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High performance liquid chromatographic analysis of phytoplankton pigments using a C_{16}-Amide column

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ABSTRACT

In this study, a reverse-phase HPLC method incorporating a ternary solvent system was developed to analyze most polar and non-polar chlorophylls and carotenoids present in phytoplankton. The method is based on an RP-C_{16}-Amide column and provided excellent peak resolution of most taxonomically important pigments and an elution profile different than C_8 or C_{18} columns provide. Analysis of mixed pigment standards, extracts of phytoplankton monocultures, and field samples showed that this method was able to resolve more than sixty pigments, ranging from very polar acidic chlorophylls to the non-polar hydrocarbon carotenes in less than 36 min. This included chlorophylls c_1, c_2 and c_3, divinyl chlorophylls *a* and *b*, the carotenoids lutein and zeaxanthin and some recently discovered pigments. The ability of this method to resolve divinyl chl *b* from monovinyl chl *b* and divinyl chl *a* from monovinyl chl *a* is particularly important for the quantification and identification of the marine cyanobacteria Prochlorococcus spp. in oceanic waters. The described protocol is sensitive and reproducible and can be used to assess the distribution and dynamics of major phytoplankton groups in marine and freshwater ecosystems.

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1. Introduction

Analysis of phytoplankton pigments by high performance liquid chromatography (HPLC) has been increasingly used to determine the composition and relative biomass of phytoplankton taxonomic groups as an alternative to microscopic analysis of phytoplankton [1–6]. The development of a single method that separates the chlorophylls and carotenoids found in marine phytoplankton has been challenging because phytoplankton pigments are present in natural samples as complex mixtures covering a wide range of structures and polarities. Moreover, some chlorophylls have very similar structures that makes them difficult to separate by most available HPLC techniques.

A review by Jeffrey et al. [7] lists a number of HPLC methods that have made significant improvements in the separation of chlorophylls and carotenoids. One of these methods was particularly significant because it could resolve more than 50 pigments in less than 30 min, including pigments of chemotaxonomic importance that traditionally were difficult to separate [8]. This method was based on an RP-C_{16}-Amide column and a ternary gradient system, and is recommended by the Scientific Committee on Oceanic Research (SCOR) for separation of phytoplankton pigments in oceanic waters. However, even this method is unable to separate important pigment pairs including chlorophyll c_1 (chl c_1) and chlorophyll c_2 (chl c_2), monovinyl (MV) and divinyl (DV) chlorophyll *a* (chl *a* and DV chl *a*, respectively), and MV and DV chlorophyll *b* (chl *b* and DV chl *b*). Divinyl chl *a* and *b* are the major photosynthetic pigments found in the marine prokaryote *Prochlorococcus marinus* and contribute substantially to the phytoplankton biomass in subtropical and tropical oceanic waters [9,10]. It is recommended that DV chl *a* and *b* be chromatographically separated from chl *a* and *b* to avoid overestimation of total chl *a* by satellite ocean color measurements [10–12].

New HPLC methods have been recently developed that combine either binary or ternary solvent systems with reversed-phase C_{18} columns [13–22] or C_8 columns [10,23–27]. Methods based on polymeric C_{18} columns improved the separation of structurally similar pigments such as chl c_1, chl c_2, and Mg-3,8-divinyl-pheoporphyrin a_5 monomethyl ester (MgDVP) [15,18,20,22], whereas methods based on monomeric C_8 columns were more successful in resolving DV chl *a* from chl *a* [10,26,27]. An HPLC technique developed by Zapata et al. [26] uses a C_8 column and a pyridine-containing mobile phase to resolve MV and DV pairs of polar chlorophylls and DV chl *a* from chl *a*. This technique also

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provides very good resolution of marker carotenoids. Despite the great selectivity of some of these methods towards carotenoids and chlorophylls, very few methods have been able to completely resolve both DV chl *a* and *b* from their MV analogues in a single analysis. Moreover, most methods are still unable to separate some important pairs of polar pigments such as chlorophyll *c* pigments and the polar derivatives of chl *a* and *b*, or failed to resolve some taxon-specific carotenoids.

The Discovery RP-Amide C_{16} column (Supelco, Bellefonte, PA), is a palmitamidopropylsilane bonded phase column designed primarily for analysis and purification of pharmaceutical products. The amide functional group gives this column some unique properties which include less hydrophobicity than C_{18} columns, compatibility with highly aqueous mobile phases, excellent retention and resolution of polar compounds, and different selectivity and elution orders than commonly used C_8 and C_{18} columns provide. These column properties should provide faster pigment analysis and improved separation of the more difficult phytoplankton pigments.

In the present study, we developed a rapid and reproducible HPLC protocol using a C_{16}-Amide column and a ternary gradient system containing aqueous ammonium acetate which is suitable for the separation and identification of a wide range of pigments found in marine phytoplankton, including DV chl *a* and *b* and some recently discovered pigments.

