BBA 45709

QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF CHLOROPHYLLS AND CAROTENOIDS FROM MARINE ALGAE

S. W. JEFFREY

C.S.I.R.O. Marine Laboratory, Box 21, Cronulla, Sydney 2230 (Australia)

(Received April 8th, 1968)

SUMMARY

Thin layers of specially prepared sucrose were used to separate the chlorophylls and carotenoids of green algae, brown algae, diatoms, dinoflagellates, chrysomonads, and the leaf pigments of higher plants. Pigments separated included chlorophylls *a*, *b* and *c*, pheophytins *a* and *b*, carotenes, lutein, violaxanthin, neoxanthin, diatoxanthin, diadinoxanthin, dinoxanthin, fucoxanthin, neofucoxanthin, peridinin, neoperidinin, and several minor dinoflagellate xanthophylls. No decomposition of pigments occurred on this adsorbent, and recoveries of 1–2 μg of various pigments, after two-dimensional chromatography, averaged 95 %. Chlorophyll degradation products, not separated on sucrose plates, were separated on thin layers of cellulose; chlorophyll *c* derivatives were separated on thin layers of polyethylene, and carotene isomers were separated on thin layers of alumina–magnesium oxide. The methods are rapid and simple, and because of the sensitivity of the method the pigment components of very small samples of algae may be determined.

INTRODUCTION

A quantitative chromatographic method for determining microgram quantities of chlorophylls and carotenoids in planktonic marine algae has long been needed. Natural populations consist predominantly of unicellular green flagellates, diatoms, dinoflagellates, chrysomonads and cryptomonads, each with their specific complement of photosynthetic pigments. A chromatographic method for determining planktonic algal pigments should separate the three chlorophylls *a*, *b* and *c*, their common degradation products (chlorophyllides, pheophytins and pheophorbides), and the 15–20 carotenoids found in these organisms, and should give quantitative recovery of each fraction after chromatographic analysis. Present methods of determining the concentrations of chlorophylls in sea-water samples use spectrophotometric[1,2] and fluorimetric[3,4] techniques. These methods are useful for survey work, but they do not distinguish adequately between the three chlorophylls *a*, *b* and *c*, and their degradation products; uncertainties in the extinction coefficients of the chlorophylls, and inherent inaccuracies in the trichromatic equations used, restrict the reliability of the values which are obtained. In the present work a search was made for a chromatographic method which would separate each pigment fraction for quantitative analysis, and

which could also be used to test the validity of the various spectrophotometric equations used for chlorophyll analyses in marine algae[2] and in higher plants[5].

Paper chromatography, which was used successfully to separate the chlorophylls and carotenoids of marine algae in cultures[6,7], sea-water samples[8], and the symbiotic algal colonies of corals and clams[9,10], proved too tedious as a routine procedure for sea-water samples. Large volumes of sea-water (40 l) were needed to obtain enough pigment (10–20 μg) for each chromatogram, and recoveries of chlorophylls after chromatography never exceeded 80 %.

Thin-layer chromatographic techniques were therefore tested in the present work, but no published method proved sufficiently quantitative without great modification. Silica gel G, used by SCHALTEGGER[11], RILEY AND WILSON[12,13], LYNN CO AND SCHANDERL[14] for chloroplast pigments, and preparations of kieselguhr used by BUNT[15], EGGER[16], and HAGER AND BERTENRATH[17], are now known to cause some degradation of chlorophylls and formation of multiple chlorophyll zones[11,14,18]. Silicious adsorbents are also known to cause oxidation and isomerization of certain carotenoids[19–21].

Organic adsorbents have been used with more success where quantitative recovery of chlorophylls was desired. BACON[22] obtained 90 % recovery of chlorophylls *a* and *b* (7–50 μg) on thin layers of cellulose, and SCHNEIDER[23] also achieved about 85 % recovery of leaf pigments from this adsorbent. MADGWICK[24,25], using glucose as adsorbent, achieved 97 % recovery of chlorophyll *a*, but variable recoveries of *b* and *c*, with 0.3–4.0 μg pigment. COLMAN AND VISHNIAC[26], and NUTTING, VOET AND BECKER[27] used thin layers of sucrose for qualitative separations of leaf pigments, but quantitative recoveries were not cited.

Columns of powdered sucrose were used by STRAIN, MANNING AND HARDIN[28] and STRAIN[29] for their classic separations of the chlorophylls and carotenoids of many classes of marine algae. Because no thin-layer method was available which duplicated these beautiful separations on a micro scale, attempts were made in the present work to develop a sucrose plate with properties similar to the sucrose column. The conditions for preparing thin layers of sucrose with stable adsorptive properties were finally worked out, and a sucrose thin-layer plate was developed which, after development in two dimensions, gave complete separation of the chlorophylls and carotenoids of green algae, brown algae, diatoms, dinoflagellates, chrysomonads and the leaf pigments of higher plants. The recovery of pigment fractions from the sucrose plate averaged 96 %, using 1.0–2.0 μg of pigment.

Of the chlorophyll degradation products, only pheophytins *a* and *b* separated well on the sucrose plate. The more polar chlorophyll derivatives, chlorophyllides *a* and *b* and pheophorbides *a* and *b*, remained close to the origin, and were not resolved. However, these fractions could be eluted and completely separated on thin layers of cellulose using a solvent system of BACON AND HOLDEN[30]. Chlorophyll *c* and pheophytin *c* each separated into two components on thin layers of polyethylene.

The present paper describes the preparation and properties of the sucrose thin-layer plate, the quantitative procedures used, and some applications of the method.

