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SEPARATION OF NON-ESTERIFIED CHLOROPHYLLS BY ION-SUPPRESSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

YUZO SHIOI*, MICHIO DOI and TSUTOMU SASA
Division of Biology, Miyazaki Medical College, Kiyotake, Miyazaki 889-16 (Japan)
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SUMMARY

Chlorophyllides and pheophorbides were separated by ion-suppression high-performance liquid chromatography using an octadecyl silica column eluted with 80–95% (v/v) methanol in water containing 13 m*M* acetic acid (final pH 4.2) as the suppressing ion. The separated pigments were detected fluorometrically with a sensitivity in the picomol range without artifact formation. This technique can be used not only for the determination of non-esterified chlorophylls, but also for esterified chlorophylls, thus enabling the simultaneous identification of chlorophylls and their derivatives.

INTRODUCTION

The technique of high-performance liquid chromatography (HPLC) is commonly used for the separation and analysis of plant pigments[1–10] because of its advantages of speed, high resolution and efficiency. However, this usage has been limited to the separation of the main chlorophylls and carotenoids. In a previous report[11] we described the successful application of reversed-phase HPLC to the separation and determination of a mixture of esterified chlorophylls and their derivatives. This system is simple and allows the rapid separation of a variety of esterified chlorophylls with high resolution and reproducibility. Although we have used the method routinely to study chlorophyll biosynthesis and degradation[12–14], it could not be used for the separation of non-esterified chlorophylls.

In this communication we present a chromatographic system using ion suppression and a reversed-phase octadecyl silica column, which enables the separation both of esterified and non-esterified chlorophylls including their derivatives by a rather simple elution system and mobile phase.

EXPERIMENTAL

Preparation and identification of pigments

Chlorophylls *a* and *b* were extracted from Swiss chard leaves with 80% (v/v) acetone. They were partially purified by precipitation with dioxane[15] and then by Sepharose CL-6B and DEAE-Sepharose CL-6B column chromatography[16].

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Chlorophyllides *a* and *b* were prepared from pure chlorophylls *a* and *b*, respectively, by the action of chlorophyllase (E.C. 3.1.1.14) which catalyzes the hydrolysis of esterified alcohols. Purified chlorophyllase was obtained from *Chlorella protothecoides*[17]. These chlorophyllide species are unstable and change into hydroxy and magnesium free derivatives even in the dark at −20°C. Protochlorophyllide was extracted from etiolated cucumber cotyledons with 80% (v/v) acetone and partially purified by phase separation[18]. Cucumbers were cultivated in wet vermiculite in the dark at 26°C.

Pheophorbides *a* and *b* and protopheophorbide were prepared by acidic treatment of the respective chlorophyllide by the method of Perkins and Roberts[19].

Chlorophyll *c* was extracted from thalli of *Undaria pinnatifida* with 80% (v/v) acetone and partially purified by phase separation[18]. *U. pinnatifida* thalli were collected on the coast of Aoshima, Miyazaki city.

The pigments thus obtained were identified by spectrophotometric analysis using a Shimadzu UV-240 spectrophotometer, and by paper chromatography in the solvent system toluene–ethanol (200:1)[20].

Chromatography

HPLC was performed with a Shimadzu LC-3A chromatograph using a Whatman Partisil-10 ODS-2 or DuPont Zorbax ODS column (250 × 4.6 mm). Pigments were eluted with 80–95% methanol in water containing a final concentration of 13 m*M* acetic acid at a flow-rate of 1.0 ml/min at 40°C. The pH value of the mixture fluctuated when a low concentration of acetic acid was added and the true pH was probably different from the value read from the meter because of the relatively non-ionic solvent. We used therefore the concentration of added acetic acid, instead of their pH. Separated pigments were detected fluorometrically by a Hitachi fluorometer, Model 650-60 and quantified by a Shimadzu Chromatopac C-R1A.

HPLC peaks were identified by comparison of their retention times and *in situ* fluorescence maxima with those obtained from authentic samples.

