Pigment Analysis of Marine Microalgae by TLC and HPLC Methods

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Abstract: Pigments extracted from marine red tide-causing microalgae, *Heterosigma akashiwo*, *Chattonella marina* (Raphidophyceae), and a diatom, *Chaetoceros ceratosporum* (Bacillariophyceae), were analysed using reversed phase thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in order to establish a rapid method for identifying algal pigments. Three raphidophyte strains showed to contain chlorophyll *a*, chlorophyll *c*, zeaxanthin, fucoxanthin, violaxanthin, and β-carotene as major pigments. On the contrary, the pigment composition of *C. ceratosporum* showed to be considerably different from that of the raphidophytes: the absence of violaxanthin, zeaxanthin, and β-carotene and the presence of 2 unidentified carotenoids. The analytical methods are useful for the rapid identification of chlorophyll and carotenoid pigments from marine microalgae and can be applied to the monitoring system of the bloom succession of marine microalgae.

Key words: Microalgae; Pigment; Chlorophyll; Carotenoid

Marine microalgae are generally beneficial to the productivity of marine ecosystems as foods for various marine animals. Among them, marine diatoms such as *Skeletonema* and *Chaetoceros* are consumed as critical foods for filter-feeding shellfish and larvae of various crustaceans and fish. However, algal blooms of various species belonging to dinoflagellates and raphidophytes which cause red-tides and produce algal toxins are harmful to wild fisheries, aquaculture and human health. In coastal regions, the changes in microalgal species composition occur frequently under various physical, chemical and biological conditions and the overall importance of diatoms, dinoflagellates and raphidophytes to the net plankton is approved[1].

Heterosigma akashiwo and *Chattonella marina* used in this study are well known red-tide algae and *Chaetoceros ceratosporum* is a popular live food source for fish and shellfish larvae in marine aquaculture. By using high-performance liquid chromatography (HPLC), numerous analyses of pigment compositions of marine microalgae have been carried out for identifying algal species and determining algal compositions in seawater samples[2-10]. Among them, Wright *et al.*[11] reported the pigment compositions for 12 microalgal species; Kohata *et al.*[12] calculated the relative abundance of *Chattonella antiqua* during the bloom based on pigment ratios obtained by multiple regression analysis of pigment concentrations.

To identify microalgal species and determine algal compositions in seawater samples, we tried to establish a rapid chromatographic method for analyzing algal pigments including chlorophylls and carotenoids.

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Materials and Methods

Microalgal Cultures

Heterosigma akashiwo Ho strain, Hy strain, and *Chattonella marina* K82 strain were isolated from Kagoshima Bay, Kagoshima Prefecture, Japan, and *Chaetoceros ceratosporum* C-16 strain was kindly provided by Dr. K. Fukami (Faculty of Agriculture, Kochi University, Japan). Microalgal cells were grown in Provasoli's enrichment seawater medium (ESS medium) at 23°C under a 12:12 LD cycle with an irradiance of 5.0×10^2 μEinstein$\cdot m^{-2}\cdot s^{-1}$ during the light period.

Pigment Extraction

Microalgal cells cultivated in 100 m*l* of ESS medium were filtered through Whatman GF/F glass fiber filter with aspiration. Pigments were extracted from the cells trapped on the filter in 10 m*l* of cold 90% acetone by grinding in a mortar. Extracts were filtered through GF/F filter to remove cellular debris. In order to avoid chromatographic artifacts, 0.4 m*l* of water was added to 1.0 m*l* of the extracts. Whole extraction process was carried out under dark and cold conditions. Extract solutions were immediately applied to thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

Pigments Used as Standards

Chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*) purchased from Wako Chemicals, Japan and carotenoids peridinin, fucoxanthin, violaxanthin (purified from marine organisms), zeaxanthin, and β-carotene (synthetic compounds) kindly provided by Dr. Y. Tanaka (Faculty of Fisheries, Kagoshima University, Japan) were used as standards for TLC and HPLC analyses.

Thin-layer Chromatography (TLC)

Two hundred and fifty microliter of the extracts and authentic pigments were applied to a KC_{18}/ODS-3 reversed phase thin-layer chromatography plate (Whatman). Chromatography plates were developed at room temperature with a solvent composed of eluent A solution (methanol-1 M ammonium acetate; 80:20, v/v) and eluent B solution (acetonitrile-acetone; 60:40, v/v) (1:1). Autofluorescence of chlorophyll was observed through 530 nm yellow optical filter by irradiation of 450 nm blue light.

