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SHORT COMMUNICATION

Determination of chlorophylls and carotenoids of marine phytoplankton: separation of chlorophyll *a* from divinyl-chlorophyll *a* and zeaxanthin from lutein

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Abstract. A rapid reverse-phase HPLC method is presented for the identification and quantification of most of the phytoplankton pigments. This method yields the resolution of divinyl-chlorophyll *a* and chlorophyll *a*, as well as the partial resolution of lutein and zeaxanthin, and of divinyl-chlorophyll *b* and chlorophyll *b*. In addition, chlorophylls $c_{1,2}$ and c_3 are well resolved. The analysis time for one sample is 20 min, which makes this method particularly suited when large numbers of samples have to be processed.

A useful HPLC method for phytoplankton pigment analysis in natural waters has to meet two main criteria: high resolution and rapidity. Indeed, most pigments of taxonomic and physiological interest have to be resolved in a single run, and the analysis time has to be the shortest possible to allow numerous samples to be processed, as now required by most sampling grids.

The introduction of reverse-phase methods (e.g. Mantoura and Llewellyn, 1983) allowed the identification of most pigments of interest in runs of less than half an hour. One problem with the older methods was the co-elution of zeaxanthin and lutein (Gieskes and Kraay, 1983). The separation of these pigments is especially important for distinguishing autotrophic prokaryotes (which contain zeaxanthin) from green algae (which contain mainly lutein). An improvement was proposed by Wright *et al.* (1991) which allowed the resolution of both pigments. At the same time, the discovery of prochlorophytes in tropical waters (Chisholm *et al.*, 1988), with a unique pigment signature (Gieskes and Kraay, 1983, 1986; Goericke and Repeta, 1992), asked for the development of still other methods to resolve divinyl-chlorophyll *a* (prochlorophytes) from chlorophyll *a* (others). These developments have included normal-phase methods (Gieskes and Kraay, 1983, 1986; Veldhuis and Kraay, 1990; Letelier *et al.*, 1993), acidification procedures (Goericke and Repeta, 1992; Partensky *et al.*, 1993) and, more recently, an RP-C18 temperature-controlled HPLC method (Van Heukelem *et al.*, 1994; Van Lenning *et al.*, 1995). These procedures are time consuming because more than a single run is required for quantifying all pigments, or they do not allow the determination of other pigments of interest, particularly carotenoids.

The reverse-phase-C8 (RP-C8) method proposed by Goericke and Repeta (1993) was a real improvement because it allowed a routine analysis which resolved most of the pigments and also provided a baseline separation of divinyl-chlorophyll

a and chlorophyll *a*, as well as a partial separation of divinyl-chlorophyll *b* and chlorophyll *b*. However, this method is time consuming (45 min), does not resolve lutein from zeaxanthin, and results in tailing peaks for the polar chlorophyll *c*.

The method presented here has been adapted from that of Goericke and Repeta (1993). To test this method, 2 l of seawater were collected at the deep chlorophyll maximum from an oligotrophic site of the tropical North Atlantic ocean (OLIGO site, France-JGOFS EUMELI 4 cruise: 21°00′N, 31°00′W) and at 5 m during a diatom bloom in the Mediterranean Sea (France-JGOFS DYFAMED site: 43°24′N, 7°52′E). Water samples were filtered through 25 mm GF/F Whatman glass fibre filters. Extraction was performed in 3 ml of methanol. Owing to the lack of chlorophyll *b* in the Atlantic samples, and in order to test the separation of divinyl-chlorophyll *b* versus chlorophyll *b*, chlorophyll *b* (purified from *Dunaliella tertiolecta*) was spiked in the extract. The separation of lutein from zeaxanthin was tested using a mixture of pigment extracts from cyanobacteria (*Synechococcus* sp.) and Chlorophyceae (*D.tertiolecta*). Prior to injection, 500 µl of extract were mixed with 250 µl of 1 M ammonium acetate. The extract was injected through a 200 µl loop into the HPLC system (LDC 4100 Constametric Pump, LDC 3100 spectromonitor set at 440 nm and 991 Waters photodiode array detector) equipped with a 3 µm Hypersil® MOS, 10 cm, 4.6 mm (ID) C8 column (Shandon). The elution was performed at a flow rate of 1 ml min^{-1} using a linear binary gradient between solvent A (MeOH:0.5 N aqueous ammonium acetate, 70:30 v/v) and solvent B (MeOH) which was programmed according to the following procedure (minutes; % solvent A, % solvent B): (0; 75,25), (1; 50, 50), (15; 0, 100), (18.5; 0, 100), (19; 75, 25). Identification of pigments was performed by comparison of on-line-collected absorption spectra with those of a library of spectra established from reference cultures.

Most of the pigments potentially observable *in situ* are reported in the two chromatograms of Figure 1. The resolution of the pigments is better (prochlorophyte pigments) than that achieved by common RP-C18 HLPC methods described in the literature. Nevertheless, the method presented here, when compared to the RP-C8 method of Goericke and Repeta (1993), offers the additional improvements listed below.

