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RAPID EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CHLOROPHYLLS AND CAROTENOIDS FROM MARINE PHYTOPLANKTON

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SUMMARY

An improved technique for analysis of photosynthetic pigments from phytoplankton is described. High efficiency extraction, based on sonication in methanol and centrifugal filtration, removes the cell debris from the initial pigment extract within 2 min of starting disruption. The pigments are separated by reversed-phase high-performance liquid chromatography, using a linear gradient from 90% acetonitrile to ethyl acetate. Carotenes, xanthophylls, chlorophylls and their degradation products are separated with greater resolution than has been previously reported in a single separation. The elution order is presented for 44 pigments from representatives of the Bacilliarophyceae, Dinophyceae, Prymnesiophyceae, Prasinophyceae, Chlorophyceae, Cryptophyceae, Rhodophyceae and Cyanophyceae.

INTRODUCTION

Chromatographic analysis of photosynthetic pigments is a powerful technique for characterization of phytoplankton populations. Most algal phyla possess distinctive carotenoids or chlorophylls which may be used both for their identification[1,2] and estimation of their abundance in mixed populations[3–5]. In addition, various chlorophyll degradation products are characteristic of senescent algae[3,6–8] or their remains in zooplankton faeces[3,9,10]. These pigments are relatively stable if the unextracted algae are stored frozen in darkness[11] permitting samples taken in remote locations to be returned to the laboratory for analysis.

Ideally, for ecological studies, a chromatographic technique should resolve all pigments in a single operation, however the diversity of pigment classes and the extreme similarity of many components make this difficult. Until recently, only two-dimensional thin-layer chromatography (TLC)[11] has achieved sufficient resolution for analyses of mixed populations. Quantitation from TLC plates is tedious and requires 0.15–1.0 μg of each pigment[11], so that 10–20 l of oligotrophic seawater is usually filtered in order to obtain sufficient material.

High-performance liquid chromatography (HPLC) offers significant advantages over TLC, including speed, automatic detection and much lower detection lim-

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its; 0.5 ng for carotenoids and 1 ng for chlorophylls with absorbance detection[12] or 84 pg for chlorophylls using fluorescence detection[13].

Recently the systems available for analysis of plant pigments were reviewed[14]. Few of these[12,15–21] separate all chlorophylls and carotenoids in a single operation and none completely resolve all pigments, lutein and zeaxanthin being particularly difficult to separate. Reversed-phase conditions are preferred to normal phase because the polar stationary phases of the latter promote pigment degradation[12].

Instability of the pigments in solution raises problems during analysis. Chlorophylls and carotenoids are degraded by heat, light, oxygen, acids and alkalis[22,23] and may spontaneously form families of isomers in solution[24]. In addition, chlorophylls are subject to degradation by intracellular chlorophyllase enzymes[25] during extraction. The resultant artefactual pigments may confound the interpretation of chromatograms by co-eluting with other pigments. Higher system efficiency is then required for separation.

The method of extraction of pigments is crucial to the success of the subsequent chromatography. Extraction conditions should rapidly inactivate chlorophyllase enzymes while minimizing pigment degradation. Rapid extraction and removal of cell debris minimizes the opportunity for isomerization and the contact time of the pigments with any enzymes remaining active. Methods previously employed for the extraction of algal pigments for chromatography include grinding[26–28], sonication[29], or incubation in various solvents[17,30,31], with extraction times ranging from a few minutes[26,28,29] to several hours[27,30,31]. In these methods, pigments are in contact with the cell debris for at least 4 min.

In the course of our attempts to improve techniques for the analysis of photosynthetic pigments from phytoplankton populations, we have addressed the problems of extraction and subsequent analysis by HPLC. We present here a simple extraction technique and HPLC system which we have found more convenient and offering greater resolution than those previously described.

EXPERIMENTAL

Phytoplankton sources

Axenic batch cultures of representative species from the Chlorophyceae (*Dunaliella tertiolecta*), Bacilliarophyceae (*Phaeodactylum tricornutum*), Dinophyceae (*Amphidinium carterae*), Prymnesiophyceae (*Pavlova lutheri*), Cryptophyceae (*Chroomonas* sp.), Rhodophyceae (*Porphyridium cruentum*), Prasinophyceae (*Tetraselmis suecica*) and Cyanophyceae (*Anabaena flos-aquae*), were maintained on a 12:12 light/dark cycle at 18°C in f/2 medium[32], or modified Brooker's medium[33] in the case of *A. flos-aquae*, the only non-marine organism. The cultures were harvested three to four weeks after subculturing.