MATERIALS AND METHODS

Algae

Cultures of marine algae were grown in Medium f of Guillard under a light intensity of 5000 lux at 14–18° (ref. 31). The uni-algal cultures studied included the

green alga *Dunaliella tertiolecta*, the diatom *Phaeodactylum tricornutum*, the dinoflagellate, Amphidinium sp., and the chrysomonad *Isochrysis galbana*. The brown alga *Sargassum flavicans* and the Siphonales alga Codium sp. were collected from Port Hacking near the Laboratory. Swiss chard leaves were obtained fresh from a local store, and were included in the study as a representative of higher plants. Sea-water samples were collected from the 50 m station outside Port Hacking, as described previously[8].

Preparation of pigment extracts

The algae from 100–200 ml of culture were harvested by centrifuging at 2000 × *g* for 3–5 min, and the supernatant was discarded. Pigments were readily released from Dunaliella, Amphidinium and Isochrysis, by extracting the cells with small volumes (3–5 ml) of 90 % acetone. After several such extractions the cell residue was colourless. Pigments were immediately transferred to diethyl ether, by adding an equal volume of ether to the combined acetone extracts, and shaking with a volume of 10 % NaCl solution at least 10 times that of the acetone extract. The pigments migrated to the ether layer, and acetone and water-soluble impurities (particularly plant acids) were removed in the aqueous phase. If this step was carried out immediately, the stability of chlorophylls and carotenoids in the samples was greatly prolonged. The ether layer was concentrated for chromatography under a stream of nitrogen. Condensed water was removed from the concentrated ether extract by centrifugation, and the sample was then made completely anhydrous by the addition of a few crystals of NaCl. Solid NaCl, unlike Na_2SO_4, did not adsorb the pigments. Alternatively, water could be eliminated from the ether by freezing at —20° for 1–2 h.

Cells which were not readily extracted with 90 % acetone (Phaeodactylum) were extracted with methanol, or deep frozen prior to extraction. Sargassum and Codium pigments were readily extracted by freezing the algal fronds at —20° for 30 min, immersing the frozen tissue in methanol for 30 sec and finally homogenizing in 100 % acetone. Subsequent chromatography showed that the pigments were not damaged by this treatment. Leaves of Swiss chard were homogenized in 100 % acetone containing a little $MgCO_3$ to neutralize the plant acids. In all cases, the acetone or methanol extracts, separated by centrifugation from the tissue residues, were immediately mixed with diethyl ether, and shaken with excess NaCl solution to wash out impurities and to concentrate the pigments in the ether phase.

Sea-water samples were treated as described previously[32]. The plankton was collected by continuous centrifugation of sea-water samples at 5000 × *g* in a continuous plankton centrifuge, and the cells were extracted with 100 % acetone for 30 min, after grinding for 5 min in a Potter–Elvehjem homogenizer with a little $MgCO_3$. Pigments were then transferred to ether as above.

For quantitative pigment determinations transfer of pigments from acetone or methanol to ether was carried out in accurately calibrated 100 ml measuring cylinders, and complete phase separation was ensured by chilling the aqueous and ether layers at —20° for 15–20 min. A loss of only 0.1–0.3 % of the extract occurred at this stage. The ether layer was made up to a measured volume in the cylinder, and an aliquot taken for chromatography. Concentration of the pigments in the aliquot to a small known volume was carried out in 10 ml graduated centrifuge tubes centrifuged if

necessary to remove condensed water, and then under N_2. The concentrated extract was dried with NaCl crystals before chromatography.

Preparation of sucrose thin-layer plates

The most important factors in the preparation of the sucrose plate were the particle size of the powdered sucrose, the presence of 5 % cornflour in the sucrose to keep the adsorbent dry and soft, the state of hydration of the sucrose before sieving and spreading, and the need to use completely anhydrous solvents for spreading the plates, spotting the extracts, and developing the chromatograms.

Powdered confectioners icing sugar (Sunny Cane brand, containing 5 % cornflour – Industrial Sugar Mills, Sydney) was dried in a shallow dish at 90° for 30 min. The dried powder was passed through a 200 mesh sieve twice, to give a fine powder with a particle size of 10–50 μ. 30 g of powder were mixed with 50 ml light petroleum (60–80°), and homogenized for 1 min at medium speed in a top-drive Sorvall Omnimixer. Plates were poured and spread immediately according to the method of LEES AND DE MURIA[33]. Commercial spreaders of the Desaga type were difficult to use with volatile solvents, because of the formation of caked adsorbent at the exit slit due to solvent evaporation. Three plates (16 cm × 12 cm) were coated with layers 0.25 mm thick from this quantity of sucrose. The plates were allowed to dry at room temperature (18–22°) for 30 min before use.

The plating procedure resulted in a thin layer of sucrose which adhered well to the glass plate, and which retained a soft powdery surface. If traces of water were present in any of the equipment used for preparing the dry powdered sugar, or if the spreading solvent was not anhydrous, a sucrose plate with a hard glassy surface resulted, with loss of adsorptive properties. Hard plates also resulted if the sucrose was not used immediately after sieving, and moisture was re-adsorbed into the dry powder, or if the initial drying at 90° was too prolonged. COLMAN AND VISHNIAC[26] spread sucrose thin layers in methanol, but in the present work this procedure resulted in hard glassy sucrose surfaces, probably due to traces of water in the methanol. Diethyl ether, used as a spreading solvent by MADGWICK[24] for glucose plates, was difficult to handle, because of extremely rapid solvent evaporation. Light petroleum (60–80°) combined the essential properties of an anhydrous solvent with one that was easy to manipulate in the pouring and spreading procedures. Hard plates could not be converted to soft plates by drying, once the plates had been made, but had always to be discarded. Soft plates with good adsorptive properties could be stored for periods from 3 to 6 weeks in a desiccator over silica gel without deterioration of the plate.