High-performance Liquid Chromatography (HPLC)

Fifty microliter of the extracts were injected to an HPLC system (Waters Alliance System) equipped with a Model 2690 programmable solvent pump module and a Model 996 photodiode array detector module (Waters, USA) according to the procedure by Garrido and Zapata[13]. The column used was a Symmetry C18 monomeric octadecylsilane column (Waters, USA; the column size, 250×4.6 mm; the particle size, 5 μm; and the pore size, 80 A) and was maintained at 25°C. A mixture of eluent A and eluent B described above was used for elution. Elution was programmed as a linear gradient from 5% to 100% of eluent B for 28 min, followed by an isocratic hold at 100% eluent B for 32 min. The flow rate was 0.5 m*l*/min. Pigments were detected by absorption at 430 nm, and absorption spectrum from 200 to 800 nm was also observed for each pigment.

Results

Pigment Analysis by Tin-layer Chromatography

In order to clarify the pigment contents of the raphidophytes *H. akashiwo* Ho and Hy strains, *C. marina* K82 strain, and the bacillariophyte *C. ceratosporum* C-16 strain, the thin-layer chromatography (TLC) analysis of the pigment extracts was carried out. Yellow, orange, and green-colored spots were observed on TLC plates as shown in Fig. 1 (A and B). Comparing Rf values and color for spots of pigments extracted from the microalgae with those of authentic carotenoids, spots b, c, g, and i were identified as fucoxanthin, violaxanthin, zeaxanthin, and β-carotene, respectively. Irradiation with 450 nm blue light revealed red-colored autofluorescence from spots a and h (Fig. 1C), suggesting that these spots were composed of chlorophylls. Spot h was identified as chlorophyll *a* (chl *a*) because the Rf value was in fair agreement with that of

authentic chl *a*. Although spot a showed no coincidence of Rf value with any standard pigments used, it is supposed to be chlorophyll *c* (chl *c*) because of its high polarity and autofluorescence. Spots d, e, and f were not identified because of the disagreement of Rf values with standard pigments used in this experiment. *H. akashiwo* Ho strain, Hy strain, and *C. marina* showed similar TLC profiles (lanes 6, 7, and 8 in Fig. 1 A and B), indicating that they have almost the same pigment compositions including chl *a*, chl *c*, fucoxanthin, violaxanthin, zeaxanthin, and β-carotene. However, *C. ceratosporum* differed in pigment composition from other 3 microalgae judging from the fact that it lacked violaxanthin, zeaxanthin, β-carotene and an unidentified pigment (spot e) whereas it contained 2 unknown carotenoids (lane 9 in Fig. 1 A and B).

Pigment Analysis by High-performance Liquid Chromatography

The extracted pigments were also applied to a high-performance liquid chromatography (HPLC) analysis. Chromatograms of 4 microalgal strains are illustrated in Fig. 2 and the retention times and absorption maxima of pigment peaks detected on HPLC are summarized in Table 1. As characteristics of adsorbent and elution solution for HPLC were equivalent to those for TLC, retention times of pigments on HPLC were reversely correlated with Rf values on TLC plates. If peaks on HPLC and spots on TLC were supposed to correspond to the same pigments, they were given the same letters, for example, peak a and spot a. Identification of the pigment peaks on HPLC was performed by comparing their retention times and absorption spectra with those of authentic pigments. For example, peak g pigment extracted from *H. akashiwo* Ho strain, Hy strain, and *C. marina* showed a retention time of 28.56 min (Fig. 2), and absorption maxima of 458 and 484 nm (Fig. 3). As these characteristics corresponded to those of authentic zeaxanthin (Fig. 3, C and D), peak g pigment was identified as zeaxanthin. In the same way major pigments detected on HPLC were identified, except for peaks a, d, e, and f (Table 1). Although there was no correspondent authentic pigment, peak a pigment was suggested to be chl *c* from the following reasons: 1) high polarity substances like chl *c* that occur as a nonesterified carboxylic acid (chlorophyllide) are rapidly eluted on reversed-phase HPLC. 2) peak a pigment showed the same absorption spectrum (λ max, 450, 584, 635 nm in elution solution) as that reported for chl *c* (λ max, 444, 581, 630 nm in acetone). TLC analysis of pigments extracted from 4 microalgal strains supported the results obtained from HPLC. The pigment compositions of *H. akashiwo* Ho strain, Hy strain, and *C. marina* were quite similar, whereas *C. ceratosporum* exhibited significant difference in pigment composition compared with the raphidophytes.