Mixed populations of Antarctic marine phytoplankton samples were collected from Prydz Bay (67°S,75°E).

Filtration and storage

Oceanic samples (5l) or algal cultures were filtered onto Whatman GF/C or Schleicher and Schüll No. 8 filters using a vacuum of 5×10^4 N · m^{-2} (0.5 atm) or less. Magnesium carbonate was not used as a filter aid due to its ability to bind

chlorophyllides and phaeophytins[29]. At sea, the filters were placed in polypropylene vials, snap frozen, and stored in liquid nitrogen for return to the laboratory. Cultured material was frozen at −18°C for about 5 min in order to aid disruption.

Extraction and purification

A conical polypropylene centrifuge tube (15 ml) was modified by cutting it off above the 7-ml mark and making a small (approximately 0.5-mm diameter) hole in the apex. The hole was sealed from the outside by stretching Nescofilm (Nippon Shoji Kaisha) tightly around the base of the tube.

The frozen filter was broken into pieces of about 5 mm across and placed in the tube. Cold methanol (4 ml, 0°C) was added, and the filter was disintegrated with a Braun Labsonic 1510 ultrasound generator (4 mm diameter probe) operated at 50 W for 30 sec. The tube was immediately placed atop a 15-ml glass centrifuge tube, and the two were centrifuged together for two minutes at 2000 rpm (850 × *g* max., lower tube) at 0°C. The extract burst the Nescofilm and collected in the lower tube, while the disintegrated filter and the cell debris were almost completely retained in the upper. The filter debris was washed by adding a further 1-ml amount of methanol and recentrifuging, most of the residual pigment being recovered in the first 0.2 ml. All operations were performed in subdued light using equipment which had been precooled to 0°C.

The crude extract was purified by passage through an octadecyl silica cartridge (C_{18} Sep-PAK, Waters Assoc.) and elution with 2 × 1.5 ml of ethyl acetate. The solution was then filtered through a 0.4-μm pore-size Millipore FH filter and usually used without concentration. Where concentration was necessary, the purified, filtered extract was transferred to diethyl ether and partitioned against cold 10% sodium chloride[3] neutralized with sodium bicarbonate. The ether fraction was evaporated to 0.1 ml in a stream of nitrogen, made up to 0.5 ml with 90% acetonitrile and filtered using a 0.22-μm pore-size teflon filter held in a Bioanalytical Systems MF-1 microfiltration assembly.

In experiments which compared the relative effectiveness of sonication and grinding, replicate filters were ground to a slurry with 3 ml of methanol in a mortar and pestle, transferred quantitatively to a Potter-Elvejhem homogenizer and ground for a further 20 sec, before centrifugal filtration and purification as described above.

Liquid chromatography

The extract (20–200 μl) was injected into a Waters Assoc. liquid chromatograph comprising M6000 and M45 pumps, a 660 solvent programmer, an U6K injector and two RCM-100 radial compression modules in series, each of which contained a Rad-Pak A cartridge (octadecyl silica, 5-μm particle-size). The first cartridge was protected by an RCSS Guard-PAK and precolumn filter. The pigments were eluted using a linear gradient from 90% acetonitrile to ethyl acetate over 20 min with a flow-rate of 2 ml · min^{-1}. One minute after elution of the final pigment (21 min), the solvent composition was returned to initial conditions over a 3-min gradient, after which a further 5 min was allowed for equilibration of the system before injection of the next sample.

Detection of pigments

Pigments were detected using the sum of absorbances at 405 and 436 nm from a Waters Assoc. 440 two-channel absorbance detector. Addition of the two channels was achieved by reversing the signal polarities of the sensor for the lower channel and selecting wavelength 1–wavelenth 2 on the upper channel. The output of the two channels could then be integrated using a Waters Data Module which integrates one signal only.

A Hewlett-Packard 8450A spectrophotometer, equipped with a Helma 15-μl flow cell, allowed pigment spectra to be obtained without the need to stop solvent flow.

Identification of pigments

Individual pigments were collected from the solvent stream and their absorption spectra in ethanol, hexane, and chloroform (in the case of carotenoids), or acetone (in the case of chlorophylls) were compared with published data. A hypsochromic shift upon acidification with HCl was used to identify 5,6-epoxy carotenoids[34]. The presence of 5,6-epoxy groups or keto groups was confirmed by re-chromatography after acidification or borohydride reduction respectively[35]. The identity of *cis*-carotenoids was confirmed by co-chromatography with products of iodine catalysis[36] of the all-*trans* carotenoid. When sufficient carotenoid was collected, electron-impact mass spectrometry was used for further identification. Unfortunately, only the molecular weight was obtained by this technique because the fragmentation pattern was masked by colourless lipids from the sample and octadecyl residues which had bled from the analytical column.