Solvent systems

The degree of resolution of the pigments depended on the composition of the solvents, and the state of hydration of the sucrose. Solvents which gave excellent resolution under most conditions were 0.8 % *n*-propanol in light petroleum (60–80°) (v/v) for the first dimension, and 20 % chloroform in light petroleum (v/v) for the second dimension. Chlorophyll *a* moved with an R_F value of approx. 0.75 in both dimensions with these solvents, and under these conditions the other pigment fractions were well separated (Table I). Occasionally under very damp or very dry climatic conditions the adsorptive properties of the plates changed slightly, with consequent

increases or decreases in the mobility of the pigments. The standard procedure adopted was to check the mobility of chlorophyll *a* with each batch of plates with the above solvents. If the R_F value of chlorophyll *a* was a little higher than 0.75 the amount of propanol and chloroform in the two solvents was lowered slightly, and if the R_F value was less than 0.75, the proportions of propanol and chloroform were increased slightly. In practice the range of concentrations was from 0.6 to 1.0 % for propanol, and 12 to 25 % for chloroform; under most normal conditions, however, 0.8 % propanol and 20 % chloroform in light petroleum gave solvent mixtures which produced the desired pigment mobilities.

150 ml solvent were placed in paper-lined chromatography tanks, and equilibration proceeded for 10–20 min. Development was carried out at room temperature in the dark, and took 5–8 min for the first dimension (11 cm) and 3–5 min for the second dimension (8 cm).

TABLE I

R_F VALUES OF CHLOROPHYLLS AND CAROTENOIDS FROM MARINE ALGAE SEPARATED IN TWO SOLVENT SYSTEMS ON SUCROSE THIN-LAYER PLATES

Pigment	*Spot No.*	*First dimension 0.8 % n-propanol–light petroleum (60–80°)*	*Second dimension 20 % chloroform–light petroleum (60–80°)*
Carotenes	1	0.97	0.97
Chlorophyll *a*	2	0.77	0.73
Chlorophyll *b*	3	0.47	0.46
Chlorophyll *c*	4	0.07	0.0
Lutein	5	0.68	0.84
Violaxanthin	6	0.28	0.53
Neoxanthin	7	0.16	0.12
Diatoxanthin	8	0.69	0.83
Diadinoxanthin	9	0.56	0.75
Fucoxanthin	10	0.34	0.50
Neofucoxanthin	11	0.34	0.15
Pale-orange dinoflagellate xanthophyll	12	0.71	0.82
Dinoxanthin	13	0.56	0.65
Yellow dinoflagellate xanthophyll	14	0.39	0.42
Neodinoxanthin	15	0.36	0.32
Peridinin	16	0.27	0.28
Neoperidinin	17	0.27	0.23
Pink-orange dinoflagellate xanthophyll	18	0.0	0.0
Pheophytin *a*	19	0.91	0.93
Pheophytin *b*	20	0.76	0.94
Pheophorbides *a* and *b*	21	0.12	0.0
Chlorophyllides *a* and *b*	22	0.10	0.0
Pheophytin *c*	23	0.0	0.0

Preparation of pigment fractions for quantitative tests

Chlorophylls and carotenoids were isolated by cellulose column chromatography or on sucrose thin-layer plates, and were dissolved in ether solution for ease of spotting. Pheophytins *a* and *b* were prepared from the parent chlorophylls by acidification[34]. Chlorophyllides *a* and *b* were obtained by incubating the green alga *D. tertiolecta* in 60 % aqueous acetone for 1 h to allow chlorophyllase activity to hydrolyse the phytol

from the parent chlorophyll[35]. Pheophorbides *a* and *b* were prepared from the chlorophyllides by acidification[34]. Purity of these chlorophyll fractions was checked by chromatography[30], and by absorption spectra[34,36].

Quantitative recovery of pigments

The pigment extract in ether solution, prepared quantitatively from a known amount of plant tissue, concentrated to a known volume under nitrogen and thoroughly dried over solid NaCl, was applied to the origin of a sucrose plate. 10 μl of extract, dispensed with a 10 μl spotting pipette (H. Pedersen, Denmark) and containing 0.5–4.0 μg of pigment, was sufficient for each chromatogram. After two-dimensional chromatography, the separated pigment zones were scraped off the thin-layer plate, using a special elution tube attached to a suction line (Fig. 1). The

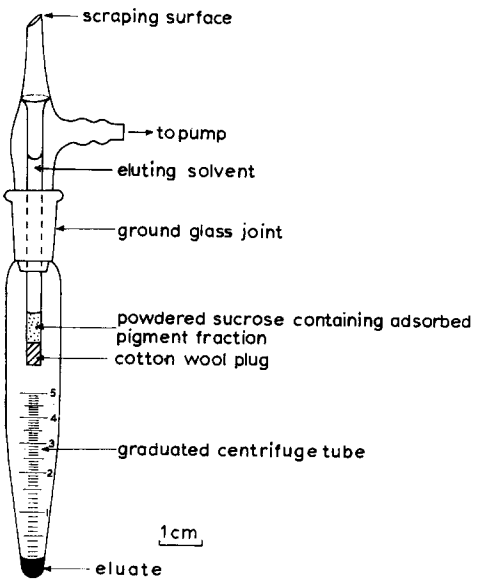

Fig. 1. Thin-layer elution tube.

powdered sucrose, containing the adsorbed pigment, was drawn down the small inner tube on to the top of the cotton wool plug, and the pigment was eluted by rinsing the tube with small volumes of the appropriate solvent. The eluate, containing the pigment, was collected in the centrifuge tube, made up to a measured volume, centrifuged if necessary to remove any particles of sucrose that may have come through the cotton plug, and the extinction of the clear solution read without delay in a Unicam SP 600 spectrophotometer. Unless submicrogram quantities of pigments were to be estimated, 1-cm cells were routinely used. A number of elution tubes were set up prior to chromatography, and the whole operation of scraping off the pigment zones and eluting the pigments was completed in about 10 min. Solutions were kept in the dark, and operations were carried out in dim light to minimize photo-oxidative breakdown of the pigments. Tests of recoveries of pure pigments from sucrose plates were made to establish the reliability of the methods used.