2. Materials and methods

2.1. Algal cultures

The algal cultures employed in this study have well-characterized pigment composition and were selected based on recommendations of Wright et al. [8]. Some alternative species available at our laboratory were also chosen for method development or for extraction of pigment standards. All cultures were purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, ME, and grown at the USEPA Atlantic Ecology Division Laboratory in Narragansett, RI, according to CCMP recommendations. The phytoplankton species include *Emiliania huxleyi* CCMP 1949 (Prymnesiophyceae), *Isochrysis galbana* CCMP 1323 (Prymnesiophyceae), *Chaetoceros muelleri* CCMP 1316 (Coscinodiscophyceae), *Micromonas pusilla* CCMP 1545 (Prasinophyceae), *Rhodomonas salina* CCMP 1170 (Cryptophyceae), *Pelagococcus subviridis* CCMP 1429 (Pelagophyceae), *Dunaliella tertiolecta* CCMP 1320 (Chlorophyceae), *Amphidinium carterae* CCMP 1314 (Dinophyceae), and *Prochlorococcus marinus* CCMP 1375 (Cyanophyceae). The selected species contained the most significant pigments found in marine phytoplankton. Algal cultures were harvested during log-phase by filtering an aliquot through 25 mm GF/F filters under vacuum. The filters were wrapped in aluminum foil and stored at −80 °C for subsequent pigment extraction and analysis.

2.2. Pigment standards

Most pigment standards were obtained from algal monoculture extracts by isolating and purifying the individual pigments according to the procedures described by Jeffrey [28] and Repeta and Bjørnland [29]. The pigment abbreviations used in this paper are those reported by SCOR. Siphonoxanthin (siphx) and siphonoein (siphn) were isolated from *Codium fragile* ssp. *tomentodoides* (Chlorophyceae). The pigments chl *a*, chl *b*, β,ε-carotene (β,ε-car) and β,β-carotene (β,β-car) were purchased from Sigma-Aldrich (Sigma), St. Louis, MO. Phaeophytin *a* (phytin *a*) and phaeophytin *b* (phytin *b*) were obtained by acidification of chl *a* and *b* solutions, respectively. Chlorophyllide *a* (chlide *a*) was prepared by enzymic de-esterification of chl *a*. Standards were quantified spectrophotometrically using appropriate extinction coefficients and then stored in the dark at −80 °C in amber glass bottles. DV chl *a* and *b* standards were purchased from Horn Point Laboratory, University of Maryland (Cambridge, MD). A spectral library was created by running several mixtures of pigment standards and algal monocultures and extracting the spectrum of each individual pigment.

Table 1
HPLC ternary solvent system program.

Time (min)	Flow rate (ml/min)	%A	%B	%C	Conditions
0	1.00	80	0	20	Injection
7	1.00	8	72	20	Linear gradient
11	1.00	5	77	18	Linear gradient
19	1.00	13	85	2	Linear gradient
30	1.00	20	80	0	Linear gradient
34	1.00	40	60	0	Linear gradient
36	1.00	80	0	20	Linear gradient

Solvents: A = 100% methanol, B = 100% acetonitrile, C = 100% aqueous ammonium acetate (0.5 M).

2.3. Field samples

Seawater samples were collected from the surface waters of Narragansett Bay, RI. The samples were pre-filtered with a 300 μm Nitex screen into one liter bottles to remove large zooplankton and particles, and transported on ice and in the dark to the laboratory. In the laboratory the samples were immediately filtered through a 25 mm GF/F glass fiber filter, wrapped in aluminum foil and stored at −80 °C for pigment extraction.

2.4. Pigment extraction

Frozen filters from algal cultures and field samples were briefly thawed, cut into small pieces and placed into small glass tubes. Three milliliters of cold acetone were added to each tube and the tubes were placed in ice. All tubes were then sonicated individually for 2 min with a Sonifier 450 ultrasonic probe (Branson Ultrasonics, Danbury, CT). Extracts were centrifuged and then filtered through a 0.45 μm Acrodisc 3 CR PTFE filter to remove small glass fiber particles and cellular debris. Prior to injection, 150 μl of each acetone extract or pigment standard were mixed with 150 μl of ammonium acetate solution (0.5 M) to improve the resolution and peak shape of polar pigments. Injection volume was 200 μl.

2.5. High performance liquid chromatograph

The HPLC system used was a Waters 2690 Alliance separations module with a 996 photodiode array detector and a 474 scanning fluorescence detector. A Waters Millennium 32 chromatography manager was used for acquisition and treatment of data. Chromatographic separations were carried out with a Supelco Discovery C_{16}-Amide column (150 mm × 4.6 mm, 5 μm particle size) maintained at 30 °C and preceded by a guard column of the same material. The solvent system consisted of methanol (solvent A), acetonitrile (solvent B) and 0.5 M ammonium acetate solution (solvent C). All solvents were HPLC grade. The gradient system used is shown in Table 1. For overnight or long term storage the column was kept in acetonitrile.

2.6. Pigment identification

Pigments were detected with the diode-array absorbance signal set at 440 and 450 nm. Chlorophylls were also detected with

the fluorescence detector (ex: 440 nm, em: 660 nm). Identification was made by comparing the retention times and spectral characteristic of sample peaks (350–700 nm) with those of authentic standards or published data. Resolution between closely eluting pigments was quantified based on retention times and peak widths using the equation $Rs = 2\Delta t(Wa + Wb)^{-1}$, where Δt is the difference in retention times and Wa + Wb is the sum of the peak widths at the baseline [14,30]. Peak pairs with resolution as low as 1.0 were considered quantifiable. A resolution of 1.5 or greater indicated baseline separation [14,31].