For comparison with unknown peaks, chlorophyllide *a* was prepared from *P. tricornutum* using endogenous chlorophyllases[25]. Phaeophorbide *a* and phaeophytins *a* and *b* were prepared by acidification of the chlorophyllide[11] and the respective chlorophylls[37]. Two-dimensional TLC[11] was also used to confirm the identity of pigments.

Reagents

Methanol, acetonitrile and ethyl acetate were HPLC-grade reagent (Waters Assoc.) and were used without purification other than filtration and degassing. Water was purified using a Millipore Milli-Q system. Diethyl ether and chloroform were purified as recommended by Davies[23].

RESULTS AND DISCUSSION

Extraction of pigments

The technique described here owes much of its simplicity and speed to the fact that the sonication tube acts as the reservoir for centrifugal filtration. The filtration can be started immediately after completion of disruption, and most of the pigments are removed from the cell debris in less than 1 min.

Methanol was chosen as the extraction solvent following the recommendations by others[38–40] who had found it to be more efficient than acetone, ethanol or dimethyl sulphoxide, although we found no significant differences with this technique.

Sonication was more effective than grinding for extracting the major pigments

from mixed phytoplankton sample, being superior both in yield and reproducibility (Table I). This is in direct contrast with recent results obtained by Chang and Rossmann[41] with freshwater algae. We attribute the difference to the power of the sonicators used. That used by Chang and Rossmann delivered 73.5 $W \cdot cm^{-2}$ compared with the 398 $W \cdot cm^{-2}$ of our needle probe. The extent to which species differences may have influenced the results is unknown.

The sonication time required for complete extraction of pigments varied between species. Pigments from *P. tricornutum* and *A. carterae* were almost completely extracted with no sonication at all, whereas those from *Chlorella* sp. required 30 sec. For the species tested here, 30 sec was adequate in all cases, averaging 95% extraction of chlorophyll *a*. Sixty seconds of sonication resulted in some allomerization of chlorophyll *a* but did not increase the yield.

No chlorophyllase activity was evident after sonication. When centrifugation of extracts of *P. tricornutum* and *D. tertiolecta* was delayed for up to 10 min after sonication, there was no significant loss of chlorophylls *a*, *b* or *c*, nor any appearance of chlorophyllides or phaeophytins. However, there was isomerization of some carotenoid peaks and the overall resolution was reduced as a result. Addition of 1% butylated hydroxytoluene[42] to the extraction solvents did not prevent this phenomenon. Thus, each sample should be injected immediately after cell disruption and filtration. The practice of preparing several samples simultaneously is not recommended unless each sample is processed independently and immediately frozen in liquid nitrogen until analysis.

Purification of the extract using an octadecyl silica cartridge was found to be essential. By removing compounds which are not eluted from octadecyl silica by ethyl acetate, this procedure prevented a rapid accumulation of material on the analytical column (much of which bound irreversibly), resulting in longer retention times, higher backpressures, and reduced column life. As a precaution, pre-column filters were cleaned every forty samples and column Guard-Paks were changed whenever the backpressure rose to 10^7 $N \cdot m^{-2}$ (1500 p.s.i.). Some inorganic material, presumably sodium chloride, precipitated on addition of ethyl acetate to extracts of marine samples. This was removed in the final filtration.

TABLE I

EXTRACTION OF PIGMENTS BY GRINDING AND SONICATION

Yield and standard error of pigments extracted in methanol from replicate filters of a mixture of cultures of *A. carterae, P. cruentum, D. tertiolecta, P. tricornutum, P. lutheri, T. suecica* and *A. flos-aquae*.

Pigment	*Grinding*		*Sonication*		*Difference (%)*
	Yield (ng)	*S.E. (%)*	*Yield (ng)*	*S.E. (%)*	
Chlorophyll *c*	183	12.3	286	1.6	56.3
Peridinin	98	7.4	141	3.5	44
Fucoxanthin	113	11.1	168	2.5	49
Diadinoxanthin	85	19.6	119	10.8	40
Chlorophyll *a*	748	7.5	1031	2.3	38
Echinone	7	16.0	10	7.9	43
β,β-Carotene	20	36.9	24	1.0	20

TABLE II

RECOVERY OF PIGMENTS AFTER ETHER CONCENTRATION

Recovery of pigments after partitioning with ether and concentration. Recoveries were standardized to a 100% recovery of β,β-carotene to allow for errors in handling small volumes. Actual recovery of β,β-carotene was 99.5% with a standard error of 10%. Peak numbers correspond to those of Fig. 2 and Table IV.

