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Simplified Technique for the Rapid Determination of Phytoplankton Pigments by Reverse-Phase High-Performance Liquid Chromatography

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A simplified technique for the rapid analysis of photosynthetic pigments in marine phytoplankton is described. The pigments extracted in N,N-dimethylformamide (DMF) are injected directly into the reverse-phase high-performance liquid chromatograph fitted with a photodiode array detector. We developed a new stepwise program; that is methanol-water (75:25 v/v), methanol, methanol-acetone (80:20) and methanol-acetone (65:35). This program gives a good separation of at least 13 carotenoids, and 10 chlorophylls and their derivatives, completed within 20 min. The quantitative precision is high; the maximum standard error was less than 6%. The stability of pigments in DMF were also considered for application of this technique to the lab work on board the ship.

1. Introduction

Chlorophyll *a* content in particulate matters has been widely used to estimate the phytoplankton biomass and productivity in natural waters, and its degradation products (chlorophyllide *a*, phaeophytin *a* and phaeophorbide *a*) are used as diagnostic indicators of physiological status of phytoplankton, detrital content and grazing processes (Lorenzen, 1967; Jeffrey, 1974; Shuman and Lorenzen, 1975; Gieskes *et al.*, 1978). Since phytoplankton have group-specific chlorophylls and carotenoids, the quantitative information on photosynthetic pigments is of ecological significance to assess taxonomic compositions in natural phytoplankton assemblages.

For the determination of photosynthetic pigments from particulate material, three points should be considered; complete extraction of pigments; stability of the extracted pigments in solvents, and method for separation and determination of pigments. On the former two points, we have reported a new extraction method for standard fluorometry using non-volatile N,N-dimethylformamide (DMF) (Suzuki and Ishimaru, 1990).

The determination of chlorophylls *a*, *b*, and *c* is routinely conducted by spectrophotometric methods (Strickland and Persons, 1972) or spectrofluorometric methods (Holm-Hansen *et al.*, 1965). However, the values obtained by these methods include some sources of error because the absorption and emission bands of chlorophyll *a* overlap with those of chlorophylls *b* and *c*, and the degradation products of chlorophylls are neither detected nor determined along with their parent chlorophylls (Garside and Riley, 1969; Jacobsen, 1978; Abayside and Riley, 1979; Brown *et al.*, 1981). The spectrophotometric method is less sensitive, so it requires a large volume of water sample. The determination of individual carotenoids (carotens and xanthophylls) are hardly achieved by these methods. For the identification and quantification of carotenoids, their

separation is needed. Recent advancements in high-performance liquid chromatographic (HPLC) technique for phytoplankton pigments seem to fulfill the third point.

Mantoura and Llewellyn (1983) developed a reverse-phase HPLC technique for rapid separation and quantification of 14 chlorophylls and their breakdown products and 17 carotenoids from acetone extracts of algal culture and natural waters. They suggested that the presence of an ion pairing reagent during the chromatographic separation was essential to obtain good resolution for chloropigments (chlorophylls, chlorophyllides and phaeophorbides). A modified technique was presented by Wright and Shearer (1984), in which photosynthetic pigments from phytoplankton were separated by HPLC using a lineal gradient elution from 90% acetonitrile to ethylacetate. Better resolution was obtained by the latter method for carotenoid pigments. But the Wright and Shearer method does not resolve the polar chlorophylls *c*1, *c*2, *c*3, and Mg-2,4 divinyl phaeoporphyrin *a*5 monomethyl ester (Mg2,4D) which coelute as a single peak, or adequately resolve the chlorophyllides *a* and *b*. A more comprehensive method has been published recently, and is used for routine work (Wright *et al.*, 1991).

We report here a simplified method for the separation, identification and quantification of the pigments by the HPLC. The pigments are extracted in DMF and directly injected after a short extraction time. In our method, no ion pairing reagent is necessary, which was once thought to be indispensable for the separation of rather polar pigments. We examined the stability of extracted pigments in DMF using this HPLC method. We found some pigments were stable in DMF at –25°C, allowing storage of samples in case immediate analysis using HPLC can not be performed.

