See also: **II/Chromatography:** Countercurrent Chromatography and High-Speed Countercurrent Chromatography: Instrumentation. **Chromatography: Liquid:** Countercurrent Liquid Chromatography. **III/Alkaloids:** Gas Chromatography; Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Amino Acids:** Gas Chromatography; Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Chiral Separations. Amino Acids and Peptides: Capillary Electrophoresis. Antibiotics:** High-Speed Countercurrent Chromatography. **Chiral Separations:** Amino Acids and Derivatives; Liquid Chromatography. **Dyes:** High-Speed Countercurrent Chromatography; Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Ion Analysis:** High-Speed Countercurrent Chromatography. **Natural Products:** High-Speed Countercurrent Chromatography. **Proteins:** High-Speed Countercurrent Chromatography.

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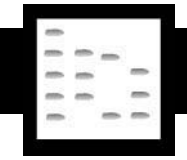
PIGMENTS



Liquid Chromatography

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Introduction

Thin-layer chromatography (TLC) represented a key development in aquatic sciences because it enabled the routine separation and quantitation of algal chlorophylls, carotenoids and their breakdown products (**Table 1** and **Figure 1**) to be obtained. These pigments can be used as markers for algal taxa, processes such as grazing or cell senescence and water masses. However, TLC methods are not easy to automate, are difficult to use in field situations and have gradually given way to liquid chromatography (LC) methods.

High performance LC (HPLC) analysis of pigments has been developed over the last 20 years. Both normal-phase (NP) and reversed-phase (RP) techniques have been used, with preference for the RP mode due to the relatively low polarity of the analysed compounds. The NP mode is now mostly used for the separation of specific pigments (e.g. monovinyl from divinyl chlorophylls) while RP-HPLC is preferred when a complete separation of all major chlorophylls and carotenoids is required. The analysis of phycobiliproteins, the other major group of algal pigments, is not yet done by chromatographic separation on a routine basis.

Within the various RP-HPLC methods, gradient elution has generally been preferred over isocratic for full pigment separation. Ion-pairing reagents, or phase buffering, have been included in a number of techniques to improve the resolution of the more polar pigments. Three groups of pigments present particular difficulties in their separation: the pigment pair lutein–zeaxanthin, the various members of the chlorophyll *c* group and the monovinyl and divinyl forms of chlorophyll *a* and *b*. Improved separation of zeaxanthin and lutein has been achieved using a nonend-capped C_{18} column (see below) and a combination of acetonitrile, methanol and ethyl acetate as mobile phase. Resolution of chlorophyll c_3 from the other compounds in the chlorophyll *c* group has been obtained by including an ammonium acetate buffer in the initial methanol mobile phase. Separation of all three forms of chlorophyll *c* has been achieved on a polyethylene column using aqeous acetone as mobile phase, as well as by using a very high ion strength solvent in combination with a high carbon loaded

