# The Analysis of Marine Algal Photosynthetic Pigments by High Performance Liquid Chromatography

A Thesis Submitted to the
UNIVERSITY OF CAPE TOWN
in fulfillment of the
requirements for the degree of
Master of Science

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September 1984

ABSTRACT

A method of analysis of the photosynthetic pigments of *in vitro* algal cultures, using reverse-phase high performance liquid chromatography (HPLC) is described. This technique was extended to analysis of photosynthetic pigments in samples from the SIBEX-I Expedition; a preconcentration technique was developed to allow a reduction in sample volume for waters of low productivity.

HPLC analysis allows accurate chlorophyll *a* determinations to be made in the presence of the spectrally similar chlorophyllide *a*, which has been found to be the major chlorophyll *a* breakdown product present in natural waters.

Comparative studies with the SCOR-UNESCO method have shown that as a result of chlorophyllide interference the chlorophyll *a* concentrations are in fact over-estimates to varying degrees depending on the productivity of the biomass.

In addition, quantification of chlorophylls *b* and *c* and the carotenoid complement allows identification of the major algal species present, and provides an indication of the physiological status of the bloom.

ACKNOWLEDGMENTS

I wish to express my appreciation

-to Professor M J Orren for support, constructive criticism and supervision of this work.

-to Pedro Monteiro, whose concept this field of study was, for providing direction, innovative ideas, and unfailing enthusiasm.

-Dave Webster and Beckman Instruments, for helpful advice, technical support and the generous loan of the HPLC and detector for use on board the M.V. S.A. Agulhas, without which this work could not have taken place.

-to Dr L. V. Shannon of the Sea Fisheries Research Institute, Cape Town, for access to their HPLC, loan of the Hewlett Packard 3390A Integrator and use of their algal cultures during the entire course of this study.

-to Caz Thomas for typing, editing and correcting this thesis.

-to Helmke Hennig for use of his pure algal cultures and ingenious practical ideas.

-to Koos Williams and John Joseph for invaluable technical assistance.

-to Simon Goudie, Alan Solomon and Daniel Garside for assistance with the diagrams.

-to Professor B R Allanson (Chief Scientist for SIBEX-I), Captain W. Leith, the officers and crew of the M.V. S.A. Agulhas.

-to the Department of Transport for funding of this project with scientific advice from SASCAR

-and to Lana, Ian, Eric, Pedro and Llewellyn, the SIBEX Night Station Team.

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# 1. INTRODUCTION

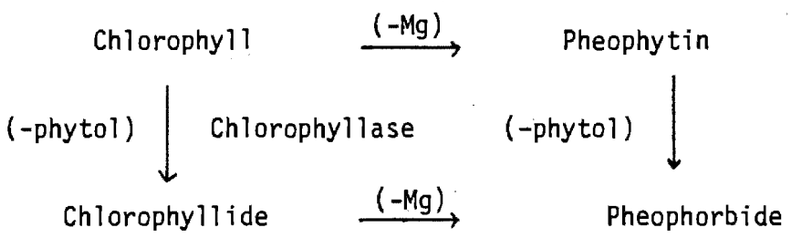
# 1 INTRODUCTION

Marine algae are the primary producers of almost all energy substrates for the entire marine stock, and estimation of their photosynthetic activity is important in evaluating the viability and carrying capacity of a particular ecosystem. Measurement of chlorophyll *a* has traditionally been used as a measure of algal biomass, and as an index for the primary production of marine phytoplankton (Gibbs, 1979). Chlorophyll *a*, a photosynthetic pigment, is found in all marine algae together with chlorophylls *b* or *c*, and several carotenoids, which are characteristic of different species. The chlorophylls are generally associated with their breakdown products, the pheophytins, pheophorbides and chlorophyllides, which are interrelated as shown in Fig. 1. These breakdown products occur in senescent algal blooms, as a result of "natural" decomposition of the parent chlorophylls, and also arise after grazing by zooplankton and bacterial enzymatic digestion of the phytoplankton cells. The ratio of breakdown product to the parent chlorophyll is hence an indication of the physiological state of the bloom.

The carotenoids are usually yellow-red isoprenoid polyene pigments, which may be subdivided into the carotenes and the xanthophylls. They offer protection against

Fig.1.

Interrelationships between chlorophyll derivatives




Jackson, 1976

photodynamic damage and allow auxiliary light absorption for photosynthesis and phototaxis (Liaeen-Jensen, 1977).

These pigments are particularly suited to spectroscopic detection, owing to their absorption of light in the visible region of the electromagnetic spectrum.

## Development of Methods of Chlorophyll a Determination

Richards with Thompson (1952) measured the absorbance of a sample at three different wavelengths for the estimation of chlorophylls a, b and c. This is referred to as the "trichromatic method". However, this method can only be applied in a system in which no pheophytins are present, because the pheophytins absorb radiation at the same wavelength range as the parent chlorophyll, although having a lower molar extinction coefficient. Lorenzen (1967) proposed the technique of acidification of the chlorophyll a, converting it to pheophytin a, and relating the change in absorbance before and after the acidification to the amount of chlorophyll a originally present.

At the SCOR-UNESCO Workshop in 1966, the existing methods of chlorophyll a determination were combined, and various empirical equations developed, which it was hoped would allow differentiation between the various chlorophylls and included a correction factor to account for the breakdown

of chlorophyll to pheophytin (Strickland & Parsons, 1972). Other workers (Jeffrey & Humphrey, 1975) have subsequently refined the UNESCO equations for various ecosystems, and spectrophotometric determination of chlorophyll is widely used today.

Yentsch and Menzel (1963) and Holm-Hansen *et al* (1965) developed a fluorometric method of chlorophyll *a* determination, which allowed a fifty to one hundred-fold increase in sensitivity. Correction for pheophytins was also made by the acidification procedure. However, an awareness that chlorophyll degradation products other than pheophytin present in senescent phytoplankton and detritus, could be causing over-estimates of the "true" chlorophyll values led to the development of separation procedures for the chlorophyll pigments.

Parsons & Strickland (1963) determined chlorophyll *c* by partitioning the pigments between an aqeuous phase and a non-polar hexane phase. Whitney and Darley (1979) adapted this method to permit more accurate determination of chlorophyll *a* in the presence of pheophytin.

Separation of the chlorophylls *a* and *b* was achieved by using paper chromatography (Jensen and Sakshaug, 1973) and quantitative reverse phase thin layer chromatography was used by Jeffrey (1974) for accurate measurement of

chlorophyll *a*. Excellent resolution was achieved fairly quickly, but this method was reported to be "too laborious for routine determinations". So the present standard method of chlorophyll *a* analysis remains the SCOR-UNESCO spectrophotometric estimation. This method incorporates several errors which lead to serious over-estimation of chlorophyll *a*, as it is based on the following assumptions:

1. That chlorophyll *a* is the major pigment present, and other pigments and breakdown products occur at insignificant levels. Hence there is no interference in a sample during a scan in the visible region.

2. That the pigment composition does not alter with species and age of the organism.

However, it has been reported that chlorophyll breakdown products occur in all natural water samples (Wun *et al*, 1980). Although the acidification method distinguishes between chlorophyll *a* and pheophytin *a*, no distinction can be made between chlorophyll *a* and chlorophyllide *a*, as they have identical absorption spectra.

All spectrophotometric and fluorometric techniques measure chlorophyllide a as "chlorophyll a" and the over-estimation of chlorophyll a is directly proportional to the chlorophyllide a abundance (Mantoura & Llewellyn, 1983). Pheophytins and pheophorbide also have identical spectral characteristics, and are similarly indistinguishable in the trichromatic technique. The chlorophyllides and pheophorbides are now thought to be important chlorophyll degradation products in addition to pheophytin (Barrett & Jeffrey, 1964; Mantoura & Llewellyn, 1983). Interference of these components will be more serious in highly productive regions, where bacterial enzymatic digestion and zooplankton predation on phytoplankton blooms will give rise to a large number of breakdown products (Glooschenko, 1972) and in the upper layer of euphotic zone during periods of high irradiance (Yentsch, 1965: Lorenzen, 1967). Chlorophyll a concentrations will also be over-estimated in senescent algal blooms, in which chlorophyllide a predominates (Jensen and Sakshaug, 1973).

The acidification technique, although allowing distinction between chlorophyll a and pheophytin a, causes a spectral shift in several carotenoids which subsequently interferes with the chlorophyll a determination (Riemann, 1978). The fluorometric determination of chlorophyll a is also subject to serious interference by chlorophyll b, which is erroneously calculated as pheophytin b (Gibbs, 1979).

It is evident that in order to overcome the problems of interference, the individual components must be separated from one another before quantification.

The method proposed here for pigment analysis employs High Performance Liquid Chromatography (HPLC). This combines the selectivity of the thin layer chromatographic methods with the rapid quantitation of the spectrophotometric and fluorometric methods of determination. HPLC has been investigated by several workers, (Shoaf, 1978; Wun et al, 1979; Brauman & Grimme, 1981; Brown et al, 1981; Falkowski & Sucher, 1981; Gieskes & Cysbert, 1983; Yuzo et al, 1983; Mantoura & Llewellyn, 1983 and Wright & Shearer, 1984), and has been recommended (Jeffrey & Lorenzen, 1980) as this method "far surpasses simple routine spectrophotometric and fluorometric methods for the complete knowledge of all photosynthetic pigments which it displays in a single operation".

## The Separation Process

The chromatographic separation of the components of a mixture occurs by virtue of differences in the equilibrium distribution (K) of the components between the mobile phase and the stationary phase. If Cs and Cm are the concentrations of a component in the stationary and mobile phases respectively, then

$$K = \frac{Cs}{Cm}$$

Elution of a component can only occur when it is distributed in the mobile phase.

The distribution coefficient is thermodynamically governed by the solvation energies of the component in either phase. The solute will preferentially distribute in the phase in which the solvated form is most thermodynamically favoured. Compounds having one or more groups with a large dipole moment will selectively interact with a mobile phase compound, such as water, which also has a large dipole moment. The sample and solvent molecules orientate themselves so that the electrostatic forces between them are at a minimum.

A sample may also interact with the solvent by hydrogen bond formation depending on its tendency to donate or accept protons, and the tendency of the solvent to complement it.

A reverse phase octadecylsilane (C18) column was chosen. This provides a non-polar stationary phase, which is compatible with the non-polar pigment molecules, and allows retention of them. The sample to be analysed (in this case the phytoplankton pigment extract) is dispersed in a relatively polar mobile phase. The sample components

are differentially partitioned between the stationary and mobile phases, according to their partition coefficients $K_1$, $K_2$,.... Kn. The chlorophylls are separated according to their different relative polarities conferred on them by the different peripheral substituent groups in the porphyrin ring.

The rate of migration of a component is then inversely proportional to its distribution coefficient: the most polar compound will have a low K value and will be eluted rapidly, having a low retention time. The least polar compound will have a high K value, which causes it to be retained more strongly in the stationary phase.

The order of elution, on the basis of relative polarity of the components can be predicted by consideration of the molecular structure: it is this that determines both the spectral characteristics - the basis of all spectrophotometric measurements - and their polar properties - which allow their sequential elution and separation on the chromatographic column (Brauman & Grimme, 1981 and Hamilton & Sewell, 1982).

The chlorophylls share the same basic structure: a porphyrin ring in which the four central nitrogen atoms are co-ordinated with a magnesium ion, to form a planar complex.

They can be subdivided on the basis of the peripheral substituent groups into classes. Chlorophylls a, b and c are the most widely distributed, and chlorophylls d, e and 'bacteriochlorophyll' also occur.

The Separation of Chlorophyll a from Chlorophyll b

The only structural difference between chlorophyll b and chlorophyll a is that chlorophyll b has a carbonyl group at position 3 as opposed to chlorophyll a, which has a methyl group at position 3 (Fig 2.). The electronegative effect of the carbonyl introduces a peripheral dipole and increases the overall polarity of the chlorophyll b molecule relative to that of the chlorophyll a molecule. Hence chlorophyll b will elute first.

Chlorophyll c: The chlorophyll c molecule (Fig. 3.)lacks the long non-polar phytol chain, and so is relatively more polar than chlorophylls a and b. It is therefore expected to elute before them (Holden, 1976 and Jackson, 1976).

Separation of Chlorophylls and Pheophytins

The pheophytins a and b are a breakdown product of chlorophylls a and b, formed by loss of the central magnesium ion, and replacement of it by two protons. This

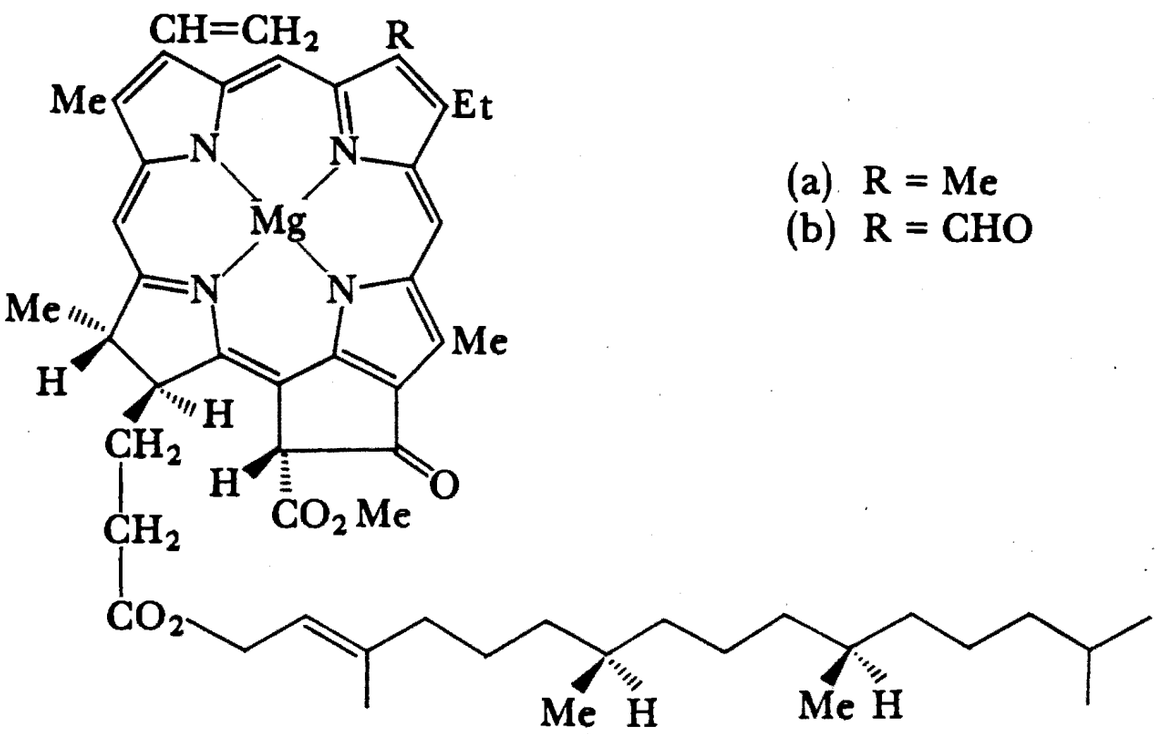
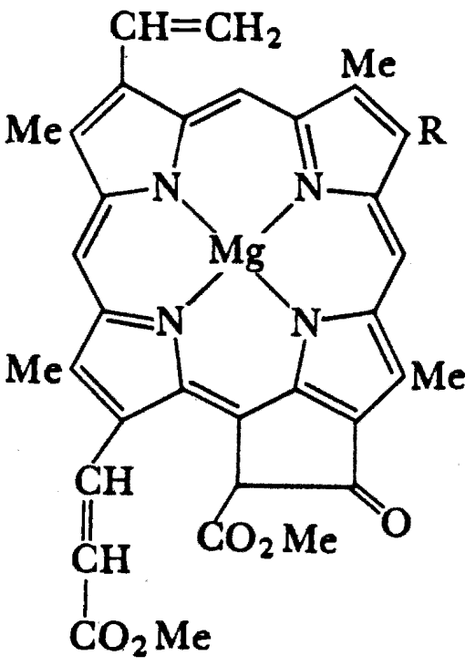



Fig 2. Chlorophylls *a* and *b*.




Chlorophyll $c_1$ R = Et
Chlorophyll $c_2$ R = CH=CH$_2$

Fig 3. Chlorophylls $c_1$ and $c_2$.

affects the relative polarity of the pheophytin in two ways:

1. The magnesium has a vacant $d^{\pi}$ orbital as a fifth co-ordination site, which may be occupied by a pair of electrons from a water molecule (Cotton & Wilkinson, 1980). Any solvent molecule with a lone pair, (eg. water, methanol, acetone) could occupy this site: hence, relative to pheophytin, with no magnesium, the chlorophyll is more easily solvated by a polar mobile phase. Its K value will therefore be lower, and it will be eluted more rapidly.

2. Presence of two protons, instead of magnesium ion causes steric hindrance, and an increase of 8% in ring diameter (Cotton & Wilkinson, 1980): this results in a decrease in relative polarity of the pheophytin which will be retained more strongly on the column (Monteiro pers. comm.).

The combination of these two effects results in a greater relative shift in affinity for solvation between the chlorophylls and the pheophytins than between the a and b classes of chlorophylls, as seen in the chromatogram of these four pigments (Fig.A.3).

Chlorophyllide a is a breakdown product of chlorophyll a (Fig. 4.) formed by loss of the phytol chain and is similar in structure to chlorophyll c. It is consequently more polar than chlorophylls a and b, and is expected to elute before them.

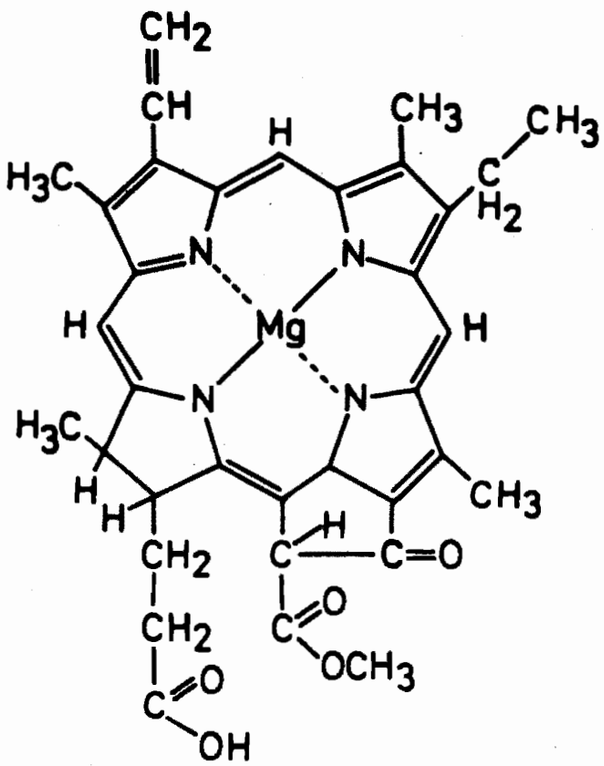



Fig 4. Chlorophyllide *a*.

Pheophorbide *a* is formed by replacement of the magnesium ion in chlorophyllide *a* by two protons, and is expected to be eluted after chlorophyllide *a*, in the same way that the pheophytins are eluted after their parent chlorophylls.

The objectives of this study were:

1. To develop a separation system for analysis of marine algal photosynthetic pigments, using *in vitro* pure cultures.

2. To identify and quantify each of the pigments, using documented special pigment compositions and the differences in spectral characteristics of the individual pigments.

3. To compare the chlorophyll *a* values obtained from HPLC analysis with values calculated by the SCOR-UNESCO spectrophotometric method.

4. To investigate the significance of pigment ratios and taxonomic applications of this method of pigment analysis.

5. To extend the method, developed using *in vitro* specimens, to natural sea water samples.

6. Interpretation of chromatograms obtained in a field exercise.

The first field work took place on SIBEX-I, the first phase of the Second International BIOMASS Experiment (BIOMASS is an acronym for Biological Investigation of Marine Antarctic Systems and Stocks). (Orren, 1984).

A comparison was made between the HPLC chlorophyll _a_ values, the SCOR-UNESCO chlorophyll _a_ measurements and $^{14}C$ primary production values, (Fogg, 1975) which were conducted simultaneously (Allanson, in preparation).

# 2. METHODOLOGY

## 2 METHODOLOGY

### 2.A Apparatus and Reagents

Pure chlorophyll a and b standards were obtained from Sigma Chemical Company (Product codes: C-6144 and C-5878 respectively). These were made up to a known volume in 90% acetone and stored at -20°C in the dark. (Mantoura & Llewellyn, 1983).

Solvents for extraction and chromatography (acetone, methanol and acetonitrile) were all of Analar grade. HPLC grade water was produced by filtering distilled water through a Norganic® trace organic removal cartridge or using freshly drawn Milli-Q® water (Waters Associates). All solvent mixture concentrations are expressed as "volume by volume" (v/v).

Filters used to collect algae from solution were Whatman GFF, of diameter 4,5 cm and porosity 0,7µm and Whatman GFC, of diameter 4,5 cm and porosity 1µm. For microfiltration of the algal extract, Millex-HV® filters, with a Durapore® membrane of porosity 0,45µm were used. For preconcentration, SEP-PAK® C-18 cartridges were used.

All chromatographic work prior to the SIBEX-I cruise was performed on a Varian 5000 High Performance Liquid Chromatograph, and a Varian UV-100 Detector.

Chromatographic work on board ship, and subsequent analysis of SIBEX-I samples was performed on a Beckman High Performance Liquid Chromatograph system, comprising: Two model 112 pumps, an Altex 210 injection valve, a 421 controller, a 15cm ODS Ultrasphere column and a 165 detector.

The Varian system uses a low pressure gradient former, in which the solvents are mixed before entering the high pressure pump and passing to the column. In a high pressure gradient forming system, the solvents are pumped at high pressure, into the mixing chamber and onto the column.

Injections of samples were made using Hamilton 10µl and 100µl syringes.

The ultrasonic bath used was a Bransonic 521 model. All spectroscopic work was performed on a Varian Spectrasan II. Peak quantification was performed using a Hewlett Packard 3390A Integrator and a Linear two-pen chart recorder.

## 2.B Laboratory Work

The following method of pigment extraction was used for all algal analyses throughout this study:
A known volume of about 10ml of algal culture (or more, if the cell density was very low) was filtered onto a GFC 4,5 cm diameter filter. This was placed in a sealed test tube, containing 10ml 90% acetone (90:10 acetone water v/v). The pigments were extracted by ultrasonification for two hours (Mantoura & Llewellyn, 1983). The water was maintained at room temperature by continual replacement of warm water by ice. The filter was then removed and the 10ml 90% acetone, containing the algal pigments filtered through a Millex HV® filter. This was resistant to acetone and removed any particulate matter which would block the pressure tubing of the chromatograph. 10µl, or 100µl if the sample was very dilute, of this extract was then injected onto the column.

Separation of the pigments extracted in this manner was then achieved by elution under the solvent conditions described in Table 2.B. This program was used for all separations on the Varian column. See Appendix A.2 for the development of the separation system.

Table 2.B: Table of Parameters used to control the gradient elution,using acetone and water as solvents,to achieve sequential elution of the pigments.

| Time | Code | Value |
|---|---|---|
| 0 | Flow | 1 |
| 0 | RSVR | BC |
| 0 | %C | 80 |
| 0 | λ | 435nm |
| 1,0 | %C | 85 |
| 4,0 | %C | 90 |
| 6,0 | %C | 95 |
| 7,9 | Flow | 1,0 |
| 8,0 | %C | 100 |
| 8,1 | Flow | 2,0 |
| 10,0 | Flow | 2,0 |
| 10,0 | %C | 100 |
| 13,0 | %C | 80 |
| 15,0 | Flow | 1,0 |

Code:

Flow = Flow Rate (ml/min ) of Mobile Phase

RSVR = Solvent Reservoir

%C = % Acetone

λ = Wavelength at which pigments detected

Peak Identification

Standards for chlorophylls a and b were commercially available, and from these, pure pheophytin standards were easily prepared. (see Appendix A.3). Hence initial identification of the peaks due to chlorophylls a and b and pheophytins a and b was achieved by spiking of the sample with the pure standard. Thereafter, retention times of these peaks were sufficiently reproducible to allow this identification. However, as no other standards were available, identification of the other pigments was made by comparison of chromatograms from algal species of known pigment composition, and use of the dual channel detector to characterise the peaks according to the different spectral properties of the pigments.

WORK ON VARIAN CHROMATOGRAPH

2.1 Peak Identification, using Algal Species

Four sets of samples were taken from different batches of culture stocks from the algae bank at the Sea Fisheries Research Institute (Cape Town). Pigment extracts were made and analysed according to the described methods (Section 2B).

The species tested were:

| CLASS | SPECIES |
|---|---|
| Bacillariophyceae: diatoms - usually yellow brown | *Phaeodactylum tricornutum* |
| | *Thalossiosira weisswellii* |
| | *Chaetoceros gracilis* |
| | *Skeletonema costatum* |
| Prasinophyceae: green flagellates | *Pyramimonas virginica* |
| | *Tetraselmis suecica* |
| Haptophyceae: yellow brown algae including coccolithophores | *Isocrisis galbana* |
| Chrysophyceae: yellow brown algae including silicoflagellates | *Pavlova lutheri* |
| | *Pseudoisocrisis paradoxa* |
| Chlorophyceae: green algae green flagellates | *Dunaliella primolecta* |
| | *Nanno saronensis* |
| | *Chlorella vulgaris* |

Identification : B Mitchell-Innes (pers. comm.)

The pigment compositions of the algal classes studied were:

| CLASS | PIGMENT |
|---|---|
| Bacillariophyceae | Chlorophylls: a and c |
| | carotenes: β and ε |
| | xanthophylls: fucoxanthin |
| | diatoxanthin |
| | diadinoxanthin |
| | neoxanthin |
| Prasinophyceae | Chlorophylls: a and b |
| | carotenes: α and β |
| | xanthophylls: zeaxanthin |
| | lutein |
| | violaxanthin |
| | neoxanthin |
| | siphonoxanthin |
| | siphonein |
| Haptophyceae | Chlorophylls: a and c |
| | carotenes: β,β-carotene |
| | common xanthophylls: diatoxanthin |
| | diadinoxanthin |
| | fucoxanthin |
| | rare xanthophylls: cryptoxanthin diepoxide |
| | cryptoxanthin |

| | |
|---|---|
| Chrysophyceae | Chlorophylls: a and c |
| | carotenes: ß |
| | xanthophylls: lutein |
| | fucoxanthin |
| | diatoxanthin |
| | diadinoxanthin |
| Chlorophyceae | chlorophylls: a and b |
| | carotenes: α ,ß,γ, lycopene |
| | xanthophylls: lutein |
| | violaxanthin |
| | neoxanthin |
| | astaxanthin |
| | zeaxanthin |

References: Liaaen-Jensen, 1977

Davies, 1976

Results: see Figs 3.1. and Table 3.1

## 2.2 *Phaeodactylum* Experiment: to identify chlorophyllide *a*

It is known that certain species of marine algae possess an extremely active chlorophyllase enzyme, capable of converting chlorophyll *a* to chlorophyllide *a*, by cleavage of the phytol chain (Barrett & Jeffrey, 1964).

Such an example is the diatom, *Phaeodactylum tricornutum* which contains chlorophylls *a* and *c*. In the preparation of pigment extracts in acetone:water solutions, cholorophyllase activity is enhanced when the proportion of water is high. It is seen to be most active in 50-60% acetone solutions, in which 90% conversion of chlorophyll *a* to chlorophyllide *a* occurs in 5 minutes. Inhibition of the enzyme occurs in 90-100% acetone solutions( Barrett & Jeffrey, 1964).

Extracts of *Phaeodactylum tricornutum* pigments were made in (i) 50% and (ii) 90% acetone:water solutions. It was expected that chlorophyllase activity in 50% acetone would be enhanced: degradation of chlorophyll *a* to form chlorophyllide *a* would occur. In 90% acetone, which inhibits chlorophyllase activity, the chlorophyll *a* would be expected to be degraded very slowly, if at all.

Comparison of the chromatograms of the extracts from the different acetone solutions should show a clear increase in the peak due to chlorophyllide *a* in the extract prepared in 50% acetone.

Method

1. Six 30ml volumes of pure *Phaeodactylum tricornutum* culture were filtered onto six GFC filters.
2. Three filters, batch "A" were ultrasonicated for two hours in 10ml 50% acetone.

   Three filters, batch "B", were ultrasonicated for two hours in 10ml 90% acetone.
3. The extracts were microfiltered through Millex HV® filters, ready for injection onto the liquid chromatograph column.
4. The following runs were made:

a. 10µl B (90% Acetone) at wavelength 665nm

b. 10µl A (50% Acetone) at wavelength 665nm

c. 10µl B (90% Acetone) at wavelength 435nm

d. 10µl A (50% Acetone) at wavelength 435nm

e. 10µl A spiked with chlorophyll *a* at wavelength 435nm

f. 10µl B (90% Acetone), acidified, at wavelength 435nm

g. 10µl A (50% Acetone), acidified, at wavelength 435nm

Runs a-d allowed initial comparisons of typical *Phaeodactylum tricornutum* chromatograms to be made.

In run "e", the 50% acetone pigment extract is spiked with chlorophyll *a* standard. It is expected that the chlorophyllase enzyme will have been liberated into the solution when acetone-induced lysis of the algal cell walls occurs.

Comparison of chromatograms made immediately after spiking with chlorophyll *a*, and again 30 minutes later should show a large chlorophyll *a* peak in the early stages, which later decreases as a corresponding chlorophyllide *a* peak increases.

It is possible that pheophorbide formation occurs: this would mean that another peak is increasing in the chlorophyllide *a* region. The chlorophyllase enzyme is specific for cleavage of the phytol chain from chlorophyll *a* and therefore formation of pheophorbide by loss of the phytol chain from any pheophytin present will not occur. However, subsequent displacement of the central magnesium ion of chlorophyllide *a* by two protons will cause formation of pheophorbide. To investigate this, solutions A and B were acidified. It was expected to be able to follow the change of chlorophyll *a* to pheophytin *a* and of chlorophyllide *a* to pheophorbide *a*, reflected as a decrease in the chlorophyll *a* and chlorophyllide *a* peak, and a corresponding increase in the pheophytin *a* and pheophorbide *a* peak. Chlorophyll *c* will also be converted to pheoporphyrin *c*. Results: Table 3.2. and Figs 3.2.

## WORK ON BECKMAN CHROMATOGRAPH

### 2.3 Dual-Wavelength Scans

It is possible to monitor two selected wavelengths simultaneously using the Beckman 165 Dual Channel Detector. This allows subtle changes in absorbance of one sample at the two wavelengths to be observed. This eliminates the possibility of such differences arising from inaccurate sample injection, degradation of the labile pigments or evaporation of the volatile solvent. This is particularly advantageous for samples containing both chlorophylls and carotenoids. Only the chlorophyll pigments absorb radiation in the 600 nm wavelength region, allowing immediate distinction between chlorophyll and carotenoid peaks.

This detector is used in Experiment 2.3 to identify the peak due to chlorophyll *c*, and to confirm that the first peak eluted was chlorophyllide *a*. An attempt was then made (Exp. 2.4) to identify the carotenoids, i.e. all those peaks which did not absorb at 665 nm, by running scans at different wavelengths, and relating increases and decreases in absorbance to their spectral maxima and minima.

Exp. 2.3: Dual-Wavelength scans of *Phaeodactylum tricornutum*

*Phaeodactylum tricornutum* was chosen, as it is known to contain chlorophyll *c* and a significant amount of chlorophyllide *a*, due to its extremely active chlorophyllase enzyme. (barret & Jeffrey, 1964). An attempt to elucidate which peaks correspond to these two

pigments was made. Extracts were made in 90% acetone so that the enzyme would be inhibited and the rate of chlorophyll a degradation would be slowed down. Natural chlorophyllide a present in the samples is sufficient to show a peak. Readings from channel 1 were recorded on the Hewlett Packard Integrator, and from channel 2 were recorded on a Linear plotter.

The following runs were made:

| Run No. | Integrator wavelength (nm) | Linear Plotter wavelength (nm) |
|---|---|---|
| 13 | 435 | 665 |
| 14 | 435 | 665 |
| 15 | 435 | 665 |
| 16 | 435 | 630 |
| 17 | 435 | 443 |

Runs 14 and 15 are consecutive runs, made 35 minutes apart, of a sample used in Run 13 spiked with chlorophyll a. It was expected that the chlorophyllase enzyme present would cause conversion of the chlorophyll a added to form chlorophyllide a. In 90% acetone, the rate of conversion is much slower, so a gradual decrease of the chlorophyll a peak, and corresponding increase of the chlorophyllide a peak may be seen to occur from Run 14 to Run 15.

Results see Fig 3.3. and Table 3.3.

## 2.4 Carotenoid Identification Using Dual-Wavelength Scanning Technique

Dual-Wavelength Scans of the pigments of other algal Species:

Nanno saronensis

Tetraselmis suecica

Pyramimonas virginica

were made at wavelengths of 435 nm and 443 nm for Tetraselmis suecica and Pyramimonas virginica, and at wavelengths of 435 nm and 453 nm for Nanno saronensis.

An attempt was made to identify peaks obtained in the chromatograms by using the documented class carotenoid distribution (see Page 23) and by comparing peak heights from the scans at the different wavelengths. This evidence was used in conjunction with the order of elution of the carotenoids as determined by Mantoura & Llewellyn (1983) Results: Table 3.4. and Fig. 3.4.

