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Highly sensitive determination of photosynthetic pigments in marine *in situ* samples by high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) method was developed by optimizing a solvent system for the highly sensitive determination of photosynthetic pigments in marine *in situ* samples. The proposed HPLC system can handle injection volumes greater than 400 μl without a significant loss in the resolution between chlorophyllide *a* and chlorophyll $c_1 + c_2$. The system also reduces the sample volumes required for the analysis of typical pigments of oligotrophic sea water to 500 ml. The elution time for all pigments is 24 min at a flow-rate of 1 ml/min and 12 min at a flow-rate of 2 ml/min using a 250 mm × 4.6 mm I.D. column.

INTRODUCTION

The determination of photosynthetic pigments and their degradation products is one of most reliable methods of estimating phytoplankton biomass in marine samples [1,2]. The chromatographic analysis of photosynthetic pigments is a powerful technique for the characterization of phytoplankton communities to provide the relative abundance of each phytoplankton class in a particular sample [3–5]. Analysis of the pigments by high-performance liquid chromatography (HPLC) has been applied successfully to samples from marine ecosystems [2–9]. However, large volumes (up to several tens of liters) of oligotrophic or tropical samples (*e.g.* chlorophyll *a* concentrations less than 1 $\mu g\ l^{-1}$) are required for pigment analysis by this method [2–8]. The time-consuming filtration of such large sample volumes has presented problems in analyses in the field. This paper shows how this problem can be overcome by using a large injection volume.

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EXPERIMENTAL

Algal culture

Clonal axenic strains of *Heterosigma akashiwo* (NIES-6), *Gephyrocapsa oceanica* (NIES-353), *Thalassiosira rotula* (NIES-328), *Skeletonema costatum* (NIES-323), *Heterocapsa triquetra* (NIES-7) and *Pyramimonas parkeae* (NIES-254), maintained in the Microbial Culture Collection of the National Institute for Environmental Studies (NIES), were used. The algal cultures were maintained on a 12:12 h light:dark cycle at 23°C in f/2 medium. Illumination was provided by daylight fluorescent lamps at a quantum flux density of 80 μE m^{-2} s^{-1}. The cultures were harvested 2–3 weeks after inoculation and were filtered onto GF/C filters (Whatman International, Maidstone, UK). The filters were refrigerated at −20°C until analysis.

Oceanic samples

Samples were taken with a 10-l Van Dorn-type bottle in the Seto Inland Sea on 20 July 1987 [10]. For the chromatographic analysis of chlorophyll (Chl) and carotenoids, 2000 ml of sea water were filtered through a Whatman CF/C filter at a field station. For preparing alloxanthin, 6000 ml of sea water were filtered in the same way. The filters were stored at −20°C until analysis at NIES.

Pigment extraction

The filtered samples were extracted in 10 ml of ice-cold 90% acetone, which had been degassed with nitrogen. The extraction was performed in the dark. Pigment extracts were obtained after homogenization of the filter with a glass grinder followed by centrifugation. The extract (25–400 μl) of the pigments was injected directly into an HPLC system after filtration with a Millipore FH filter (0.5 μm pore size, Nihon Millipore, Tokyo, Japan).

Column liquid chromatographic analysis

The HPLC system was essentially the same as that described previously (LC-6A pumps controlled by SCL-6A, Shimadzu, Kyoto, Japan) [11] except for a solvent system for linear-gradient elution. The gradient-elution was performed as follows: from 100% solvent A (ion-pairing solution–water–acetone–acetonitrile, 5:25:20:50) to 100% solvent B (acetone–ethyl acetate, 50:50) in a 20 min, followed by an isocratic hold at 100% B over 5 min at a flow-rate of 1.0 ml min^{-1}. In a fast-flow mode, each time period of gradient elution was shortened to half of the original period, and the flow-rate was maintained at 2.0 ml min^{-1}. The ion-pairing solution [1] consisted of tetrabutylammonium hydroxide (10 ml of a 0.5 *M* solution) and ammonium acetate (7.7 g) made up to 100 ml with distilled water. The solution was neutralized with acetic acid to pH 7.1. The solvent system was chosen to allow a large injection volume (up to 400 μl) to reduce the sampling volume in the field. The two solvents were degassed by helium throughout the analyses. The HPLC column used in this work was a 250 mm × 4.6 mm I.D. Whatman Partisil ODS-3, 5 μm (packed by Chemco Scientific, Osaka, Japan) column protected by a CSK I guard column with ODS packing (Whatman, Clifton, NJ, USA).