Table 1 Major algal chlorophyll and carotenoid pigments

Name	*Molecular formula*	*Occurrence*
Chlorophylls		
Chlorophyll *a* (monovinyl)	$C_{55}H_{72}N_4O_5Mg$	All photosynthetic algae and higher plants
Divinyl chlorophyll *a*	$C_{55}H_{70}N_4O_5Mg$	Some prochlorophytes
Chlorophyll *b* (monovinyl)	$C_{55}H_{70}N_4O_6Mg$	Green algae, symbiotic prochlorophytes and higher plants
Divinyl chlorophyll *b*	$C_{55}H_{68}N_4O_6Mg$	Some prochlorophytes
Chlorophyll c_1	$C_{35}H_{30}N_4O_5Mg$	Chromophyte algae, brown seaweeds
Chlorophyll c_2	$C_{35}H_{28}N_4O_5Mg$	Chromophyte algae, brown seaweeds
Chlorophyll c_3	$C_{36}H_{28}N_4O_7Mg$	Some prymnesiophytes, chrysophytes and diatoms
Mg-3,8-divinyl phytoporphyrin-132-methyl carboxylate	$C_{35}H_{30}N_4O_5Mg$	Some prasinophytes
Chlorophyll degradation products		
Chlorophyllide *a*	$C_{35}H_{34}N_4O_5Mg$	Senescent algae
Pheophytin *a*	$C_{55}H_{74}N_4O_5$	Algal and plant detritus (also small concentration in photosynthetic reaction centres)
Pheophorbide *a* + same types of degradation products for the other chlorophylls	$C_{35}H_{36}N_4O_5$	Zooplankton faecal pellets and marine detritus
Carotenoids		
Alloxanthin	$C_{40}H_{52}O_2$	Cryptophytes
Astaxanthin	$C_{40}H_{52}O_4$	Some green algae + marine animals
19′-Butanoyloxyfucoxanthin	$C_{46}H_{64}O_8$	Pelagophytes, some prymnesiophytes and dinoflagellates
β,γ-Carotene	$C_{40}H_{56}$	Minor pigment in green and red algae and in cryptophytes
β,β-Carotene	$C_{40}H_{56}$	Green and chromophyte algae and higher plants
Crocoxanthin	$C_{40}H_{54}O$	Cryptophytes
Diadinoxanthin	$C_{40}H_{54}O_3$	Diatoms, prymnesiophytes, some chrysophytes and dinoflagellates
Echinenone	$C_{40}H_{54}O$	Cyanobacteria + some green algae
Fucoxanthin	$C_{42}H_{58}O_6$	Diatoms, prymnesiophytes, brown seaweeds, some dinoflagellates
19′-Hexanoyloxyfucoxanthin	$C_{48}H_{68}O_8$	Many prymnesiophytes, some dinoflagellates
Lutein	$C_{40}H_{56}O_2$	Green algae, red seaweeds, higher plants
Neoxanthin	$C_{40}H_{56}O_4$	Brown seaweeds, chrysophytes, green algae, higher plants
Peridinin	$C_{39}H_{50}O_7$	Photosynthetic dinoflagellates
Prasinoxanthin	$C_{40}H_{56}O_4$	Some prasinophytes
Siphonein	$C_{52}H_{78}O_5$	Green algae and siphonous green seaweeds
Vaucheriaxanthin (ester)	$C_{40}H_{56}O_5$	Xanthophytes and eustigmatophytes
Violaxanthin	$C_{40}H_{56}O_4$	Green algae, brown seaweeds and higher plants
Zeaxanthin	$C_{40}H_{56}O_2$	Cyanobacteria, green algae, prochlorophytes, most chrysophytes and raphidophytes

fully end-capped C_{18} column. The resolution of mono- from divinyl chlorophyll *a* and *b* can be obtained on a C_8 column or by varying the temperature of a polymeric C_{18} column (see below).

The most complete, up-to-date information about the analysis of pigments, particularly for the use of aquatic scientists, can be found in a recent Scientific Committee on Oceanic Research (SCOR) UNESCO monograph edited by Jeffrey *et al*. This book covers sample collection, methods for pigment extraction and analysis, with emphasis on HPLC methods, comparisons with nonchromatographic methods, preparation of pigment standards and a key for identification of the various algal pigments.

Selection of Columns

Most methods attempting to separate the majority of pigments in a single step have used RP-HPLC with C_{18} columns (**Figure 2**). Presently, the most popular C_{18} columns for pigment analysis are the end-capped, monomer-coated C_{18} columns (**Table 2**), although these may not be the best for the separation of polar carotenoids. End-capping deactivates the remaining free silanol groups after the monomeric coverage. Columns from different companies differ in their carbon load (amount of bound C_{18}) and the type and extent of end-capping.

The separation of monovinyl from divinyl chlorophyll *a* and *b* was first achieved on NP silica columns, but these methods did not become very popular because of the reactivity of the stationary phase, the incompatibility of the extraction solvents with the technique and the lack of resolution of the polar pigments. These were replaced by C_8 columns, which can separate mono- from divinyl chlorophyll *a* and *b* but do not resolve all the chlorophyll *c* compounds (**Figure 3**).

The latest improvement in this field has been the development of columns which have multiple levels of C_{18} bound to the silica (often called polymeric octadecylsilica columns). These allow the most