## 2.C SIBEX FIELD WORK

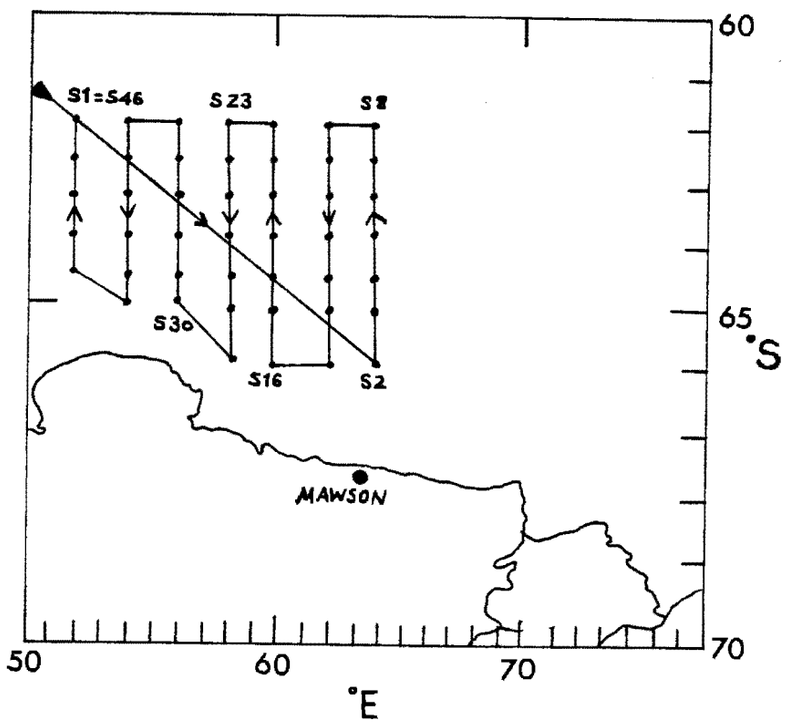
The pigment concentrations of the algal extracts used so far were much higher than those likely to be encountered in the SIBEX region (Fig S.1.). Reported concentrations of chlorophyll *a* in other regions of the Southern Ocean were of the order of 0,2 $ngm^{-3}$ (Allanson *et al*, 1981 and Eduardo, 1982). A method of preconcentration of the algal pigments was developed, using the Waters C-18 SEP-PAK® cartridges (see Appendix A.4.), following Eskins & Dutton, 1979. This procedure allowed the algal pigments filtered from two litres of seawater and extracted in 10ml 90% acetone, to be concentrated into 0,5ml acetone. This 4000-fold concentration of the pigments prior to injection was sufficient to allow detection of them by the Beckman 165 spectrophotometric detector.

### Sampling Procedure

Two litre samples of seawater were taken from Niskin oceanographic sampling bottles at the surface and depths of 20,30,50,75,100,150 and 200 metres at every daylight station, so that the HPLC chlorophyll values could be related to the productivity values and the SCOR-UNESCO chlorophyll *a* values, calculated by Allanson *et al* (pers. comm.). Each sample was filtered through a GFF filter, under subdued light conditions. If analysis was to be performed on board, each filter was ultrasonicated for two

Fig. S.1. Map showing location of the SIBEX 1 sampling grid

Forty-six stations were sampled, beginning and ending at S = 1/46.




Allanson, 1984

hours in 10ml 90% acetone, and the pigment extract filtered on a Millex HV® 0,5µm filter and concentrated to yield 0,5ml of pigment concentrate as described (section 2.C.). 100µl of this extract is injected onto the column for analysis. An irreversible blockage of the column during the cruise necessitated the storage of the algal filtrate on the GFF filter, until shore-based analysis could resume. Each filter was wrapped in aluminium foil, labelled and frozen in liquid nitrogen. Pigment samples remain intact when frozen in liquid nitrogen for up to 3 months (Wright pers. comm.). On return to shore, the frozen filter is placed in 10ml 90% acetone, and ultrasonicated for 2 hours and the analysis continued in the manner described.

Results: Section 3.C

Quantification of Chlorophyll c

As yet, no pure standard chlorophyll *c* is commercially available, and so quantification of chlorophyll *c* cannot simply be achieved by setting up a calibration curve, as for chlorophyll *a*. HPLC elution does not separate chlorophyll *c* into its two components, chlorophyll $c_1$ and chlorophyll $c_2$ (Jeffrey, 1969, 1972).

Two methods of chlorophyll *c* quantification are proposed:

Analysis of the magnesium content of the chlorophyll *c* eluant fraction, collected from the column, by atomic absorption spectroscopy. One mole of chlorophyll *c* molecules contains one mole of magnesium ions, and so the molarity of the magnesium will be equivalent to that of the chlorophyll. This was tested by making a series of pure standard chlorophyll *a* solutions of concentrations 10,5,2 and 1 $mgl^{-1}$ in 90% acetone. Analysis of the magnesium content of these standards gave accurate and quantitative estimates of the chlorophyll *a* concentrations (Monteiro pers. comm.).

However, magnesium is one of the major elements in seawater and occurs at a concentration of about 1,290 ppm. (Brewer, 1975). Hence the estimation of the magnesium content of chlorophyll *c* in regions where chlorophyll *a*

concentrate = 0,1 $mgl^{-1}$ is subject to serious contamination. Excess $Mg^{2+}$ ions present in seawater may be removed from natural samples by injecting pigment extract, in 10ml 90% acetone, onto a C-18 SEP-PAK®. This cartridge is washed with water of pH7, to remove any excess free $Mg^{2+}$ ions. Problems may arise if other organic complexes of magnesium are present in the seawater, as these may be retained on the SEP-PAK® cartridge, and then eluted in acetone and analysed for the magnesium content. This is related to the chlorophyll c content of the original sample. Further investigations will have to be made in order to establish this as a possible method of chlorophyll c quantification.

An alternative method of calculating chlorophyll c concentration is to compare the absorbance at 443nm of the chlorophyll a and chlorophyll c peaks. The areas of the peaks in the chromatogram are a function of their characteristic absorbances, E , at the wavelength at which they are detected.

The concentration of chlorophyll c, (Chl c) is calculated:

$$(\text{Chl}\underline{c}) = \frac{(\text{Area Chl}\underline{c}\text{ peak}) \times \alpha(\text{Chl}\underline{a})}{(\text{Area Chl}\underline{a}\text{ peak})}$$

where $\alpha$ = extinction coefficient of chl a at 435nm

The concentration of chlorophyll a can be calculated from a calibration curve of chlorophyll a standard scanned at 443nm, and the area at this wavelength related to concentration (Kleppel & Pieper, 1984).

# 3. RESULTS

## 3.A. RESULTS OF LABORATORY WORK

### 3.1. Identification of the Peaks from Comparative Scans of Several Algal Species See Table 3.1. and Figs. 3.1.

Peaks 5,6,7 and 8 were identified as chlorophylls *b*, *a* and pheophytins *b* and *a* respectively by injection and co-elution of these standards.

Peak 9 was identified as ß-carotene. This peak was the only one to occur in all chromatograms, other than that due to chlorophyll *a*, and ß-carotene is the only pigment other than chlorophyll *a* and pheophytin *a*, common to all algal species.

In all chromatograms of algae known to contain chlorophyll *c*, "peak 1" was sharp and intense, and had a pronounced shoulder. In other chromatographic separations of photosynthetic pigments (Mantoura & Llewellyn, 1983; Wright & Shearer, 1984), chlorophyll *c* is seen to elute immediately after chlorophyllide *a*, so it is possible that the shoulder on the chlorophyllide *a* peak arises from chlorophyll *c*. However, peak "3" occurs as an intense peak in all samples expected to contain chlorophyll *c*, and as a small peak in some of the chromatograms of the Chlorophyceae species. It was discovered during the course of these investigations, that all the "pure" algal cultures had been contaminated with diatoms, and this

explains the anomalous appearance of peak "3", if it is due to chlorophyll *c*, in the chromatograms of Chlorophyceae. If peak "3" is not chlorophyll *c*, it must arise from a carotenoid common to Bacillariophyceae, Haptophyceae, and Chrysophyceae, and is then fucoxanthin. From this experiment it is not possible to positively identify chlorophyllide *a*, chlorophyll *c* or fucoxanthin. However, chlorophyllide *a* is the most acidic component (page 13), and chlorophyll *c* is structurally similar and likely to be eluted soon after chlorophyllide *a*. This leads to the conclusion that peak 1 is chlorophyllide *a*, peak 2 is chlorophyll *c* and therefore peak 3 is fucoxanthin. See summary of proposed peak identities on page 48.

Peak "2" is a pigment that occurs only in Prasinophyceae and Chlorophyceae, and is either zeaxanthin, neoxanthin or lutein. Of these, zeaxanthin is the most polar and is therefore expected to elute before neoxanthin or lutein. Hence peak "2" is postulated to arise from zeaxanthin.

Peak "4" is a pigment common to the classes Prasinophyceae and Chlorophyceae, and is thought to be violaxanthin.

Table 3.1. Distribution of pigments among the species investigated

Until positively identified, the peaks have been called "Peaks 1 - 9".

| ALGAL SPECIES | Chlide *a* Peak 1 | Peak 2 | Peak 3 | Peak 4 | Chl *b* Peak 5 | Chl *a* Peak 6 | Peak 7 | Peak 8 | Peak 9 | ALGAL CLASS |
|---|---|---|---|---|---|---|---|---|---|---|
| *P. tricornutum* | Xs | | X | | | X | | X | X | |
| *C. gracilis* | Xs | | X | | | X | | X | X | Bacillariophyceae |
| *S. costatum* | Xs | | X | | | X | | X | X | |
| *P. virginica* | X | X | | X | X | X | X | X | X | Prasinophyceae |
| *T. suecica* | X | X | | X | X | X | X | X | X | |
| *I. galbana* | Xs | | X | | | X | | X | X | Haptophyceae |
| *P. lutheri* | Xs | | X | | | X | | X | X | Chrysophyceae |
| *P. paradoxa* | Xs | | X | | | X | | X | X | |
| *N. saronensis* | X | X | (X) | X | X | X | | X | X | |
| *C. vulgaris* | X | X | | X | X | X | | X | X | Chlorophyceae |
| *D. primolecta* | X | X | (X) | X | X | X | | X | X | |

"X" indicates that the peak occurred in the Chromatograph of that species.
"s" indicates "shoulder" on Peak 1

Table 3.0.: Table of Carotenoid Distribution among Algal Classes

| | *Fucoxanthin* | *Violaxanthin* | *Zeaxanthin* | *Lutein* | *Neoxanthin* | *Diadinoxanthin* | *Diatoxanthin* | *Antheraxanthin* |
|---|---|---|---|---|---|---|---|---|
| Bacillariophyceae | x | | | | | x | x | |
| Prasinophyceae | | x | x | x | x | | | |
| Haptophyceae | x | x | | | | x | x | |
| Chrysophyceae | x | x | | | | | | x |
| Chlorophyceae | | x | x | x | x | | | |

References: Liaaen-Jensen, 1977
Davies, 1976

Figs. 3.1. Typical Chromatograms of Pigment Extracts from each species

Figs. 3.1.A. Chromatograms from species in class Bacillariophyceae

Figs. 3.1.B. Chromatograms from species in class Prasinophyceae

Figs. 3.1.C. Chromatograms from species in class Haptophyceae

Figs. 3.1.D. Chromatograms from species in class Chrysophyceae

Figs. 3.1.E. Chromatograms from species in class Chlorophyceae

Fig 3.1.A(i) *Phaeodactylum tricornutum*

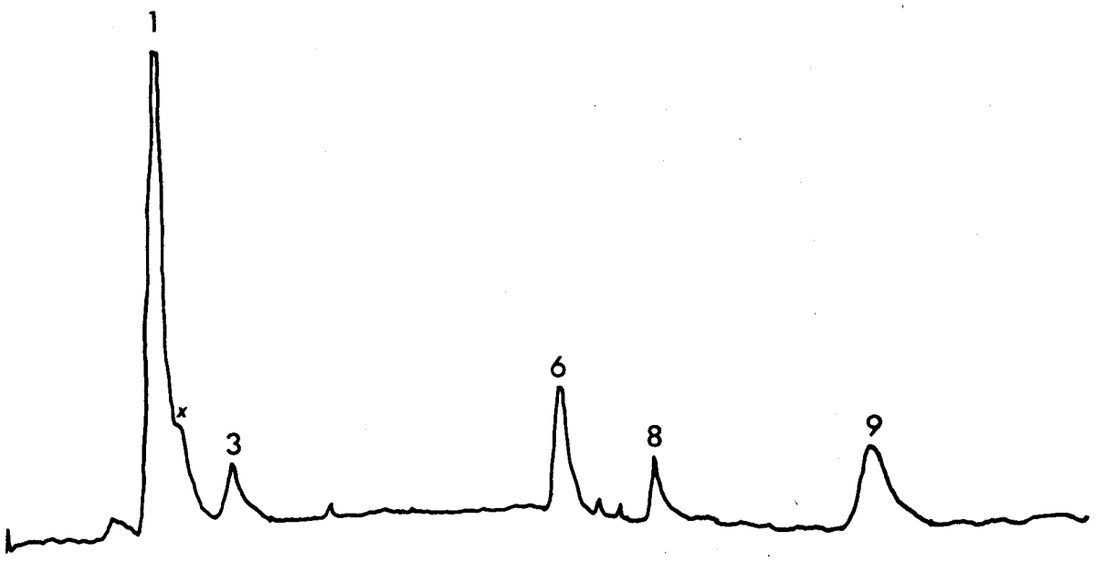



Fig 3.1.A(ii) *Chaetoceros gracilis*

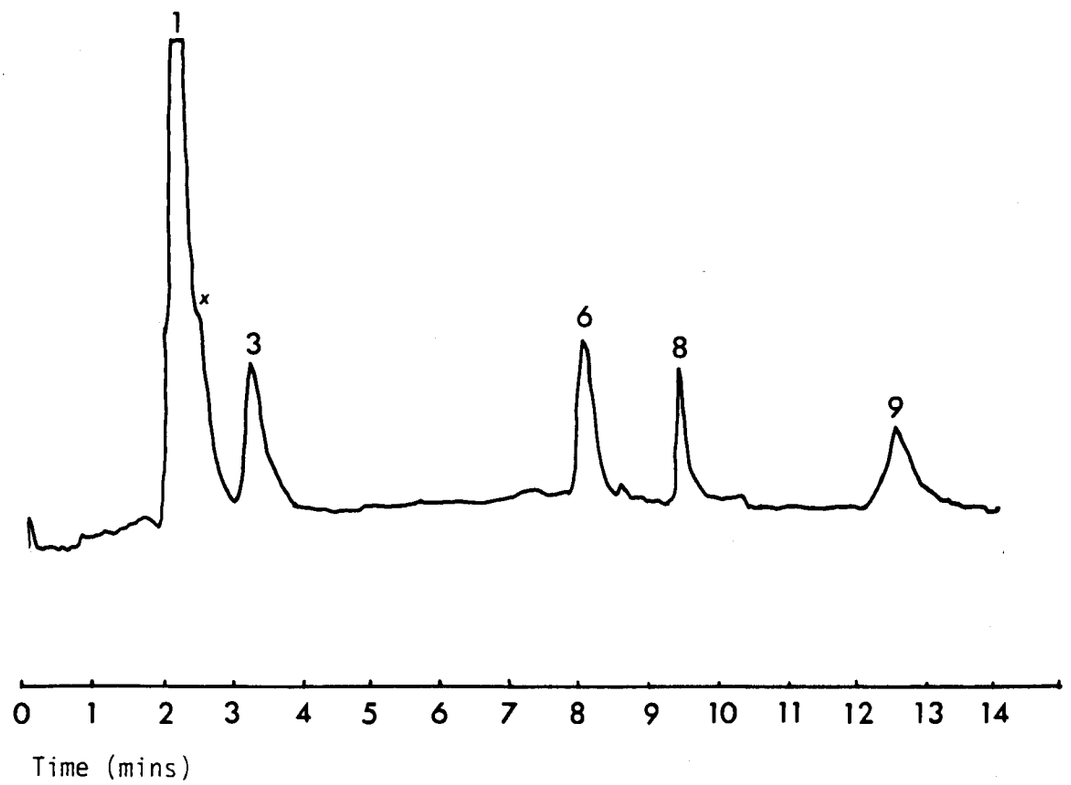


Fig 3.1.A(iii) *Skeletonema costatum*

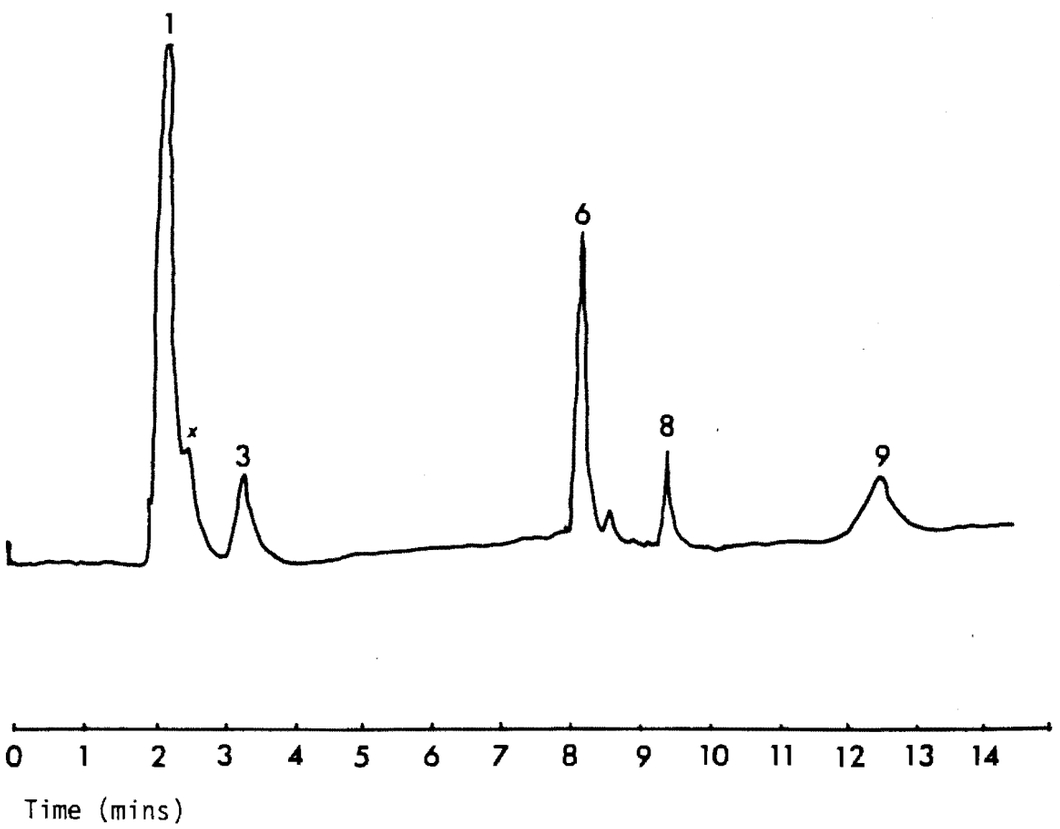


Fig 3.1.B(i) *Pyramimonas virginica*

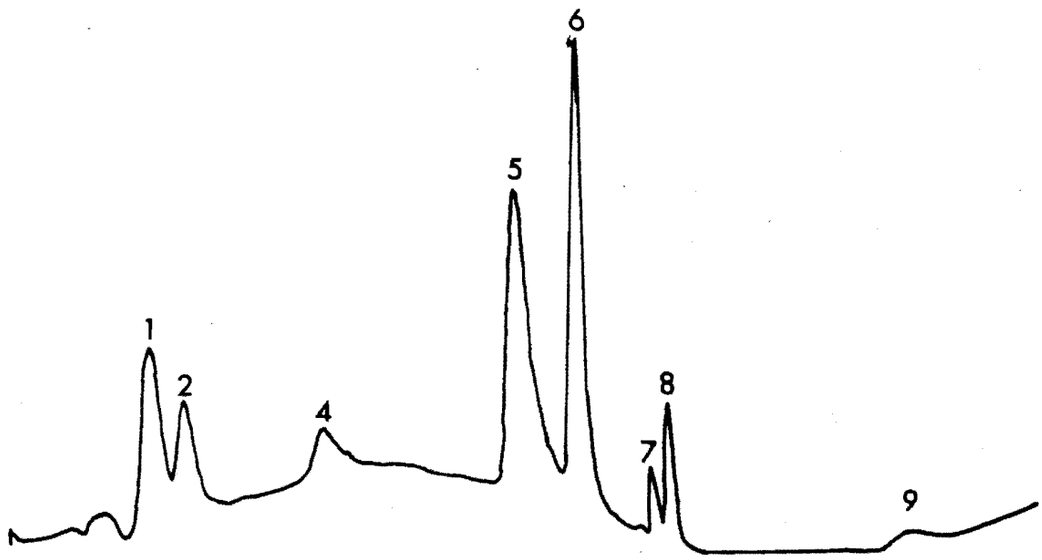



Fig 3.1.B(ii) *Tetraselmis suecica*

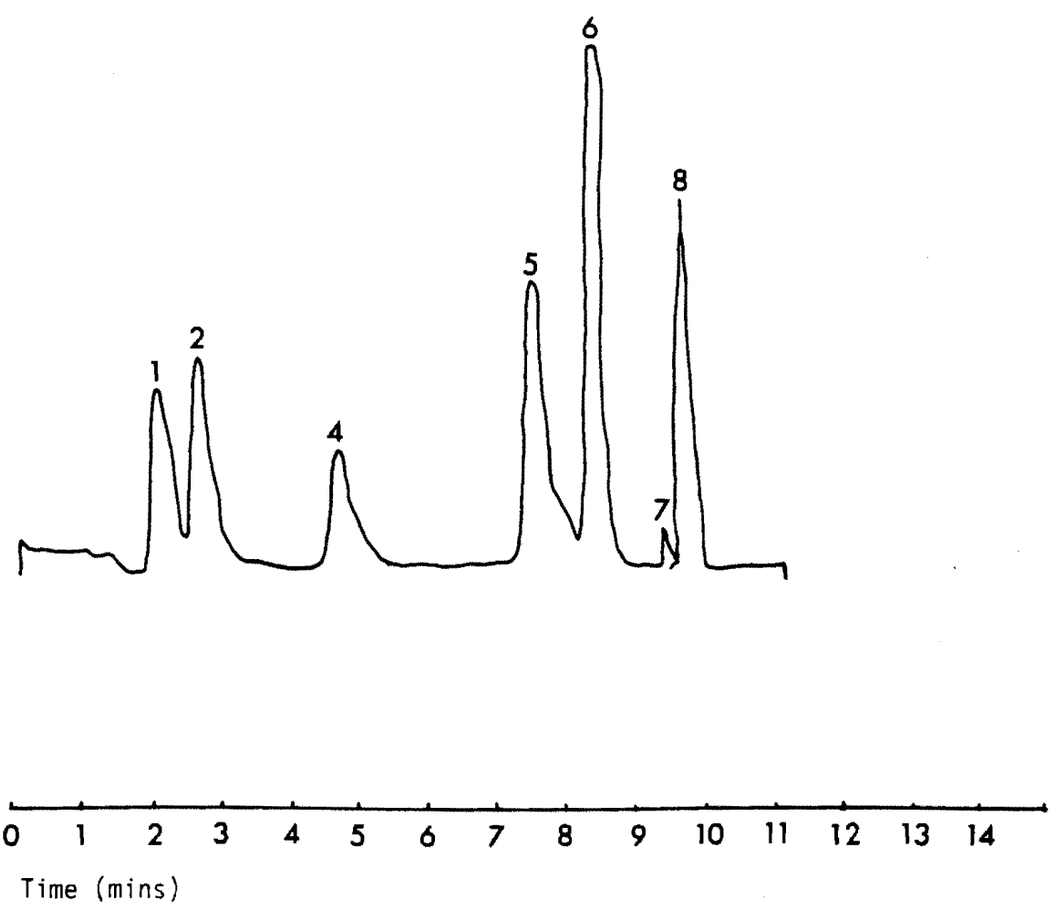


Fig 3.1.C *Isocrisis galbana*

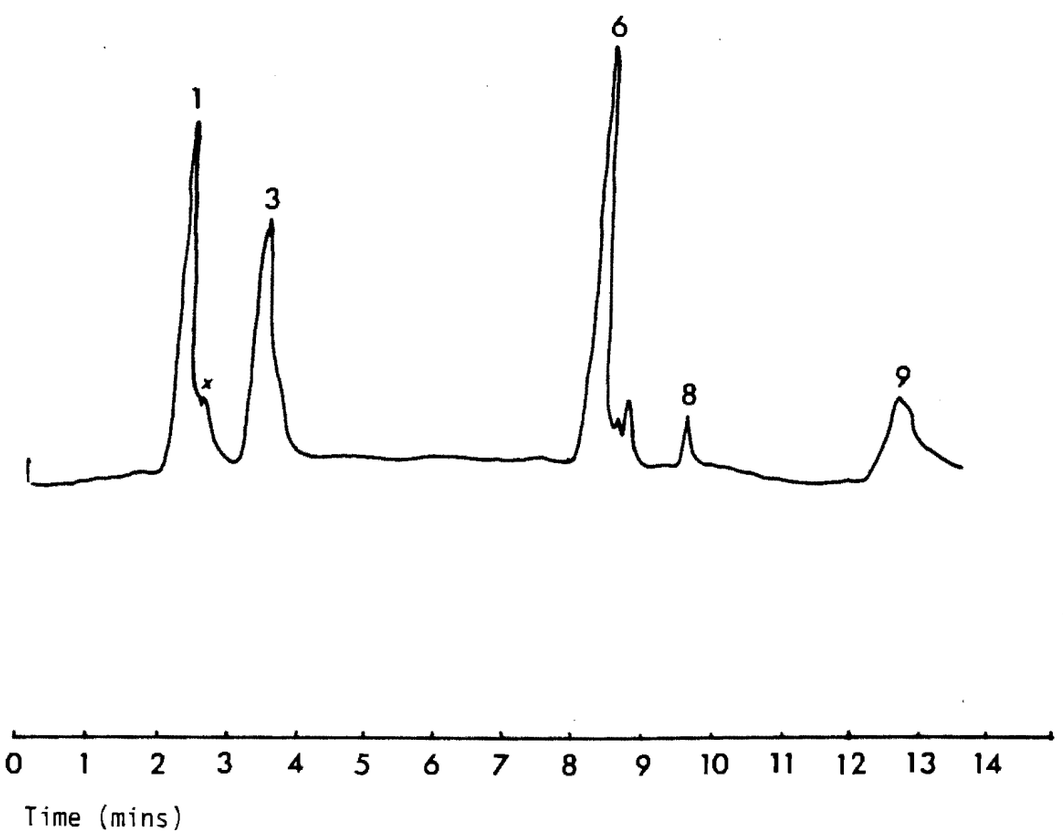


Fig 3.1.D(i) *Pavlova lutheri*

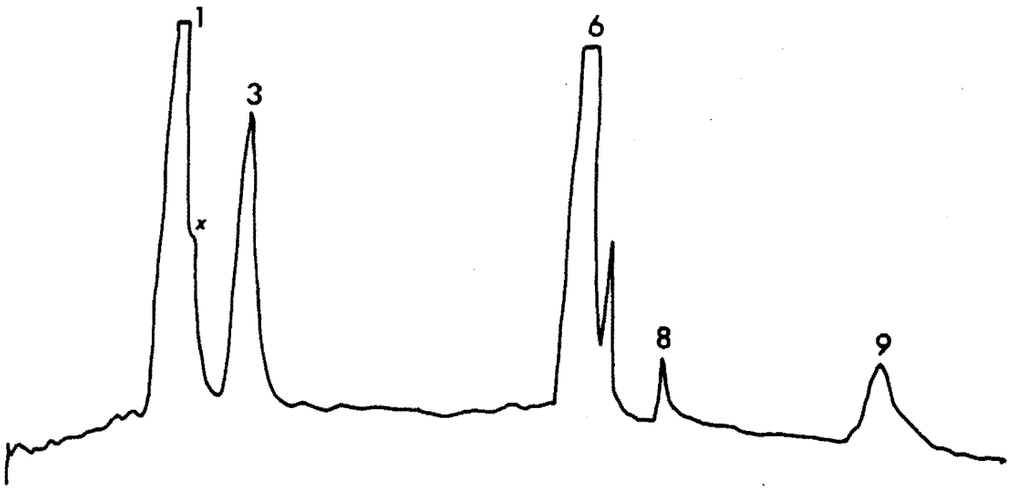



Fig 3.1.D(ii) *Pseudocrisis paradoxa*

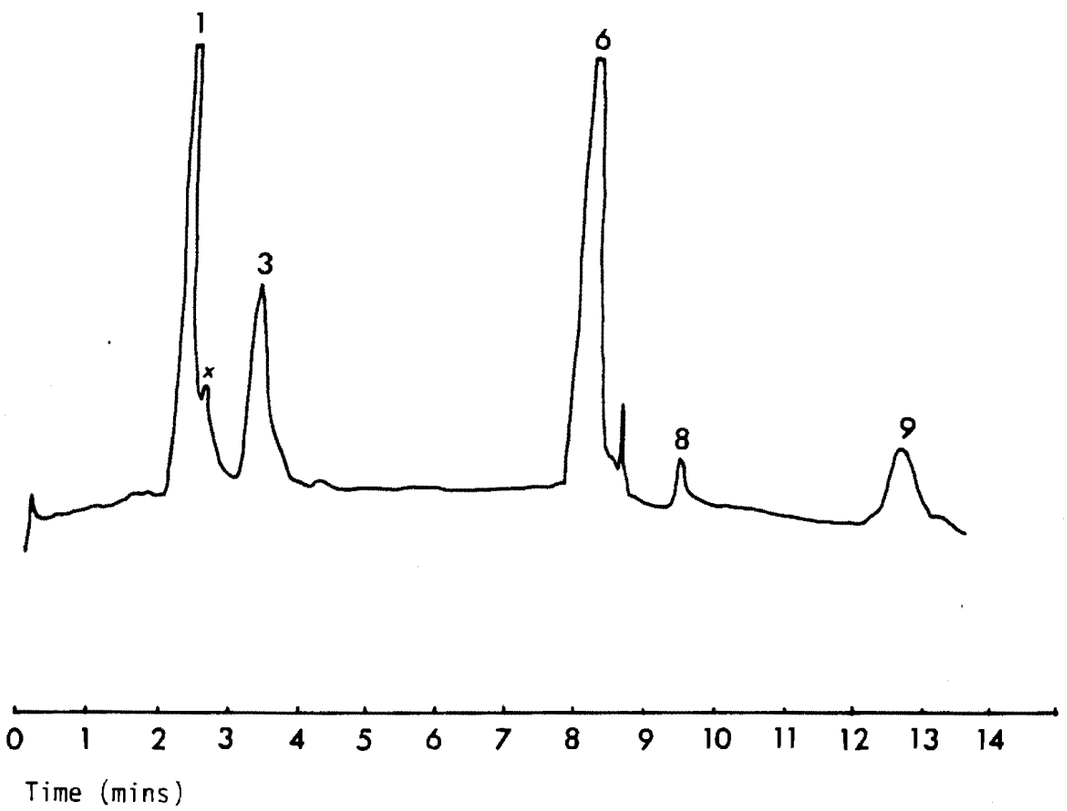


Fig 3.1.E(ii) *Chlorella vulgaris*



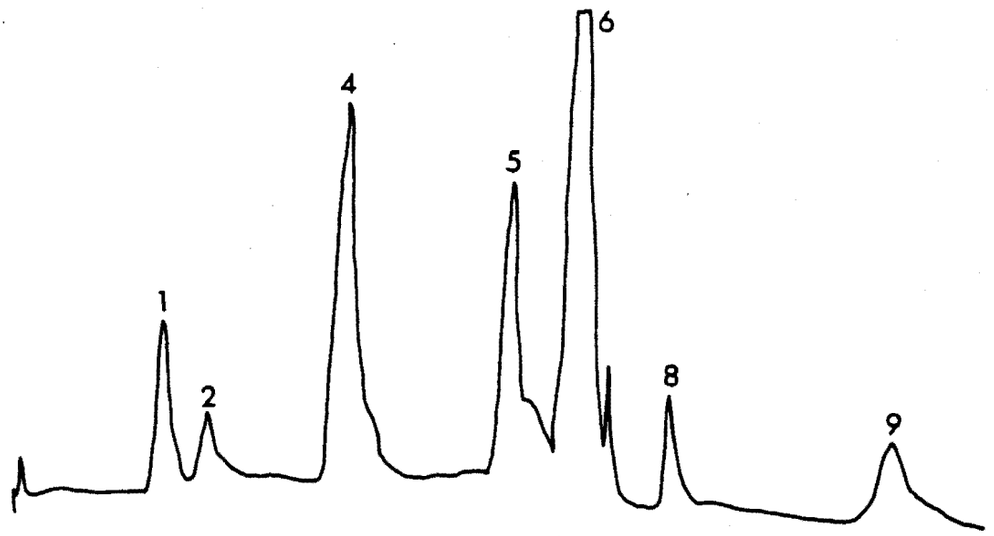



Fig 3.1.E(i) *Nanno saronensis*

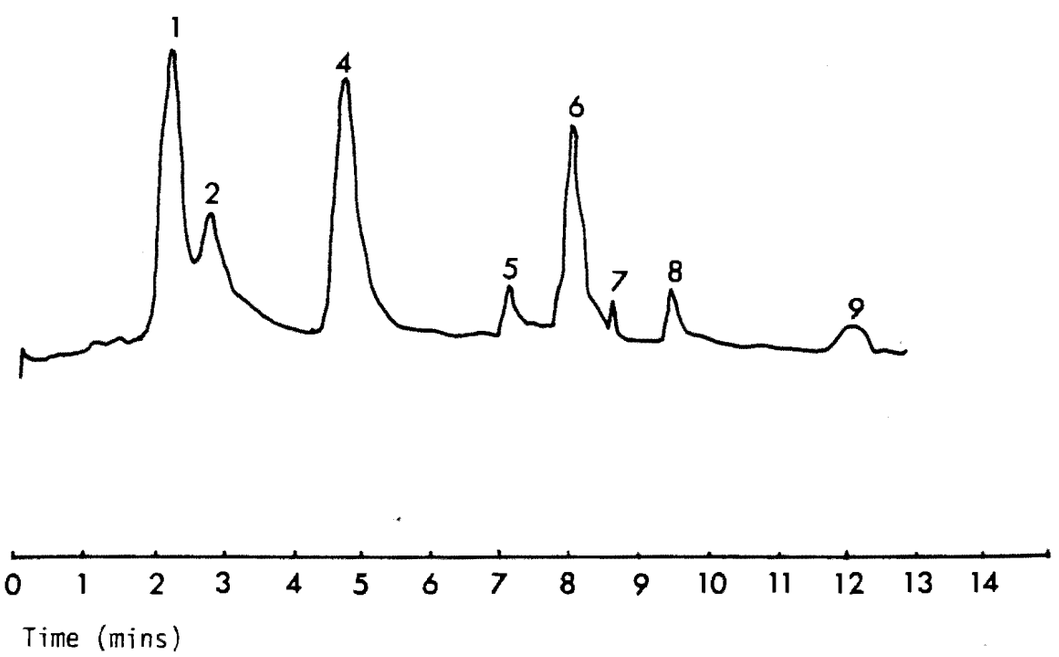
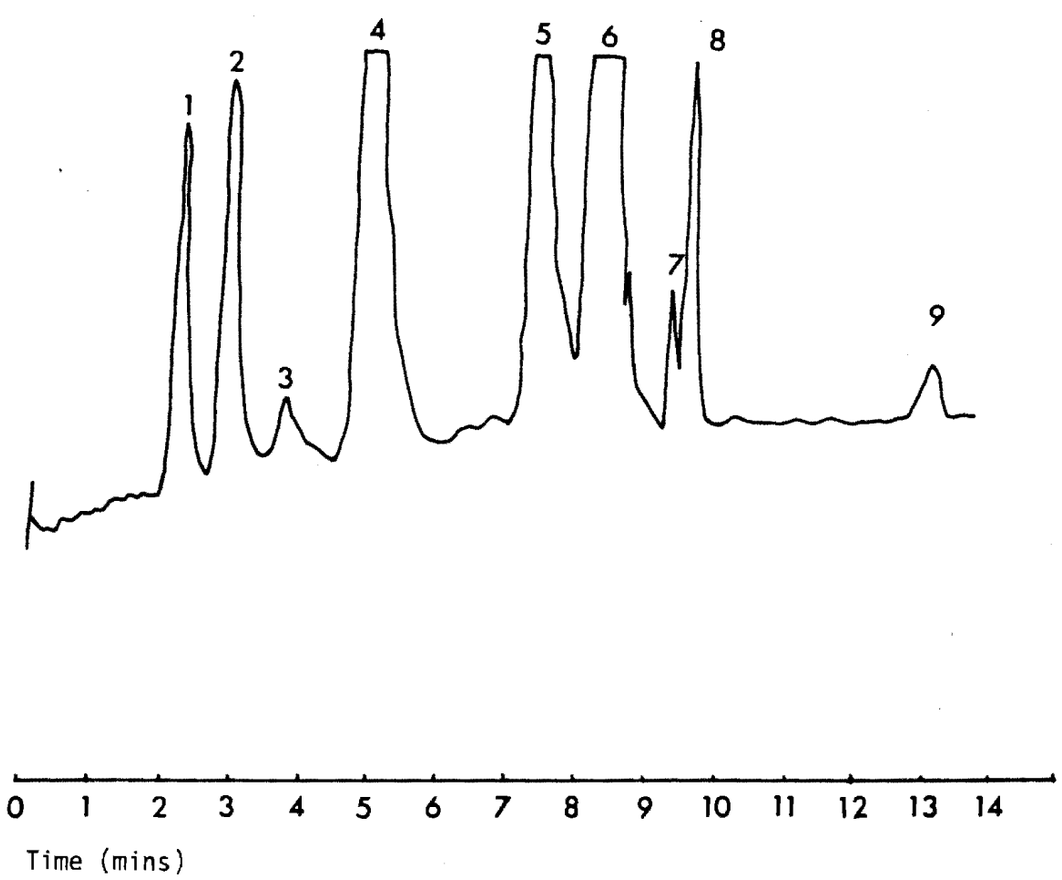