2. Materials and Methods

2.1 Phytoplankton sources

Eleven clone cultures belonging to 7 algal classes were used as reference algae: *Skeletonema costatum* strain 1 and 2, *Phaeodactylum tricornutum*, *Chaetoceros socialis* (Bacillariophyceae), *Alexandrium tamarense* strain OK875-1 (Dinophyceae), *Dunaliella primolecta* (Chlorophyceae), *Platymonas* sp. strain PLATP (Prasinophyceae), *Isochrysis galbana*, *Pavlova lutheri* (Haptophyceae), *Chroomonas salina* strain 3C (Cryptophyceae), *Synechococcus* sp. strain WH6501 (Cyanophyceae). *S. costatum* and *C. socialis* were isolated from the surface water of Tokyo Bay. The algae were grown in the *f*/2 medium (Guillard and Ryther, 1962) at 15°C with occasional shaking under an illumination of 20–40 μE m^{-2} from fluorescent tubes with a 12:12 hr. light-dark cycle. Natural phytoplankton samples were collected on the Cruise KH91-3 of the Hakuho-Maru, University of Tokyo from 9 May to 8 June, station B (25°N, 165°E).

2.2 Extraction of pigments

At the late logarithmic phase of growth, algal cells were harvested by a gentle filtration in vaccuo through a 47 mm Whatman GF/F filter for *Synechococcus*, and GF/C filter for the other cultures and natural samples. The filter was cut into small pieces of about 5 mm across, and placed in a 10 ml grass tube. One ml of cold DMF was added. After standing for >30 min. at 5°C in the dark, the solvent was poured, together with the filter pieces, onto an Evergreen 3045 filter column set on a glass centrifuge tube, and was centrifuged for 5 min. at 4000 rpm at 0°C. The extract was injected into the HPLC after filtration through a Millipore Column guard FH filter unit (0.5 μm pore size).

2.3 Liquid chromatography

The DMF extract (100 μl) was injected, without concentration, into a Shimadzu LC-4 liquid chromatograph system fitted with a 125 × 4 mm column packed with octadecyl silica of 5 μ in particle size (Merk LiChrospher 100 RP-18e). The column was protected by a 4 × 4 mm guard column (Merk LiChrospher 100 RP-18e). The pigments were eluted successively with methanol-water (75:25 v/v) for 4 min., methanol for 1 min., methanol-acetone (80:20 v/v) for 6 min. and methanol-acetone (65:35 v/v) for 9 min. at a flow rate of 1 ml min. $^{-1}$, not using linear gradient systems.