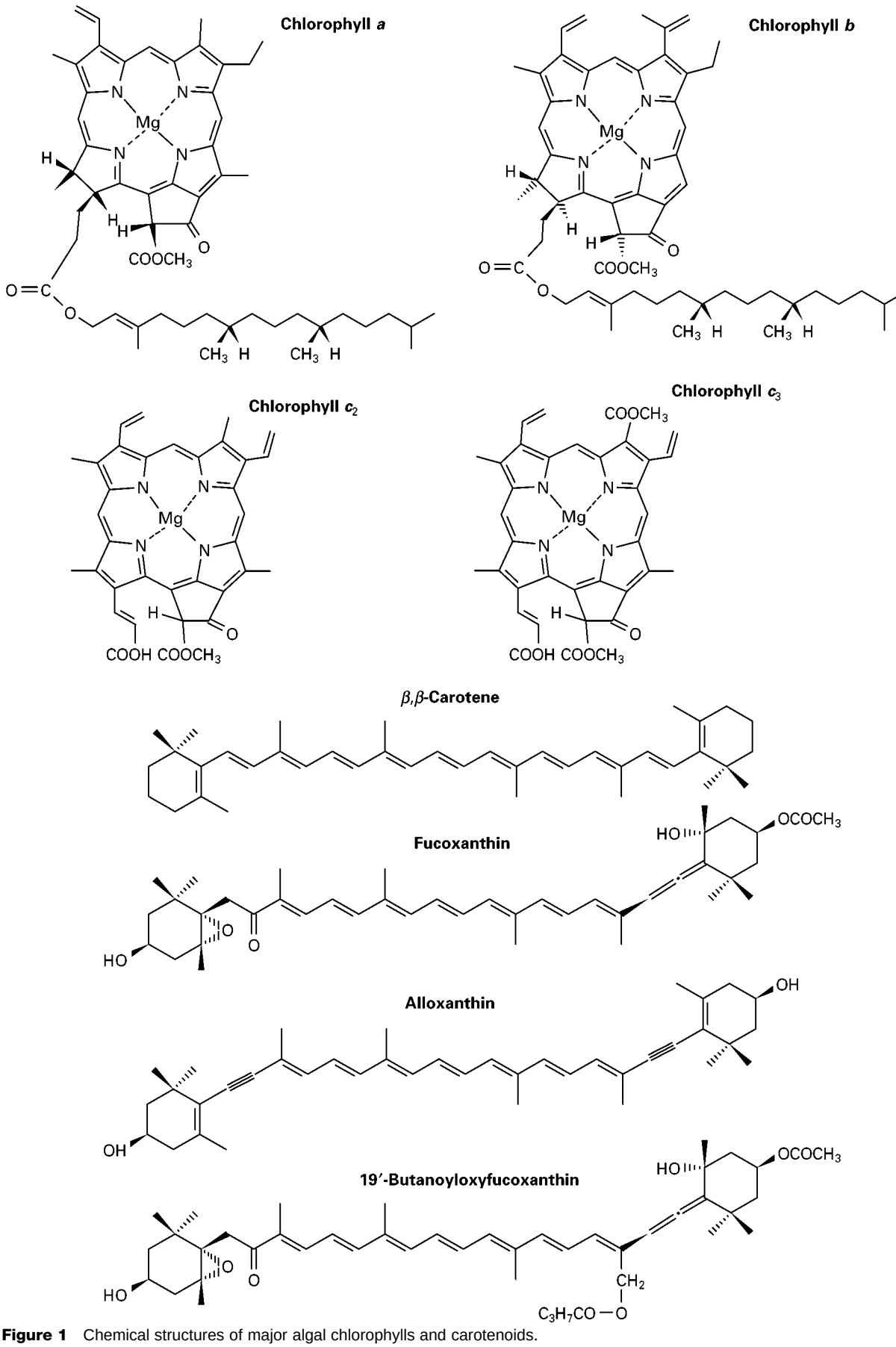

Figure 1 Chemical structures of major algal chlorophylls and carotenoids.

