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Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C_8 column and pyridine-containing mobile phases

Manuel Zapata[1,*], Francisco Rodríguez[1], José L. Garrido[2]

[1]Centro de Investigacións Mariñas, Consellería de Pesca, Xunta de Galicia, Apdo.13, 36620 Vilanova de Arousa, Spain
[2]Instituto de Investigacións Mariñas de Vigo (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain

ABSTRACT: A high-performance liquid chromatographic (HPLC) method based on a reversed-phase C_8 column and pyridine-containing mobile phases was developed for the simultaneous separation of chlorophylls and carotenoids. The method is selective enough to resolve monovinyl (MV) and divinyl (DV) pairs of polar chlorophylls and DV chlorophyll *a* (chl *a*) (the marker pigment for the prokaryote *Prochlorococcus marinus*) from chl *a* (the MV analogue). Only the pair DV chl *a*/chl *b* was not resolved. This resolution capability for chlorophylls was only previously achieved using polymeric C_{18} columns in combination with ammonium acetate or pyridine-containing mobile phases. The proposed method also allows the separation of taxon-specific carotenoids belonging to 8 algal classes, including some critical pigment pairs for previous HPLC methods using C_{18} columns. The method employs a binary gradient, so it can be used with both low-pressure and high-pressure mixing instruments. Method transferability was tested using 3 HPLC systems. Only a slight adjustment of gradient profile was required to obtain similar results with HPLC equipment having different dwell volumes. The selectivity of the method towards some recently discovered chlorophyll and carotenoid pigments makes it especially suitable for studying not only field samples, but also for re-examining the pigment composition of different algal classes.

KEY WORDS: HPLC pigment analysis · Phytoplankton pigments · C_8 column · Pyridine-containing mobile phases Chemotaxonomy

INTRODUCTION

The chemotaxonomic assessment of phytoplankton populations present in natural waters requires good biochemical markers and very efficient analytical tools. The analysis of photosynthetic pigments by high-performance liquid chromatography (HPLC) fulfils the above requirements as it allows the separation and quantification of taxon-specific chlorophylls and carotenoids, some of them present in seawater samples in trace amounts. The outstanding importance of HPLC-based phytoplankton pigment analysis in oceanographic studies has led to the publication of a comprehensive monograph in which both modern analytical methods and their application to biological oceanography were reviewed exhaustively (Jeffrey et al. 1997b).

The photosynthetic pigments of phytoplankton in natural samples appear as very complex mixtures whose separation has challenged analytical methods for decades. On the one hand, they cover a wide range of molecular structures, showing very different polarities (from the acidic chlorophylls to the non-polar hydrocarbon carotenes). On the other hand, some chlorophylls and carotenoids are difficult to separate as they only differ in the presence or position of a double bond (e.g. monovinyl [MV] and divinyl [DV] chlorophyll pairs, β,β-carotene and β,ε-carotene and their isomeric xanthophyll derivatives).

*E-mail: mzapata@cimacoron.org

The classical HPLC methods were based on reversed-phase octadecylsilica (ODS, C_{18}) columns and gradient elution with aqueous methanol (Gieskes & Kraay 1983) or aqueous acetonitrile (Wright & Shearer 1984) as initial mobile phase. To retain the most polar acidic chlorophylls either the ion-pair reagent tetrabutylammonium acetate (Mantoura & Llewellyn 1983) or a simple ammonium acetate buffer solution (Zapata et al. 1987) was incorporated to the aqueous methanol mobile phase.

Combining advantages of earlier methods, Wright et al. (1991) developed a ternary gradient HPLC system which made use of the retention capacity of ammonium acetate-containing mobile phase (Zapata et al. 1987) and the special selectivity of acetonitrile-based eluents for carotenoid separation (Wright & Shearer 1984). Wright et al.'s (1991) method was employed as a standard protocol in international oceanographic programs (Joint Global Ocean Flux Study: JGOFS, UNESCO 1994), and recommended by SCOR Working Group 78 (Wright & Jeffrey 1997). Although these methods achieved good separation for most phytoplankton carotenoids, none of them was able to separate acidic chlorophylls, with co-elution of chl c_1, chl c_2 and Mg-3,8-divinyl-pheoporphyrin a_5 monomethyl ester (MgDVP). After the discovery of the marine prokaryote *Prochlorococcus marinus* (Chisholm et al. 1988, 1992) a new drawback was added, as these methods are not able to separate DV chls *a* and *b* (the marker pigments for *P. marinus*, Goericke & Repeta 1992) from the MV (chls *a* and *b*) analogues.

Once the simultaneous separation of pigments of different polarities had been obtained, the next step was to improve the HPLC methods by increasing their capacity to resolve photosynthetic pigments with very similar structures. Thus, the resolution of polar and non-polar chls *c* was improved by using a high carbon-loaded C_{18} column (Kraay et al. 1992), polymeric C_{18} columns (Garrido & Zapata 1993, Van Heukelem et al. 1994, Van Lenning et al. 1995), and by increasing the mobile phase selectivity with changes in the solvents or the ion-pair reagent (Garrido & Zapata 1996, 1997).

The use of monomeric octylsilica (OS, C_8) columns was first introduced by Goericke & Repeta (1993) to achieve the resolution of chl *a* and DV chl *a*. That method and further modifications, which employed the same stationary phase (Vidussi et al. 1996, Barlow et al. 1997), still failed in the separation of chl *c*-related pigments. In a recent paper (Rodríguez et al. 1998), we showed that using adequate gradient profiles and injection conditions, monomeric C_8 columns can separate acidic chlorophylls simultaneously with other chlorophylls and carotenoids. However, this method, which obtained good results in the analysis of pigment composition from many unialgal cultures, failed in the separation of certain pigment pairs from natural samples, especially those composed by chls *c* and chl *a* acidic derivatives. To overcome such a problem, and knowing that the use of pyridine as the eluent modifier provides enhanced selectivity towards certain polar chlorophylls and carotenoids (Garrido & Zapata 1996, 1997, Zapata et al. 1998), we have evaluated the performance of pyridine-containing mobile phases on monomeric C_8 stationary phase.

Here we present an HPLC method for the analysis of phytoplankton pigments which combines a C_8 column with an optimised mobile phase including an aqueous pyridine solution as an ion-pair reagent. The results obtained from analysis of unialgal culture extracts, complex pigment mixtures and natural samples show that the new method is able to separate, in a single run, most polar and non-polar chlorophylls and most taxon-specific carotenoids found in marine phytoplankton.

MATERIALS AND METHODS

HPLC. Method development was performed using Waters Alliance HPLC equipment (System 1), including a 2690 separations module (low-pressure mixing system) and a Waters 996 diode-array detector (1.2 nm optical resolution) interfaced with a Waters 474 scanning fluorescence detector by means of a Sat/In analog interface. To verify the transferability of the new method to other HPLC systems, 2 additional instruments (Systems 2 and 3) were also employed. HPLC System 2 was a Beckman System Gold including a model 126 programmable solvent module (high-pressure mixing system), a model 168 diode-array detector (2 nm optical resolution) and a Rheodyne 7725i injection valve fitted with a 200 µl loop. HPLC System 3 was a Waters modular system (high dwell volume) including a Waters 600 S controller, a Waters 616 pump (low-pressure mixing system), a Waters 717 Plus autosampler (200 µl loop) and a Waters 996 diode-array detector (1.2 nm optical resolution).

Stationary phase. Analytical separations were performed using a Waters Symmetry C_8 column (150 × 4.6 mm, 3.5 µm particle size, 100 Å pore size). The column was thermostatted at 25°C by means of a refrigerated circulator water bath (Neslab RTE-200) connected to an HPLC column water jacket (Alltech).

Mobile phases. Eluent A was a mixture of methanol:acetonitrile:aqueous pyridine solution (0.25 M pyridine, see below) (50:25:25 v:v:v) while eluent B was either B1, methanol:acetonitrile:acetone (20:60:20 v:v:v), or B2, acetonitrile:acetone (80:20 v:v). Organic solvents employed to prepare mobile phases were HPLC-grade. The aqueous pyridine solution (0.25 M)

was prepared as follows: 10 ml of acetic acid and 20 ml of pyridine (Merck) were added to 900 ml of milli-Q (Millipore) water in a 1 l flask and mixed using a magnetic stirrer. Acetic acid was then added dropwise until the pH was 5.0. The mixture was diluted to 1000 ml with water (final pyridine concentration 0.248 M) and the pH rechecked. All procedures were performed in a fume hood. The pyridine solution was filtered (0.45 µm GHP Gelman filter) after mixing with methanol and acetonitrile (eluent A). Different gradient profiles were adjusted for minimising differences of equipment dwell volume (see Table 1). The flow rate was fixed at 1 ml min^{-1}.