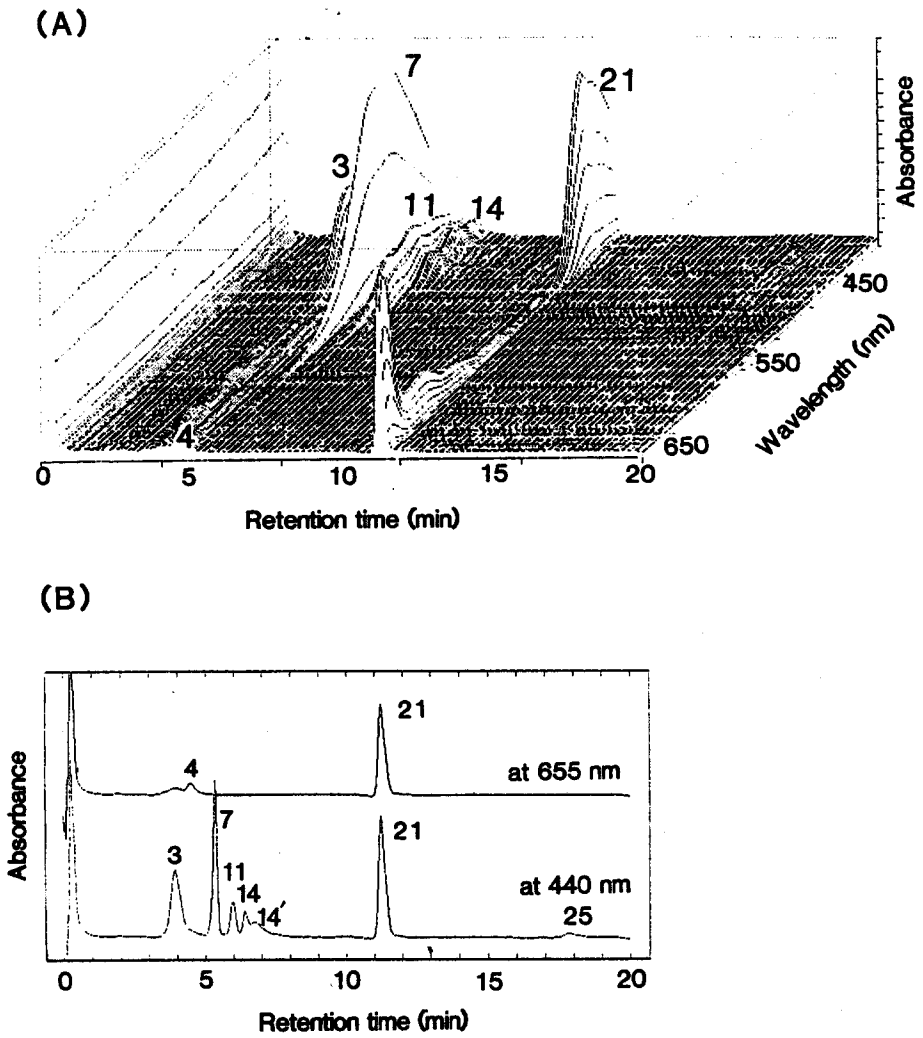

Fig. 1. Chromatograms of chlorophyll and carotenoid pigments extracted with N,N-dimethylformamide from *Skeletonema costatum* strain 1. (A) 3D plot of absorption spectra (wavelength; 380–670 nm) as a function of retention time, and (B) chromatograms detected at 655 nm (upper) and at 440 nm (lower) were obtained simultaneously. For peak numbers, refer to Fig. 2.

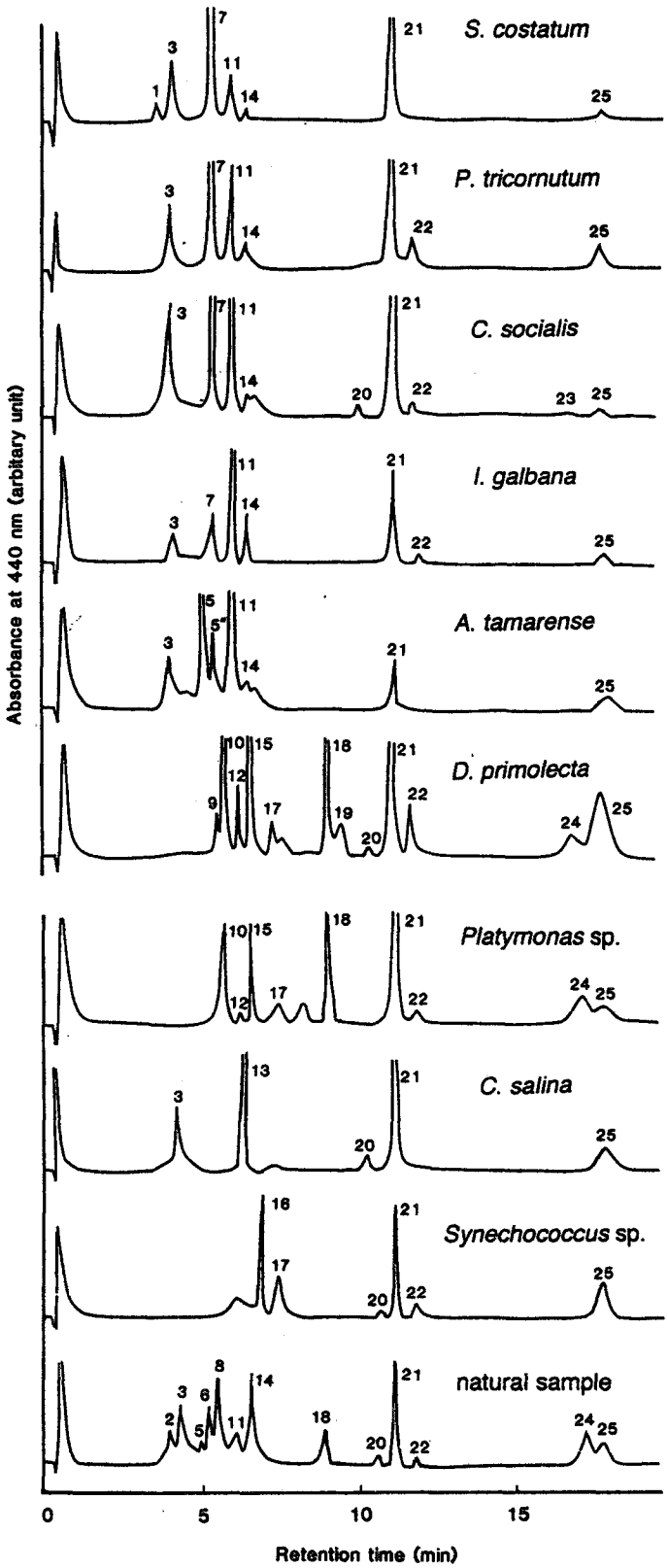
S. costatum
P. tricornutum
C. socialis
I. galbana
A. tamarense
D. primolecta
Platymonas sp.
C. salina
Synechococcus sp.
natural sample
Absorbance at 440 nm (arbitary unit)
Retention time (min)
0
5
10
15