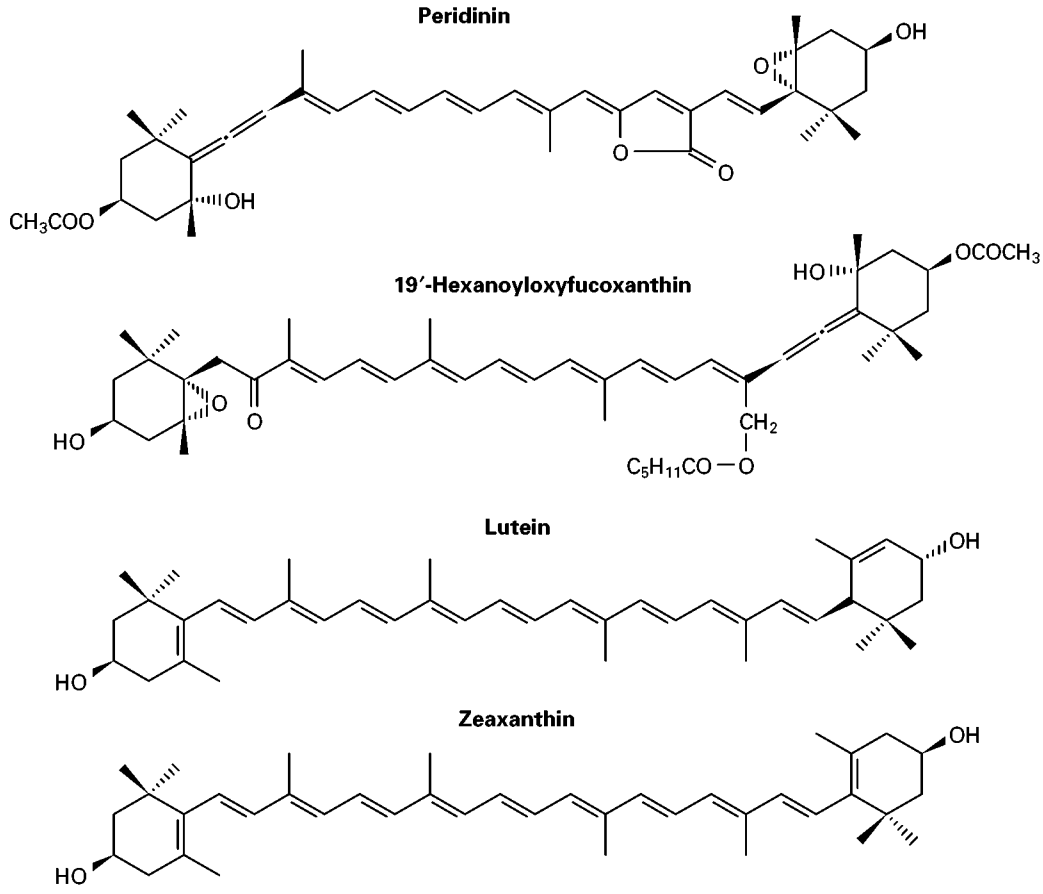

Figure 1 *Continued*

complete separation of chlorophylls and carotenoids but these coatings are more sensitive to temperature than monomeric columns, and hence the columns need to be thermostated. The work on marine pigments conducted in the laboratory of Zapata and Garrido has been instrumental in these developments (**Figure 4**). Recently, research scientists in the field of food science have developed a polymeric triacontyl (C_{30}) column which provides adequate retention of polar carotenoids (e.g. good separation of lutein and zeaxanthin) as well as much improved separation of the nonpolar carotenes (**Figure 5**).

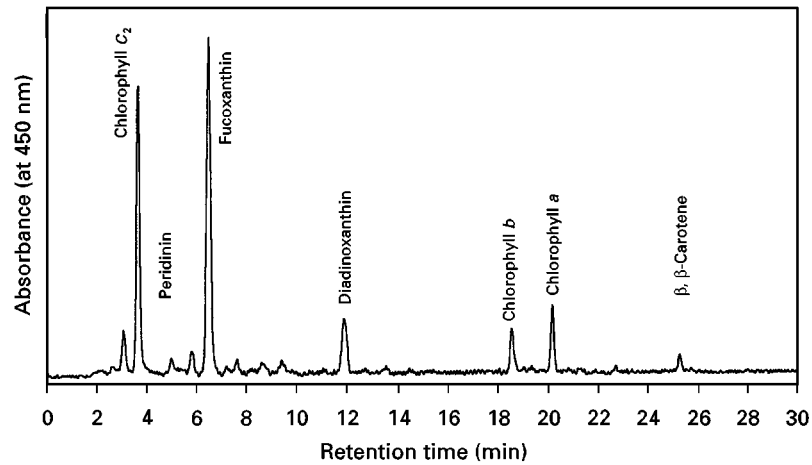

Figure 2 Analysis of pigments from ice algae from the Arctic (Roy, unpublished). Column 5 μm. Spherisorb ODS-2 (C_{18}). Separation conditions: 50 : 50 of (85% methanol–15% ammonium acetate 0.5 moL L^{-1}) : (90% acetonitrile–10% water) to 30 : 70 of (90% acetonitrile–10% water) : ethyl acetate over 33 min; 0.8 mL min^{-1}. Temperature 27°C.

