Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C₈ column and pyridine-containing mobile phases

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ABSTRACT: A high-performance liquid chromatographic (HPLC) method based on a reversed-phase C₈ 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 C₁₈ 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 C₁₈ 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 · C₈ 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 carotenoids). 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).
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, chl c₂ and Mg-3,8-divinyl-pheoporphyrin a₅ 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, C₈) 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 (Rodriguez et al. 1998), they showed that using adequate gradient profiles and injection conditions, monomeric C₈ 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 C₈ stationary phase.

Here we present an HPLC method for the analysis of phytoplankton pigments which combines a C₈ 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 C₈ column (150 x 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 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* ALIV-IEO (Dinophyceae) from the Instituto Espanol de Oceanografia, Vigo, Spain; *Emiliania huxleyi* NIOZ CH 24 (Prymnesiophyceae) from the Netherlands Institute for Sea Research, Texel, The Netherlands; *Pavlova gyraus* 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 (Cyanophyceae),*Dunaliella tertiolecta* ICMA (Chlorophyceae), and *Tetraselmis suecica* ICMA (Prasinophyceae) from the Instituto de Ciencias Marinas de Andalucia (CSIC), Cadiz, 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 Investigaciones Marinas, 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), *Dinameliella 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 *Pyrococcus provocati* 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 siphonin. 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 'Hesperides', 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 filter (nominal pore size 0.7 μm). 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 c₃, 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: \(R_s = 2(R_{t2} - R_{t1})/W\), where \(R_{t2}\) and \(R_{t1}\) 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 gyrrans 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 (Rodríguez 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 (\(\Delta \%B \, \text{min}^{-1}\)) 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 siphonine 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 \(R_t = 11.46\) 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)

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(b) Analytical gradient protocol (HPLC System 3)

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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 $R_t = 24.08$ min, SD = 0.19 min, n = 16, RSD = 0.80%), chl $a$ (mean $R_t = 33.15$ 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 ($R_t > 1.40$) for the pigment pairs chl $c_3$ (peak 41)/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* AL1V-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,) between pigment pairs is indicated when R,< 1.40. Wavelengths given in parenthesis denote shoulders. MgDVP: Mg-3,8-divinyl-pheoporphyrin a5 monomethyl ester.

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<td>417 441 470</td>
</tr>
<tr>
<td>29</td>
<td>Antheroxanthin</td>
<td>25.38</td>
<td>0.8 (28/29) (421) 446 474</td>
</tr>
<tr>
<td>30</td>
<td>Alloxanthin</td>
<td>26.25</td>
<td>(426) 452 482</td>
</tr>
<tr>
<td>31</td>
<td>Diatoxanthin</td>
<td>26.90</td>
<td>(426) 453 481</td>
</tr>
<tr>
<td>32</td>
<td>Monadoxanthin</td>
<td>27.07</td>
<td>0.80 (31/32) (423) 447 476</td>
</tr>
<tr>
<td>33</td>
<td>Zeaxanthin</td>
<td>27.49</td>
<td>(426) 453 478</td>
</tr>
<tr>
<td>34</td>
<td>Lutein</td>
<td>27.65</td>
<td>0.80 (33/34) (422) 446 475</td>
</tr>
<tr>
<td>35</td>
<td>Dihydroxylutein</td>
<td>28.00</td>
<td>(405) 429 454</td>
</tr>
<tr>
<td>36</td>
<td>Siphonein</td>
<td>29.37</td>
<td>458</td>
</tr>
<tr>
<td>37</td>
<td>Unknown carotenoid from Tetraselmis suecica</td>
<td>29.71</td>
<td>(423) 449 476</td>
</tr>
<tr>
<td>38</td>
<td>Unknown carotenoid from T. suecica</td>
<td>30.45</td>
<td>(422) 448 476</td>
</tr>
<tr>
<td>39</td>
<td>Crocoxanthin</td>
<td>31.11</td>
<td>(422) 447 476</td>
</tr>
<tr>
<td>40</td>
<td>Chlorophyll b allomer</td>
<td>31.28</td>
<td>0.8 (39/40) (422) 447 476</td>
</tr>
<tr>
<td>41</td>
<td>Unknown carotenoid from Prochlorococcus marinus</td>
<td>31.42</td>
<td>422 447 476</td>
</tr>
<tr>
<td>42</td>
<td>DV chlorophyll b</td>
<td>31.58</td>
<td>1.02 (41/42) 470 600 648</td>
</tr>
<tr>
<td>43</td>
<td>Chlorophyll b</td>
<td>31.62</td>
<td>&lt;0.5 (42/43) (426) 452 482</td>
</tr>
<tr>
<td>44</td>
<td>Chlorophyll b epimer</td>
<td>31.87</td>
<td>462 599 650</td>
</tr>
<tr>
<td>45</td>
<td>Non-polar chlorophyll c from E. huxleyi</td>
<td>32.39</td>
<td>455 584 633</td>
</tr>
<tr>
<td>46</td>
<td>Chlorophyll a allomer</td>
<td>32.63</td>
<td>430 615 662</td>
</tr>
<tr>
<td>47</td>
<td>DV chlorophyll a</td>
<td>32.83</td>
<td>441 616 666</td>
</tr>
<tr>
<td>48</td>
<td>Chlorophyll a</td>
<td>33.15</td>
<td>431 617 662</td>
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<tr>
<td>49</td>
<td>Chlorophyll a epimer</td>
<td>33.48</td>
<td>430 615 664</td>
</tr>
<tr>
<td>50</td>
<td>β,y-carotene</td>
<td>34.25</td>
<td>(436) 461 492</td>
</tr>
<tr>
<td>51</td>
<td>Unknown carotenoid from Micromonas pusilla</td>
<td>34.32</td>
<td>&lt;0.5 (50/51) (418) 442 470</td>
</tr>
<tr>
<td>52</td>
<td>ω,β-carotene</td>
<td>35.52</td>
<td>420 441 470</td>
</tr>
<tr>
<td>53</td>
<td>β,ε-carotene</td>
<td>35.74</td>
<td>0.88 (52/53) (422) 447 475</td>
</tr>
<tr>
<td>54</td>
<td>β,β-carotene</td>
<td>35.95</td>
<td>0.88 (53/54) (426) 452 477</td>
</tr>
</tbody>
</table>
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), Chlde a (peak 8), chl c2 (peak 10) and chl c1 (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 & Liaaeen-Jensen 1995, Egeland et al. 1995) such as uriloidc (Url peak 15), micromonal (Micr, peak 22), micromonal (Micral, peak 25) and dihydrofrotein (Dihydro toxin, 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 (R4 = 1.08), Zea/Lut (R4 = 0.80). An unknown polar chl c-like pigment (peak 7), with spectral characteristics similar to chl c2 (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 (ε,ε-Car, peak 52), elutes before the other carotenones.