Chlorophylls *a* and *b* and pheophytins *a* and *b* were eluted quantitatively with acetone, chlorophyll *c*, chlorophyllides *a* and *b*, and pheophorbides *a* and *b* with methanol, carotene with light petroleum, and xanthophylls with ethanol. Extinction coefficients for the calculation of pigment concentrations were:

Chlorophyll *a*	(acetone)	$\varepsilon_{663\ nm}$	92 l/g·cm	(ref. 37)
Chlorophyll *b*	(acetone)	$\varepsilon_{645\ nm}$	53.5 l/g·cm	(ref. 37)
Chlorophyll *c*	(methanol)	$\varepsilon_{635\ nm}$	15.2 l/g·cm	(ref. 38)
Carotene	(light petroleum)	$\varepsilon_{450\ nm}$	250.5 l/g·cm	(ref. 39)
Lutein	(ethanol)	$\varepsilon_{447\ nm}$	255.0 l/g·cm	(ref. 40)
Violaxanthin	(ethanol)	$\varepsilon_{441\ nm}$	225.0 l/g·cm	(ref. 40)
Neoxanthin	(ethanol)	$\varepsilon_{438\ nm}$	227.0 l/g·cm	(ref. 40)
Peridinin	(ethanol)	$\varepsilon_{475\ nm}$	132.5 l/g·cm	(ref. 9)
Fucoxanthin	(ethanol)	$\varepsilon_{452\ nm}$	160 l/g·cm	(ref. 41)

Other carotenoids, whose extinction coefficients are unknown, were calculated using the extinction coefficient of carotene.

Spectrophotometry

Identification of chlorophylls and carotenoids was carried out by eluting the pigments after chromatography in an appropriate solvent, and measuring the absorption spectrum in a Unicam SP 700 recording spectrophotometer. The instrument was calibrated with Hg and H lines, and was considered accurate to 1 nm. For routine estimations of pigment concentrations, a Unicam SP 600 spectrophotometer was used.

Other thin-layer systems

Chlorophyll decomposition products (prepared as described above) which did not separate on sucrose plates were separated on thin layers of cellulose powder (Macherey Nagel, MN300) using 20 % acetone in light petroleum (b.p. 60–80°)[30]. Other types of cellulose powder (Whatman CC41, and Macherey Nagel 300HR) were not satisfactory. The latter adsorbent caused appreciable conversion of chlorophylls to pheophytins during chromatography.

Cellulose (MN300) also provided a useful alternative to sucrose in the two-dimensional system. Chromatographic separations obtained using 2 % *n*-propanol in light petroleum, and 25 % chloroform in light petroleum were similar to those obtained with the sucrose plate. The zones, however, were a little more diffuse, and recovery of the pigments from the cellulose powder was not quite so accurate. However, in very damp atmospheric conditions, cellulose powder provided a useful alternative to the sucrose plate, which needs the maintenance of dry conditions for successful results.

Thin layers of polyethylene (Dow Chemical Co.) were used for the separation of the two components, both of chlorophyll *c* and pheophytin *c* (ref. 42). This polyethylene system also provided an alternative method for separating the degradation products of chlorophyll *a*. The plates were prepared by homogenizing 15 g of polyethylene powder in 90 ml acetone for 1 min in a Sorvall Omnimixer at medium speed. The plates were spread, and dried at room temperature for 1 h before use. Polyethylene thin layers could be stored in a desiccator over silica gel for about 10 days, without deterioration of the plate.

Thin layers of Al_2O_3–MgO (3:1, w/w) were used to separate carotene isomers according to the method of CHAPMAN[43]. The solvent was 4 % ethyl acetate in hexane.

Routine chromatographic procedures

The procedure used for the complete examination of pigments in a natural sample was first to chromatograph the extract on a sucrose plate to see the overall chlorophyll and carotenoid pattern, and the presence or absence of zones of chlorophyll decomposition products. If material in addition to chlorophyll *c* was present at the origin, the entire origin zone was eluted from the sucrose plate with methanol, and rechromatographed on cellulose and polyethylene plates. This procedure separated chlorophyllides *a* and *b*, and pheophorbides *a* and *b* from chlorophyll *c*. If the nature of the carotene isomers was needed, the carotene fraction was first separated on a sucrose plate, eluted with ether, and re-run on the Al_2O_3–MgO plate[43] to resolve the isomers. Sometimes extracts of phytoplankton contained large amounts of lipid material which interfered with the sucrose chromatography by remaining at the origin in the first solvent system and holding back some pigment fractions. This effect could be completely overcome by reversing the order of the solvents used, since the chloroform–light petroleum solvent removed the lipid from the origin, and all pigment fractions then separated normally. By a combination of the sucrose, cellulose, polyethylene and magnesium oxide–alumina thin-layer systems successful resolution of all pigment components was obtained.