RESULTS

Chromatographic behaviour of non-esterified chlorophylls

In the absence of suppressing ion, the peaks of non-esterified pigments gradually disappeared during successive analyses on a reversed-phase column[11]. To clarify this phenomenon, the chromatographic behaviour of non-esterified chlorophylls was examined using a Whatman Partisil ODS-2. The mobile phase, ranging in pH from 4.2 to 8.0, contained different suppressing ion species such as acetic acid, sulphuric acid and hydrochloric acid. Of these, acetic acid (13 m*M*, pH 4.2) was found to be optimal for resolving the pigments. In addition, no degradation product was detected under these conditions.

Fig. 1 shows the effect of acetic acid concentration on the capacity factors, k', a measure of the retention of chlorophylliade *a* and protochlorophyllide. In the presence of 13 m*M* acetic acid (pH 4.2), the capacity factors of chlorophyllide *a* and protochlorophyllide were 0.668 and 1.41, respectively, these values gradually increased with increasing acetic acid concentration down to 1 m*M*. Omission of acetic acid resulted in no separation and an abrupt increase in the capacity factors. Also,

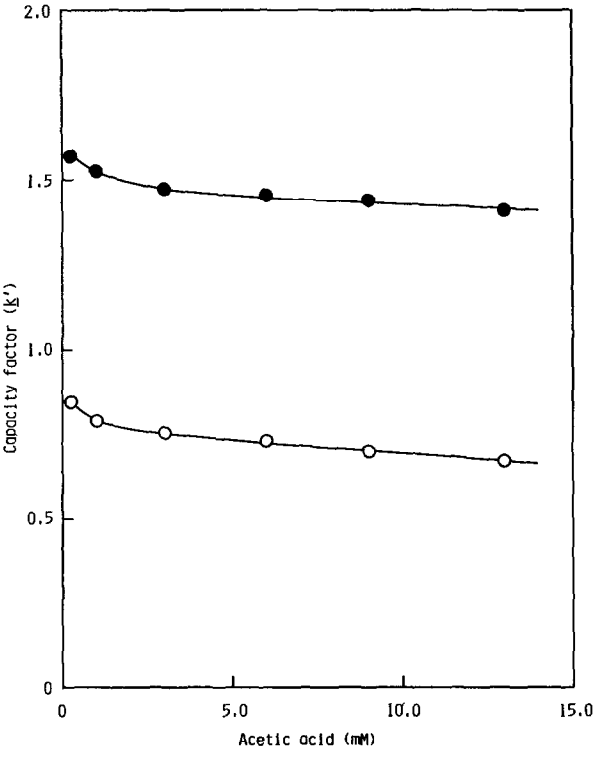

Fig. 1. Effect of the suppressing ion (acetic acid concentration) on the capacity factors, k', of chlorophyllide *a* (○) and protochlorophyllide (●). The capacity factors of the separated pigments were estimated after elution with methanol–water (95:5) containing the indicated concentrations of acetic acid at a flow-rate of 1.0 ml/min at 40°C. The pigments were detected fluorometrically using excitation and emission wavelengths at 430 and 650 nm, respectively. The capacity factors of the separated pigments were calculated as in Table I.

disappearance of the peaks was observed in the following analyses. These results suggest that, in the absence of a suppressing ion, the pigments were strongly retained probably due to their selective adsorption to the support as noted previously[11] (see Discussion).

The resolution calculated from the separation of structurally similar pigments, chlorophyllide *a* and protochlorophyllide (*cf.*, Fig. 1), sharply increased with increasing acetic acid concentration (Fig. 2) and reached 1.5 at 13 m*M*, which is adequate for normal separation. Thus, the separation of the non-esterified chlorophylls on a reversed-phase column depends strongly on the presence of suppressing ion (pH) in the mobile phase.