Algal cultures. Two sets of algal cultures were employed during this study. The first one was used in our laboratories for HPLC method development and evaluation, and was selected to include the most diagnostic pigments and algal classes found in marine phytoplankton: *Alexandrium minutum* AL1V-IEO (Dinophyceae) from the Instituto Español de Oceanografía, Vigo, Spain; *Emiliania huxleyi* NIOZ CH 24 (Prymnesiophyceae) from the Netherlands Institute for Sea Research, Texel, The Netherlands; *Pavlova gyrans* CCMP 608 (Prymnesiophyceae), *Prochlorococcus marinus* CCMP 1375 (Cyanophyceae) and *Pelagococcus subviridis* CCMP 1429 (Pelagophyceae) from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, ME, USA; *Rhodomonas baltica* ICMA (Cryptophyceae), *Dunaliella tertiolecta* ICMA (Chlorophyceae), and *Tetraselmis suecica* ICMA (Prasinophyceae) from the Instituto de Ciencias Marinas de Andalucía (CSIC), Cádiz, Spain; *Micromonas pusilla* CCAP 1965/4 (Prasinophyceae) from the Culture Collection of Algae and Protozoa, Oban, UK; and *Skeletonema costatum* Sk-1 (Bacillariophyceae) from the Centro de Investigacións Mariñas, Vilanova de Arousa, Spain.

All cultures except *Prochlorococcus marinus* were grown on f/2 enriched seawater medium (Guillard & Ryther 1962) under 12:12 h L:D cycle with an irradiance of 42 µmol photons m^{-2} s^{-1} during the light period. Temperature was maintained at 16 ± 1°C. *P. marinus* CCMP 1375 was grown as described by Moore et al. (1995).

In an additional study performed at the CSIRO Marine Laboratories in Hobart, Australia, the method was transferred to other HPLC equipment (System 3) and the following SCOR reference microalgal cultures (Jeffrey & LeRoi 1997) were analysed: *Amphidinium carterae* CS-212 (Dinophyceae), *Dunaliella tertiolecta* CS-175 (Chlorophyceae), *Emiliania huxleyi* CS-57 (Prymnesiophyceae), *Pavlova lutheri* CS-182 (Prymnesiophyceae), *Pelagococcus subviridis* CS-99 (Pelagophyceae), *Phaeodactylum tricornutum* CS-29 (Bacillariophyceae), *Porphyridium cruentum* CS-25 (Rhodophyceae), and *Pycnococcus provasoli* CS-185 (Prasinophyceae). Culture conditions were as described by Jeffrey & LeRoi (1997). All cultures were harvested during the exponential phase of growth by filtering under reduced vacuum onto 25 mm diameter Whatman GF/F filters.

The macroalga *Codium tomentosum* (Chlorophyceae) was employed as source of siphonaxanthin and siphonein. Isolated pigments were injected in HPLC System 1 to establish retention time and spectral information.

Field samples. Seawater samples were obtained from different regions. During the FRUELA 96 cruise (January 1996) on board the RV 'Hespérides', a sample was collected from Gerlache Strait (64° 20' S, 61° 48' W, at 5 m depth) near the Antarctic Peninsula. A sample from oligotrophic waters was collected (May 1999) from eastern subtropical North Atlantic (33° 03' N, 21° 16' W, at the deep chlorophyll maximum depth: 80 m). Seawater samples were filtered through a 47 mm diameter Whatman GF/F filters. An estuarine sample was collected (November 1998) from the Ría of Arousa (Galician coast, NW Spain). Seawater was size-fractionated by sequential filtration through 47 mm diameter Whatman GF/D filter (nominal pore size 2.7 µm) and a Whatman GF/F filter (nominal pore size 0.7 µm).

Filters were kept frozen prior to HPLC analysis. The FRUELA sample was stored at −30°C for 2 yr, the North Atlantic sample at −80°C for 10 d and filters from the estuarine sample at −30°C for 1 d.

Pigment extraction. Frozen filters from algal cultures and natural samples were extracted in a Teflon-lined screw-capped tube with 5 ml 95% methanol (2 ml for the oligotrophic sample) using a stainless steel spatula for filter grinding. The tube was then placed in a beaker with ice and water, and the whole set placed in an ultrasonic bath for 5 min. Extracts were then filtered through 25 mm diameter polypropylene syringe filters (MFS HP020, 0.2 µm pore size) to remove cell and filter debris. An aliquot (1 ml) of methanol extract was mixed with 0.2 ml of water (0.4 ml for SCOR culture extracts) to avoid the shape distortion of earlier eluting peaks (Zapata & Garrido 1991). Each sample was injected just after water addition, as a decrease in non-polar pigment concentrations was observed when diluted extracts were held inside the refrigerated autosampler (4°C) prior to injection. The injection volume was 200 µl. All samples were prepared under subdued light.

Pigment detection and identification. Chlorophylls and carotenoids were detected by diode-array spectroscopy (350 to 750 nm). Chlorophylls were also detected by fluorescence (Ex [excitation]: 440 nm, Em [emission]: 650 nm). Absorbance chromatograms were

extracted at different wavelengths (430, 440 and 450 nm). Pigments were identified by co-chromatography with authentic standards and by diode-array spectroscopy (wavelength range: 350 to 750 nm, 1.2 nm spectral resolution). Each peak was checked for spectral homogeneity using the Millenium software (Waters) algorithms, and the absorption spectrum was compared with a spectral library previously created. Pigment standards were isolated from microalgal cultures or seaweeds of well-known pigment composition, purified by semi-preparative HPLC, and transferred into standard solvents following protocols described by Jeffrey (1997) and Repeta & Bjørland (1997). Novel compounds, such as 4-keto-19'-hexanoyloxyfucoxanthin (Egeland et al. in press) and MV chl c_3, were isolated and characterised as previously described (Garrido & Zapata 1998).

Resolution (R_s) between a peak and the preceding one was calculated by means of Millenium System Suitability software (Waters) using the following equation: $R_s = 2(Rt_2 - Rt_1)/W$, where Rt_2 and Rt_1 are the retention times of 2 adjacent peaks, and W is the sum of peak widths at baseline. As resolution was measured for peaks eluting in the same absorbance chromatogram, mixtures of several culture extracts were injected when necessary for obtaining adjacent peaks.

Pigment nomenclature and abbreviations suggested by SCOR WG 78 (Jeffrey & Mantoura 1997) were used. A prefix indicating structural variations of well-known pigments (e.g. chls *a*, *b*, and c_3) was used for designating novel compounds (i.e. DV chls *a* and *b*, MV chl c_3). For the chlorophylls whose molecular structure has not yet been elucidated, the nomenclature includes the pigment type and the species name where it was first detected (e.g. chl *c* from *Pavlova gyrans* or non-polar chl *c* from *Emiliania huxleyi*).

RESULTS

Mobile phase composition

The optimal composition of eluents A and B was studied by isocratic elution. Mixtures of isolated polar and non-polar chlorophylls were used as resolution probes for optimising mobile phases A and B, respectively. The aqueous component in eluent A (0.25 M ammonium acetate or 0.25 M pyridine solutions, employed in parallel experiments) was fixed at 25% in volume, a proportion previously found to be optimal for the retention capacity of C_8 columns (Rodriguez et al. 1998). The percentage of acetonitrile and methanol in mobile phase A was varied from 75:0 (v:v) to 0:75 (v:v), and the resolution of acidic chlorophylls measured in each case. The best resolutions for MV and DV pairs of polar chlorophylls were obtained using methanol:acetonitrile (50:25 v:v). For any combination of organic solvents the mobile phases, including pyridinium acetate, always provided better results than those containing ammonium acetate.

Mobile phase B was initially methanol, but the resolution of non-polar chlorophylls (i.e. chl *b*, non-polar chl *c* from *Emiliania huxleyi*, DV chl *a*, and chl *a*) was improved when acetonitrile was added in increasing proportions. This change produced an unexpected increase in retention time that was compensated for by the addition of acetone to increase the solvent strength. The best results were obtained when mobile phase B was acetonitrile:methanol:acetone (60:20:20, v:v:v).

Elution gradient

To optimise the gradient profile, different slopes in the rate of change from mobile phase A to mobile phase B (Δ %B min^{-1}) were evaluated. The mobile phase change rate was kept slow at the beginning of the analysis to ensure a good separation of acidic chlorophylls and the most polar carotenoids. The gradient steepness was then increased to achieve the necessary solvent strength for the elution of non-polar pigments. The optimum gradient and mobile phases for the 3 HPLC systems employed are shown in Table 1 After all other chromatographic conditions were fixed, a further comparison between mobile phase A containing pyridine (Fig. 1A) or ammonium acetate (Fig. 1B) was performed. The pyridine-containing mobile phase A shows better selectivity not only for the anionic (acidic) chlorophylls but also for the group of fucoxanthin-related carotenoids.