RESULTS

Chromatograms of pigments on thin layers of sucrose from different classes of algae are given in detail in Fig. 2. Complete separation of all major pigment fractions was obtained with the green alga, diatom, dinoflagellate, chrysomonad, brown alga and the leaf pigments of a higher plant (Swiss chard). The order of resolution of the pigments was similar to that of the paper chromatography method[6], except that xanthophylls which showed overlapping on paper were completely resolved on the sucrose plate (*e.g.* fucoxanthin and neofucoxanthin, peridinin and neoperidinin, dinoxanthin and diadinoxanthin). In the case of the dinoflagellates several new minor carotenoid zones were observed. These were characterized by absorption spectra and chromatographic behaviour[9]. R_F values of the chlorophylls and carotenoids in the two solvent systems are shown in Table I. The systems were standardized with chlorophyll *a*, which was adjusted with appropriate solvents to move with R_F values of approx. 0.75 in each dimension. Good resolution of all other pigments was achieved with this mobility of chlorophyll *a*.

Absorption maxima of isolated pigment fractions are given in Table II. These are compared with published maxima of the same compounds isolated by other methods. The absorption maxima were closely similar whether pigments were separated on sucrose columns[28], paper[6], or sucrose thin-layer plates.

Since planktonic algae of oceanic sea-waters are usually mixed populations of diatoms, chrysomonads (with similar pigment composition) and dinoflagellates, a chromatogram of mixtures of pigments from these organisms is shown in Fig. 3A. In addition, coastal sea-waters may also contain green flagellates, and a chromatogram of a mixture of a green alga, diatom and dinoflagellate is shown in Fig. 3B. Good

TABLE II

ABSORPTION MAXIMA OF CHLOROPHYLLS AND CAROTENOIDS SEPARATED ON SUCROSE THIN-LAYER PLATES

Pigment	*Published maxima (nm)*	*Ref.*	*Maxima found (nm)*	*Solvent*	*Organisms*
Chlorophyll *a*	662, 430	34	662, 430	Diethyl ether	*Sargassum flavicans*
Chlorophyll *b*	644, 455	34	645, 455	Diethyl ether	*Dunaliella tertiolecta*
Chlorophyll *c*	633, 447	34	635, 449	Methanol	*Sargassum flavicans*
Carotenes ($\alpha+\beta$)	428*, 452, 480	44	428*, 452, 479	Diethyl ether	*Sargassum flavicans*
			428, 451, 478	Diethyl ether	Swiss chard
Lutein	446, 476	40	447, 475	Ethanol	Swiss chard
Violaxanthin	419*, 441, 470	20	418*, 441, 472	Ethanol	Swiss chard
Neoxanthin	413*, 437, 465	20	415*, 438, 467	Ethanol	Swiss chard
Diatoxanthin	453, 481	28	453, 481	Ethanol	*Phaeodactylum tricornutum*
Diadinoxanthin	448, 478	28	448, 479	Ethanol	*Phaeodactylum tricornutum*
Dinoxanthin	441, 471	28	440, 473	Ethanol	Gymnodinium
Fucoxanthin	453	28	452	Ethanol	*Sargassum flavicans*
Neofucoxanthin A and B	446 447	28	443	Ethanol	*Sargassum flavicans*
Peridinin	475	28	474	Ethanol	Amphidinium sp.
Siphonein	465	29	467	Ethanol	Codium sp.
Siphonaxanthin	450	29	452	Ethanol	Codium sp.

* Inflection.

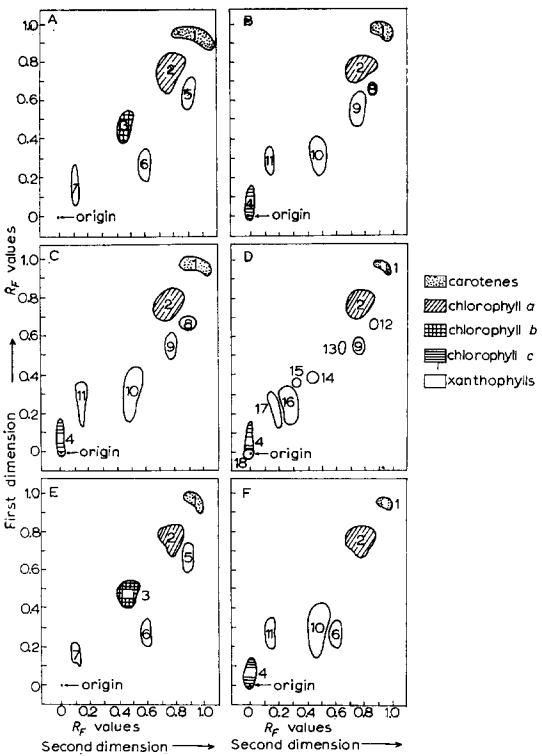

Fig. 2. Two-dimensional chromatograms of pigments in marine algae separated on sucrose thin-layer plates. A. Swiss chard. B. *I. galbana*. C. *P. tricornutum*. D. Amphidinium sp. E. *D. tertiolecta*. F. *S. flavicans*. Chromatographic solvent system: first dimension, 0.8% *n*-propanol in light petroleum (60–80°); second dimension, 20% chloroform in light petroleum. 1, carotenes (orange); 2, chlorophyll *a* (blue-green); 3, chlorophyll *b* (olive-green); 4, chlorophyll *c* (light-green); 5, lutein (yellow); 6, violaxanthin (yellow); 7, neoxanthin (yellow); 8, diatoxanthin (pale-orange); 9, diadinoxanthin (yellow); 10, fucoxanthin (orange); 11, neofucoxanthin (orange); 12, unknown dinoflagellate xanthophyll (pale-orange); 13, dinoxanthin (yellow); 14, unknown dinoflagellate xanthophyll (yellow); 15, neodinoxanthin (yellow); 16, peridinin (brick-red); 17, neoperidinin (brick-red); 18, unknown dinoflagellate xanthophyll (pink-orange).

resolution of the pigment fractions was obtained in both cases, except for some overlapping of lutein, diatoxanthin and the pale-orange dinoflagellate xanthophyll (No. 12). Chromatogram C (Fig. 3) shows the pigments of a sea-water sample taken during a bloom of the dinoflagellate Ceratium.