Peak	*Identity*	*Recovery (%)*	*S.E. (%)*
1	Chorophyllide *a*	83	10
2	Chlorophyll *c*	86	18
9	Fucoxanthin	90	10
12	Neoxanthin	80	22
14	*cis*-Fucoxanthin	93	31
19	Violaxanthin	90	2
21	Diadinoxanthin	91	13
24	Antheraxanthin	118	18
27	Lutein	100	12
32	Chlorophyll *b*	100	14
35	Chlorophyll *a* allomer	146	56
36	Chlorophyll *a*	92	3
40	Phaeophytin *a*	2556	134
42	β,ψ-Carotene	116	16
44	β,β-Carotene	(100)	–

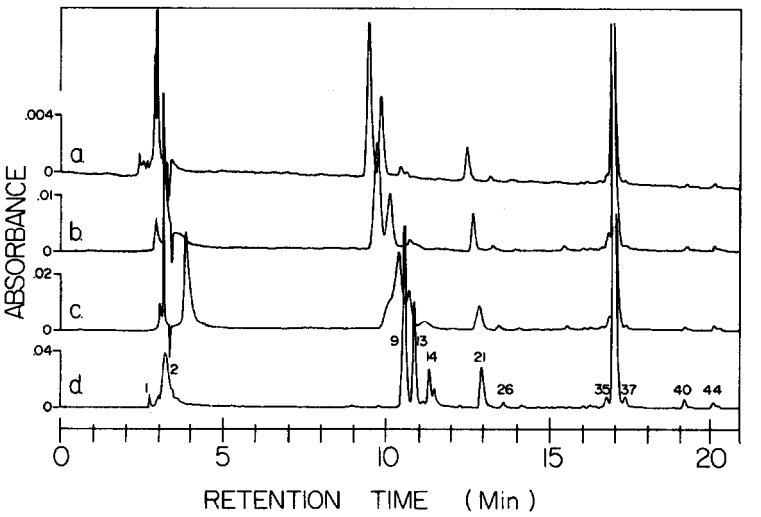

Fig. 1. Resolution of pigments from a natural phytoplankton sample from the Southern Ocean. Traces a, b, c show the results of injecting 40, 100 and 200 μl, respectively, of unconcentrated extract. Trace d represents 40 μl of extract concentrated by diethyl ether partitioning. Peak identities are given in Table IV.

For quantitative work, the final extract was used without concentration, since the ether partitioning method gave incomplete recovery and promoted degradation of chlorophyll *a* to phaeophytin *a* (Table II). This occurred even though the aqueous phase had been neutralized. Since the extract contains approximately 40% ethyl acetate, large volumes could not be injected without causing peak spreading (Fig. 1a–c). Substantial loss of resolution occurred if 200 μl or greater was injected. For dilute samples concentration by ether partitioning was necessary despite its serious disadvantages. Fig. 1d shows the improved resolution and sensitivity obtained by this procedure, but also the significant increase in the proportions of *cis*-fucoxanthin and phaeophytin *a* (peaks 14 and 40, respectively). We are presently investigating other methods of sample concentration.

A potential disadvantage of this practice is that in the absence of a partitioning step, a significant amount of chloride is carried through into the final extract and injected into the liquid chromatograph. We are aware of the risk of halide-induced corrosion, but no such problems have been encountered to date after the injection of more than one thousand marine samples. As a precautionary measure, we periodically purge the instrument with distilled water.

Liquid chromatography

Our chromatographic system is based on that of Eskins and Dutton[19], who used a gradient from 90% methanol to ethyl acetate. We found that this system gave adequate resolution for most pigments, but did not reliably separate chlorophyllide *a* from chlorophyll *c* except on aged columns. Furthermore, the early eluting peaks (notably peridinin) were low and broad and thus difficult to detect and integrate.

In attempts to improve overall resolution and peak shapes, a number of solvent systems were compared. The resolution of various pairs of pigments with these systems and heights for some representative peaks are given in Table III. For quantitative work, a resolution value of at least 1.0 is required[43]. This table also shows the improvement obtained with two RCM-100 columns connected in series. Although the interconnection would contribute to peak spreading, the number of theoretical plates, and therefore the height of peaks, was greater with this arrangement. Peak spreading may have been reduced if a single 20-cm column had been available.