Pigment composition of microalgal cultures

The HPLC chromatograms (System 1 and mobile phase B1) of pigment extracts from 10 microalgal cultures are shown in Fig. 2. Most pigments of the species studied have been characterised in the literature (Egeland 1996, Jeffrey et al. 1997a). Table 2 lists the microalgal pigments detected, as well as siphonaxanthin and siphonein standards, in increasing elution order. The resolution for pigment pairs is only indicated when $R_s < 1.40$. Spectral characteristics of pigments in the mobile phase are also included.

Variability in retention time between injections was evaluated using 3 pigments eluting at different regions of the chromatogram: chl c_2 (mean retention time Rt = 11.46 min, standard deviation [SD] = 0.15 min, n = 17, relative standard deviation [RSD] = 1.28 %), diadino-

Table 1. Gradient profile and mobile phase composition employed with different HPLC systems. (System 1: Waters Alliance; System 2: Beckman System Gold; and System 3: Waters 600)

(a) Analytical gradient protocol (HPLC Systems 1 and 2)

Time (min)	A: Methanol:acetonitrile:aqueous pyridine (50:25:25 v:v:v) % A	B1: Methanol:acetonitrile:acetone (20:60:20 v:v:v) % B
0	100	0
22	60	40
28	5	95
38	5	95
40	100	0

(b) Analytical gradient protocol (HPLC System 3)

Time (min)	A: Methanol:acetonitrile:aqueous pyridine (50:25:25 v:v:v) % A	B2: Acetonitrile:acetone (80:20 v:v) % B
0	100	0
18	60	40
22	0	100
38	0	100
40	100	0

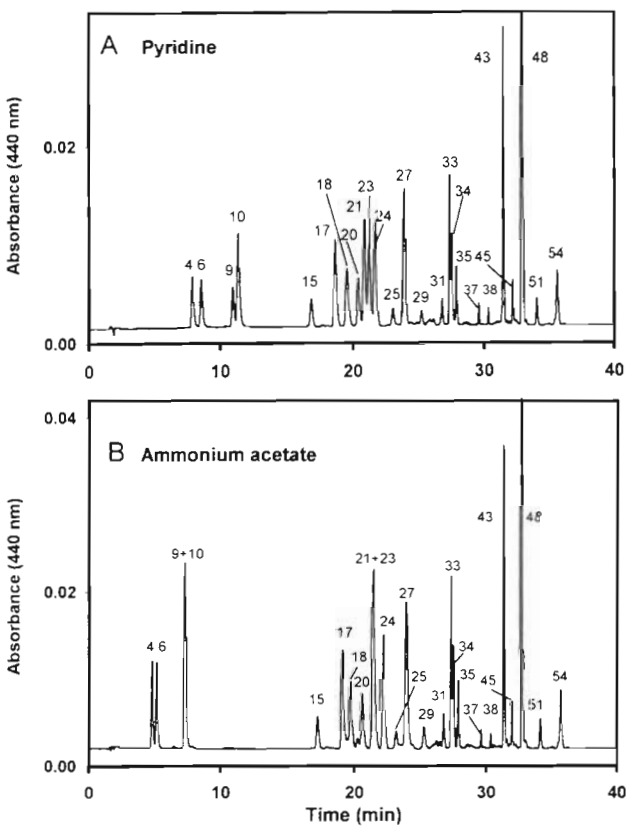


Fig. 1. Effect of (A) pyridine (pyridinium acetate) and (B) ammonium acetate solutions as aqueous components of mobile phase A on pigment resolution. Sample was a mixed methanol extract of *Emiliania huxleyi* NIOZ CH 24, *Micromonas pusilla* CCAP 1965/4 and *Tetraselmis suecica* ICMA. HPLC System 1. Detection by absorbance at 440 nm. Peak identifications as in Table 2

xanthin (mean Rt = 24.08 min, SD = 0.19 min, n = 16, RSD = 0.80%), chl *a* (mean Rt = 33.15 min, SD = 0.13 min, n = 19, RSD = 0.38%).

Several inversions in elution order, compared with that on monomeric C_{18} columns (see Wright & Jeffrey 1997, Table 12.2), are observed. Such is the case for the pigment pairs chl c_3 (peak 4)/chlorophyllide a (Chlide *a*, peak 8); prasinoxanthin (Pras, peak 20)/19'-hexanoyloxyfucoxanthin (Hex-Fuco, peak 24); violaxanthin (Viola, peak 23)/Hex-Fuco (peak 24); diadinochrome (Diadchr, peak 26)/diadinoxanthin (Diadino, peak 27); Diadino (peak 27)/dinoxanthin (Dino, peak 28); Diadino (peak 27)/monadoxanthin (Monado, peak 32); zeaxanthin (Zea, peak 33)/lutein (Lut, peak 34); crocoxanthin (Croco, peak 39)/chl *b* (peak 43); and non-polar chl *c* (peak 45)/chl *a* (peak 48) (see Table 2).

Relevant capabilities of the proposed method can be observed in the chromatogram of selected species. The chromatogram of *Emiliania huxleyi* NIOZ CH 24 (Fig. 2A) illustrates the resolution of acidic chlorophylls, with baseline separation ($R_s \geq 1.40$) for the pigment pairs chl c_3 (peak 4)/MV chl c_3 (peak 6) and MgDVP (peak 9)/chl c_2 (peak 10). The separation of fucoxanthin (Fuco, peak 17) and its acyloxy derivatives: 19'-butanoyloxyfucoxanthin (But-fuco, peak 16, detected in trace amount) and Hex-fuco (peak 24) is also achieved. A carotenoid recently characterised by Egeland et al. (in press) as 4-keto-19'-hexanoyloxyfucoxanthin (4k-Hex-fuco, peak 21), and an unidentified carotenoid (peak 19) eluting before the novel pigment, were baseline resolved. Finally, the non-polar chl *c* from *E. huxleyi* (peak 45), first detected by Nelson & Wakeham (1989), eluted well separated from chl *a* (peak 48).