Fig. 2.

2.4 Detection and quantification of pigments

The elution pattern was spectroscopically monitored using a Shimadzu SPD-M6A photodiode array detector over a wavelength from 380–670 nm (Fig. 1). The eluting peaks were also monitored simultaneously by absorbance at 440 nm and 655 nm. During elution, each peak was sampled when needed to identify the pigment, then transferred to standard solvents and its visible absorption spectrum was recorded (Shimadzu MPS 2000). In an ordinary run, peak identification was based on retention time and absorption spectrum obtained by the diode array.

Amounts of pigments were estimated from the areas under the peak of the individual components. Calibrations were made by using the standard samples. Chlorophyll *a*, chlorophyll *b* and β-carotene were purchased from Sigma Chemicals. Purity of these pigments was examined by HPLC. Chlorophyll *c*, fucoxanthin and diadinoxanthin were extracted from *P. tricornutum* and *S. costatum* and purified by HPLC in laboratory. Chlorophyll *c*3, 19′-butanoyloxyfucoxanthin and 19′-hexanoyloxfucoxanthin were referred to their absorption spectra obtained from *Gymnodinium mikimotoi* (Suzuki and Ishimaru, 1992). Quantity of pigments in the standard samples were determined using the extinction coefficients reported in the literature (Mackinney, 1941 for chlorophylls *a* and *b*; Jeffrey and Hamphrey, 1975 for chlorophyll *c*; Goodwin, 1955 for β-carotene and diadinoxanthin; Jensen, 1966 for fucoxanthin).

3. Results and Discussion

3.1 Extraction of photosynthetic pigments

We used DMF as an extracting solvent, because it gives rise to a higher extraction efficiency from phytoplankton, either cultured or in nature, compared with 90% acetone (Suzuki and Ishimaru, 1990).

Methanol is known to be an extracting solvent, but can cause the formation of artifacts (e.g. esterification, epimerization and allomerization of chlorophylls, Mantoura and Llewellyn, 1983). It can also cause artifactual distortion of chromatographic peaks (Zapata and Garrido, 1991), and the production of carotenoid derivatives (Khachik *et al.*, 1988).

3.2 Chromatography

In choice of mobile phases, a number of solvent systems were compared on the basis of overall resolution and peak shapes; a special attention was paid to simplicity and rapidity. We found the best program for mobile phase described in the above section. Figure 1 shows a separation pattern of pigments isolated from *S. costatum* by our protocol. In order to demonstrate a clear separation of a phaeophorbide *a*-like component, the pigments were extracted from the strain 1 of *S. costatum* which was known to have a high activity of chlorophyllase and produce phaeophorbide *a*-like derivative during filtration processes (Suzuki and Fujita, 1986). Cells were

Fig. 2. Chromatograms of chlorophyll and carotenoid pigments extracted with N,N-dimethylformamide immediately after filtration. Peak identities: 1) chlorophyllide *a*; 2) chlorophyll *c*3; 3) chlorophyll *c*1 + *c*2; 4) phaeophorbide *a*; 5) peridinin; 5′) peridinin derivative; 6) 19′-butanoyloxyfucoxanthin; 7) fucoxanthin; 8) 19′-hexanoyloxyfucoxanthin; 9) neoxanthin; 10) violaxanthin; 11) diadinoxanthin; 12) antheraxanthin; 13) alloxanthin; 14) diatoxanthin; 14′) diatoxanthin derivative; 15) lutein; 16) zeaxanthin; 17) unidentified carotenoid; 18) chlorophyll *b*; 19) chlorophyll *b* epimer; 20) chlorophyll *a* allomer; 21) chlorophyll *a*; 22) chlorophyll *a* epimer; 23) phaeophytin *a*; 24) carotene (cf. α-carotene); 25) β-carotene.

