The use of HPLC for the characterization of phytoplankton pigments

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Summary

HPLC is still the technique of choice for the analysis and characterization of phytoplankton pigments. In this chapter we describe procedures for sample preparation and pigment extraction, and the use of octyl silica columns and pyridine-containing mobile phases to separate chlorophylls and carotenoids. The identification of pigments on the basis of their retention times and visible spectra, the preparation of pigment standards and the quantitative analysis by either external or internal standard procedures are also described.

Key words: Carotenoids, Chlorophylls, HPLC, Octyl silica, Photodiode array detector, Phytoplankton

1. Introduction

The analysis of photosynthetic pigments has become a source of essential information for studies of the physiology and ecology of marine microalgae. Examples of applications of detailed pigment information include the study of changes in natural plankton populations (associated for example with climate change), the ground-truthing of satellite derived algal biomass estimations, the photosynthetic responses to the changing aquatic light environment, or the trophic transfer from primary producers.

The photosynthetic pigments of algae belong to three chemical families: phycobiliproteins, carotenoids and chlorophylls (Chls) ***(1)***. Whereas phycobiliproteins are water-soluble, carotenoids and Chls are lipid-soluble. Most of the time, the term "phytoplankton pigment analysis" refers to the joint analysis of chlorophylls and carotenoids, because both types of compounds occur in all photosynthetic algae, they are easily extracted into organic solvents and can be detected with high sensitivity in the visible range.

Chls and carotenoids are present in different algal taxa with variable degrees of specificity: some of them occur in several algal classes whereas others are restricted to one or only a few algal groups, making them unambiguous chemotaxonomic markers.

Liquid chromatography is the method of choice for the analysis of algal Chls and carotenoids. The analysis of complex algal pigment extracts, especially those derived from phytoplankton samples from natural waters, still constitute a challenge for chromatographic techniques. Algal Chls and carotenoids span a wide range of

polarities in which some of them only differ by small structural features (in some cases, only the position of a double bond).

An excellent short course on phytoplankton pigments analysis is available on internet ***(2)***, but for those readers interested either in broad knowledge on pigment biology or in detailed information on their analysis, a recent and comprehensive monograph is strongly recommended ***(3)***. That book, together with the preceding one ***(4)***, cover most aspects in pigment characterization, chemotaxonomy and applications in oceanography. The latest advances in chromatographic analysis of algal pigments have been recently reviewed ***(5)***. In this chapter we describe a specific protocol for the analysis of algal pigments based on that proposed by Zapata et al. ***(6)***. Other protocols exist (e.g. the one developed by Van Heukelem and Thomas ***(7)***) but here we describe the one we are most familiar with. The advantages and disadvantages of these and other alternative methods have been discussed elsewhere ***(1,8)***.

2. Materials

2.1 Sample preparation

1. Sampling: Seawater is filtered on glass fibre filters (GF/F type, nominal pore size 0.7 μm). A regulated vacuum pump is needed (*see* **Note 1**).

2. Pigment extraction solvent: Prepare extraction solvent by either mixing 9 volumes of acetone with 1 volume of water or by mixing 9.5 volumes of methanol (MeOH) with 0.5 volumes of water (*see* **Note 2**). Use graduate cylinders and HPLC grade solvents.

3. Extraction: Use Pyrex® screw cap glass tubes with polytetra-fluoroethylene (PTFE) lined caps. A centrifuge and a probe sonicator or an ultrasonic bath can be necessary,

depending on the extraction procedure (see 3.1.2 and **Note 2**). Use PTFE syringe filters with 0.22 μm pore size to clarify extracts before injection in the HPLC system.

2.2 Chromatographic equipment

1. Pumps: A pumping system able to deliver at least binary gradients is needed. This can be achieved either with a high pressure mixing system, or with a four-solvent, low-pressure mixing gradient pump.

2. Detection: A diode-array detector is essential for pigment characterization, especially in natural waters samples. Fluorescence detection (very sensitive and selective towards Chls) can be used in addition to diode-array.

2.3 Columns

1. Octylsilica (C_8) stationary phases with particle size 3.5 μm or less should be used, packed in columns of 150 mm × 4.6 mm.

2.4 Solvents

1. Use HPLC-grade MeOH, acetonitrile (AcN), acetone and water. Pyridine and acetic acid must be of reagent grade or better (*see* **Note 2**).

