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Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton

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ABSTRACT: Using a ternary gradient system, over 50 carotenoids, chlorophylls and their derivatives were separated from marine phytoplankton. Only 2 pairs of carotenoid pigments (19'-butanoyloxyfucoxanthin and siphonaxanthin, and 19'-hexanoyloxyfucoxanthin and 9'-*cis*-neoxanthin) and 3 chlorophylls (chlorophylls c_1, c_2 and Mg 2,4 divinyl pheoporphyrin a_5 monomethyl ester [Mg2,4D]) were not resolved. Pigment chromatograms are presented for 12 unialgal cultures from 10 algal classes important in the marine environment: *Amphidinium carterae* Hulbert (Dinophyceae); *Chroomonas salina* (Wislouch) Butcher (Cryptophyceae); *Dunaliella tertiolecta* Butcher (Chlorophyceae); *Emiliania huxleyi* (Lohmann) Hay et Mohler and *Pavlova lutheri* (Droop) Green (Prymnesiophyceae); *Euglena gracilis* Klebs (Euglenophyceae); *Micromonas pusilla* (Butcher) Manton et Parke and *Pycnococcus provasolii* Guillard (Prasinophyceae); *Pelagococcus subviridis* Norris (Chrysophyceae); *Phaeodactylum tricornutum* Bohlin (Bacillariophyceae); *Porphyridium cruentum* (Bory) Drew et Ross (Rhodophyceae), and *Synechococcus* sp. (Cyanophyceae). A chromatogram is also given of a complex mixture of over 50 algal pigments such as might be found in a phytoplankton field sample. This method is useful for analysis of phytoplankton pigments in seawater samples and other instances where separations of complex pigment mixtures are required.

INTRODUCTION

A number of methods for the determination of phytoplankton pigments are available which have various levels of sophistication and accuracy. These range from widely used simple spectrophotometric and fluorometric methods for chlorophylls *a*, *b* and *c* and their Mg-free derivatives (Holm-Hansen et al. 1965, Lorenzen 1967, Strickland & Parsons 1972, Jeffrey & Humphrey 1975, Lorenzen & Jeffrey 1980) to chromatographic methods which include thin-layer chromatography (Jeffrey 1981), high performance thin-layer chromatography (HPTLC; Wright & Jeffrey 1987) and high performance liquid chromatography (HPLC; reviewed by Roy 1987). The chromatographic methods separate an array of chlorophylls and carotenoids, which act as indicators of microscopic plant biomass, and biological markers for algal types, recycling processes and productivity measurements (Jeffrey 1974, Hooks et al. 1988, Bjørnland & Liaaen-Jensen 1989, Strom & Welschmeyer 1991).

Although a number of HPLC methods for phytoplankton pigments have been published over the past few years, no one method is ideal for all pigments. Techniques for the separation of chlorophylls and their derivatives include those of Mantoura & Llewellyn (1983), Bidigare et al. (1985), Gieskes & Kraay (1986a), and Zapata et al. (1987), while the best methods for carotenoid resolution are those of Wright & Shearer (1984) and Repeta & Gagosian (1982). The last method has the disadvantages of using a silica column which causes degradation of chlorophylls, while the Wright & Shearer method does not resolve the polar chlorophylls c_1, c_2, c_3 and Mg-2,4 divinyl phaeoporphyrin a_5 mono-

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methyl ester (Mg2,4D) which coelute as a single peak, or adequately resolve the chlorophyllides *a* and *b*. A single method, suitable for the full range of important chlorophylls, chlorophyll derivatives and taxonomically significant carotenoids found in phytoplankton was urgently needed, particularly for international oceanographic programs currently underway for climate change studies (e.g. the Joint Global Ocean Flux Study).

Aware of the need to evaluate and compare pigment methods, the Scientific Committee for Oceanic Research (SCOR) founded 'Working Group 78: Measurement of Photosynthetic Pigments in Seawater', whose objectives included: (1) comparing spectrophotometric, fluorometric and HPLC methods for chlorophyll *a* determination; (2) recommending a simple isocratic HPLC shipboard technique for chlorophylls and derivatives; (3) evaluating gradient HPLC techniques for detailed separations of chlorophylls and carotenoids.

For the third objective, the Working Group members who attended the SCOR Chlorophyll Workshop at the CSIRO Marine Laboratories, November, 1988 used the method of Wright & Shearer (1984) for carotenoids and non-polar chlorophylls, and added to it an initial solvent system used by Welschmeyer & Hoepffner (in press) for separating polar chlorophylls. An improved method resulted which separates more than 50 chlorophylls, carotenoids, their derivatives and isomers from marine phytoplankton in one ternary gradient system. This paper gives details of the HPLC method and examples of chromatograms from reference algae. Quantitative analyses form part of the SCOR Workshop Report, and are not duplicated here. While several carotenoids and 3 chlorophyll *c* pigments (Jeffrey 1989) are not resolved, this new method provides oceanographers with a significantly improved system for the analysis of phytoplankton pigments in the sea.

MATERIALS AND METHODS

Algal cultures. Because of the instability and commercial non-availability of pure algal pigment standards, the SCOR Working Group 78 recommended that the source of such standards should be reference algal cultures whose pigments had been fully characterised. A small number of species were selected (Table 1) which together contained most significant pigments known to be found in marine phytoplankton (Table 2). Additional cultures to those recommended by SCOR were included for the present work to ensure that a full range of algal classes was represented.

The cultures were maintained in the CSIRO Algal Culture Collection (Jeffrey 1980). Table 1 lists clonal designation, CSIRO Culture Code number, culture medium, temperature and light intensities used for growth as well as references to the chemical identification of pigments. All cultures were grown in stationary 125 ml Erlenmeyer flasks containing 75 ml culture medium. Illumination was provided by banks of Philips 'daylight' fluorescent tubes, beneath glass shelves supporting the culture flasks. Light irradiances (12 : 12 h light : dark cycles) were measured in the culture medium with a Biospherical Optics light meter. Culture stocks were transferred every 7 to 10 d.

Culture harvest. Cells were harvested before the end of log phase and 10 ml culture aliquots were taken for pigment analysis. These were filtered through 25 mm Whatman GF/F filters under low vacuum (e.g. $\leq$ 100 mm Hg) and rinsed with filtered medium or seawater. The filter was extracted immediately, or was folded, placed in a labelled cryotube and immediately frozen in liquid nitrogen. Several replicates of each culture were made. The time taken from filtration to liquid nitrogen was not more than 30 s, and for the experiments described in this paper, storage in liquid nitrogen was not more than 1 to 10 d.