Discussion

By using reverse-phase TLC and HPLC systems, major pigments from 4 microalgal strains could be sufficiently separated and identified with some exceptions. Comparing the pigment contents of 4 strains, raphidophytes *H. akashiwo* Ho and Hy strains, and *C. marina*, contained quite similar compositions of chlorophylls and carotenoids. Three raphidophyte strains used in this study are found to contain chl *a*, chl *c*, zeaxanthin, fucoxanthin, violaxanthin and β-carotene. Kohata *et al.*[12] reported that *C. antiqua* possessed chl *a*, chl *c*, fucoxanthin, violaxanthin and β-carotene as major pigments but not zeaxanthin. On the other hand, bacillariophyte, *C. ceratosporum*, was found to be distinguished from the raphidophyte strains in pigment composition because of its absence of zeaxanthin and violaxanthin and the presence of unknown pigments. Some investigators demonstrated that bacillariophyte species have dominantly chl *a*, chl *c*, fucoxanthin, β-carotene, diadinoxanthin, and diatoxanthin. In view of this and our results it is suggested that two unidentified carotenoid pigments obtained from *C. ceratosporum* on TLC (spots d and f) and HPLC (peaks d and f) are diadinoxanthin and diatoxanthin, respectively. These facts indicate that the pigment compositions are usuful as criteria of algal identification