Table 2 Selection of columns for HPLC pigment analysis

Type of column	*Carbon load*	*End-capping*	*Dimensions (mm)*	*Particle size (μm)*	*Type of analysis*
Reversed-phase (monomeric)					
Spherisorb ODS-2 (C_{18})	12%	Yes	250 × 4.6	5	Algal pigments
Ultrasphere C_{18}	12%	Yes	250 × 4.6	5	Plant pigments
Zorbax ODS	20%	No	250 × 4.6	5	Lutein/zeaxanthin
RSil C_{18}	16%	Yes	150 × 4.6	5	Chlorophyll c_1, c_2, c_3
Hypersil MOS-2 (C_8)	6–7%	Yes	100 × 4.6	3	MV and DV chlorophyll and lutein/zeaxanthin
Reversed-phase (polymeric)					
Vydac 201 TP	9%	No	250 × 4.6	5	MV and DV chlorophyll and chlorophyll c_1, c_2, c_3
Asahipak ODP-50	17%	No	250 × 4.6	5	MV and DV chlorophyll
Lichrospher PAH	21%	No	250 × 4.6	5	Chlorophyll c_3, MgDVP, phytol chlorophyll *c*
Normal-phase					
Rosil	10%	No	150 × 4.6	5	MV and DV chlorophyll *a* and *b*

MV, Monovinyl; DV, divinyl; MgDVP, Mg-3,8-divinyl phytoporphyrin-132-methyl carboxylate.

Mobile Phases

The most common mobile phases for pigment analysis currently use two to three solvents in a gradient

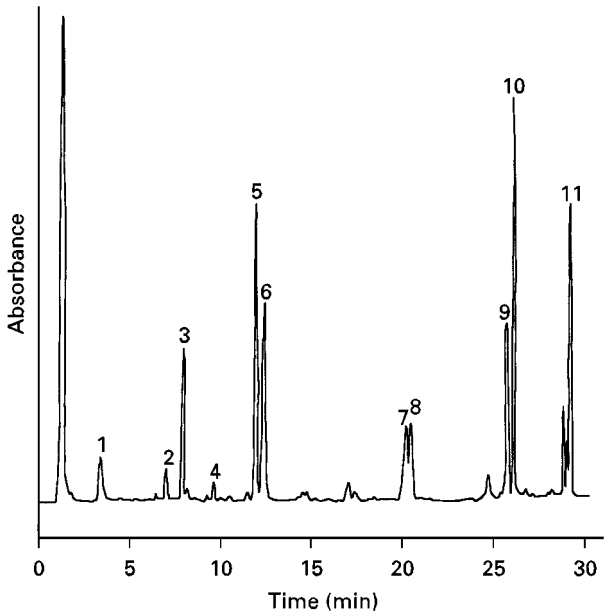

Figure 3 Analysis of pigments from algal cultures (*Dunaliella tertiolecta, Synechococcus* sp. and *Prochlorococcus* sp.). Column (3 μm) Shandon Hypersil MOS2 (C_8). Separation conditions: 75 : 25 of (70% methanol–30% ammonium acetate 1 mol L^{-1}): methanol to 100% methanol over 32 min; 1 mL min^{-1}. Peaks detected at 440 nm: 1, chlorophyll *c*-like; 2, neoxanthin; 3, violaxanthin; 4, antheraxanthin; 5, zeaxanthin; 6, lutein; 7, divinyl chlorophyll *b*; 8, chlorophyll *b*; 9, divinyl chlorophyll *a*; 10, chlorophyll *a*; 11, α- and β-carotene. (Reproduced with permission from Barlow RG, Cummings DG and Gibb SW (1997) Improved resolution of mono- and divinyl chlorophylls *a* and *b* and zeaxanthin and lutein in phytoplankton extracts using reverse phase C-8 HPLC. *Marine Ecology Progress Series* (*Inter-Research*) 161: 303–307.)