Field samples. The sample illustrated was collected from 90 m depth at 51°54′ S, 73°14′ E in the Southern Ocean in the vicinity of Heard Island on 9 June 1990. Seawater (1 to 5 l) was filtered through a 25 mm diameter Whatman GF/F filter. This was folded, blotted dry and stored in a cryotube under liquid nitrogen and returned to the laboratory for HPLC. The particular sample illustrated was stored for 3 mo before analysis. No discernible changes occur during this time (SCOR Working Group 78, unpubl.).

Pigment extraction: cultures. The SCOR Working Group 78 report will separately consider in detail the optimum methods for extraction of pigments from cultures and field samples. The frozen filter containing the algal sample was cut into small slices (several mm $\times$ 1 cm) and was ground for 30 s in a motorized grinder with a teflon pestle in 2.7 ml 100% acetone. Water (0.3 ml) was then added to make up to 90% acetone, and the sample reground. Methanol could have been used as extraction solvent, and often is for field samples (see below). The results of comprehensive tests of solvents and extraction protocols will be described in the SCOR report. The filter plus acetone extract was transferred to a plastic centrifuge tube cut down to 5 ml, which had been 'needle-pierced' at the bottom (Wright & Shearer 1984) and covered with a 5 mm circle of GF/F filter. The cut-down centrifuge tube containing the extract was then placed in the top of a 10 ml conical glass centrifuge tube, and both tubes centrifuged in tandem for 3 min at 700 $\times$ *g*. The clear green-yellow extract was collected in the lower glass centrifuge tube, while the filter debris remained in the upper tube. The

Table 1. Microalgal species used for reference pigments and culture conditions used for their growth

Species	Class	Clonal designation and origin	CSIRO culture code	Culture medium[a]	Growth temperature (°C)	Light intensity ($\mu E\ m^{-2}\ s^{-1}$)	Reference to chemical analysis of pigments[b]
Amphidinium carterae[c]	Dinophyceae	AMPH1, Plymouth 450	CS-212	G	20	80	Johansen et al. (1974)
Chroomonas salina[c]	Cryptophyceae	CCMP 56/00/00	CS-174	fE	20	80	Pennington et al. (1985)
Dunaliella tertiolecta[c]	Chlorophyceae	CCMP	CS-175	f_2	20	80	Jeffrey (1968)[e]
Emiliania huxleyi[c]	Prymnesiophyceae	BT6	CS-57	f_2	20	80	Arpin et al. (1976)
Euglena gracilis	Euglenophyceae	Univ. NSW, strain Z	CS-66	MBL/NB_2	20	80	Bjørnland et al. (1989)
Micromonas pusilla	Prasinophyceae	UTEX LB991	CS-86	G	20	80	Ricketts (1966a, b, 1970)[e]
Pavlova lutheri	Prymnesiophyceae	CCMP	CS-182	f_2	20	80	Berger et al. (1977)
Pelagococcus subviridis[c]	Chrysophyceae	East Aust. Current	CS-99	f_2	20	80	Bjørnland et al. (1989)
Phaeodactylum tricornutum[c]	Bacillariophyceae	Plymouth (1052/1)	CS-29	f_2	20	80	Pennington et al. (1988)
Porphyridium cruentum	Rhodophyceae	Halifax, Canada	CS-25	f_2	20	80	Stransky & Hager (1970)[e]
Pycnococcus provasolii[c, d]	Prasinophyceae	CCMP, omega 48-23AX	CS-185	f_2	20	80	Foss et al. (1984)
Synechococcus sp.	Cyanophyceae	DC2	CS-197	f_2	17.5	20	Guillard et al. (1985)

[a] Media code: f = Guillard & Ryther (1962); f_2 = ½ dilution of f medium with seawater; MBL/NB_2 = Guillard's MBL in Nichols (1973); fE = f + EDTA (Jeffrey 1980); G = Loeblich & Smith (1968)

[b] Analysis in the cited reference may have been done on a different clone or strain to that used in the present work

[c] Reference algae recommended by SCOR Working Group 78

[d] Previously known as Prasinophyte omega 48-23AX (Guillard et al. 1991)

[e] Reinvestigation by modern methods required

Table 2. Major pigments in reference cultures

Peak no.	Pigment	*Amphidinium carterae*	*Chroomonas salina*	*Dunaliella tertiolecta*	*Emiliania huxleyi*	*Euglena gracilis*	*Micromonas pusilla*	*Pavlova lutheri*	*Pelagococcus subviridis*	*Phaeodactylum tricornutum*	*Porphyridium cruentum*	*Pycnococcus provasolii*	*Synechococcus* sp.
2	Carotenoid P 468[a]	+											
4	Carotenoid P 457[a]	+											
5	Chlorophyll c_3[b]				+				+				
6a	Chlorophyll c_2	+	+		+				+				
6a	Chlorophyll c_1 + c_2							+		+			
6c	Mg 2,4D[c]						+					+	
7	Peridinin	+											
9	19′-butanoyloxyfucoxanthin								+				
10	Fucoxanthin				+[d]			+	+	+			
14	9′-*cis*-neoxanthin			+		+	+					+	
15	19′-hexanoyloxyfucoxanthin				+								
18	Unknown siphonaxanthin-like						+						
19	Prasinoxanthin											+	
21	Violaxanthin			+			+					+	
23	Dinoxanthin	+											
26	Diadinoxanthin	+			+	+		+	+	+			
29	Antheraxanthin			+			+						
30	Alloxanthin		+										
31	Monadoxanthin		+										
32	Diatoxanthin	+			+			+	+	+			
33	Lutein			+								+	
34	Zeaxanthin			+			+				+	+	+
37	Chlorophyll *b*			+		+	+					+	
39	Crocoxanthin		+										
41	Chlorophyll *a*	+	+	+	+	+	+	+	+	+	+	+	+
49	β, Ψ-carotene (= γ)			+								+	
50	ε,ε-carotene								+				
51	β,ε-carotene (= α)		+	+							+		
52	β,β-carotene (= β)	+	+	+	+	+	+	+	+	+		+	+

[a] Following the nomenclature of Johansen et al. (1974)
[b] Chemical confirmation of structure by Fookes & Jeffrey (1989)
[c] Chemical confirmation of structure by Fookes & Jeffrey (unpubl.)
[d] Fucoxanthin is a minor pigment in *Emiliania*

homogenizer and pestle were rinsed with 1 ml 90 % acetone, which was added to the filter debris in the upper tube, and recentrifuged. The clear combined extracts from the lower tube were then transferred to a 5 ml volumetric flask and made up exactly to 5 ml with 90 % acetone to ensure volumetric accuracy. After filtering through a Millex-SR 0.5 µm filter (Millipore), an aliquot of the extract (about 25 µl) was injected immediately into the liquid chromatograph for pigment analysis.