collected on a GF/C filter and allowed to stand for 20 min. at room temperature to promote decomposition of chlorophyll *a* to the phaeophorbide *a*-like derivative. The phaeophorbide *a*-like compound (4 in Fig. 1) was separated without using any ion pair reagent. A peak (14′) which was eluted just after diadinoxanthin (14 in Fig. 1) was a carotenoid degradation product formed by the same treatment. Identification of pigments was aided by the comparison of the two chromatograms (440 nm and 655 nm) and the spectra obtained from the diode array detector. We identified 10 chlorophylls and their derivatives, and 13 carotenoids from the various classes of algae. Chlorophyllide *a* (1 in Fig. 2) was distinguished from chlorophyll *c*3 (2) and *c*1 + *c*2 (3) based on difference in retention time. Since chlorophyll *c*1 and *c*2 were not resolved from each other in the present study, both pigments were expressed as the sum of chlorophyll *c*1 +*c*2. Other chlorophyll derivatives (chlorophyll *a* allomer (20) and chlorophyll *a* epimer (22) or chlorophyll *b* epimer (19)) were separated from their parent chlorophylls (chlorophyll *a* (21) and chlorophyll *b* (18)). Good resolution was also attained regarding carotenoids of major significance in ecological studies, such as peridinin (5), 19′-butanoyloxyfucoxanthin (6), fucoxanthin (7), 19′-hexanoyloxyfucoxanthin (8), diadinoxanthin (11), diatoxanthin (14), lutein (15), and zeaxanthin (16). The pigments of natural samples were identified based on absorption spectra of each peak (Fig. 3). The pigments shown above were well resolved in natural samples.

The limits of detection for chlorophylls and carotenoids were found to be approximately 5 ng and 1 ng respectively. Filtration volumes needed for natural water samples depended on pigments concerned. Generally, in the open ocean 15–20 liter is sufficient for the pigments mentioned above to obtain absorption spectra.

3.3 Quantification

The amounts of pigments injected to HPLC were linearly correlated with the areas under individual peaks in absorbance unit in the chromatogram (Fig. 4). Standard errors for estimation of individual pigments were less than 5% for chlorophyll pigments and carotenoid, as shown in Table 1.

3.4 Stability of pigments during storage in DMF extracts

In the last part, we checked the stability of extracted pigments in the DMF solution. In 1986, we reported that chlorophylls isolated one strain of S. costarum was degraded after a short period of extraction (Fig. 1), thus, a selection of extracting solvent and the stability of pigments became

Table 1. Relative standard errors of determination of chlorophyllous and carotenoid pigments. Pigments were extracted from cultured *Skeletonema costatum* cells with N,N-dimethylformamide, separated by HPLC, and peak areas in chromatograms were measured. Five determinations were made.

Pigments	Relative standard error (%)
chlorophyll *a*	5.3
chlorophyll *a* allomer	3.1
chlorophyll *a* epimer	2.7
chlorophyll *c*	2.9
fucoxanthin	2.7
diadinoxanthin	3.2
diatoxanthin	5.5

critical. In our previous paper (Suzuki and Ishimaru, 1990), we reported that chlorophyll *a* was stable more than 1 month in DMF. Since it was determined by fluorometrically, information was not available concerning how fast chlorophyll *a* is converted into chlorophyllide *a* and their epimeric and allomeric forms. Thus, we checked it by using our new method.

We adopted two different storage conditions and compared the degradation patterns; the extracted pigments were stored in darkness either at 5°C or –25°C and the variations of amount