3. Results

3.1. Mobile phase and elution gradient

The ternary solvent system used in this study consisted of 100% methanol as eluant A, 100% acetonitrile as eluant B, and 100% aqueous ammonium acetate (0.5 M) as eluant C. The gradient conditions used for separation of polar pigments are shown in Table 1. An initial seven minute gradient from 80% A to 72% B improved the resolution of polar chlorophylls (c_1, c_2, and c_3), while the rate of change of solvent C (aqueous ammonium acetate) remained very slow for the first 11 min to optimize the retention capacity of the C_{16}-Amide column and also improve the separation of early eluting chlorophylls and carotenoids. The separation of most carotenoids and non-polar chlorophylls was better when the percentage of acetonitrile remained considerably higher than the percentage of methanol. Improvements in the separation of DV chl *a* and *b* from their MV analogues were particularly noteworthy. Zapata et al. [26] had also found an improvement in the resolution of non-polar chlorophylls when acetonitrile was used in increasing proportions. With the C_{16}-Amide column, the high proportion of acetonitrile produced such a great increase in the retention times of MV and DV chl *a* and *b*, that the elution order of these chlorophylls changed relative to the non-polar carotenoids β,ε-car and β,β-car. Methanol was gradually increased to provide the necessary solvent strength for elution of the non-polar pigments in less than 36 min. By manipulating the proportion of acetonitrile and methanol, the elution order and resolution of the non-polar chlorophylls and carotenoids were optimized. The optimal resolution for DV chl *a* and *b* occurred when β,ε-car and β,β-car eluted between DV chl *b* and chl *a*.

3.2. Pigment standard mixtures

Mixtures of pigment standards were used to evaluate the gradient conditions for separation of important pigments. These test mixtures had the advantage of eliminating interferences from unknown or less important peaks in the initial stages of method development, and helped identify resolution problems in some groups of pigments. The elution gradient was optimized where poor separation was observed to achieve a resolution of at least one or greater. The pigments were chosen by degree of importance with regard to the algal class diversity and abundance [27]. Twenty-eight pigments were selected for the test mixtures, including a few secondary carotenoids and derivatives of chl *a* and *b*.

A chromatogram of a standard mixture with all 28 pigments is shown in Fig. 1. The resolution achieved for some taxonomically important chlorophylls and carotenoids, such as the pairs chl c_1/chl c_2 (Rs = 1.2), phaeophorbide *a* (phide *a*)/peridinin (perid) (Rs > 1.5), siphx/19′-butanoyloxy fucoxanthin (but-fuco) (Rs > 1.5), fucoxanthin (fuco)/9′-*cis*-neoxanthin (neo) (Rs > 1.5), prasinoxanthin (pras)/violaxanthin (viola) (Rs = 1.1), lutein (lut)/zeaxanthin (zea) (Rs = 1.3, Rs > 1.5), and β,ε-car/β,β-car (Rs > 1.5) which traditionally have been more difficult to separate, was particularly significant. Table 2 lists all the pigments found in the phytoplankton species and standard mixtures in increasing elution order along with their respective SCOR abbreviations and UV–vis spectral characteristics in the HPLC eluant. The resolution between peaks was determined from mixtures of algal extracts. Lycopene was not detected in any samples and chlorophyll *b*′ may co-elute with chlorophyll *a* allomer when present. The co-elution of chlorophyll *b*′ and chlorophyll *a* allomer could be further investigated using spectral data if desired.

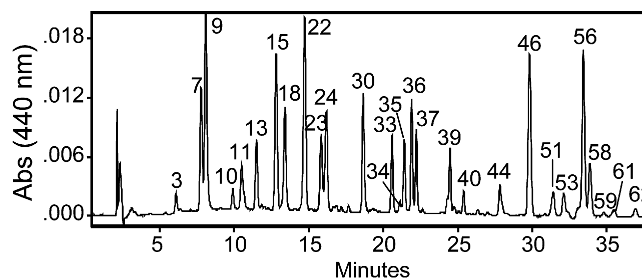

Fig. 1. HPLC chromatogram of a mixture of pigment standards. Peak numbers correspond to pigments listed in Table 2.

3.3. Pigment analysis of algal monocultures

Pigment signatures from nine well-characterized species of phytoplankton representing eight algal classes are shown in Fig. 2. The excellent peak resolution found in the chromatograms of the selected species contributed to an easy identification of most pigments, including some recently discovered novel pigments (micromonol (microl) and micromonal (micral)) [32–34].