Pigment extraction: field samples. The procedure for field samples was slightly different from that of the cultured samples because of the need to keep extraction volumes to a minimum and hence achieve a higher final concentration of pigments. Filters were cut into small pieces (several mm × 1 cm) and sonicated for 30 s in the cut down centrifuge tubes using 1.5 ml methanol (or acetone) and a Braun Labsonic 1510 equipped with a 4 mm diameter probe, operated at 50 W. The bottom needle-hole in the centrifuge tube was securely covered with a single layer of Nescofilm or Parafilm to prevent solvent leakage during sonication. During centrifugation (as above), the film burst allowing the extract to collect in the lower tube. The extract was filtered (Millex-SR 0.5 µm) and then, immediately before injection, was diluted with water to 80 % MeOH (or 66 % acetone if acetone was used). 125 µl of the diluted extract was injected. In general, the field samples were much less concentrated than the culture samples, hence a larger injection volume was required. However, if a large aliquot of strong solvent (e.g. methanol or 90 % acetone) was injected, then the pigment bands, particularly the early ones, were too broad and retention times decreased. Dilution of the extract, as mentioned above, increased the affinity of pigments for the column during the loading step, resulting in sharper peaks and allowing greater overall loading than possible with the undiluted extract. It is important that the extract is not diluted until immediately before injection, since highly aqueous extracts are not stable and pigment losses (adsorption and precipitation, particularly the more hydrophobic pigments) occur on standing.

It was suggested by an anonymous reviewer that the addition of 2 % ammonium acetate buffer to the methanol extraction solvent improved the height and sharpness of the chlorophyll *c* peaks. This has been confirmed in our laboratories.

High performance liquid chromatography. Three HPLC instruments were used for comparison of separation efficiencies: (1) a Waters Associates liquid chromatograph comprising M6000A, M45 and M501 pumps, Valco injector, Hewlett-Packard 8450A diode array spectrophotometer, and a Waters 440 absorbance detector, connected via a Waters System Interface Module to a microcomputer running Waters Maxima software; (2) a Varian Model 5000 liquid chromatograph with Varian UV50 variable wavelength detector, Varian Fluorichrome fluorescence detector, and Varian 'Vista' 402 data module; and (3) a Spectraphysics HPLC comprising an SP8800 ternary pump, Spectra-Focus detector and a Gilson 231 autosampler (200 µl loop) with samples refrigerated to −10 °C. Chromatograms from the Varian 5000 were used in Figs. 1 & 2, and from the Spectraphysics instrument in Fig. 3. Reversed phase columns used were Spherisorb ODS2, 25 cm × 4.6 mm ID, 5 µm particle size, packed by Australian Government Analytical Laboratories, Melbourne (90 000 to 100 000 plates m^{-1}). Similar resolution was obtained with commercial columns such as Waters Resolve C_{18} and Activon Ultratechsphere, although some C_{18} columns tested were not as efficient, particularly in the ability to resolve lutein and zeaxanthin.

Pigment detection was at 436 nm (Figs. 1 to 3) for all chlorophylls and carotenoids and 405 nm for phaeophytin *a* and phaeophorbide *a* (not shown). The solvent systems used were as follows:

Solvent A: 80:20 methanol : 0.5 M ammonium acetate (aq.; pH 7.2 v/v)
Solvent B: 90:10 acetonitrile (210 nm UV cut-off grade) : water (v/v)
Solvent C: ethyl acetate (HPLC grade)

Flow rate was 1 ml min^{-1}. The gradient systems used are shown in Table 3. In the analytical system an initial 4 min gradient from 100 % A to 100 % B provided improved separation of polar compounds. The program returned to initial conditions and re-equilibrated for 5 min before the next sample injection. Table 3a shows the gradient used on the Varian system to obtain the results presented in Figs. 1 & 2. Table 3b shows the modified procedure for the Spectraphysics system. In this case, the slope of the gradient for Solvent C was reduced between 2.6 and 13.6 min for the region of the chromatogram between fucoxanthin and chlorophyll *a* (increasing the resolution in the most complex region of the chromatogram) and increased elsewhere to reduce the overall time for analysis. Table 3c shows the shutdown protocol whereby the column is washed in the strongest solvent (ethyl acetate) to remove any retained material before being shutdown overnight or for storage. The start-up protocol is the reverse of this program.

Methanol, acetonitrile and ethyl acetate were HPLC grade reagents from Waters and BDH (Hypersolve), used without further purification other than filtration and degassing by sonication. Water was purified using a Millipore Milli-Q system. Ammonium acetate was A.R. grade.

Table 3. HPLC solvent system programs

Time (min)	Flow rate (ml min^{-1})	% A	% B	% C	Conditions
a. Analytical gradient protocol (Waters and Varian systems)					
0	1.0	100	0	0	Injection
4	1.0	0	100	0	Linear gradient
18	1.0	0	20	80	Linear gradient
21	1.0	0	100	0	Linear gradient
24	1.0	100	0	0	Linear gradient
29	1.0	100	0	0	Equilibration
b. Modified protocol on Spectraphysics systems					
0	1.0	100	0	0	Injection
2	1.0	0	100	0	Linear gradient
2.6	1.0	0	90	10	Linear gradient
13.6	1.0	0	65	35	Linear gradient
20	1.0	0	31	69	Linear gradient
22	1.0	0	100	0	Linear gradient
25	1.0	100	0	0	Linear gradient
30	1.0	100	0	0	Equilibration
c. Shutdown protocol					
0	1.0	100	0	0	Analysis complete
3	1.0	0	100	0	Linear gradient
6	1.0	0	0	100	Linear gradient
16	1.0	0	0	100	Washing
17	0	0	0	100	Shut down; linear gradient

Pigment identification. Pigments were identified by co-chromatography with authentic standards prepared for the SCOR Carotenoid Workshop (for details see SCOR report, in prep.), by diode array spectroscopy during elution, and by transferring HPLC fractions to standard solvents and comparing their visible absorption spectra with reference standards (Foppen 1971, Davies 1976, Wright & Shearer 1984, SCOR Carotenoid Workshop Report in prep.). A Shimadzu Model RPI recording spectrophotometer was used for UV-visible spectroscopy of purified pigments.

Preparation of additional standard pigments. Chlorophyll derivatives and carotenoids not found in the standard algal cultures were prepared separately. Chlorophyllides *a* and *b* were obtained from cultures of *Phaeodactylum tricornutum* (chlorophyllide *a*) and *Dunaliella tertiolecta* (chlorophyllides *a* and *b*), by incubating harvested algae in 50 % acetone in the dark to allow the endogenous chlorophyllase to hydrolyse the parent chlorophylls (Barrett & Jeffrey 1964, 1971, Lorenzen & Jeffrey 1980). Spectroscopically pure pigments were then obtained by HPLC.

