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CHLOROPHYLL ANALYSIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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The separation and determination of chlorophylls by high-performance liquid chromatography (HPLC) is described. Chlorophylls and their derivatives were separated by reversed-phase HPLC based on hydrophobic interaction between solute and support, using an octadecyl silica column and elution with 100% methanol. Separated pigments were detected fluorometrically with a sensitivity in the picomole range: the fluorescence response was linear over a wide pigment concentration range. Resolution of five chlorophylls *a* and four protochlorophyll species esterified with different alcohols was achieved within 22 min in a single experiment. This method can be used for the determination of chlorophyll *b*, bacteriochlorophyll *a* esters and products synthesized from chlorophyll, but not for nonesterified pigments, i.e., chlorophyllide, protochlorophyllide and chlorophyll *c*. The chromatographic mobility of chlorophyll *a* esterified with different alcohols increases with increasing number of carbon atoms in the esterifying alcohols. The plots obtained from the logarithm of the capacity factor (k') of these pigments versus the numbers of carbon atoms of the alcohol molecule gave a straight line, thus permitting the estimation of the chain length of unknown pigment esterifying alcohols. This HPLC separation technique did not cause the formation of artifacts. The deviation of the individual retention time for each pigment is less than $\pm 0.5\%$, thus making this method suitable for the rapid identification and quantification of unknown pigments.

Introduction

The identification and determination of plant pigments has relied largely on the use of paper, thin-layer or column chromatography followed by spectroscopic examination and determination of the isolated pigments [1]. These methods, although often useful, are not suitable for the separation of structurally very similar compounds.

Recently, the technique of HPLC has been employed for the separation and determination of plant pigments [2–9] because of such advantages as speed, high resolution and sensitivity. These useful characteristics of HPLC have led to the detection of minute quantities of previously undetected components in extracts of plant pigments. The HPLC analysis, however, has previously been largely concerned with determination of the main chlorophylls and carotenoids.

During the course of our studies on chlorophyll formation [9–11], we required a simple and rapid method for the separation and identification of chlorophylls and their precursor pigments. Previously, we reported successful applications of reversed-phase HPLC to the separation and determination of the mixture of PChls esterified with different alcohols from inner seed coats of three

Abbreviations: Chl, chlorophyll; PChl, protochlorophyll; BChl, bacteriochlorophyll; F, farnesol; G, geraniol; GG, geranylgeraniol; DHGG, dihydrogeranylgeraniol; THGG, tetrahydrogeranylgeraniol; P, phytol; HPLC, high-performance liquid chromatography. $Chl_{alcohol(G,F,GG,DHGG,THGG,P)}$, chlorophyll esterified with the respective alcohols.

Cucurbitaceae [12]. Here, we describe an HPLC technique with high resolution and versatility for the separation and determination of various chlorophyll esters, including some pigments synthesized from chlorophylls, and BChl *a*. This simple method is suitable for the identification of unknown chlorophyll species in the crude extract of plant tissues at picomole levels and is both rapid and highly reproducible.

Materials and Methods

Preparation and identification of chlorophylls

Chl *a* and *b* were extracted from spinach leaves with 80% acetone and were partially purified by precipitation with dioxane [13] and further purified by Sepharose CL-6B and DEAE-Sepharose CL-6B column chromatography [14]. These purified chlorophylls were used for the following preparations: chlorophyllides and chlorophyll derivatives. For instance, methylchlorophyllide *a*, ethylchlorophyllide *a*, Chl a_G, Chl a_F and Chl a_{GG} were prepared from the pure Chl *a* and the respective alcohol by the operation of chlorophyllase (EC 3.1.1.14) which catalyzes hydrolysis and transesterification of different alcohols as described previously [9]. Purified chlorophyllase was obtained from *Chlorella protothecoides* [10].