The absorbance was measured at 440 nm (Shimadzu, SPD-6AV) and fluorescence detection (Shimadzu RF-540 spectrofluorophotometer) was used to aid in

the identification of the chloropigments. The spectrofluorophotometer was set at an excitation wavelength of 440 nm (20 nm slit width) and an emission wavelength of 660 nm (40 nm slit width). Peak heights and peak area were measured (Shimadzu C-R3A Chromato-data processor) on the absorbance trace. Supplemental identification of pigments was carried out from absorbance spectra (every 2 nm from 350 to 668 nm) obtained with a photo-diode array detector (Shimadzu, SPD-M6A) and a data-processing system (PC9801RA5, NEC, Tokyo, Japan).

Pigment standards and solvents

Authentic chlorophyll (Chl) *a*, Chl *b*, and *β*-carotene were purchased from Sigma (St. Louis, MO, USA). Standard samples of carotenoids other than alloxanthin were obtained from the axenic cultures described earlier. Alloxanthin was obtained from the oceanic sample. Pigment extracts of the algal cultures and the oceanic sample were obtained in the same way as for the HPLC samples. The extract was transferred to diethyl ether and concentrated under a nitrogen gas stream. The pigments were isolated on reversed-phase C_8 thin-layer chromatography (TLC) plates (20 × 5 cm, KC8, Whatman) developed with 98–80% aqueous methanol in a saturated-type TLC box (Model HPS-204, hanging plate type, Advantec Toyo Kaisha, Tokyo, Japan) at room temperature. The pigments were scraped from the plates and redissolved in 90% acetone or ethanol. The standard solutions of pigments, the absorption spectra of which had been recorded with a Hitachi 220A spectrophotometer and standardized using reported specific absorption coefficients [12], were injected into the HPLC system to provide pigment identification and quantitative data from the HPLC chromatogram (Table I).

Chlorophyllide *a* was obtained from the enzymatic degradation of Chl *a* [13] with chlorophyllase extracted from the acetone powder of *Citrus unshiu* fruits [14]; the powder was a gift from Dr. K. Shimokawa. Phaeophytin *a* was prepared by acidification of Chl *a* [2].

Acetone, acetonitrile, ethanol and ethyl acetate were HPLC-grade reagents (Wako, Osaka, Japan) and were used without purification other than filtration and degassing. Water was purified using a Millipore Milli-Q system.

RESULTS AND DISCUSSION

The chromatographic and spectroscopic properties of the algal pigments are listed in Table I and compared with those reported previously. The purities of the peaks in Table I were calculated by averaging two correlation factors at the upslope and downslope; the correlation factor was defined as [15]:

$$\text{Correlation factor} = \frac{[\sum x \cdot y - (\sum x \cdot \sum y)]^2}{\left[\sum x^2 - \frac{(\sum x \cdot \sum x)}{n}\right]\left[\sum y^2 - \frac{(\sum y \cdot \sum y)}{n}\right]}$$

The values x and y are the measured absorbances in the peak-top and upslope (or downslope) spectrum at the same wavelength; n is the number of data points and $\sum$ is the sum of the data.