Phaeophytins *a* and *b* and phaeophorbides *a* and *b* were prepared by acidification (1 M HCl) of acetone solutions of the corresponding chlorophylls and chlorophyllides respectively, which were then transferred to diethyl ether and washed against several changes of water to remove the acid (Lorenzen & Jeffrey 1980).

19'-Hexanoyloxy- and 19'-butanoyloxyfucoxanthin were obtained from cultures of *Emiliania huxleyi* and *Pelagococcus subviridis* respectively, and purified on Merck HPTLC RP-8 plates (Wright & Jeffrey 1987).

Peridinin was prepared from *Amphidinium carterae* by chromatography of pigment extracts on Merck HPTLC RP-8 plates (1 dimension, solvent system methanol : water = 19 : 1 (v/v)). Diadinochrome was obtained by acid-catalyzed rearrangement of diadinoxanthin ex *Euglena gracilis*. Siphonein and siphonaxanthin were isolated from the siphonous alga *Codium fragile* by HPTLC as described for peridinin above. Echinenone (synthetic) and lycopene (from tomatoes) were gifts from Hoffmann-La Roche, Basel, to Dr S. Liaaen-Jensen, Trondheim, Norway. Canthaxanthin was a synthetic standard provided by Dr D. Repeta. Ethyl 8'-β-apocarotenoate, purchased from Fluka Chemicals, USA, was suggested by Repeta as a possible quantitative internal standard and it was included in these tests to ensure that it did not co-chromatograph with known marker pigments. Another synthetic carotenoid, *trans*-β-apo-8'-carotenal, purchased from Sigma Chemical Co., was included as an internal standard in the field sample. All pigments were stored in the dark at −20 °C or in liquid nitrogen until used. In our experience, if the solvent remains frozen the pigments are stable indefinitely, otherwise pigments degrade over a period of days to weeks.

Pigment recovery. Recovery of pigments was measured by comparing peak areas for peridinin, lutein, chlorophyll *b*, chlorophyll *a*, and β,β-carotene using the gradient and column described above with those obtained using isocratic buffered methanol (first phase of the gradient) and stainless steel tubing instead of the column (5 replicates). Figures were not corrected for differences in extinction coefficients in the different mobile phases.

RESULTS

Chromatograms of 12 unialgal pigment extracts taken from log phase cultures are shown in Fig. 1 Excellent resolution of almost all individual pigments was achieved for each species.

Fig. 2 shows the resolution of a comprehensive pigment mixture prepared by pooling extracts from 7 reference algae supplemented with additional carotenoid and chlorophyll derivatives; giving a suite of over 40 pigments. Thirty-nine pigments were resolved with 37 securely identified. Table 4 lists the pigments separated in increasing elution order, together with a summary of their UV-visible spectral characteristics. Full

visible spectra in appropriate solvents (including HPLC eluant) are available in the SCOR Working Group 78 Carotenoid Report (in prep.).

At the polar end of the chromatogram, the phytol-free chlorophyll derivatives chlorophyllide *b*, chlorophyllide *a* (Fig. 1a) and chlorophyll c_3 (Fig. 1e) were almost completely resolved from chlorophyll c_1 + c_2 + Mg2,4D which coeluted as a single peak (Figs. 1b & 2).

In the complex central region of the chromatogram, most of the known carotenoid bio-markers (peridinin, fucoxanthin, prasinoxanthin, violaxanthin, alloxanthin, lutein, zeaxanthin, canthaxanthin and siphonein) were adequately resolved. Only 2 pairs of carotenoids were not separated: 19'-butanoyloxyfucoxanthin from siphonaxanthin, and 19'-hexanoyloxyfucoxanthin from 9'-*cis*-neoxanthin, although the individual components of each pair could be distinguished spectrally by diode array detection. Many minor carotenoid components were also resolved and identified (Figs. 1 & 2 and Table 4).

At the non-polar end of the chromatogram, the phytolated chlorophylls *a* and *b*, the phaeophytins, and the less-polar carotenoids and carotenes were resolved. In fresh extracts, the native chlorophylls predominated with a very small proportion of chlorophyll *a* allomer, epimer and trace unknown chlorophyll *a* derivatives (less than 1 %). We cannot determine whether such trace pigments are present in situ, or are formed during the short (1 to 10 d) low temperature storage period or the short 'extraction to injection' period. The internal carotenoid standard, ethyl 8'-β-apocarotenoate, separated as a single peak between chlorophyll *b* and chlorophyll *a* (Fig. 2). The other internal standard, *trans*-β-apo-8'-carotenal, co-chromatographed with canthaxanthin, eluting before chlorophyll *b* (Fig. 3). The hydrocarbons, lycopene (ψ,ψ-carotene) and β,ψ-carotene, were completely resolved. β,ε-Carotene eluted as the leading shoulder of the β,β-carotene peak. *Cis*-β,β-carotene eluted as the trailing shoulder of the β,β-carotene peak (see Figs. 1a & 2).

Recovery of pigments from the column was excellent. Recoveries measured were: peridinin, 102 %; lutein, 107 %; chlorophyll *b*, 100 %; chlorophyll *a*, 107 %; and β,β-carotene, 98 %. Recovery figures greater than 100 % may reflect the different extinction coefficients in the mobile phases used.