Pheophytins *a* and *b* were prepared by acidic treatment of the respective chlorophyll by the method of Perkins and Roberts [15]. Chl C_{10} epimer, Chl *a'* and *b'* were prepared by dissolving in 1-propanol and heating at 50°C for 15 min [16]. 10-Hydroxychlorophyll *a* was prepared from Chl *a* by dissolving in absolute methanol and exposing to air for 48 h at room temperature [1]. Pyrochlorophyll *a* was prepared from Chl *a* by dissolving in pyridine and heating in a water bath at 100°C for 48 h in a sealed tube [1].

Chl *a* and PChls esterified with different C_{20} alcohols were extracted with acetone/methanol (7 : 2, v/v) from irradiated (10 min with a 20 W fluorescence tube) 15-day-old etiolated leaves of *Phaseolus vulgaris* and purified by DEAE-Sepharose CL-6B column chromatography as a mixture of Chl *a* and PChl esters. *P. vulgaris* was cultivated in wet vermiculite in the dark at 25 ± 1°C.

BChl *a* esters were extracted with 80% acetone from the cells of *Rhodopseudomonas palustris* and purified by repeated DEAE-Sepharose CL-6B column chromatography. Each component was finally separated by cellulose thin-layer chromatography using a methanol/methylene chloride/water mixture (100 : 18 : 20, v/v/v) [17] and analyzed for their esterifying alcohol by gas chromatography as described in the following section. *R. palustris* was grown semianaerobically at 30°C for 3 days under continuous illumination (10000 lux) in a medium by Van Niel [18].

The chlorophylls thus obtained were identified by cellulose thin-layer chromatography [17] in the solvent system noted above and by paper chromatography using a solvent mixture of toluene/ethanol (200 : 1, v/v) [19].

Liquid chromatography

HPLC was carried out with a model LC-3A (Shimadzu, Kyoto), equipped with column oven, using a Du Pont Zorbax octadecyl silica (ODS) column (250 × 4.6 mm diameter). Chlorophylls were eluted with 100% methanol at a flow rate of 1.0 or 1.5 ml per min at 40°C and detected fluorometrically by a fluorescence spectrophotometer, model 650-60 (Hitachi, Tokyo) connected with an 18 µl flow cell. The parameters used for identification are described in the legend to the figures. The slit width was 15 nm for both excitation and emission and an appropriate recorder range (5–500) was used. Chromatogram peaks were quantified by a computing integrator, Chromatopack C-R1A (Shimadzu).

Identification of pigment esterifying alcohols

The purified pigment dissolved in benzene was saponified by refluxing for 2 h after addition of an equal volume of 90% methanol (v/v) containing 10% KOH (w/v). Alcohols were extracted with diethyl ether after addition of 3 vol. distilled water. The resulting ethereal solution of the alcohol was passed through anhydrous sodium sulfate layered on a glass filter and then evaporated to dryness under vacuum. The alcohol residue was finally dissolved in a small volume of *n*-hexane for gas chromatographic analysis.

The alcohols were identified and estimated using a gas chromatograph, model GC-7A (Shimadzu), equipped with a flame ion detector. The carrier gas was nitrogen (60 ml/min) and a silica capillary

column (12 m × 0.24 mm inside diameter) packed with polyethylene glycol 20M on Uniport B was used. The sample (2–5 μl) was injected with the use of a solvent cut system at 170°C oven temperature (injection temperature 190°C). Chromatograms were recorded and quantified by a Chromatopack C-R1A. Under these conditions, retention times of the alcohols (in min) were phytol, 13.02; tetrahydrogeranylgeraniol, 19.22; dihydrogeranylgeraniol, 22.83 and geranylgeraniol, 29.03. Standard phytol was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Geranylgeraniol was a gift from Nippon Roche Research Center (Kamakura). Dihydro- and tetrahydrogeranylgeraniol were prepared from purified Chl *a* esterified with those alcohols in the extract of irradiated etiolated kidney bean leaves (*P. vulgaris*) [6].