As shown in Fig. 3, the chromatographic mobility of the pigments strongly depends on the polarity of the mobile phase. The logarithm of the capacity factor of each pigment increased linearly with increasing polarity of the eluent. The individual plots gave parallel lines, indicating that the ratio of the capacity factor, α, was not changed with increasing mobile phase polarity. This means that the retention of the pigments can be changed arbitrarily depending on the mobile phase polarity and that

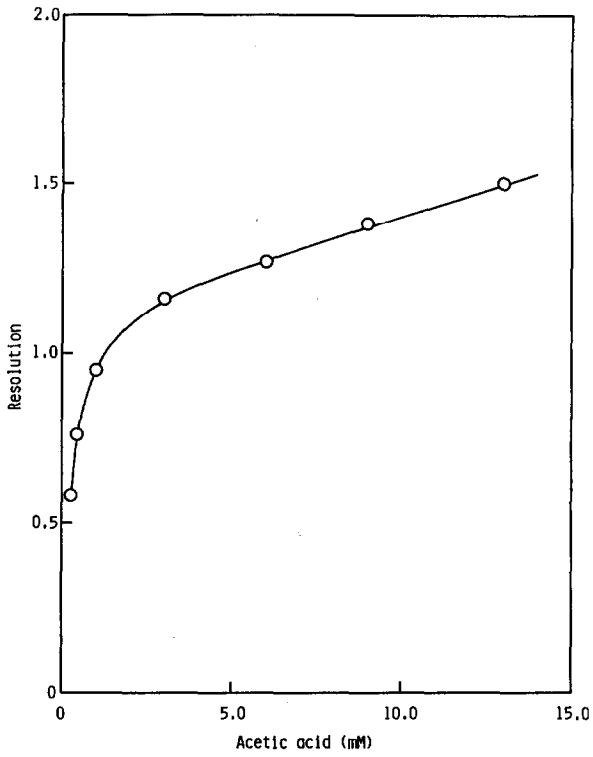

Fig. 2. Effect of suppressing ion (acetic acid concentration) on the resolution between chlorophyllide *a* and protochlorophyllide. Chromatographic conditions and pigment detection as in Fig. 1. The resolution is given by $R_s = 2\Delta t_R/(w_1 + w_2)$, where Δt_R is the difference in retention times between the peaks and w_1 and w_2 are the band widths of the chlorophyllide and protochlorophyllide peaks, respectively.

the order of pigments elution is constant independent of polarity. For instance, the mixture of chlorophyllide *b* species could not be separated with methanol–water (95:5) containing 13 m*M* acetic acid (pH 4.2). However, they could be resolved fairly well by increasing the mobile phase polarity (see Fig. 5). The polarity of the mobile phase is thus an important factor in attaining rapid resolution of the pigments in this system.

Separation of chlorophylls

Fig. 4 shows the separation of a mixture of chlorophyllide *a*, protochlorophyllide and pheophorbide *a* on a Whatman ODS-2 column using a methanol–water (95:5) containing 13 m*M* acetic acid as the mobile phase (pH 4.2). These pigments were separated in less than 20 min without artifact formation. Monovinyl and divinyl derivatives of chlorophyllide *a* and protochlorophyllide could not be separated under the conditions used, although chlorophyll *c* species can be separated as described below.

A mixture of chlorophyllide *b* pigments was separated with a methanol–water (85:15) containing 13 m*M* acetic acid (Fig. 5). Peak 1 is probably a chlorophyllide