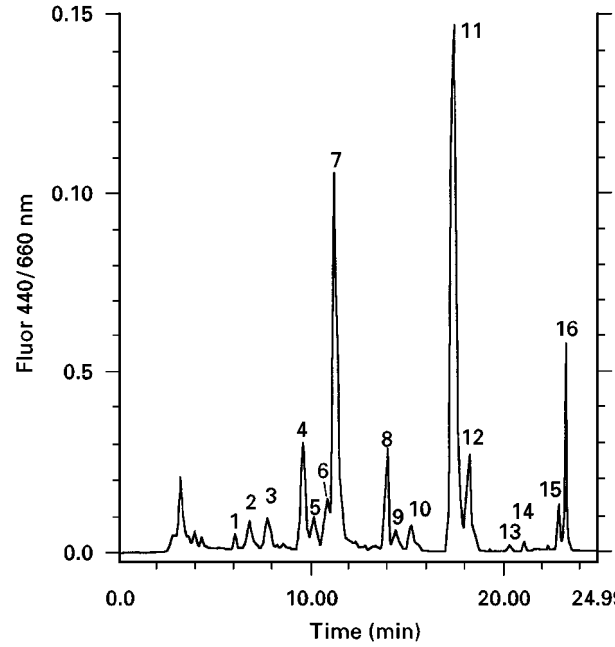

Figure 4 Analysis of pigments from marine phytoplankton. Column (5 μm) Vydac 201 TP. Separation conditions: 65 : 35 of (45% methanol–35% acetonitrile–20% aqueous pyridine, 0.25 mol L^{-1}) : acetone to 100% acetone over 24 min; 1.2 mL min^{-1}. Temperature 27°C. Peaks detected by their fluorescence (excitation = 440 nm, emission = 660 nm): 1, chlorophyll *c*-like; 2, chlorophyll *c*-like; 3, divinyl protochlorophyllide *a*; 4, chlorophyll c_1; 5, monovinyl chlorophyll c_3; divinyl chlorophyll c_3; 7, chlorophyll c_2; 8, chlorophyll *b*; 9, chlorophyll b_2; 10, chlorophyll *a* allomer; 11, chlorophyll *a*; 12, chlorophyll a_2; 13, chlorophyll *a* isomer; 14, chlorophyll a_2 isomer; 15, phytol-substituted chlorophyll *c*-like pigment; 16, phytol-substituted chlorophyll *c*-like pigment. (Reproduced with permission from Garrido JL and Zapata M (1997) Reversed-phase high-performance liquid chromatographic separation of mono- and divinyl chlorophyll forms using pyridine-containing mobile phases and a polymeric octadecylsilica column. *Chromatographia* 44: 43–49.)

