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NOTE

Improved resolution of mono- and divinyl chlorophylls *a* and *b* and zeaxanthin and lutein in phytoplankton extracts using reverse phase C-8 HPLC

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ABSTRACT: A reverse phase HPLC (high performance liquid chromatography) technique using a binary solvent system with a linear gradient on a Hypersil® MOS2 C-8 column is described. As well as the resolution of key chemotaxonomic chlorophyll and carotenoid pigments, baseline separation of mono- and divinyl chlorophyll *a* and of lutein and zeaxanthin, and partial separation of mono- and divinyl chlorophyll *b* are achieved in a total analysis time of less than 30 min. The method provides an optimal balance between analyte resolution and sample throughput and is hence well suited to the analysis of oceanographic samples.

KEY WORDS: HPLC · C-8 column · Improved resolution · Chlorophylls · Carotenoids

The pioneering work of Gieskes & Kraay (1983) and Mantoura & Llewellyn (1983) has led to the widespread use of reverse phase high performance liquid chromatography (HPLC) techniques for the determination of phytoplankton pigments. HPLC is now the method of choice for obtaining accurate measurements of chlorophyll *a* and a range of accessory pigments in phytoplankton extracts. Since many of these pigments have strong chemotaxonomic associations such HPLC techniques provide incisive information in the study of a variety of marine processes.

Various improvements in the HPLC techniques have been introduced over the years and the establishment of a SCOR (Scientific Committee for Ocean Research) working group on pigments led to the publication of a method in which over 50 carotenoids, chlorophylls and their derivatives could be separated and quantified (Wright et al. 1991). More recently, all the results of the working group were published in a monograph (Jeffrey et al. 1997). Nevertheless, one of the problems with some current techniques is that certain key diagnostic pigments co-elute: lutein, a marker of chlorophytes, co-elutes with zeaxanthin which is used to indicate the presence of the prokaryotic cyanobacteria and prochlorophytes, and the resolution of divinyl chlorophylls *a* and *b* from their mono-vinyl analogues is often problematic. Since divinyl chlorophylls *a* and *b* are markers of the recently discovered prochlorophytes (Chisholm et al. 1988), their determination may be used to improve our understanding of the oceanic distribution and role of prochlorophytes.

Wright et al. (1991) separated lutein and zeaxanthin using a ternary gradient elution system on a C-18 column but failed to resolve the divinyl and mono-vinyl chlorophylls. The separation of lutein and zeaxanthin was also achieved using binary gradients on wide-pore polymeric C-18 columns (Van Heukelem et al. 1992, Garrido & Zapata 1993a, b), while the use of temperature controlled HPLC on the polymeric C-18 columns facilitated the separations of lutein and zeaxanthin and of mono- and divinyl chlorophylls *a* and *b* (Van Heukelem et al. 1994, Van Lenning et al. 1995). However, these latter techniques are time consuming, requiring more than one run to produce quantifiable results.

A significant improvement in separating mono- and divinyl chlorophylls was achieved by Goericke & Repeta (1993) using a C-8 reverse phase column. This method also allowed resolution of most of the other key diagnostic pigments, except that of lutein and zeaxanthin, and has been adapted to investigate the distribution of phytoplankton pigments in the Mediterranean Sea (Barlow et al. 1997a) and the Arabian Sea (Barlow et al. 1997b). Recently, Vidussi et al. (1996) reported an

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adaptation of the C-8 technique of Goericke & Repeta (1993) involving alteration of the polarity of the solvent system and modification of the binary gradient. This technique achieved good resolution of mono- and divinyl chlorophyll *a* as well as partial resolution of mono- and divinyl chlorophyll *b* and lutein and zeaxanthin in 20 min. We have modified the method of Vidussi et al. (1996) to attain complete baseline resolutions of mono- and divinyl chlorophyll *a* and of lutein and zeaxanthin. This note summarizes our improved method and its application to natural phytoplankton assemblages.