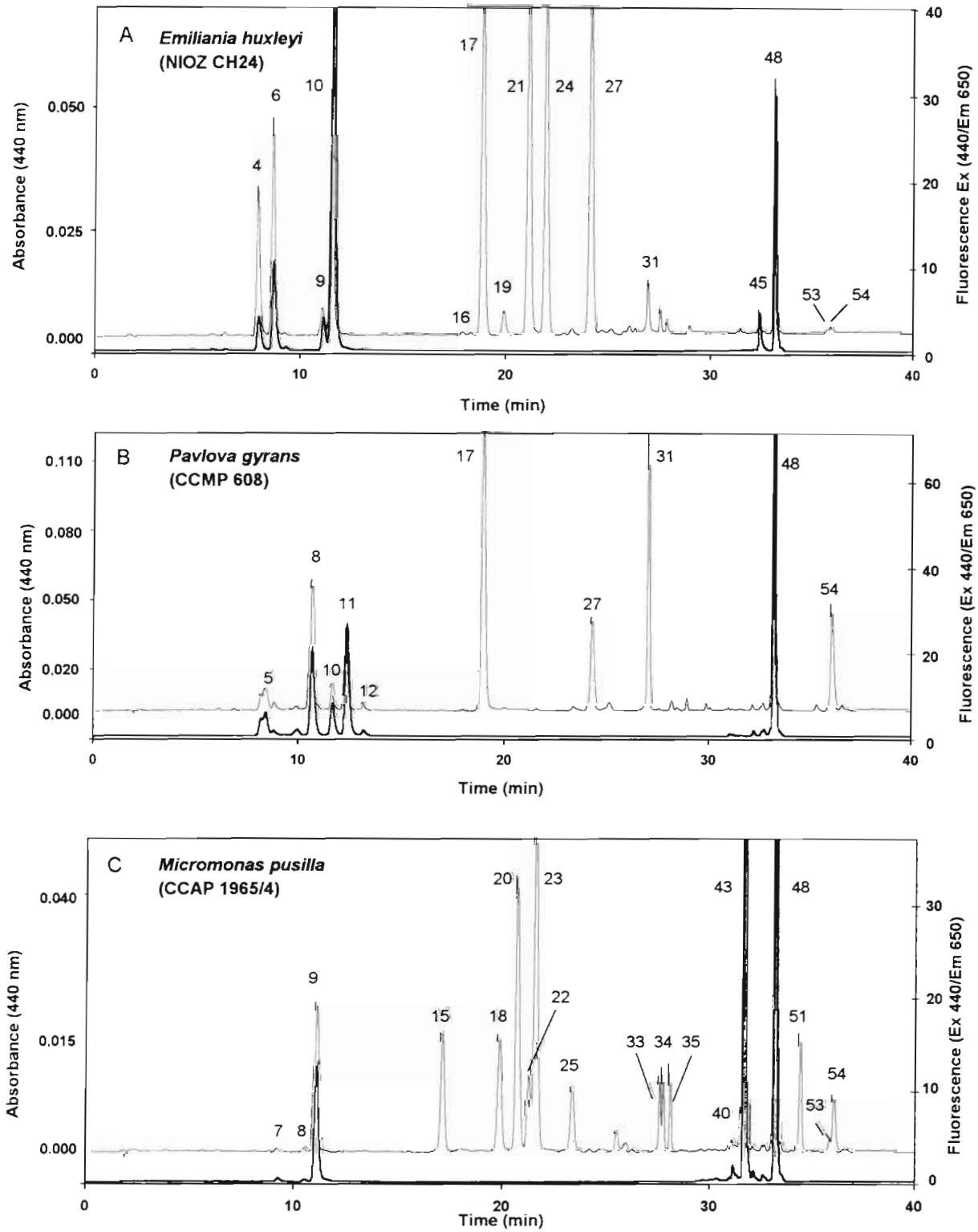


Fig. 2. Pigment composition from the algal cultures (A) *Emiliania huxleyi* NIOZ CH 24, (B) *Pavlova gyrans* CCMP 608, (C) *Micromonas pusilla* CCAP 1965/4, (D) *Pelagococcus subviridis* CCMP 1429, (E) *Skeletonema costatum* Sk-1-CIMA, (F) *Dunaliella tertiolecta* ICMA, (G) *Rhodomonas baltica* ICMA, (H) *Prochlorococcus marinus* CCMP 1375, (the insert shows a chromatogram of a mixture of *P. marinus* and *D. tertiolecta* ICMA obtained by using a modified gradient profile), (I) *Alexandrium minutum* ALLY-IE, and (J) *Tetraselmis suecica* ICMA. HPLC System 1. Detection by absorbance at 440 nm (thin trace) and fluorescence at Ex 440/Em 650 nm (thick trace). Peak identifications as in Table 2

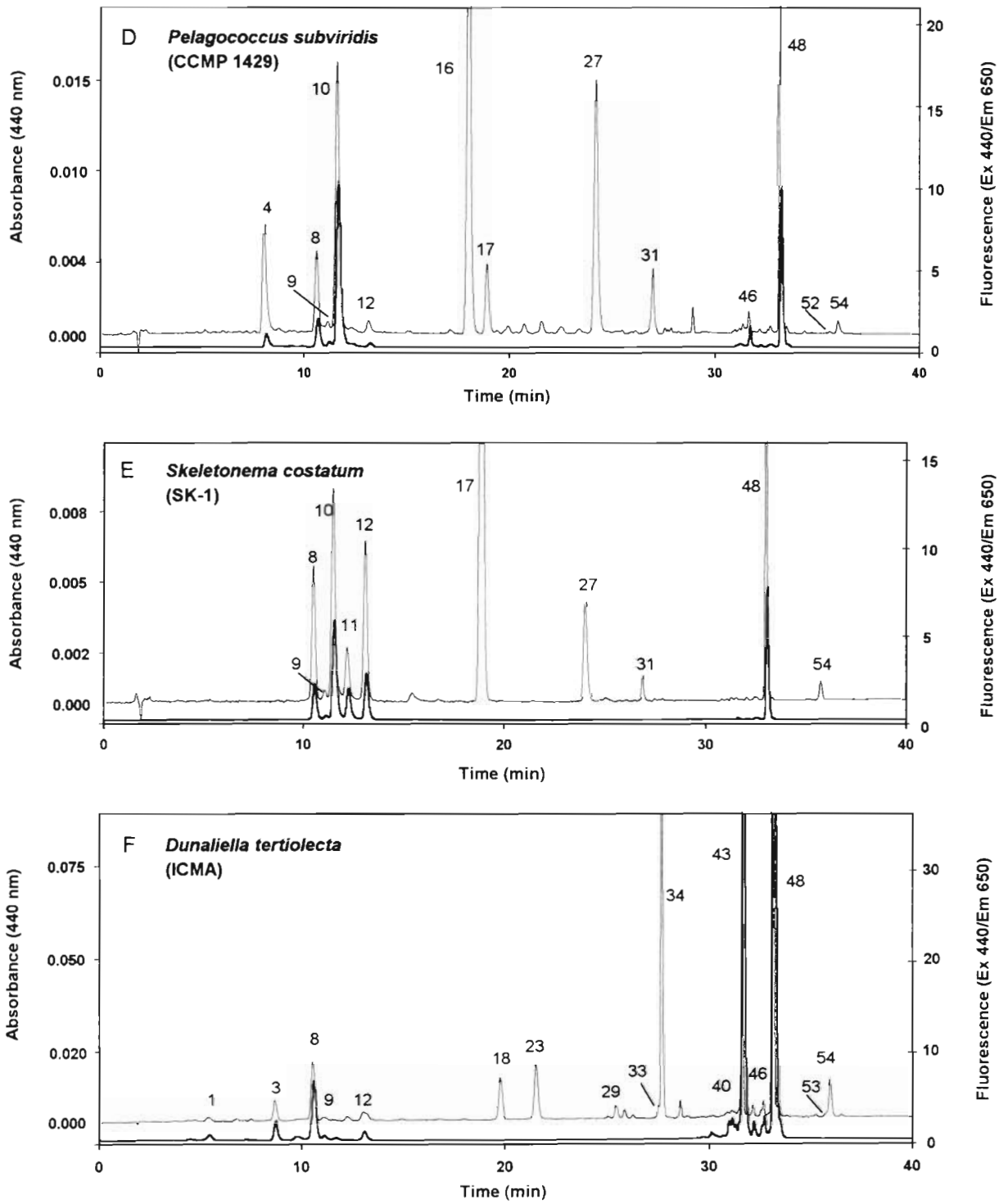


Fig. 2. (continued)

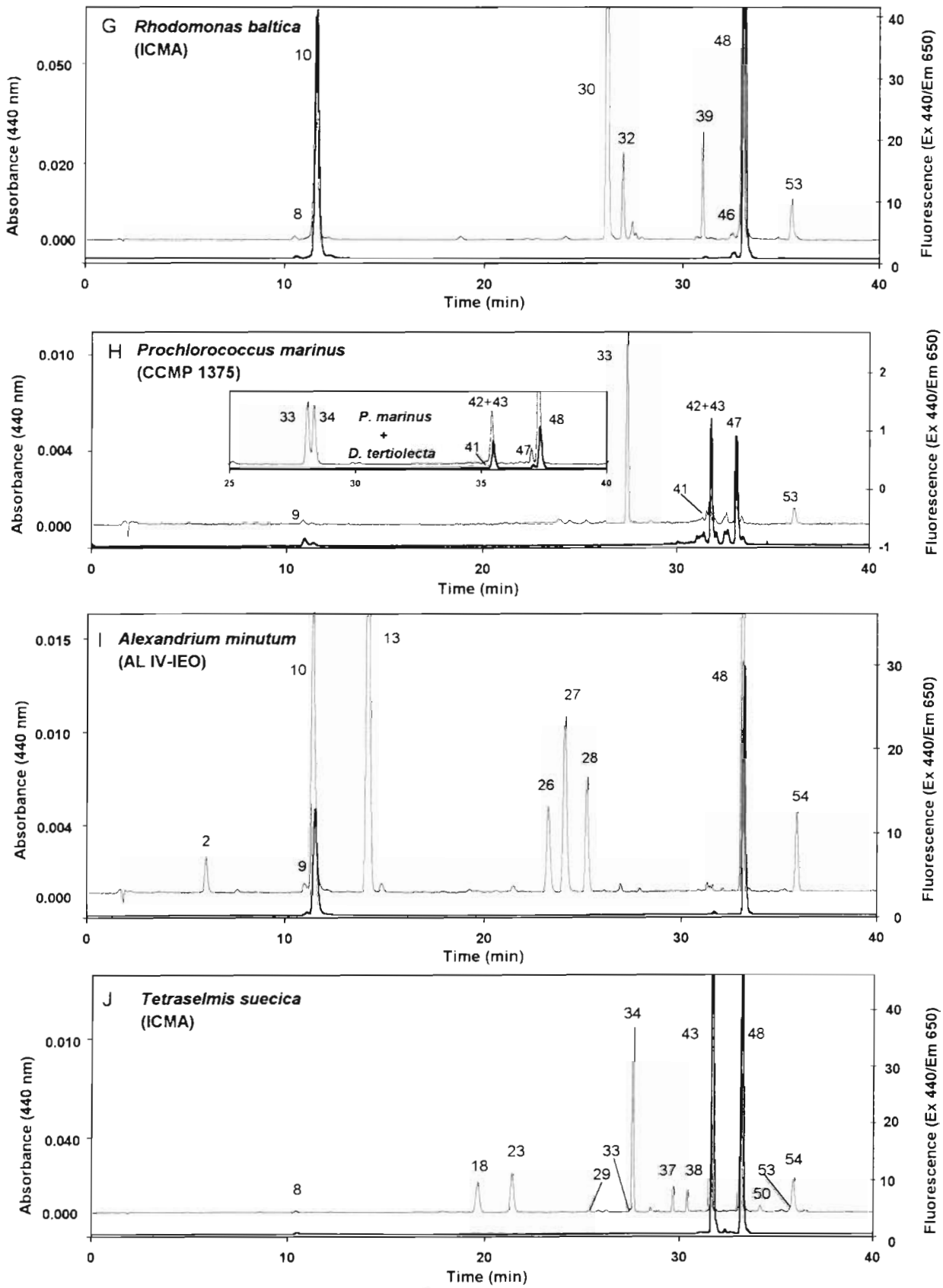


Fig. 2. (continued)