The chromatogram of the prymnesiophyte *Emiliania huxleyi* (Fig. 2a) shows the resolution of polar chlorophylls. Chlorophyll c_3 (chl c_3) (4) was quantifiably resolved from the phytol free chl *a* derivative chlide *a* (3) (Rs > 1.1), while MV chl c_3 (5) coeluted with chl c_3 as a single peak. Mg-3,8-divinyl pheoporphyrin a_5 monomethyl ester (MgDVP) (8) was found coeluting on the shoulder of chl c_2 (9). The carotenoids fuco (15) and 19′-hexanoyloxyfucoxanthin (hex-fuco) (22) achieved complete resolution (Rs > 1.5). Two carotenoids (peaks 16 and 19) eluting between fuco and hex-fuco and a doublet eluting after hex-fuco are baseline separated. Peak 16 was tentatively identified as 19′-pentanoyloxyfucoxanthin (pent-fuco) and peak 19 was identified as 4-keto-hexanoyloxyfucoxanthin (4k-hex-fuco) based on their spectral characteristics [35]. The doublet eluting after hex-fuco at 15.86 min and 16.02 min has spectral characteristics similar to that of *cis*-19′-hexanoyloxyfucoxanthin [35]. We suspect the peak coeluting with chl *a* (56) is chlorophyll c_2 monogalactosyldiacylglyceride ester (chl c_2-MGDG) (55), based on spectral characteristics similar to that of published data [36]. Separation of the hydrocarbons β,ε-car (51) and β,β-car (53) was also achieved with the two carotenoids eluting ahead of chl *a*. The non-polar chlorophyll *c*-like pigment reported in *Emiliania huxleyi* [19,37] was not detected in this chromatogram.

The chromatogram of the prymnesiophyte *Isochrysis galbana* (Fig. 2b) is dominated by the carotenoids fuco (15) and diadinoxanthin (diadino) (30). MgDVP was not detected and was probably coeluting with chl c_2 under these conditions. Two unknown carotenoids (41, 47) (λ_{max} 463, 448 nm) and trace amounts of a non-polar chlorophyll *c*-like pigment (54), completely resolved from chl *a*, were detected. We suspect this is chl c_1-galactoglyceride ester. Chl c_2-MGDG was not detected, and was most likely coeluting with chl *a* under the conditions employed as was found in *Emiliania huxleyi*.

The chromatogram of *Chaeotoceros muelleri* (Fig. 2c) has the typical pigment composition of diatoms. Chlorophylls c_1 (7) and c_2 (9)

Table 2
Elution order of pigments, SCOR abbreviation, resolution between peaks and visible absorption.

Maxima in HPLC Eluant

Peak number	Pigment	SCOR Abbreviation	Rs[a] (peaks)	Retention time (min)	Absorption maxima (nm)		
1	Chlorophyllide *b*	Chlide *b*		4.03	469	602	649
2	Peridininol	Peridininol		5.57		478	
3	Chlorophyllide *a*	Chlide *a*		6.09	432	617	665
4	Chlorophyll c_3	Chl c_3	1.1 (3/4)	6.33	452	586	626
5	Monovinyl chl c_3	MV chl c_3		6.40	451	585	626
6	P-457	P-457	0.7 (5/6)	6.57		462	
7	Chlorophyll c_1	Chl c_1		7.78	440	578	630
8	Mg-3,8-divinyl pheoporphyrin a_5 monomethyl ester	MgDVP	0.8 (7/8)	7.95	439	576	628
