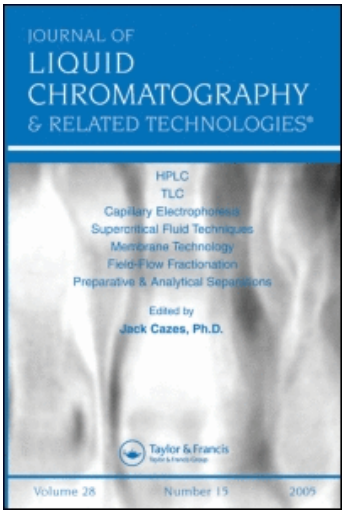

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THE USE OF HIGH PRESSURE LIQUID CHROMATOGRAPHY FOR THE IDENTIFICATION AND PREPARATION OF PIGMENTS CONCERNED IN PHOTOSYNTHESIS

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ABSTRACT

The chlorophylls and carotenoids present in preparations from chloroplasts of marine algae can be extracted and separated by high pressure liquid chromatography (H.P.L.C.). The reverse-phase columns; Partisil 10 ODS (Whatman), μ Bondapak C18 and μ Bondapak CN (Waters Associates) gave good separation of the different pigments. Addition

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of the ion-pairing agent tetrabutylammonium phosphate to the methanol/water solvents gave improved separations with complex mixtures and identification was facilitated by examination of the column eluant at 440nm where all the pigments absorbed and at 650nm where only chlorophylls absorbed. The method allowed the isolation of individual pigments for further study.

INTRODUCTION

During work on the isolation of photo-active complexes from marine algae[1] it became necessary to have a means of analysing the pigments present. Methods used to isolate these complexes involve extraction with strong buffers or detergents and chromatography on various columns, and sometimes inactivated the previously photo-active material. It was suspected that this was due to alteration of the chlorophylls present in the complexes, e.g. from chlorophyll a to its chlorophyllide (loss of the phytol group) or to pheophytin (by loss of Mg) or to pheophorbide (minus both phytol and Mg). Such changes make little difference to the absorption spectra of the protein-pigment complexes and might not be observed. Although TLC methods have been devised for separating the pigments of marine algae[2], most workers have found difficulty in working with sucrose plates or in obtaining the particular grade of powdered polythene required for this work. Shoaf[3] described a method for the rapid separation of chlorophylls a and b by reverse-phase HPLC and a micro-method was published using partition HPLC on silica-gel columns showing separation of chlorophylls, their degradation products and of the carotenoids[4]. Carotenoids are believed to be important in photo-active processes and therefore the separation of carotenoids and chlorophylls in the same sample would be an

advantage. The separation of carotenoids and their many isomers was achieved by partition HPLC on silica gel[5]. Chlorophylls c_1 and c_2 occur in marine algae[6] and we found that chlorophyll c was strongly absorbed on silica gel and difficult to elute. For this reason investigations were made, and are reported here, into methods of separating chlorophylls a, b and c and their associated carotenoids. The use of ion-pairing agents gave improved separations in some instances. The advantage of the HPLC method was that it could be used as a preparative method for the isolation of individual pigments.

MATERIALS AND METHODS

Chlorophylls a and b, and β carotene were obtained from Sigma (Sigma Chemical Co. St. Louis) Chlorophyll c_2 was isolated from Amphidinium carterae and c_1 and c_2 were isolated from Phaeodactylum tricornutum as described by Jeffrey[7]. Chlorophyllide a was prepared by incubating cells of Phaeodactylum tricornutum in 30% acetone at 40°C for 2 hrs and isolating the pigment on a cellulose column[8]. Pheophytin was prepared by acid treatment of chlorophyll a[9].

All solvents used were of ANALAR quality and for HPLC were degassed and filtered through a Pyrex filter holder and a 0.45μm filter (Millipore Corporation). Pigments were isolated from marine algae or from protein-pigment complexes[1] by pouring the materials into 30 volumes of a mixture of acetone, dimethyl sulphoxide 3:1 $^{v}/_{v}$, kept at -40°C. After keeping at this temperature with occasional shaking for 1 hr, the mixture was brought to 2°C, centrifuged and the supernatant evaporated by a stream of N_2 to 3 mls, water 1 ml and ethyl ether 4 mls were added and the pigments extracted into the ether. The ether solution

was dried over anhydrous Na_2SO_4 and concentrated to a small volume (0.1 - 1.0mg chlorophyll per ml). All operations were conducted in a dim light.