Table 2. Peak identification table. Resolution (R_s) between pigment pairs is indicated when $R_s < 1.40$. Wavelengths given in parenthesis denote shoulders. MgDVP: Mg-3,8-divinyl-pheoporphyrin a_5 monomethyl ester

Peak no.	Pigment	Retention	R_s (peaks)	Maxima in eluant (nm)		
0	(Solvent front)	1.93				
1	Chlorophyllide *b*	5.43		466	601	648
2	Peridininol	6.06			483	
3	Methyl-chlorophyllide *b*	7.19		464	601	647
4	Chlorophyll c_3	7.94		457	588	628
5	Chlorophyll *c* from *Pavlova gyrans*	8.27	0.82 (4/5)	457	586	635
6	MV Chlorophyll c_3	8.66	0.85 (5/6)	449	584	628
7	Unknown chlorophyll *c*	9.20		450	583	631
8	Chlorophyllide *a*	10.46		430	581	663
9	MgDVP	11.01		438	575	627
10	Chlorophyll c_2	11.44		452	583	633
11	Chlorophyll c_1	12.14		448	580	631
12	Methyl-chlorophyllide *a*	13.13		430	581	663
13	Peridinin	14.20			473	
14	Siphonaxanthin	14.76			453	
15	Uriolide	17.03			452	475
16	19'-butanoyloxyfucoxanthin	17.94			446	469
17	Fucoxanthin	18.87			449	
18	9'-cis-neoxanthin	19.62		413	437	465
19	Unknown carotenoid from *Emiliania huxleyi*	19.72	<0.5 (18/19)		446	469
20	Prasinoxanthin	20.46			455	
21	4-keto-19'-hexanoyloxyfucoxanthin	20.92	1.27 (20/21)		447	470
22	Micromonol	20.99	<0.5 (21/22)	(405)	429	454
23	Violaxanthin	21.32	1.08 (22/23)	416	440	470
24	19'-hexanoyloxyfucoxanthin	21.75	1.21 (23/24)		446	469
25	Micromonal	23.24			462	
26	Diadinochrome	23.27	<0.5 (25/26)	(406)	429	457
27	Diadinoxanthin	24.11		(422)	446	476
28	Dinoxanthin	25.22		417	441	470
29	Antheraxanthin	25.38	0.8 (28/29)	(421)	446	474
30	Alloxanthin	26.25		(426)	452	482
31	Diatoxanthin	26.90		(426)	453	481
32	Monadoxanthin	27.07	0.80 (31/32)	(423)	447	476
33	Zeaxanthin	27.49		(426)	453	478
34	Lutein	27.65	0.80 (33/34)	(422)	446	475
35	Dihydrolutein	28.00		(405)	429	454
36	Siphonein	29.37			458	
37	Unknown carotenoid from *Tetraselmis suecica*	29.71		(423)	449	476
38	Unknown carotenoid from *T. suecica*	30.45		(422)	448	476
39	Crocoxanthin	31.11		(422)	447	476
40	Chlorophyll *b* allomer	31.28	0.8 (39/40)	462	598	646
41	Unknown carotenoid from *Prochlorococcus marinus*	31.42		422	447	476
42	DV chlorophyll *b*	31.58	1.02 (41/42)	470	600	648
43	Chlorophyll *b*	31.62	<0.5 (42/43)	462	599	648
44	Chlorophyll *b* epimer	31.87		462	599	650
45	Non-polar chlorophyll *c* from *E. huxleyi*	32.39		455	584	633
46	Chlorophyll *a* allomer	32.63		430	615	662
47	DV chlorophyll *a*	32.83		441	616	665
48	Chlorophyll *a*	33.15		431	617	662
49	Chlorophyll *a* epimer	33.48		430	615	664
50	β,ψ-carotene	34.25		(436)	461	492
51	Unknown carotenoid from *Micomonas pusilla*	34.32	<0.5 (50/51)	(418)	442	470
52	ε,ε-carotene	35.52		420	441	470
53	β,ε-carotene	35.74	0.88 (52/53)	(422)	447	475
54	β,β-carotene	35.95	0.88 (53/54)	(426)	452	477

The resolution of other polar chlorophylls is shown in the chromatogram of *Pavlova gyrans* CCMP 608 (Fig. 2B) where the chl *c*-like pigment (peak 5), first detected in *P. gyrans* by Fawley (1989), Chlide *a* (peak 8), chl c_2 (peak 10) and chl c_1 (peak 11) were baseline separated. Although peak 5 (chl *c*-like pigment) is not symmetric, it is spectrally homogeneous.

Some recently characterised carotenoids from Prasinophyceae (Egeland & Liaaen-Jensen 1995, Egeland et al. 1995) such as uriolide (Uri, peak 15), micromonol (Microl, peak 22), micromonal (Micral, peak 25) and dihydrolutein (Dihydrolut, peak 35) are detected in *Micromonas pusilla* CCAP 1965/4 (Fig. 2C). Although the separation of major peaks Pras (peak 20) and violaxanthin (Viola, peak 23) seems good, other carotenoids are only partially resolved: Microl/Viola (R_s = 1.08), Zea/Lut (R_s = 0.80). An unknown polar chl *c*-like pigment (peak 7), with spectral characteristics similar to chl c_2 (see Table 2), was detected in this strain of *M. pusilla*.

The chromatogram of *Pelagococcus subviridis* CCMP 1429 (Fig. 2D) shows a major peak of But-fuco (peak 16), usually employed as a marker pigment for the class Pelagophyceae (Andersen et al. 1993), eluting ahead of Fuco (peak 17). A minor peak, identified as ε,ε-carotene (εε-Car, peak 52), elutes before the other carotenes.

The chromatogram of the diatom *Skeletonema costatum* Sk-1 (Fig. 2E), whose chlorophyllase activity promotes the conversion of chl *a* (peak 48) into Chlide *a* (peak 8) (Jeffrey & Hallegraeff 1987), shows the separation of this acidic derivative and its methyl ester (peak 12), probably generated during the extraction process using methanol as solvent.

Another species showing high chlorophyllase activity is *Dunaliella tertiolecta* ICMA, whose chromatogram (Fig. 2F) shows the presence of Chlide *b* (peak 1), Chlide *a* (peak 8), and their methyl esters (peaks 3 and 12, respectively). Trace amounts of Zea (peak 33) eluted ahead of Lut (peak 34) achieving a partial resolution (R_s = 0.80). Its characteristic monocyclic β,ψ-carotene (βψ-Car, peak 50), was detected in trace amount eluting before β,ε-carotene (βε-Car, peak 53).

In the chromatogram of *Rhodomonas baltica* ICMA (Fig. 2G) the marker pigment alloxanthin (Allo, peak 30) was baseline resolved from monadoxanthin (Monado, peak 32); and Croco (peak 39) and βε-Car (peak 53) were also detected.

The chromatogram of the cyanobacterium *Prochlorococcus marinus* CCMP 1375 (Fig. 2H) shows MgDVP (peak 9), Zea (peak 33, the major carotenoid), an unknown carotenoid (peak 41, spectrally similar to βε-Car) eluting before a peak containing DV chl *b* (peak 42, the major component) plus chl *b* (peak 43, detected as a minor component eluting at the final part of the DV chl *b* peak), and peaks corresponding to DV chl *a* (peak 47) and βε-Car (peak 53).

A mixture of methanol extracts from *Prochlorococcus marinus* and *Dunaliella tertiolecta* ICMA was used to study the effect of different gradient profiles to resolve the critical pair DV chl *b*/chl *b* (see insert in Fig. 2H). Although the gradient steepness applied at minute 22 was changed from 40–95% B in 6 min (~Δ 10%B min^{-1}, standard conditions) to 40–95% B in 12 min (~Δ 5% B min^{-1}), the pigment pair remained unresolved. However, an improvement in the resolution was observed for the pigment pairs Zea/Lut (from R_s = 0.80 to R_s = 1.08) and DV chl *a*/chl *a* (from R_s = 1.42 to R_s = 1.48).

The chromatogram of the toxic dinoflagellate *Alexandrium minutum* AL1V-IEO (Fig. 2I) shows an inversion in elution order (with respect C_{18} columns) for the pigment pairs: Diadchr (peak 26)/Diadino (peak 27), and Diadino (peak 27)/Dino (peak 28).

Two unknown carotenoids (peaks 37 and 38, Fig. 2J) with similar visible absorbance spectra (see Table 2) were detected in *Tetraselmis suecica* ICMA. Considering both spectral information and chromatographic behaviour the carotenoids were tentatively identified as loroxanthin esters having a different fatty acid composition.