Results

Fig. 1 shows the separation of isolated Chl *a* and PChl esters from illuminated etiolated leaves of *P. vulgaris* on a Zorbax ODS column using 100% methanol as moving phase. Nine separated pigments were obtained in less than 22 min. Four peaks, 5, 7, 8 and 9, were observed both in illuminated and nonilluminated samples and these pigments belonged to PChl species and were identified by their fluorescence emission maxima at 637–639 nm and spectral properties. The other five peaks, 1–4 and 6, observed only in irradiated samples, showed fluorescence emission maxima at 667–669 nm which suggested that they were Chl *a* species. Spectral properties and retention times of the above PChl peaks were consistent with their identification as $PChl_{GG}$ (peak 5), $PChl_{DHGG}$ (peak 7), $PChl_{THGG}$ (peak 8) and $PChl_P$ (peak 9): the retention times and spectral properties of these four PChls from inner seed coats of three Cucurbitaceae have been previously described [12]. Peak 1 could be detected only when the pigment was extracted with an acetone/methanol mixture and the retention time was identical with that of synthesized methylchlorophyllide *a*. Methylchlorophyllide *a* was formed from chlorophyll or Chl *a* during the acetone/methanol extraction by the action of chlorophyllase. Chlorophyllase has been shown to be very active in aqueous acetone solution [9]. Similarly, peaks 2 and 6 were identified by retention times and spectral properties to be Chl a_{GG} and Chl a_P, respectively (cf. Fig. 2). It can be seen that the mobility of the pigments decreases with decreasing number of double bonds in the esterifying alcohol molecule, as can be seen in the

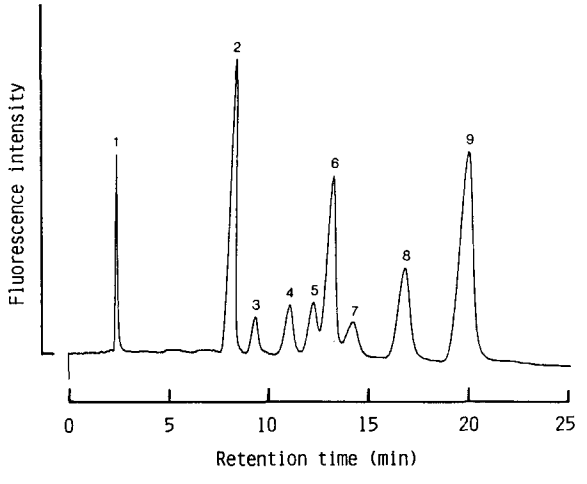

Fig. 1. Separation of isolated Chl *a* and PChl esters by HPLC. Partially purified chlorophylls from an acetone/methanol extract of illuminated etiolated leaves of *P. vulgaris* (see Materials and Methods) were eluted from a Zorbax ODS column with 100% methanol at a flow rate of 1.5 ml per min (pressure approx. 75 kg/cm^2) at 40°C and detected by fluorescence emission measurements (excitation 433 nm, emission 650 nm). (1) Methylchlorophyllide *a*, (2) Chl a_{GG}, (3) Chl a_{DHGG}, (4) Chl a_{THGG}, (5) $PChl_{GG}$, (6) Chl a_P, (7) $PChl_{DHGG}$, (8) $PChl_{THGG}$, (9) $PChl_P$.

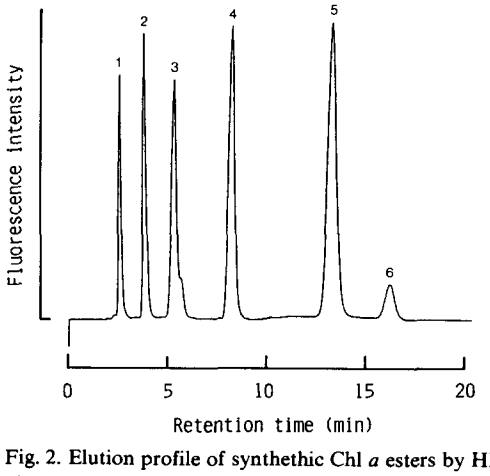

Fig. 2. Elution profile of synthethic Chl *a* esters by HPLC. The pigments were eluted with 100% methanol at a flow rate of 1.5 ml per min (pressure approx. 75 kg/cm^2) at 40°C and were detected by fluorescence emission measurements (excitation 428 nm, emission 672 nm). (1) Ethylchlorophyllide *a*, (2) Chl a_G, (3) Chl a_F, (4) Chl a_{GG}, (5) Chl a_P, (6) Chl a'_P.