A Waters analytical high pressure liquid chromatograph (Waters Associates Pty. Ltd., Chippendale, N.S.W. 2008, Australia) equipped with a Model 660 solvent programme accessory was used for these separations. A variable wavelength detector (made by Mr. J.E. Jordan of this laboratory) was used at either 440 nm or 650 nm. When pigments were to be isolated, the eluate from the columns was collected by means of a Gradipore MK2 fraction collector (Gradipore Pty. Ltd., Scientific Instruments, Artarmon, Sydney). The columns used were stainless steel 4.6 x 250 mm and packed with one of the following particles: Partisil 10 ODS (Whatman Ltd., Maidstone, England), μ Bondapak CN (Waters Associates), μ Porasil (Waters Associates). When ion-pairing was used tetrabutylammonium phosphate (Pic A Waters Associates) or 1 heptane sulphonic acid (Pic B) was added to methanol/water mixtures to produce 5mM solutions.

RESULTS AND DISCUSSION

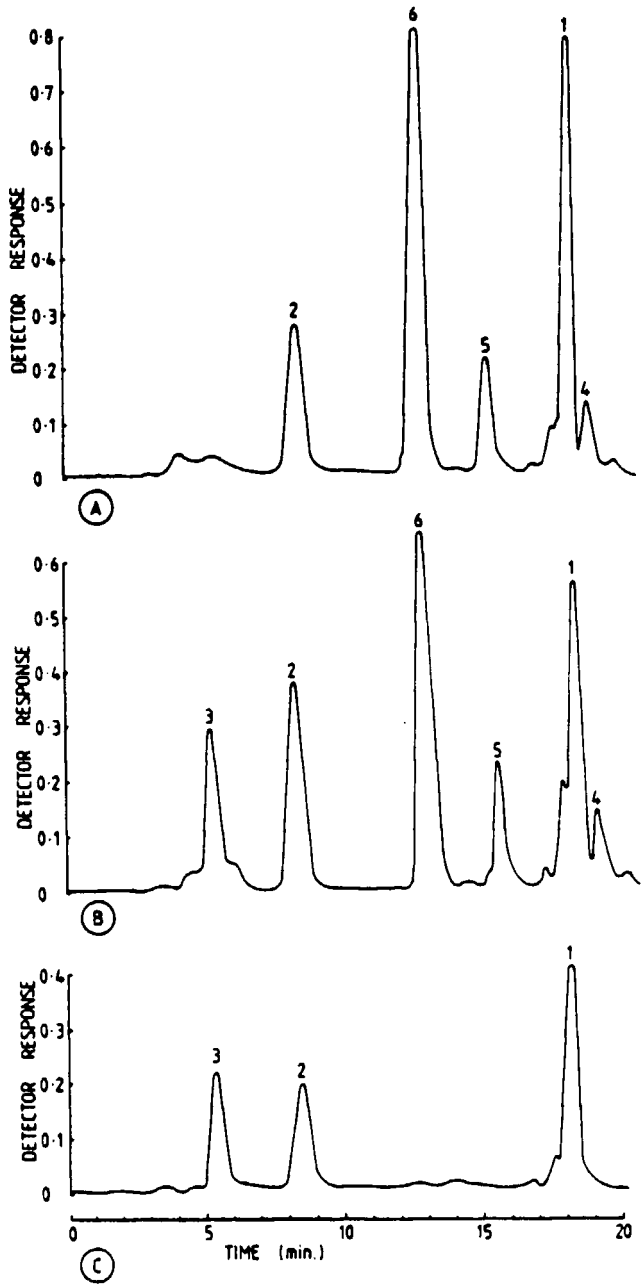
The use of the detector at 440 ± 10 nm serves to detect all three chlorophylls, by their Soret bands, and also the carotenoids (fig. 1a). Chlorophylls and their derivatives are detected at 650 ± 10 nm at which wavelength the carotenoids are transparent (fig. 1c). The use of these two wavelengths assisted in identifying the different pigments. The pigments from freshly harvested Phaeodactylum tricornutum, separated on the reverse phase Partisil 10 ODS by a methanol/water gradient, are shown in fig. 1a. After breaking the cells and isolating the chloroplast membranes[1] it was found that the chlorophyllase, known to be present in

this organism[8], had partially degraded the chlorophyll a to chlorophyllide a (peak 3 fig. 1b and 1c). A corresponding reduction in photo-activity had occurred. Since chlorophyllase occurs sporadically in marine algae[10] it is important that the integrity of the pigments be studied during the isolation of photosynthetic pigment complexes.

The solvent programme of a linear gradient 70:30 $^{v}/_{v}$ to 95:5 $^{v}/_{v}$ methanol/water gave the best separation with the pigments of Phaeodactylum tricornutum. The different peaks were collected as they emerged from the column and identified by their absorption spectra[2] and also for the carotenoids by TLC on polyamide (Merck) plates in seven different solvent systems[11].

