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Short communication

# Determination of chlorophylls by reversed-phase high-performance liquid chromatography with isocratic elution and the column-switching technique

Koichi Saitoh*, Izumi Awaka, Nobuo Suzuki

*Department of Chemistry, Faculty of Science, Tohoku University, Sendai, 980-77, Japan*

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**Abstract**

A reversed-phase high-performance liquid chromatographic (HPLC) system was designed for the separation and determination of chlorophylls and their degradation products. Two separation columns packed with octadecyl-bonded silica gel (ODS) and octadecyl-bonded vinyl alcohol copolymer (ODP) were employed with the aid of a column-switching technique. The ODS column (1 cm) functioned for the separation of very hydrophobic compounds, such as chlorophyll *a*, chlorophyll *b*, pheophytin *a* and pheophytin *b*, and the ODP column (15 cm) for separating the compounds of low hydrophobicity, such as chlorophyll $c_1$, chlorophyll $c_2$, pheoporphyrin $c_1$, pheoporphyrin $c_2$, pheophorbide *a* and pheophorbide *b*. The HPLC system could cope with a variety of hydrophobicities of chlorophyll pigments by means of isocratic elution with methanol–phosphate buffer (pH 3) (92:8, v/v). Application to several seaweed samples was demonstrated.

## 1. Introduction

The determination of chlorophylls and their degradation products, such as pheophytins and pheophorbides, in natural samples from sea, lake, sediment and other sources gives valuable information about the biological processes occurring in these environments [1,2]. In oceanography, the chlorophyll abundance is an important measurement for estimating phytoplankton biomass [3]. The degradation products from chlorophylls found in a sediment are expected to act as paleoindicators of the nature of sedimentary input or living biomass composition [4].

Spectrophotometry and spectrofluorimetry, which are traditional and still popular methods for chlorophyll analysis [5], are not always effective with the complex range of chlorophylls and related compounds found in natural samples owing to the similarity of their spectral characteristics. Reversed-phase high-performance liquid chromatography (RP-HPLC) has become a promising means for the separation of chlorophylls, including chlorophyll-*a*, -*b* and -*c* (Chl-*a*, -*b* and -*c*), and also their demetallated forms, such as pheophytin-*a* and -*b* (Pheo-*a* and -*b*), and phytyl-free demetallated forms, such as pheophorbide-*a* and -*b* (Phor-*a* and -*b*) [6–8]. The successful resolution of Chl-*c* into Chl-$c_1$ and Chl-$c_2$ has been reported [9,10].

There are considerable differences in polarity (or hydrophobicity) among chlorophyll com-

* Corresponding author.

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Fig. 1. Chlorophylls and its degradation products. Chl-*a* (A, $R_1$ = methyl); Chl-*b* (A, $R_1$ = formyl); Chl-$c_1$ (B, $R_2$ = ethyl); Chl-$c_2$ (B, $R_2$ = vinyl).

pounds owing to their molecular structures (see Fig. 1). Gradient elution is a popular and effective mode of HPLC to cope with a variety of compounds. However, it requires sophisticated instrumentation for changing the mobile phase composition in accordance with a preset programme. Isocratic elution is a convenient and desirable chromatographic mode from the viewpoint of the simplicity of the equipment. This paper describes the feasibility of a column-switching technique for the HPLC separation of chlorophylls with isocratic elution.

## 2. Experimental

### 2.1. Chlorophyll pigment standards

Chl-*a* and -*b* were extracted with acetone from fresh spinach and precipitated by adding dioxane and water [11]. The chlorophylls were purified by preparative RP-HPLC using an octadecyl-bonded silica gel (ODS) column (particle size 30–50 μm, 36 cm × 24 mm I.D.) (Fuji Gel RQ-2; Fuji, Tokyo, Japan) with methanol–acetone (70:30, v/v). Chl-$c_1$ and -$c_2$ were extracted with acetone from the fresh brown seaweed *Undaria pinnatifida* (Phaeophyceae) obtained at Onagawa Bay, Miyagi, Japan. After preliminary separation by liquid–liquid partitioning and then by preparative chromatography on an octadecyl-bonded silica gel column [10], Chl-$c_1$ and -$c_2$ were purified by HPLC on an ODP column (5 μm, 15 cm × 6 mm I.D.) (Asahipak ODP-50; Asahi Kasei, Kawasaki, Japan) with methanol–phosphate buffer (pH 3) (90:10, v/v).

Demetallated forms of chlorophylls were prepared by treatment of corresponding chlorophylls as detailed previously, for Pheo-*a* and -*b* and Phor-*a* [11] and pheoporphyrin $c_1$ and $c_2$ (Pheo-$c_1$ and -$c_2$) [10].