Mixed algal extracts

The performance of the method with mixed culture extracts simulating phytoplankton populations of field samples was also evaluated. Three regions of the resulting chromatograms — polar end, central region and non-polar end — were examined in detail (Fig. 3).

The behaviour of polar chlorophylls can be illustrated by a mixture of *Emiliania huxleyi* NIOZ CH 24 and *Pavlova gyrans* CCMP 608 (Fig. 3A). The high resolution (R_s = 2.31) for the pigment pair chl c_3 (peak 4)/MV chl c_3 (peak 6), allows the separation of chl *c* from *P. gyrans* (peak 5) between them, while Chlide *a* (peak 8) and MgDVP (peak 9) elute baseline separated after them, followed by the pair chl c_2 (peak 10) and chl c_1 (peak 11), also well resolved (R_s = 2.09).

Most of the taxon-specific carotenoids elute at the central part of the chromatogram. This is illustrated when a mixture of methanol extracts from *Emiliania huxleyi* NIOZ CH 24, *Micromonas pusilla*, *Pelagococcus subviridis* CCMP1429 and *Rhodomonas baltica* is analysed (Fig. 3B). Several carotenoids usually employed as marker pigments for different algal classes are separated: Hex-fuco (peak 24) for Prymnesiophyceae, But-fuco (peak 16) for Pelagophyceae, Allo (peak 30) for Crytophyceae, and Pras (peak 20) for Prasinophyceae.

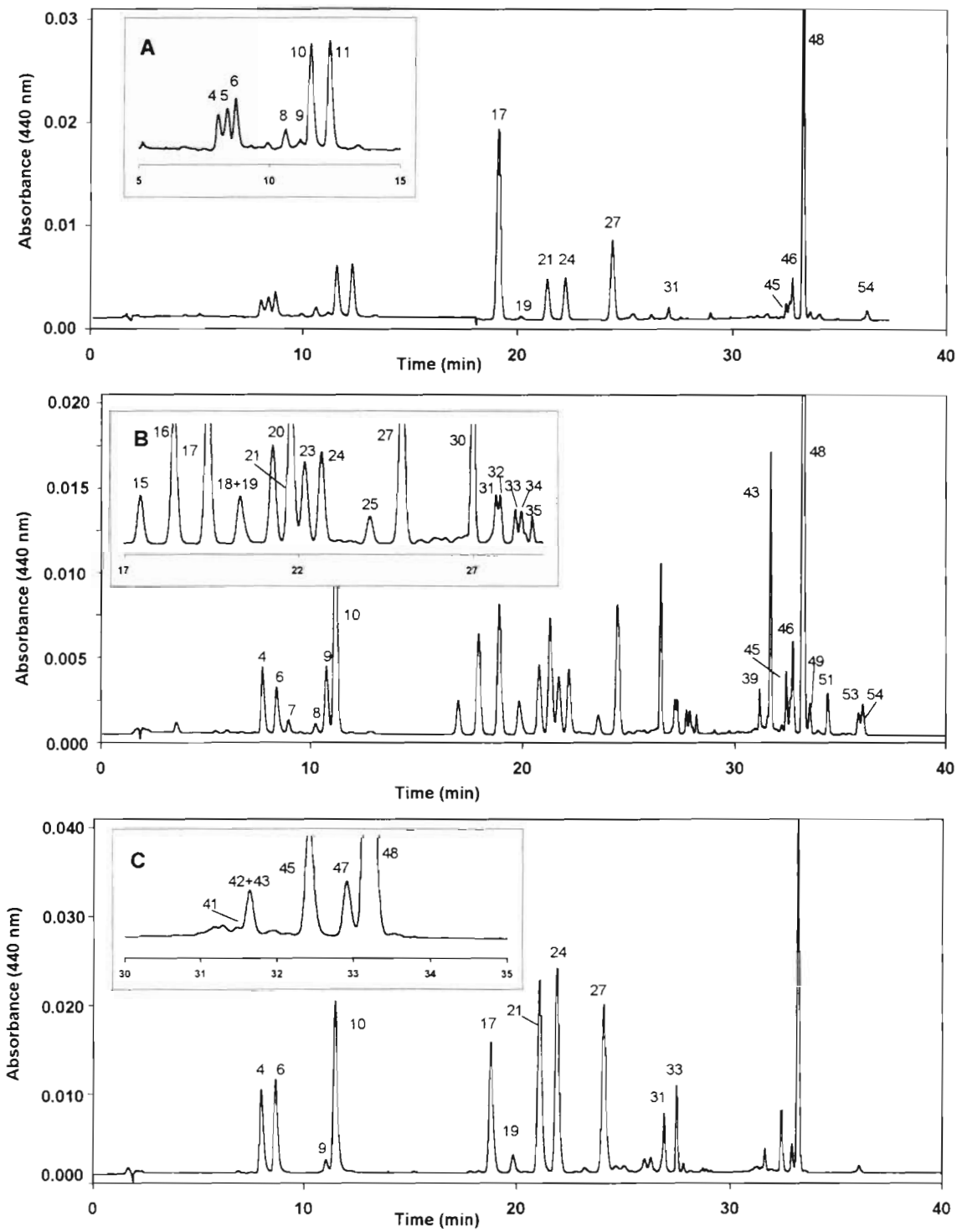


Fig. 3. Chromatograms (HPLC System 1) with inserts covering 3 polarity ranges. (A) Mixed pigment extract from *Emiliania huxleyi* NIOZ CH 24 and *Pavlova gyrans* CCMP 608 (the insert shows the resolution of polar chl *c* pigments, Chlide *a* and MgDVP), (B) mixed pigment extract from *E. huxleyi* NIOZ CH 24, *Rhodomonas baltica* ICMA, *Micromonas pusilla* CCAP 1965/4, and *Pelagococcus subviridis* CCMP 1429 (the insert shows the resolution of major carotenoids used as marker pigments); and (C) mixed pigment extract from *E. huxleyi* NIOZ CH 24 and *Prochlorococcus marinus* CCMP 1375 (the insert shows the resolution of non-polar chl *c* from *E. huxleyi*, DV chl *a* and chl *a*). Detection by absorbance at 440 nm. Peak identification as in Table 2

Finally, the non-polar end of the chromatogram from a mixture of methanol extracts from *Prochlorococcus marinus* (Cyanophyceae) and *Emiliania huxleyi* NIOZ CH 24 (Prymnesiophyceae) (Fig. 3C) shows the coelution of DV chl *b* (peak 42) and chl *b* (peak 43) present in *P. marinus* as trace amounts; the non-polar chl *c* from *E. huxleyi* NIOZ CH 24 (peak 45) is well resolved (Rs > 1.50) from DV chl *a* (peak 47), and DV chl *a* (peak 47) is separated (R_s = 1.47) from chl *a* (peak 48).

Natural samples

The chromatogram of a sample from Gerlache Strait close to the Antarctic Peninsula (Fig. 4A) shows pigments from diatoms: chl c_2 (peak 10), chl c_1 (peak 11) and Fuco (peak 17); and from chlorophytes: Neo (peak 18), Viola (peak 23), chl *b* (peak 43) and an unknown carotenoid (peak 38) tentatively identified as a loroxanthin ester. A carotenoid spectrally similar to loroxanthin was detected co-eluting with Neo (peak 18), as confirmed by the characteristic spectra of both carotenoids observed at initial and final parts of the peak. It is remarkable that the occurrence of Lut (peak 34) was only in trace amounts, since this pigment usually appears in higher amounts associated with chl *b*. Microscopic observations indicated the dominance of *Pyramimonas* sp. (Prasinophyceae) as a major component (M. Varela pers. comm.).

The chromatogram from a sample collected from oligotrophic waters of eastern subtropical North Atlantic (33° 03' N, 21° 16' W) shows a very complex pigment composition (Fig. 4B) including DV chl *a* (peak 47), the marker pigment of the cyanobacterium *Prochlorococcus marinus* (contributing 40% of total chl *a*), Zea (peak 33), DV chl *b* (peak 42) and βε-Car (peak 53). The chromatogram also contains pigments from haptophytes as chl c_3 (peak 4), non-polar chl *c* (peak 45) and Hex-fuco (peak 24), as well as But-fuco (peak 16) and Fuco (peak 17), probably associated with pelagophytes. Other minor pigments such as Uri (peak 15), Pras (peak 20) and the unknown carotenoid (peak 51) associated with prasinophytes (Egeland et al. 1995), Perid (peak 13) associated with dinoflagellates, and Allo (peak 30) associated with cryptophytes, were also identified. At the non-polar end of the chromatogram, DV chl *b* (peak 42) and chl *b* (peak 43) elute together as a single peak, while the non-polar chl *c* from *Emiliania huxleyi* (peak 45) is well resolved (R_s > 1.50) from DV chl *a* (peak 47) and the latter appears well separated (R_s = 1.42) from chl *a* (peak 48).