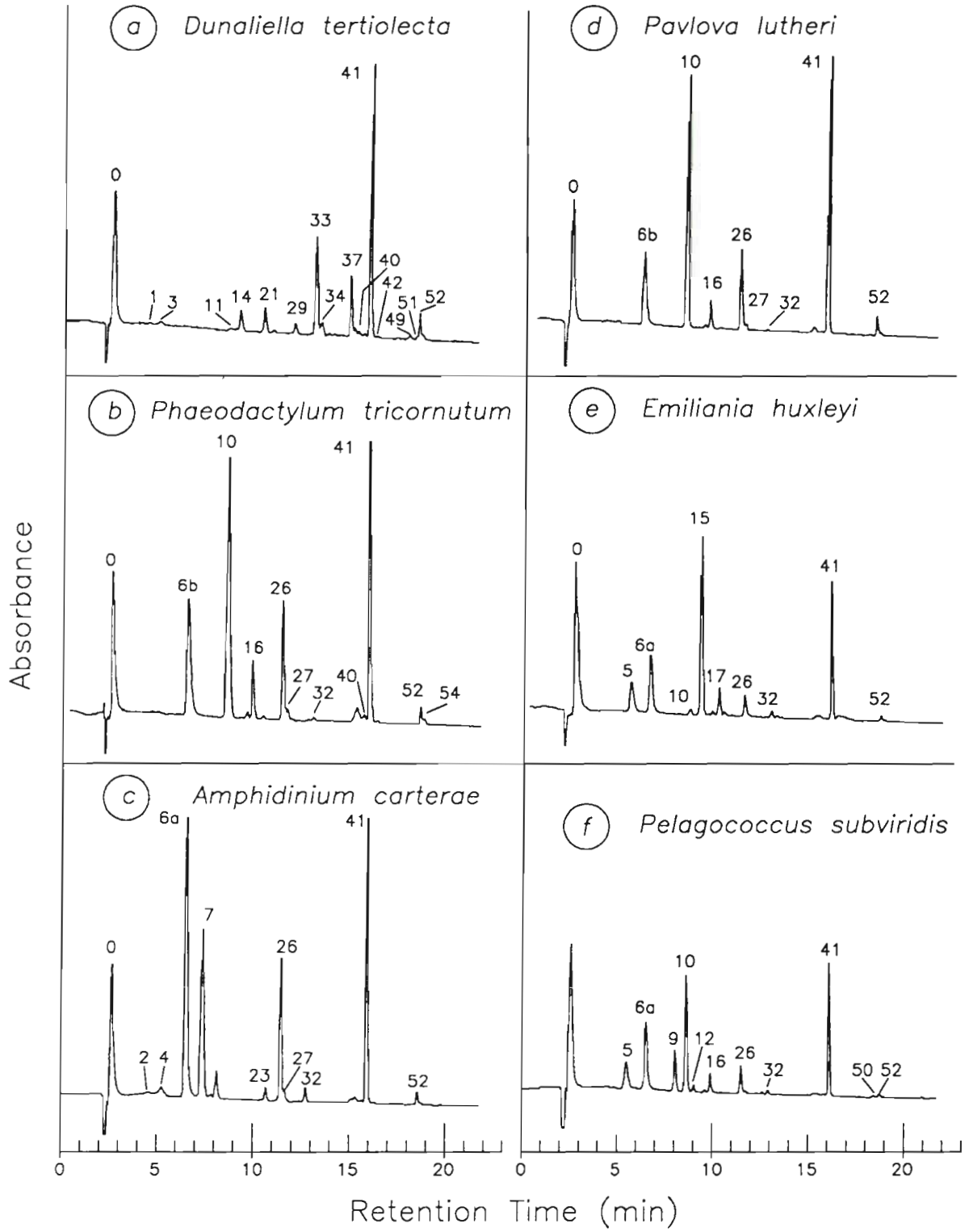
In each of the single algal chromatograms, the native all-*trans* carotenoids predominated, with only small amounts of *cis*-isomers. Note that in *Dunaliella tertiolecta* (Fig. 1a), and all other green algae to our knowledge, neoxanthin is present as the 9'-*cis* isomer in fresh extracts (Bjørnland 1990). The *trans* isomer appears in the extract within hours of preparation.

Most of the pigments of the species examined have been characterised with modern spectrometric methods and require no detailed discussion here (cf. Table 1). Exceptions are those of *Dunaliella tertiolecta* (Fig. 1a), *Porphyridium cruentum* (Fig. 1l) and the widely-distributed prasinophyte *Micromonas pusilla* (Fig. 1h). The last species contains the chlorophyll *c*-like pigment Mg2,4D (Fookes & Jeffrey unpubl.), and a range of carotenoids differing from those of normal chlorophytes (see *Dunaliella tertiolecta*, Fig. 1a), and prasinoxanthin-containing prasinophytes (*Pycnococcus provasolii*, Fig. 1g). Two significant carotenoid components in *M. pusilla* had siphonaxanthin-like spectra; the first one (peak 18) almost co-chromatographed with prasinoxanthin but differed from it spectrally in lacking any trace of a shoulder at about 475 nm. It is possible that minor contaminants may be altering its apparent spectrum. In some cultures, a leading shoulder was observed on peak 18, having maxima at (406), 429 and 455 nm. A similar shoulder was observed on the neoxanthin peak with maxima at 447 and 470 nm. The identity of these pigments is presently being studied by Dr S Liaaen-Jensen and co-workers, Trondheim, Norway.

Two phaeophorbide *a*-like peaks co-eluted with carotenoids but did not interfere with detection of the carotenoids because they did not absorb at 436 nm. The phaeophorbides can be detected without interference from carotenoids using fluorescence (excitation 407 nm, emission 672 nm for phaeophorbide *a*; excitation 432 and emission 659 nm for phaeophorbide *b*); or absorption at 405 or 665 nm.

Fig. 3 shows the resolution of chlorophylls and carotenoids from a field sample from the Southern Ocean in the vicinity of Heard Island. Note that the field sample was analysed with the Spectraphysics HPLC system and the retention times do not exactly match those of the Varian HPLC system, listed in Table 4. The absence of chlorophyll *a* epimer and chlorophyllides indicated that little degradation had occurred. The chromatogram shows a good resolution of the major pigments. Apart from chlorophyll *a*, the chromatogram is dominated by fucoxanthin, its 19'-hexanoyloxy- and 19'-butanoyloxy-derivatives, chlorophylls c_3 and c_1+c_2, and diadinoxanthin. Smaller amounts of peridinin, chlorophyll *b*, prasinoxanthin, and antheraxanthin were present along with traces of alloxanthin and zeaxanthin.

Examination of the samples by light microscopy showed them to be dominated by small diatoms (consistent with the abundance of fucoxanthin), a numerically smaller dinoflagellate population (indicated by peridinin) with many unidentifiable flagellates. The pigment composition suggests that the flagellates comprised mainly prymnesiophytes (chlorophyll c_3, fucoxanthin, 19'-hexanoyloxyfucoxanthin and 19'-butanoyloxyfucoxanthin) together with smaller populations


a Dunaliella tertiolecta
0
1 3
11
14 21
29
33
34
37
40
42
49
51 52
41
b Phaeodactylum tricornutum
0
6b
10
16
26
27 32
40
41
52 54
c Amphidinium carterae
0
2 4
6a
7
23
26
27 32
41
52
d Pavlova lutheri
0
6b
10
16
26
27 32
41
52
e Emiliania huxleyi
0
5
6a
10
15
17 26
32
41
52
f Pelagococcus subviridis
5
6a
9
10
12
16
26
32
41
50 52
Absorbance
0 5 10 15 20
Retention Time (min)