2. Preparation of pyridinium acetate (pH = 5.0) stock solution (0.25 M): Add 10 mL of acetic acid and 20 mL of pyridine to 900 ml of HPLC grade water in a 1 L beaker, mixing with a magnetic stirrer. Continuously monitor pH with a pH-meter. Add acetic acid drop-wise until pH value is 5.0. Transfer the mixture to a 1 L volumetric flask and adjust volume (the final pyridine concentration is 0.248 M).

3. Preparation of pyridinum acetate working solution (0.025 M): Dilute ten times the stock solution with HPLC grade water. Filter this solution through a 0.45 μm filter before use (*see* **Note 3**).

2.5 Pigment standards

1. Commercial pigment standards: pigments can be obtained from several companies, including Carotenature, Chromadex, DHI Lab Products, Frontier Scientific Inc., Sigma-Aldrich and VWR. DHI Lab Products (Hørsholm, Denmark) offers solutions of typical marine algae pigments that can be used directly for HPLC calibration. Standards can also be isolated from reference algal cultures.

2. Reference algal cultures: If you wish to isolate pigment standards from algal cultures, recommendations have been made for phytoplankton unialgal cultures of known pigment composition (Roy et al. ***(9)***). Extracts from these cultures can also serve to fix retention times and on-line spectra of their characteristic pigments.

3. Isolation of pigment standards: Longer columns with larger size C_8 particles (5 μm) can be used in the preparative work for the isolation and purification of pigment standards. Octadecylsilica (C_{18}) solid phase extraction cartridges (for example Waters SeP-Pak, Waters, Milford, MA,) are used for concentration. A source of dry N_2 gas is needed.

4. Internal standard (if calibration with an internal standard is used -see section 3.5 below-): trans-β-8'-apocarotenal (Sigma Chemical Company, St. Louis, MO, USA). Other internal standards (such as vitamin E acetate) are available.

5. Quantification: A UV-Vis spectrophotometer is needed to determine the concentration of pigment solutions. Optical glass cuvettes with tight PTFE stoppers should be used to avoid solvent evaporation.

3. Methods

3.1. Precautions

1. Photosynthetic pigments are sensitive towards light, oxygen, heat, acids and alkalis. All operations should be done under dim light (a secure green light can be used) and keeping reasonable laboratory temperature. Perform all solvent handling operations in a fume hood. Wear appropriate gloves and protective glasses.

3.1. Sample preparation

1. Sampling: Filter seawater or algal culture samples onto glass fibre filters making sure that vacuum is not higher than 200 mm Hg (*see* **Note 4**). Deep freeze the filters immediately and keep them frozen until extraction (*see* **Note 5**).

2. Pigment extraction: Place frozen filters from algal cultures or natural samples in PTFE-lined screw-capped tubes, then add the extraction solvent (acetone:water 9:1, v/v, or MeOH:water 9.5:0.5; see **Note 2**) to each tube: at least 5 mL if 47 mm diameter filters were used to collect the plankton sample, a minimum of 3 mL for 25 mm filters or not less than 1.5 mL for 13 mm ones. If using an internal standard for calibration (see section 3.5 below), add it at this point. Grind the filter using a stainless steel spatula. Place the tube in an ultrasonic bath with ice and water for 5 min. Centrifuge for 5 min at 3500 × *g* (this step can be omitted, see **Note 2**). Alternatively, use an ultrasonic probe to disrupt the filter and break the retained cells: put the probe (set at

50 W) inside the solvent and move it up and down for 60 seconds, while keeping the tube in a beaker with ice to prevent heating. Centrifuge for 5 min at 3500 × *g* (see **Note 2**). Whichever procedure is used, filter extracts through a 0.22 µm PTFE syringe filter before injecting them into the HPLC system.

3.2 Chromatography

1. Injection: Mix aliquots of pigment extracts with water (one volume of 90% aqueous acetone extract with 0.4 volumes of water or one volume of 95% aqueous MeOH extract with 0.2 volumes of water(*see* **Note 6**)) to avoid shape distortion of earlier eluting peaks ***(10)*** and inject the sample immediately (*see* **Note 6**). If a programmable autosampler is available, program it to perform water mixing, preferably in the sample loop. In this case, water addition can be divided into two volumes, to be loaded in the loop before and after the sample extract (*see* **Note 6**).