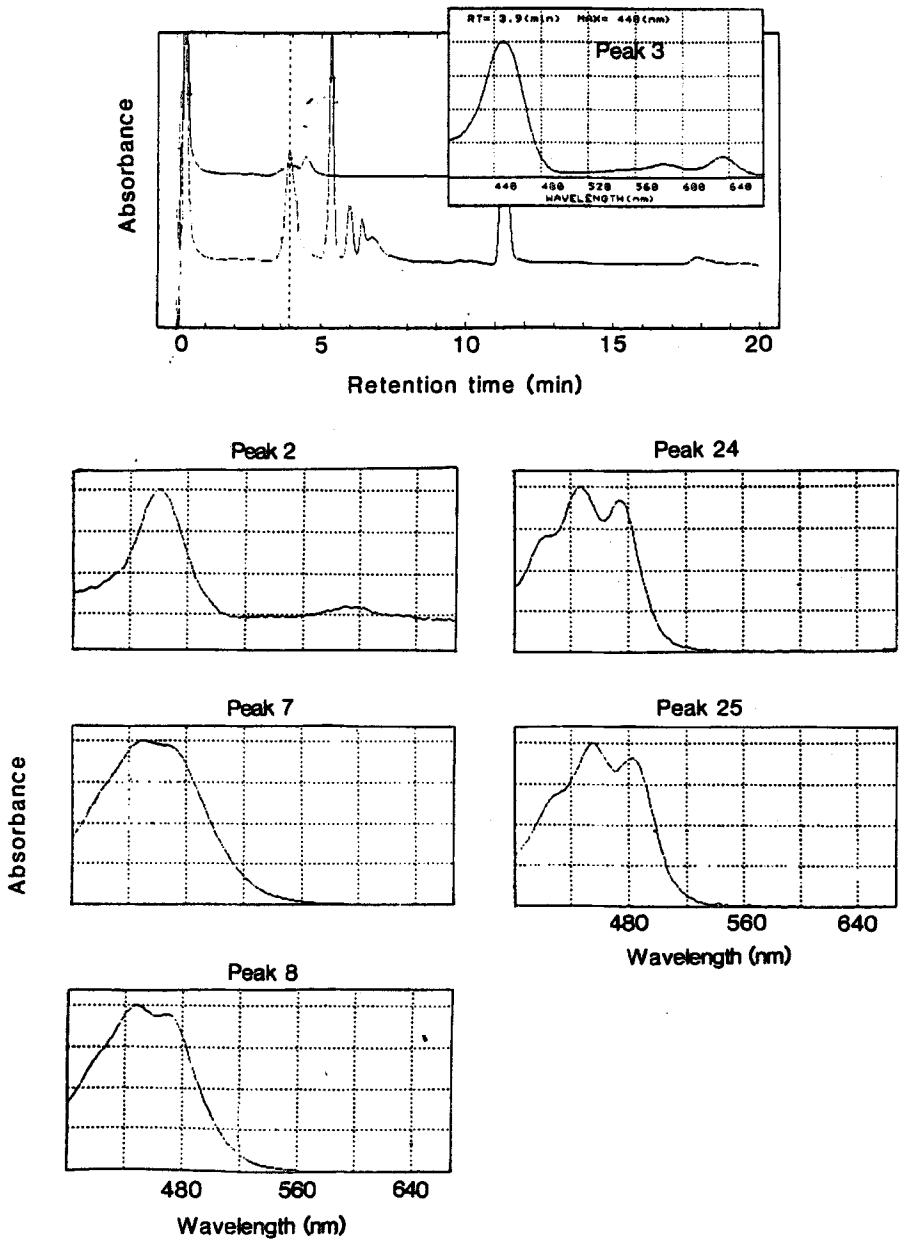

Fig. 3. Absorption spectra of some pigments of *S. costatuma* and natural sample. The spectra were obtained by a photodiode-array detector. For peak number, refer to Fig. 2.

Table 2. Temporal variations of chlorophyllous and carotenoid pigments during storage in N,N-dimethylformamide. Pigments were extracted with DMF from cultured phytoplankton cells, and determined by HPLC after indicated times of storage in the dark at 5°C or −25°C. Pigments concentrations relative to the initial concentrations of respective pigments (%) are given.