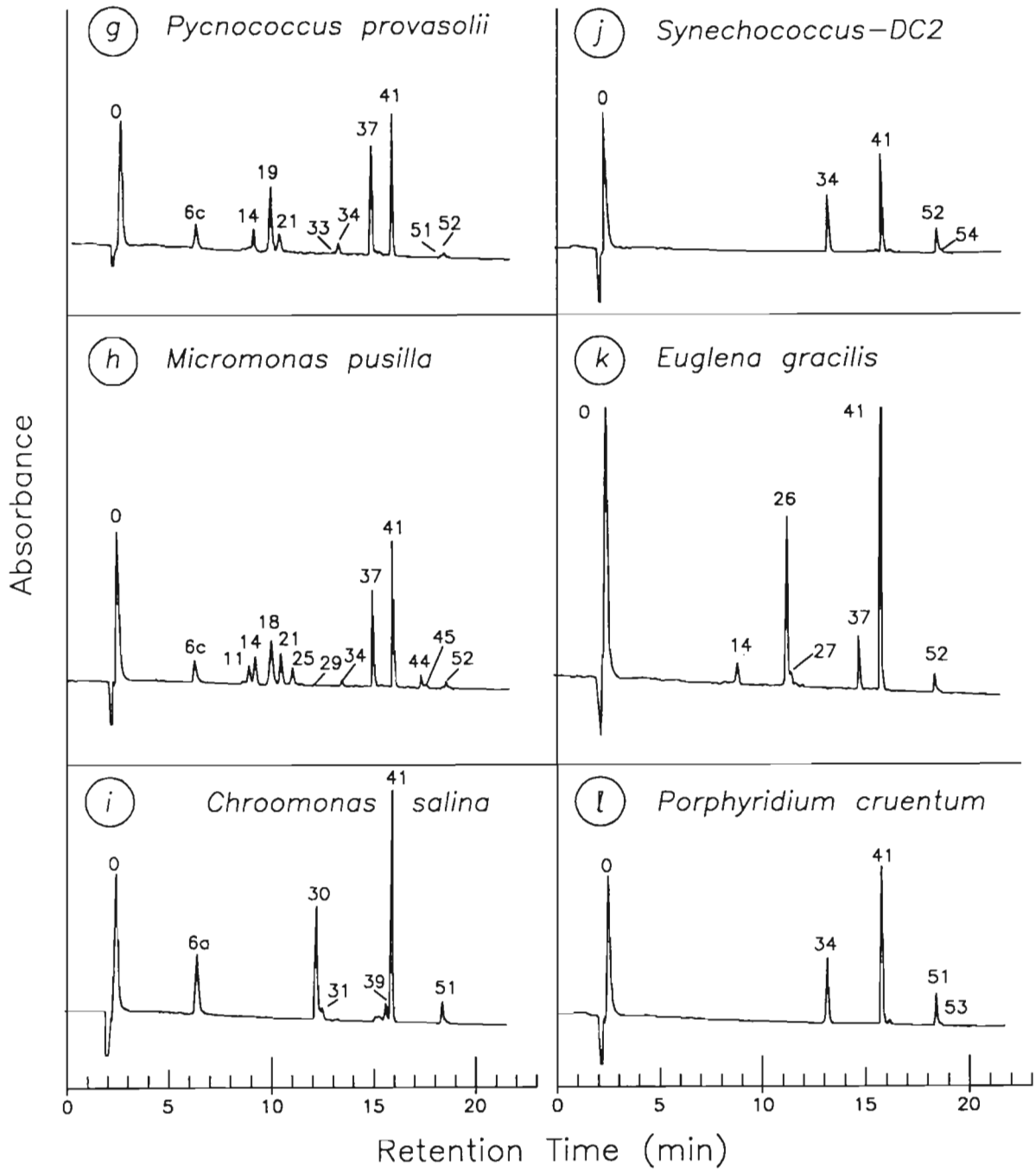


Fig. 1. HPLC traces of pigment separations of unialgal cultures of 12 reference microalgae: (a) *Dunaliella tertiolecta* (green alga); (b) *Phaeodactylum tricornutum* (diatom); (c) *Amphidinium carterae* (dinoflagellate); (d) *Pavlova lutheri* (prymnesiophyte); (e) *Emiliania huxleyi* (prymnesiophyte); (f) *Pelagococcus subviridis* (chrysophyte); (g) *Pycnococcus provasolii* (prasinophyte); (h) *Micromonas pusilla* (prasinophyte); (i) *Chroomonas salina* (cryptomonad); (j) *Synechococcus* sp. (cyanophyte); (k) *Euglena gracilis* (euglenoid); and (l) *Porphyridium cruentum* (red alga). For identity of pigment fractions see Table 4. Chromatograms obtained with the Varian system

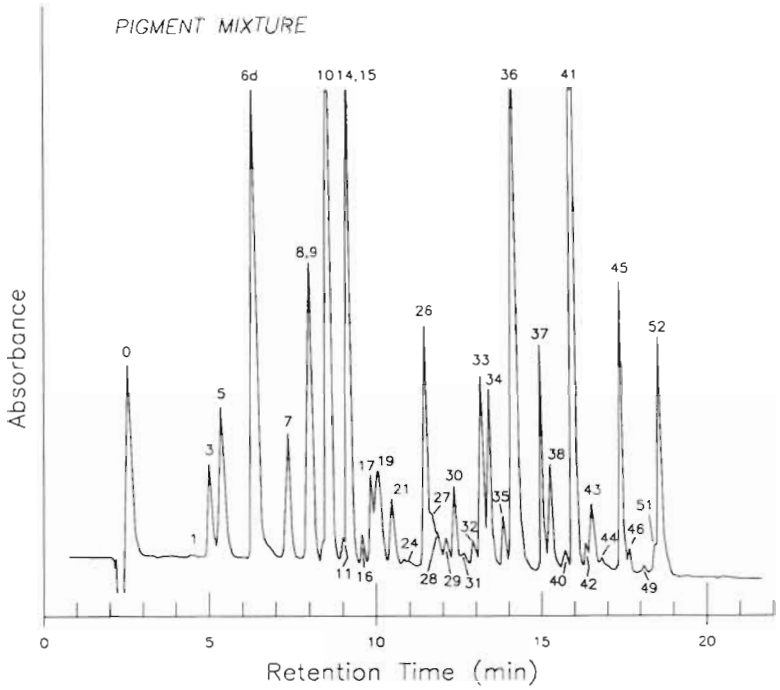


Fig. 2. HPLC traces of a mixed pigment extract from algal cultures of *Dunaliella tertiolecta* (green alga), *Phaeodactylum tricornutum* (diatom), *Synechococcus* sp. (cyanophyte), *Pycnococcus provasolii* (prasinophyte), *Chroomonas salina* (cryptomonad), *Emiliania huxleyi* (prymnesiophyte), *Pelagococcus subviridis* (chrysophyte), and the authentic carotenoids diadinochrome, siphonein, siphonaxanthin, echinenone, cantha-xanthin, lycopene, peridinin and the internal synthetic standard ethyl 8'-β-apocarotenoate. For identity of pigment fractions see Table 4. Chromatogram obtained with the Varian system