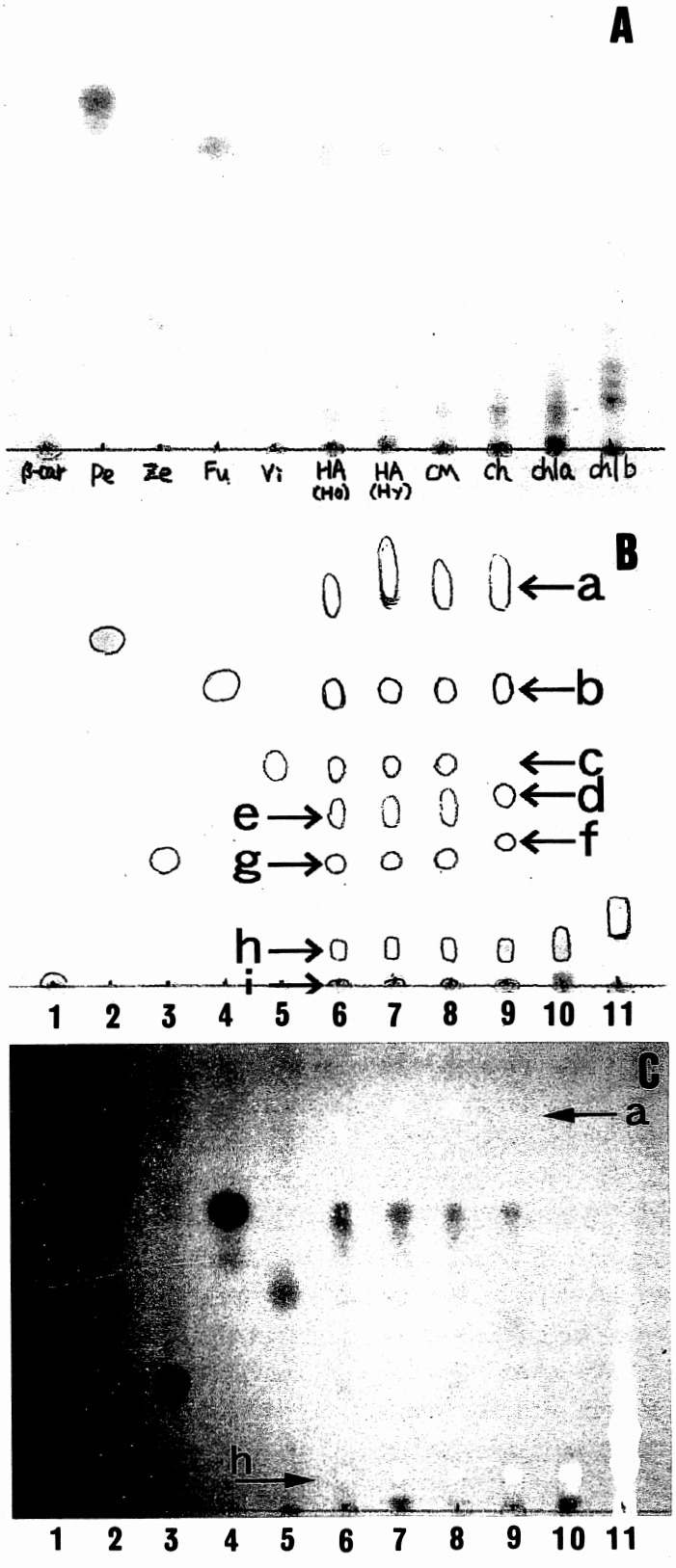

Fig. 1. TLC plates of pigments extracted from microalgal strains and authentic pigments. A and B plates are visible light profiles on TLC. Pigment spots are indicated by arrows with small alphabetical letters.; lane 1, β-carotene (β-car); lane 2, peridinin (Pe); lane 3, zeaxanthin (Ze); lane 4, fucoxanthin (Fu); lane 5, violaxanthin (Vi); lane 6, *H. akashiwo* (Ho) extract; lane 7, *H. akashiwo* (Hy) extract; lane 8, *C. marina* (CM) extract; lane 9, *C. ceratosporum* (Ch) extract; lane 10, chlorophyll *a* (chla); lane 11, chlorophyll *b* (chlb). C plate is the autofluorescent profile on TLC under irradiation of 450 nm blue light. Pigment samples are the same as A and B plates.