TABLE I

ANALYTICAL SEPARATIONS OF CHLOROPHYLLS AND THEIR DERIVATIVES BY HPLC

Chromatography was performed under the conditions described in the text. The chlorophylls were eluted with 100% methanol at a flow rate of 1.5 ml per min at 40°C. Retention times were read out directly from a Chromatopack C-RIA and are expressed as the mean values of four to six experiments. The capacity factor, k', is given by $k' = (t_R - t_0)/t$, where t_R and t_0 are the retention times of retained and unretained solute in the given system, respectively. The ratio of the capacity factor, α_1, is calculated by $k'_1/k'_{\text{Chl } a \text{ (phytol)}}$. α_2 is estimated as k'_1/k'_{phytol} in each group of the pigments.

Chlorophylls	Retention time (min)	k'	α_1	α_2
Methylchlorophyllide *a*	2.46	0.482	0.07	0.07
Ethylchlorophyllide *a*	2.52	0.518	0.08	0.08
Chlorophyll a_G	3.74	1.25	0.18	0.18
Chlorophyll a_F	5.27	2.17	0.32	0.32
Chlorophyll a_{GG}	8.04	3.84	0.56	0.56
Chlorophyll a_{DHGG}	9.29	4.60	0.67	0.67
Chlorophyll a_{THGG}	11.03	5.65	0.83	0.83
Chlorophyll a_P	13.03	6.85	1.00	1.00
Chlorophyll a'_P	15.80	8.52	1.24	1.24
Pheophytin a_P	53.87	31.45	4.59	4.59
10-Hydroxychlorophyll a_P	10.62	5.40	0.79	0.79
Pyrochlorophyll a_P	17.85	9.75	1.42	1.42
Chlorophyll b_P	7.25	3.37	0.49	1.00
Chlorophyll b'_P	8.14	3.90	0.57	1.16
Pheophytin b_P	34.85	19.68	2.87	5.84
$\text{Protochlorophyll}_{GG}$	12.12	6.30	0.92	0.58
$\text{Protochlorophyll}_{DHGG}$	14.10	7.49	1.09	0.69
$\text{Protochlorophyll}_{THGG}$	16.62	9.01	1.32	0.83
$\text{Protochlorophyll}_P$	19.69	10.86	1.59	1.00
Protopheophytin_P	58.05	33.97	4.96	3.13
Bacteriochlorophyll a_{GG}	4.36	1.63	0.24	0.56
Bacteriochlorophyll a_{DHGG}	4.93	1.97	0.29	0.68
Bacteriochlorophyll a_{THGG}	5.61	2.38	0.35	0.82
Bacteriochlorophyll a_P	6.45	2.89	0.42	1.00
Bacteriochlorophyll a'_P	7.28	5.62	0.82	1.17
Bacteriopheophytin a_P	27.52	15.58	2.27	5.39

elution profiles of the pigments. The log k' values of peaks 3 and 4 were in good agreement with those values of dihydro- and tetrahydrogeranylgeraniol obtained from the plots of log k' against their number of double bonds for Chl a_{GG} and Chl a_P [12]. Thus, peaks 3 and 4 were identified as Chl a_{DHGG} and Chl a_{THGG}, respectively. These results were further confirmed by gas chromatographic analysis of the esterifying alcohol of each peak; the alcohol moiety of peaks 3 and 4 showed retention times of 22.83 and 19.22 min and these were identical with those of authentic samples of dihydro- and tetrahydrogeranylgeraniol, respectively. Furthermore, the ratio of capacity factor in each group of pigments, α_2 of these Chl *a* species was coincident with those of PChl and BChl *a* esters. The retention time, capacity factor (k') and ratio of capacity factors (α_1 and α_2) of chlorophylls and their derivatives are presented in Table I.