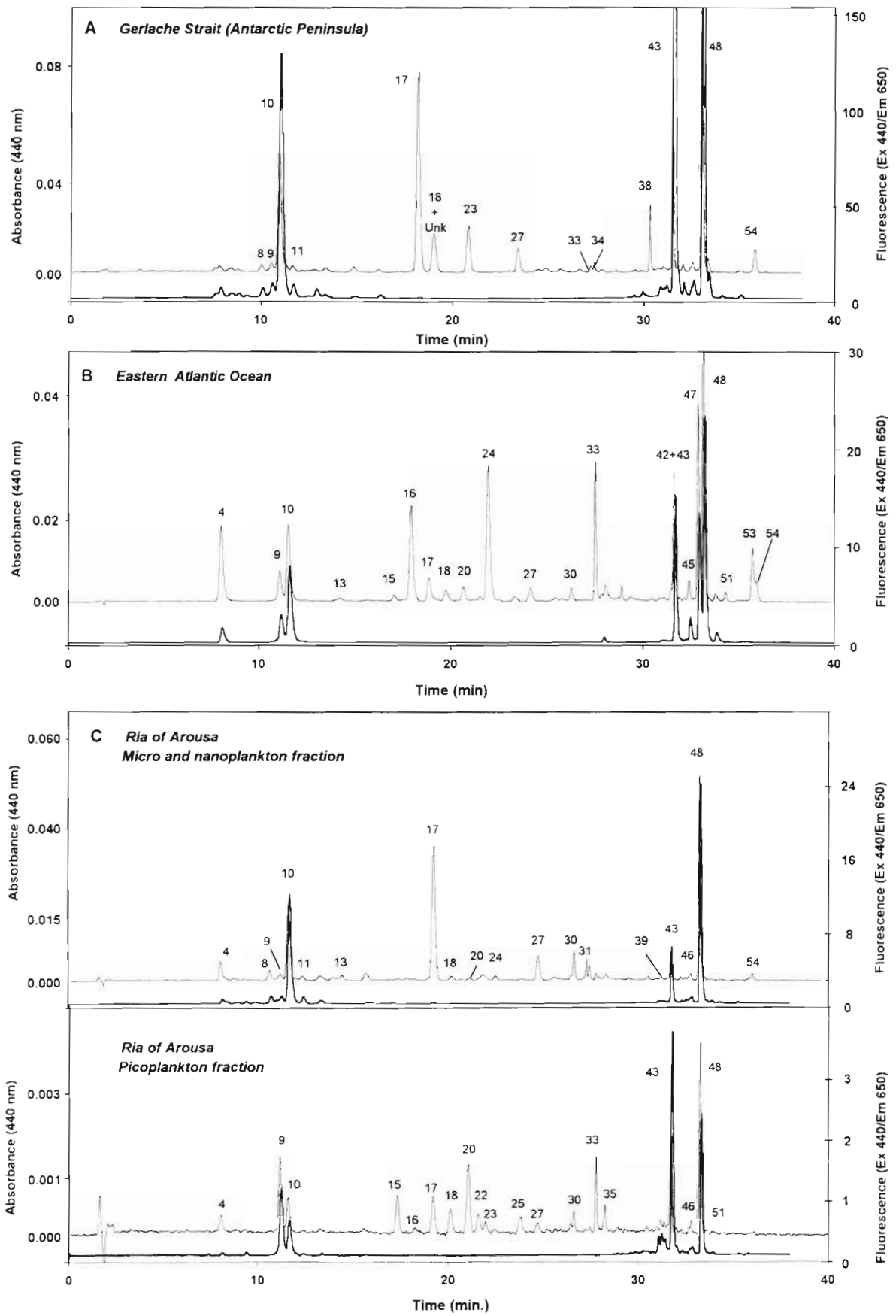
The phytoplankton of a seawater sample collected from Ría of Arousa (Galician coast, NW Spain) was fractionated into 2 size categories. The chromatogram of the nano- and microplankton size-fraction (Fig. 4C, upper trace) shows pigments associated with diatoms: chl c_2 (peak 10) and chl c_1 (peak 11), and Fuco (peak 17) as the major carotenoid. Chl c_3 (peak 4), chl c_2 (peak 10), Fuco (peak 17), Hex-fuco (peak 24) and But-fuco (peak 16) could be related with the algal classes Pelagophyceae and Prymnesiophyceae. The presence of chl *b* (peak 43) and minor peaks of Neo (peak 18) and Pras (peak 20) was associated with the class Prasinophyceae and Perid (peak 13) with Dinophyceae.

The chromatogram of the picoplankton fraction (Fig. 4C, lower trace) shows the dominance of pigments associated with different algal classes such as Prasinophyceae (MgDVP [peak 9], chl *b* [peak 43], uriolide [peak 15], Neo [peak 18], Pras [peak 20], Viola [peak 23], micromonal [peak 22] and dihydrolutein [peak 35]) and Cryptophyceae (Allo [peak 30]). The combined presence of chl c_3 (peak 4), chl c_2 (peak 10), But-fuco (peak 16) and Fuco (peak 17) in samples lacking Hex-fuco (peak 24) and non-polar chl *c* (peak 45) could be attributed to members of the class Pelagophyceae. This pigment diversity reflects the complexity of the eukarotic picoplankton community.

Method transferability

The transferability of the proposed method between low-pressure mixing (HPLC System 1) and high-pressure mixing (HPLC System 2) instruments was checked in our laboratories using the same mixture of algal pigments, operators and chemicals. A good agreement was observed between systems (Fig. 5A,B). However, when the method was transferred to the other low-pressure mixing equipment (HPLC System 3) employed at the CSIRO Marine Laboratories in Hobart, Australia, during a collaborative study, a slight adjustment was required to equal the resolution capacity of HPLC Systems 1 and 2. A modified gradient profile (see Table 1) was applied to correct differences between equipment dwell volumes (4 ml higher than System 1, as informed by the manufacturer). In addi-

Fig. 4. Chromatograms (HPLC System 1) of phytoplankton pigments from seawater samples collected from (A) Gerlache Strait (64° 20' S, 61° 48' W, at 5 m depth) close to the Antarctic Peninsula, (B) eastern subtropical North Atlantic (33° 03' N, 21° 16' W), sample from deep chlorophyll maximum layer (80 m depth); and (C) Ría of Arousa (Galician coast, NW Spain), integrated profile (15 m depth). Pigment composition of micro- and nanoplankton fraction (upper traces) and pigment composition of picoplankton fraction (lower traces). Detection by absorbance at 440 nm (thin trace) and fluorescence Ex 440/Em 650 nm (thick trace). Peak identifications as in Table 2


A Gerlache Strait (Antarctic Peninsula)
B Eastern Atlantic Ocean
C Ria of Arousa
Micro and nanoplankton fraction
Ria of Arousa
Picoplankton fraction
Absorbance (440 nm)
Fluorescence (Ex 440/Em 650)
Time (min)
Time (min.)