Natural phytoplankton populations contain living and senescent cells, so that chlorophyll degradation products may be important components of some samples. The position of the chlorophylls, pheophytins, chlorophyllides and pheophorbides (prepared as described above) on the sucrose plate are shown in Fig. 3D. Although the chlorophylls and pheophytins were well resolved, chlorophyllides, pheophorbides and chlorophyll *c* remained at the origin. These components could be eluted with methanol, and rechromatographed on a cellulose plate, using 20% acetone in light petroleum[30] as the solvent system. Fig. 4 shows that good resolution of all chlorophyll *a* and *b* components, and partial resolution of chlorophyll *c* components, was obtained with the cellulose thin-layer plate.

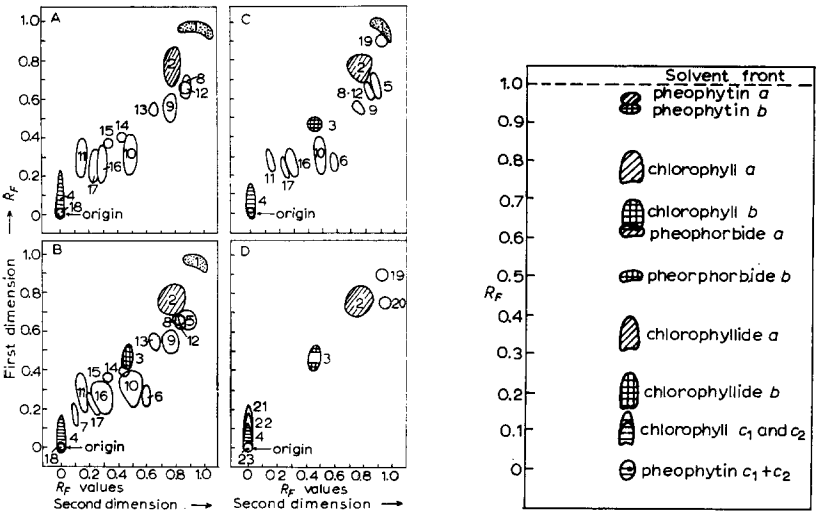

Fig. 3. Two-dimensional chromatograms of mixtures of marine algal pigments separated on sucrose thin-layer plates. A. *P. tricornutum* and Amphidinium sp. B. Phaeodactylum, Amphidinium and *D. tertiolecta*. C. Sea-water sample containing many dinoflagellates (Ceratium sp.). D. Chlorophylls and derivatives. Chromatographic solvent systems and pigment zones as for Fig. 2. In addition: 19, pheophytin *a* (grey-green); 20, pheophytin *b* (yellow-grey); 21, pheophorbides *a* and *b* (grey); 22, chlorophyllides *a* and *b* (green); 23, pheophytin *c* (light-brown).

Fig. 4. Separation of chlorophyll derivatives on thin layers of cellulose. Solvent system: 20% acetone in light petroleum (60–80°).

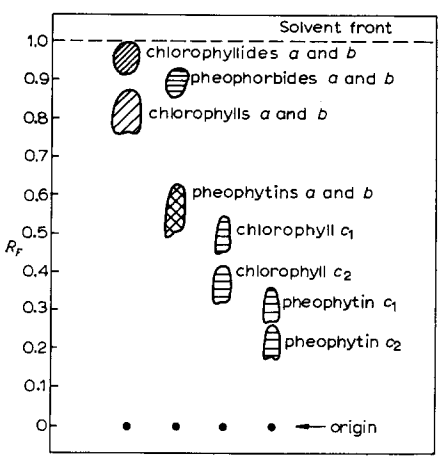

Fig. 5. Separation of chlorophyll derivatives on thin layers of polyethylene. Solvent system: acetone–water (9:1, v/v).

Chlorophyll *c* and pheophytin *c* each separated into two components on thin layers of polyethylene (Fig. 5). These have been designated chlorophylls c_1 and c_2, and pheophytins c_1 and c_2 (ref. 42). Resolution of all four fractions was not absolute, since some overlapping of chlorophyll c_2 and pheophytin c_1 occurred. Polyethylene thin layers also provided an alternative method of separating chlorophyll *a*, pheo-

phytin *a*, chlorophyllide *a* and pheophorbide *a* into discrete zones. Since the *b* chlorophyll derivatives ran in the same position as the *a* chlorophylls on polyethylene, this system was only useful for samples containing chlorophylls *a* and *c*, but no *b*. It provided an additional method of studying the chlorophyll decomposition products of sea-water samples, when no *b* derivatives were present.

Quantitative recoveries of a number of pigments applied to sucrose plates in known amounts (0.5–3.0 μg) are given in Table III. The recoveries averaged 96% for microgram amounts of all pigments tested in the two-dimensional system. Some

TABLE III

QUANTITATIVE RECOVERIES OF SOME CHLOROPHYLLS AND CAROTENOIDS AFTER CHROMATOGRAPHY ON THIN LAYERS OF SUCROSE

Pigment	*Amount applied* (μg)	% *Recoveries after 2-dimensional chromatography**
Chlorophyll *a*	2.58	96
Chlorophyll *b*	1.00	95
Chlorophyll *c*	2.76	95
Chlorophyllide *a*	2.58	95
Pheophorbide *a*	1.32	98
Carotene	1.00	95
Lutein	0.40	95
Fucoxanthin	1.59	97
Peridinin	0.86	95

* Means of 3 determinations (S.E. $\pm$ 2.5%).