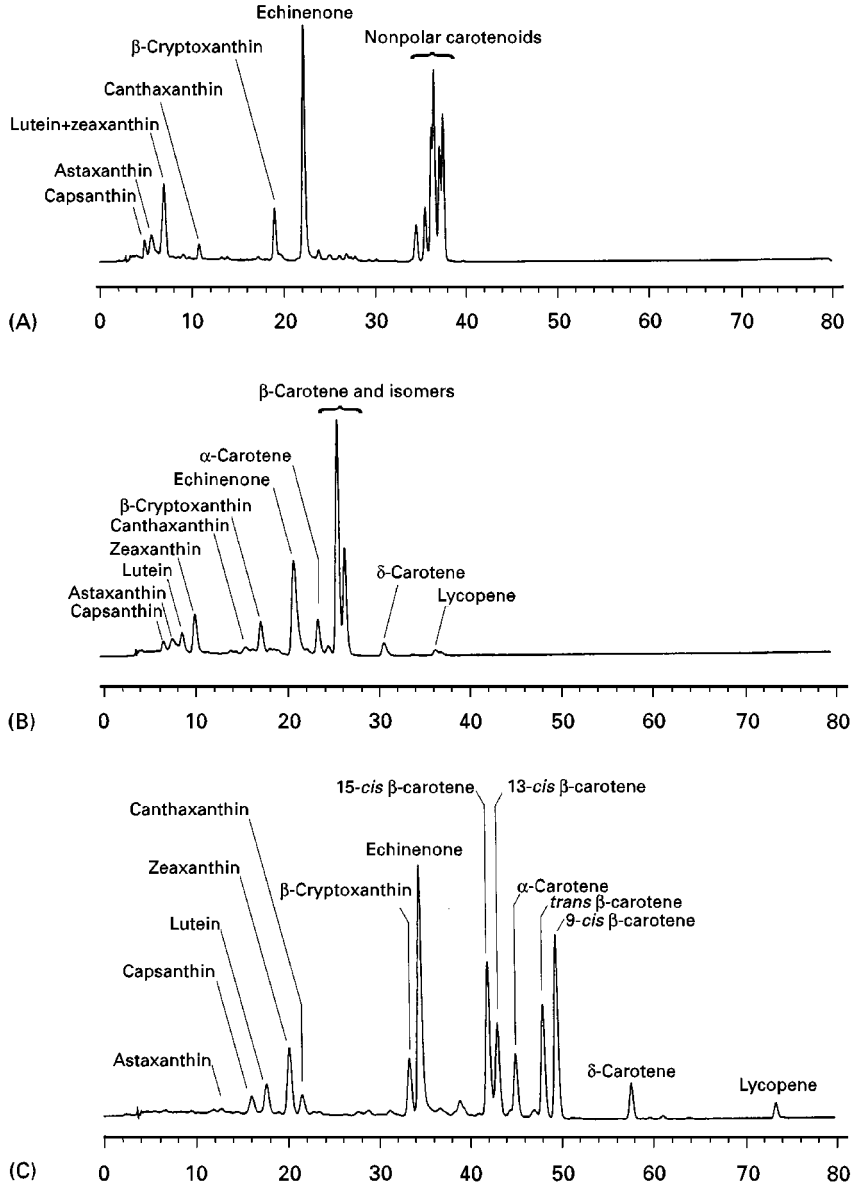

Figure 5 Separations of carotenoid standards on commercial (A) monomeric and (B) polymeric C_{18} columns, as well as (C) the polymeric C_{30} column. Separation conditions: 81 : 15 : 4 to 6 : 90 : 4 methanol–methyl-*tert*-butyl ether–water over 90 min; 1 mL min^{-1}. Temperature 20°C. (Reproduced with permission from Sander LC, Sharpless KE, Craft NE and Wise SA (1994) Development of engineered stationary phases for the separation of carotenoid isomers. *Analytical Chemistry* 66: 1667–1674.)

elution mode. Initial conditions often include 80–90% methanol buffered with ammonium acetate, followed by 90% acetonitrile. Ethyl acetate or acetone is commonly used as the third solvent to elute the less polar pigments such as the pheophytins. The newer methods use the same solvents, except that an organic modifier is added to the methanol/acetonitrile eluent. Various five-carbon amino compounds have been tested as ion pair reagents, with aqueous pyridine showing the best results with the Vydac 201 TP column. Again, temperature control is essential when using these columns.

Detection

Detection of compounds eluting from HPLC columns is normally done by recording the absorbance (chlorophylls and carotenoids) or the fluorescence (chlorophylls) of algal pigments. Three types of detectors are commonly used for absorbance: fixed wavelength, variable wavelength and full spectrum absorbance detector. The fixed wavelength models are the most sensitive, but the least informative of the three. Full spectrum instruments are now a general requirement of any HPLC set-up for pigment analysis. They

include photodiode array detectors and rapid scanning instruments. Both allow the full absorbance spectrum of each peak to be collected without stopping the flow, facilitating identification of peaks, to the detriment, however, of sensitivity. Instruments with a lamp that has high output in the visible range (e.g. tungsten) are preferred for pigment analysis because of their increased sensitivity.

Fluorescence detectors are either of the filter type, with a band pass excitation filter and a long pass emission filter, or dual monochromator instruments. The filter type is often sufficient for most uses, especially if used in conjunction with an absorbance detector. The use of fluorescence detection along with absorbance detection allows confirmation of the identity of chlorophyll-type compounds since only these fluoresce. Fluorescence detection is also often preferred for quantitative use because of its great sensitivity.

Standards

Few pigment standards were available commercially, until recently (**Table 3**). However, the high cost of these standards, and the unavailability of a number of them, has prompted researchers to prepare in-house standards, using well-defined algal cultures. Guidelines for the preparation of standards can be found in the specialized literature, and summarized in the recent SCOR UNESCO monograph on algal pigments in oceanography.

Applications

The analysis of photosynthetic pigments is instrumental in numerous studies of the photosynthetic system of bacteria, plants and algae. It is also used in the fields of agriculture and food chemistry, in studies of food processing, and in the use of natural food-colouring agents. In aquatic sciences, algal pigments are used as an index of algal biomass and as biomarkers of different taxonomic groups of algae, particularly when HPLC analysis is applied. This information can then serve in the monitoring of red tides (toxic algal blooms), in studies of aquatic food webs in relation to harvestable resources or in the study of water masses in oceanography. The recent development of satellite remote-sensing of the colour of the oceans, related to their pigment composition and concentration, allows a synoptic perspective previously unobtainable from ship studies. Present studies are developing algorithms that can estimate primary production over wide areas of the oceans from remotely sensed (e.g. sea-viewing wide field-of-view sensor (SeaWiFS)) chlorophyll data.