9	Chlorophyll c_2	Chl c_2	<0.5 (8/9)	8.07	446	582	631
10	Phaeophorbide *a*	Phide *a*		9.91	410	607	664
11	Peridinin	Perid		10.48		475	
12	Peridinin isomer	Perid isom		11.49		467	
13	Siphonaxanthin	Siphx	<0.5 (12/13)	11.50		450	
14	19′-Butanoyloxyfucoxanthin	But-fuco		11.91		446	471
15	Fucoxanthin	Fuco		12.77		449	465
16	19′-Pentanoyloxyfucoxanthin	Penta-fuco		13.10		446	468
17	Uriolide	Uri	<0.5 (16/17)	13.16		452	474
18	9′-*cis*-neoxanthin	Neo	0.7 (17/18)	13.40	417	442	469
19	4-keto-19′-hexanoyloxyfucoxanthin	4k-hex-fuco		13.84		446	471
20	Unknown carotenoid from *I. galbana* and *C. muelleri*			14.16		445	464
21	Unknown carotenoid from *I. galbana* and *C. muelleri*			14.64		443	
22	19′-hexanoyloxyfucoxanthin	Hex-fuco		14.68		445	471
23	Prasinoxanthin	Pras		15.79		454	
24	Violaxanthin	Viola	1.1 (23/24)	16.11	417	440	471
25	Unknown carotenoid from *M. pusilla*			16.57	406	439	464
26	Micromonol	Microl		16.99		429	455
27	Dinoxanthin	Dino		17.57	419	443	471
28	Micromonal	Micral		18.12		454	
29	Diadinochrome	Diadchr		18.20	(408)	432	457
30	Diadinoxanthin	Diadino		18.66	(424)	448	478
31	Antheraxanthin	Anth		19.64	(425)	448	474
32	Unknown carotenoid from *C. muelleri*			19.70		442	
33	Alloxanthin	Allo		20.59	(431)	454	483
34	Monadoxanthin	Monado		21.19	(425)	448	477
35	Diatoxanthin	Diato		21.41	(426)	454	481
36	Lutein	Lut		21.91	425	446	474
37	Zeaxanthin	Zea	1.3 (36/37)	22.21	(430)	452	480
38	Dihydrolutein	Dihydro-lut		22.29		429	455
39	Siphonein	Siphn		24.47		457	
40	Crocoxanthin	Croco		25.39	(426)	448	477
41	Unknown carotenoid from *I. galbana*			25.84		463	
42	Unknown carotenoid from *P. marinus*			26.19	(424)	448	475
43	Lycopene	Lyco		27.83	445	473	503
44	Chlorophyll *b* allomer	Chl *b* allom		28.79	462	598	648
45	Unknown carotenoid from *M. pusilla*			29.04	420	443	471
46	Chlorophyll *b*	Chl *b*		29.84	462	598	648
47	Unknown carotenoid from *I. galbana*			30.09		448	
48	Divinyl chlorophyll *b*	DV chl *b*		30.40	473	602	650
49	Chlorophyll *a* allomer	Chl *a* allom		31.18	431	615	661
50	Chlorophyll *b*′	Chl *b*′	<0.5 (49/50)	31.40	460	599	650
51	β,ε-carotene	β,ε-car	<0.5 (50/51)	31.78	(422)	448	475
52	Divinyl chlorophyll *b*′	DV chl *b*′		31.98	472	604	650
53	β,β-carotene	β,β-car	<0.5 (52/53)	32.12	(428)	454	480
54	Non-polar chlorophyll *c*-like from *I. galbana*			32.70	451	582	631
55	Chlorophyll c_2-MGDG	Chl c_2-MGDG		33.20	451	586	626
56	Chlorophyll *a*	Chl *a*		33.43	431	616	661
57	Divinyl chlorophyll *a*	DV chl *a*	1.3 (56/57)	33.79	442	617	663
58	Phaeophytin *b*	Phytin *b*	<0.5 (52/53)	33.88	436	596	651
59	Chlorophyll *a*′	Chl *a*′		34.81	431	618	664
60	Divinyl chlorophyll *a*′	DV chl *a*′		35.22	443	619	662
61	Phaeophytin *b*′	Phytin *b*′	<0.5 (55/56)	35.49	436	596	653
62	Phaeophytin *a*	Phytin *a*		36.92	409	607	664