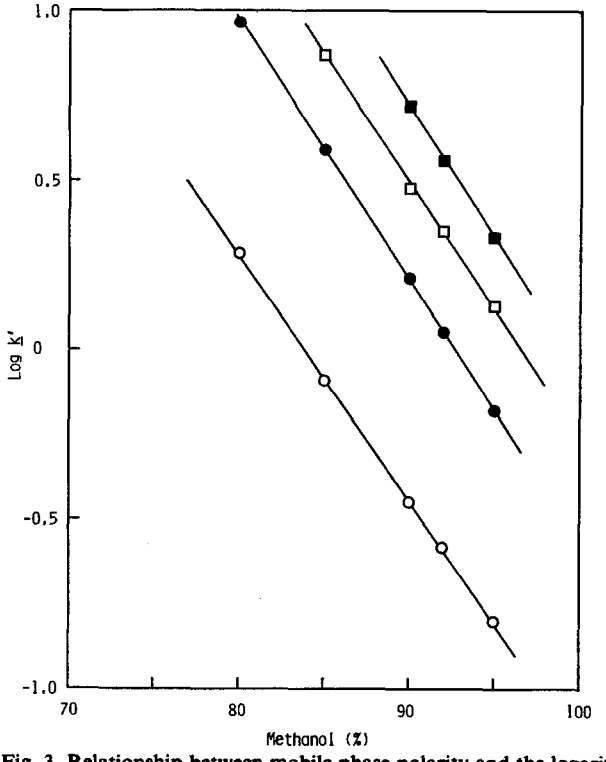

Fig. 3. Relationship between mobile phase polarity and the logarithm of the capacity factor of the separated pigments. The pigments were eluted with the indicated percentages of methanol–water containing 13 m*M* acetic acid (pH 4.2). Other chromatographic conditions as in Fig. 1. The capacity factor of each pigment was calculated as in Table I. ○, Chlorophyllide *b*; ●, chlorophyllide *a*; □, protochlorophyllide; ■, chlorophyll c_2.

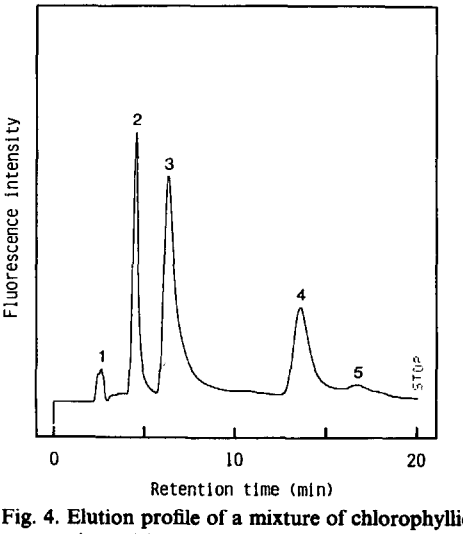

Fig. 4. Elution profile of a mixture of chlorophyllide *a*, protochlorophyllide and pheophorbide *a* by ion-suppression HPLC. The pigments were eluted with methanol–water (95:5) containing 13 m*M* acetic acid (pH 4.2). Other chromatographic conditions and pigment detection methods as in Fig. 1. Peaks: 1 = unretained pigments; 2 = chlorophyllide *a*; 3 = protochlorophyllide; 4 = pheophorbide *a*; 5 = pheophorbide *a'*.

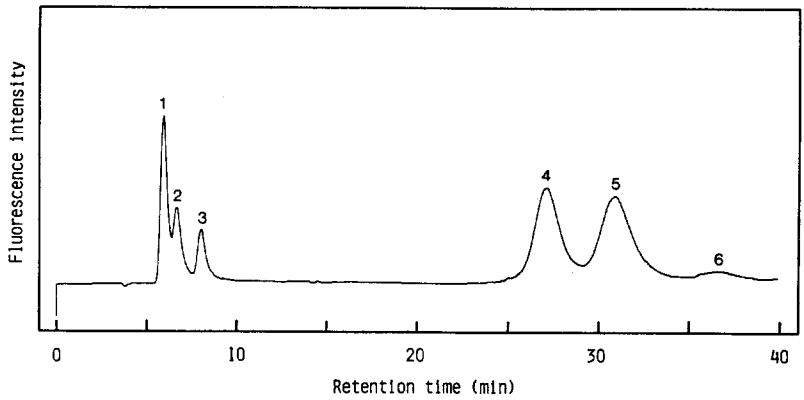

Fig. 5. Elution profile of a mixture of chlorophyllide *b* and pheophorbide *b* by ion-suppression HPLC. The pigments were eluted with methanol–water (85:15) containing 13 m*M* acetic acid (pH 4.2) at a flow-rate of 1.0 ml/min at 40°C. Pigments were detected by fluorescence measurements (excitation 440 nm; emission 650 nm). Peaks: 1 = 10-hydroxychlorophyllide *b*; 2 = chlorophyllide *b*; 3 = chlorophyllide *b'*; 4 = 10-hydroxypheophorbide *b*; 5 = pheophorbide *b*; 6 = pheophorbide *b'*.

b derivative, 10-hydroxychlorophyllide *b*. It was usually found in the chlorophyllide fractions prepared from chlorophyll *b* by the action of chlorophyllase. Peaks 2 and 3 were identified as chlorophyllide *b* and its 10 epimer, chlorophyllide *b'*, respectively, peaks 4–6 as pheophorbide species. More intense and sharper peaks were obtained by use of two-step gradient elution with 80% and 88% methanol–water mixtures containing 13 m*M* acetic acid (pH 4.2).