First, the elution time of divinyl-chlorophyll *a* and chlorophyll *a* is <17 min, which is equivalent to the elution time of chlorophyll *a* on most of the common RP-C18 systems, but shorter than the 34 min elution time on the RP-C8 system reported by Goericke and Repeta (1993). This time reduction was achieved in several ways: (i) by shortening the time required to reach 100% methanol; (ii) by using, at the beginning of the run, a less polar solvent system (22.5% water) than Goericke and Repeta (1993) used (25% water); (iii) by reducing the polarity of the solvent during the first minute of the run. These procedures bring the chlorophylls *c* closer to pigments with moderate polarity (carotenoids with the elution time from peridinin to zeaxanthin) while having practically no effect on the resolution of chlorophyll and divinyl-chlorophyll compounds. Second, this method allows a better resolution of the polar chlorophyll $c_1 + c_2$ and c_3 (Figure 1). This better resolution was achieved by increasing the amount of ammonium acetate in solution A, together with the use of methanol for extraction instead of acetone and the addition of the ion-pairing reagent to the sample extract. Third, this

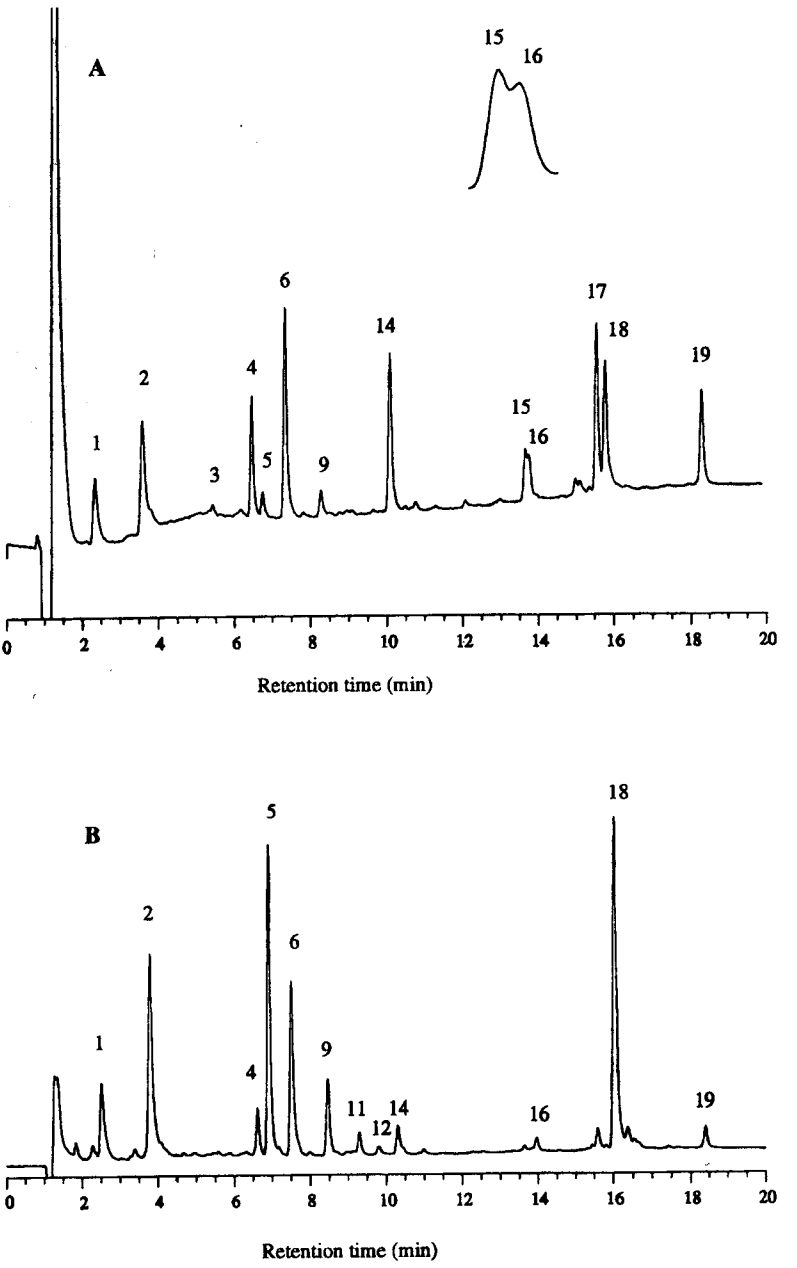

Fig. 1. Two 440 nm pigment chromatograms showing the achieved resolution for various phytoplankton assemblages. (**A**) Typically oligotrophic conditions (Eumeli, OLIGO site: 21°00′N, 31°00′W). The insert shows the resolution of divinyl-chlorophyll *b* and chlorophyll *b*. (**B**) Typical diatom bloom conditions in the Mediterranean Sea (DYFAMED site: 43°24′N, 7°52′E). 1: chlorophyll c_3; 2: chlorophyll $c_1 + c_2$; 3: peridinin; 4: 19′-butanoyloxyfucoxanthin; 5: fucoxanthin; 6: 19′-hexanoyloxyfucoxanthin; 9: diadinoxanthin; 11: alloxanthin; 12: diatoxanthin; 14: zeaxanthin; 15: divinyl-chlorophyll *b*; 16: chlorophyll *b*; 17: divinyl-chlorophyll *a*; 18: chlorophyll *a*; 19: α- and β-carotene.

method is capable of resolving, at least partially, lutein from zeaxanthin (Figure 2), which co-elute under the conditions used by Goericke and Repeta (1993).