Peaks 2 in figs. 1 and 2 were identified as chlorophyll c. Various column packings and solvent mixtures were used in attempts to separate c_1 from c_2. Although these two pigments can just be separated by liquid chromatography on a cellulose column[7], none of the commercially available HPLC column packings gave any separation. Chlorophylls c_1 and c_2 were strongly absorbed during partition chromatography on silica gel (μ Porasil) using hexane, isopropanol mixtures[4] and could then only be eluted by 100% methanol. Neither of the ion-pairing agents added to solvents on the reverse phase "C18" column (Partisil 10 ODS) were able to resolve c_1 from c_2. On the μ Bondapak CN column (Waters) the pattern obtained was similar to the reverse phase "C18" column, except peaks eluted up to 15 mins were very sharp but later peaks were not well separated e.g. β carotene from chlorophyll a.

The ion-pairing agent tetrabutylammonium phosphate (Pic A) was particularly useful for separating peaks that were not well separated



in methanol/water solvents on the reverse-phase "C18" column. Figure 2a shows such a separation of lutein (peak 7) from chlorophyll a (peak 1) using this ion-pairing agent. In methanol/water solvents and using various gradients these two peaks did not separate. The pigments of Pavlova lutheri are rich in lutein and diadinoxanthin and these carotenoids were readily isolated from the extract by HPLC. Fig. 2b shows the pigments present in a dinoflagellate Amphidinium carterae. Although the conditions specified allow the separation of the pigments

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FIGURE 1

Pigments from Phaeodactylum tricornutum examined on a reverse-phase "C18" column (Partisil 10 ODS) with a gradient 70:30 to 95:5 $^{v}/_{v}$ methanol, water. Flow rate 2m/min. "A" freshly isolated cells, detector at 440nm; "b" chloroplast membranes (440nm); "c" chloroplast membranes, detector at 650nm.

Peak identification:

(1) chlorophyll a
(2) chlorophyll c_1 and c_2
(3) chlorophyllide a
(4) β carotene
(5) Diadinoxanthin
(6) Fucoxanthin
(7) Lutein
(8) Dinoxanthin
(9) Peridinin
(10) Neoperidinin
(11) unidentified yellow pigment.

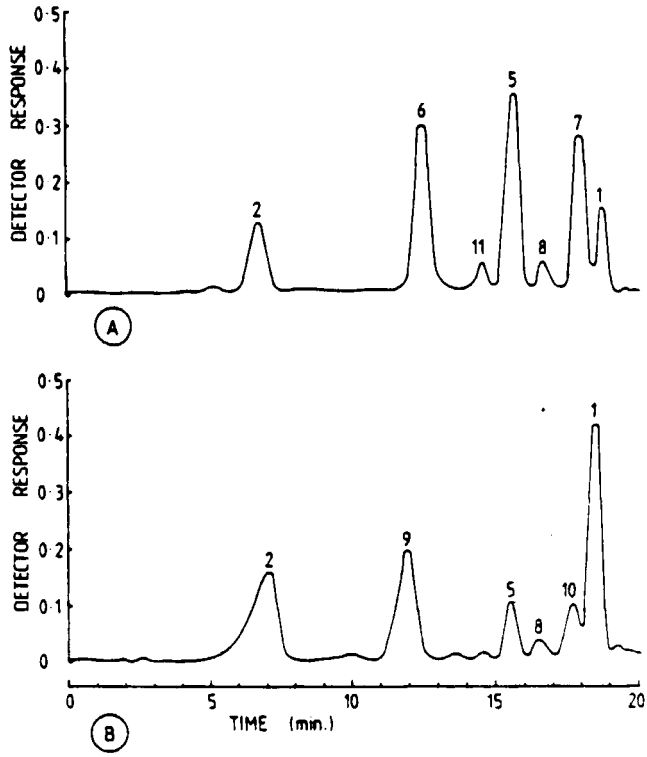

FIGURE 2

Pigments from (a) Pavlova lutheri and (b) Amphidinium carterae examined on a reverse-phase "C18" column (Partisil 10 ODS) with a gradient 70:30 to 95:5 $^{v}/_{v}$ methanol, water containing 5mM tetrabutylammonium phosphate ("Pic A"). Detector at 440nm, peak identification as in fig. 1.

of a single organism, it is recommended that when examining the pigments of a mixed population of marine alge, that the "C18" and μ Bondapak CN columns be tried with a programme of 60:40 to 95:5 $^{v}/_{v}$ methanol/water, 2ml/min for 30 min, with and without tetrabutylammonium phosphate and with the detector set at the two wavelengths 440 and 650 nm. In this

manner most of the pigments of mixed cultures were separable. The areas of the peaks cannot be used for quantitative analysis since the pigments have different ε max. at these wavelengths. However the eluant from the column can be collected and the pigments estimated from their known spectroscopic absorption data.

In summary, the pigments concerned in photosynthesis can be readily separated and identified by HPLC.

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