Fig. 6 shows the separation of chlorophyll *c* species with methanol–water (95:15) containing 13 m*M* acetic acid (pH 4.2). Two peaks showing *in situ* fluores-

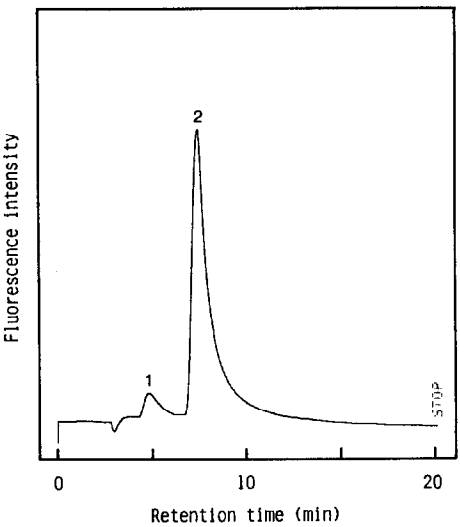

Fig. 6. Separation profile of partially purified chlorophyll *c* species by ion-suppression HPLC. The pigments were eluted with methanol–water (95:5) containing 13 m*M* acetic acid (pH 4.2) at a flow-rate of 1.0 ml/min at 40°C and were detected fluorometrically using excitation and emission wavelengths of 450 and 640 nm, respectively. Peaks: 1 = chlorophyll c_1; 2 = chlorophyll c_2.

cence excitation and emission wavelengths at 449–652 nm (peak 1) and at 454–655 nm (peak 2) were observed. These excitation maxima coincide with those of chlorophyll c_1 (peak 1) and c_2 (peak 2), respectively, but the emission maxima are shifted by about 20 nm to longer wavelengths. This was probably caused by the formation of aggregates as described by Jeffrey[21]. Thus, these peaks are tentatively identified as chlorophyll c_1 (peak 1) and c_2 (peak 2).

The retention times, k' values and α values of chlorophylls are presented in Table I. As shown, this HPLC technique can be used not only for the determination of non-esterified chlorophylls, but also for the esterified chlorophylls. The separation and identification of esterified chlorophylls by HPLC were described earlier[11].

TABLE I

SEPARATION OF CHLOROPHYLLS BY ION-SUPPRESSION HPLC

Chlorophylls were eluted with the indicated percentages of methanol–water containing 13 m*M* acetic acid (pH 4.2) at a flow-rate of 1.0 ml/min at 40°C. Other chromatographic conditions as in the text. Retention times, t_R, were read directly from a Chromatopac C-R1A and expressed as the mean values from four to five experiments. The capacity factor, k' is given by $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of retained and unretained solutes, respectively. The ratio of capacity factors, α, is calculated by $k'/k'_{\text{Chlorophyllide } a}$ in methanol–water (95:5).

Chlorophyll	*Methanol (%)*	t_R *(min)*	k'	α
Chlorophyllide *a*	95	4.4	0.654	1.00
Chlorophyllide *b*	85	7.9	1.97	–
Chlorophyllide *b'*	85	9.1	2.42	–
Protochlorophyllide	95	6.4	1.41	2.16
Pheophorbide *a*	95	13.5	4.08	6.24
Pheophorbide *a'*	95	16.6	5.24	8.01
Pheophorbide *b*	90	14.6	4.48	–
	85	30.6	10.50	–
Pheophorbide *b'*	90	16.4	5.17	–
	85	35.8	12.46	–
Protopheophorbide	95	14.4	4.41	6.74
Chlorophyll c_1	95	4.7	0.767	1.17
Chlorophyll c_2	95	7.3	1.74	2.66
Chlorophyll a_{GG}	95	27.3	9.26	14.16
Chlorophyll a_{DHGG}	95	32.6	11.26	17.22
Chlorophyll a_{THGG}	95	40.9	14.38	21.99
Chlorophyll a_{Phytol}	95	49.2	17.50	26.76