Given the potential and the wide range of applicability of this method, significant improvement can be made in assessing phytoplankton biomass quantification and distribution. Using this method, prochlorophyte biomass can be routinely and precisely quantified using divinyl-chlorophyll *a* as marker. Until now, prochlorophyte records based on pigment signatures have been limited to the tropical oligotrophic ocean (Goericke and Repeta, 1993; Letelier *et al.*, 1993; Claustre and Marty, 1995; Suzuki *et al.*, 1995). However, using cytometric signatures, prochlorophytes have also been reported in rather unexpected seasons or localities, e.g. during winter in the Mediterranean Sea (Vaulot *et al.*, 1990). In such cases, where the ratio of chlorophyll *a*/divinyl-chlorophyll *a* may be very high, the presence of divinyl-chlorophyll *a* may be masked using classical

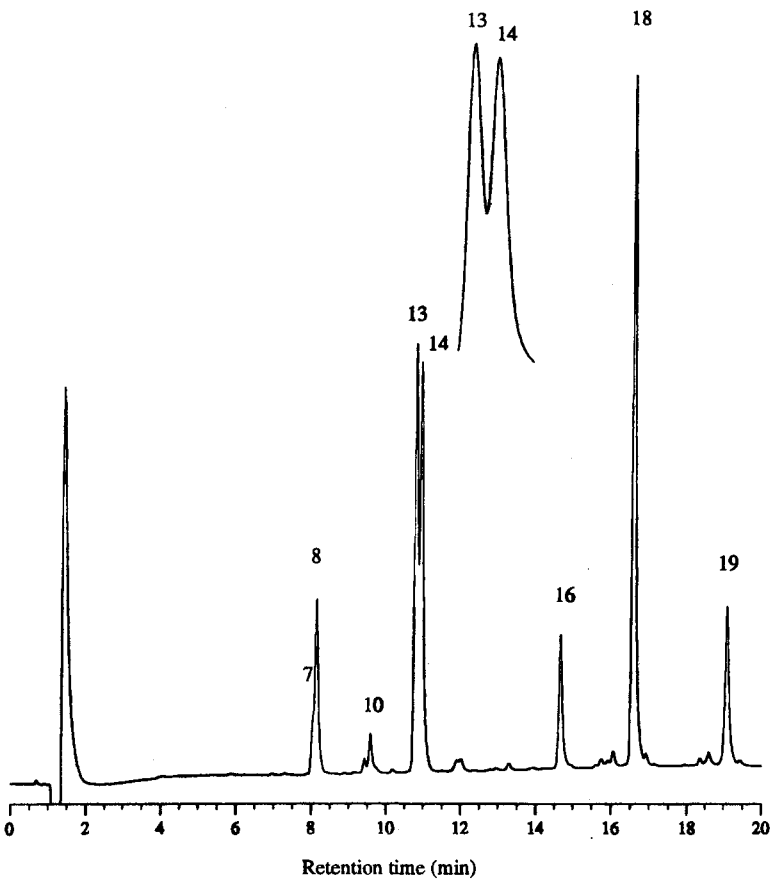

Fig. 2. Chromatogram at 440 nm highlighting the resolution of zeaxanthin and lutein on a mixture of *D.tertiolecta* and *Synechococcus* sp. 7: 9′-*cis*-neoxanthin; 8: violaxanthin; 10: antheraxanthin; 13: lutein; 14: zeaxanthin; 16: chlorophyll *b*; 18: chlorophyll *a*; 19: α- and β-carotene.

RP-C18 systems, making the method presented here an indispensable alternate tool.

Prochlorophytes contribute to the chlorophyll *b* signal together with divinyl-chlorophyll *b*, especially in high-light conditions (Goericke and Repeta, 1993; Partensky *et al.*, 1993; Moore *et al.*, 1995) which prevents the use of chlorophyll *b* as a marker of green algae. The resolution of zeaxanthin from lutein, although partial, overcomes this problem by using lutein as a marker of green algae.

Besides refining the procedure for chemotaxonomic determinations, this method can be applied efficiently towards the understanding of phytoplankton dynamics. Dilution experiments combined with HPLC (Burkill *et al.*, 1987), as well as pigment labelling (Gieskes and Kraay, 1989; Goericke and Welschmeyer, 1993), are methods aimed at determining specific gross growth rates, and these experiments will benefit from increased resolution of important taxonomic pigments.

Finally, combined with the resolution improvements, the short run time required by this method allows the processing of numerous samples, thus allowing the concomitant sampling frequency required by actual research, especially at the mesoscale or sub-mesoscale. Therefore, RP-C8 HPLC methods, as presented here, should be more widely used to assess better the distribution and dynamics of the major phytoplankton groups in the ocean.

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