Fig 3.1.E(iii) *Dunaliella primolecta*



Summary of Proposed Peak Identities From Experiment 2.1.

| | |
|---|---|
| Peak 1 | Chlorophyllide *a* |
| shoulder on peak 1 | Chlorophyll *c* |
| Peak 2 | Zeaxanthin |
| Peak 3 | Fucoxanthin |
| Peak 4 | Violaxanthin |
| Peak 5 | Chlorophyll *b* |
| Peak 6 | Chlorophyll *a* |
| Peak 7 | Pheophytin *b* |
| Peak 8 | Pheophytin *a* |
| Peak 9 | β-carotene |

3.2 Results of Experiment 2.2: *Phaeodactylum tricornutum* Experiment. See Table 3.2. on page 53.

Results of Scans 4a and 4b at 665nm

An attempt was made to scan the extracts at this wavelength, in order that the chlorophyll-related peaks could be isolated. However, after one successful scan of a 90% acetone extract (Fig. 3.2.a.) excessive baseline drift prevented any further work at this wavelength.

Results of Scans 4c and 4d at 435nm

The extract in 90% acetone (B) gave a chromatogram with three main peaks (Fig. 3.2.c.):

At 2,17 minutes, with a pronounced shoulder (peak 1)

At 3,38 minutes, (peak 2) and

a chlorophyll a peak at 8,16 minutes (peak 4).

The extract in 50% acetone (A) also showed three peaks (Fig. 3.2.d.): A peak at 2,17 minutes (peak 1) which was of a greater area than the corresponding peak from sample B

A peak at 3,38 minutes(peak 2), also of greater area than the corresponding peak in sample B.

A chlorophyll a peak (peak 4) of smaller area than the peak from sample B.

The decrease in the chlorophyll a peak in the 50% acetone extract is attributed to the enhanced effect of the chlorophyllase enzyme in this medium. The increased peaks at 2,17 minutes (peak 1) and 3,38 minutes (peak 2) could arise from chlorophyllide a and pheophorbide a.

Results of Scan 4e (Spiking of (A) with Chlorophyll a) at 435 nm

Chromatograms were run (i) immediately after spiking and (ii) 30 minutes later.

Comparison of the chromatograms (Fig. 3.2.e(i) and e(ii)) shows a clear decrease in the intensity of the chlorophyll a peak (peak 4) during the thirty minutes. The peak at

3,30 minutes (peak 2) remains constant in intensity and that at 2,11 minutes (peak 1) remains constant in area. These results indicate that the peak at 2,11 minutes (peak 1) is due to chlorophyllide a, because of the increase in area of this peak as the chlorophyll a peak area decreases. In chromatogram e(i) a shoulder (x) is present on peak 1. It seems that this shoulder is overlapped when high concentrations of peak 1 are present. It is thought that this peak is due to chlorophyll c, which is not properly resolved from chlorophyllide a in these chromatograms.

Results of Scans 4f and 4g (Acidification of samples A and B) at 435 nm

This experiment was designed to investigate the formation of pheophorbide a.

In the 90% acetone extract, chlorophyll a was converted to pheophytin a (peak 5) (Fig. 3.2.f(i)).

After thirty minutes, (Fig 3.2.f(ii), the pheophytin peak had disappeared and the peak areas of peaks 1 and 2 decreased. This was possibly due to the breakdown of the pigments to smaller compounds which do not absorb visible radiation (Holden, 1976).

In the 50% acetone extract, no chlorophyll a or pheophytin a peaks were seen immediately after acidification (Fig. 3.2.g(i)).

The peak retention times had shifted slightly, due to warming of the laboratory.

The shoulder on peak 1 (x), (at 1,99 mins) separated from this peak, on acidification (Fig. 3.2.g(ii)), to form a better resolved peak at 2,64 minutes (peak x´). This is postulated to arise from conversion of chlorophyll c (the "shoulder x" on peak 1) to pheoporphyrin c in the presence of excess protons, which replace the magnesium ion. This results in a decrease in polarity and hence the pheoporphyrin c peak is eluted slightly later than chlorophyll c, allowing better resolution of these two peaks.

The area of peak 1 (at 1,99 mins) decreases after thirty minutes: this would occur if chlorophyllide a is converted to pheophorbide a.

The acidification also caused a double peak to appear at 3,08 (peak 2) and 3,28 minutes(peak 3). After thirty minutes, the area of peak 3 increases. The area of peak 2 remains constant. It is proposed that the 3,08 minute peak (peak 2) is due to a carotenoid which is stable under acidification and the 3,28 minute peak (peak 3) is due to pheophorbide a.

Summary of Proposed Peak Identities From Experiment 2.2.

| Peak No. | Identity |
|---|---|
| 1 | Chlorophyllide *a* |
| x | Chlorophyll *c* |
| x´ | Pheoporphyrine *c* |
| 2 | Stable carotenoid |
| 3 | Pheophorbide *a* |
| 4 | Chlorophyll *a* |
| 5 | Pheophytin *a* |

Table 3.2.

Results of Phaeodactylum tricornutum Experiment Varian Instrument

| | Time of Elution (mins) | Peak Area | Peak No. |
|---|---|---|---|
| a.Chlorophyll *a* | 8,09 | | 1 |
| b.Baseline deteriorated no peaks seen | | | |
| c. | 2,17 | 223 000 ± 6 000 | 1 |
| | 3,38 | 42 000 ± 1 000 | 2 |
| | 8,16 | 69 000 ± 2 000 | 4 |
| d. | 2,17 | 840 000 ± 20 000 | 1 |
| | 3,38 | 147 000 ± 4 000 | 2 |
| | 8,16 | 6 500 ± 200 | 4 |
| e.Spiking 50% acetone extract with chlorophyll *a* (i) immediately after spiking | 2,11 | 710 000 ± 20 000 | 1 |
| | 3,30 | 159 000 ± 4 000 | 2 |
| | 8,10 | 97 000 ± 3 000 | 4 |
| (ii) 30 mins. later | 2,23 | 890 000 ± 20 000 | 1 |
| | 3,45 | 154 000 ± 4 000 | 2 |
| | 8,18 | 34 300 ± 900 | 4 |

| | | | |
|---|---|---|---|
| f.Acidification of 90% | 2,05 | 338 400 ± 9 000 | 1 |
| acetone extract (i) | 3,45 | 48 000 ± 1 000 | 2 |
| | 9,15 | 7 700 ± 200 | 5 |
| (ii) 30 mins. later | 2,04 | 86 000 ± 2 000 | 1 |
| | 3,44 | 7 400 ± 200 | 2 |
| g.(i) Acidified | 1,99 | 740 000 ±20 000 | 1 |
| 50% acetone extract | 3,08 | 47 000 ± 1 000 | 2 |
| | 3,29 | 98 000 ± 3 000 | 3 |
| (ii) 30 mins. later | 2,00 | 670 000 ±17 000 | 1 |
| | 2,64 | 166 000 ± 4 000 | x |
| | 3,09 | 47 000 ± 1 000 | 2 |
| | 3,30 | 126 000 ± 3 000 | 3 |

Figs. 3.2. Peak Identities for Chromatograms 3.2.

1 Chlorophyllide *a*

x Chlorophyll *c*

x´ Pheoporphyrin *c*

2 Stable Carotenoid

3 Pheophorbide *a*

4 Chlorophyll *a*

5 Pheophytin *a*

Fig 3.2.(a) 90% Acetone extract at wavelength 665 nm.

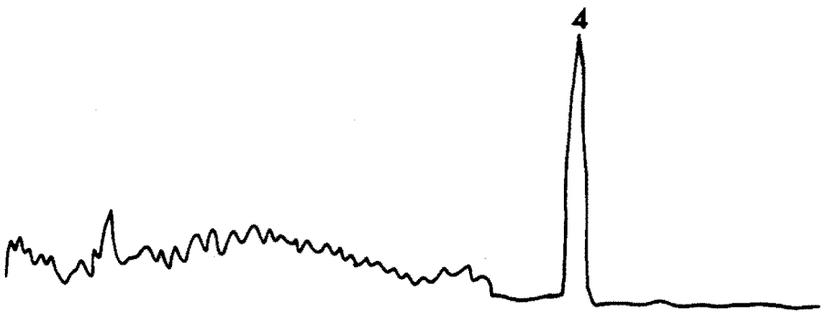



Fig 3.2.(c) 90% Acetone extract at wavelength 435 nm.

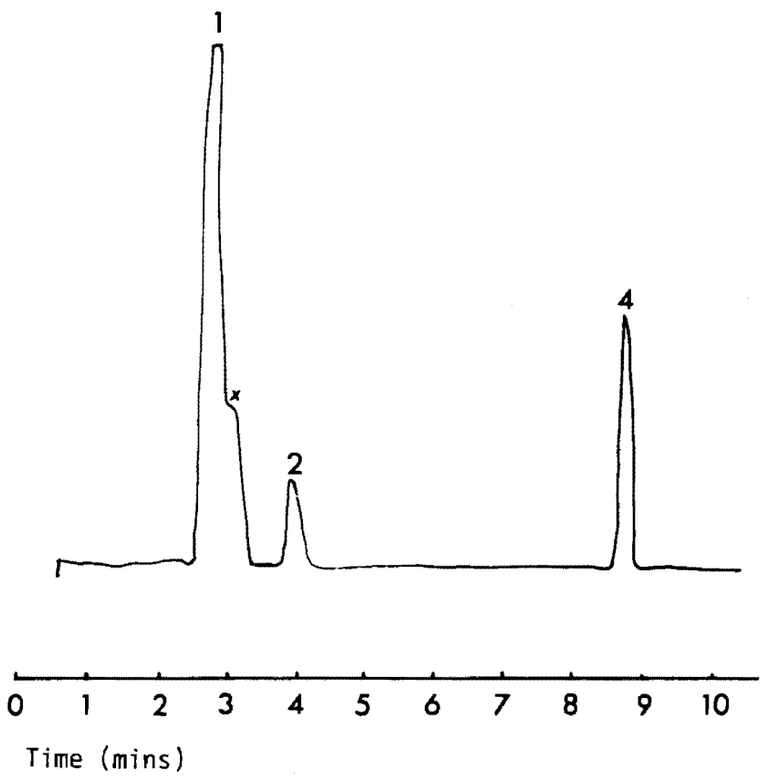


Fig 3.2.(d) 50% Acetone extract at wavelength 435 nm.

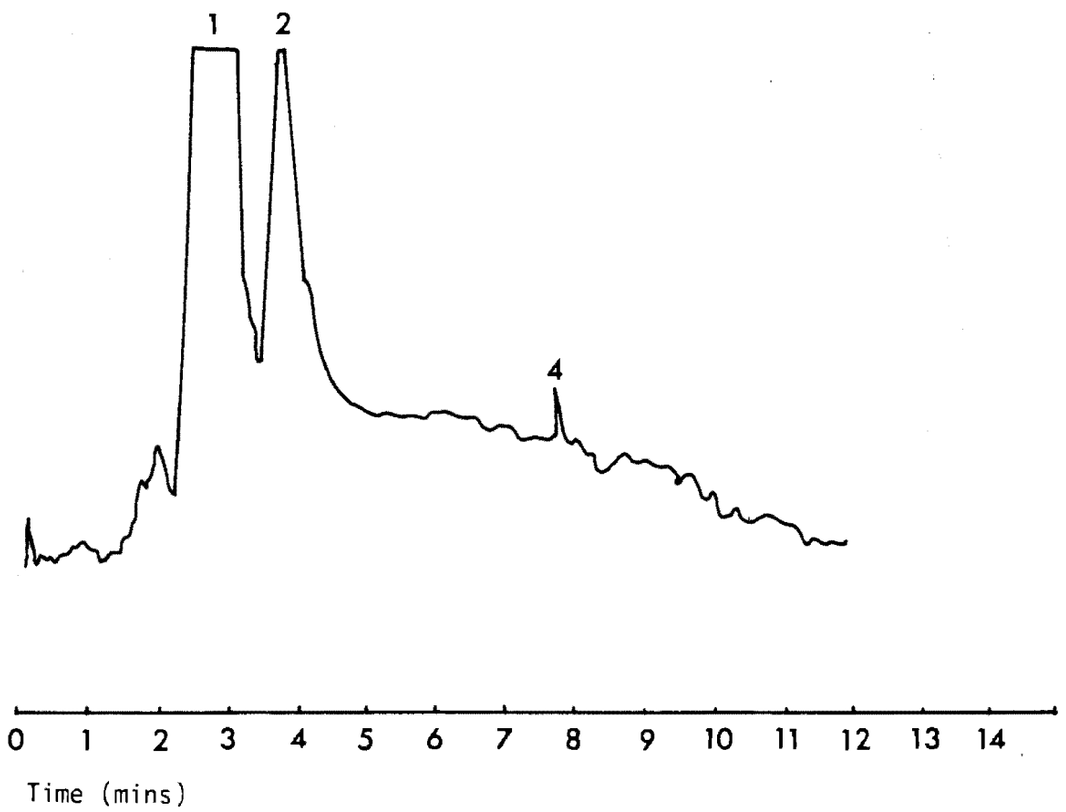


Fig 3.2.(e)i.50% Acetone extract spiked with chlorophyll *a*. 

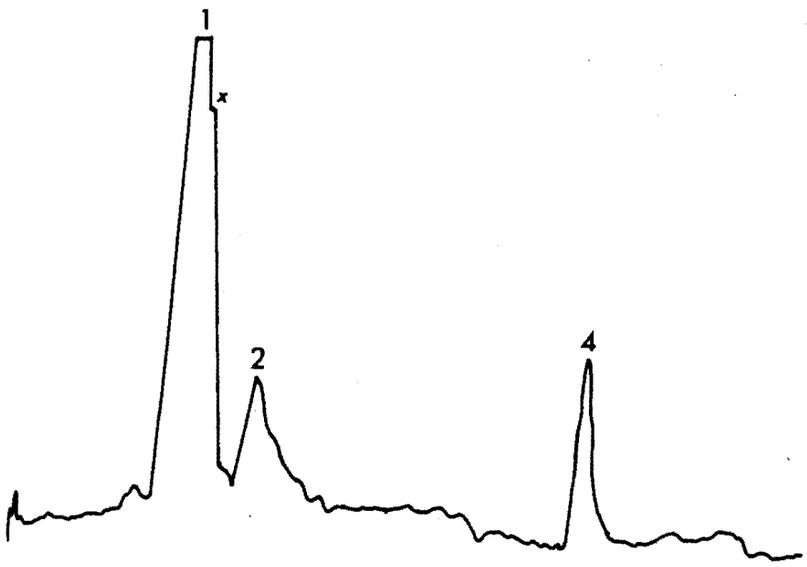



Fig 3.2.(e) ii.Sample (i) after 30 minutes.

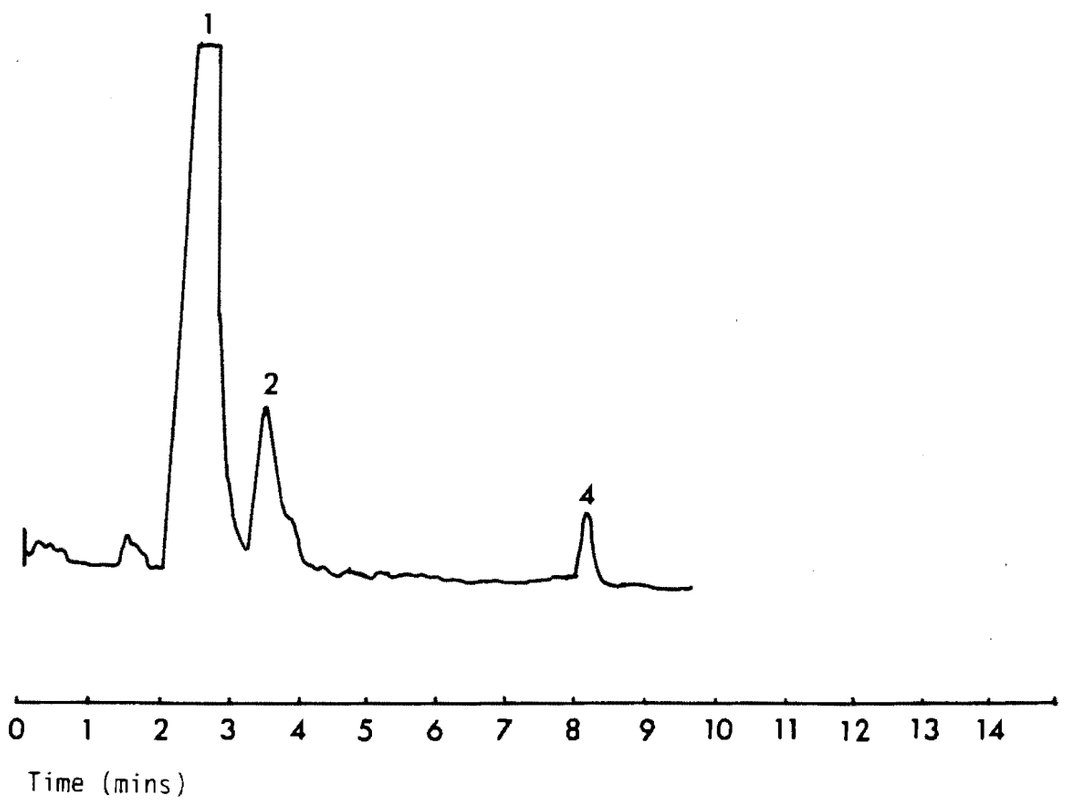


Fig 3.2.(f) i.90% Acetone extract,acidified.

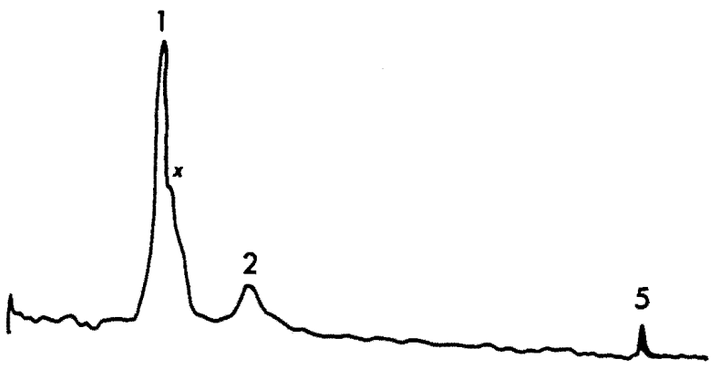



Fig 3.2.(f) ii.Sample(i) after 30 minutes.

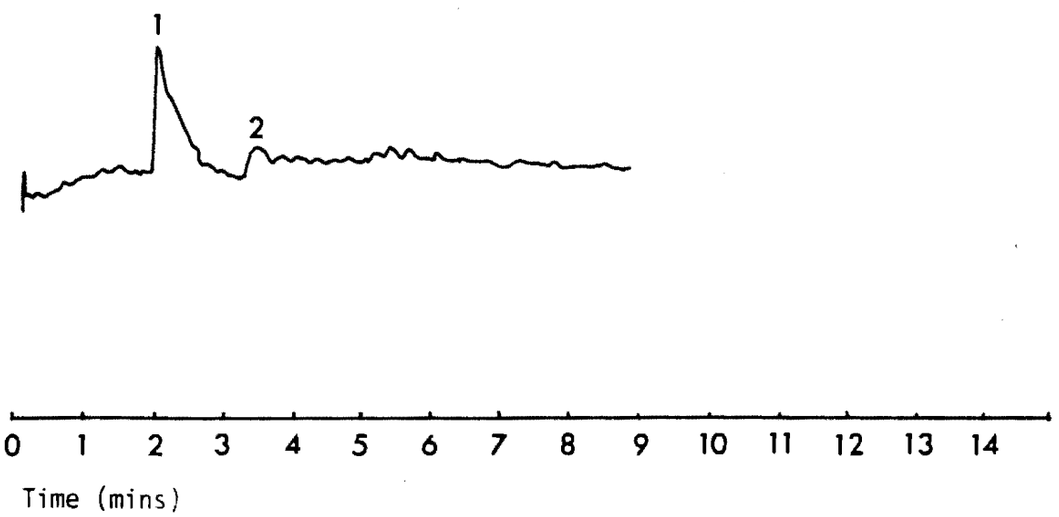


Fig 3.2.(g) i.50% Acetone extract,acidified.

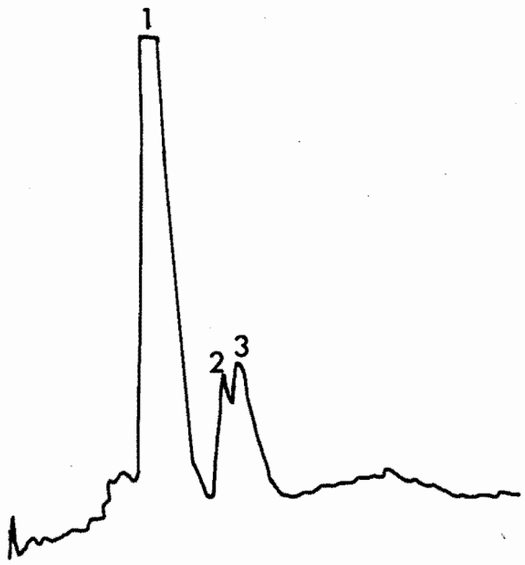
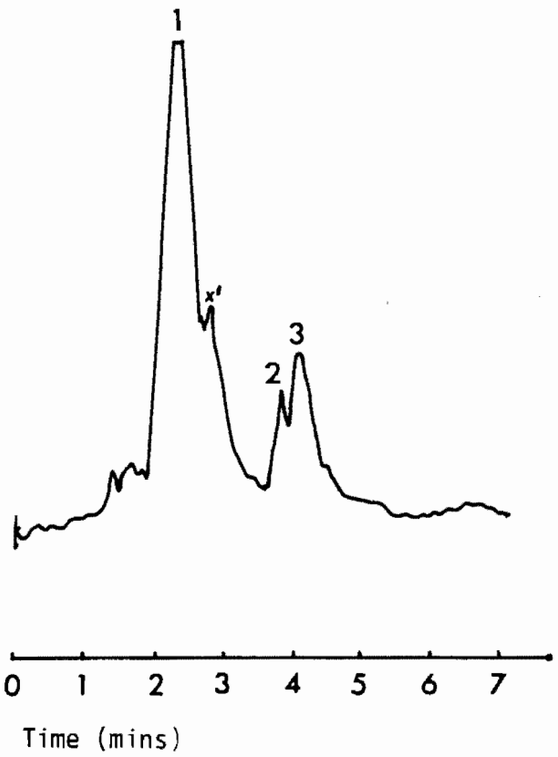



Fig 3.2.(g) ii.Sample (i) after 30 minutes.



## 3.3. Results of Dual-Wavelength Scans of Pheodactylum tricornutum

Results: See Table 3.3.(page 65), Figs. 3.3. (page 66) and Table A.2. (page 155)

Examination of the relative peak intensities indicates that the peak at 0,96 minutes is due to chlorophyllide a and the peak at 1,21 minutes is due to chlorophyll c. These assumptions can be supported by the following evidence:

1. Scans 13b, 14b, 15b at 665nm show peaks at 0,96; 6,95; 7,31; 7,63; 7,07 and 9,56 minutes. Only these peaks can be chlorophyll related - ie contain the porphyrin ring and hence absorb radiation at this wavelength.

2. The peak at 7,63 minutes is chlorophyll a
   9,65 minutes is pheophytin a
   7,07, 6,60 and 6,35 minutes are anomers and epimers of chlorophyll a (Mantoura & Llewellyn, 1983)

The remaining peak absorbing at 665nm is the very intense sharp peak at 0,96 minutes. It is expected that this peak arises from chlorophyllide a, whose specific absorption coefficient at a wavelength of 665nm is 127, which would account for the large area of this peak. If the 0,96 minute peak was due to chlorophyll c, whose specific absorption coefficient at this wavelength is very small (~44)

the amount of chlorophyll c present would have to be significantly greater than the amount of chlorophyll a present ($E\alpha$ = 87) to give rise to a peak such as that at 0,96 minutes. This is unlikelv since Jeffrey (1963) has reported that in most diatoms, the ratio of the pigment chlorophyll a:chlorophyll c is 2:1.

3. Comparing run 14b at 665nm, (made 5 minutes after spiking with chlorophyll a) with run 15b at 665nm (of the same samples 35 minutes after spiking) shows that the chlorophyll a peak area decreased and the 0,96 minute peak area increased. The chlorophyllide a:chlorophyll a peak area ratio in run 14 is 0,20, and in run 15, is 0,29, indicating that a significant amount of chlorophyll a has been converted to chlorophyllide a. This could arise from the slow conversion of chlorophyll a to chlorophyllide a, due to chlorophyllase activity, which would be reflected by a diminishing chlorophyll a and an increasing chlorophyllide a peak and contributes further evidence to the effect that the peak at 0,96 minutes is due to chlorophyllide a. If this peak was due to chlorophyll c, its height should remain constant, as chlorophyll c is not attacked by chlorophyllase.

4. The wavelength of maximum absorbance of chlorophyll c is 630nm, and in a scan at this wavelength (16b), the peak at 0,96 minutes diminishes relative to the same peak in a scan at 665nm (15b). It is therefore not chlorophyll c. A new peak at 1,21 minutes appears: this is possibly chlorophyll c.

5. The wavelength of maximum absorbance of chlorophyll c in the Soret band region is 443nm (Table A.2., page 155). At this wavelength, the specific absorption coefficient of chlorophyll c is 21,1. A scan at this wavelength ( 17b) shows a 12-fold increase in peak height relative to the scan at 630nm (16). The peak at 0,96 minutes only shows a 4-fold increase in intensity relative to the corresponding peak at 630nm (16b) and is relatively less intense than the 0,96 minute peak at 665nm (16b), which confirms that this peak is not due to chlorophyll c.

6. Considering the chromatograms at 435nm (13a, 14a and 15a) it is seen that when peak 1 is very intense, peak 2 is not resolved. In chromatograms 14a and 15a, in which the sample is more dilute, this large peak has been resolved into two peaks "peak 1 and peak 2", at 0,96 and 1,21 minutes, which have been assigned to chlorophyllide a and chlorophyll c respectively.

Comparing the ratios of the peak areas confirms that the first peak in run 13a is a combination of peaks due to chlorophyllide a and chlorophyll c and that these peaks are not resolved if the sample is very concentrated:

Summary of Results of Experiment 2.3

The first peak eluted is chlorophyllide a, followed closely by chlorophyll c. In this solvent program, these two pigments are separated, except when the sample is very concentrated.

Table 3.3. *Phaeodactylum tricornutum* Results

| Peak Identity | λ435nm Run 13 | Run 14 | Run 15 | Run 16 | Run 17 | λ665nm Run 13 | Run 14 | Run 15 | λ630nm Run 16 | λ443nm Run 17 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0,92 | 0,81 | 0,81 | 0,81 | | 0,77 | 0,81 | | 0,73 |
| chlide *a* | 0,86 | 1,15 | 0,95 | 0,96 | 0,94 | 0,86 | 0,92 | 0,95 | 0,96 | 0,88 |
| chl *c* | 1,81 | 1,85 | 1,20 | 1,21 | 1,73 | | | | 1,21 | 1,63 |
| | 2,26 | 2,32 | 1,85 | 1,79 | 2,22 | | | | | 2,06 |
| | 2,51 | | 2,33 | 2,26 | 2,51 | | | | | 2,27 |
| | 2,80 | | 2,56 | 2,55 | | | | | | 2,73 |
| | 3,07 | | | | | | | | | |
| | | 7,04 | 6,99 | 6,95 | 7,02 | | 7,04 | 6,99 | | 6,35 |
| | | 7,32 | 7,31 | 7,31 | 7,24 | | 7,32 | 7,31 | 7,31 | 6,60 |
| chl *a* | 7,53 | 7,62 | 7,62 | 7,63 | 7,48 | 7,53 | 7,62 | 7,62 | 7,63 | 6,86 |
| | | 7,83 | 7,84 | | 7,66 | | 7,82 | | 7,84 | 8,81 |
| phe *a* | 9,57 | 9,62 | 9,66 | 9,65 | 9,35 | | 9,62 | | | |

Table 3.3. shows the tabulation of peak retention times recorded during Runs 13 - Runs 17 for the four wavelengths 435, 665, 630 and 443 nm.

The retention times corresponding to each pigment can be read from the table horizontally.