TABLE IV

QUANTITATIVE DETERMINATIONS OF PIGMENTS IN MARINE ALGAE SEPARATED ON SUCROSE THIN-LAYER PLATES*

Pigment	μg/0.1 ml *packed cell volume*		
	Dunaliella tertiolecta	*Phaeodactylum tricornutum*	*Amphidinium sp*
Chlorophyll *a*	172	128	252
Chlorophyll *b*	154	—	—
Chlorophyll *c*	—	53	244
Carotenes	58	42	24
Lutein	102	—	—
Violaxanthin	30	—	—
Neoxanthin	27	—	—
Diatoxanthin	—	4	—
Diadinoxanthin	—	36	45
Dinoxanthin	—	—	33
Fucoxanthin	—	52	—
Neofucoxanthin	—	10	—
Peridinin	—	—	205
Neoperidinin	—	—	62
Dinoflagellate xanthophylls 12, 14, and 18	—	—	<10
Total chlorophylls	326	181	496
Total carotenoids	217	144	379

* Cultures used for these determinations were 2–3 weeks old.

of the error occurred in the application of the pigment to the plate, since pigments spotted and eluted immediately without chromatography, averaged 98 % recovery. The chromatographic and elution procedures caused only very small additional errors, since after 1 dimension recovery averaged 97 %, and after 2 dimensions, 96 % (Table III). Table IV shows the concentration of chlorophylls and carotenoids in a green alga, a diatom and a dinoflagellate, analysed by sucrose thin-layer chromatography. This study simply shows the use of the method, which now permits quantitative determinations of pigment concentrations in algae under different physiological conditions to be made.

The chlorophylls and carotenoids from several other groups of algae not found in the phytoplankton were also studied on sucrose plates. The Siphonales alga Codium, and the brown alga *S. flavicans*, gave pigment separations similar to those obtained by paper chromatography[10] and sugar columns[29]. Absorption maxima of the Siphonales xanthophylls, siphonein and siphonaxanthin, separated on thin layers of sucrose, are given in Table II.

DISCUSSION

In spite of many methods which use silicious adsorbents for qualitative separations of chlorophylls and carotenoids in plant extracts, the instability of some compounds on silica gel and kieselguhr preparations precluded use of such adsorbents for quantitative procedures. The success of the sucrose plate as a quantitative micromethod was due to the stability of the pigments on this adsorbent, the complete resolution of major chlorophyll and carotenoid components, and the ease of elution of pigment fractions after chromatographic separation. Thin layers of sucrose with stable adsorptive properties required care in their preparation, but if the conditions needed to produce fine, dry layers of sucrose were followed, well-resolved chromatograms resulted.

No breakdown of pigments on the plates was detected. However, DAVIES[45] has cautioned against the use of chloroform as a solvent for carotenoids because of the danger of traces of acid in the chloroform causing isomerization of 5,6-epoxides to furanoid epoxides. Such changes were not detected with the AnalaR chloroform used in this and previous work for the second solvent system.

The pigment components of the various algae studied were identical with those previously described for these organisms[6], except in the case of the dinoflagellates in which three new minor xanthophyll zones were found. These xanthophylls occurred both in symbiotic and free-living dinoflagellates, and are described in more detail elsewhere[9]. In addition to the separation of chlorophylls and carotenoids from planktonic algae—diatoms, dinoflagellates, chrysomonads and green flagellates—the pigment components of a Siphonales alga (Codium), a brown sea-weed (*S. flavicans*), and a higher plant (Swiss chard) were also well separated on the sucrose thin-layer system.

Since the sucrose method needs only a few μg of pigment for each chromatogram, pigment analyses of algae from special situations, as well as those from cultures, are now open to study. Previously, quantitative estimates of phytoplankton in seawater samples were too tedious to obtain by paper chromatography, but with the sucrose plate much smaller volumes of sea-water (10–15 l) provided ample material for chromatographic estimation. Micro-algae growing on rock surfaces have also been

examined by sucrose thin-layer techniques (S. B. HAVEN AND S. W. JEFFREY, unpublished), and the composition of the algal flora was deduced from pigment components found in very small samples. Algal pigments in the intestinal tracts of minute zooplankton animals (*e.g.* the salp, *Thalia democratica*) have also been studied (A. C. HERON AND S. W. JEFFREY, unpublished). Information was obtained concerning algal types used as food by these animals, and the nature of the degradative changes occurring to the chlorophylls and carotenoids within the gut.

Because chlorophylls are recovered quantitatively from sucrose thin-layer plates, this system provides an alternative method to spectrophotometric and fluorimetric methods of chlorophyll assay. In fact it may now be possible to check the accuracy of the spectrophotometric equations used for chlorophylls *a* and *b* (refs. 5, 34, 37), for chlorophylls *a* and *c* (G. F. HUMPHREY AND S. W. JEFFREY in ref. 7) and chlorophylls *a*, *b* and *c* (refs. 1, 2). Since chlorophyll decomposition products are readily separated from the parent compounds by the methods described here, but are estimated together with the chlorophylls by spectrophotometric methods, more accurate estimates of chlorophyll concentrations in natural populations of algae are now possible. The method may also be useful as a sensitive check on the purity of chlorophyll preparations, since minute traces of decomposition products can be detected on the plate under ultraviolet light.