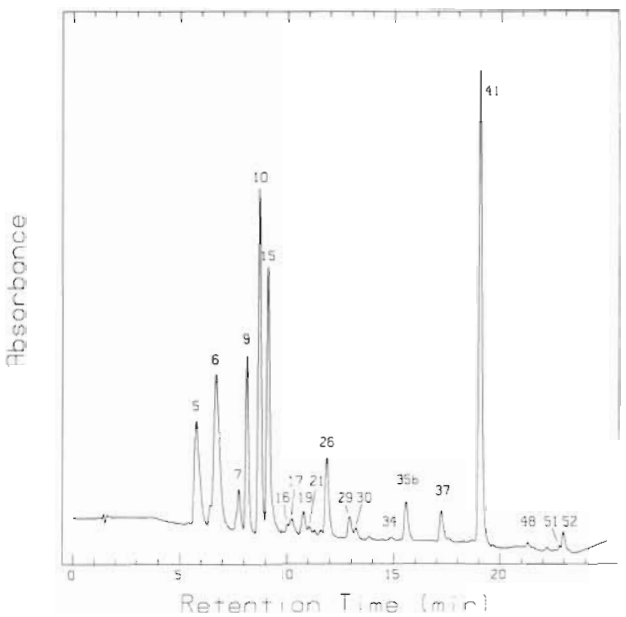


Fig. 3. HPLC trace of phytoplankton sample from the Southern Ocean (51°54' S, 73°14' E, 9 June 1990). For identity of pigment fractions see Table 4. Chromatogram obtained with the Spectraphysics system

of prasinophytes (chlorophyll *b*, prasinoxanthin and antheraxanthin) and cryptomonads (alloxanthin).

DISCUSSION

In the development of this HPLC method, 12 species from 10 algal classes were examined (Table 1). While this range is comprehensive, one algal class with representatives widely distributed in the tropical ocean but not included here is the Prochlorophyceae. No culture of an isolated representative was available at the time of the workshop. Prochlorophytes contain a chlorophyll *a*-like pigment instead of chlorophyll *a* (Chisholm et al. 1988, Veldhuis & Kraay 1990), now identified as 8-desethyl, 8-vinyl chlorophyll *a* (Goericke & Repeta in press). These 2 pigments can be separated in normal phase HPLC systems (Gieskes & Kraay 1983a), but coelute in reverse phase systems unless converted to their respective pheophytins, a technique subject to interference by an unknown carotenoid, according to an anonymous reviewer.

The present method has a number of advantages over other published methods for phytoplankton pigments (Roy 1987), in that separation of over 50 key carotenoids, chlorophylls and chlorophyll derivatives is achieved in one simple ternary gradient system.

In comparison with the technique of Wright & Shearer (1984) the resolution of polar chlorophylls and carotenoids has been enhanced, while retaining the resolution of later-eluting pigments. Of particular importance is the pigment pair lutein and zeaxanthin, not separated by Mantoura & Llewellyn (1983) or Gieskes & Kraay (1986b). The new method retains the almost baseline resolution for this pigment pair of Wright & Shearer (see Fig. 2), similar to that of Ben-Amotz et al. (1982), although it does not match the excellent separation achieved by Zapata et al. (1987). However, Zapata's resolution of other carotenoids is inferior to our improved method, as shown by compar-

Table 4. Elution order of pigments from standard cultures and their visible absorption characteristics

Peak no.	Pigment	Retention time (min)	Acetone Maxima (nm)			Acetone % III : II[a]	Eluant Maxima (nm)			Eluant % III : II[a]
0	(Solvent front)	2.56								
1	Chlorophyllide *b*	4.48	453	598	645					
2	Carotenoid P468[b]	4.50						470		
3	Chlorophyllide *a*	5.01	430	577	617					
			663							
4	Carotenoid P457[b]	5.11						458		
5	Chlorophyll c_3	5.38	452	584	627					
6a	Chlorophyll c_2	6.40	449	581	629		446	580	626	
6b	Chlorophyll $c_1 + c_2$	6.40		- - -						
6c	Mg2,4D	6.40	437	575	625					
6d	Chlorophyll $c_1 + c_2$ + Mg2,4D	6.40		- - -						
7	Peridinin	7.42		474				472		
8	Siphonaxanthin	8.11		445				442	(466)	
9	19'-butanoyloxyfucoxanthin	8.11		445	470	44	(415)	444	469	42
10	Fucoxanthin	8.70		446				446	(466)	
11	*trans*-neoxanthin	9.11					416	441	470	100
12	9'-*cis*-19'-butanoyloxy-fucoxanthin	9.12		440	464	33				
13	Neochrome	9.21					400	422	450	90
14	9'-*cis*-neoxanthin	9.31	415	439	467	65	412	436	466	88
15	19'-hexanoyloxyfucoxanthin	9.31		444	470	48	418	445	469	38
16	*cis*-fucoxanthin	9.68						444	(464)	
17	*cis*-19'-hexanoyloxy-fucoxanthin	9.97								
18	Unknown siphonaxanthin-like	10.12						453		
19	Prasinoxanthin	10.20		451				450	(470)	
20	Phaeophorbide *a*	10.39	410	505	535					
			609	666						
21	Violaxanthin	10.59	419	443	472	96	416	440	470	101
22	Phaeophorbide *a*-like[c]	10.62	410	505	535					
			609	666						
23	Dinoxanthin	10.76	418	442	471	86	415	440	470	87
24	*cis*-prasinoxanthin	11.11								
25	Unknown siphonaxanthin-like	11.15						456		
26	Diadinoxanthin	11.61					422	446	476	64
27	Diadinochrome I	11.79		431	458	63	406	430	458	76
28	Diadinochrome II	11.96					406	428	458	76
29	Antheraxanthin	12.24					(423)	446	476	67
30	Alloxanthin	12.51		454	484	48	(428)	454	484	63
31	Monadoxanthin	12.78					(428)	448	480	40
32	Diatoxanthin	13.08		454	483	42	(424)	452	482	33
33	Lutein	13.36	425	446	476	68	(422)	446	476	64
34	Zeaxanthin	13.59		454	481	32	(428)	454	478	11
35	Canthaxanthin	14.00		472				472		
35b	*trans*-β-apo-8'-carotenal	14.00		456						
36	Siphonein	14.36		452				455		34
37	Chlorophyll *b*	15.15	453	598	645		456	596	646	
38	Ethyl 8'-β-apocarotenoate	15.43		444				446	(467)	–
39	Crocoxanthin	15.87					(427)	449	480	58
40	Chlorophyll *a* allomer	15.87					425	616	664	
41	Chlorophyll *a*	16.15	412	431	581		431	618	665	
				616	663					
42	Chlorophyll *a* epimer	16.53					431	618	666	
43	Echinenone	16.74		461				464		
44	Unknown carotenoid	16.99					420	443	471	59
45	Lycopene	17.59	448	474	505	77	438	472	505	70
46	Phaeophytin *b*	17.68								
47	*cis*-lycopene	17.84					365, 444	470	502	
48	Phaeophytin *a*	18.56	410	505	535		408 504	534 608	666	
			609	666						
49	β, Ψ-carotene	18.26		464	495	54	438	462	496	44
50	ε,ε-carotene	18.40					416	440	471	94
51	β,ε-carotene	18.64		447	475	55	(425)	446	476	60
52	β,β-carotene	18.76		453	480	13	427	462	480	18
53	*cis*-β,ε-carotene	18.83					(419)	442	470	37
54	*cis*-β,β-carotene	18.94					(422)	446	470	