Figs. 3.3. Peak Identities for chromatograms from experiment 2.3

1 Chlorophyllide *a*

2 Chlorophyll *c*

3 Chlorophyll *a*

Fig 3.3. Run 13(a) Wavelength 435nm

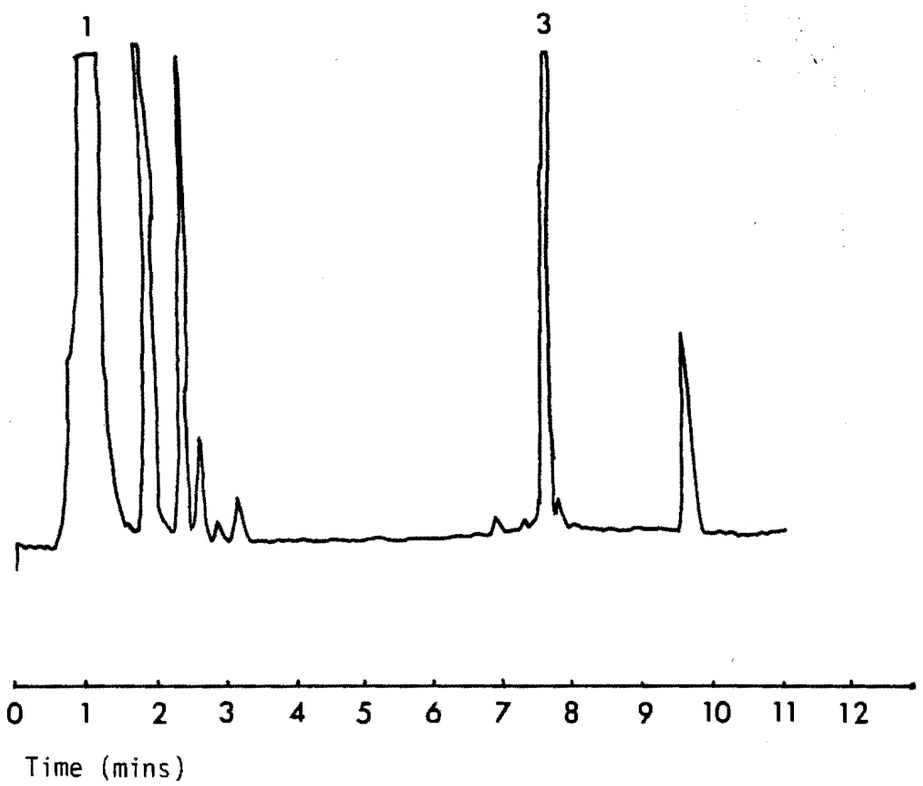


Fig 3.3. Run 13(b) Wavelength 665nm

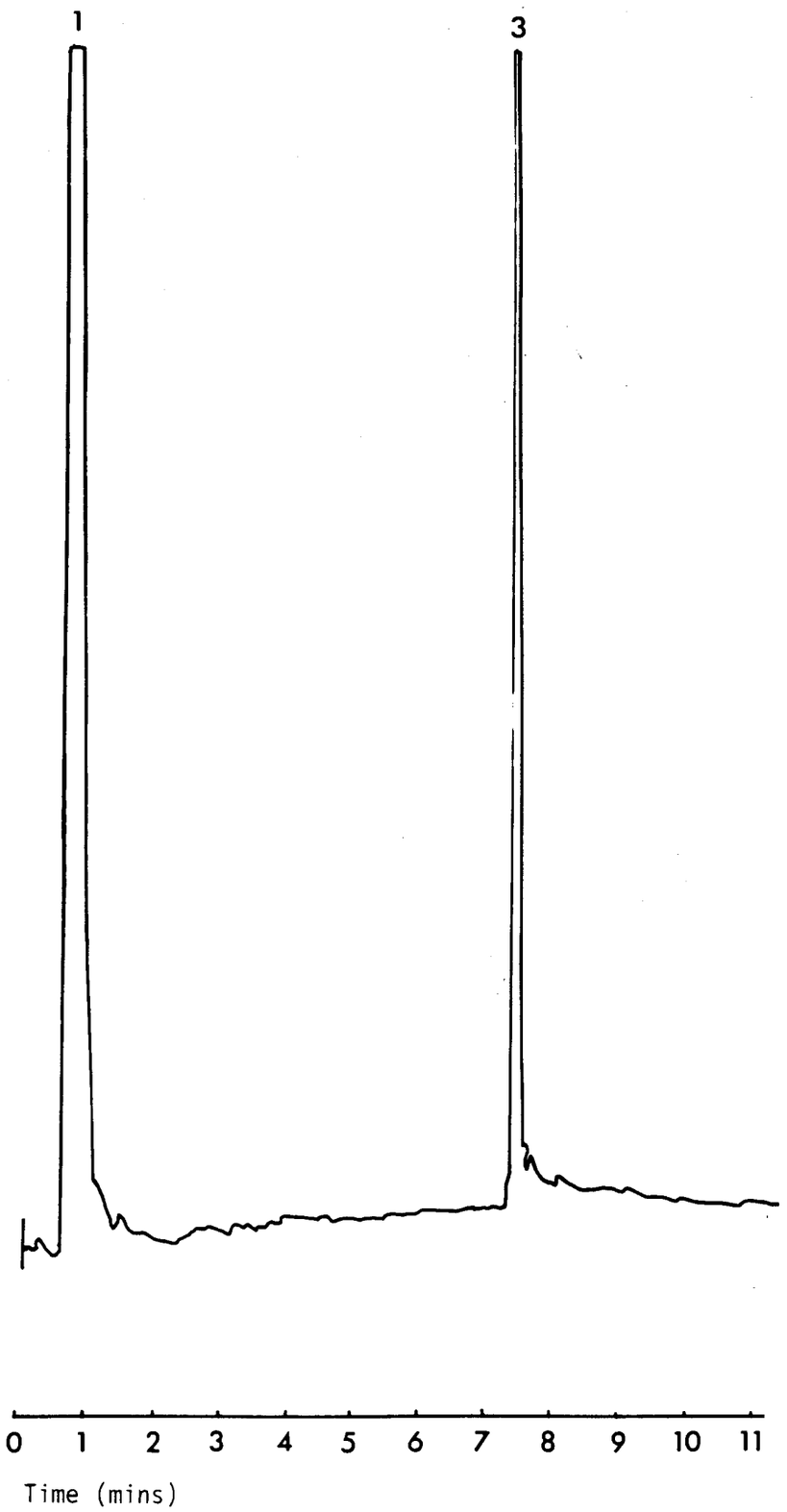
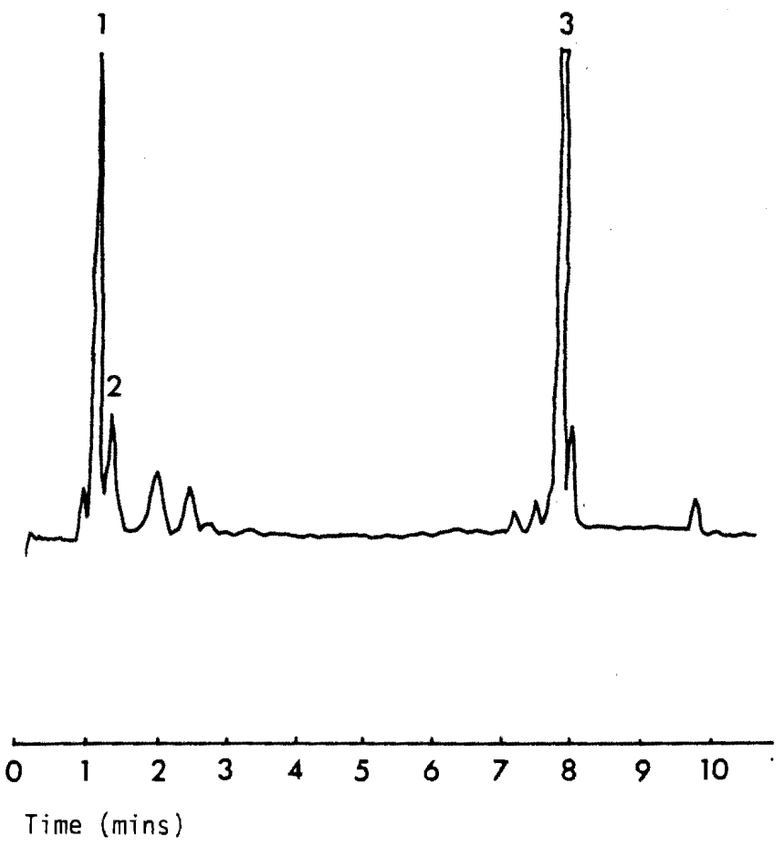


Fig 3.3. Run 14(a) Wavelength 435nm



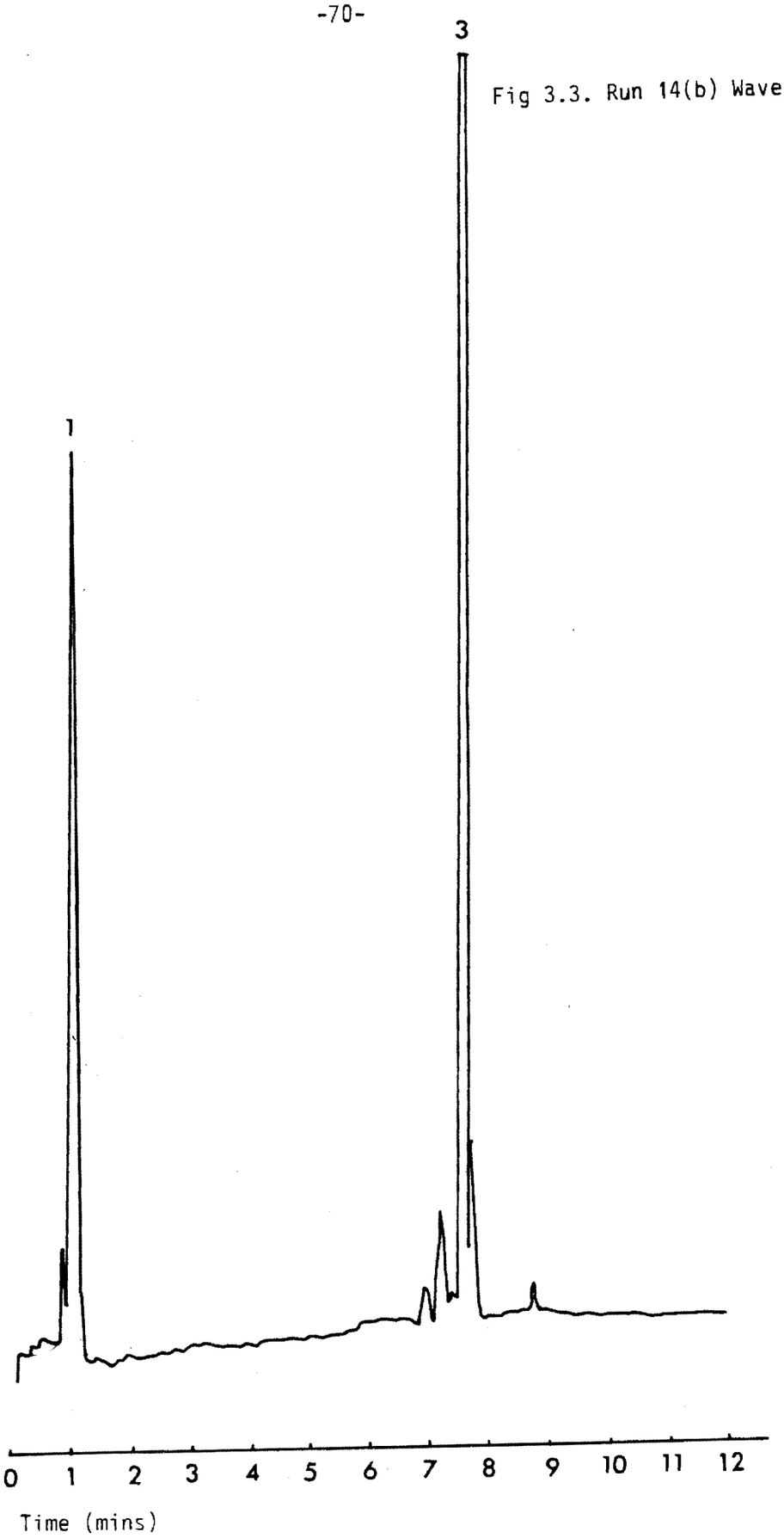



Fig 3.3. Run 14(b) Wavelength 665nm

Fig 3.3. Run 15 (a) Wavelength 435nm

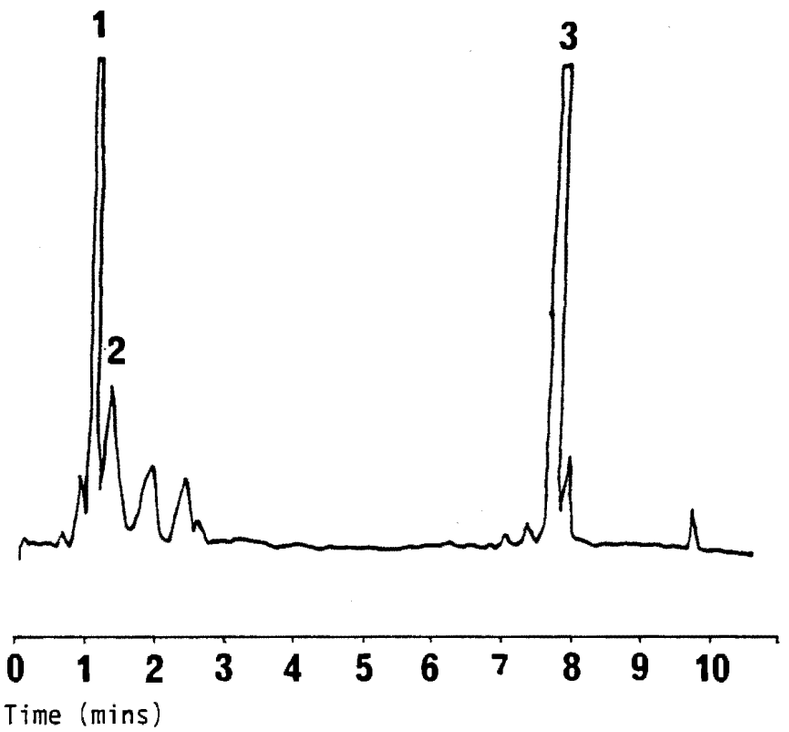


Fig 3.3. Run 15 (b) Wavelength 665nm

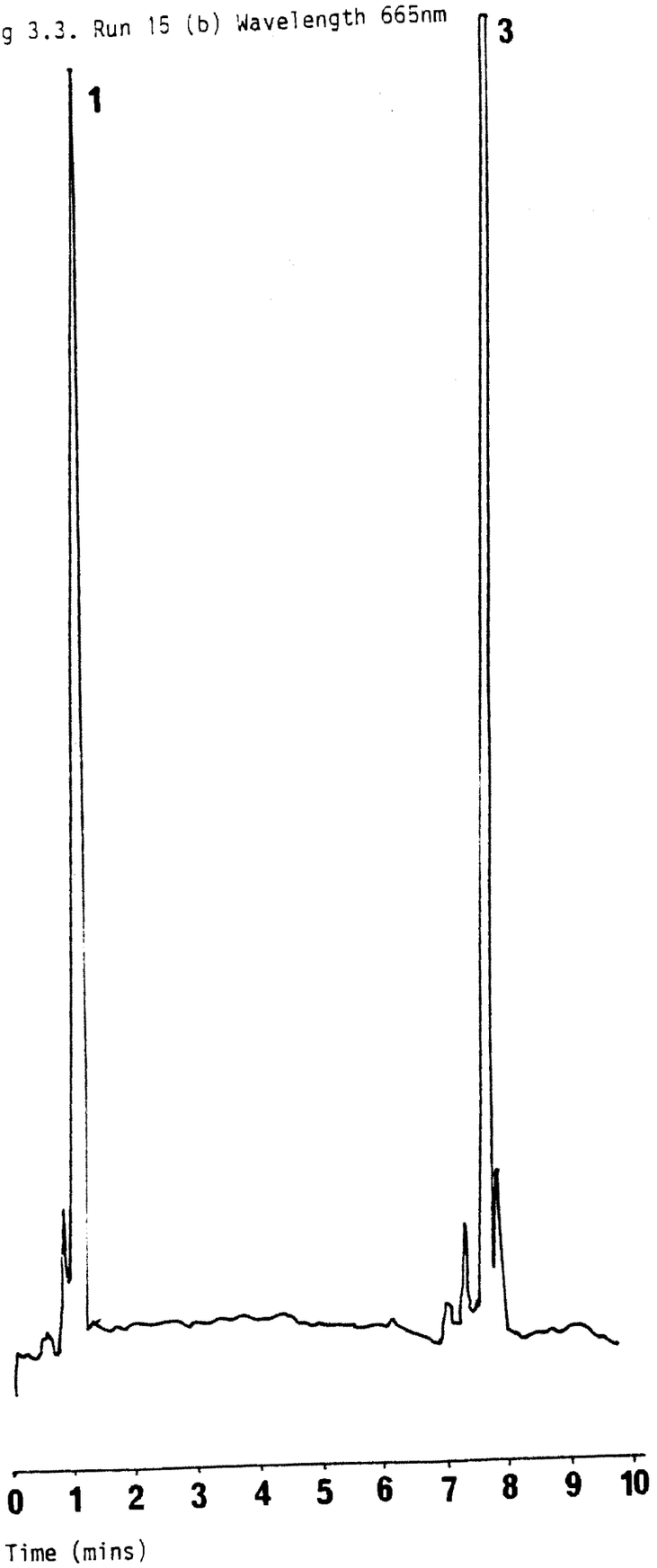


Fig 3.3. Run 16(a) Wavelength 435nm

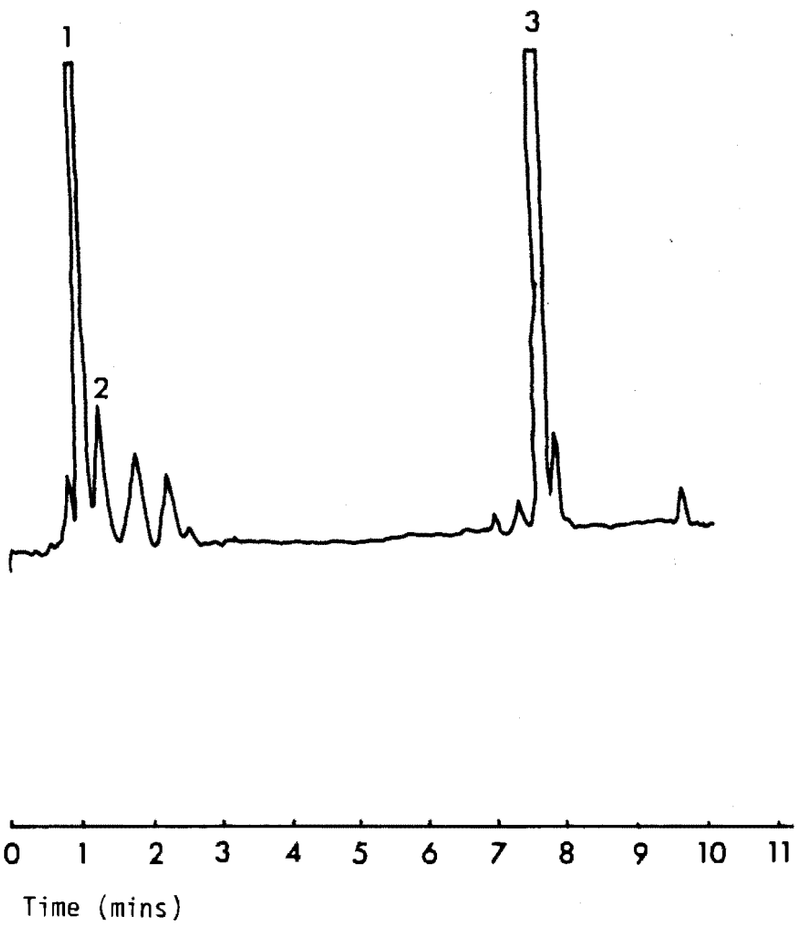


Fig 3.3. Run 16(b) Wavelength 630nm

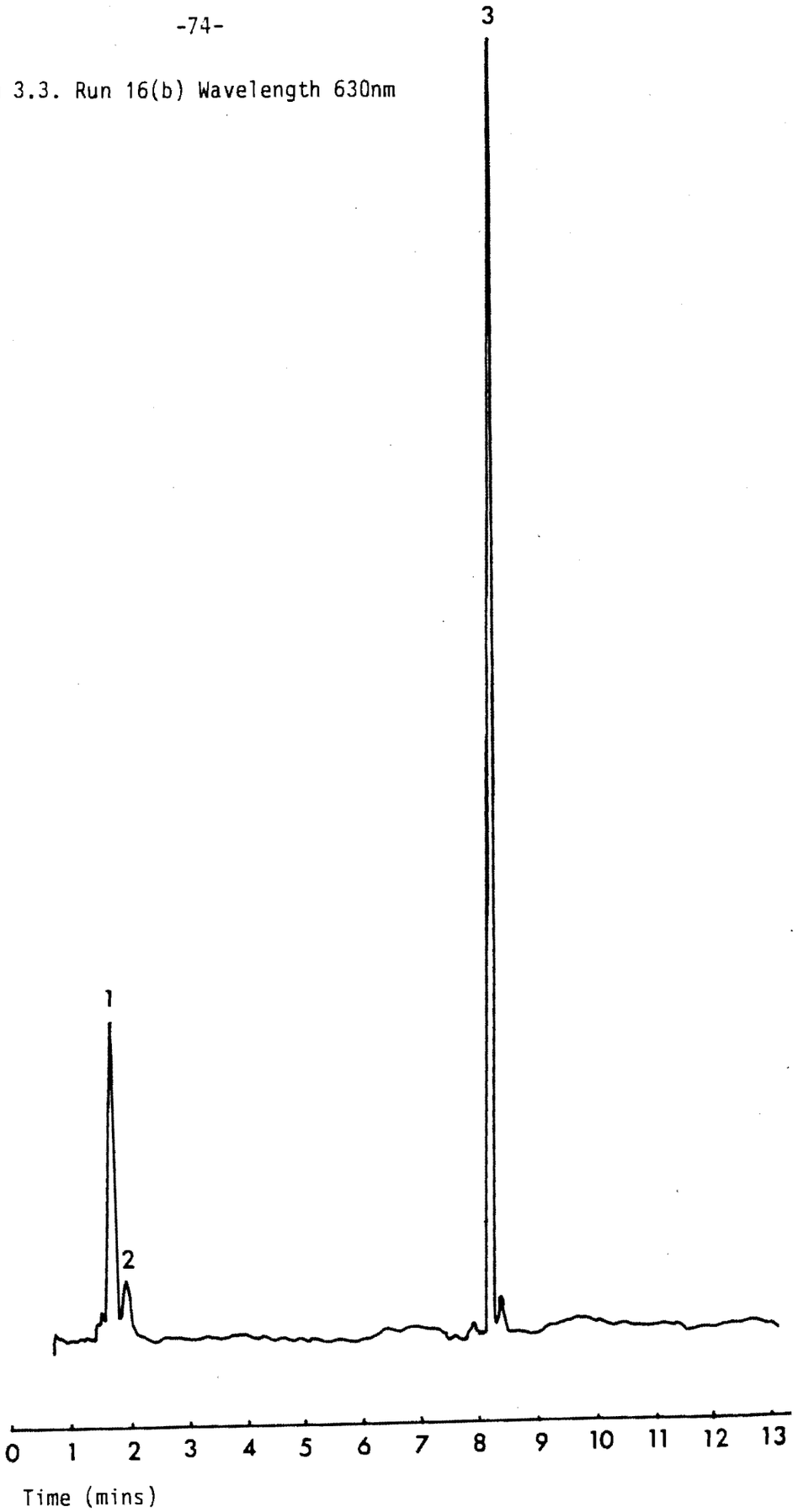


Fig 3.3. Run 17(a) Wavelength 435nm

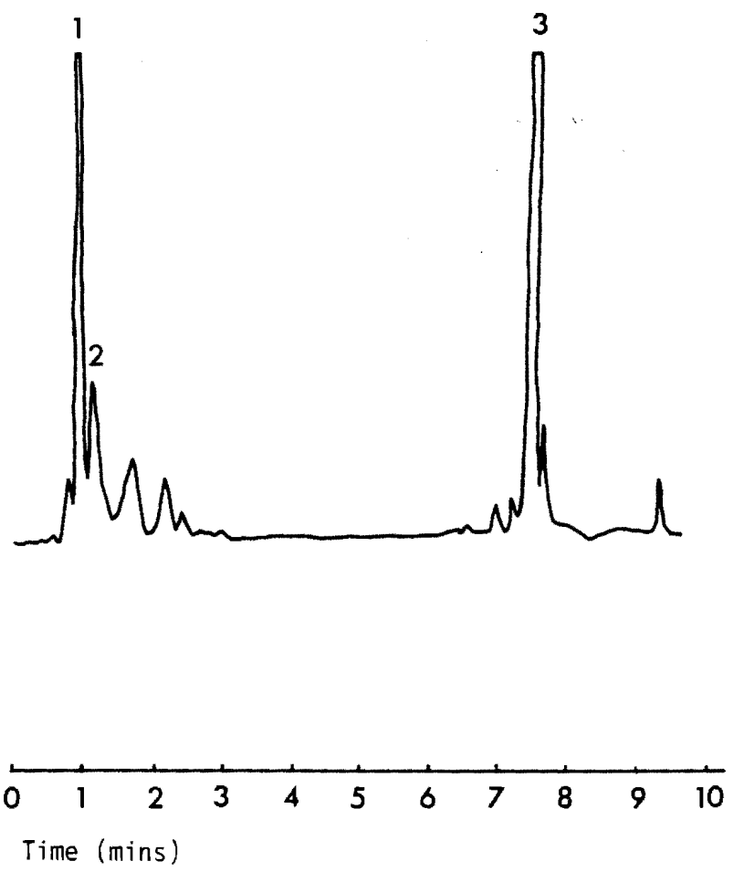
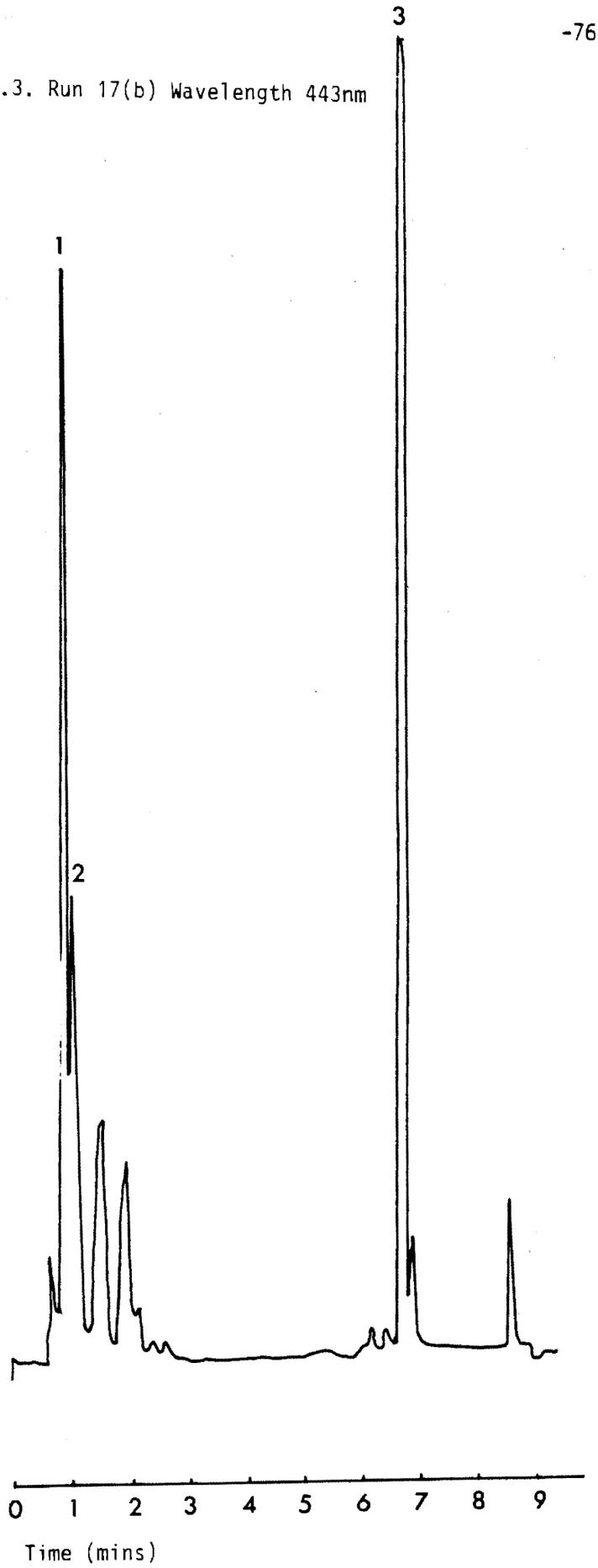


Fig 3.3. Run 17(b) Wavelength 443nm



## 3.4. Results of Experiment to Determine the Identities of the Carotenoid Peaks

### Characterisation of Pigment Peaks

See Table 3.4(i) and 3.4(ii) and Figs. 3.4

A table of peaks was compiled and labelled A to U so that they could be inspected on the basis of whether they were common to all species or to be found in only one. Relative peak heights were compared at the different wave-lengths: this allowed the spectral characteristics of the different carotenoids to be used in their identification. This information was used in conjunction with the order of elution of the carotenoids as determined by Mantoura & Llewellyn (1983).

1. Chlorophyllide *a* and chlorophyll *c* have been identified in *Phaeodactylum tricornutum* (Liaaen-Jensen, 1977). Chlorophyll *c* is not expected to occur in *Nanno saronensis*, *Tetraselmis suecica* or *Pyramimonas virginica* but arises here as a result of contamination of the stock cultures by diatoms (Mitchell-Innes, pers. comm.).

2. β-carotene is common to all algal species (Liaaen-Jensen, 1977). It is known to fluoresce in the 400nm region, and this is noted as a negative absorbance, recorded on the chromatogram after this peak occurs.

3. Diadinoxanthin and diatoxanthin are found in Phaeodactylum tricornutum, but not in Nanno saronensis or Tetraselmis suecica (Liaaen-Jensen, 1977). They are assigned to their respective peaks in the order of elution described by Mantoura & Llewellyn (1983).

4. Astaxanthin was found only in Nanno saronensis.

5. Siphonein was only found in Pyramimonas virginica (Liaaen-Jensen, 1977).

6. Neoxanthin is common to all four species. Peak "I" was assigned to neoxanthin rather than peak "F", as neoxanthin is reported to elute after diatoxanthin (Mantoura & Llewellyn, 1983).

7. Zeaxanthin, violaxanthin and lutein are all found in Nanno saronensis, Tetraselmis suecica and Pyramimonas virginica. Zeaxanthin has a wavelength of maximum absorbtion at 450nm. Violaxanthin and lutein have a wavelength of maximum absorbance of 440nm and 444nm respectively. Of the three peaks common to the species, Tetraselmis suecica, Nanno saronensis and Pyramimonas virginica peaks G, J, and M, only peak G increased in intensity at the wavelength 453nm, relative to scans at 435 and 443nm. Peak J was assigned to violaxanthin and peak M to lutein, in accordance with the order of elution described by Mantoura & Llewellyn (1983).