The solvent system of Eskins and Dutton generally gave better resolution and peak shape with two columns (system D) than one (system A), however with two columns, the high viscosity of the initial solvent resulted in backpressures close to the rated limit of the RCM columns ($16.5 \cdot 10^6$ N $\cdot$ m^{-2}) and some failures were experienced. Modification of the initial solvent by the addition of 6% tetrahydrofuran (system E) gave somewhat better resolution but backpressures were still unacceptably high. In order to reduce the viscosity and hence improve column efficiency, acetonitrile was substituted for methanol. This resulted in more reliable resolution of chlorophyllide *a* from chlorophyll *c* and gave improved resolution of the other pigments as well. The best combination of resolution and peak shape was obtained with 90% acetonitrile as the initial phase using two columns (system G). This gave excellent results across the whole chromatogram with well-formed early peaks and acceptable backpressure. When one column was used (system C) the resolution was still excellent, but the early peaks were broader and lower. System G of Table III was used for all subsequent work, and is the system described in the Experimental section.

TABLE III

PERFORMANCE OF VARIOUS SOLVENT SYSTEMS

Performance indicators of analytical systems using 1 or 2 columns and various solvent pairs. The initial solvent was as indicated below. A linear gradient over 20 min to 100% ethyl acetate was used in all cases, with a flow-rate of 2 ml/min. Dashes indicate that the resolution was unmeasurable. Abbreviations: MeOH, methanol; CH_3CN, acetonitrile; 84:10:6, methanol, water, tetrahydrofuran in these proportions by volume; chlide, chlorophyllide *a*; chl, chlorophyll; perid/inin; fucox/anthin; neox/anthin; diad/inoxanthin; anth/eraxanthin; lut/ein; zeax/anthin; $\beta,\psi/\beta$, β-car, β,ψ- and β, β-carotene respectively.

	System						
	A	*B*	*C*	*D*	*E*	*F*	*G*
	Initial solvent						
	90% MeOH	*80% CH_3CN*	*90% CH_3CN*	*90% MeOH*	*84:10:6*	*80% CH_3CN*	*90% CH_3CN*
	Number of columns						
	1	*1*	*1*	*2*	*2*	*2*	*2*
Resolution of pigment pairs							
Chlide/chl *c*	2.71	–	2.50	1.79	–	–	2.38
Perid/fucox	4.55	3.80	3.55	5.36	6.17	4.15	4.27
Fucox/neox	2.30	0.56	1.75	2.33	2.64	1.25	2.83
Diad/anth	1.33	2.56	2.73	1.48	1.50	2.61	2.68
Lut/zeax	–	0.63	1.17	–	–	0.50	1.20
Chl *b*/chl *a*	5.55	4.58	4.89	5.25	5.43	4.47	4.52
β, $\psi/\beta,\beta$-car	1.39	2.95	2.75	1.52	1.81	2.65	2.91
Peak height (mm)							
Peridinin	24	44	33	54	8	63	51
Fucoxanthin	55	83	54	83	27	94	86
Chl *b*	66	102	86	70	58	89	90
Initial Backpressure ($\times 10^6$ N · m^{-2})	6.9	3.8	3.3	14.5	13.8	8.3	7.58

Chromatograms of pigment extracts from representatives of various algal phyla and a mixture thereof are shown in Fig. 2. The pigment numbering corresponds with that of Table IV, where pigment identities, retention times and spectral data are given.

There was excellent separation of most pigments. Chlorophyllide *a* (peak 1) was well resolved from two chlorophyll *c* peaks (peaks 2 and 3), which did not correspond to separate peaks of chlorophylls *c1* and *c2*. On the basis of spectra in eluent, peak 2 contained both chlorophylls *c1* and *c2*, with chlorophyll *c2* unresolved, but eluting slightly ahead of chlorophyll *c1*. Peak 3 contained unidentified isomers of chlorophylls *c* which had spectra identical to those of the native chlorophylls.

The carotenoids of major significance in ecological studies, peridinin (peak 8), fucoxanthin (peak 9), diadinoxanthin (peak 21) and lutein (peak 27), were all com-