To examine the reproducibility of separation of the experimental system routinely used, retention times of some Chl *a* and PChl esters were measured for a series of successive separations and the results (Table II) indicate an experimental error in determination of the retention time and the C.V. of the retention times of less than 0.5%. Furthermore, retention times were reproducible for the range 2–50 μl injection volumes. The quantitative recovery, estimated by the ratio of peak areas to

TABLE II

REPRODUCIBILITY OF CHLOROPHYLL AND PChl SPECIES DURING SIX SUCCESSIVE SEPARATIONS

The chromatographic conditions are the same as those in Fig. 1. C.V., coefficient of variation.

Run	Retention time (min)				
	Methylchlorophyllide *a*	Chl a_{GG}	$PChl_{GG}$	Chl a_P	$PChl_P$
1	2.45	8.04	12.11	13.03	19.66
2	2.46	8.03	12.10	13.03	19.67
3	2.45	8.04	12.14	13.02	19.69
4	2.45	8.04	12.12	13.02	19.67
5	2.46	8.04	12.13	13.03	19.69
6	2.46	8.05	12.13	13.03	19.73
Mean	2.46	8.04	12.12	13.03	19.69
S.D.	0.005	0.006	0.013	0.005	0.023
C.V. (%)	0.203	0.072	0.107	0.036	0.117

chlorophyll concentration (μg), tended to decrease as injection volume increased, but the decrease was only 4% at the top of the range (50 μl).

The calibration curves for Chl *a* and *b* show that the recovery of Chl *a* and *b* from the column as measured by fluorescence emission is linear over a wide range of chlorophyll concentrations (up to 100 pmol) applied to the HPLC column. While this range can be extended to 10 nmol, quantitative analysis required a minimum of 2 pmol for Chl *a* and 4 pmol for Chl *b* (data not shown).

The effect of the alcohol side chain on the chromatographic mobility of pigments was studied with mixtures of Chl *a* esterified with different alcohols (Fig. 2). The concentration ratio and the retention times of each pigment in a mixture were compared with those obtained from the individual experiments with authentic pigment samples. Thus, peaks 1–6 were identified as ethylchlorophyllide *a*

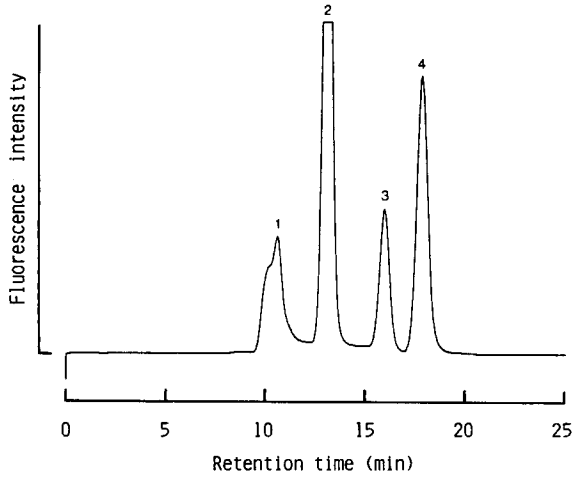

Fig. 3. The HPLC elution profile of products synthesized from Chl a_P. Chromatographic and detection conditions are the same as those in Fig. 2. (1) 10-Hydroxychlorophyll a_P, (2) Chl a_P, (3) Chl a'_P, (4) Pyrochlorophyll a_P.