In summary, the sucrose thin-layer plate provides a chromatographic system that duplicates on a micro scale the classical separations of STRAIN, MANNING AND HARDIN[28] and STRAIN[29] of algal pigments on sucrose columns. Complete separation of chlorophylls *a*, *b* and *c*, pheophytins *a* and *b*, and the carotenoids carotene, lutein, violaxanthin, neoxanthin, diatoxanthin, diadinoxanthin, dinoxanthin, fucoxanthin, neofucoxanthin, peridinin, neoperidinin, and four additional minor xanthophyll fractions from dinoflagellate extracts was achieved. Chlorophyllides *a* and *b*, and pheophorbides *a* and *b*, which were not separated from each other or chlorophyll *c* on the sucrose plate were well resolved on thin layers of cellulose, and the two components of chlorophyll *c* (to be described in detail elsewhere) were separated on thin layers of polyethylene. Carotenoid hydrocarbons (α-, β-, γ- and ε-carotenes) which formed one zone on the sucrose plate were separated on the alumina–magnesium oxide plate of CHAPMAN[43]. By judicious use of the various systems presented, qualitative and quantitative information on the major chlorophyll and carotenoid pigments of marine algal populations, may now be obtained.

REFERENCES

1 F. A. RICHARDS AND T. G. THOMPSON, *J. Marine Res. Sears Found.*, 11 (1952) 156.
2 SCOR–UNESCO, *Monographs on Oceanographic Methodology*, Vol. 1, 1966, p. 11.
3 C. S. YENTSCH AND D. W. MENZEL, *Deep Sea Res.*, 10 (1963) 221.
4 O. HOLM-HANSEN, C. J. LORENZEN, R. W. HOLMES AND J. D. H. STRICKLAND, *J. Conseil Conseil Perm. Intern. Exploration Mer*, 30 (1965) 3.
5 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
6 S. W. JEFFREY, *Biochem. J.*, 80 (1961) 336.
7 S. W. JEFFREY AND M. B. ALLEN, *J. Gen. Microbiol.*, 36 (1964) 277.
8 S. W. JEFFREY, *Australian J. Marine Freshwater Res.*, 16 (1965) 307.
9 S. W. JEFFREY AND F. T. HAXO, *Biol. Bull.*, in the press.
10 S. W. JEFFREY, *Biol. Bull.*, in the press.
11 K. H. SCHALTEGGER, *J. Chromatog.*, 19 (1965) 75.
12 J. P. RILEY AND T. R. S. WILSON, *J. Marine Biol. Assoc. U.K.*, 45 (1965) 583.
13 J. P. RILEY AND T. R. S. WILSON, *J. Marine Biol. Assoc. U.K.*, 47 (1967) 351.

14 D. Y. C. LYNN CO AND S. H. SCHANDERL, *J. Chromatog.*, 26 (1967) 442.
15 J. S. BUNT, *Nature*, 203 (1964) 1261.
16 K. EGGER, *Planta*, 58 (1962) 664.
17 A. HAGER AND T. BERTENRATH, *Planta*, 58 (1962) 564.
18 M. F. BACON, *Biochem. J.*, 101 (1966) 34C.
19 H. H. STRAIN, J. SHERMA, F. L. BENTON AND J. KATZ, *Biochim. Biophys. Acta*, 109 (1965) 23.
20 H. H. STRAIN, J. SHERMA AND M. GRANDOLFO, *Anal. Chem.*, 39 (1967) 926.
21 J. W. NELSON AND A. L. LIVINGSTON, *J. Chromatog.*, 28 (1967) 465.
22 M. F. BACON, *J. Chromatog.*, 17 (1965) 322.
23 H. A. W. SCHNEIDER, *J. Chromatog.*, 21 (1966) 448.
24 J. C. MADGWICK, *Deep Sea Res.*, 12 (1965) 233.
25 J. C. MADGWICK, *Deep Sea Res.*, 13 (1966) 459.
26 B. COLMAN AND W. VISHNIAC, *Biochim. Biophys. Acta*, 82 (1964) 616.
27 D. NUTTING, M. VOET AND R. BECKER, *Anal. Chem.*, 37 (1965) 445.
28 H. H. STRAIN, W. M. MANNING AND G. HARDIN, *Biol. Bull.*, 86 (1944) 169.
29 H. H. STRAIN, *Chloroplast Pigments and Chromatographic Analysis*, 32nd Annual Priestly Lectures, Pennsylvania State University, 1958.
30 M. F. BACON AND M. HOLDEN, *Phytochemistry*, 6 (1967) 193.
31 B. R. GRANT, *J. Gen. Microbiol.*, 48 (1967) 379.
32 S. W. JEFFREY, *Limnol. Oceanog.*, in the press.
33 T. M. LEES AND P. J. DE MURIA, *J. Chromatog.*, 8 (1962) 108.
34 J. H. C. SMITH AND A. BENITEZ, in K. PAECH AND M. TRACEY, *Modern Methods of Plant Analysis*, Vol. 4, Springer, Heidelberg, 1955, p. 142.
35 J. BARRETT AND S. W. JEFFREY, *Plant Physiol.*, 39 (1964) 44.
36 A. S. HOLT AND E. E. JACOBS, *Am. J. Botany*, 41 (1954) 710.
37 L. P. VERNON, *Anal. Chem.*, 32 (1960) 1144.
38 S. W. JEFFREY, *Biochem. J.*, 86 (1963) 313.
39 T. W. GOODWIN, in K. PAECH AND M. TRACEY, *Modern Methods of Plant Analysis*, Vol. 3, Springer, Heidelberg, 1955, p. 272.
40 H. H. STRAIN, *Carnegie Inst. Wash. Publ.*, 490 (1938).
41 A. JENSEN, *Acta Chem. Scand.*, 20 (1966) 1728.
42 S. W. JEFFREY, in preparation.
43 D. J. CHAPMAN, Ph.D. Dissertation, University of California, San Diego, Calif., 1966.
44 J. M. ANDERSON, Ph.D. Dissertation, University of California, Berkeley, Calif., 1959.
45 B. H. DAVIES, in T. W. GOODWIN, *Chemistry and Biochemistry of Plant Pigments*, Academic Press, New York, 1965, p. 489.