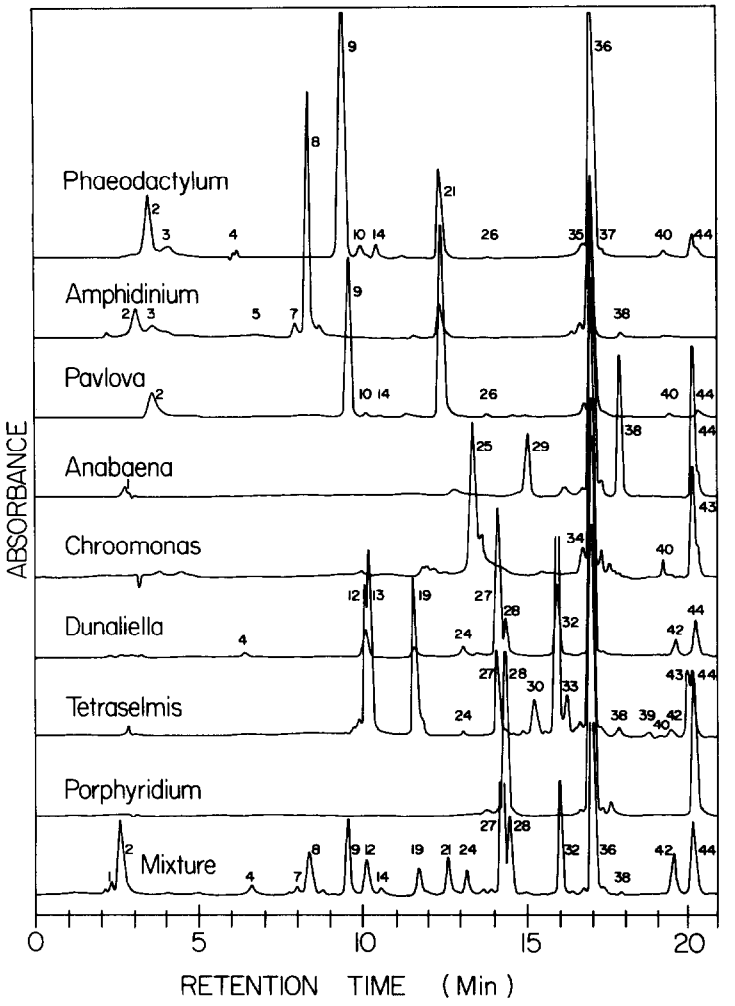

Fig. 2. Chromatograms of pigment extracts from various algae. Peak identities are given in Table IV.

TABLE IV

ELUTION ORDER OF THE PIGMENTS

Identities, retention times (t_R) and spectral data for pigments shown in Fig. 2. 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 % III/II ratio[67]. $CHCl_3$, pet. eth. and E/HCl refer to chloroform, petroleum ether, and ethanol containing 0.05 *M* HCl.

Peak No.	t_R *(min)*	*Pigment*	*Mol. wt.*	*Spectral data*				*Solvent*	*Published data*				
				Maxima (nm)			*Peak ratio*		*Maxima*			*Peak ratio*	*Ref.*
1	2.26	Chlorophyllide *a*	–	430	616	664	1.3	Eluent					
								Diethyl ether	428		662		46
2	2.56	Chlorophylls *c*	–	442	580	630	9.8	Eluent					
				444	580	630	7.7	Acetone	444	581	630	6.71	47
3	3.11	Chlorophylls *c'*	–	442	578	628		Eluent					
4	5.82	Chlorophyllide *a* derivative	–	430	616	662	1.15	Eluent					
5	6.54	Chlorophyllide *a* derivative	–	428		664	1.08	Eluent					
6	7.69	Phaeophorbide *a*	–	408	608	666	2.04	Eluent					
								Diethyl ether	408	609	667	2.07	48
7	7.93	*cis*-Peridinin ?	–	334	464			Eluent					
8	8.33	Peridinin	630		470			Eluent					
					458	476*	–	Ethanol		472		–	49
					466	492*	–	$CHCl_3$					
				430*	454	484	86	Hexane	431*	454	484		30
9	9.53	Fucoxanthin	–		446	468*		Eluent					
					446	466*		Ethanol	426*	449	456*		50
				422	446	475	41	Hexane	425	449	478		18
					460	486*		$CHCl_3$		460	478*		51
10	10.09	*cis*-Fucoxanthin	–	325	440	466*							
11	10.09	Phaeophorbide *a* derivative	–	408		664	3.11	Eluent					
12	10.12	Neoxanthin	600	412	437	466	91	Eluent					
				410	436	464	93	Ethanol	416	440	468	89	52
				408	432	462	89	Hexane	415	435	462		23
				418	442	472	84	$CHCl_3$	423	448	476		52
				398	420	446		E/HCl					
13	10.24	Trihydroxy-β,ε-carotene?	–	420*	444	472	41	Eluent					
								Ethanol	425*	445	470	38	52
14	10.37	*cis*-Fucoxanthin	–	329	444	464*		Eluent					
15	10.37	Neochrome	–	400	422	450	92	Eluent					
								Ethanol	401	424	451		53
16	11.19	Phaeophorbide *a* derivative	–	410	606	666	2.1	Eluent					
17	11.38	Phaeophorbide *a* derivative	–	408	612	669	2.4	Eluent					
18	11.25	Myxoxanthophyll	–	450	473	504	64	Eluent					
				448	473	504	61	Ethanol	448	473	503	64	54