2. Elution: If a high-pressure mixing system is employed, mix solvents in a graduate cylinder to prepare two mobile phases, as follows: for eluent A mix MeOH:AcN:0.025 M aqueous pyridinium acetate (pH 5.0) (50:25:25, by volume). Eluent B is composed of MeOH:AcN:acetone (20:60:20; v:v:v). Filter both eluents through a 0.45 µm filter before use. Set flow rate at 1 mL.min^{-1}. Program the gradient profile according to Table 1.

If a quaternary low-pressure mixing system is available there is no need to prepare mixed eluents. Place each solvent in an eluent line. Set flow rate at 1 mL.min^{-1}. Program the gradient profile according to Table 2 (*see* **Note 7**).

3. Detection: Program the diode array detector to get a full visible spectrum (at least from 400 to 700 nm) at each chromatographic point. Monitor the chromatogram at a wavelength that allows general detection of Chls and carotenoids (435-440 nm). Chromatograms at different wavelengths can also be obtained, allowing either a general or selective detection (see "Pigment identification" section below). Many systems can provide a chromatographic trace summing the response at several wavelengths, which increases the sensitivity but reduces the selectivity of the detection. If fluorescent detection is employed, use broad excitation and emission bandwidths to increase sensitivity and detection of all Chl derivatives.

3.3 Pigment standards

1. Preparation of standards: Wet SPE cartridge with 5 mL acetone and then 5 mL of a water:acetone mixture (7:3, v/v). Collect pigment fractions from preparative separation after passing HPLC detector. Immediately before processing, dilute fractions by adding half their volume of water, then pass through the cartridge. Observe the formation of a coloured zone in the cartridge. If the pigment passes through, add more water to the eluate and pass it again through the cartridge (*see* **Note 8**). Eliminate excess solvent by blowing the cartridge with N_2 until completely dry. Then quickly elute the pigment with acetone (or any other adequate solvent in which extinction coefficients are available ***(11)***) (*see* **Note 9**).

2. Characterization of standards. Check the purity of each standard by injecting an aliquot in the HPLC system. If more than one peak appears, or if the spectrum is not homogeneous at different times of peak elution (*see* **Note 10**), a further purification step with a different chromatographic system is needed (*see* **Note 11**).

3.4 Pigment identification

Identify pigments by a) their retention behaviour (compare pigment retention times with those of standards (or pigment profiles of standard cultures) -see Figure 1. Considering that the same retention time can be shared by several pigments if they co-elute in the same peak (*see* **Note 10**) further identification criteria are needed: b) visible spectrum (compare the spectrum of each peak in the sample with that of the corresponding pigment standard eluting at the same retention time). Examine the full visible spectrum in different sections of each peak, for its characterization in terms of pigment identity and peak purity (*see* **Note 12**). For comparison purposes, reference retention times and spectral data are available ***(1-4, 6-7, 11-13)***. Selective detection can be achieved by extracting chromatograms at selected wavelengths (e.g., 435, 470 and 665 nm ***(1)***) (*see* **Note 13**).

3.5 Quantitative analysis

1. Prepare a primary standard solution of each pigment in a solvent in which extinction coefficients are available (usually acetone or ethanol ***(11)***). Determine the concentration spectrophotometrically (absorbance reading at the coefficient wavelength should be between 0.1 and 0.8 absorbance units), taking care of keeping tubes or volumetric flasks and spectrophotometer cuvettes tightly stopped between transfers to minimize solvent evaporation.

2. System calibration, external standard method ***(14)***. Prepare a "concentrated pigment mixture" by carefully mixing known volumes of primary standards (concentration approx. 2.5 $\mu g.mL^{-1}$) and adjusting to a known volume with acetone: water (9:1, v/v). Then, prepare a series of at least six *working solutions* by diluting

different volumes of the "concentrated pigment mixture" to the same final volume with acetone: water (9:1, v/v). These solutions should be prepared in different ranges of concentrations, depending on the expected concentration of pigments in the samples. Hence, different ranges of concentrations will be necessary for oligotrophic or eutrophic waters ***(15)***. Inject each *working solution* into the HPLC system in triplicate (water must be added to each working standard just prior to injection, as for samples – see 3.2.1). Derive a response factor for each pigment (f_p) as the inverse of the slope of the regression line of peak area (A_p) against the weight (ng) of pigment injected (w_p) ***(16)***.

3. System calibration, internal standard method ***(14)***. Operate as described above, but incorporate the same amount of an internal standard primary solution to each *working solution* (with growing concentrations of pigments to be analysed in the series, while the internal standard concentration remains constant). Recommended internal standard concentration in each *working solution* should be in the middle of the range covered by the other pigments. Inject as described above. For each pigment standard, the relative response factor (f_p^{is}) can be calculated as the inverse of the slope of the regression of the ratio of areas of the pigment and internal standard (A_p/A_{is}) against the ratio of their corresponding weights in the injection (w_p/w_{is}).