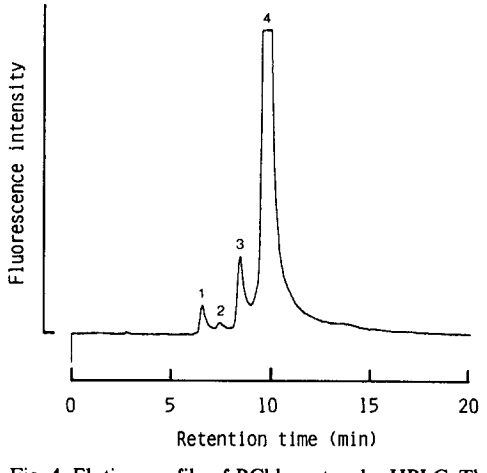

Fig. 4. Elution profile of BChl *a* esters by HPLC. The pigments were eluted with 100% methanol at a flow rate of 1.0 ml per min (pressure approx. 60 kg/cm^2) at 40°C and were detected by fluorescence emission measurements (excitation 370 nm, emission 780 nm). (1) BChl a_{GG}, (2) BChl a_{DHGG}, (3) BChl a_{THGG}, (4) BChl a_P.

(peak 1), Chl a_G (peak 2), Chl a_F (peak 3), Chl a_{GG} (peak 4), Chl a_P (peak 5) and Chl a'_P (peak 6). As can be deduced from the data of Fig. 2, the plot of the log of the capacity factor, k', of different esters of Chl *a* (see Table I) against the number of carbon atoms in the alcohol moiety results in a straight line, indicating that this technique can be used not only to separate pigments differing only in their esterifying alcohol but also to identify this alcohol (data not shown). Increasing the number of carbon atoms in the esterifying alcohol of Chl *a* resulted in a decrease in mobility (Fig. 2). A similar result was obtained in the PChl esters isolated from inner seed coats of Cucurbitaceae [12].

The elution profile of the mixture of newly synthesized pigment products is shown in Fig. 3. Peaks 1–4 corresponded to 10-hydroxychlorophyll a_P (peak 1), Chl a_P (peak 2), Chl a'_P (peak 3) and pyrochlorophyll a_P (peak 4) in terms of retention time of each sample. The shoulder of peak 1 showed the presence of a minor component which is tentatively identified as 10-methoxychlorophyll a_P, since the spectral properties of both pigments are the same [1] and it was produced during the preparation of 10-hydroxychlorophyll *a* [1].

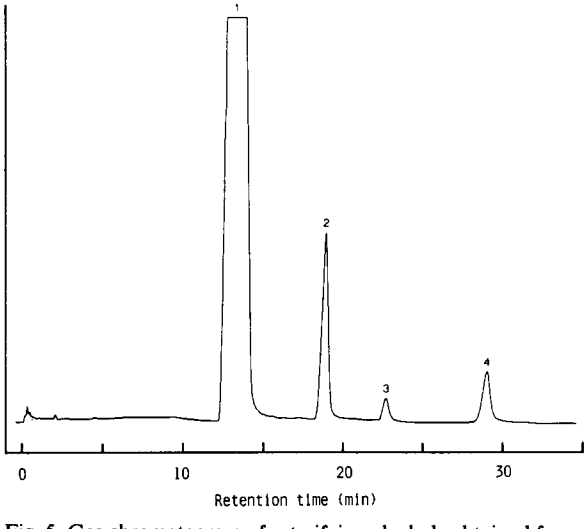

Fig. 5. Gas chromatogram of esterifying alcohols obtained from a mixture of BChl *a* esters. Preparation of the alcohols by saponification of the pigments and gas chromatographic conditions employed are described in the text. (1) Phytol, (2) tetrahydrogeranylgeraniol, (3) dihydrogeranylgeraniol, (4) geranylgeraniol.

Fig. 4 shows the elution profile of BChl *a* esters from *R. palustris*. One prominent and three minor peaks were observed. The spectra of all these pigments were identified to that of BChl *a*. To identify the different esterifying alcohols, the mixture of pigments was saponified and the alcohols analyzed by gas chromatography (Fig. 5). Comparison of the retention times with those of authentic standards shows the presence of four alcohols, geranylgeraniol, dihydrogeranylgeraniol, tetrahydrogeranylgeraniol and phytol. Thus, the pigments were readily identified as BChl a_{GG} (peak 1), BChl a_{DHGG} (peak 2), BChl a_{THGG} (peak 3) and BChl a_P (peak 4) by the concentration ratios between pigments and alcohols. To confirm these results further, peaks 1 and 4 were isolated by thin-layer chromatography [17], saponified and subjected to gas chromatographic analysis. Peak 1 had a retention time of 29.0 min and was identified by comparison with an authentic sample as geranylgeraniol. Similarly, peak 4 showed a retention time of 12.98 min identifying it as phytol. Peaks 1–4 were identified, therefore, as BChl a_{GG} and BChl a_P, respectively. By the procedure described above for the determination of equivalent double bonds, peaks 2 and 3 were confirmed as BChl a_{DHGG} and BChl a_{THGG}, respectively. A similar elution profile was obtained from the acetone extract of *R. spheroides*.