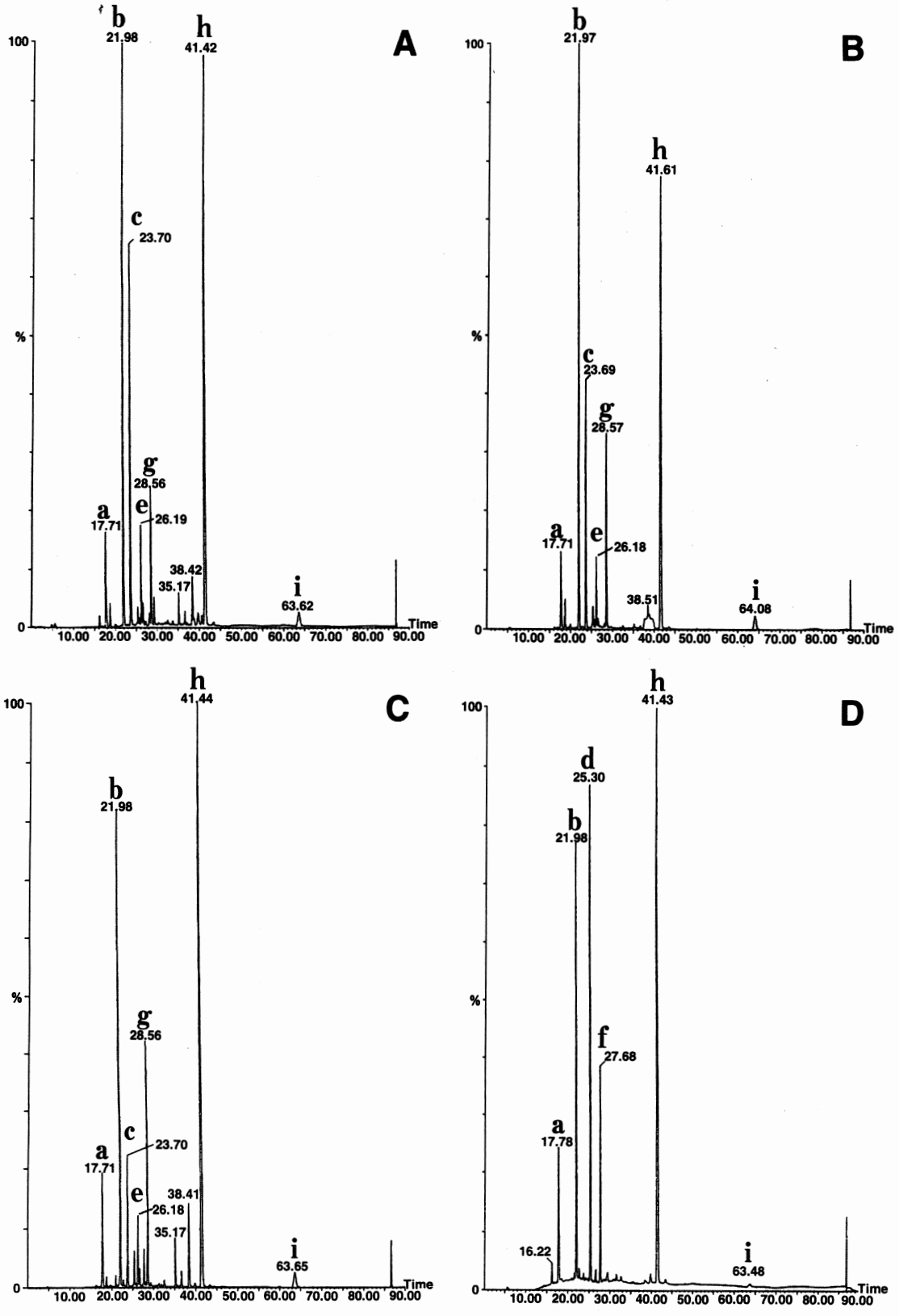

Fig. 2. HPLC chromatograms of pigments extracted from microalgal strains. A, *H. akashiwo* Ho strain; B, *H. akashiwo* Hy strain; C, *C. marina* K82 strain; D, *C. ceratosporum* C-16 strain. Major pigment peaks are indicated by small alphabetical letters.