4. Determining the concentration of pigments in the samples with the external standard method ***(14)***: Use the external standardization equation to calculate Cp, the concentration of a pigment in seawater (ng.L^{-1}), from its peak area (A_p) and response factor (f_p):

$$Cp = \frac{A_p . f_p . v_{ext} . 10^3}{v_{inj} . v_{filt} . B}$$

where v_{ext}, v_{inj}, and v_{filt} denote the volumes of extract (mL), injected sample (µL) and filtered seawater sample (L). *B* is the dilution factor (<1.0), calculated as the ratio of the extract volume and the sum of extract and water added prior to injection.

5. Calculating the concentration of pigments in the samples with the internal standard method ***(14)***:

$$Cp = \frac{A_p . w_{is} . f_p^{is}}{A_{is} . v_{filt}}$$

where A_p is the peak area, w_{is} denotes the mass (ng) of internal standard added to the sample and v_{filt} is the volume of filtered seawater sample (L) (*see* **Note 14**)

Notes

1. Filters with different nominal pore size (GF/D, GF/C) can be used to collect cells of different sizes. Fractionated samples can be achieved employing sequentially several of these filters. On the other hand, polycarbonate filters are commonly used in oceanography to size-fractionate phytoplankton. These filters can either be used directly to extract pigments (only if MeOH is used as extraction solvent -see **Note 2**-, due to the limited chemical resistance of polycarbonate to other solvents,) or they can be used to pre-filter a water sample which is then filtered on GF/F, providing information on the smaller size categories of phytoplankton (see ***(17)*** for a recent update on filtration, storage and extraction).

2. Different solvents have been used to extracts pigments from algal cells retained on the filter (see ***(17)***). 90% aqueous acetone is still the most commonly used solvent, but aqueous 95% MeOH (see ***(6)***) is also being used with good results ***(18)***. As methanolic extracts have been claimed to promote Chl allomerization upon prolonged storage ***(17)***, samples should be immediately injected after extraction. Sparging the sample vial with an inert gas to displace air can help reduce pigment alteration when samples have to stand in a refrigerated autosampler for several hours ***(18)***. Dimethylformamide, alone or in combination with other solvents, has also been used with very good extraction efficiency, but many laboratories are reluctant to use this solvent because of its toxicity ***(17)***. If the filters are extracted by grinding with a spatula followed by placing them in a ultrasonic bath, filter debris still contain rather large fibres and centrifugation can be omitted as most debris do not pass the luer tip of the syringe barrel, thus not clogging the filter. When a sonifier probe is used to disrupt the filter a slurry is produced so centrifugation or prefiltration is necessary before a final filtration onto 0.22 μm filters. Filter disruption can be done directly in the syringe ***(2, 4)***.

3. The original method of Zapata et al. ***(6)*** used 0.25 M pyridine solution in the eluents, but further work showed that the same chromatographic performance can be achieved with 0.025 M. The stock solution is stable at ambient temperature for months. The working solution tends to contaminate (fungal growth has been observed). Prepare it fresh each two or three days. Purge the chromatographic tubing with MeOH when not in use. If the working solution is not to be used for several days, keep it refrigerated and re-filter before the next use.

4. Highly concentrated samples (cultures, field samples from estuarine or coastal waters) can result in filter clogging. Prolonged filtration with (partially) clogged filters can promote mechanical disruption of algal cells. Filtration times (or volumes) must be adjusted in a compromise between extract concentration and sample integrity, but should not be more than 20 minutes.

5. Immediate freezing is best achieved with liquid nitrogen (dry shippers are convenient for this), but quick storage in a -80 ºC freezer normally suffices. Both are adequate for long term conservation of the samples. Dry ice can be an alternative for quick freezing and short term storage, but avoid contact of the filters with vapours from dry ice (they can acidify pigments).

6. Mixing with water is necessary to match injection solvent viscosity to that of eluent, thus avoiding peak shape distortion for less retained pigments due to “viscous fingering” phenomena ***(19)***. Losses of low polarity pigments can occur due to their limited solubility in aqueous solvents ***(20)***. Mixing in the injection loop minimizes these losses as any insoluble pigment will be recovered as solvent polarity decreases with the development of the gradient. MeOH:water mixtures show higher viscosities than acetone:water ones, so that the step of water addition can be omitted when MeOH:water (95:5, v/v) is used as extraction solvent and injection volumes do not exceed 100 μL.