TABLE IV

Peak No.	t_R (min)	Pigment	Mol. wt.	Spectral data				Solvent	Published data				
				Maxima (nm)			Peak ratio		Maxima			Peak ratio	Ref.
19	11.72	Violaxanthin	600	417	440	470	89	Eluent					
				416	440	470	87	Ethanol	417	440	470	93	56
				414	436	468	85	Hexane	415	440	470	95	55
				424	450	480	87	$CHCl_3$	426	449	478		56
				383	400	426		E/HCl	378	400	424		57
20	12.15	Auroxanthin	–	383	402	426	103	Eluent					
								Ethanol	380	402	428		51
21	12.58	Diadinoxanthin	582	424	448	478	74	Eluent					
				424*	446	476	39	Ethanol	424*	446	476		30
				422	444	474	38	Hexane	421*	445	475		30
				431	454	486	54	$CHCl_3$	432	455	482		56
				406	428	456		E/HCl	405*	428	456		58
22	12.73	Diadinochrome	–	407	429	459	74	Eluent					
23	12.92	Phaeophorbide *a* derivative	–	410	610	668	2.5	Eluent					
24	13.18	Antheraxanthin	584	421*	448	475	62	Eluent					
				424*	444	474	63	Ethanol	422	444	472	54	52
				420*	442	470	65	Hexane	420*	443	471	49	55
				432*	456	484	58	$CHCl_3$	430	456	484		56
				404*	426	452		E/HCl					
25	13.43	Alloxanthin	–	430*	452	481	50	Eluent					
								Ethanol	430*	453	483		59
26	13.45	Diatoxanthin	–	424	448	476	25	Eluent					
				424*	448	475	23	Ethanol	425*	449	475	25	56
				424	447	475	29	Hexane		450	479	48	60
				432*	458	488	15	$CHCl_3$	433*	458	486		56
27	14.26	Lutein	568	422*	446	474	65	Eluent					
				424*	446	474	63	Ethanol	422	445	474	62	52
				420	444	472	69	Hexane	420	445	474	74	60
				432*	456	484	60	$CHCl_3$	428	454	483		61
				424*	446	472		E/HCl					
28	14.49	Zeaxanthin	–	426*	452	480	32	Eluent					
				424*	450	478	31	Ethanol	428*	450	478	26	52
				422*	448	476	43	Hexane	426*	450	480		23
				432	462	488	25	$CHCl_3$	434	459	488		56
29	15.04	Canthaxanthin	564		476			Eluent					
								Ethanol		474			52
30	15.54	Unknown	–	422	448	476	36	Eluent					
31	15.94	Chlorophyll *d*-like	–	450	650	690	3.6	Eluent					
								Diethyl ether	447	643	688		62
32	16.04	Chlorophyll *b*	–	456	598	646	3.0	Eluent					
				464	600	650	2.5	Ethanol					
								Acetone	453	598	645		64
33	16.46	Unknown	–	422	448	476	42						
34	16.70	Crocoxanthin ?	–	427*	447	478	68	Eluent					
								Ethanol	421*	443	472	62	50

(*Continued on p. 292*)

TABLE IV (*continued*)

Peak No.	t_R (min)	Pigment	Mol. wt.	Spectral data				Solvent	Published data				
				Maxima (nm)			Peak ratio		Maxima			Peak ratio	Ref.
35	16.82	Chlorophyll *a* allomer	–	428	614	662	1.07	Eluent					
				428	616	664		Ethanol					
								Pet.-eth.	428	614	662		48
36	17.06	Chlorophyll *a*	–	430	617	664	1.17	Eluent					
				430	616	666		Acetone	430	618	665		63
37	17.41	Chlorophyll *a* epimer	–	430		668	1.8	Eluent					
								Diethyl ether	428	614	661	1.24	64
38	17.97	Echinone	550		464			Eluent					
								Ethanol		461			52
39	18.32	Phaeophytin *b*	–	434	599	652	4.0	Eluent					
								Diethyl ether	433	599	654	4.81	48
40	19.32	Phaeophytin *a*	–	408	504	666	2.2	Eluent					
								Diethyl ether	408	503	667	2.14	48
41	19.42	Lycopene	–	447	474	506	66	Eluent					
								Ethanol	443	472	502		65
42	19.64	β,ψ-Carotene	–	436*	462	494	37	Eluent					
				438*	460	490	42	Ethanol	440*	460	489	23	52
				436	458	488	49	Hexane	431	462	494		51
				448*	472	504	35	$CHCl_3$	449*	472	504		52
43	20.14	β,ε-Carotene	–	423	447	475	88	Eluent					
								Ethanol	423	444	473	61	52
44	20.24	$\beta\beta$-Carotene	–	426*	452	478	20	Eluent					
				422*	448	472	10	Ethanol	425*	450	477		66
				425*	444	474	14	Hexane	427*	448	475		30
				438*	460	484	2	$CHCl_3$		460	485*		30