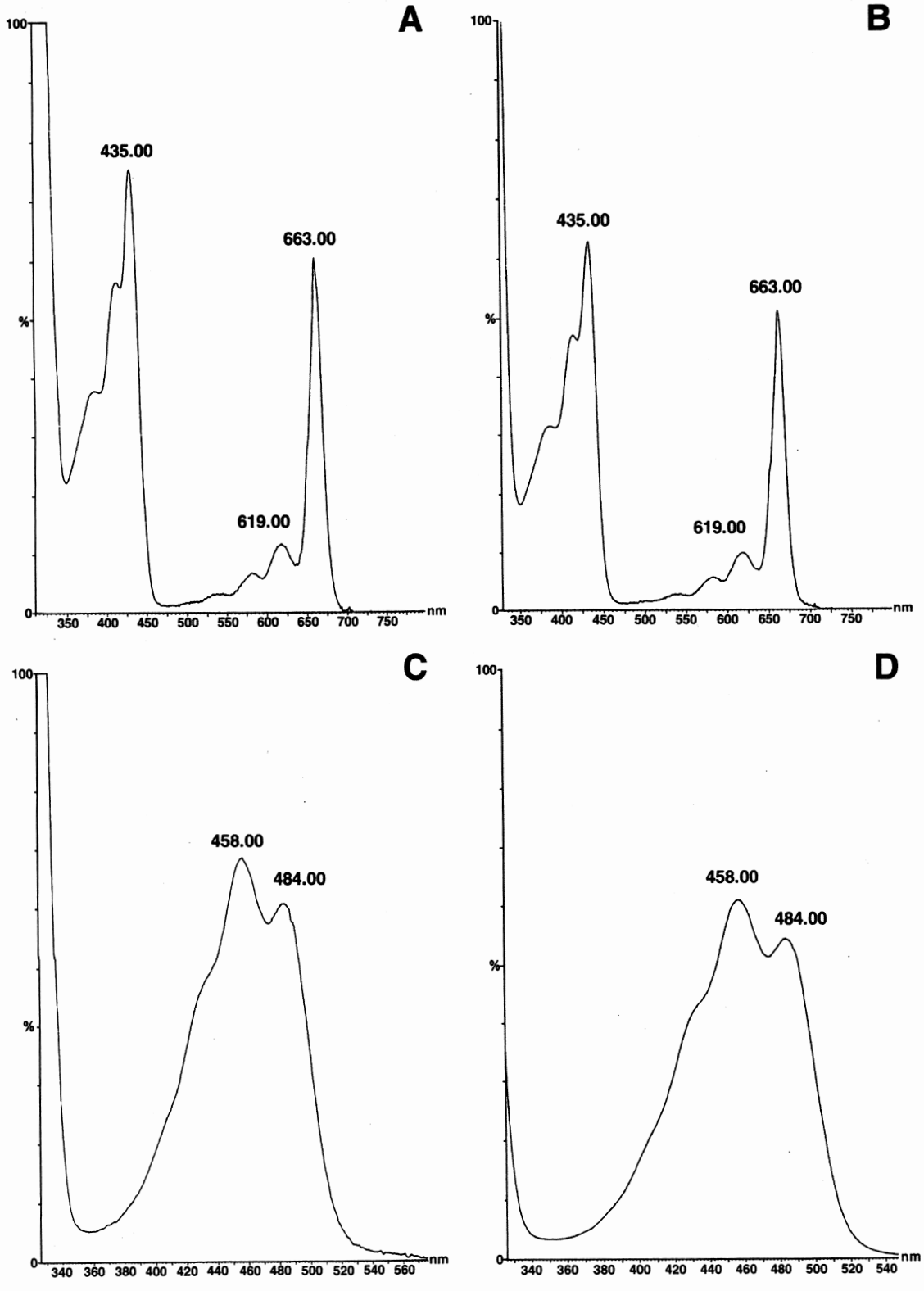

Fig. 3. Absorption spectra of pigment peaks on HPLC chromatograms. A, peak h (RT, 41.61 min) from *H. akashiwo* Hy strain; B, authentic chlorophyll *a* (41.46 min); C, peak g (28.57 min) from *H. akashiwo* Hy strain; D, authentic zeaxanthin (28.57 min).

Table 1. Retention times and absorption maxima of pigments extracted from microalgae isolated from Kagoshima Bay

Pigments	Peaks on HPLC	Standards	*H. akashiwo* (Ho)	*H. akashiwo* (Hy)	*C. marina*	*C. ceratosporum*
		λ max (nm)	λ max (nm)	λ max (nm)	λ max (nm)	λ max (nm)
Chlorophyll *c*	a	-	17.71 (451,585,633)	17.71 (450,585,633)	17.71 (450,584,633)	17.78 (448,584,633)
Fucoxanthin	b	21.96* (453)*	21.97 (452)	21.98 (453)	21.98 (453)	21.98 (453)
Violaxanthin	c	23.69 (422,446,475)	23.69 (421,445,475)	23.70 (422,446,475)	23.70 (422,446,475)	-
Unknown carotenoids	d	-	-	-	-	25.30 (452,482)
Unknown carotenoids	e	-	26.18 (452,480)	26.19 (452,481)	26.18 (452,480)	-
Unknown carotenoids	f	-	-	-	-	27.68 (458,486)
Zeaxanthin	g	28.57 (458,484)	28.57 (458,484)	28.56 (458,484)	28.56 (458,484)	-
Chlorophyll *b*		37.54 (460,598,647)	-	-	-	-
Chlorophyll *a*	h	41.46 (435,619,663)	41.61 (435,619,663)	41.42 (435,619,663)	41.44 (435,618,663)	41.43 (435,620,663)
β-Carotene	i	63.86 (459,485)	64.08 (458,488)	63.62 (458,483)	63.65 (458,483)	63.48 (459,488)

* The upper values are retention times (min) on HPLC and lower ones are maximum absorbances (nm).

at a class level as reported by many investigators. Raphidophyte species are known as causative organisms of red tides and the monitoring systems of their bloom formation are required to be developed. The rapid analytical methods of algal pigments used in this study should contribute as one of the identification and monitoring systems for algal blooms in marine environments.

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TLC および HPLC 法による海産微細藻類色素の分析

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鹿児島湾から分離したラフィド藻，*Heterosigma akashiwo* 2株と *Chattonella marina* 1株および養殖飼料用珪藻 *Chaetoceros ceratosporum* 1株の藻類色素を抽出し，逆相 TLC および HPLC 法を用いて分析した。ラフィド藻の3株はクロロフィル *a* と *c*，ゼアキサンチン，フコキサンチン，ビオラキサンチン，β-カロチンを含有し，非常に類似した色素組成をしていた。一方，珪藻株はゼアキサンチン，ビオラキサンチン，β-カロチンを含有せず，未同定の2つのカロテノイド色素を有しており，ラフィド藻とはかなり異なった色素組成を示した。本研究で用いた色素分析法は簡便迅速であり，海産微細藻類の同定およびモニタリングに応用できることが示された。