7. When using gradients with high-pressure or low-pressure mixing systems some differences in retention times can be observed that are caused by the different "dwell volume" (the volume between the point of mixing and the column) in the two systems (in gradient elution, the actual composition in the gradient program is delayed in

reaching the column, because it has to pass through the dwell volume). Retention times will be higher on low-pressure systems that normally have larger dwell volumes. In consequence, changes in gradient profiles and/or eluent composition can be necessary to adjust retention and resolution with different HPLC systems ***(6)***.

8. The amount of water used to achieve the retention of pigment fractions in the solid phase extraction cartridge can vary depending on the compound and the composition of the solvent in which it is eluted from the preparative fraction. In some cases excess water causes the pigment to pass unretained through the cartridge due to the formation of micelles. If this is observed, a reduction in water content can promote retention.

9. This procedure can also be used for the preparative isolation of unknown pigments for further characterization by structural techniques (mass spectrometry, nuclear magnetic resonance), but the co-occurrence of non absorbing lipids usually requires additional purification.

10. Check homogeneity of spectrum at least at mid-upslope, top and mid-downslope of each peak.

11. Different elution can be achieved by changing the stationary phase. Polymeric octadecylsilica columns (e.g. Vydac TP, The Separations Group, Hesperia, California, USA) can be used for this purpose ***(8)***.

12. Spectral checking in different regions of the peak does not always ensure peak purity. This can be the case if the impurity appears at very low concentration, if the

spectrum of the impurity is very similar to that of the main pigment in the peak or if a total coelution (identical retention profile) happens.

13. At 435 nm most pigments are detected, except pheophytin *a*, pheophorbide *a* and their derivatives; 470 nm excludes Chl *a* derivatives from the chromatogram; and 665 nm detects only Chl *a,* pheophytin *a*, pheophorbide *a* and their derivatives.

14. Extraction and injection volumes are not included in the equation.

References

1. Wright SW, Jeffrey SW (2006) Pigment markers for phytoplankton production. In Volkman JK (Ed) Marine Organic Matter. Handbook of Environmental Chemistry, vol. 2 N. Springer, Heidelberg, pp 71-104
2. Wright SW (2005) Analysis of phytoplankton populations using pigment markers.http://www.antarctica.gov.au/__data/assets/pdf_file/0007/21877/ml_38651523287037_analysis20of20phytoplankton20pigments.pdf. Accessed 1 Nov 2013
3. Roy S, Llewellyn CA, Egeland E, Johnsen G (eds)(2011) Phytoplankton Pigments – Characterization, Chemotaxonomy and Applications in Oceanography. Cambridge University Press. Cambridge
4. Jeffrey SW, Mantoura RFC, Wright SW (eds) (1997) Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods. UNESCO Publishing. Paris
5. Roy S, Garrido JL (2013) Pigments |Liquid chromatography. In Reedijk J (ed) Reference Module in Chemistry, Molecular sciences and Chemical Engineering. Elsevier, Waltham, MA. doi: 10.1016/B978-0-12-409547-2.04878-2

6. Zapata M, Rodríguez F, Garrido JL (2000) Separation of chlorophylls and carotenoids from marine phytoplankton: A new HPLC method using a reversed-phase C_8 column and pyridine-containing mobile phases. Mar Ecol Prog Ser 195: 29-45

7. Van Heukelem L, Thomas CS (2001) Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. J. Chromatogr A 910: 31-49.

8. Garrido JL, Airs R, Rodríguez F, Van Heukelem L, Zapata M (2011) New HPLC separation techniques. In: Roy S, Egeland ES, Johnsen G, Llewellyn CA (eds) Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography. Cambridge University Press, Cambridge, pp. 165-194

9. Roy S, Wright SW, Jeffrey SW (2011) Phytoplankton cultures for standard pigments and their suppliers. In: Roy S, Egeland ES, Johnsen G, Llewellyn CA (eds) Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography. Cambridge University Press, Cambridge, pp. 653-657

10. Zapata M, Garrido JL (1991) Influence of injection conditions in reversed-phase high-performance liquid chromatography of chlorophylls and carotenoids. Chromatographia 31: 589-594