Fig 3.4. Run 1(a) *Nanno saronensis* at 453nm

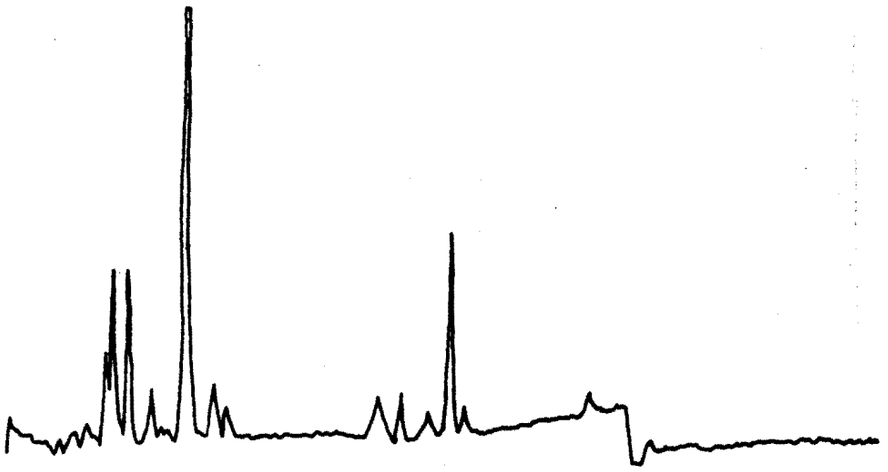


Fig 3.4. Run 1(b) *Nanno saronensis* at 435nm

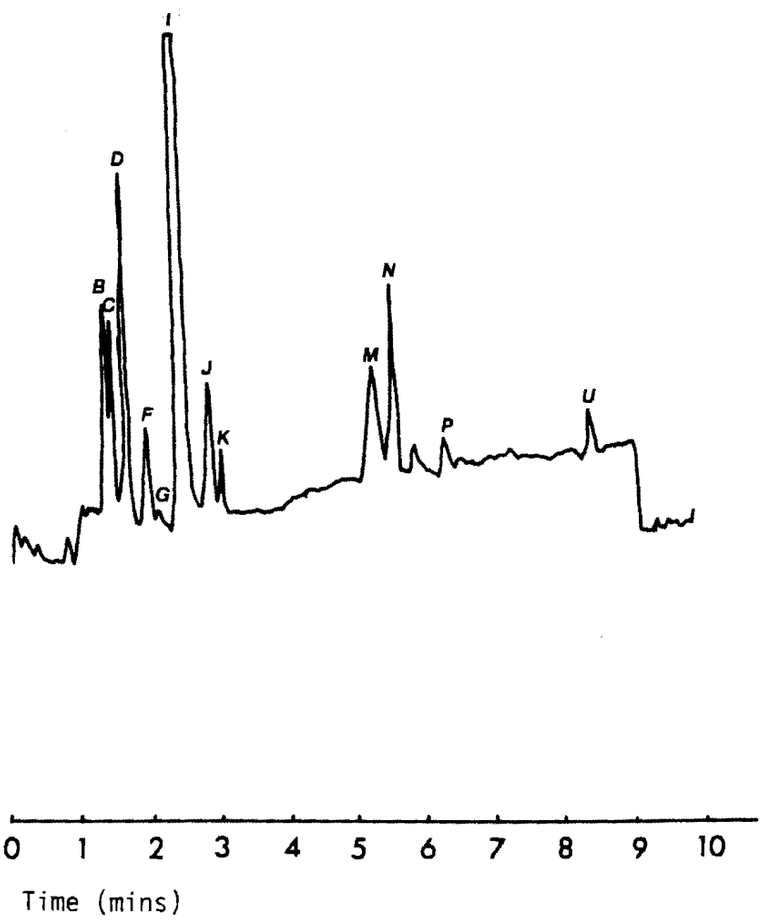


Fig 3.4. Run 2(a) *Pyramimonas virginica* at 443nm

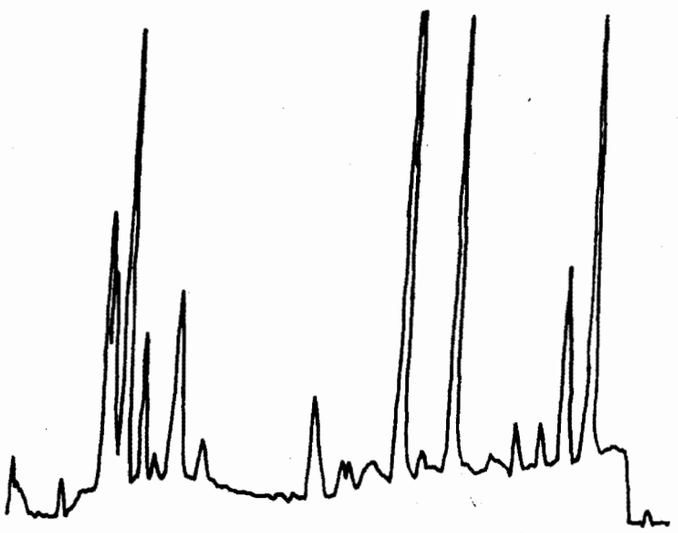


Fig 3.4. Run 2(b) *Pyramimonas virginica* at 435nm

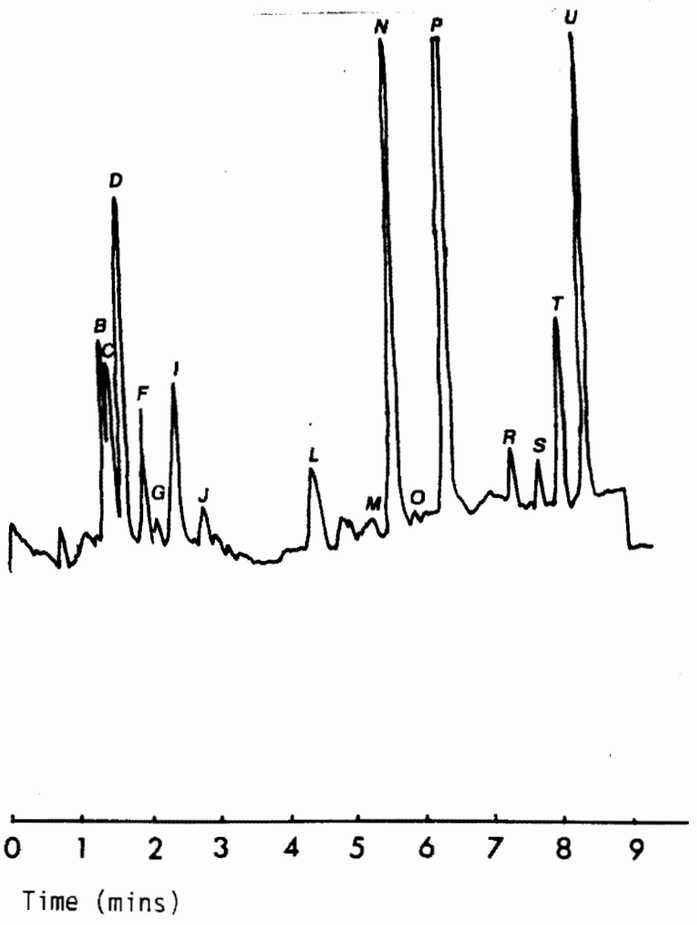


Fig 3.4. Run 3(a) *Phaeodactylum tricornutum* at 443nm

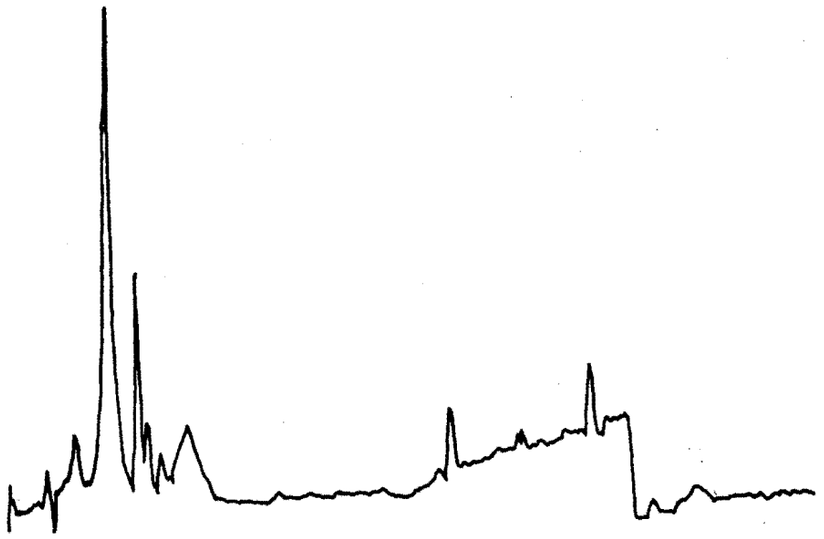


Fig 3.4. Run 3(b) *Phaeodactylum tricornutum* at 435nm

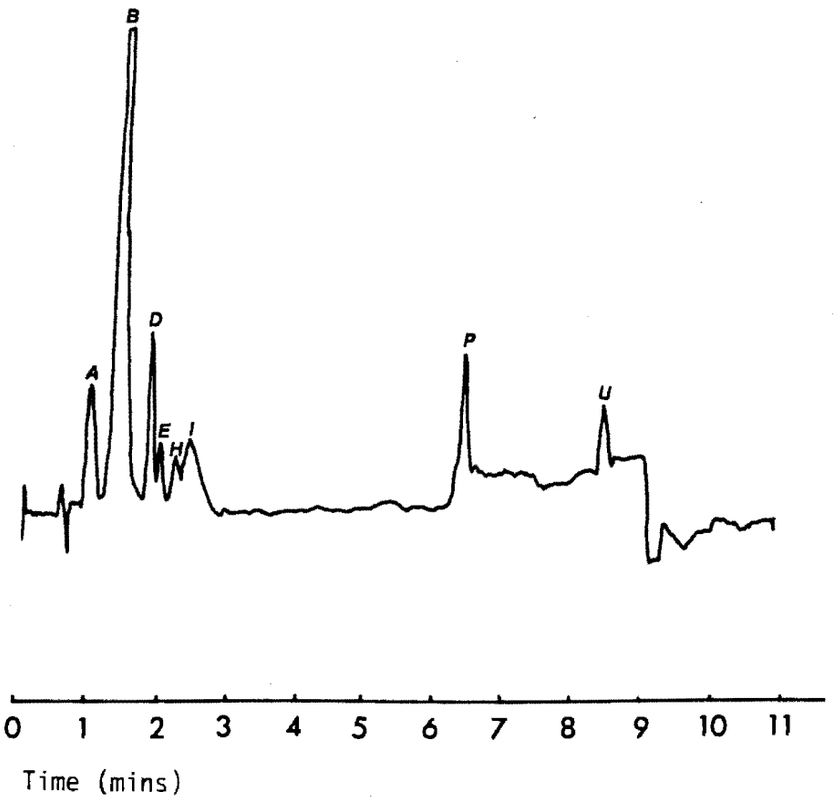


Fig 3.4. Run 4(a) *Tetraselmis suecica* at 443nm

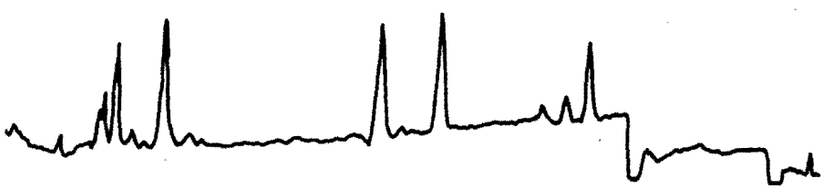


Fig 3.4. Run 4(b) *Tetraselmis suecica* at 435nm

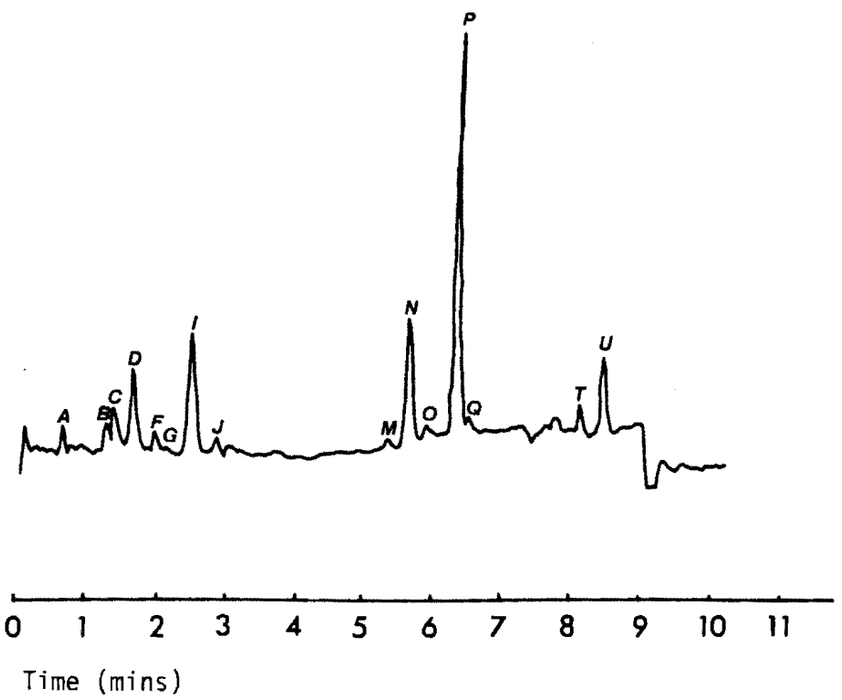


Table 3.4.i. Tabulation of Peak Retention Times recorded from scans of the four algal species at the wavelengths 435nm, 453nm and 443nm

Twenty-one different pigments were identified as shown.

| Peak No. | *P.t* | *N.s* | *P.v* | *T.s* | Peak Identity |
|---|---|---|---|---|---|
| A | 0,57 | | | 0,56 | Solvent Front |
| B | 1,37 | 1,43 | 1,39 | 1,05 | Chlorophyllide *a* |
| C | | 1,52 | 1,48 | 1,20 | Chlorophyllide *a'* |
| D | 1,79 | (1,75) | (1,66) | (1,56) | Chlorophyll *c* |
| E | 1,95 | | | | Diadinoxanthin |
| F | | 2,09 | 1,94 | 1,90 | Unidentified |
| G | | 2,26 | 2,12 | 2,08 | Zeaxanthin |
| H | 2,18 | | | | Diatoxanthin |
| I | 2,36 | 2,58 | 2,43 | 2,39 | Neoxanthin |
| J | | 2,99 | 2,83 | 2,59 | Violaxanthin |
| K | | 3,18 | | | Astaxanthin |
| L | | | 4,41 | | Siphonein |
| M | | 5,37 | 5,26 | 5,24 | Lutein |
| N | | 5,68 | 5,58 | 5,52 | Chlorophyll *b* |
| O | | | 5,95 | 5,81 | Chlorophyll *b'* |
| P | 6,28 | 6,38 | 6,32 | 6,20 | Chlorophyll *a* |
| Q | | | | 6,60 | Chlorophyll *a'* |
| R | | | 7,28 | | Pheophytin *b* |
| S | | | 7,69 | | Pheophytin *a* |
| T | | | 8,01 | 8,03 | α-carotene |
| U | 8,36 | 8,39 | 8,35 | 8,36 | β-carotene |

Numbers indicate Retention Time of the Peak in minutes

*P.t* = *P. tricornutum* *N.s* = *N. saronensis*

*P.v* = *P. virginica* *T.s* = *T. suecica*

Table 3.4.ii : Table Showing Documented Pigment Composition of the Algal Species: *Phaeodactylum tricornutum*, *Nanno Saronensis*, *Pyramimonas virginica* and *Tetraselmis suecica*

| CLASS / SPECIES | *Phaeodactylum tricornutum* | *Nanno saronensis* | *Pyramimonas virginica* *Tetraselmis suecica* |
|---|---|---|---|
| PIGMENT | | | |
| Chlorophyll *a* | X | X | X |
| Chlorophyll *b* | | X | X |
| Chlorophyll *c* | X | | |
| α carotene | | X | X |
| β carotene | X | X | X |
| fucoxanthin | X | | |
| diatoxanthin | X | | |
| diadinoxanthin | X | | |
| neoxanthin | X | X | X |
| zeaxanthin | | X | X |
| lutein | | X | X |
| violaxanthin | | X | X |
| astaxanthin | | X | |

References: Liaaen-Jensen (1977)
Davies (1976)

Summary of Results of Laboratory Work

The resolution of the peaks using a low pressure gradient former is inferior to the resolution of peaks from a high pressure gradient system. In a chromatogram from the high pressure mixing system, the peaks have high "height to half width" ratios, the chlorophyllide *a* and chlorophyll *c* peaks are resolved and the chlorophyll *a* rearrangement products are separated and identified.

In chromatograms from the low pressure mixing system, the peaks are broad, and the chlorophyll *c* peak appears as a "shoulder" on the chlorophyllide *a* peak - ie it is unresolved.

Tentative identification of the pigments other than chlorophylls *a* and *b* and pheophytins *a* and *b* has been achieved. Positive confirmation of these pigment identities will be made by stopped flow rapid scanning of the spectral characteristics of each peak, in conjunction with two-dimensional TLC. This technique is used by Jeffrey & Humphrey, 1975; Mantoura & Llewellyn, 1983 and Wright & Shearer, 1984, for collection and identification of the pigments.

# 3.B SIBEX RESULTS

Pressure Build-up on the HPLC Column

Soon after the commencement of on-board pigment analyses, it was found that the column back-pressure gradually increased, until it became impossible to continue. The sample filters were stored in liquid nitrogen until a new column was obtained and analysis resumed ashore. It was suspected, at this stage, that failure to filter all samples prior to injection may have caused the blockage.

However, the problem recurred during the shore-based analysis, despite rigorous adherence to all preliminary filtration procedures. Changing and cleaning of the frits (small filters at the top and bottom of the column, whose function is to remove particulate matter before it enters the column) did not relieve the problem. Hence the blockage appeared to result from precipitation of a particular component in the stationary phase at some stage during the gradient elution.

The blockage of the column, apart from causing constantly increasing back-pressure, also caused the retention times of the peaks to slowly increase during the runs. The retention time of the chlorophyll a peak increased from 6,61 minutes in the initial runs, to 7,04 minutes in the final chromatograms. Figs. S.3. and S.7.

It was also seen that the retention times of the chlorophyllide a peaks were extremely irregular and very distorted. This suggested that extra interactive forces are occurring on the column, in addition to those responsible for the normal Gaussian shaped peaks (Fig. S.7)

Wright & Shearer, (1984) attributed this problem to the accumulation of "lipids and non-polar material" on the column, and reported that purification of the samples by elution from a C-18 SEP-PAK® in ethyl acetate removed these compounds and alleviated the pressure build-up, although it was still necessary for the Guard Pak® columns to be routinely replaced during the analyses.

During sample preparation (Section 2C) all samples were eluted from a SEP-PAK® cartridge as part of the preconcentration procedure. Acetone is relatively less polar than ethyl acetate, and would be more selective against the non-polar lipids. However, despite this procedure, column back-pressure was seen to increase very quickly. This suggests that the effect does not arise from the binding of lipid material to the column packing.

It is known that chlorophyll a molecules can polymerise to form molecules of colloidal dimensions in solvents such as dodecane (Cotton & Wilkinson, 1980). Chlorophyllide a

molecules would also be capable of polymerising more readily, due to the absence of the phytol chain. Ideal conditions for chlorophyllide a polymerisation exist as the pigments are carried in 100% acetone onto the stationary phase, as a concentrated band, followed immediately by 80% acetone which provides the water necessary for the polymerisation process to occur via hydrogen bonding to the carbonyl functional group and coordination of the water molecule to the magnesium ion (Fig. 5). The resulting colloidal particles will be retained in the column and hence increase the resistance to mass transfer of the mobile phase, manifesting itself in increase in back pressure. The elution systems developed by Mantoura & Llewellyn, (1983) and Wright & Shearer, (1984), do not use water as a component of the mobile phase. Hence polymerisation cannot occur to the same extent. It is planned in future, to substitute another solvent - probably methanol - for the water used in the gradient system described in this work.

Kleppel and Pieper (1984) used a C-8 column for their xanthophyll analysis, and found that purification of the sample by elution in 90% methanol from a SEP-PAK® C-18 cartridge prevented any build-up of back-pressure on the column. It is possible that this could arise from the ability of the polymerised chlorophyllide a to travel through the C-8, but not through the C-18 packing.

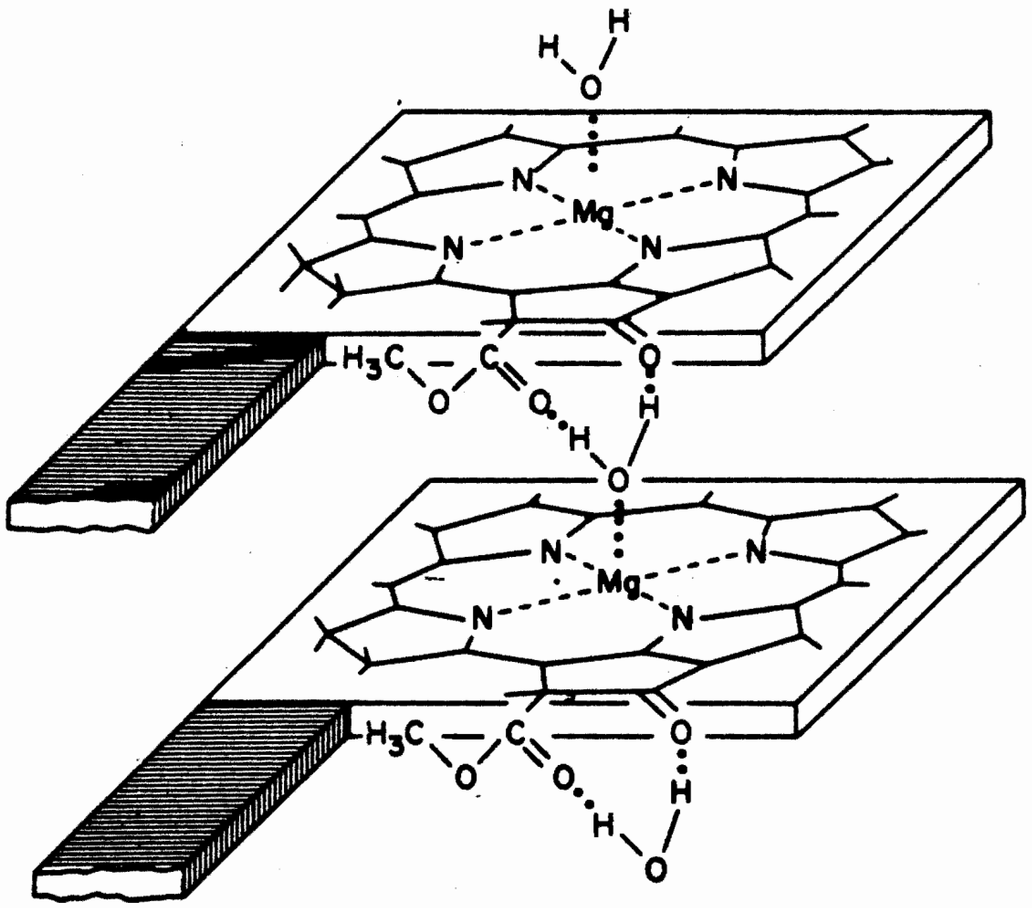



Fig 5. Structure illustrating the chlorophyll *a*-water-chlorophyll *a* interaction.The dimensions of the ring and phytyl chain are not to scale. Chlorophyllide *a* molecules do not possess the bulky phytol chain and readily participate in hydrogen-bonding with water in a similar manner.

It is apparent that the chlorophyll a molecules do not participate in the polymerisation as quantitative elution was observed during the calibration procedure. This irreproducibility of retention time in the SIBEX chromatograms means that the chlorophyllide a peak is not absolutely certain and quantification of it maybe inaccurate since some may have been retained on the column. However, on the basis of the peak identification experiments, tentative chlorophyllide a concentrations were calculated and compared with the chlorophyll a and primary productivity measurements. A correction factor may be introduced when the necessary work has been completed.

SIBEX Results:

1. Assuming that the chlorophyllide a peak has been correctly identified, it is seen that all samples contain chlorophyllide a, which appears to be the major breakdown product of chlorophyll a, in preference to pheophytin a. (Figs. S.2. - S.7.)

2. No samples contain chlorophyll b. This indicates a total absence of blue-green algal species and an algal population consisting entirely of diatoms. (Figs. S.2. - S.7)

3. Direct comparison of the HPLC values with the trichromatic spectrophotometric chlorophyll *a* values is difficult, as the samples for the two methods of chlorophyll *a* determination were taken from two separate casts of Niskin bottles at every station. The depths at which the samples were taken were different for the two casts, which were made several hours apart. Stratification of algal blooms can account for significant changes in algal density within a depth of 5 - 10 metres (Fogg, 1975).

4. The chlorophyll *a* and chlorophyllide *a* values were compared by calculation of the Mole Ratio:

$$\frac{\text{Moles Chlorophyll } a \text{ m}^{-3}}{\text{Moles Chlorophyllide } a \text{ m}^{-3}}$$

A high mole ratio indicates that a higher proportion of "active" chlorophyll *a* is present. A low mole ratio is an indication that most of the chlorophyll *a* is present in its degraded form, chlorophyllide *a*. Such a situation would be representative of a senescent algal bloom, or one which has been subject to intense predation by zooplankton or bacterial degradation.

5. The only trend common to nearly all stations was a chlorophyll *a* maximum at 75 - 100 metres. The average

chlorophyll a concentration was 0,08 mgm-$^3$. The chlorophyll a concentrations at the southernmost stations at the edge of the pack ice increased, and the average chlorophyll a concentration for stations 30,40 and 42 was 0,12 mgm-$^3$. (See Fig. S.1., page 32 and SIBEX-I Results, page 109).

The primary productivity values were not in good agreement with either the chlorophyll a concentrations measured by HPLC, or those measured by the trichromatic method (Allanson, 1984). However, a correlation seems to exist between the Mole Ratio values and the primary productivity values.

The Mole Ratio values are calculated from the HPLC results and the productivity values are calculated from the trichomatic chlorophyll a values. Since these two sets of values originated from different samples, no statistical correlation can be made. However, the vertical profiles have been used to compare trends in the different sets of values. As yet, the proposed relationship can neither be proved nor disproved, but can serve as the basis of further studies. The "correlation" is illustrated by some specific examples

Stations 38 and 40 (See Figs. S.9. and S.10)

Both stations have similar chlorophyll a profiles(Fig.S.9(ii) and S.10(ii)): the values are fairly constant, with a maximum at 50 metres. However, the productivity values and the chlorophyllide values for these stations are completely different.

At station 38, a high Mole Ratio at the surface occurred in conjunction with high primary productivity values with correspondingly low values at 20 metres.

At station 40 the chlorophyllide a values are higher than the chlorophyll a values at all depths, and the Mole Ratios are consistently low. Specific productivities are also correspondingly low.

Stations 34 (See Figs. S.8.)

This station has two chlorophyll a maxima in the water column - one at 30 metres and another at 100 metres. The Mole Ratio at the 30 metre maximum is high (chlorophyllide a values at this depth are low), corresponding with high specific productivity values at this depth. The Mole Ratio at 100 metres is low and the specific productivity at these depths correspondingly low.

This information suggests that the chlorophyll a maximum at 30 metres arises due to a productive bloom, containing predominantly active chlorophyll a. However, the chlorophyll a maximum at 100 metres arises due to an accumulation of detrital matter and faecal pellets at the pycnocline. However, the proportion of degraded chlorophyll a, ie chlorophyllide a, that accumulates at this depth is high, giving rise to low Mole Ratios and low productivity. (See Fig. S.12).

Discussion of Chromatograms (Figs. S.4. - S.7.)

Phytoplankton samples were collected twice daily in bongo net hauls. Some samples were preserved in liquid nitrogen and the pigments extracted and analysed so that a typical pigment structure of the phytoplankton population could be obtained (Fig. S.2.). Peaks due to the pigments chlorophyllide a, chlorophyll c and chlorophyll a can be seen.

Figs. S.3., S.4. and S.7. are examples of different chromatograms, showing (Fig. S.3.) a chromatogram in which chlorophyllide a and chlorophyll c are well resolved and (Fig. S.4.) a chromatogram in which the peaks due to chlorophyllide a and chlorophyll c overlap. Fig. S.7. is an example of a chromatogram in which the chlorophyllide a and chlorophyll c peaks are distorted. The chlorophyll a

peak has been split into two components and its retention time increased to 7,01 minutes.

Samples of dense algal blooms were taken from pieces of ice, and from the water surrounding the ice, and were analysed for chlorophyll a and chlorophyllide a, and productivity values measured (Figs. S.5 and S.6.)

The Mole Ratios of the pack ice algae and the algae living in water surrounding the ice were 1,03 and 2,38 respectively and specific productivity values in mgC/mgChl$\underline{a}$ $hr^{-1}$ were 0,80 and 2,24 indicating a high proportion of "active" chlorophyll a and a highly productive algal population.

Figs. S2 - S7: Typical Chromatograms selected from SIBEX I Data

Peak Identities

1 Chlorophyllide *a*

2 Chlorophyll *c*

3 Chlorophyll *a*

Fig S.2. Two examples of pigments extracted from diatoms collected in the Bongo net

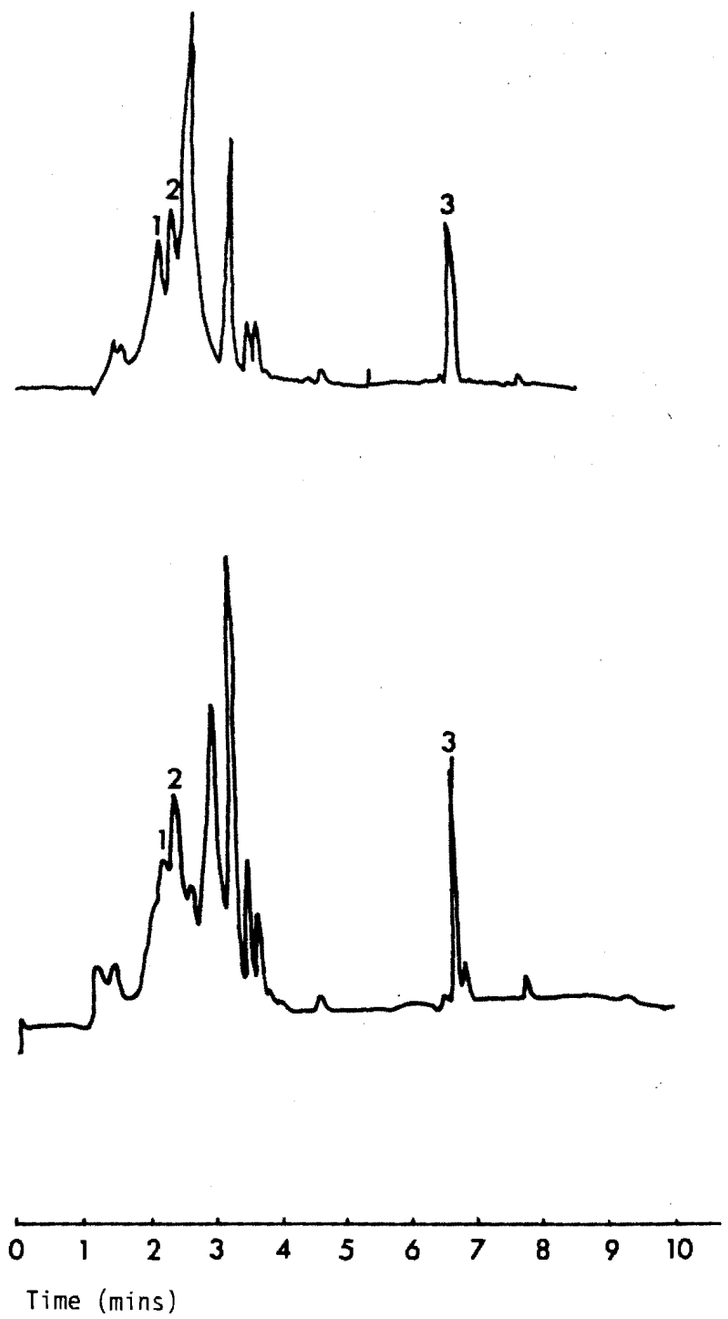


Fig S 3. A chromatogram from Station 32,at 30 metres, in which chlorophyllide *a* and chlorophyll *c* are well resolved.The retention time of chlorophyll *a* is 6,65 minutes.

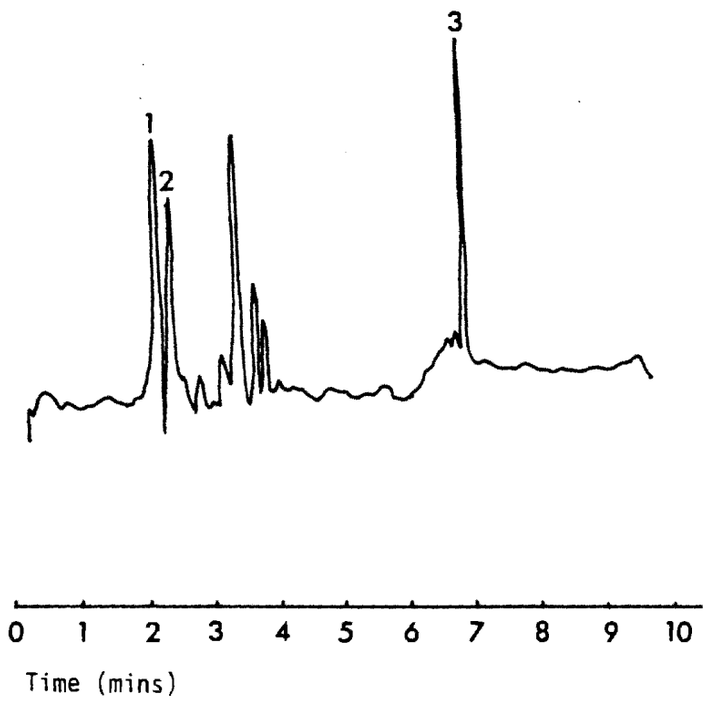


Fig S.4. A chromatogram from station 34, at 200m. The chlorophyllide *a* concentration is high and so the chlorophyll *c* peak is not clearly resolved.

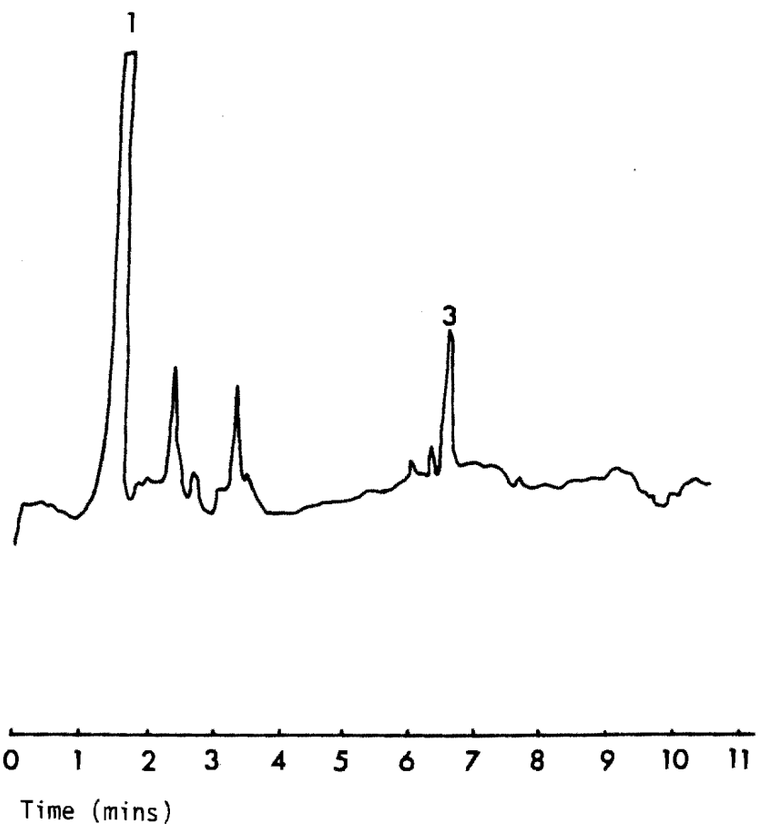


Fig S 5. Pigments from phytoplankton living in water between ice floes.