Pigments	Storage time (days)								
	at 5°C					at −25°C			
	7	15	21	30	60	30	60	90	180
	Phaeodactylum tricornutum								
Chlorophyll *a*	92	94	81	80	—	103	—	—	83
Chl. *a* + Chl. *a* epimer	99	101	100	88	—	105	—	—	98
Chlorophyll *c*	—	98	103	70	—	98	—	—	104
Fucoxanthin	98	103	101	100	—	101	—	—	96
Diadinoxanthin	94	82	83	81	—	80	—	—	86
β-carotene	99	101	100	104	—	101	—	—	97
	Dunaliella primolecta								
Chlorophyll *a*	99	84	82	80	—	93	—	—	81
Chl. *a* + Chl. *a* epimer	104	94	94	92	—	88	—	—	83
Chlorophyll *b*	90	79	80	83	—	98	—	—	75
Chl. *b* + Chl. *b* epimer	103	95	93	89	—	90	—	—	85
Violaxanthin	102	91	91	89	—	79	—	—	75
Lutein	102	98	97	96	—	101	—	—	87
	Synechococcus sp.								
Chlorophyll *a*	82	82	80	80	—	95	—	—	81
Chl. *a* + Chl. *a* epimer	92	96	92	89	—	93	—	—	85
Zeaxanthin	97	102	101	95	—	101	—	—	93
	Skeletonema costatum strain 1								
Chlorophyll *a*	94	87	83	79	67	103	98	94	—
Chl. *a* + Chl. *a* epimer	102	100	98	94	68	105	101	100	—
Chlorophyll *c*	97	98	96	100	81	104	97	104	—
Fucoxanthin	101	95	98	93	79	105	101	98	—
Diadinoxanthin	104	102	88	91	83	92	89	90	—
	Skeletonema costatum strain 2								
Chlorophyll *a*	94	83	82	76	67	99	96	92	—
Chl. *a* + Chl. *a* epimer	95	86	88	90	82	101	100	97	—
Chlorophyll *c*	103	—	98	97	97	100	105	104	—
Fucoxanthin	97	89	97	91	81	105	105	100	—
Diadinoxanthin	101	97	94	98	99	91	92	96	—

—: Not determined.

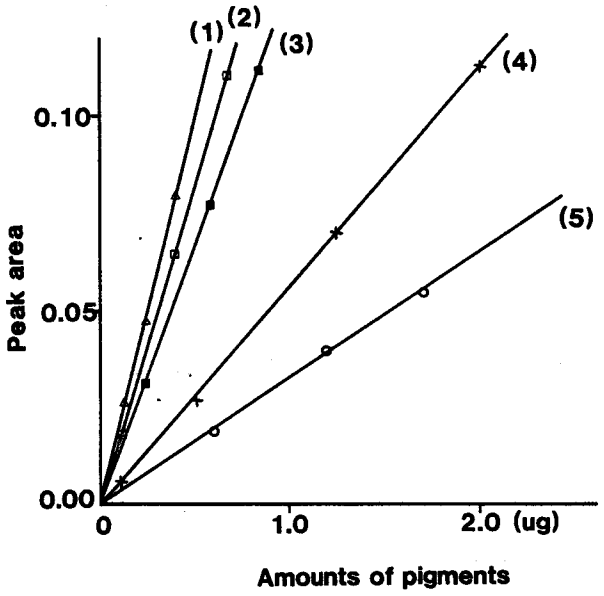

Fig. 4. Relationship between injected amounts of pigments and peak areas in chromatograms. (1) diadinoxanthin, (2) chlorophyll *c*1 + *c*2, (3) fucoxanthin, (4) chlorophyll *a*, (5) chlorophyll *b*.

of individual pigments were successively determined by HPLC (Table 2). The test was not made for pigments with low abundance because of possible errors in estimation. Both chlorophylls and carotenoids were stable except for diadinoxanthin; their concentration remained practically unchanged during one month storage at –25°C. Although stability of chlorophyll *a* was lower at 5°C, sum of chlorophyll *a* and its epimer was rather stable for 2 or 3 weeks. While the storage of pigment samples at 5°C seems to be unrealistic, our examination suggests a possible alternative of the storage for a short period of days without a freezer.

Chlorophyll *a* epimer increased with time, and then decreased in the later period of the storage. This suggests formation of epimeric products and the progress of their further degradation. Although the examination was limited to the selected pigments, our results indicate that, although the analysis of the photosynthetic pigments is better completed as soon possible after harvest, the extracts can be kept at low temperature (e.g. –25°C) when their storage is needed.

4. Conclusion

The method described here is simple, rapid and reproducible. Since the pigments extracts are directly injected into the HPLC column, the possibility of the breakdown of the pigments and of the formation of artifacts during the analysis is considered to be minimal. The short elution times of 20 min. facilitates the handling of many samples. DMF has the advantages of rapid extraction and stability of pigments; Pigments are well extracted by soaking in 1 hr., and can be stored at –25°C in the dark before analysis. The method is practical and suitable for routine use on board ship.

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