Methods. Various microalgal cultures, as recommended by the SCOR working group (Jeffrey et al. 1997), were maintained and used in the method development, including: *Amphidinium carterae*, *Chroomonas salina*, *Dunaliella tertiolecta*, *Emiliania huxleyi*, *Pelagococcus subviridis*, *Synechococcus* sp. and *Prochlorococcus* sp. Cultures were filtered through 25 mm GF/F glass fibre filters (Whatman) and extracted in 2 to 5 ml 90% acetone by ultrasonication and centrifugation. Extracts were loaded into a Thermo Separations autosampler (capable of cooling pigment extracts to 2°C) and mixed with 1 M ammonium acetate (1:1, v/v) prior to injection onto a Shimadzu HPLC system (dual LC-6B pumps; SCL-6B controller). The column was a 3 µm Shandon Hypersil® MOS2 (endcapped), C-8 (6.2 to 6.8% carbon), 120 Å pore size, 100 × 4.6 mm and maintained at 30°C. Pigments were separated at a flow rate of 1 ml min^{-1} by a linear gradient programmed as follows (minutes; % solvent A; % solvent B): (0; 75; 25), (1; 50; 50), (20; 30; 70), (25; 0; 100), (32; 0; 100). The column was then reconditioned to original conditions over a further 7 min. Solvent A consisted of 70:30 (v/v) methanol:1 M ammonium acetate and solvent B was 100% methanol. Pigments were detected by absorbance at 440 nm using a Shimadzu SPD-6AV spectrophotometric detector. Chromatographic data was processed using the Pye-Unicam 4880 software and pigments identified by retention time and on-line visible spectroscopy using a Waters 990 diode array detector. Chlorophyll *a* and *b*, and α- and β-carotene standards were obtained from Sigma Chemical Co., and divinyl chlorophyll *a* and *b* from R. Bidigare (University of Hawaii, Honolulu). Standards for the other pigments were purchased from the Water Quality Institute (VKI), Hørsholm, Denmark. The limits of detection for all analytes were of the order of 1.0 ng l^{-1}.

Results and discussion. Figs. 1 & 2 illustrate the resolution of phytoplankton pigments detected in mixed microalgal cultures and show baseline separation of mono- and divinyl chlorophyll *a* and of lutein and zeaxanthin, and partial separation of mono- and divinyl chlorophyll *b*. In our method, zeaxanthin (peak 5, Fig. 1 and peak 10, Fig. 2) eluted ahead of lutein (peak 6, Fig. 1 and peak 11, Fig. 2). This contrasts with Vidussi et al. (1996) who report zeaxanthin to elute after lutein.

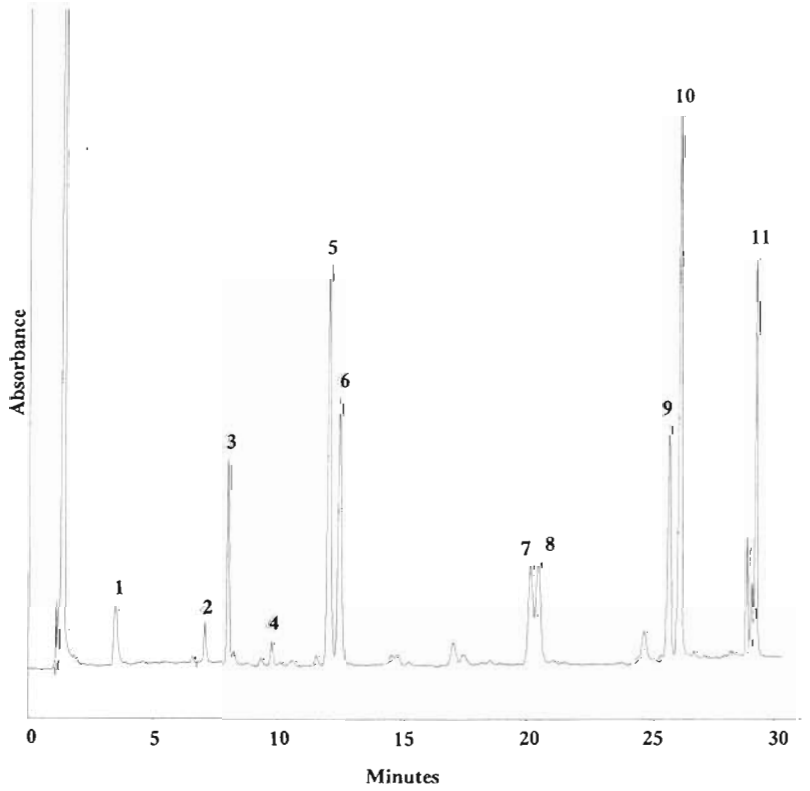

Fig. 1. Absorbance chromatogram of a mixture of *Dunaliella tertiolecta*, *Synechococcus* sp. and *Prochlorococcus* sp. Pigment identities are: (1) chlorophyll *c*-like pigment; (2) neoxanthin; (3) violaxanthin; (4) antheraxanthin; (5) zeaxanthin; (6) lutein; (7) divinyl chlorophyll *b*; (8) chlorophyll *b*; (9) divinyl chlorophyll *a*; (10) chlorophyll *a*; (11) α- and β-carotene