### 2.2. Chromatographic system

An isocratic elution HPLC system (Twincle; JASCO, Tokyo, Japan) equipped with a UV–visible absorption detector (SPD-6AV; Shimadzu, Kyoto, Japan) and a photodiode-array detector (modified Shimadzu SPD-M6A, wavelength range 300–800 nm) was used. Columns packed with ODS (Inertsil ODS-2; GL Science, Tokyo, Japan) (particle diameter 5 μm, 15 cm × 4.6 mm I.D.) and ODP (5 μm, 15 cm × 4.6 mm I.D.) (Asahipak ODP-50) were used for a preliminary retention study. A short ODS column (5 μm, 1 cm × 4.6 mm I.D.) was combined with the 15-cm ODP column in the HPLC system using a column-switching technique. The mobile phase was methanol–buffer solution (1.24 m*M* phosphoric acid–8.76 m*M* sodium dihydrogenphosphate, pH 3) (92:8, v/v) and the flow-rate was 1.0 ml/min. The detection wavelength was 450 nm unless indicated otherwise.

## 3. Results and discussion

### 3.1. Retention characteristics

It has been reported that Chl-*a* shows a longer retention than Chl-*b* on an ODS column working in the reversed-phase mode, and a similar retention trend held for the *a*–*b* pairs of pheophytins and pheophorbides [8,11]. There was almost no difference in the retention of Chl-$c_1$ and Chl-$c_2$ on an ODS column with a

methanol–water mobile phase, whereas a sharp difference appeared on an ODP column, Chl-$c_1$ showing a smaller retention than Chl-$c_2$ [11]. It was reasonable to use an ODP column for chlorophyll analysis with recognition of Chl-$c_1$ and -$c_2$. However, compounds possessing phytyl groups, such as Chl-*a* and -*b*, and also Pheo-*a* and -*b*, were retained so strongly on this column that they could not be eluted with the mobile phase effective for the resolution of Chl-*c* [e.g., methanol–buffer (pH 3) (92:8, v/v)]. The retention of each compound of interest on ODS was smaller than that on ODP with an identical composition of the mobile phase, as shown in Fig. 2a and b. Accordingly, ODS was regarded as being preferable to ODP, particularly for the separation of the phytylated compounds in a short time.

### 3.2. Column-switching technique

In order to permit the analysis of chlorophyll compounds in one run in a short time by isocratic elution, a selective decrease in retention times was required for hydrophobic compounds possessing phytyl groups. This problem was solved in this work by changing the effective separation column from the long ODP column (150 mm × 4.6 mm I.D.) to the short ODS column (10 mm × 4.6 mm I.D.) at a suitable time in each run by means of a column-switching technique. The mobile phase conditions remained identical throughout the analysis, i.e., methanol–phosphate buffer (pH 3) (92:8, v/v) at a flow-rate of 1.0 ml/min.

A sample solution was injected into the 1-cm ODS column that led to the 15-cm ODP column. Phytyl-free chlorophyll compounds, such as Chl-$c_1$, Chl-$c_2$, Pheo-$c_2$ and Phor-*a*, passed through the ODS column in short time without effective separation and flowed into the ODP column, whereas more hydrophobic compounds possessing phytyl groups, such as Chl-*a*, Pheo-*a*, Chl-*b* and Pheo-*b*, were retained on the former column. At this time ($t_1$), the column-switching valve was activated so as to bypass the ODS column and to allow the effective separation of the phytyl-free compounds in the ODP column. When the separation of these compounds was finished (at time $t_2$), the valve was driven so that the ODS column followed the ODP column, and phytylated compounds were resolved in the ODS column. After completion of an analysis, the order of connection of these columns was reset for subsequent analysis. The feasibility of the column-switching technique was demonstrated in the separation of several chlorophyll standards, as shown in Fig. 3a.

### 3.3. Application to seaweed samples

Green seaweeds (Chlorophyceae), *Monostroma nitidum* and *Bryopsis plumosa*, and brown seaweeds (Phaeophyceae), *Undaria pin-*

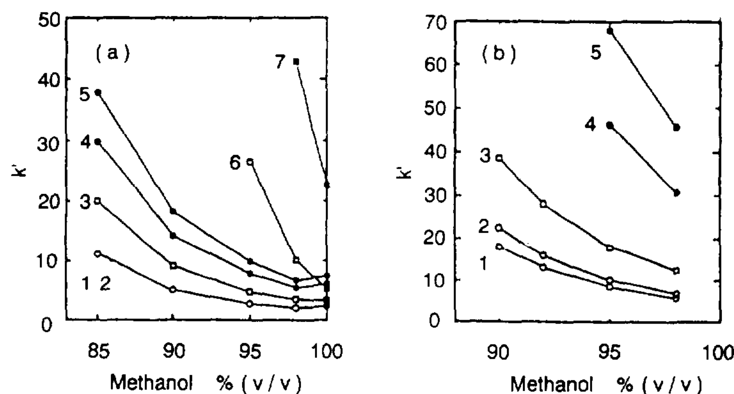


Fig. 2. Capacity factor ($k'$) versus methanol content of the mobile phase on (a) ODS and (b) ODP. Compounds: 1 = Chl-$c_1$; 2 = Chl-$c_2$; 3 = Phor-*a*; 4 = Pheo-$c_1$; 5 = Pheo-$c_2$; 6 = Chl-*a*; 7 = Pheo-*a*.