* Indication of shoulder.

pletely resolved. Of particular note was the resolution of zeaxanthin (peak 28) from lutein. These compounds differ only in the double bond configuration of one end group, and their resolution has not been previously reported in reversed-phase HPLC.

A number of chlorophyll derivatives were associated with chlorophylls *b* and *a* (peaks 32 and 36, respectively), the most important of which were the chlorophyll *a* allomer (peak 35) and epimer (peak 37). These usually comprised about 3–4% of the chlorophyll *a* peak in extracts of healthy cultures. At least four other chlorophyll derivatives eluted between chlorophylls *a* and *b*. An interesting minor component (peak 31), which resembled the chlorophyll *d* of Manning and Strain[44], was found in extracts of *P. tricornutum*. This was probably a chlorophyll *a* derivative.

When chlorophyllide *a* was acidified, peak 6 was the major product. This was taken to be phaeophorbide *a*. A number of other pigments had phaeophorbide-like spectra. Peak 11 was a minor product of the acidification reaction, peaks 16 and 17 were found in the faeces of salps which had been feeding on natural phytoplankton

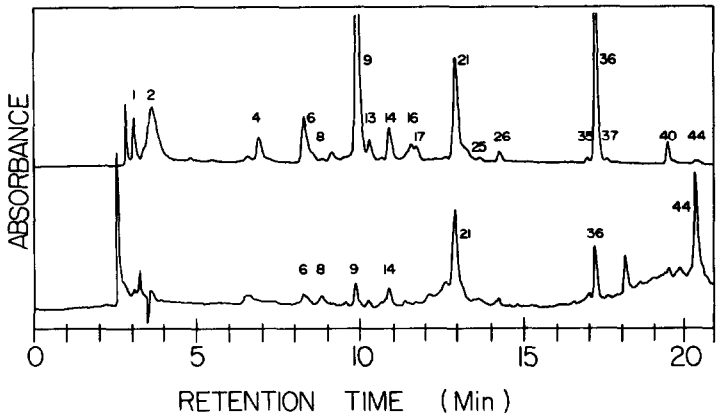

Fig. 3. Chromatograms of pigment extracts from phytoplankton ingested by zooplankton. Upper trace, faeces of the salp, *Salpa thompsoni*; lower trace, gut contents of the krill, *Euphausia superba*. Peak identities are given in Table IV.

(chiefly diatoms), whilst peak 23 was present in extracts of gut contents of the krill, *Euphausia superba*. These peaks have not yet been fully characterized but are believed to be phaeophorbide *a* derivatives.

An acid-stable carotenoid (peak 13), which has been tentatively identified as trihydroxy-β,ε-carotene, eluted closely behind neoxanthin in extracts from *T. suecica*. This pigment was also found in some marine samples from the Southern Ocean (*e.g.* Fig. 1). It may represent a useful marker for the Prasinophyceae.

The carotenes were not fully resolved. β,ψ-Carotene (peak 42), which was unexpectedly abundant in *D. tertiolecta*, eluted ahead of β,ε-carotene (peak 43) and β,β-carotene (peak 44) which were incompletely resolved. The trailing shoulder on the β,β-carotene peak resembled the 15-*cis* isomer spectroscopically[45].

Appraisal of the method

We have found the method described here to be simple, rapid, and reproducible, being suitable for analyses of oceanic phytoplankton samples (Fig. 1), as well as zooplankton gut contents and faeces (Fig. 3).

The limits of detection for chlorophyll *a* and fucoxanthin were found to be approximately 1.0 and 0.3 ng respectively. The variability of the entire method, from filtration of the algae to integration of the chromatogram, was 2–3% for the major pigments.

Whilst the extraction technique would require modification for use with samples other than phytoplankton, the chromatographic system reported here offers greater resolution of carotenoids and chlorophylls than others published to date, and as such is likely to be useful in studies where chromatography of plant pigments is employed.

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