[a] Pairs of peaks with resolution (Rs) < 1.5. Wavelengths in parenthesis indicate shoulders.

are considered adequately resolved (Rs = 1.2) for identification and quantification purposes. Fuco (15), diadino (30) and chl *a* (56) are the other major pigments. Trace amounts of diatoxanthin (diato) (35) were also found. Two unknown carotenoids (21, 32) (443, 442 nm maximum wavelengths) were detected.

The chromatogram of *Micromonas pusilla* (Fig. 2d) reveals a group of carotenoids in the central part of the chromatogram dominated by pras (23) and a coeluting pair of carotenoids (Rs = 0.7). One was spectrally identified as neo (18) and the other tentatively identified as uriolide (uri) (17). The novel pigments microl (26) and micral (28) were also present. These latter carotenoids were recently discovered in prasinophytes [32,33]. Viola (24) was partially resolved from pras (23) (Rs = 1.1) while an unidentified minor carotenoid (25) (λ_{max} 439 nm) eluted between viola and microl

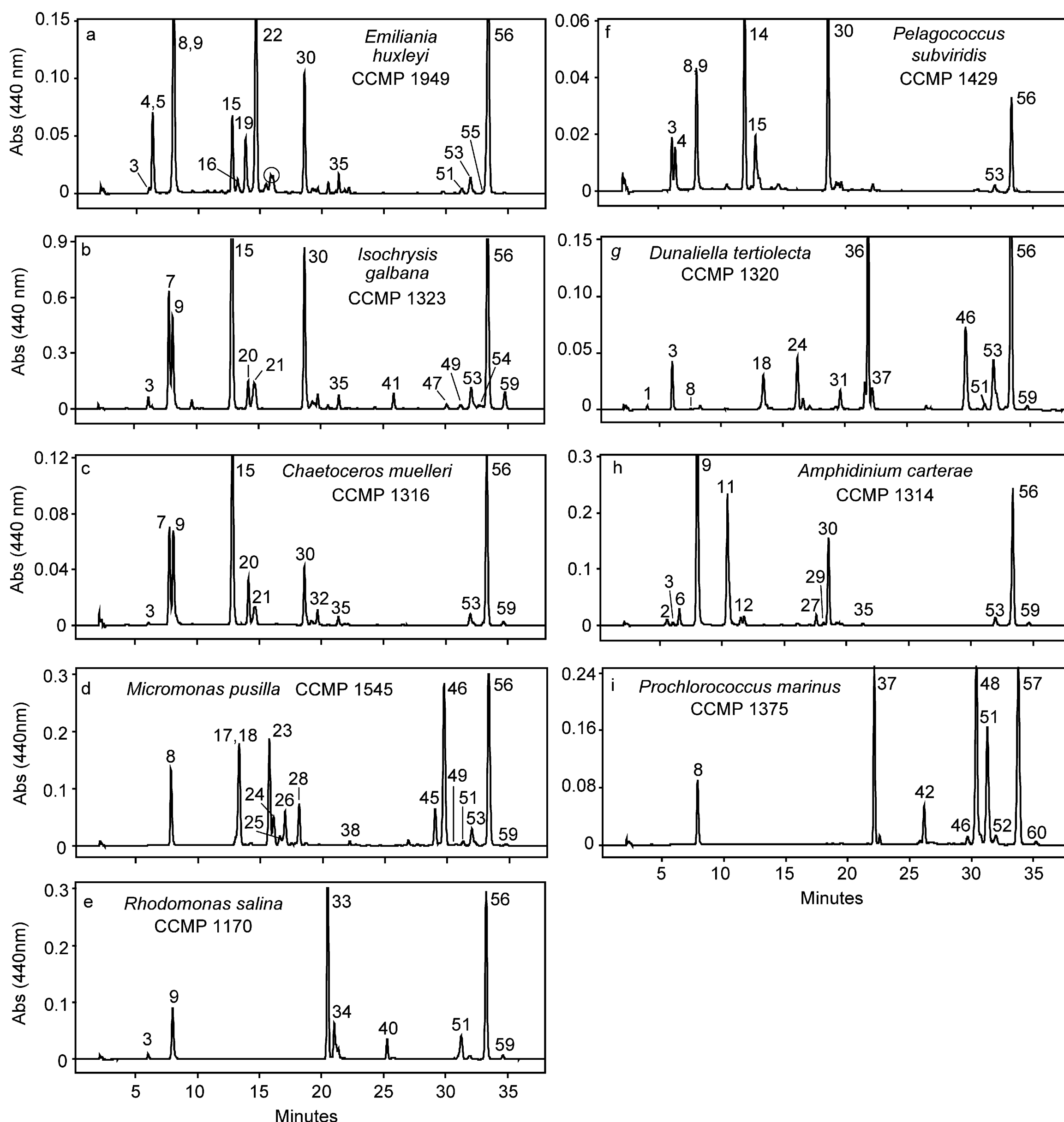

Fig. 2. HPLC chromatograms of acetone extracts of algal monocultures from various classes analyzed with the C_{16}-Amide column on the Alliance system: (a) *Emiliania huxleyi*, (b) *Isochrysis galbana*, (c) *Chaetoceros muelleri*, (d) *Micromonas pusilla*, (e) *Rhodomonas salina*, (f) *Pelagococcus subviridis*, (g) *Dunaliella tertiolecta*, (h) *Amphidinium carterae*, (i) *Prochlorococcus marinus*. Detection was by absorbance at 440 nm. Peak numbers refer to pigments listed in Table 2.

partially coeluting with the latest. Trace amounts of the important carotenoid, dihydrolutein (dihydro-lut) (38), were detected. Another unknown carotenoid (45) (λ_{max} 443 nm), fully resolved from chl *b* (46), had similar spectral characteristics to those measured by Zapata et al. [26].

The chromatogram of *Rhodomonas salina* (Fig. 2e) is dominated by the carotenoid alloxanthin (allo) (33), the marker pigment for cryptophytes, completely resolved from monadoxanthin (monado) (34). Crocoxanthin (croco) (40), β,ε-car (51), and trace amounts of chl *a*′ (59) were also detected.

The pigment composition of *Pelagococcus subviridis* is shown in the chromatogram of Fig. 2f. The major peak observed is diadino (30) and the marker pigment but-fuco (14) elutes before fuco (15). The pigment pair chlide *a* (3)/chl c_3 (4) is partially resolved (Rs = 1.1) in the initial part of the chromatogram. Trace amounts of β,β-car (53) were also present.

The chromatogram of *Dunaliella tertiolecta* (Fig. 2g) reveals three major carotenoids (neo (18), viola (24), and lut (36)) eluting in the central part of the chromatogram with zea (37) eluting as a small peak almost completely resolved from lut (Rs = 1.3). The polar chlorophyll derivatives chlorophyllide *b* (chlide *b*), as a minor peak (1), and chlide *a* (3) were detected. β,β-car (53), chlorophyll *a*′ (chl *a*′) (59), and trace amounts of chlorophyll *b*′ (chl *b*′) were present. The unresolved shoulder on β,β-car (53) has spectral characteristics of carotene but could not be confidently identified.

The first peak in the chromatogram of *Amphidinium carterae* (Fig. 2h) reveals peridininol (2) completely resolved from chlide *a* (3). Perid (11), the marker pigment for dinoflagellates, eluted after chl c_2 (9) while trace amounts of the minor pigment dinoxanthin (dino) (27) were completely resolved from diadino (30). The minor carotenoid P-457 (6) was also detected.