[a] See Ke et al. (1970)
[b] Following the nomenclature of Johansen et al. (1974)
[c] Probably pyrophaeophorbide *a* (unverified)

Table 5. Summary of major signature pigments for algal types and processes in the ocean

Pigment	Algal type or process	Source
Chlorophyll *a*	All photosynthetic microalgae, except prochlorophytes	1
8-desethyl, 8-vinyl chlorophyll *a*	Prochlorophytes	2, 3
Chlorophyll *b*	Chlorophytes, prasinophytes, euglenophytes, prochlorophytes	1, 2
Chlorophyll *c* family	Chromophyte algae	4
c_1	Most diatoms, some prymnesiophytes, some chrysophytes	4, 5, 6
c_2	Most marine chromophyte algae (except some chrysophytes)	4, 5, 6
c_3	Some prymnesiophytes, chrysophytes, diatoms	4, 7, 8
Mg 2,4-divinylpheoporphyrin a_5 monomethyl ester (Mg2,4D)	Some prasinophytes	9, 10
Fucoxanthin	Diatoms, prymnesiophytes, chrysophytes, raphidophytes, a few dinoflagellates	1, 11
19′-hexanoyloxyfucoxanthin 19′-butanoyloxyfucoxanthin	Some chrysophytes, prymnesiophytes, 1 diatom, a few dinoflagellates	8, 11, 12, 13
Peridinin	Most photosynthetic dinoflagellates	14, 15
Zeaxanthin	Cyanobacteria (blue-green algae), prochlorophytes, green-algae	16, 17
Fucoxanthin and violaxanthin	Some chrysophytes	11, 18
Alloxanthin	Cryptomonads	19
Prasinoxanthin	Some prasinophytes	20
Lutein	Green algae (chlorophytes and some prasinophytes)	1
Phaeophytin *a* Phaeophorbide *a*	Grazed phytoplankton; fecal pellets, sediments	1, 21
Chlorophyllide *a*	Extraction artefact, chlorophyllase-containing species	22
Unidentified chl *a* derivatives	Senescent microalgae	23
Pyrophaeophorbide *a* Mesophaeophorbide *a*	Sediments	24

Sources:
1, Jeffrey (1974); 2, Chisholm et al. (1988); 3, Goericke & Repeta (in press); 4, Jeffrey (1989); 5, Stauber & Jeffrey (1988); 6, Andersen & Mulkey (1983); 7, Jeffrey & Wright (1987); 8, Vesk & Jeffrey (1987); 9, Ricketts (1966a); 10, Fookes & Jeffrey (unpubl.); 11, Bjørnland & Liaaen-Jensen (1989); 12, Wright & Jeffrey (1987); 13, Bjørnland et al. (1989); 14, Jeffrey et al. (1975); 15, Johansen et al. (1974); 16, Guillard et al. (1985); 17, Gieskes et al. (1988); 18, Withers et al. (1981); 19, Pennington et al. (1985); 20, Foss et al. (1984); 21, Vernet & Lorenzen (1987); 22, Jeffrey & Hallegraeff (1987); 23, Hallegraeff & Jeffrey (1985); 24, Mantoura (unpubl.)

ing the resolution of *Dunaliella tertiolecta* pigments (their Fig. 4B, and our Fig. 1a).

Further advantages of the method are that no ion pairing reagent is necessary as in the method of Mantoura & Llewellyn (1983) and Bidigare et al. (1985), and no expensive radial compression module is used as in the original method of Wright & Shearer (1984). Further, our early method trials, using 2 different liquid chromatographs (a Waters system and a Varian system), showed only slightly different retention times between the 2 instruments. Some differences in retention times but not retention sequence occurred in the Spectraphysics system (Fig. 3) which had a different dead volume between the pump and column.

Apart from the 39 pigments resolved in Fig. 2, 2 important fucoxanthin derivatives, 19′-hexanoyloxyfucoxanthin and 19′-butanoyloxyfucoxanthin, were not chromatographically separated from 9′-*cis* neoxanthin and siphonaxanthin respectively. Both 19′-acyloxyfucoxanthins are important indicators of particular prymnesiophytes, chrysophytes and dinoflagellates (see Table 5). The use of a diode array detector allows the pigments to be resolved spectroscopically, if not chromatographically, and this would seem an essential adjunct to pigment identification of field samples. To our knowledge, these pigment pairs have not been resolved in other reverse-phase HPLC systems.

While chlorophyll c_3 was clearly resolved from other chlorophyll *c* pigments, the important chlorophylls c_1, c_2 and the chlorophyll *c*-like prasinophyte pigment Mg2,4D could not be resolved from each other, similar to other HPLC systems using octadecylsilica columns (Mantoura & Llewellyn 1983, Bidigare et al. 1985, Zapata et al. 1987, Gieskes & Kraay 1988). The only known HPLC method capable of resolving these 4 chlorophyll *c* pigments is that of Jeffrey & Brown (unpubl.; described in Jeffrey 1989), which uses a special polyethylene column. It could be used in conjunction with the present method to obtain more detail on the chlorophyll *c* pigments present. Confirmation that the prasinophyte chlorophyll *c*-like pigment is Mg2,4D and not chlorophyll c_1 as was suggested by Wilhelm

(1987) has come from spectral and chemical data (Jeffrey 1989, Fookes & Jeffrey unpubl.). Indeed, a fifth chlorophyll *c*-like pigment was recently characterised from a prasinophyte (Jeffrey 1989, Fookes & Jeffrey unpubl.).

Our improved method presented here fulfils an objective of SCOR Working Group 78 to recommend an HPLC method for resolution of phytoplankton chlorophylls and carotenoids in seawater and cultures of algae. Due to the complexity of pigment composition in field samples, caution should be exercised in the identification of pigments. Retention times, spectral analysis, chemical properties, and co-chromatography with known standards from reference cultures should all be included to confirm identifications, wherever possible.

Using HPLC techniques, important pigments characteristic of algal classes, groups of species or processes occurring in phytoplankton populations (Table 5) can be determined, but for quantitative assessment of the biomass of taxonomic groups the variability of pigment ratios must be taken into account mathematically (Gieskes & Kraay 1983b, Gieskes et al. 1988). If definitive taxonomic characterization of individual species is required, HPLC should be complemented by other techniques such as light and electron microscopy of non-destructively preserved samples.

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