The reproducibility of the separation in this system is dependent on several factors. The variation of the retention times approached 2%, and the method results in slightly poorer reproducibility compared to the earlier system[11] using a single elution. This is undoubtedly due to the fact that the retention strongly depends on the mobile phase polarity. In addition, the resolution is greatly dependent on the amount of acetic acid added, *i.e.*, pH (see Fig. 2), although there is little effect on retention above 1 m*M* (see Fig. 1). To examine the reproducibility and readily identify the pigment, we used protochlorophyllide as a standard sample because of its greater

stability compared with other chlorophyllides and because of the simplicity of its preparation from etiolated seedlings by sugar column chromatography[19].

The calibration curves for chlorophyllide *a* and protochlorophyllide show that the recovery of the pigments from the column as measured by fluorescence emission is linear over a wide range of chlorophyllide concentrations applied to the HPLC column. Quantitative analysis required a minimum of 0.1 pmol for chlorophyllide *a* and 0.2 pmol for protochlorophyllide (data not shown).

DISCUSSION

The method described not only enables rapid identification and direct quantification of non-esterified chlorophylls, but also esterifed chlorophylls in crude extracts of plant tissues at picomol levels. Thus, it allows simultaneous separation of most chlorophylls and their derivatives. Considering the efficiency and resolution, a linear or step gradient of 80–100% methanol in water (pH 4.2) is suitable for routine experiments. For instance, we used the following program for the separation of chlorophyll *b* species: methanol–water (80:20) (pH 4.2) (15 min), methanol–water (88:12) (pH 4.2) (20 min) and 100% methanol (15 min). As found previously[11], the major advantage of this method is the simple, rapid analysis and the minimum loss of pigments. It is of interest in this context that contradictory reports concerning the photoreduction of protochlorophyll to chlorophyll *a* are mainly due to the procedures used to separate protochlorophylls from protochlorophyllides[14].

Unfortunately, under the conditions used here, monovinyl and divinyl derivatives of the pigments were eluted as a single peak. In contrast to these chlorophyllides, chlorophyll *c* species having the same structure at the C-2 and C-4 positions as the former pigments could be resolved under the same conditions. Although there is little information on the relationship between structure and separation mechanism, except for esterified alcohols[11], the structural differences in the chlorophyll *c* species, *i.e.*, an acrylic acid residue at the C-7 position and its aggregation in the methanol–water mobile phase, seem to be relevant to the resolution of these pigments.

Our observations on the chromatographic behaviour of the pigments in this system show that the non-esterified pigments are not retained, but they are adsorbed onto the support in the column as reflected in the disappearance of their peaks in the absence of suppressing ion. The Partisil ODS-2 stationary phase contains 25% residual unreacted hydroxyl groups which cause high and selective adsorption of ionized solutes[22]. When the ionization of free hydroxyl groups or pigments is suppressed at low pH, the pigments are retained on the support. Subsequently, they can be separated by their different hydrophobic interactions with the mobile phase and the chemically bonded silica support.

A similar separation was obtained by the ion-pairing method with 80–95% methanol-water containing 2 m*M* tetra-*n*-butylammonium phosphate at pH 7.0. This method is a reasonable alternative to the ion-suppression method, but it is not suitable with columns such as Partisil ODS-2 and Zorbax ODS. Ion-pairing reagents, especially quaternary bases, are known to attack non-capped surfaces of silica gel[23] and therefore shorten the column life.

Column stability is an important factor for reproducibility in routine separations. In this system, loss of column stability is chiefly due to the accumulation of

lipids from crude extracts, so that washing the column with absolute methanol after each analysis is recommended to prevent column deterioration. Fortunately, the column can be regenerated by successive washings with the following solutions: water; methanol; chloroform; methanol; water; 0.1 *M* sulphuric acid; water[24]. Because of its stability and efficiency, we used a Zorbax ODS column for routine experiments[11].

In conclusion, the experimental system described not only allows the rapid and efficient separation of non-esterified chlorophylls, but also simultaneous separation of a variety of esterified chlorophylls without artifact formation.

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