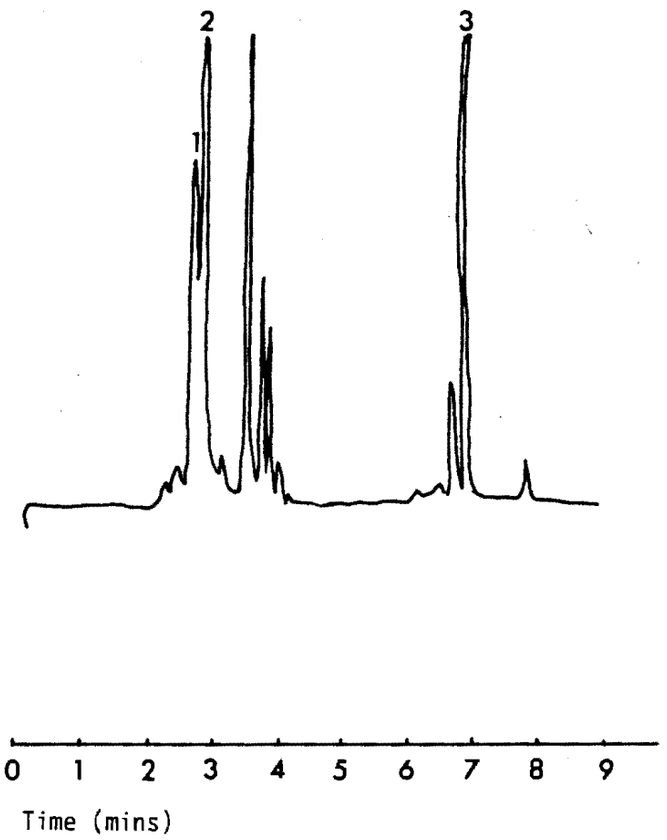


Fig S 6. Pigments from phytoplankton collected from a melted ice floe.

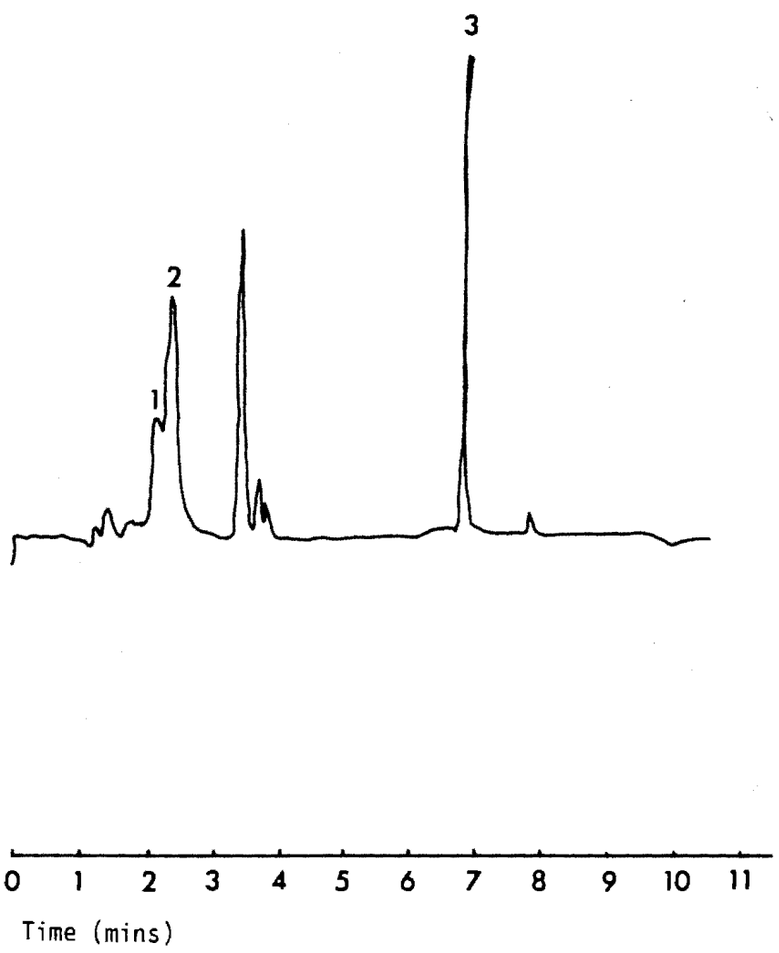
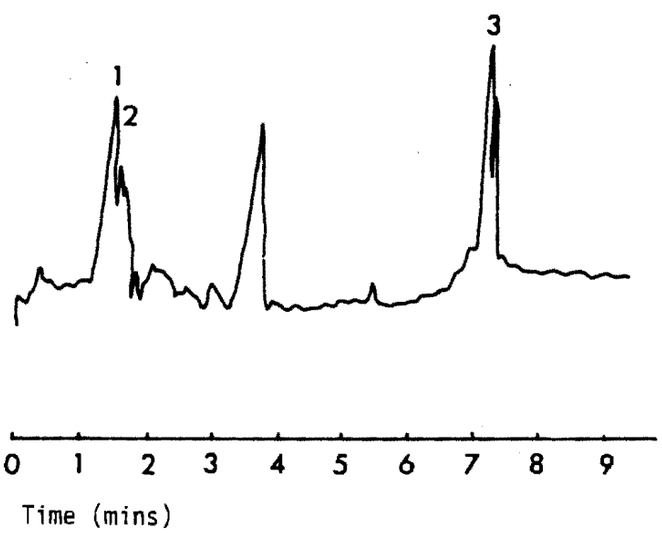


Fig S 7. An example of a chromatogram in which the initial peaks are distorted and the chlorophyll $a$ retention time has increased to 7,01 minutes.



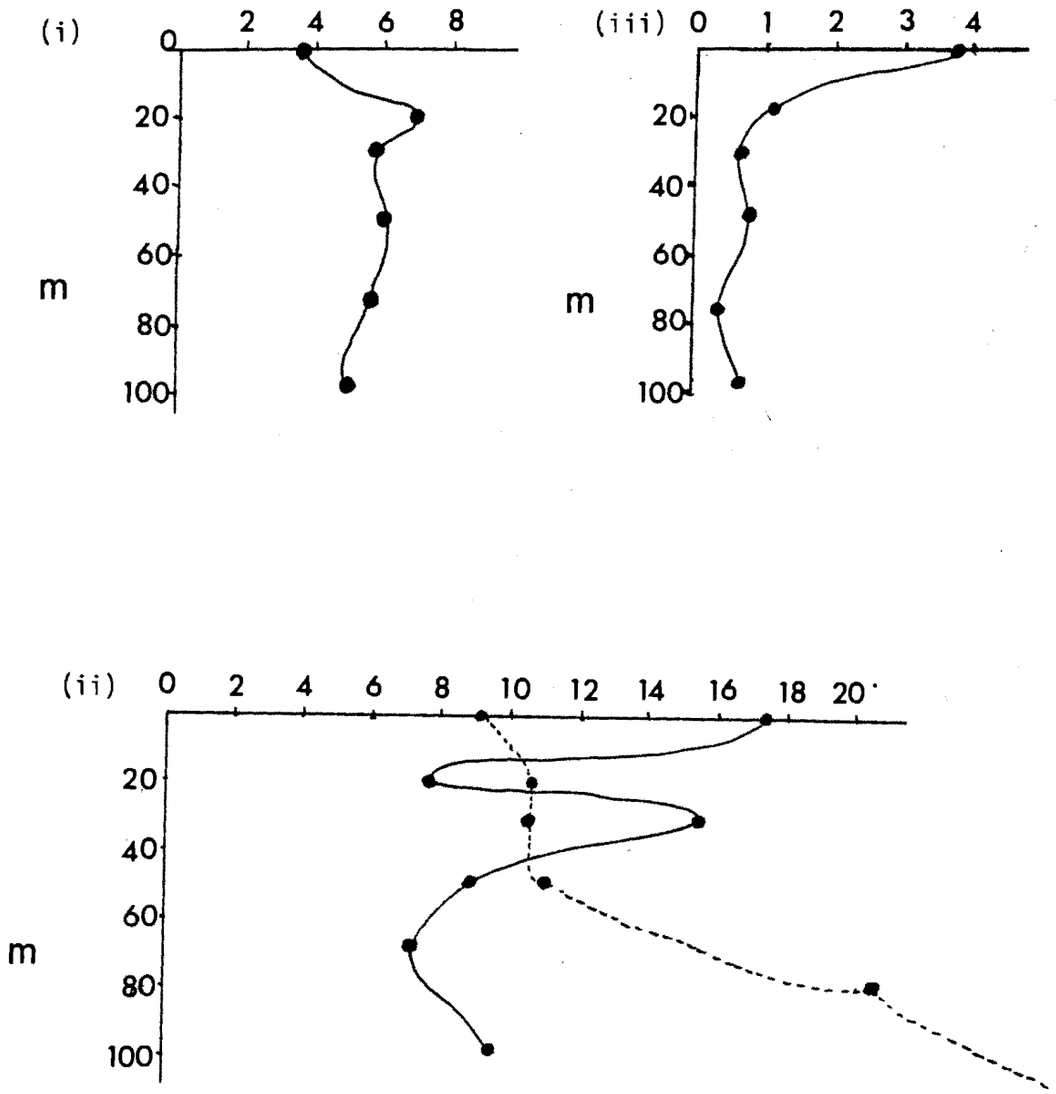



Fig S.8. SIBEX Station 34

(i) SCOR-Unesco Chlorophyll *a* values ($mg.m^{-3} \times 10^{-2}$)

(ii) HPLC Chlorophyll *a* values —and Chlorophyllide *a*-- ($mg.m^{-3} \times 10^{-2}$)

(iii) Primary Productivity values (mg C/mg Chl *a* $hr^{-1}$)

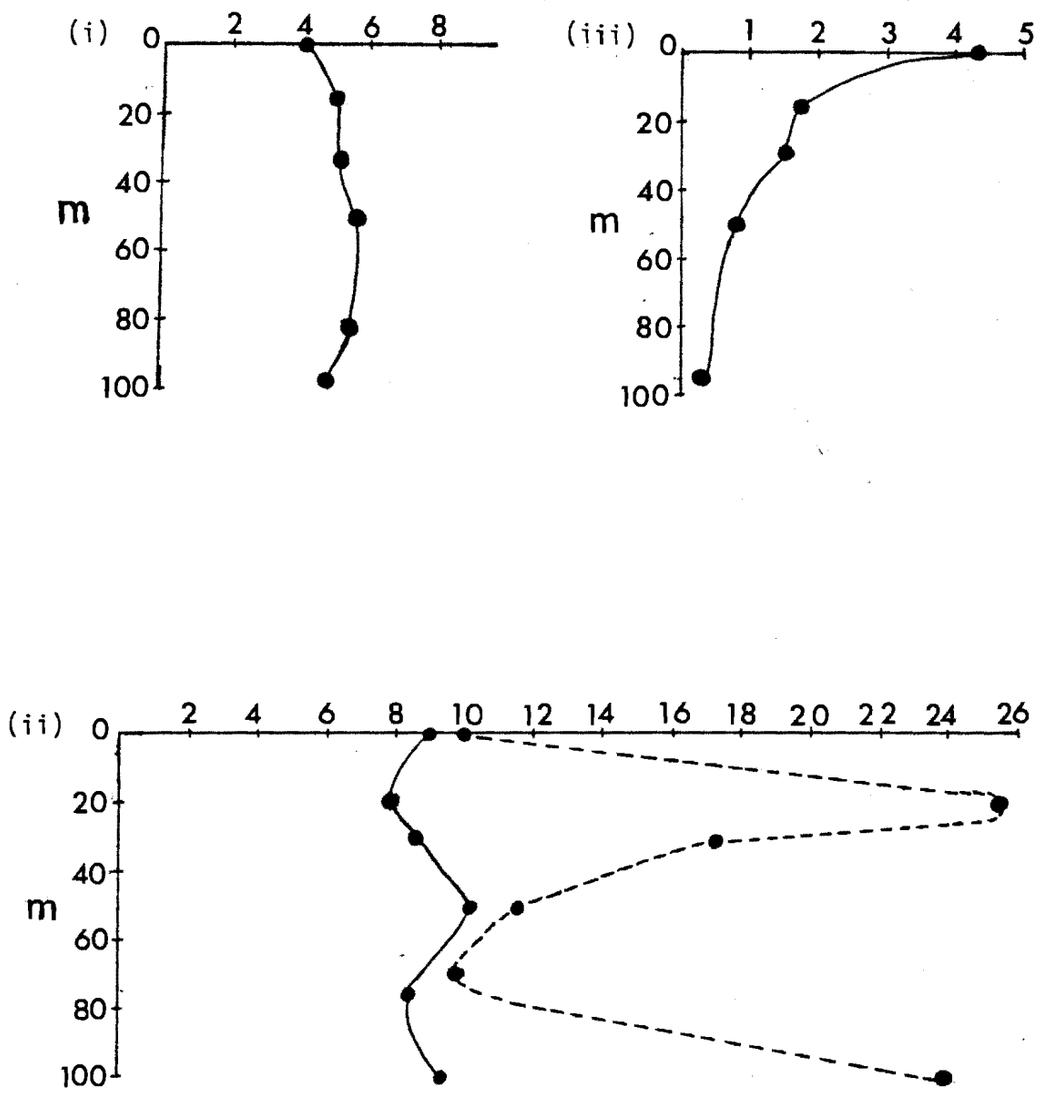



Fig. S.9. SIBEX Station 38

(i) SCOR-Unesco Chlorophyll *a* values ($mg.m^{-3} \times 10^{-2}$)

(ii) HPLC Chlorophyll *a* values — and Chlorophyllide *a* --- ($mg.m^{-3} \times 10^{-2}$)

(iii) Primary Productivity values (mg C/mg Chl *a* $hr^{-1}$)

STATION 34

| HPLC Determinations | | | | SCOR-UNESCO Determinations | | |
|---|---|---|---|---|---|---|
| Depth(m) | Chl$a$ | Chlide$a$ | Mole Ratio | Depth(m) | Chl$a$ | Specific Productivity |
| 0 | 0,175 | 0,092 | 1,90 | 0 | 0,035 | 3,57 |
| 20 | 0,078 | 0,105 | 0,51 | 15 | 0,065 | 1,07 |
| 30 | 0,158 | 0,104 | 1,05 | 30 | 0,057 | 0,62 |
| 50 | 0,090 | 0,110 | 0,56 | 50 | 0,059 | 0,64 |
| 75 | 0,073 | 0,202 | 0,25 | 88 | 0,054 | 0,37 |
| 100 | 0,096 | 0,317 | 0,21 | 100 | 0,050 | 0,55 |
| 150 | 0,054 | no value | - | | | |
| 200 | 0,054 | 0,105 | 0,35 | | | |

STATION 38

| HPLC Determinations | | | | SCOR-UNESCO Determinations | | |
|---|---|---|---|---|---|---|
| Depth(m) | Chl$a$ | Chlide$a$ | Mole Ratio | Depth(m) | Chl$a$ | Specific Productivity |
| 0 | 0,091 | 0,099 | 0,63 | 0 | 0,044 | 4,29 |
| 20 | 0,081 | 0,255 | 0,22 | 15 | 0,052 | 1,80 |
| 30 | 0,086 | 0,173 | 0,34 | 30 | 0,055 | 1,46 |
| 50 | 0,101 | 0,116 | 0,60 | 50 | 0,058 | 0,75 |
| 75 | 0,084 | 0,097 | 0,63 | 88 | 0,055 | 0,37 |
| 100 | 0,094 | 0,238 | 0,28 | 100 | 0,052 | 0,21 |
| 150 | 0,065 | - | - | | | |
| 200 | 0,058 | 0,087 | - | | | |

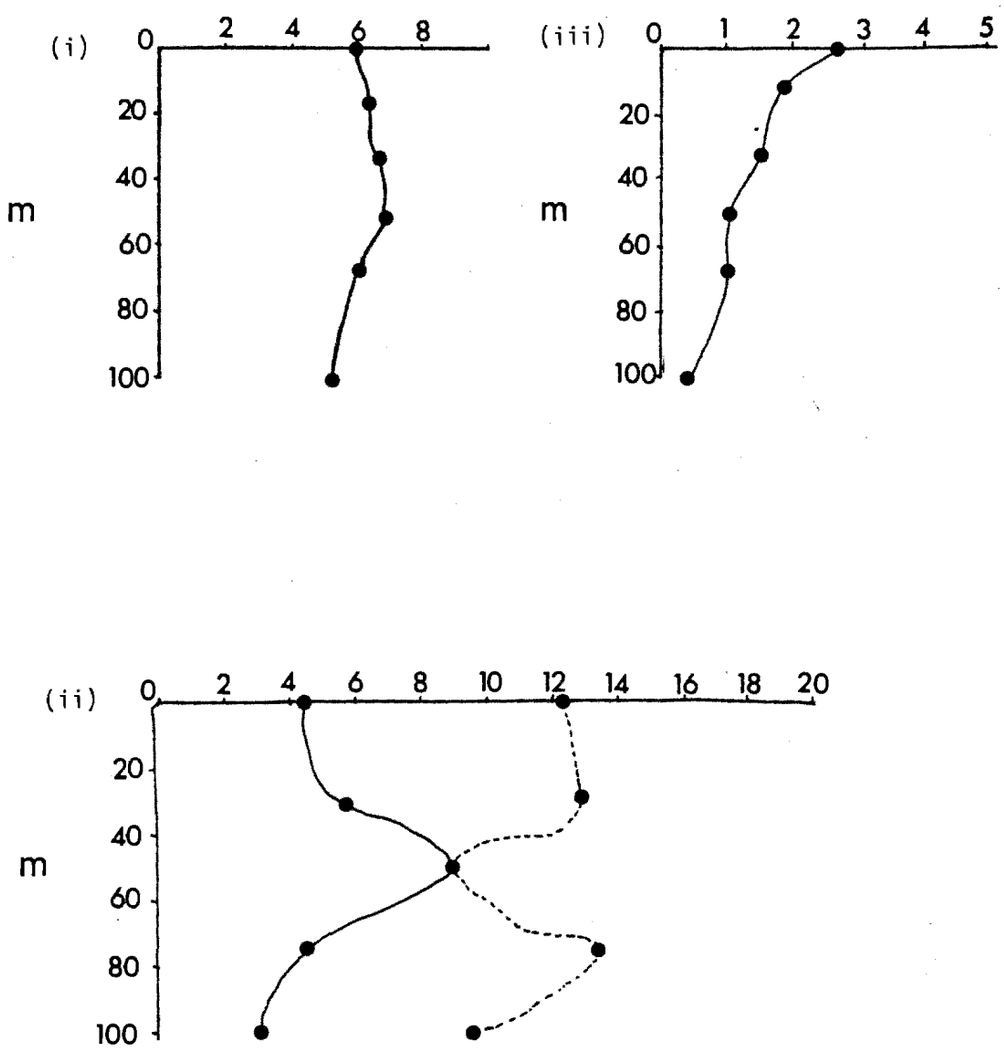



Fig S.10. SIBEX Station 40

(i) SCOR-Unesco Chlorophyll *a* values ($mg.m^{-3} \times 10^{-2}$)

(ii) HPLC Chlorophyll *a* values —— and Chlorophyllide *a* ---

($mg.m^{-3} \times 10^{-2}$)

(iii) Primary Productivity values (mg C/mg Chl *a* $hr^{-1}$)

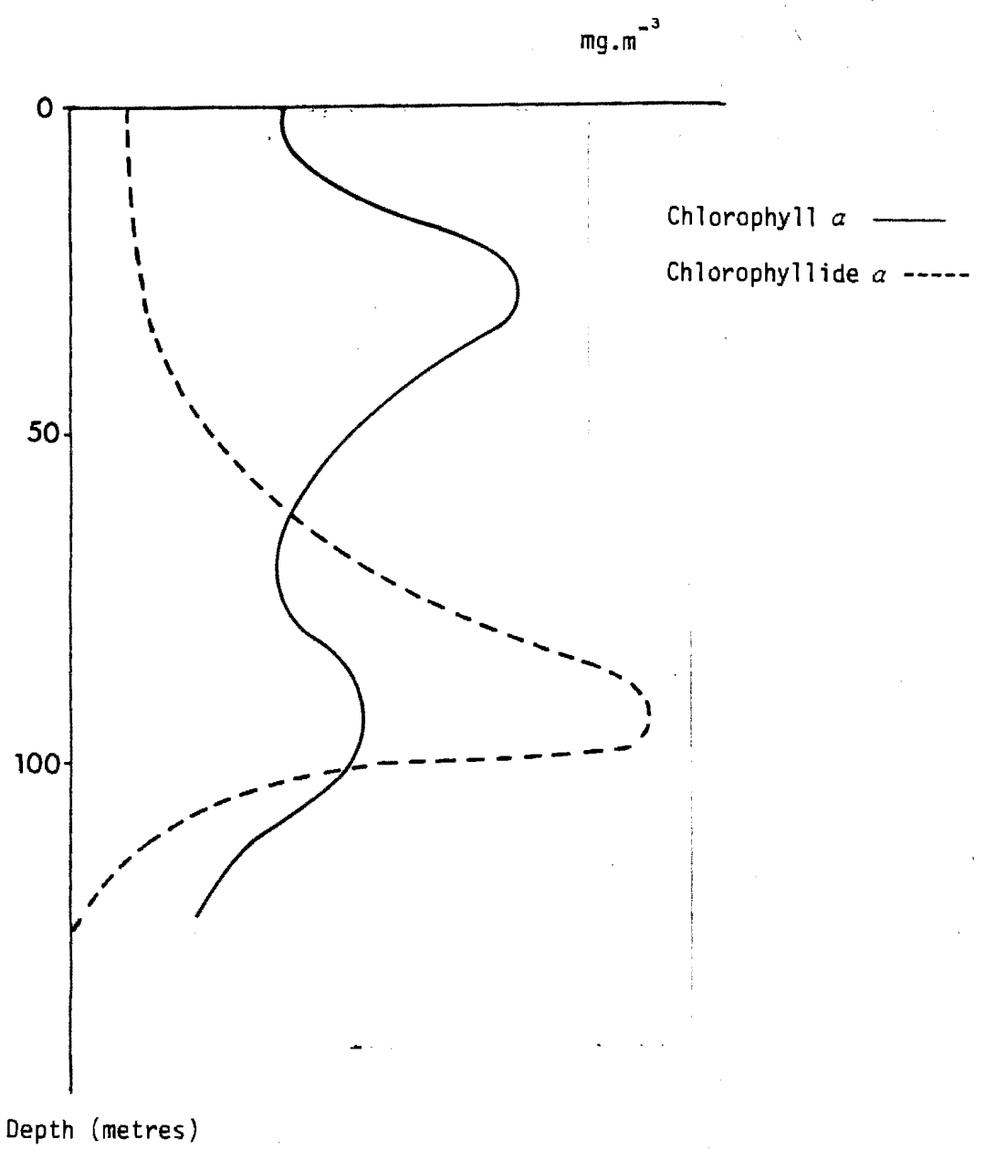
STATION 40

| HPLC Determinations | | | | SCOR-UNESCO Determinations | | |
|---|---|---|---|---|---|---|
| Depth(m) | Chl$a$ | Chlide$a$ | Mole Ratio | Depth(m) | Chl$a$ | Specific Productivity |
| 0 | 0,044 | 0,123 | 0,25 | 0 | 0,060 | 2,56 |
| 20 | - | - | - | 16 | 0,64 | 1,91 |
| 30 | 0,057 | 0,129 | 0,30 | 31 | 0,066 | 1,60 |
| 50 | 0,090 | 0,088 | 0,70 | 51 | 0,068 | 1,06 |
| 75 | 0,045 | 0,135 | 0,23 | 67 | 0,060 | 1,08 |
| 100 | 0,031 | 0,096 | 0,23 | 102 | 0,052 | 0,38 |
| 200 | 0,021 | - | - | | | |

STATION 40

| HPLC Determinations | | | | SCOR-UNESCO Determinations | | |
|---|---|---|---|---|---|---|
| Depth(m) | Chl*a* | Chlide*a* | Mole Ratio | Depth(m) | Chl*a* | Specific Productivity |
| 0 | 0,044 | 0,123 | 0,25 | 0 | 0,060 | 2,56 |
| 20 | - | - | - | 16 | 0,64 | 1,91 |
| 30 | 0,057 | 0,129 | 0,30 | 31 | 0,066 | 1,60 |
| 50 | 0,090 | 0,088 | 0,70 | 51 | 0,068 | 1,06 |
| 75 | 0,045 | 0,135 | 0,23 | 67 | 0,060 | 1,08 |
| 100 | 0,031 | 0,096 | 0,23 | 102 | 0,052 | 0,38 |
| 200 | 0,021 | - | - | | | |

Fig S.12 Schematic diagram showing the relationship between chlorophyll *a* and chlorophyllide *a* in the euphotic zone.



SIBEX-I RESULTS See Fig. S.1

All concentrations expressed in $mgm^{-3}$

STATION 17 65° 20.50S; 60° 4.44E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,049 | - | - |
| 10 | 0,070 | 0,062 | 1,13 |
| 20 | 0,059 | 0,026 | 1,56 |
| 30 | 0,046 | 0,045 | 0,70 |
| 50 | 0,043 | 0,027 | 1,10 |
| 75 | 0,045 | 0,022 | 1,40 |
| 100 | 0,062 | 0,030 | 1,42 |

STATION 22 62° 0.82S; 60° 0.40E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,079 | - | - |
| 10 | 0,056 | - | - |
| 20 | - | 0,051 | - |
| 30 | 0,064 | 0,072 | 0,61 |
| 50 | 0,088 | 0,087 | 0,70 |
| 75 | 0,153 | 0,148 | 0,70 |
| 100 | 0,090 | 0,248 | 0,25 |

STATION 24 62° 42.03S; 58° 2.56E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,049 | 0,048 | - |
| 20 | 0,032 | - | - |
| 30 | - | 0,038 | - |
| 50 | 0,039 | 0,027 | ~1 |
| 75 | 0,070 | 0,049 | ~1 |
| 200 | - | 0,043 | - |

STATION 26 65° 59.44S; 58° 0.24E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,028 | - | - |
| 20 | 0,044 | 0,029 | 1,04 |
| 30 | - | - | - |
| 50 | 0,044 | 0,059 | 0,51 |
| 75 | 0,063 | 0,095 | 4,58 |
| 100 | 0,107 | 0,249 | 0,30 |

STATION 30 65° 21.61S; 55° 58.96E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,198 | 0,105 | 1,29 |
| 20 | 0,092 | 0,118 | 0,54 |
| 30 | 0,201 | 0,444 | 0,31 |
| 50 | 0,363 | - | - |
| 75 | 0,118 | 0,231 | 0,03 |
| 100 | 0,066 | 0,150 | 0,30 |
| 150 | - | - | - |
| 200 | 0,048 | - | - |

STATION 32 64° 1.23S; 56° 1.43E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,215 | 0,435 | 0,34 |
| 20 | 0,066 | 0,119 | 0,43 |
| 30 | 0,087 | 0,130 | 0,46 |
| 50 | 0,078 | 0,114 | 0,47 |
| 75 | 0,136 | 0,145 | 0,65 |
| 100 | 0,124 | 0,089 | 0,98 |
| 200 | - | 0,117 | - |

STATION 34 62° 41.11S; 58° 1.18E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,175 | 0,092 | 1,90 |
| 20 | 0,078 | 0,105 | 0,51 |
| 30 | 0,158 | 0,104 | 1,05 |
| 50 | 0,090 | 0,110 | 0,56 |
| 75 | 0,073 | 0,202 | 0,25 |
| 100 | 0,096 | 0,317 | 0,21 |
| 150 | 0,054 | - | - |
| 200 | 0,054 | 0,105 | 0,35 |

STATION 36 62° 1.08S; 54° 53.86E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,103 | 0,108 | 0,66 |
| 20 | 0,059 | 0,127 | 0,32 |
| 30 | 0,097 | 0,120 | 0,56 |
| 50 | 0,105 | 0,110 | 0,66 |
| 75 | 0,066 | 0,121 | 0,75 |
| 100 | 0,124 | 0,095 | 0,89 |
| 150 | 0,078 | 0,165 | 0,33 |

STATION 38 63° 19.84S; 53° 56.48E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,091 | 0,099 | 0,63 |
| 20 | 0,081 | 0,255 | 0,22 |
| 30 | 0,086 | 0,173 | 0,34 |
| 50 | 0,101 | 0,116 | 0,60 |
| 75 | 0,084 | 0,097 | 0,63 |
| 100 | 0,094 | 0,238 | 0,28 |
| 150 | 0,065 | - | - |
| 200 | 0,058 | 0,087 | - |

STATION 40 64° 40.82S; 53° 58.35E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,044 | 0,123 | 0,25 |
| 20 | - | - | - |
| 30 | 0,057 | 0,129 | 0,30 |
| 50 | 0,090 | 0,088 | 0,70 |
| 75 | 0,045 | 0,135 | 0,23 |
| 100 | 0,031 | 0,096 | 0,22 |
| 200 | 0,021 | - | - |

STATION 42 64° 40.47S; 51° 55.71E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | - | - | - |
| 20 | 0,154 | 0,098 | 0,10 |
| 30 | - | - | - |
| 50 | 0,212 | 0,302 | 0,43 |
| 75 | 0,106 | - | - |
| 100 | 0,274 | 0,678 | 0,28 |
| 150 | 0,057 | - | - |
| 200 | - | - | - |

STATION 46 62° 0.60S; 52° 0.25E

| Depth(m) | Chlorophyll *a* | Chlorophyllide *a* | Molar Ratio |
|---|---|---|---|
| 0 | 0,063 | - | - |
| 20 | - | - | - |
| 30 | 0,091 | - | - |
| 50 | 0,029 | - | - |
| 75 | 0,170 | 0,908 | 0,02 |
| 100 | 0,051 | 0,327 | 0,36 |
| 150 | 0,055 | 0,114 | 0,31 |
| 200 | - | 0,891 | 0,04 |

"SIBEX I" Melted Pack Ice

| HPLC | | | SCOR-UNESCO | |
|---|---|---|---|---|
| Chlorophyll *a* | Chlide *a* | Mole Ratio | Chlorophyll *a* | Specific Productivity |
| 0,94 | 0,63 | 1,03 | 10,363 | 0,80 |

"SIBEX P" Water Between Ice Floes

| HPLC | | | SCOR-UNESCO | |
|---|---|---|---|---|
| Chlorophyll *a* | Chlide *a* | Mole Ratio | Chlorophyll *a* | Specific Productivity |
| 3,32 | 0,96 | 2,38 | 1,156 | ~2,24 |

Diatoms from Bongo Nets

| HPLC | | |
|---|---|---|
| Chlorophyll *a* | Chlorophyllide *a* | Mole Ratio |
| 0,40 | 1,33 | 0,27 |

All concentrations expressed in mg $m^{-3}$

Specific Productivity expressed in mgC/mgChl*a* $hr^{-1}$

# 4. DISCUSSION

# 4. DISCUSSION

## 4.A. Discussion of Methodology

The various procedural steps of sampling, filtration and extraction of the algal pigments are extremely important stages which influence the quality of the final pigment analysis.

Sampling: As discussed, the only problem was that samples for HPLC analysis were taken from a different cast to those for trichromatic analysis. The ideal situation for intercalibration of the two methods would be to take the samples from the same Niskin bottle. However, the Niskin bottle capacity is limited to eight litres, and if more than six litres of sea water is required for the trichromatic analysis the only way in which samples could be taken simultaneously would be to lower two bottles to the same depth.

Filtration: Whatman GFF filters were used, as these have been specified as the standard type of filter to be employed in algal pigment analysis (Marchant & Wright, in prep.). Magnesium carbonate treatment is not applied as it does not significantly improve the quality of the extract, and has been reported to adsorb pheopigments (Daley et al, 1973).

Extraction: 90% acetone is commonly used as an extraction solvent, but Marker (1972), has proposed methanol as more efficient. However, a chromatographic comparison by Mantoura & Llewellyn (1983), showed that during extraction into methanol, chlorophyllase activity was enhanced and formation of several chlorophyll a rearrangement products occurred. Hence, 90% acetone was used in all pigment extractions during this study. It was observed that the concentrations of the pigment extracts from the algal classes Chlorophyceae and Cyanophceae were generally lower than for other algal classes, as reported by Marker, (1972). Nusch, (1980) proposed the use of ethanol as an extraction solvent, as it is as efficient as methanol and chlorophyll a rearrangement products are not formed.

A chromatographic study of the extraction efficiency of ethanol, and its effect on the chlorophyllase enzyme, is presently underway.

Following Mantoura & Llewellyn (1983) all samples were placed in an ultrasonic bath for two hours. This is reported to be superior in yield and reproducibility to grinding of the cells (Wright & Shearer, 1984). However, Wright & Shearer suggest the use of an ultrasonic probe for 30 seconds, to extract the pigments with minimal formation of chlorophyll a allomerisation products.

Separation System: The major difference between the two types of chromatographic systems used is in the type of gradient forming system. (See page 18).