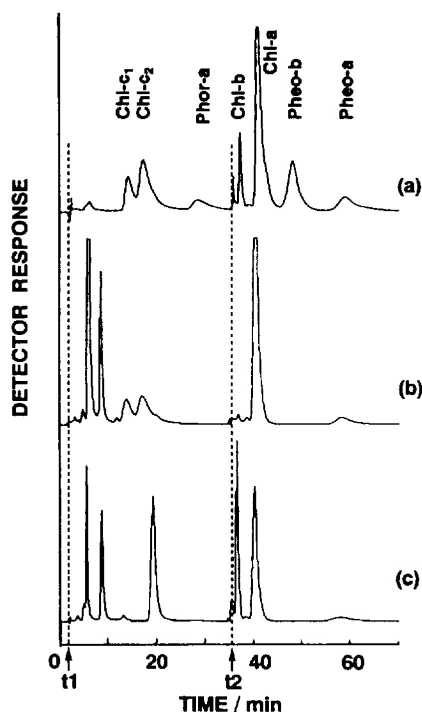


Fig. 3. Chromatograms for (a) chlorophyll standards, (b) extract from *Undaria pinnatifida* and (c) extract from *Monostroma nitidum*. Mobile phase, MeOH–buffer (pH 3) (92:8, v/v); flow-rate, 1.0 ml/min; detection wavelengths, 440 nm for Chl-*a* and -*b*, 450 nm for Chl-$c_1$ and -$c_2$ and 410 nm for Pheo-*a*, Pheo-*b* and Phor-*a*. $t_1$, $t_2$ = Timings of column switching.

*natifida*, *Laminaria religiosa* and *Sargassum sagamianum*, were sampled at Oshika (Pacific coast), Miyagi, Japan, and stored at −20°C until used. Chlorophyll pigments were extracted from a sample (0.2–3 g) with cold methanol (10 ml per gram of wet sample) in triplicate. The extract was passed through a PTFE membrane filter (0.5 μm), followed by dilution to the desired volume (10–100 ml) of test solution using the HPLC mobile phase. The extraction residue was dried at 85°C for 2 h and was then weighed.

A 10-μl aliquot of a test solution was applied to the HPLC system. In order to detect the compounds of interest with high sensitivity, the detection wavelength was changed automatically: 440 nm for Chl-*a* and Chl-*b*, 410 nm for Pheo-*a* and Phor-*a* and 450 nm for Chl-$c_1$, Chl-$c_2$, Pheo-$c_1$ and Pheo-$c_2$. The peak identification was supported by the UV–visible spectra obtained with the photodiode-array detector.

Chl-*a*, Chl-*b* and Pheo-*a* were detected in all green seaweed samples, and Chl-*a*, Pheo-*a*, Chl-$c_1$ and Chl-$c_2$ in brown seaweeds. Typical examples of chromatogram are shown in Fig. 3b and c. Several chlorophylls and related compounds were determined by comparing their peak areas with those measured for standards. The results for different seaweeds are given in Table 1. The values given in parentheses are the relative

Table 1
Determination of chlorophylls in seaweed samples collected at Oshika, Miyagi, Japan

| Seaweed | Date | Mean (mg/g)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Chl-*a* | Chl-*b* | Chl-$c_1$ | Chl-$c_2$ | Pheo-*a* | $(c_1/c_2)$[b] |
| *Undaria pinnatifida* | 11 July 1991 | 8.4 (0.68) | ND[c] – | 1.2 (1.7) | 1.6 (11) | 1.4 (4.4) | 0.75 |
| *Laminaria religiosa* | 28 April 1990 | 4.0 (15) | ND – | 0.41 (1.6) | 0.99 (0.7) | 2.6 (0.1) | 0.42 |
| *Sargassum sagamianum* | 11 July 1991 | 4.5 (3.4) | ND – | 0.53 (5.6) | 1.1 (6.0) | 1.7 (5.6) | 0.50 |
| *Bryopsis plumosa* | 11 July 1991 | 6.0 (0.8) | 5.6 (0.3) | ND – | ND – | 0.49 (4.4) | – |

[a] Mean of three determinations, based on dry mass of extraction residue, with R.S.D. (%) in parentheses.
[b] Ratio of Chl-$c_1$ to Chl-$c_2$.
[c] ND = Not detected.

standard deviations calculated from three measurements for each.

In conclusion, the column-switching technique has enabled HPLC to cope with the wide range of hydrophobicity of chlorophyll compounds even using the isocratic elution mode. The distinctive measurements of Chl-$c_1$ and -$c_2$ are expected to give useful information similarly to those for Chl-*a* and -*b* for determining biomass and pollution in the environment and also for the determination of biological activity.

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