Apart from the carotenoids zea (37) and β,ε-car (51), the chromatogram of *Prochlorococcus marinus* (Fig. 2i) is dominated by the marker pigments DV chl *a* (57) and *b* (48). Chl *b* (46) is present as a minor pigment completely resolved from DV chl *b* and eluting after an unidentified carotenoid. The polar chlorophyll MgDVP (8) elutes

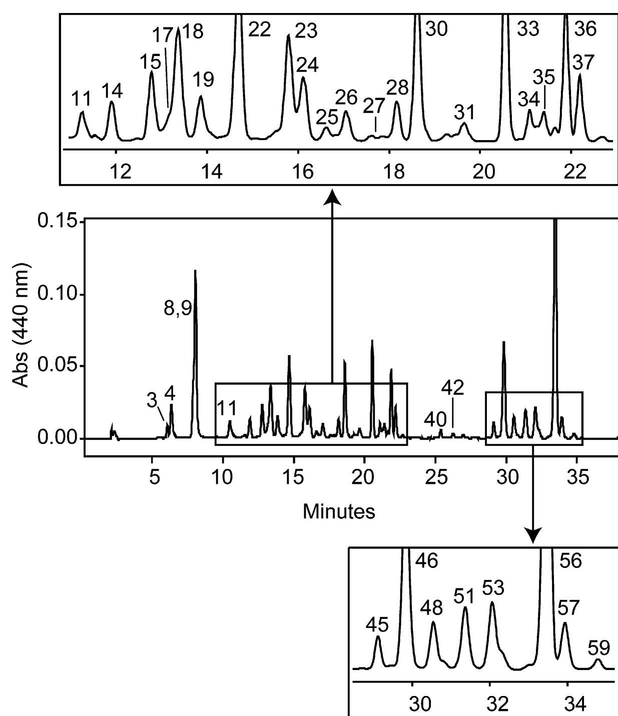

Fig. 3. HPLC chromatogram of mixed pigment extracts from *Emiliania huxleyi, Micromonas pusilla, Rhodomonas salina, Dunaliella tertiolecta, Pelagococcus subviridis, Prochlorococcus marinus, and Amphidinium carterae.* The central insert highlights the resolution of the most important carotenoids. The right insert shows the resolution for pigments chl b/DV chl b and chl a/DV chl a.

in the initial part of the chromatogram. The major photosynthetic pigment chl *a* was not present.

3.4. Pigment analysis of mixed algal cultures and field samples

The capabilities of the proposed method in resolving complex pigment mixtures was further evaluated by combining extracts from *Emiliania huxleyi*, *Micromonas pusilla*, *Rhodomonas salina*, *Amphidinium carterae*, *Dunaliella tertiolecta*, *Pelagococcus subviridis* and *Prochlorococcus marinus*. Fig. 3 shows the pigment separation in the algal mixture. In the initial part of the chromatogram the phytol-free chlorophyll derivative chlide *a* (3) and the acidic chlorophyll c_3 (4) were adequately resolved while MgDVP (8) and chl c_2 (9) coelute. The insert in the central region of the chromatogram highlights the resolution of the most important carotenoids (perid (11), but-fuco (14), fuco (15), neo (18), hex-fuco (22), pras (23), viola (24), diadino (30), allo (33), lut (36), and zea (37)) found in marine phytoplankton. Of particular significance is the excellent resolution of fuco and its acyloxy derivatives including the novel carotenoid 4k-hex-fuco (19). The resolution of the pair lut (36)/zea (37) (Rs = 1.3) and the complete separation of the new pigments micral (28) and microl (26) from adjacent carotenoids is also noteworthy. Apart from neo (18) and uri (17), which coelute in a single peak, all other carotenoids are quantifiably resolved (Rs > 1.5).

Fig. 3 (non-polar end of the chromatogram, right insert) highlights the excellent resolution achieved for the pigment pairs chl *b* (46)/DV chl *b* (48) (Rs > 1.5) and chl *a* (56)/DV chl *a* (57) (Rs = 1.3). The carotenoids β,ε-car (51) and β,β-car (53) were also completely separated (Rs > 1.5) but coelute with chl *b* epimer (chl *b*′) or chl *a* allomer when these chlorophyll derivatives are present.

The capability of the method in determining the pigment composition of phytoplankton populations in field samples was evaluated in water samples from Narragansett Bay, RI. Fig. 4 shows the chlorophylls and carotenoids present in a water sample taken

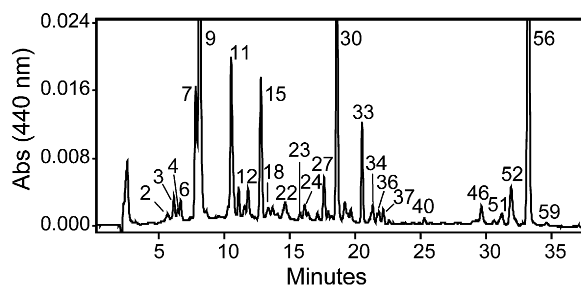

Fig. 4. HPLC chromatogram of phytoplankton pigments extracted from a seawater sample collected in the upper part of Narragansett Bay, RI, near the Providence River. Pigment identity in Table 2.

from the upper part of the bay near the Providence River. Besides chl *a* (56), the chromatogram is dominated by pigments associated with dinoflagellates (perid (11), diadino (30), and chl c_2 (9)), diatoms (fuco (15), chl c_1 (7), chl c_2, and diadino), and cryptophytes (allo (33)). Minor peaks of hex-fuco (22) and chl c_3 (4), which are usually associated with prymnesiophytes, were also identified. Traces of peridininol (2), pras (23), neo (18), viola (24), lut (36), and zea (37) were detected along with small amounts of chl *b* (46), indicating the presence of green algae (prasinophytes, chlorophytes and euglenophytes) and possibly cyanobacteria.

