revision of many previously published conclusions drawn from the elaborated pigment composition until now.

A special problem lies within the determination of the ratio of chlorophyll *a* to chlorophyll *b*, a common parameter with respect to the characterization of the physiological status of green plants and the differentiation of partial structures of the photosynthetic apparatus. The usual spectroscopic determination [1–3,47] relies on the treatment of a total extract as a two-component system yielding two equations which take into consideration the absorbance at the two wavelength maxima of chlorophyll *a* and chlorophyll *b*, respectively. But only the wavelength maximum of chlorophyll *a* can be derived directly from the absorption spectrum of the extract; the absorption maximum of chlorophyll *b* on the other hand is represented by a shoulder and must therefore be determined before. As pointed out by Ziegler and Egle [42], a deviation of only 1 nm from the fixed wavelength maximum of chlorophyll *b* causes an error of 15%. The accurateness of the equations is furthermore a function of the quantitative relation between the two components, being seriously disturbed at high ratios of chlorophyll *a* to chlorophyll *b*. A third argument against the spectroscopic determination results from the inability of the method to distinguish between the chlorophylls *a* and *b* and their isomerization and degradation products.

The experimental system described in this work permits the separation of chlorophylls within the short analysis time of 10 min (Fig. 4B) without the danger of producing artefacts. On the contrary, chlorophyll decomposition products are readily separated from the parent compounds. rp-HPLC is especially usefull when the analysis has to be performed on a microscale isolation and when high ratios of chlorophyll *a* to chlorophyll *b* are to be expected.

These results clearly demonstrate that HPLC with nonpolar stationary phases can be used for the rapid and efficient analysis of plant pigments. This technique appears to be superior to the conventional TLC and paper chromatography with respect to selectivity, sensitivity, accuracy and expense of time.

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