TABLE I

CHROMATOGRAPHIC AND SPECTROSCOPIC PROPERTIES OF ALGAL PIGMENTS

No.	Pigment	Retention time (min)	Solvent	Maximum wavelengths (nm)			Peak ratio[a]	Purity[b]	Ref.
				I	II	III			
1	Chlorophyllide *a*	7.03	Eluent[c]	429	616	664	1.1	0.9999	TW[e]
			Diethyl ether	429	615	662	1.2		
			Eluent[d]	430	616	664	1.3		2
			Diethyl ether	428		662		29	
2	Chl *c*[f]	8.71	Eluent	442	580	630	7.5	0.9999	TW
			Acetone	446	580	630	8.1		
			Eluent	442	580	630	9.8		2
			Acetone	444	581	630	6.7		30
3	Peridinin	11.34	Eluent		472			1.0000	TW
			Ethanol		471				
			Eluent		470				2
			Ethanol		472				31
4	Neoxanthin	11.85	Eluent	413	437	465	87	0.9999	TW
			Ethanol	413	437	466	88		
			Eluent	412	437	466	91		2
			Ethanol	410	436	464	93		2
5	Fucoxanthin	12.40	Eluent		447	(466)		1.0000	TW
			Acetone		446	467	5.4		
			Eluent		446	(468)			2
			Acetone		446	470	3.3		20
6	Loroxanthin	12.77	Eluent		445	474	51	0.9999	TW
			Ethanol	(420)	446	474	47		21
7	19′-Hexanoyloxy-fucoxanthin	13.30	Eluent		444	467	28	1.0000	TW
			Acetone		445	470	36		
			Acetone		445	471	44		20
8	Violaxanthin	13.45	Eluent	416	440	468	91	0.9999	TW
			Ethanol	417	441	471	96		
			Eluent	417	440	470	89		2
			Ethanol	417	440	470	93		32
9	Diadinoxanthin	14.46	Eluent	(422)	446	475	63	1.0000	TW
			Ethanol	424	448	478	55		
			Eluent	424	448	478	74		2
			Ethanol	(424)	446	476	39		2
10	Antheraxanthin	14.92	Eluent	426	445	471	54	1.0000	TW
			Ethanol	422	445	474	49		
			Eluent	(421)	448	475	62		2
			Ethanol	422	444	472	54		33
11	Alloxanthin	15.33	Eluent	(426)	452	481	41	0.9996	TW
			Ethanol	427	454	484	45		
			Eluent	(430)	452	481	50		2
			Ethanol	(430)	453	483			34
12	Diatoxanthin	15.78	Eluent		453	481	57	0.9989	TW
			Ethanol	(427)	453	481	28		
			Eluent	424	448	476	25		2
			Ethanol	428	452	479			12

TABLE I *(continued)*

No.	Pigment	Retention time (min)	Solvent	Maximum wavelengths (nm)			Peak ratio[a]	Purity[b]	Ref.
				I	II	III			
13	Lutein	16.22	Eluent	(421)	445	473	58	0.9999	TW
			Ethanol	(421)	447	475	55		
			Eluent	(422)	446	474	65		2
			Ethanol	422	445	474	62		33
14	Zeaxanthin	16.23	Eluent	(425)	450	477	29	1.0000	TW
			Ethanol	426	452	480	52		
			Eluent	(426)	452	480	32		2
			Ethanol	(428)	450	478	26		33
15	Chl *b*	19.64	Eluent	456	597	646	2.9	0.9999	TW
			Acetone	458	596	646	2.9		
			Eluent	456	598	646	3		2
			Acetone	453	598	645			35
16	Chl *a*	20.50	Eluent	429	616	663	1.2	0.9999	TW
			Acetone	430	618	665	1.2		
			Eluent	430	617	664	1.2		2
			Acetone	430	618	665			36
17	Phaeophytin *a*	22.30	Eluent	407	504	666	2.4	1.0000	TW
			Acetone	409	506	667	2.3		
			Eluent	408	504	666	2.2		2
			Dicthyl ether	408	503	667	2.1		37
18	β-Carotene	23.07	Eluent		451	476	17	0.9993	TW
			Ethanol	(425)	448	475	21		
			Eluent	(426)	452	478	20		2
			Ethanol	(425)	450	477			38

[a] For chlorophylls and their derivatives, the peak ratio is that of the Soret band absorbance divided by the maximum absorbance in the red region. For carotenoids, the peak ratio refers to the percentage III/II ratio [39].
[b] The purity of the peaks was calculated by averaging two correlation factors at the up- and downslopes.
[c] The pigments were eluted using a linear gradient from 100% solvent A (ion-pairing solution- water–acetone–aceto nitrile, 5:25:20:50) to 100% solvent B (acetone–ethyl acetate, 50:50) in a 20-min period.
[d] The pigments were eluted using a linear gradient from 90% acetonitrile to ethyl acetate over 20 min.
[e] This work.
[f] Chlorophyl $c_1 + c_2$.

It has been elucidated that there are many types of Chl *c* whereas Chl c_2 and/or c_1 are commonly observed in marine algae [16,17]. This system was not able to resolve Chl c_1 from c_2. The sum of Chl c_1 and c_2 is represented as Chl *c* in this work (Table I).