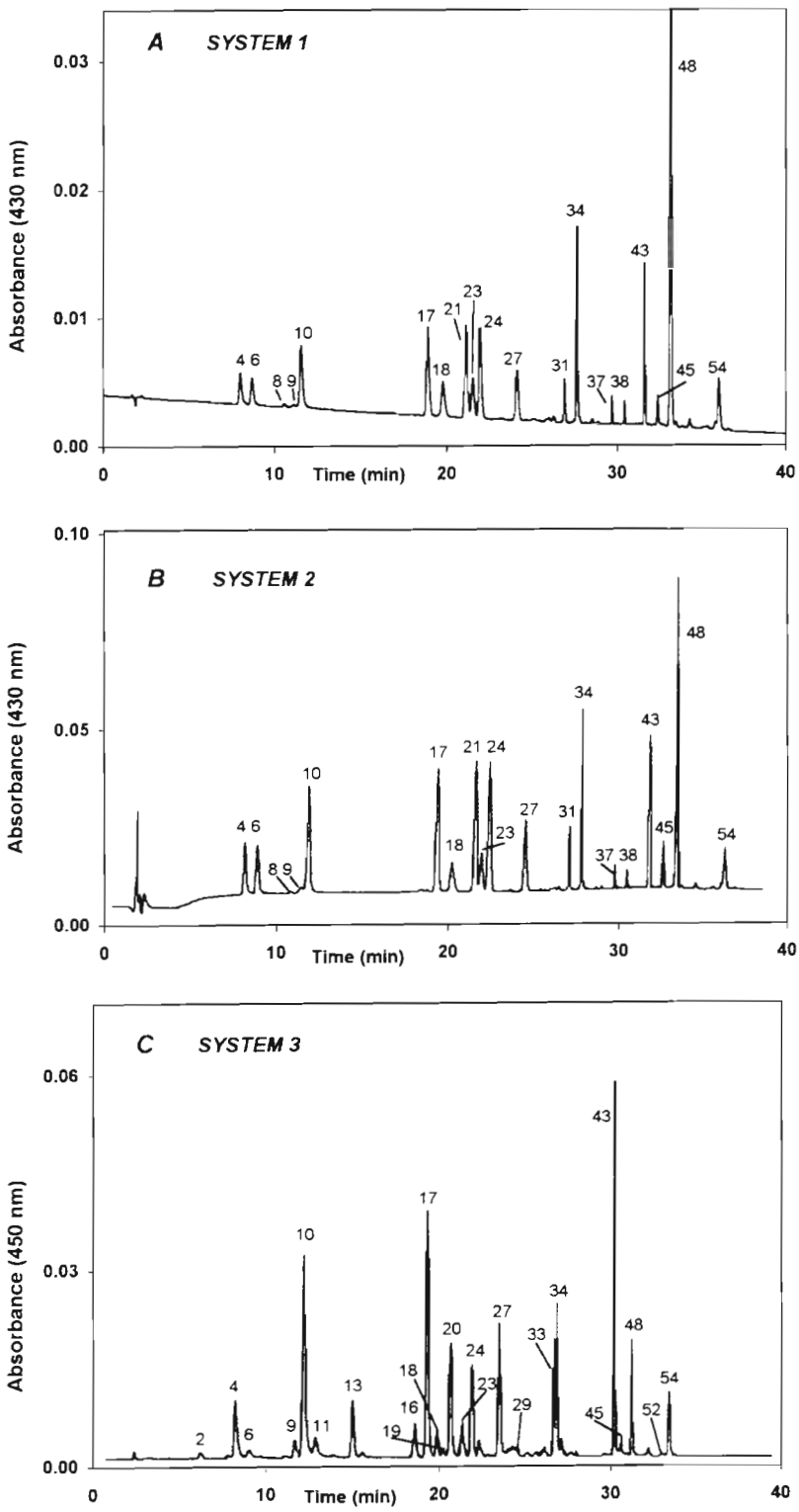


Fig. 5. Method transferability. Chromatograms of a mixed methanol extract from *Emiliania huxleyi* NIOZ CH 24 and *Tetraselmis suecica* ICMA obtained with (A) a low-pressure mixing solvent with low dispersion design (HPLC System 1) and (B) a high-pressure mixing solvent (HPLC System 2). Detection by absorbance at 430 nm. (C) HPLC chromatogram of a mixed methanol extract from the following SCOR reference cultures: *Amphidinium carterae* (CS-212), *Dunaliella tertiolecta* (CS-175), *Emiliania huxleyi* (CS-57), *Pavlova lutheri* (CS-182), *Pelagococcus subviridis* (CS-99), *Phaeodactylum tricornutum* (CS-29), *Porphyridium cruentum* (CS-25) and *Pycnococcus provasoli* (CS-185). HPLC System 3 and mobile phase B2. Detection by absorbance at 450 nm. Peak identification as in Table 2

tion, the methanol of mobile phase B was substituted with acetonitrile (eluent B2). After that, a mixture of selected SCOR reference cultures was analysed and a similar retention time and resolution were obtained (see Fig. 5C).

DISCUSSION

Chromatographic aspects

The advantages of using pyridinium acetate instead of ammonium acetate as a mobile phase additive seem to be based on the fact that the pyridinium ion not only acts as a more hydrophobic ion-pair reagent (increasing the retention of acidic chlorophylls), but also as a real mobile phase modifier, affecting the selectivity towards both neutral (carotenoids and esterified chlorophylls) and charged compounds (dephytylated chl *a* and *b* derivatives and acidic chl *c* pigments). This results in the improved separation of fucoxanthin-related carotenoids, where the pyridine-containing mobile phase allows the resolution of 4-k-Hex-fuco (peak 21) from Viola (peak 23). Similarly, polar chlorophylls show an increase in retention time and a better resolution between DV/MV chl *c* pigment pairs. A possible explanation for this effect could rely on π-π interactions established between the aromatic ring of pyridine and the aromatic chlorophyll macrocycles or the polyene system in the carotenoids.

Different mobile phase combinations can be used if the method is to be applied to different kinds of samples. For example, the mobile phase B2 was optimum for the pigment analysis of Haptophyta (Zapata et al. unpubl.).

About method transferability we stress that different HPLC instruments, even employing the same gradient mixing principle (high or low pressure systems), could have different dwell volumes, so any HPLC method developed for one system may require slight changes to provide a similar performance on another system.

One of the main features that a method should have is the capacity of being reproduced by different analysts, laboratories, columns, instruments and reagents (Snyder et al. 1997). Methods based on ternary elution gradients cannot be reproduced on a 2-pump high-pressure gradient mixing system, equipment which is still very popular and widespread. The binary gradient method proposed can be implemented both in high and low pressure mixing systems (Fig. 5).

From the method development stage to routine analysis of cultures and natural samples, four C_8 Symmetry columns (belonging to different lots) have been used with a remarkable reproducibility. The particle size (3.5 µm) of this column seems to be a good compromise between the efficiency of 3.0 µm particle size and the lower back pressure of 5 µm particle size C_8 columns. The separation capability of the proposed method could vary if other monomeric C_8 columns are employed. The selection of a column has to be an informed decision, based on the knowledge of stationary phases properties such as bonding chemistry, pore size, surface area, coverage and carbon load. The column used in this study was selected after considering the results of a previous study (Rodríguez et al. 1998) in which we evaluated the performance of 4 commercial C_8 monomeric columns for resolution of MV and DV pairs of chl *c*s.

Separation of polar and non-polar chlorophylls

The proposed method allows the simultaneous resolution of pigments belonging to the chl *c* family and Chlides *a* and *b*, in the same chromatographic run in which carotenoids, non-polar chlorophylls and carotenes are analysed. This ability deserves special emphasis, as the detailed study of distribution patterns of chl *c* pigments into several taxonomic groups has been hampered due to previous analytical limitations.

The simultaneous separation of polar and non-polar chl *c* pigments was previously achieved employing polymeric C_{18} columns in which their special shape-selectivity governed the elution order (Garrido & Zapata 1997). On these columns divinyl forms that have planar structures (e.g. chl c_2 and chl c_3) elute after their monovinyl counterparts (e.g MV chl c_3 and chl c_1), whose molecules are more voluminous. In the proposed method the elution order seems to be controlled by subtle differences in the overall polarity of the molecule, eluting the slightly more polar DV chl forms before their MV counterparts.

Separation of carotenoids

Besides separating polar and non-polar chlorophylls, the method shows a good resolution towards carotenoids. This includes the separation of Fuco and its well-known acyloxy derivatives (But-fuco and Hex-fuco) from the novel 4-keto-19'-hexanoyloxyfucoxanthin (Egeland et al. 1999), first detected in *Emiliania huxleyi* using polymeric C_{18} columns (Garrido & Zapata 1998). The presence in this carotenoid of a novel end-group with 3 oxygenated functions (-keto, -hydroxy- and -epoxy groups), explains why this compound elutes before Hex-fuco. Studies on the distribution pattern of 4-k-Hex-fuco into several taxonomic groups, and natural samples, are currently in progress (Zapata et al. unpubl.).

Natural samples

The proposed method improves the resolution of a wide range of pigments present in field samples, achieving a baseline separation (R_s > 1.40) of polar chlorophylls (including Chlide *a* and *b* and the diverse family of chl *c* pigments), non-polar chlorophylls (except the critical pair DV chl *b*/chl *b*), and excellent resolution of many taxonomically significant carotenoids in a reasonable run time of 36 min.

The coelution of DV chl *b*/chl *b* does not hamper the identification of *Prochlorococcus marinus* in seawater samples, since DV chl *a* is well separated from chl *a*. In addition, DV chl *b* is a less specific marker pigment for *P. marinus* since some cultured isolates are able to synthesise chl *b* as a response to high irradiance (Moore et al. 1995). A recent study has identified surface ecotypes of *P. marinus* with low DV chl *b*/DV chl *a* ratio co-existing, at intermediate water depths, with deep-water ecotypes characterised by high DV chl *b*/DV chl *a* ratio (Moore et al. 1998). The coelution of chl *b* and DV chl *b* in a single peak could be a drawback if the contribution of algal classes as Chlorophyceae, Euglenophyceae and Prasinophyceae has to be evaluated when DV chl *a*-containing cyanobacteria are present.

A matrix factorisation program (Chemical taxonomy: CHEMTAX) recently developed by Mackey et al. (1996) is able to resolve such limitations. It exploits the capability of the chemotaxonomic approach to infer the contribution of different algal groups to natural phytoplankton assemblages. This approach has been successfully applied to HPLC pigment data obtained from field samples (Wright et al. 1996, Mackey et al. 1998, Pinckney et al. 1998), allowing the quantitative estimation of algal class abundance from marker pigments.

The combination of new HPLC methods, able to separate additional marker pigments, and the new generation of mathematical tools for interpreting the HPLC pigment data, will provide invaluable information about the variability of phytoplankton populations from different oceanic regions.

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