Discussion

The reversed-phase HPLC on a Du Pont Zorbax ODS column was suitable for the separation of a variety of chlorophylls derived from plant and bacterial extracts. The esterified chlorophylls were separated with high resolution within 22 min without leading to the formation of artifacts (Fig. 1 and Table I). In addition, the chromatography was highly reproducible, permitting the use of this technique for rapid analysis of a variety of esterified chlorophylls (Table II). This system is not suitable for the separation of pheophytins, because of poor resolution and exceedingly long retention times (Table I). The only limitation is the inability to separate nonesterified pigments including, for instance, chlorophyllide, pheophorbide and Chl *c* groups; however, some batches of the ODS col-

umn supplied by Du Pont were capable of rough separations of these pigments with an 80–90% methanol/water mixture (v/v), but the retention characteristics were poorly reproducible and very unstable. This instability may be due to the adsorption of nonesterified pigments to the support. Our preliminary experiments showed that the retention capacity could be recovered by washing with 0.1% sodium dodecyl sulfate (w/v) solution, but reproducibility of the retention time was not good. Conversely, however, the separation of esterified pigments was not affected when the ability to separate nonesterified pigments was lost. The ODS column was quite stable and was used for more than 600 samples over a 1 year period with excellent reproducibility. Recently, two reports have appeared on the separation of nonesterified pigments using a micro C_{18} column and linear gradients of methanol/water and ethyl acetate [8] and using Whatman PXS 1025 ODS-2, Magnum 9 series and step gradients of methanol/water [20].

The mechanism of separation of the HPLC technique described here is based on the weak hydrophobic interaction of the pigment between the moving phase and chemically bonded silica support as is frequently noticed in the reversed-phase HPLC [21]. The driving force for interaction between the pigments and the moving phase depends fundamentally on the species and the magnitude of the dispersion forces such as London dispersion. Furthermore, the degree of interaction between two compounds is generally interpreted as a parameter of 'polarity' between solute and the moving phase. Thus, the degree of polarity of the moving phase, which is determined by the composition and indicated as Hildebrand solubility parameter, is an important factor in separations by this system. The elution system (100% methanol) used in this study is, however, rather simple, but is apparently sufficient for the separation of esterified pigments (except for pheophytin species), since decreasing the polarity decreases the retentivity of main the chlorophyll pigments.

Our observations in this work show that the pigments are separated according to their lipophilicity and retention behavior is very sensitive to small variations of chemical structure (Figs. 1 and 2). Similar results have been reported in structurally related herbicides and plant pigments in *Chlorella* by Braumann and Grimme [7,22].

Our data give some practical suggestions on the elution behavior of chorophyll pigments. The mobility of chlorophylls esterfied with different alcohols increases with increasing number of carbon atoms in esterifying alcohol molecule (Fig. 2). In the case of chlorophylls with alcohols of a single carbon chain length but containing different numbers of double bonds, the mobility increases as the number of double bonds decreases (Figs. 1, 4 and Table I).

Conclusion

The experimental system described in this study allows the rapid separation of a variety of chlorophylls with high resolution, reproducibility and efficiency. The high sensitivity of fluorescence detection and the simplicity of the present method allow the attractive possibility of utilizing this technique for analyzing plant and bacterial extracts. Consequently, this procedure appears to be a reasonable alternative to the more conventional methods employing paper and thin-layer chromatography. The method is being used routinely in our laboratory to study chlorophyll biosynthesis and degradation.

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