11. Egeland ES (2011) Data sheets aiding identification of phytoplankton carotenoids and chlorophylls. In: Roy S, Egeland ES, Johnsen G, Llewellyn CA (eds) Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography. Cambridge University Press, Cambridge, pp. 665-822

12. Zapata M, Rodríguez F, Garrido JL, Jeffrey SW, Jeffrey SW, Clementson L (2004) Photosynthetic pigments in 37 species (65 strains) of Haptophyta: implications for oceanography and chemotaxonomy. Mar Ecol Prog Ser 270: 83-102
13. Zapata M, Fraga S, Rodríguez F, Garrido JL (2012) Pigment-based chloroplast types in dinoflagellates. Mar Ecol Prog Ser 465: 33-52
14. Mantoura RFC, Repeta D (1997) Calibration methods for HPLC. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods. UNESCO Publishing. Paris, pp. 407-428
15. Van Heukelem L, Hooker SB (2011) The importance of the quality assurance plan for method validation and minimizing uncertainties in the HPLC analysis of phytoplankton pigments. In: Roy S, Egeland ES, Johnsen G, Llewellyn CA (eds) Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography. Cambridge University Press, Cambridge, pp. 195-242
16. Hooker SB, Van Heukelem L (2011) A symbology and vocabulary for an HPLC lexicon. In: Roy S, Egeland ES, Johnsen G, Llewellyn CA (eds) Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography. Cambridge University Press, Cambridge, pp. 243-256
17. Pinckney JL, Millie DF, Van Heukelem L (2011) Update on filtration, storage and extraction solvents. In: Roy S, Egeland ES, Johnsen G, Llewellyn CA (eds) Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography. Cambridge University Press, Cambridge, pp.
18. Alou-Font E, Mundy CJ, Roy S, Gosselin M, Agustí S (2013) Snow cover affects ice algal pigment composition in the coastal Artic Ocean during spring. Mar Ecol Prog Ser 474: 89-104

19. Rousseaux G, Martin M, De Wit A (2011) Viscous fingering in packed chromatographic columns: non-linear dynamics. J. Chromatogr A 1218: 8353-8361

20. Latasa M, van Lenning K, Garrido JL, Scharek R, Estrada M, Rodríguez F, Zapata M (2001) Losses of chlorophylls and carotenoids in aqueous acetone and MeOH extracts prepared for RPHPLC analysis of pigments. Chromatographia 53: 385-391

Figure captions

Figure 1. High-performance liquid chromatogram of pigments from marine phytoplankton (mixed pigment standard from DHI + Monovinyl (MV) Chlc_3 + Chl c_1), obtained with the method of Zapata et al. (2000) monitored at 435 nm (upper trace), 470 nm (middle trace) and 665 nm (lower trace). Peak identification: 1, Chl c_3; 2, MV-Chl c_3; 3, Chlorophyllide *a*; 4, Mg-2,4-divinylpheopropyrin a_5 monomethyl ester (Mg-DVP) ; 5, Chl c_2; 6, Chl c_1; 7, Peridinin; 8, Peridinin-like unknown carotenoid; 9, 19'-butanoyloxyfucoxanthin; 10, Fucoxanthin; 11, Neoxanthin; 12, Prasinoxanthin; 13, Violaxanthin; 14, 19'-hexanoyloxyfucoxanthin; 15, Diadinoxanthin; 16, Antheraxanthin; 17, Diatoxanthin; 18, Alloxanthin; 19, Zeaxanthin; 20, Lutein; 21, Divinyl (DV)- Chl *b* + Chl *b*; 22, DV- Chl *b* epimer; 23, MGDG- Chl c_2; 24, DV- Chl a; 25, Chl *a*; 26, Chl *a* epimer; 27, β, ε-Carotene; 28, β,β-Carotene

Table headings

Table 1. Gradient profile for binary chromatographic systems

Table 2. Gradient profile for quaternary chromatographic systems

Table 1

Time (min.)	% A MeOH: AcN: 25 mM aq. pyridinium acetate (pH 5) (50:25:25 v:v:v)	%B MeOH:AcN:Acetone (20:60:20 v:v:v)
0	100	0
22	60	40
28	5	95
38	5	95
40	100	0

Table 2

Time (min.)	% MeOH	% AcN	% 25mM aq. pyridinium acetate (pH 5)	% Acetone
0	50.0	25.0	25.0	0.0
22	38.0	39.0	15.0	8.0
28	21.4	58.3	1.3	19.0
38	21.4	58.3	1.3	19.0
40	50.0	25.0	25.0	0.0