Chromatograms of the pigment extracts from various algal classes are shown in Fig. 1. The chlorophyllide *a* (peak 1) was resolved well from the Chl *c* peak (peak 2). The carotenoids of major significance in ecological studies, peridinin (peak 3), fucoxanthin (peak 5), diadinoxanthin (peak 9) and lutein (peak 13), were all completely

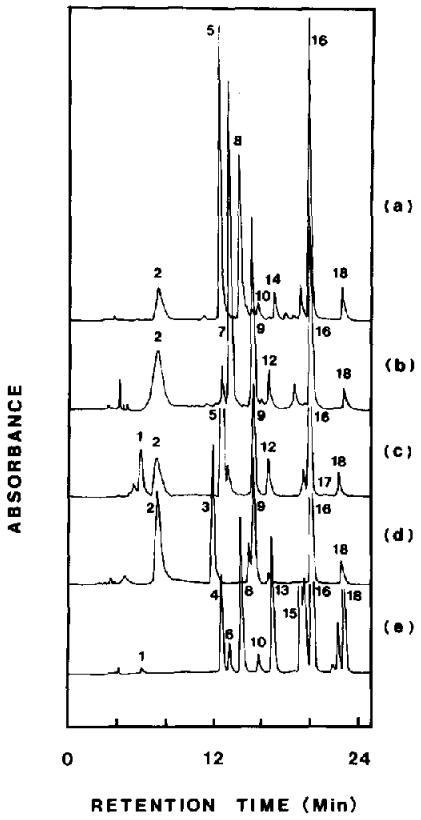

Fig. 1. HPLC absorbance (440 nm) chromatograms of various algae: (a) *Heterosigma akashiwo* (Raphidophyceae); (b) *Gephyrocapsa oceanica* (Haptophyceae); (c) *Thalassiosira rotula* (Bacillariophyceae); (d) *Heterocapsa triquetra* (Dinophyceae); (e) *Pyramimonas parkeae* (Prasinophyceae). Peak identities are given in Table I.

resolved. Neoxanthin (peak 4) of *Pyramimonas parkeae* was presumably in a *cis*-form [18], according to the maximum wavelength [19]. Alloxanthin and 19'-hexanoyloxyfucoxanthin had the same absorbance spectra as those reported previously [2,20]. Most peaks on the chromatogram of the oceanic sample were identified (Fig. 2c). Several HPLC systems have been reported to provide better pigment separation for green algae and higher plants than that in this work [21–23]. However, less attention has been paid in these systems to the separation of chlorophyllide *a* from Chl *c*, because green algae and plants lack Chl *c*. This separation is essential for analyzing marine aquatic ecosystems [1–7], large parts of which consist of many algal classes containing Chl *c*. In this system, the separation was taken into account even with a large sample injection volume (Fig. 2, Table II).

For dilute samples, concentration by ether partitioning has been necessary despite its serious disadvantages. It has been reported that improved resolution and sensitivity are obtained by this procedure, but also that there is a significant increase in the proportions of *cis*-fucoxanthin and phaeophytin *a* [2]. For quantitative work,

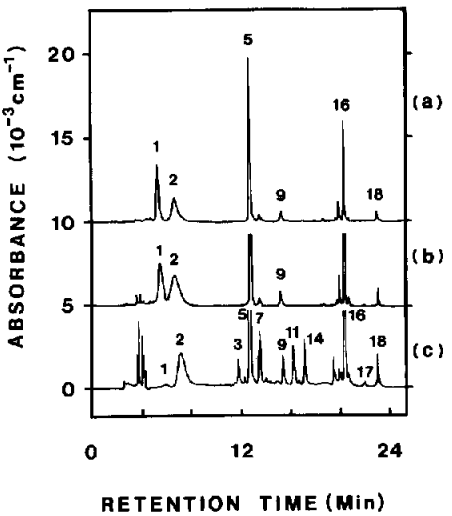

Fig. 2. Effect of injection volume on resolution of pigments on an absorbance (440 nm) chromatogram. (a) 20 μl injection of a *Skeletonema costatum* extract; (b) 400 μl injection of a *S. costatum* extract; (c) 400 μl injection of a field sample from the Seto Inland Sea. The chromatograms (a) and (b) are shifted by 10 and 5 $\times 10^{-3}$ cm^{-1}, respectively, for convenience. Peak identities are the same as those in Fig. 1.

the final extract was used without concentration, as the ether partitioning method gave an incomplete recovery and promoted the degradation of Chl *a* to phaeophytin *a* [2,24].

To minimize the loss of pigments during analytical procedures, it is recommended to inject the final extract directly into an HPLC system. Previous carotenoid analyses by HPLC have required sea water samples of more than 10 l, which is inconvenient for handling during field surveys. To increase the sensitivity of carote-

TABLE II

COMPARISON OF THE RESOLUTION BETWEEN CHLOROPHYLLIDE *a* AND CHLOROPHYLL c_1+c_2 ACCORDING TO INJECTION VOLUMES

The resolution of each separation was calculated by $R_s = 2(t_{R2}-t_{R1})/(w_1+w_2)$, where t_R and w are the retention times and peak widths of each pigment, respectively.