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 Chlde b (peak 1), Chlde 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 (R4 = 0.80). Its characteristic monocyclic β,β-carotene (β,β-Car, peak 50), was detected in trace amount eluting before β,ε-carotene (β,ε-Car, peak 53).

In the chromatogram of *Rhodomonas baltica* ICMA (Fig. 2G) the marker pigment allooxanthin (Allo, peak 30) was baseline resolved from monodoxanthin (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 (~Δ10%B min⁻¹, standard conditions) to 40–95% B in 12 min (~Δ5%B min⁻¹), the pigment pair remained unresolved. However, an improvement in the resolution was observed for the pigment pairs Zea/Lut (from R4 = 0.80 to R4 = 1.08) and DV chl a/chl a (from R4 = 1.42 to R4 = 1.48).

The chromatogram of the toxic dinoflagellate *Alexandrium minutum* AL1V-EO (Fig. 2I) shows an inversion in elution order (with respect C18 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. 2I) 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 (R4 = 2.31) for the pigment pair chl c3 (peak 4)/MV chl c1 (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 c3 (peak 10) and chl c1 (peak 11), also well resolved (R4 = 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 Cryophyceae, 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 pyramis* 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* (Cyano phycaceae) 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 ($R_s > 1.50$) 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$_1$ (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 lorcroxanthin ester. A carotenoid spectrally similar to lorcroxanthin 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 ß-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 de 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 ð (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), urilolide (peak 15), Neo (peak 18), Pras (peak 20), Viola (peak 23), micromonal (peak 22) and dihydrodolitein (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 nanoplanckton 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.
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 (increas-
ing the retention of acidic chlorophylls), but also as a
real mobile phase modifier, affecting the selectivity
towards both neutral (carotenoids and esterified chlo-
rophylls) and charged compounds (dephtylated 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 chloro-
phylls show an increase in retention time and a better
resolution between DV/MV chl c pigment pairs. A pos-
sible explanation for this effect could rely on π-π in-
teractions established between the aromatic ring of pyri-
dine 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 sam-
ple. 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 ana-
lysts, 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 C5 Sym-
metry 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 com-
promise between the efficiency of 3.0 μm particle size
and the lower back pressure of 5 μm particle size C5
columns. The separation capability of the proposed
method could vary if other monomeric C5 columns are
employed. The selection of a column has to be an
informed decision, based on the knowledge of station-
ary phases properties such as bonding chemistry, pore
size, surface area, coverage and carbon load. The col-
umn used in this study was selected after considering
the results of a previous study (Rodriguez et al. 1998) in
which we evaluated the performance of 4 commercial
C5 monomeric columns for resolution of MV and DV
pairs of chl cs.

**Separation of polar and non-polar chlorophylls**

The proposed method allows the simultaneous reso-
lution 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 caro-
tenes 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 C18 columns in which their special shape-
selectivity governed the elution order (Garrido & Zap-
ata 1997). On these columns divinyl forms that have
planar structures (e.g. chl c2 and chl c3) elute after their
monovinyl counterparts (e.g. MV chl cy and chl c1),
whose molecules are more voluminous. In the pro-
posed method the elution order seems to be controlled
by subtle differences in the overall polarity of the mol-
ecule, 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 caro-
tenoids. This includes the separation of Fuco and its
well-known acyloxy derivatives (But-fuco and Hex-
fuco) from the novel 4-keto-19'-hexanoyloxyfucoxan-
thin (Egeland et al. 1999), first detected in Emiliania
huxleyi using polymeric C18 columns (Garrido & Zap-
ata 1998). The presence in this carotenoid of a
novel end-group with 3 oxygenated functions (-keto,
-hydroxy- and -epoxy groups), explains why this com-
pound elutes before Hex-fuco. Studies on the distribu-
tion 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 ($R > 1.40$) of polar chlorophylls (including Chlde 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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LITERATURE CITED


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