Table 3 Pigment standards available commercially

Pigment	*Company*	*Location*
Chlorophylls		
Chlorophyll *a*	Sigma Chemical Company	St Louis, Missouri, USA
	Fluka Chemie AG	Buchs, Switzerland
	Aldrich Chemical Co. Ltd	Milwaukee, Wisconsin, USA
Chlorophyll *b*	Sigma Chemical Company	St Louis, Missouri, USA
	Fluka Chemie AG	Buchs, Switzerland
	Aldrich Chemical Co. Ltd	Milwaukee, Wisconsin, USA
Chlorophyll c_2	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Carotenoids		
Alloxanthin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Astaxanthin	Sigma Chemical Company	St Louis, Missouri, USA
19′-Butanoyloxy-fucoxanthin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Canthaxanthin	Fluka Chemie AG	Buchs, Switzerland
	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
β,γ-Carotene	Sigma Chemical Company	St Louis, Missouri, USA
β,β-Carotene	Sigma Chemical Company	St Louis, Missouri, USA
	Fluka Chemie AG	Buchs, Switzerland
	Aldrich Chemical Co. Ltd	Milwaukee, Wisconsin, USA
Diadinoxanthin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Diatoxanthin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Echinenone	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Fucoxanthin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
19′-Hexanoyloxy-fucoxanthin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Lutein	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Lycopene	Sigma Chemical Company	St Louis, Missouri, USA
Myxoxanthophyll	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Neoxanthin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Peridinin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Prasinoxanthin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Violaxanthin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark
Zeaxanthin	Int. Agency for ^{14}C determ.	Hørsholm, Denmark

Future Developments

Future advances in the field of pigment chromatography can be expected from:

1. development of new RP-HPLC columns, particularly the polymeric ones, and associated testing of the effect of temperature on the separation of pigments with these columns. HPLC equipment will also need to be more closely temperature-controlled to meet this need
2. advances in the field of mass spectrometry, such as electron impact, field desorption and fast atom bombardment, that permit volatilization of small quantities of pigments, and the coupling of mass spectrometry with HPLC (LC-MS) or with supercritical fluid chromatography (SFC-MS). These new methods should be particularly useful in studies dealing with degraded or derived pigments, such as fossil pigments found in sediments or transformation of pigments in food, agricultural, physiological or ecological studies.

See Colour Plate 114.

See also: **II / Pigments:** Thin-Layer (Planar) Chromatography. **Terpenoids: Liquid Chromatography.**

Further Reading

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Thin-Layer (Planar) Chromatography

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General Introduction

Colour and Pigments

The colours found in natural organisms are of two types: those which are due to structural effects, and those which result from the presence of pigments. The two types of colouring often occur together. Examples of structural colour are to be found in the scales of fish, the wings of butterflies and the hair of many animals, although structural colour is also to be found in the plant world. The apparent colour depends on the interplay of these effects. It is thus important to realize at the outset that only the colour due to the pigments can be extracted. However, even then the *in vivo* and *in vitro* colours can be vastly different, since the pigments themselves show different colours according to the environment in which they occur – whether in fat globules or aqueous complexes.

Importance

Colour is used by almost all organisms to communicate in one form or another, for example, the defensive colours of insects, the attractive colours of flowers and fruit. The way in which various organisms produce pigments is genetically determined, and thus related organisms have similar pigment patterns and biologists use pigments to classify organisms at all levels. The commercial value of agricultural and particularly horticultural products is often closely related to their colour.

Natural Pigment Structures

The number of main pigment groups is relatively small. While various pigment groups are of vastly different skeletal types, there is usually little variation of the skeleton within each group and in many cases the possible group of pigments involved can be arrived at based on the observed colour in nature. Thus, while the green colour of chlorophylls is immediately recognizable, the typical flavonoids provide pale yellow colours, and the anthocyanidins, a special group of flavonoid salts, are usually responsible for red-blue colours in plants. The deep yellow to orange red shades resulting from the presence of carotenoids are also readily identified. Variations within a particular group are usually due to two factors: the degree of oxidation and the presence of substituents.

Extraction Methods

The different types of pigment vary greatly in polarity and in their sensitivity to chemical reagents, and thus require different extraction methods. It is best to avoid the use of acids or bases unless necessary, and