Solvent A composition[a]	Resolution				Ref.
	Injection volumes (μl)				
	100	200	300	400	
P[b]–DW[c]–acetone–CH_3CN (5:25:20:50)	1.32	1.33	1.23	1.12	This work
P–DW–methanol (5:5:90)	1.72	1.13	0.69	0.63	11
P–DW–methanol (10:10:80)	1.54	NC[d]	NC	NC	1

[a] Other experimental conditions were the same as those of this work.
[b] Ion-pairing solution (see text).
[c] Distilled water.
[d] Peak leading is too large to calculate the resolution.

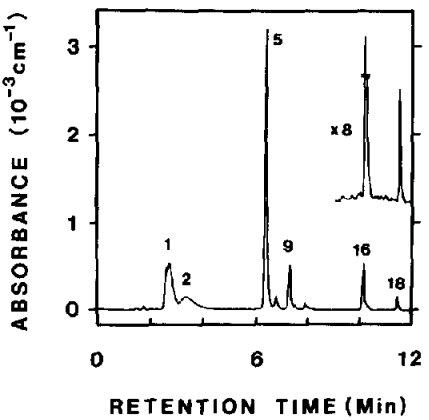

Fig. 3. HPLC absorbance (440 nm) chromatogram of an injection of a 400 μl sample of diluted *S. costatum* extract in the fast-flow mode. Peak identities are the same as those in Fig. 1. Content of β-carotene (peak 18) is 0.23 ng.

noid analysis by HPLC, one choice is to increase the sensitivity of the UV detector. However, it will probably be many years before the sensitivity of the detector is improved sufficiently, unless completely different procedures are developed.

Another way to increase the sensitivity is to increase the injection volume. With the usual analytical column of 250 × 4.6 mm I.D. packed with 5-μm ODS-bonded silica gel, the pigment extract injection volume chosen has been 20–50 μl [1–7] at a flow-rate of 1.0 ml min^{-1}. This HPLC system allows an injection volume of greater than 400 μl (Fig. 2) without a significant loss of resolution between chlorophyllide *a* and Chl *c* (Table II).

Peak spreading was commonly observed at an early retention time on the chromatogram and depended on the difference in solvent strength [25] between the extracts and mobile phases. Acetone has been used as an extract solvent because it reduces the chlorophyllase activity [23,26]. The mobile phases were therefore pre-

TABLE III

DETECTION LIMITS AND REQUIRED SAMPLE VOLUME OF THIS HPLC ANALYSIS

Pigment	Detection limit[a] (ng)	Concentration in sea water (μg l^{-1})	Least required sample[b] volume (ml)
Chl *a*	0.2	(i) 0.20[c]	25
		(ii) 0.67[d]	7
Chl *c*	0.5	(ii) 0.20	63
Fucoxanthin	0.4	(i) 0.02	500
		(ii) 0.14	71
β-Carotene	0.2	(i) 0.02	250
		(ii) 0.02	250

[a] Corresponds to $S/N = 10$ on an HPLC chromatogram.
[b] 400 μl injection of 10 ml extract.
[c] Oligotrophic sea water (from Gieskes and Kraay [6]).
[d] Oceanic sample of this work.

pared to permit the simultaneous separation of the pigments, allowing a large injection volume of the 90% acetone extracts without only significant decay in the shape of the peaks at early retention times.

The viscosities of acetone, acetonitrile and ethyl acetate, which are used in this HPLC system, are lower than that of methanol. Thus the flow-rate of this system could be twice that of methanol systems. The HPLC chromatogram, which was obtained at twice the flow-rate and half the gradient elution time, was similar to that obtained under the original conditions (Fig. 3). The column pressure with the fast-flow mode was less than 160 kgf cm^{-2} ($1.6 \cdot 10^7$ N m^{-2}). The higher flow-rate requires a shorter elution time without significant peak spreading. This makes it potentially suitable for field work and continuous surveys.

Simultaneous and rapid separation of pigments by HPLC has been achieved in gradient-elution modes. However, gradient elution sometimes causes a marked baseline drift, which prevents the measurement of small peaks on the chromatogram [27,28]. In this analysis, the baseline of the absorbance trace at 440 nm remained constantly low, thus allowing highly sensitive observations to be made (Fig. 3). The rise in sensitivity reduces the sample volumes needed for field surveys (Table III). Thus this solvent system ensures rapid and highly sensitive analyses and is convenient for use in the field, especially for oceanic samples.

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