4. Discussion

Most recently developed HPLC methods have successfully used polymeric C_{18} columns or monomeric C_8 columns to improve the separation of phytoplankton pigments in marine samples. The most relevant factor that distinguishes this current method from previous ones is the choice of a C_{16}-Amide column for chromatographic separation of algal pigments. The choice was made based on the performance of this column in resolving DV chl *a* and *b* from their MV counterparts during preliminary evaluation tests. Under the gradient conditions used, the C_{16}-Amide column proved to be more selective towards the neutral carotenoids and esterified chlorophylls than the more polar dephytylated chl *a* and *b* derivatives and acidic chl *c* pigments. The elution profile was also similar to C_{18} columns.

In comparison with the method of Wright et al. [8], the resolution of polar chlorophylls has been improved with the pairs chlide *a*/chl c_3 and chl c_1/chl c_2 partially resolved (Rs = 1.1). However, the proposed method is limited in its ability to resolve MgDVP from chl c_2, which has been successfully separated by other methods [22,26,27].Van Lenning et al. [38] also found it difficult to separate MgDVP in the presence of chl c_1 and c_2. In the absence of chl c_1, we were able to detect small concentrations of MgDVP coeluting with chl c_2 as shown in Fig. 3 and in the chromatograms of *Emiliania huxleyi* and *Pelagococcus subviridis*. In the absence of both chl c_1 and c_2, we were able to positively identify MgDVP in the prasinophyte *Micromonas pusilla* [39] and the cyanophyte *Prochlorococcus marinus*. Although MgDVP is mostly associated with prasinophytes, its coelution with chl c_2 does not prevent the identification of this class of phytoplankton in marine samples because pras is the biomarker for most prasinophytes and it was quantifiably resolved from viola (Rs = 1.1).

MV chl c_3 was found but not fully resolved from chl c_3 in *Emiliania huxleyi*. According to Zapata et al. [36], MV chl c_3 (considered a minor pigment) can be a useful marker under bloom conditions in addition to hex-fuco, the marker pigment for prymnesiophytes [40]. Chl c_2-MGDG was detected on the shoulder of the chl *a* peak in *Emiliania huxleyi* but was not detected in *Isochrysis galbana*. We suspect that chl c_2-MGDG is coeluting with chl *a* under the conditions employed and the C_{16}-Amide column was not able to completely

separate chl c_2-MGDG from chl *a*. Zapata et al. [41] have successfully resolved chl c_2-MGDG from chl *a* on monomeric C_8 and polymeric C_{18} columns.

Few methods can achieve the resolution obtained by this method for key diagnostic carotenoids. The excellent resolution of fuco, its acyloxy derivatives (but-fuco and hex-fuco) and the novel pigment 4k-hex-fuco equal that of pyridine-containing mobile phases used by Garrido and Zapata [22] and Zapata et al. [26]. This method has also improved the resolution of lut and zea relative to these methods. The separation of these two pigments is important for distinguishing autotrophic prokaryotes from green algae (prasinophytes and chlorophytes).

Methods developed for analysis of complex pigment mixtures are generally unable to separate DV chl *b* from chl *b*. Although this does not hamper the identification of *Prochlorococcus marinus* in natural samples, it prevents the use of chl *b* as a marker for green algae (chlorophytes, prasinophytes, and euglenophytes) in waters where prochlorophytes are present. DV chl *a* is a more specific marker for prochlorophytes and it can be resolved from chl *a* by several methods. HPLC techniques that cannot separate DV chl *a* from chl *a* in cases where they are present together will compromise the sum of chl *a*. If these pigments are not separated, the use of chl *a* as an indicator of phytoplankton biomass will become difficult [9]. The method developed in this study is capable of baseline separation of DV chl *b* and chl *b*, and achieves almost complete separation for the pair DV chl *a*/chl *a* (Rs = 1.3), demonstrating the additional capabilities of this method to identify and quantify these pigments and better discriminate the phytoplankton class with which they are associated.

Mendes et al. [42] were able to separate chl *a* from DV chl *a* in standards but not from natural samples. Chlorophyll *a* and DV chl *a* were fully resolved while chl *b* and DV chl *b* were partially resolved on a C_8 column-based RP-HPLC system in *Prochlorococcus marinus* [10]. We have demonstrated that DV chl *a* and *b* can be separated using our method on *Prochlorococcus marinus* and the mixed algal culture extracts. To our knowledge this is one of very few methods that can resolve in a single run both non-polar DV chlorophylls from their MV analogues along with most other important diagnostic carotenoids and chlorophylls.

In summary, we have described a reverse phase HPLC method using a C_{16}-Amide column which provides improved resolution of most chemotaxonomically important carotenoids and chlorophylls, particularly DV chlorophyll *b* and *a*. The resolution improvements combined with the relatively short run time makes this method suitable for assessing the distribution and dynamics of the major phytoplankton groups in marine and freshwater ecosystems.

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