In low pressure mixing systems, the time for a change in gradient, initiated in the mixing chamber, to reach the head of column will be three times longer than in the high pressure mixing system. This allows more subtle control of changes in the mobile phase when using the high pressure mixing system and consequently produces better resolution of the initial peaks, which can be seen on comparison of chromatograms from both systems (Figs.A.11 and A.13., page 149 and 151).

Detection Systems: The detection system used determines the quality of the information provided by the chromatogram. The dual-channel detection system allowed identification of several peaks, because it was possible to detect peaks in a sample at two different wavelengths simultaneously. It is difficult for small differences in absorbance at different wavelengths to be measured in consecutive runs, as reproducible injections cannot always be made, and even if they are, the solvent (acetone) tends to evaporate slowly, meaning that the sample concentration tends to increase slightly during a long series of runs.

A fluorescence detector would allow better quantification of the chlorophyll components because of its greater sensitivity. Detection limits for chlorophyll a, using a spectrophotometric detector are 0,1 $\mu g l^{-1}$ and using a fluorescence detector are 0,1 $ng l^{-1}$, after preconcentration (Mantoura & Llewellyn, 1983).

Many chlorophyll breakdown products - the pheophytins and pheophorbides - were not detected in our chromatograms, as the spectrophotometric detector was not sufficiently sensitive. A fluorescence detector is required for the quantification of these pigments, and will allow better evaluation of ratios of the various chlorophyll breakdown products.

Preconcentration System: More accurate quantification would result from elution of the concentrate into a graduated vessel, so that precisely 1,0 ml or 0,5 ml could be eluted. Use of a more sensitive detection system would allow elution of 1,0 ml of concentrate, providing sufficient sample for triplicate analyses to be performed,

allowing statistical analysis of the reproducibility of the injections and the error incorporated in this method.

## 4.B. Discussion of Results of Laboratory-based Experiments

The major advantages of the immediate information provided by this method of pigment analysis are:

1. An accurate measure of viable or "active" chlorophyll a is made. It has been reported that the trichromatic method over-estimates the amount of chlorophyll a in marine systems by as much as 75% (Mantoura & Llewellyn, 1983).

2. The chlorophyllide a content of the system can be measured.

3. The sampling volume in marine waters of low productivity can be reduced from 5 litres (necessary for SCOR-UNESCO analyses) to 2 litres, due to the 4000-fold preconcentration method using SEP-PAK® cartridges.

4. The correction factor, employed in spectrophotometric measurements and estimated by measuring absorbance at 750nm, need not be applied in the HPLC determination of chlorophyll a. This factor is used to partially correct for sample turbidity, which arises from cell matter

present in the acetone solution, which has not been removed by the centrifugation process (Strickland & Parsons, (1972). This is because all samples are micro-filtered before injection onto the HPLC, and all suspended matter removed, to prevent blockage of the column and pressure tubing.

5. The chromatogram provides an exact representation of the chlorophyll and carotenoid composition of a sample. Pigment composition, together with the cytology and ultra-structure, and biochemical criteria are used to classify the algae into their respective classes and species (Liaaen-Jensen, 1977). Hence the use of HPLC for pigment analysis has great potential as a chemotaxonomic tool, as it is possible to identify key class-specific pigments in the algae (Mantoura & Llewellyn, 1983). However, a system providing high resolution is essential as shown by comparison of the chromatograms produced by the two different types of gradient forming systems.

## 4.C. Discussion of SIBEX Results

1. It was expected that the HPLC chlorophyll *a* values would be lower than the trichromatic chlorophyll *a* values, because of the differentiation between chlorophyll *a* and chlorophyllide *a* possible by the chromatographic method. However, HPLC chlorophyll *a* values were sometimes higher than the SCOR-UNESCO chlorophyll *a* values. An inter-calibration using pure standard chlorophyll *a* showed estimates of chlorophyll *a* by the two methods to be in good agreement. Further tests, using pure algal cultures, will be conducted in order to ascertain whether the sum of individual absorbances of chlorophyll *a* and chlorophyllide *a*, as measured by the HPLC method, are equivalent to the total absorbance due to these compounds, as measured by the trichromatic method.

2. In some instances anomalously high productivity values were recorded at the lower levels of the euphotic zone, which could not be equated to apparent levels of chlorophyll *a* present (Allanson, pers. comm.). It is thought that these may be related to the presence of accessory pigments which enhance the absorption of shorter wavelength radiation, which penetrates to these depths (Shimura & Fujita, 1975). Fucoxanthin is such a pigment and its presence is suspected in most samples. As yet, the peak due to fucoxanthin has not been positively

identified in our chromatograms. However, once this has been achieved, together with the identification of several other accessory pigments, a series of studies on chromatic adaption of algae to different levels in and below the euphotic zone are planned.

3. The apparent correlation between the Mole Ratio values and the Primary Productivity values opens another area of research which may help to provide more specific information concerning the phytoplankton processes and the physiological state of the bloom.

## 4.D Other Applications of This Technique and, Proposed Fields for Further Investigation

This technique may be used to elucidate other aspects of the response of the phytoplankton pigment complement to stress factors in the marine ecosystem. An experimental model will be set up to study the effect of bacterial grazing and enzymatic digestion of the phytoplankton on their pigment structure.The variation of pigment ratios during the different growth phases of synchronous cultures will also be studied (Jensen & Sakshung, 1973).

HPLC analysis has been used to observe chemical changes occurring in vertically sinking organic matter (Repeta & Gargosian, 1982 and Komar et al, 1981). Carotenoids being

labile and source specific, are suitable for use as organic tracers to identify short term degradation processes and to investigate the role of rapid transformation processes in carbon and nitrogen cycling and benthic nutrition. HPLC does not cause degradation of the pigments during analysis - hence the products observed are known to be representative of species in the natural environment and not artefacts of the analytical procedure.

Phytoplankton xanthophylls in the gut content of copepods have been analysed by HPLC (Kleppel & Pieper, 1984). The chlorophyll and pheophytin content is measured simultaneously, using fluorometric techniques. The author proposes that the xanthophylls are associated with recently ingested biomass, as the pheophytin/chlorophyll ratios are low. However, because fluorometric techniques were used in the chlorophyll analysis, no account has been taken of the chlorophyllide a present. Analysing all pigments by HPLC will allow distinction between chlorophyll a and the chlorophyllide a, and may lead to a different conclusion.

HPLC pigment analysis is becoming increasingly widely recognised and used. However, much work is still required in order to establish and standardise optimum sampling, storage and extraction conditions.

# 5. CONCLUSION

# 5 CONCLUSION

A system that separates the carotenoid and chlorophyll pigments extracted from *in vitro* cultures of marine algal species has been developed. Positive identification of peaks due to chlorophyll *a*, chlorophyll *b*, chlorophyll *c*, pheophytin *a*, pheophytin *b*, chlorophyllide *a* and ß-carotene has been achieved. The other peaks have been assigned to pigments according to their spectral characteristics, special distribution and order of elution. The method was extended to analysis of natural sea water samples, for which a preconcentration technique was developed to allow measurement of the low chlorophyll concentrations characteristic to the Southern Ocean and small sample volumes (~2000 ml).

Work is in progress to determine whether ethanol is a more efficient extraction solvent than acetone, and to establish the period of time for which samples may be stored in liquid nitrogen without decomposition occurring. The problem of column blockage during natural sample analysis is to be studied. Use of a guard-column will be adopted as a standard procedure, and a methanol-acetone solvent system developed. It is postulated that chlorophyllide *a* polymerisation is responsible for blockage of the column, and will not occur as readily in the absence of water.

Chromatographic pigment analysis is shown to provide an accurate measure of viable chlorophyll *a* concentrations which is not possible using the SCOR-UNESCO trichromatic method. HPLC analysis of natural samples has shown that chlorophyllide *a*, not pheophytin *a*, is the major chlorophyll *a* breakdown product. Hence all chlorophyll *a* values measured by the SCOR-UNESCO method have been overestimated by a factor related to the amount of chlorophyllide *a* present. It is seen that even in highly productive systems, such as the ice-bound algae, there is a significant amount of chlorophyllide *a* present. Anomalous "chlorophyll *a*" maxima at the lower regions of the euphotic zone can also be attributed to measurement of chlorophyllide *a* present as active chlorophyll *a*. Hence the "acid-ratio" test, used to distinguish chlorophyll *a* from pheophytin *a* in SCOR-UNESCO chlorophyll measurements does not yield any significant information concerning the physiological state of the algal bloom: the chlorophyll *a*/chlorophyllide *a* ratio appears to give a better indication of this.

The chromatogram also provides details of the carotenoid content of the algal population which allows identification of the major algal classes present. For such work it is essential to use a system which provides high resolution - ie a high pressure gradient former. In addition, this technique may be applied to the study of

phytoplankton processes, such as the role of auxilliary pigments in photosynthetic mechanisms, and the effect of zooplankton grazing on the algal pigment complement.

This thesis has conclusively shown that HPLC analysis of marine algal photosynthetic pigments provides quantitative and qualitative knowledge of the pigment structure. This should allow more accurate estimation of productive biomass than any other method. In addition, as it is primarily a separation technique it has many potential applications in studies of the dynamic processes occurring in marine ecosystems, which have until now, been confounded by the variety and complexity of organic species present.

# APPENDIX

# A. APPENDIX

## A.1. Development of a Separation System of the Standards, Chlorophyll a, Chlorophyll b, Pheophytin a and Pheophytin b

An isocratic run with 100% methanol was chosen for initial trials since the pigments are soluble in methanol, and it is compatible with both the column and the detector.

The wavelength chosen for detection of the compounds was 665nm, the wavelength of maximum absorbance of pure chlorophyll a in 90% Acetone.

Initial work on the liquid chromatograph focussed on elimination of excess baseline drift. A possible reason for this drift was precipitation of a water-soluble component in the detector flow cell during elution. The detector uses a deuterium discharge lamp which emits radiation in the range from 190-600nm. The intensity of this emission decreases dramatically above 400nm, and the wavelength of 665nm is near the upper detection limit of the photomultiplier tube, leading to increased signal noise and drift (Orren, pers. comm.). Flushing the column and flow cell with water at regular intervals alleviated the precipitation problem, but to avoid spectral problems, it was decided to use a wavelength of 435nm. This is the

λmax of the Soret band of chlorophyll a. The baseline was stable at this wavelength, and the molar absorption coefficients of chlorophylls b and c and pheophytins a and b are higher in this region thus increasing the sensitivity of the detector. Use of this wavelength also has the advantage that the carotenoids may be detected simultaneously.

Once a stable baseline was established, it was possible to develop a solvent system that would achieve resolution of the four standards in the shortest possible time. The available solvents, and their relative polarities, according to the Waters©Polarity Index were:

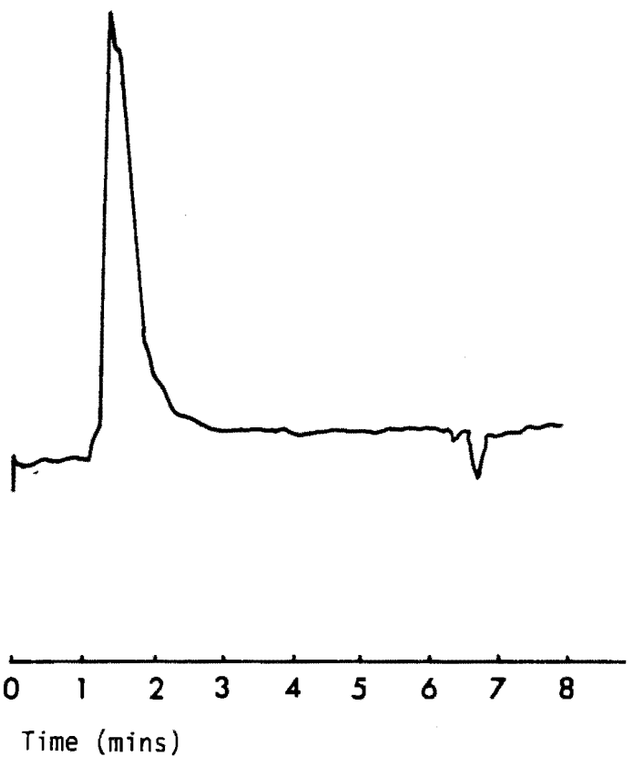
| Solvent | Polarity |
|---|---|
| Water | 9,0 |
| Methanol | 6,6 |
| Acetone | 6,2 |
| Acetonitrile | 5,4 |

In 100% methanol elution only chlorophylls a and b were eluted. The pheophytins were retained in the stationary phase, since methanol is too polar a solvent (Fig A.1.).

In 100% acetone the pigments were eluted very quickly but were unresolved (Fig A.2.). No pigments were eluted with 100% acetonitrile - suspected contamination of this solvent with water raised its polarity to give anomalously high retention times.

Fig A.1. chlorophyll *a* (peak 2) and chlorophyll *b* (peak 1) eluted in 100% MeOH


Fig A.2. All pigments co-eluted in 100% Acetone



These results indicated that the ideal range of polarity for resolution of the pigments could be attained by using a combination of methanol and acetone.

Isocratic runs of methanol:acetone ($^{v}/v$) solvents were investigated:

(i) 50:50 methanol:acetone

(ii) 80:20 methanol:acetone

(iii) 90:10 methanol:acetone

at flow rates (a) 1 ml $min^{-1}$

(b) 2 ml $min^{-1}$

The higher flow rate gives reduced retention times. Column theory predicts a loss of efficiency at higher flow rates, but in this range (for a 2,6mm, internal diameter column, with the optimum flow rates 0,5 - 2 ml $min^{-1}$) the van Deemter curve is almost horizontal, so only marginal loss in efficiency accompanies increasing flow rate (Hamilton & Sewell, 1982).

From the chromatograms arising from the various runs, it was seen that the chlorophylls a and b were resolved in 70:30 methanol:acetone at a flow rate of 1 ml $min^{-1}$.

Pheophytins a and b were resolved in 80:20 methanol:acetone at a flow rate of 2 ml $min^{-1}$. These were combined to form a gradient elution (Table A.1.), which successfully resolved all four standards (Fig. A.3.).

Table A.1.

| Time (mins) | Solvent Composition | Flow Rate ($mlmin^{-1}$) |
|---|---|---|
| 0 | 70:30 MeOH:Acetone | 1 |
| 3 | 80:20 MeOH:Acetone | 2 |
| 4,7 | 80:20 MeOH:Acetone | 2 |

## A.2. Development of Separation System for an in vitro Pigment Extract

Pigments from an *in vitro* culture of *Dunaliella primolecta* were extracted as described and eluted using the gradient developed to separate the standard chlorophylls (Table A.1.). Several unresolved peaks occurred before chlorophylls *a* and *b* (Fig. A.4.), and so a different gradient system had to be developed, to resolve these peaks.

Four solvent systems were tested:

1. 100% Methanol at flow rates 0,5 ml $min^{-1}$, 1 ml $min^{-1}$ and 2 ml $min^{-1}$. Adequate resolution was only achieved at a flow rate of 0,5 ml $min^{-1}$, but retention times were too long.

2. Methanol:Water (Fig. A.5.) This system was investigated to establish whether resolution of the new peaks could be achieved by addition of a more polar solvent: because these peaks were eluted before chlorophyll *a* they must be relatively more polar. They were expected to be chlorophyllide and pheophorbide *a*, chlorophyll *c* and some carotenoids.

Combinations tried were: (i) 98:2 Methanol:Water
(ii) 95:5 Methanol:Water
(iii) 90:10 Methanol:Water

Fig A.3. The final gradient, giving resolution of the four standards: chlorophyll *a* (peak 2), chlorophyll *b* (peak1), pheophytin *a* (peak 4) and pheophytin *b* (peak 3).

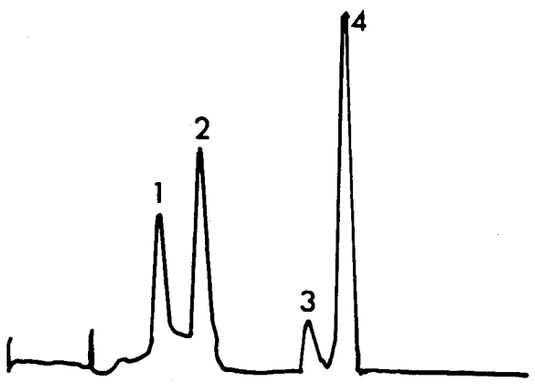



Fig A.4. The pigments extracted from *Dunaliella primolecta* eluted under the gradient in which the four standards are resolved.

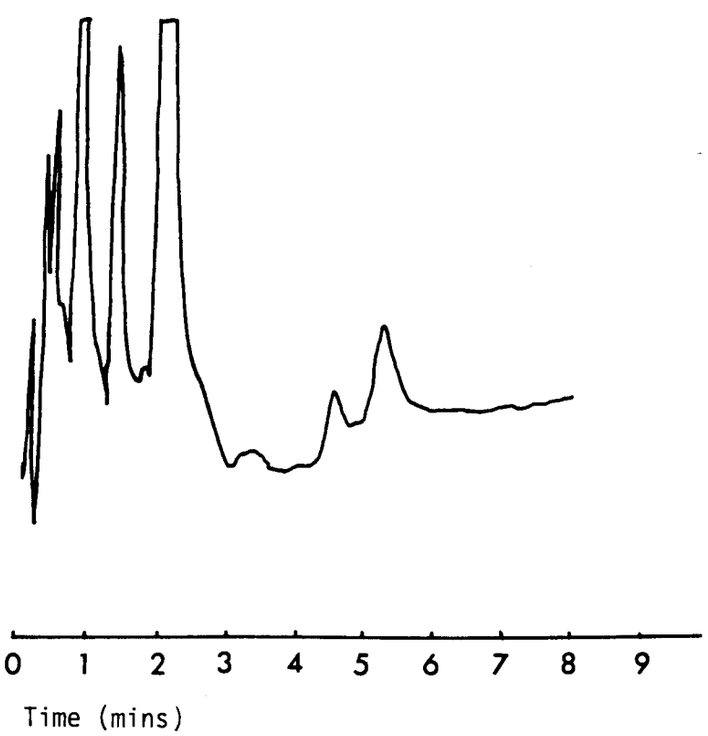
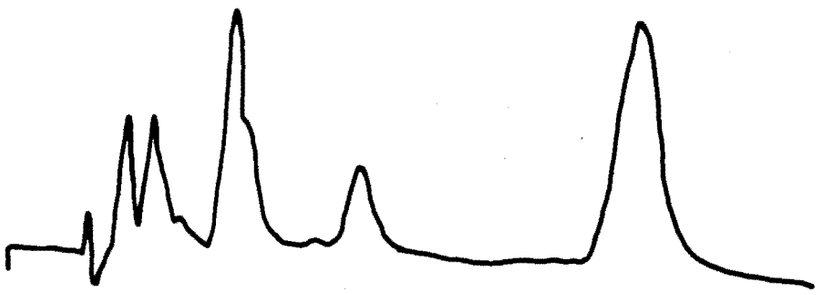
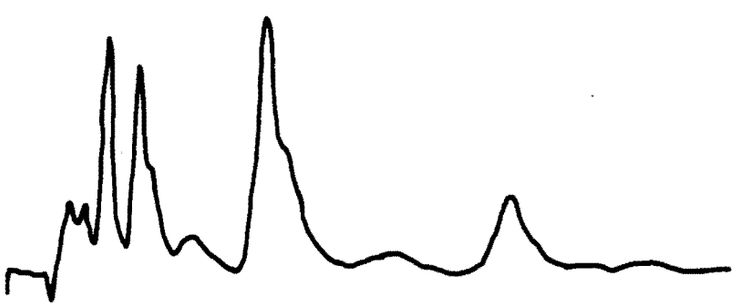


Fig A.5. Methanol: water solvent systems

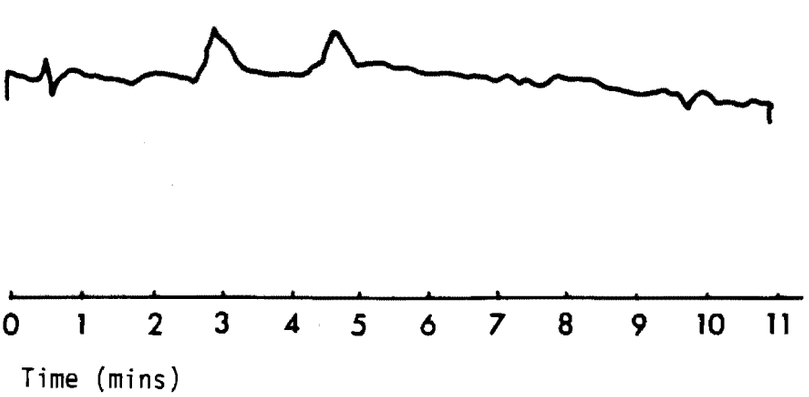
(i) MeOH: $H_2O$ 98:2



(ii) MeOH: $H_2O$ 95:5



(iii) MeOH: $H_2O$ 90:10



All combinations of solvent were too polar to allow elution of any peaks after chlorophyll b.

In (i) the initial peaks were eluted too close to the unretained peak. Resolution of these peaks was achieved in (ii), but the retention times were too long to be of practical use. Increasing the percentage of water to 10% caused distortion of all the peaks. This is because there is a positive heat of mixing, and a high volume contraction when methanol and water mix, giving rise to a solution of very high viscosity. This anomaly is observed as a rise in column back pressure, due to the high viscosities.

3. Methanol:Acetone (Fig. A.6.)

(i) 98:2 Methanol:Acetone Flow rate = 0,5 ml $min^{-1}$

(ii) 90:10 Methanol:Acetone Flow rate = 1 ml $min^{-1}$

(iii) 95:5 Methanol:Acetone Flow rate = 1 ml $min^{-1}$

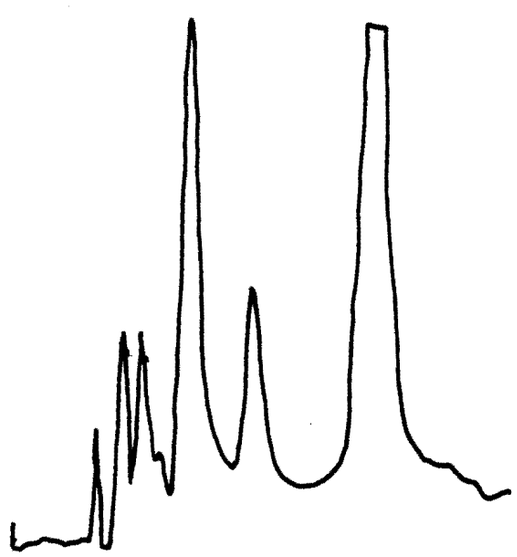
In (i) the components were resolved, but retention times were too long, due to the low flow rate.

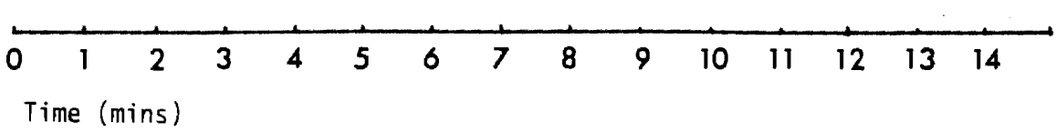
In (ii) and (iii) all peaks were eluted close to the unretained peak, meaning that these solvents were not sufficiently polar. As methanol:water solutions were too viscous, an acetone:water system was investigated.

Fig A.6. Methanol: acetone solutions

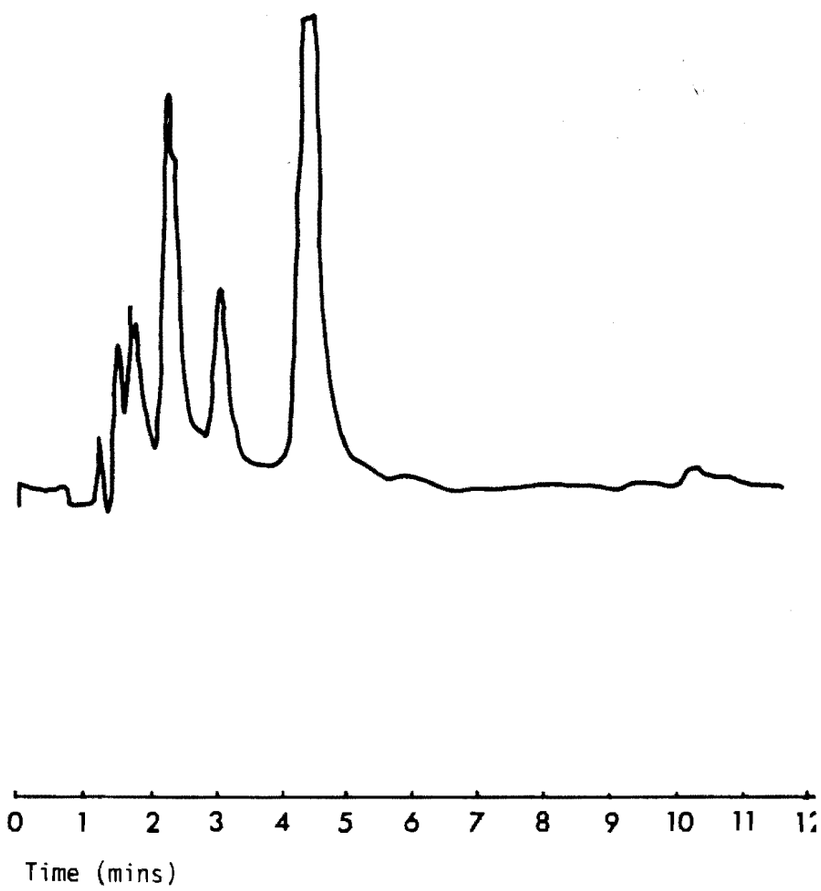
(i) MeOH: Ac=0 98:2

(ii) MeOH: Ac=0 95:5





(iii) MeOH: Ac=0 90:10



4. Acetone:Water (Fig. A.7.). Because the relative polarities of acetone and water are so different, varying compositions will offer a wide range of solvent polarities. Combinations investigated were:

(i) 75:25 Acetone:Water Flow rate 1 ml $min^{-1}$

(ii) 80:20 Acetone:Water Flow rate 2 ml $min^{-1}$

(iii) 90:10 Acetone:Water Flow rate 2 ml $min^{-1}$

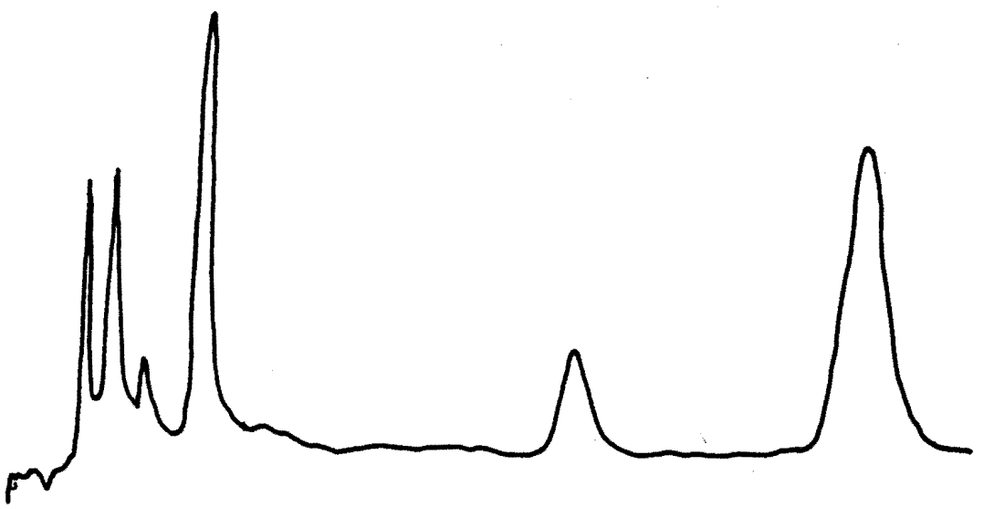
No satisfactory result was achieved. It was clear that an isocratic solvent system would not achieve resolution of the peaks in a suitable time.

Two gradient systems were tested (Fig. A.8.). Both gradient systems produced chromatograms in which the initial peaks were well resolved, but the final peaks not sufficiently resolved.

A "convex" gradient system will theoretically solve this problem - ie a gradient in which the rate of change of polarity is faster at the beginning of the chromatogram and slower at the end. Some resolution may be lost initially, but because the change in polarity is more subtle towards the end of the chromatogram, resolution of components with only slight differences in relative polarity is possible.

Fig A.7. Acetone: water solutions

( i) Ac=0: $H_2O$ 80:20



(ii ) Ac=0: $H_2O$ 90:10

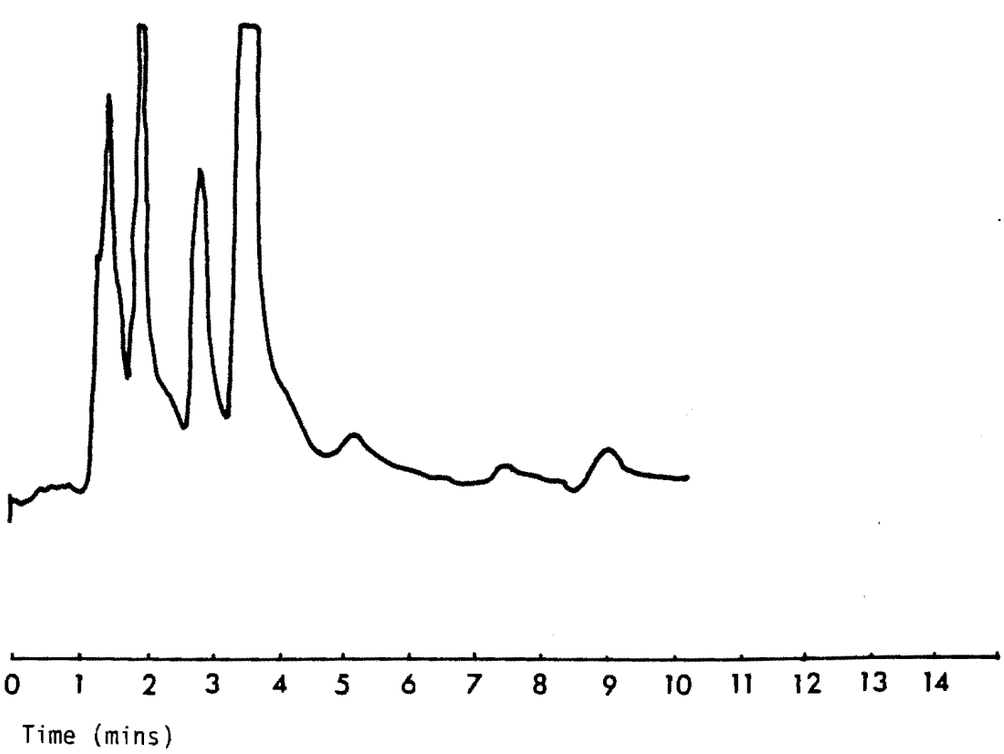
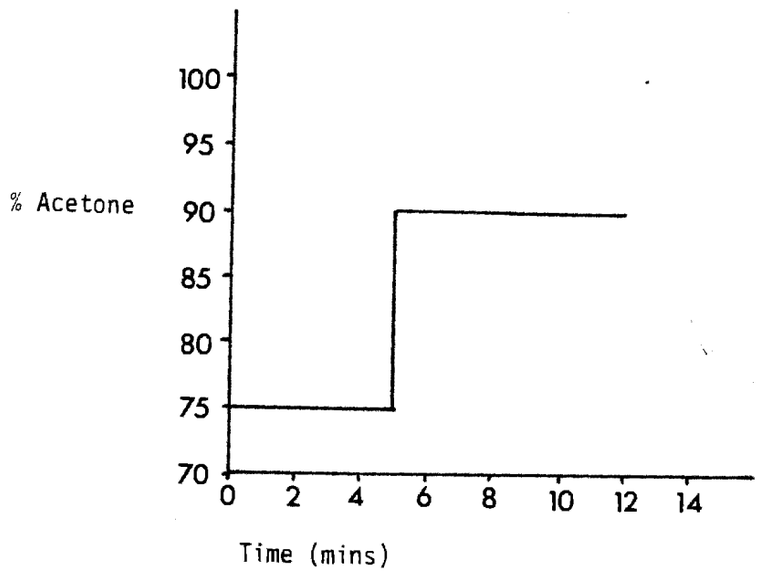
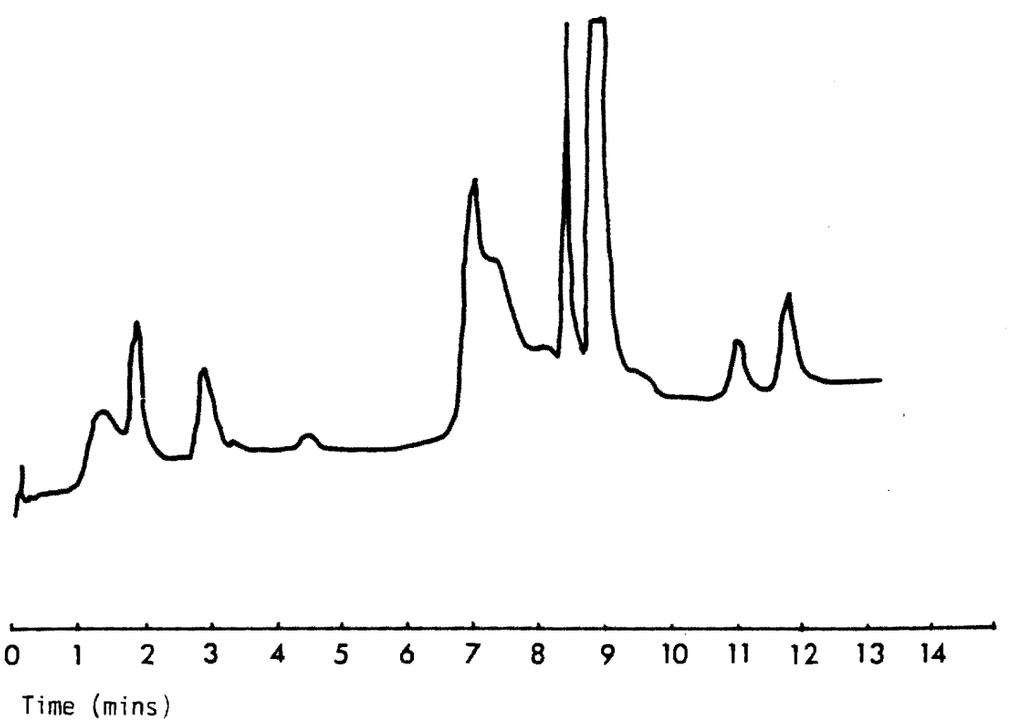


Fig A.8. Acetone: water gradient systems.