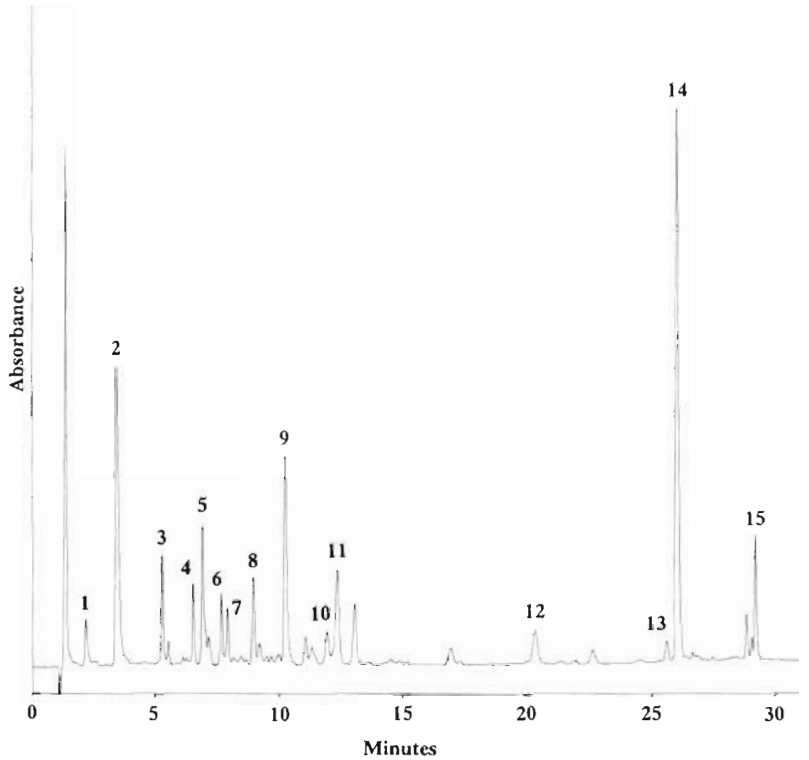

Fig. 2. Absorbance chromatogram of a mixture of *Dunaliella tertiolecta*, *Amphidinium carterae*, *Emiliania huxleyi*, *Pelagococcus subviridis*, *Chroomonas salina* and *Prochlorococcus* sp. Pigment identities are: (1) chlorophyll c_3; (2) chlorophylls c_1 and c_2; (3) peridinin; (4) 19'-butanoyloxyfucoxanthin; (5) fucoxanthin; (6) 19'-hexanoyloxyfucoxanthin; (7) violaxanthin; (8) diadinoxanthin; (9) alloxanthin; (10) zeaxanthin; (11) lutein; (12) chlorophyll *b*; (13) divinyl chlorophyll *a*; (14) chlorophyll *a*; (15) α- and β-carotene

The improved resolution of zeaxanthin and lutein in our method was achieved by increasing the run time of the chromatographic separations and by changing the linear gradient to reach 70% B at 20 min and 100% B at 25 min and hold 100% B isocratically for a further 7 min. The chlorophyll *c*-like pigment in Fig. 1 appeared to be Mg 3,8 divinyl-phaeoporphyrin *a*5 (Mg 3,8 DVP *a*5) as reported by Goericke & Repeta (1992). However, further analysis needs to be undertaken to positively confirm this identification.

The alteration in the run time and gradient also resulted in achieving baseline separations of mono- and divinyl chlorophyll *a* (peaks 9 and 10, Fig. 1; peaks 13 and 14, Figs. 2 & 3) and an improvement in the degree of separation of mono- and divinyl chlorophyll *b* achieved by Vidussi et al. (1996) and Goericke & Repeta (1993) (peaks 7 and 8, Fig. 1; peaks 10 and 11, Fig. 3). The method also achieved good resolution of the other major chlorophyll and carotenoid pigments of interest (Figs. 1 & 2) although it did not separate chlorophylls c_1, c_2 and Mg 3,8 DVP *a*5, nor could we resolve prasinoxanthin from 19'-hexanoyloxyfucoxanthin. However, prasinoxanthin is usually found in very low concentrations in the open ocean (Everitt et al. 1990, Letelier et al. 1993), while 19'-hexanoyloxyfucoxanthin is one of the major carotenoids detected in phytoplankton populations (Barlow et al. 1997a, b). If separation of all known chlorophyll *c* compounds is desired then the methods of Kraay et al. (1992), Van Lenning et al. (1995) and Zapata & Garrido (1997) using C-18 columns, temperature control and long run times may be employed.