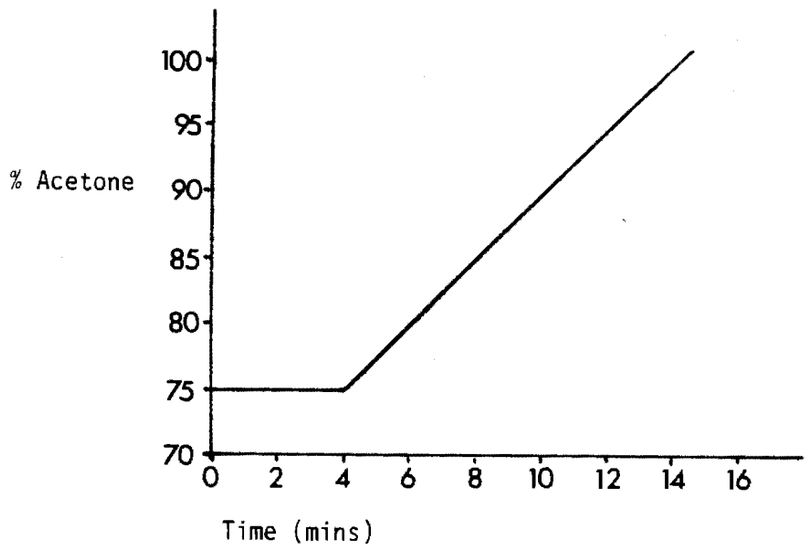
(i) Graph showing solvent gradient I




(ii) Resolution achieved by gradient I



(iii) Graph showing solvent gradient II




(iv) Resolution achieved by gradient II

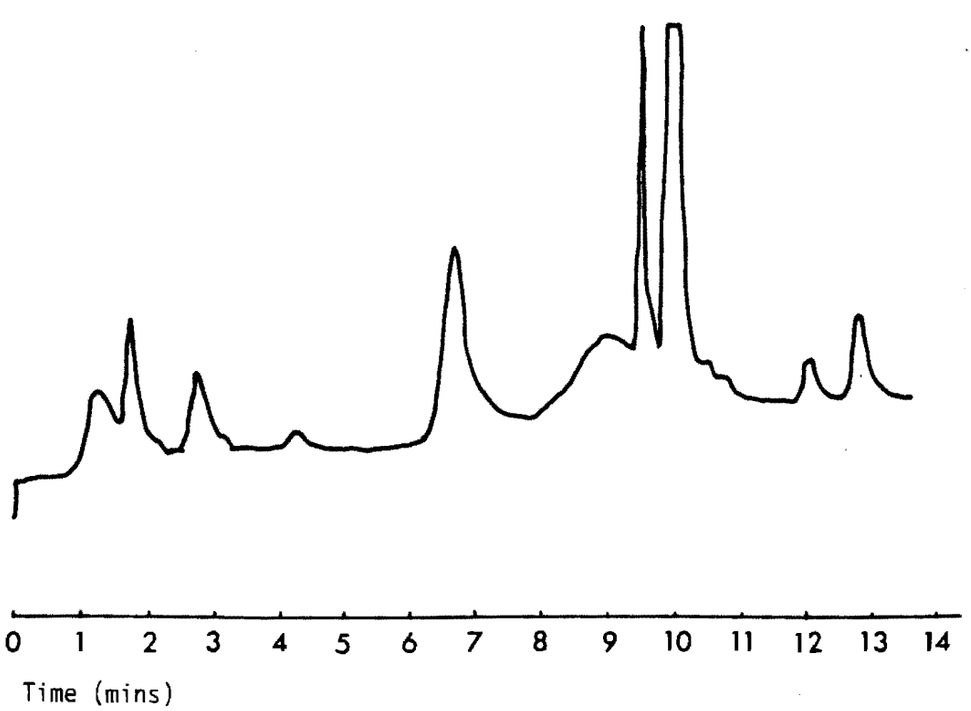
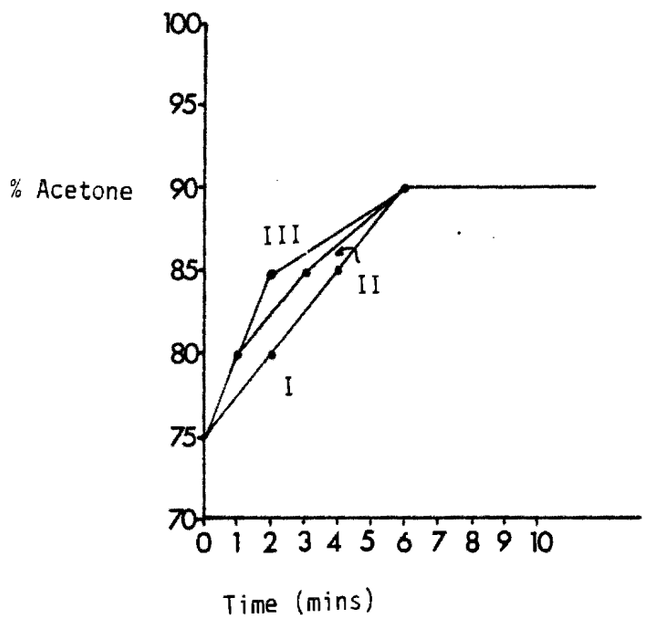
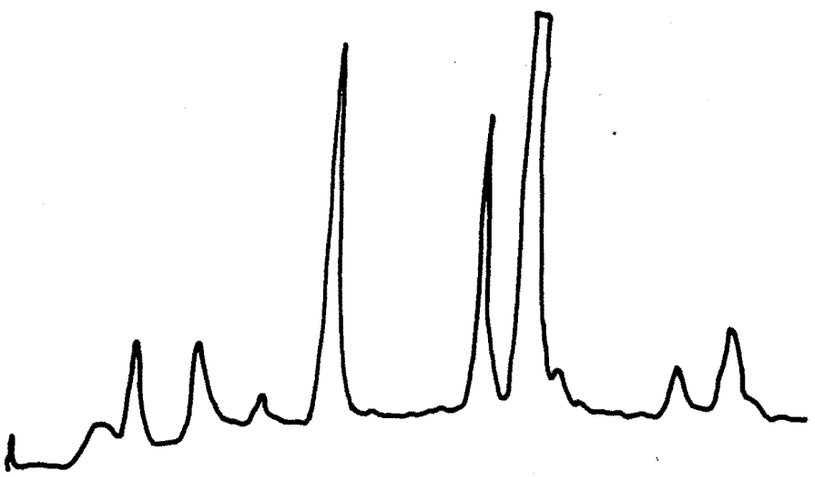


Fig A.9. Convex Gradient Systems

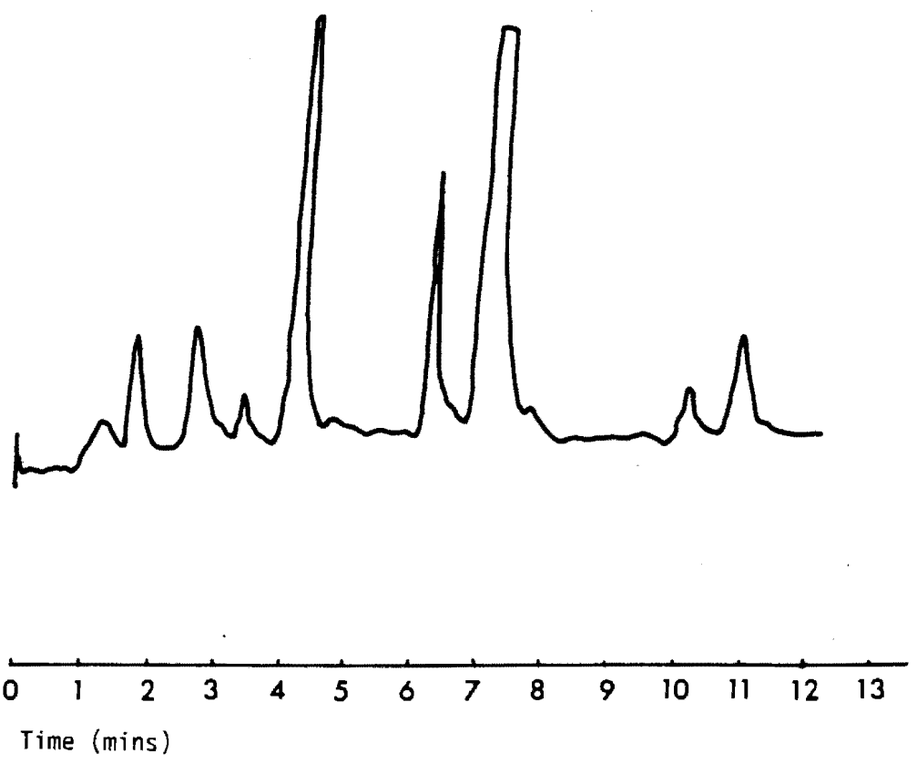
(i) Graph showing convex solvent gradients I, II, III



(ii) Resolution achieved by gradient I



(iii) Resolution achieved by gradient II



(iv) Resolution achieved by gradient III

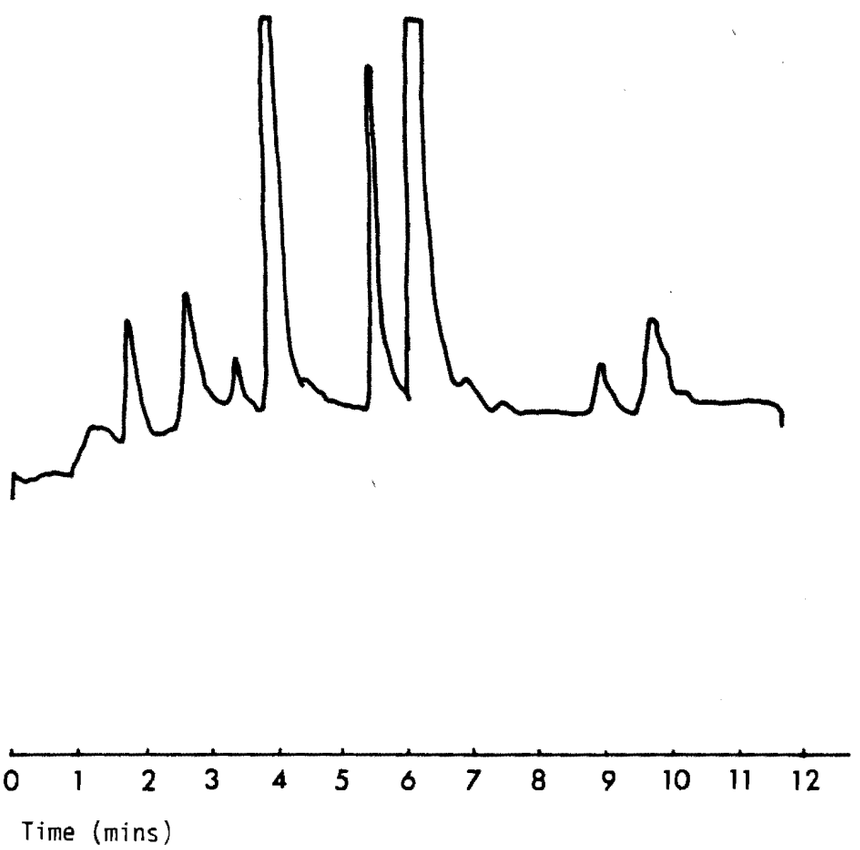
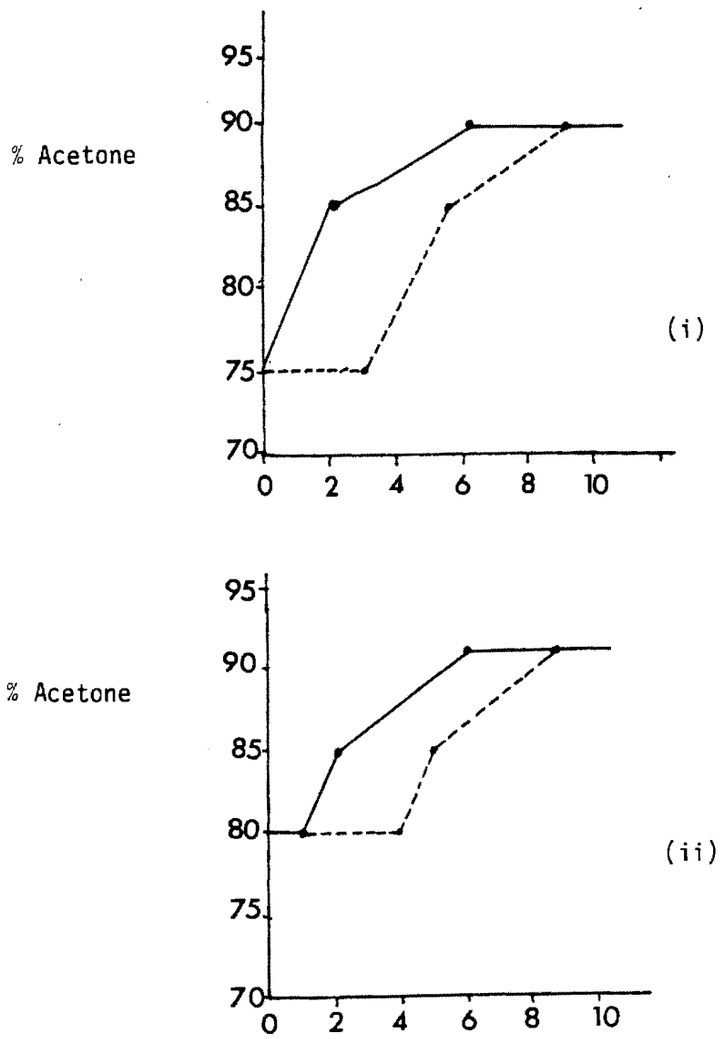


Fig A.10.(i) Graph showing the difference between the programmed solvent gradient and the actual solvent gradient

Programmed gradient: ————

Actual gradient: ---------

Fig A.10.(ii) Graph showing that starting programmed gradient at a higher solvent strength and holding for these conditions for one minute approaches more closely the desired change in actual solvent strength



Three convex gradient systems were tested (Fig. A.9.). The overall resolution was improved, but retention times were still too long. The Varian chromatograph has a low pressure mixing system so there is a time lag of three minutes from the initiation of the solvent change by microprocessor and the time the new solvent composition reaches the top of the column. Consequently, the first three minutes of a run will be unaffected by a gradient (Fig. A.10.(i)) and resolution of initial peaks is more difficult to control.

One method of overcoming this problem, and of approaching the theoretical gradient as closely as possible, is to increase the concentration of the non-polar solvent and hold these conditions for the first minute. (Fig. A.10(ii)).

Several gradients were tested, and the one which achieved the best resolution of the pigments in the shortest time selected. (Fig. A.11.).

A slightly different gradient system was developed for elution of the pigments on the Beckman HPLC. This instrument has a high pressure mixing system allowing better control of initial gradient changes (Table A.3).

## A.3 Preparation of the Pheophytin Standards

Pheophytin *a* and pheophytin *b* standards were prepared by allowing a solution of the respective chlorophyll to stand for 20 minutes, after acidification with two drops of 1 *M* nitric acid. This ensures complete conversion of the parent chlorophyll to the pheophytin - ie replacement of the magnesium ion in the porphyrin ring by two protons (Holden, 1976). However, these acidified samples cannot be injected directly onto the column, because the silanol bonds between the carbon chains and the silica beads of the column packing are destroyed at such low pH values. If such a sample was used to spike an algal extract, in order to identify the pheophytin peak, the excess protons would cause degradation of sample chlorophylls and chlorophyllides, altering the natural pigment composition. These protons can be removed by the method described, using a C-18 Waters SEP-PAK® cartridge: the cartridge is wet with water, a polar solvent. The acidified pheophytin in 90% acetone is then injected onto the cartridge. The pheophytin remains preferentially on the non-polar C-18 stationary phase, rather than the polar mobile phase. The excess protons however can be solvated by the polar water molecules and are removed by flushing the cartridge with 10 ml water. The pheophytins are finally eluted from the cartridge in acetone, and may be injected onto the column in this form.

Fig A.11. (i) Final gradient system chosen

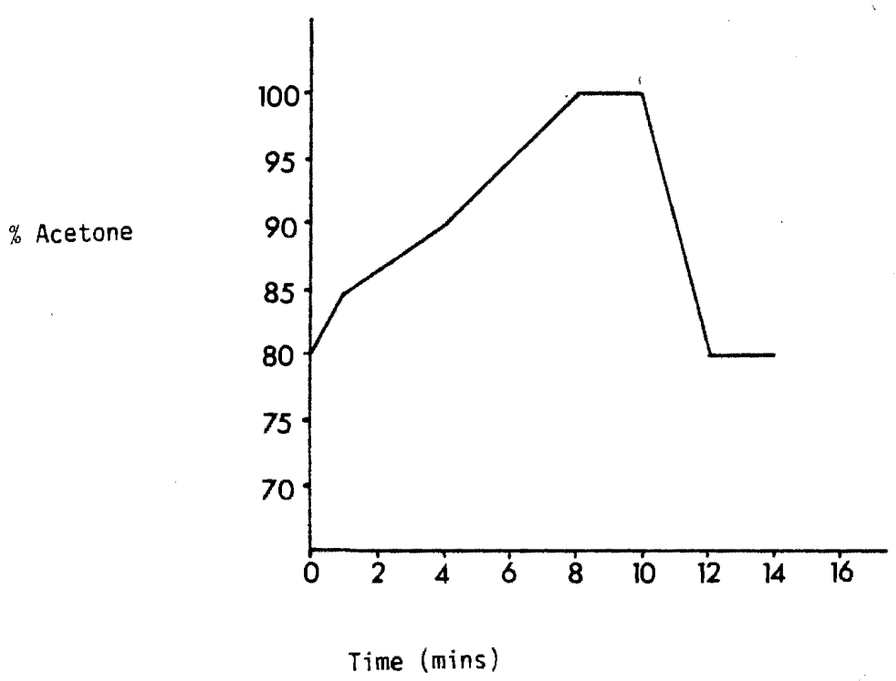


Fig A.11. (ii) The optimum resolution of the pigments of *Dunaliella primolecta*

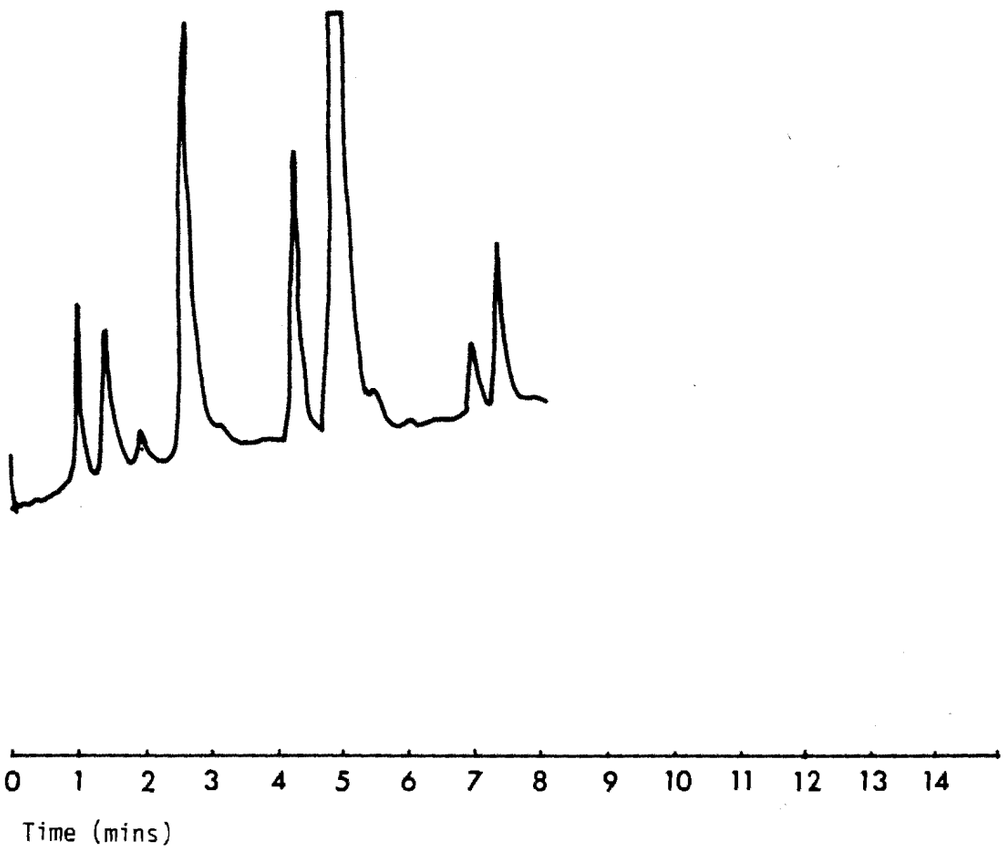


Table A.3. See Fig. A.12. and A.13.

Program controlling gradient for pigment elution on BECKMAN HPLC

| Time | Function | Value | Duration |
|---|---|---|---|
| 0,00 | Flow rate | 0,5mlmin$^{-1}$ | |
| 0,00 | %B | 18% | 2 mins |
| 2,00 | %B | 15% | 1 min |
| 2,00 | Flow rate | 2mlmin$^{-1}$ | |
| 3,00 | %B | 10% | 1 min |
| 4,00 | %B | 0% | 2 mins |
| 8,00 | %B | 20% | 2 mins |
| 13,00 | Alarm | | |

Initial Conditions %B = 20

Reservoir B = Water

Reservoir A = Acetone

Flow Rate = Flow rate of mobile phase (mlmin$^{-1}$)

A slightly different gradient system was developed for elution of pigments on the Beckman HPLC. This has a high pressure mixing system and allows more subtle control of elution of initial peaks.

Fig A.12. Gradient elution programme for Beckman HPLC

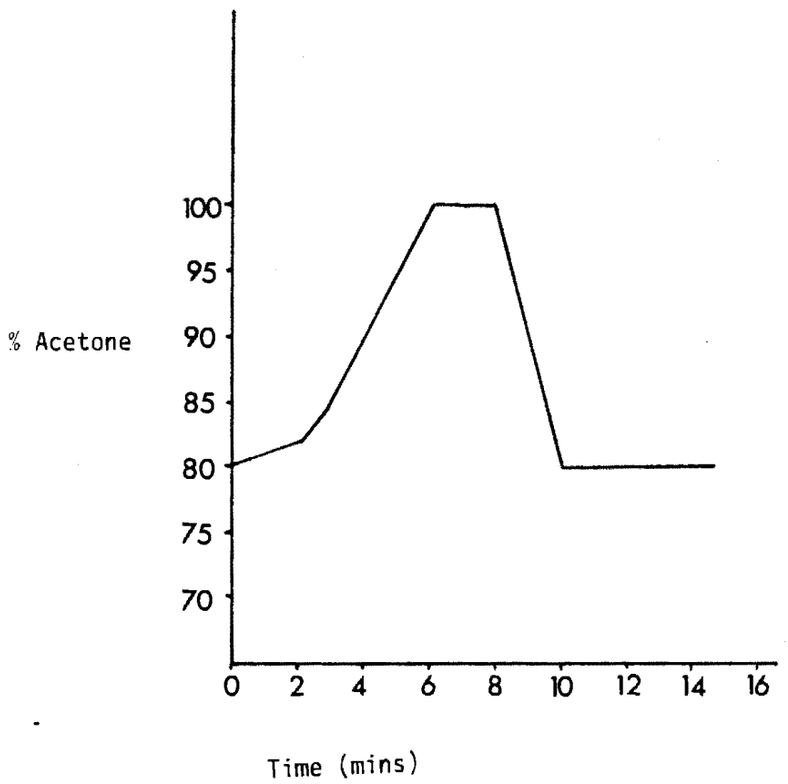
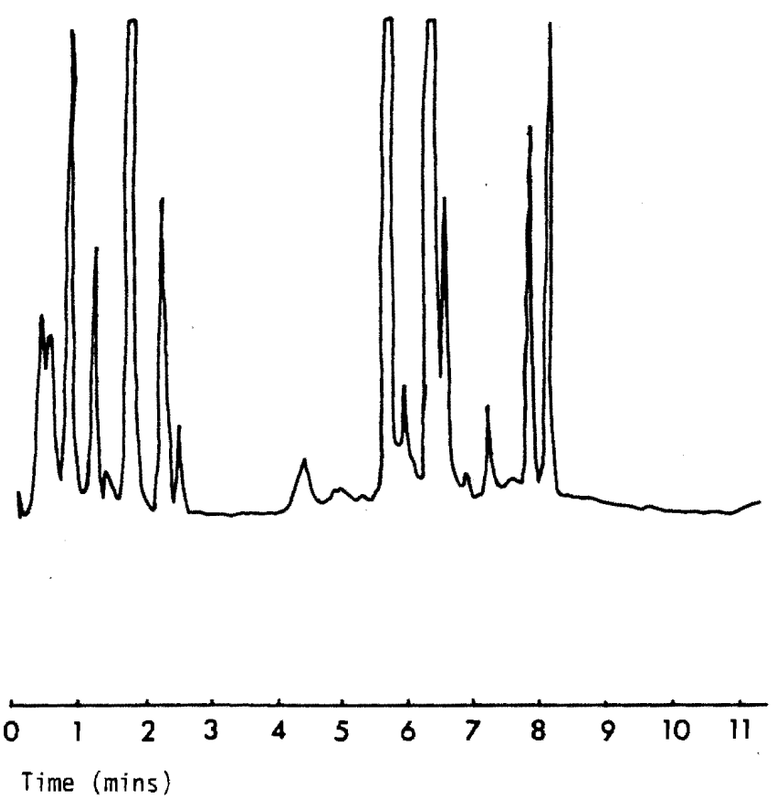


Fig A.13.Optimum resolution of *Dunaliella primolecta* pigments on the Beckman HPLC



## A.4. Preconcentration Procedure, using Waters SEP-PAK®C-18 Cartridges

The cartridge is wet with water and the sample, in 10 ml 90% acetone, injected through a 0,5 m Millex-HV®filter onto the cartridge. The pigments present in the 10 ml 90% acetone are retained on the stationary phase. They are finally eluted in 0,5 ml acetone, and this concentrate injected onto the column. Pigments from two litres of seawater are concentrated into 0,5 ml acetone effecting a 4000-fold concentration (After Eskins & Dutton, 1979).

## A.5. Spectrophotometric Calibration

A scan of each standard was made on the Varian Spectrascan II, between 350 and 750 nm, using 90% acetone as a blank. Glass cuvettes of pathlength 0,01 m were used. The molar absorbance coefficients of the standard solutions could be calculated from the absorbance values at the wavelength of maximum absorbance. The observed specific extinction coefficient at 665 nm was 98,9 and at 435 nm was 114.

## A.6. Spectroscopic Study of the Standards

A spectroscopic scan of each of the standards chlorophyll *a*, chlorophyll *b*, pheophytin *a* and pheophytin *b* was made, in order to check their purity, and that their observed absorbance maxima agreed with values reported (Jeffrey & Lorenzen, 1980). Chlorophyll *a* and *b* standards were made up to a concentration of approximately 10mg $l^{-1}$ in 90% acetone, and pheophytin standards prepared from these, as described (Appendix A.3.).

Results: The characteristic spectra for all standards were observed: See Fig. A.14. (Vernon, 1960; Jeffrey, 1972; Jeffrey & Humphrey, 1975 and Jeffrey & Lorenzen, 1980)

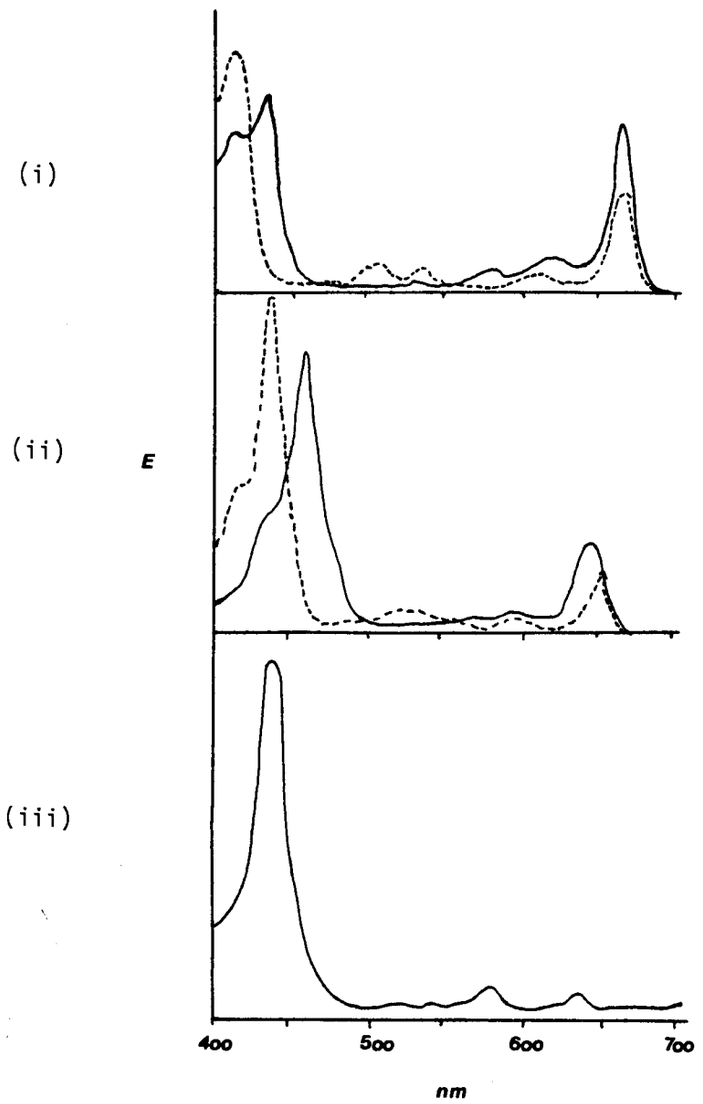



Fig A.14. Absorbtion spectra of:

(i) Chlorophyll *a* —— and pheophytin *a* ---

(ii) Chlorophyll *b* —— and pheophytin *b* ---

(iii) Chlorophyll *c* ——

in 90% Acetone

Table A.2. Table of Extinction Coefficients in 90% Acetone

| Pigment | M.W. | (nm) | 2m x 10-$^{4}$ | Eα. |
|---|---|---|---|---|
| Chl a | 893,4 | 435 | 6,7 | |
| | | 665 | 7,8 | 87,67 |
| Chl b | 907,46 | 453 | 9,8 | |
| | | 647 | 3,2 | 51,36 |
| Phe a | 869,16 | 410 | 8,5 | |
| | | 665 | 3,4 | 51,2 |
| Phe b | 883,15 | 434 | 6,4 | |
| | | 643 | 2,3 | |
| Chlide a | 614,97 | 665 | | 127 |
| Chl c | 611 | 443 | 19,4 | 318 |
| | | 630 | | 44,8 |
| Chl $c_2$ | 609 | 443,8 | 22,8 | 374 |
| | | 630,9 | | 40,4 |

Em = Molar Extinction Coefficient

Eα = Specific Extinction Coefficient

(Vernon, 1960; Jeffrey, 1972; Jeffrey & Humphrey, 1975 and Jeffrey & Lorenzen, 1980)

KEY: Chl = Chlorophyll Phe = Pheophytin Chlide = Chlorophyllide

A7. Study of the Reproducilibity of the Varian Liquid Chromatograph

Nine consecutive injections of a chlorophyll a standard were made, and the peak areas,at 435nm, were estimated by the HP 3390A Integrator. The areas obtained were:

| | |
|---|---|
| | 189 140 |
| | 183 960 |
| | 186 400 |
| | 192 960 |
| | 197 220 |
| | 193 620 |
| | 194 000 |
| | 199 930 |
| | 195 250 |
| Mean | 192 497,8 |
| S | 5 125,8 |
| RSD | 2,6% |

A8. Study of Reproducibility of the Beckman Liquid Chromatograph

Five consecutive injections of a chlorophyll a standard were made, and the peak areas at 435 nm estimated by the HP 3390A Integrator. The areas obtained were:

| | |
|---|---|
| | 36 936 |
| | 39 033 |
| | 37 335 |
| | 37 426 |
| | 36 968 |
| Mean | 37 539 |
| S | 862,6 |
| RSD | 2,3% |

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