We have used the method presented here to analyse samples collected on Atlantic Meridional Transect cruises between the United Kingdom and the Falkland Islands. A representative chromatogram of a 70 m sample from the oligotrophic South Atlantic gyre (7.50° S, 29.75° W) shows satisfactory resolution of a range of diagnostic pigments, as illustrated in Fig. 3. It should be noted that the longer retention times in Fig. 3 compared to Figs. 1 & 2 are the result of the use of a 20 mm guard column during analysis of field samples which was not used during method development on cultures.

Fig. 3 demonstrates that divinyl chlorophylls *a* and *b* were more abundant than mono-vinyl chlorophylls *a* and *b*, and with the dominance of zeaxanthin, it appears that the prokaryotes *Synechococcus* sp. and *Prochlorococcus* sp. were the major components of the phytoplankton community. The detection of 19'-hexanoyloxyfucoxanthin, 19'-butanoyloxyfucoxanthin, fucoxanthin and peridinin indicated that other nanoflagellates, diatoms and dinoflagellates were present in lower concentrations.

In summary, we have described a reverse phase HPLC method using a C-8 column which provides improved resolution of mono- and divinyl chloro-

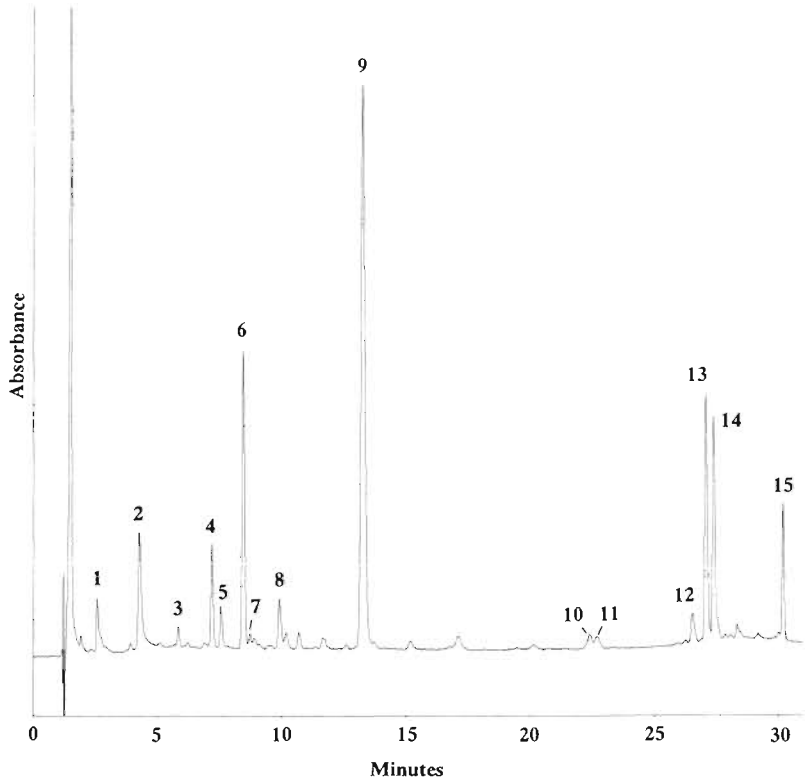

Fig. 3. Absorbance chromatogram of a phytoplankton sample from 70 m in the oligotrophic South Atlantic gyre (7.50° S, 29.75° W). Pigment identities are: (1) chlorophyll c_3; (2) chlorophylls c_1 and c_2; (3) peridinin; (4) 19'-butanoyloxyfucoxanthin; (5) fucoxanthin; (6) 19'-hexanoyloxyfucoxanthin; (7) violaxanthin; (8) diadinoxanthin; (9) zeaxanthin; (10) divinyl chlorophyll *b*; (11) chlorophyll *b*; (12) chlorophyll *a* allomer; (13) divinyl chlorophyll *a*; (14) chlorophyll *a*; (15) α- and β-carotene

phylls *a* and *b*, and lutein and zeaxanthin, as well as satisfactory separation of other key pigment biomarkers within 30 min. Although slower than the 20 min analysis time of Vidussi et al. (1996), it is faster than the 40 min run time of Goericke & Repeta (1993). Providing a pragmatic balance between good analyte resolution and acceptable sample throughput, this method is hence suitable for application to the analysis of oceanographic samples.

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