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REVIEW

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CHLOROPIGMENTS

SUZANNE ROY*
Department of Oceanography, Dalhousie University, Halifax, Nova Scotia, B3H 4J1 (Canada)
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CONTENTS

1. INTRODUCTION

The measurement of chlorophyll pigments and derivatives has traditionally been very important in oceanography because of the specific character of these algal pigments and their relative ease of measurement, especially using fluorescence. However, over the last 10 years, dissatisfaction has been expressed with the various methods for measuring chloropigments[1]. The principal concerns rested with interference of various chloropigments and the non-uniformity of different technical details among users. This was clearly expressed in the SCOR/Unesco report by Lorenzen and Jeffrey[2]. They noted that chromatography was the only acceptable method to obtain a precise knowledge of pigment composition.

Various forms of chromatography have been used through the years for pigment analysis, *viz.*, column[3], paper and thin-layer chromatography (TLC)[4,5]. Extensive reviews on the TLC of chloropigments have been published by Šestak[6,7]. However, these techniques have not found much popularity in oceanography because they are difficult to use at sea. The situation changed with the introduction of high-performance liquid chromatography (HPLC) in this field. Many different HPLC techniques have been used for chloropigment analysis in the last 10 years. Both normal-phase (NP) and reversed-phase (RP) HPLC techniques have been tried, although the reversed-phase mode seems to be favored by most researchers now. This review summarizes this information, concentrating on applications to the marine environment.

* Present address: INRS-Océanologie, 310 avenue des Ursulines, Rimouski, Québec, Canada G5L 3A1.

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TABLE 1

NORMAL-PHASE CHROMATOGRAPHY

F = flow-rate; P = pressure; n.a. = not available.

Ref.	*Analysis*	*Column*		*Mobile phase*		*Detection*		*Time (min)*
		Stationary phase	*Dimensions*	*Composition*	*F and/or P*	*Instrument*	*Wavelength (nm)*	
8	Natural porphyrins and Chl derivatives	Pellicular Si; Corasil II porous Si; Merckosorb SI 60 or Partisil 5, 10 μm	2 (90 × 0.22 cm)	20% ethyl acetate in light petroleum	F = 0.7 ml/min	Cecil variable-wavelength spectrophotometer	412 or 434	10–30
15	Algal Chl a and b, Phe a and b	Silica gel H (Sigma)	300 × 3 mm	20% acetone in ligroin	F = 2 ml/min, P = 1000 p.s.i.	LDC fluorimeter	n.a.	20
11	Spinach Chl a, a′, b and b′, Phe a and b and carotenoids	Silica gel SS-05, 0.5 μm	65 × 0.5 mm	Step gradient: 1% isopropyl alcohol in hexane for 20 min, 2% for 30 min, 5% for 12 min and 10% until 75 min	F = 16 μl/min	Uvidec UV detector	380	75
9	Spinach Chl a and b and carotenoids	Nucleosil 50–5, 5 μm	250 × 3 mm	10% ethanol in isooctane	F = 1 ml/min	Schoeffel 770 UV–visible spectrophotometer	445	15
16	Algal Chls and carotenoids	Partisil 10	300 × 0.45 mm	75% light petroleum–23.25% acetone–1.5% DMSO–0.25% dimethylamine for 35 min; change to 30:40:3:27% methanol for 30 min for polar pigments	F = 2 ml/min, P = 14 kg/cm²	Cecil CE-373 visible range spectrophotometer	440	60

14	Deep sea sediments, Chl diagenesis products	Spherisorb 3 μm silica	250 × 4.6 mm	10% acetone in hexane or gradient elution with acetone–hexane	F = 1–1.2 ml/min, P = 1000 p.s.i.g.	Variable-wavelength detector	n.a.	20
12	Higher plants Chl a and b	Spherisorb 5 μm	25 cm	Step gradient: 50% benzene in hexane for 39 min; 6% acetone in benzene from 40 to 52 min; 8% from 53 to 63 min; 16% from 64 to 80 min	F = 1 ml/min	Schoeffel FS970 spectrofluorimeter	425 (ex.)	80
17	Algal Chl a and b	LiChrosorb Si 60, 5 μm	200 × 2 mm	2,2,4-Trimethylpentane–acetone (5:2)	F = 0.5 ml/min, P = 1000 p.s.i.	LDC spectromonitor I (UV–visible)	440, 645, 665	10
18	Algal Chls and carotenoids	μPorasil	30 cm	75% light petroleum–22.75% acetone–2% DMSO–0.25% dimethylamine; change to 83% of above–11% methanol for polar pigments	F = 1 ml/min F = 2 ml/min	Waters 440 spectrophotometer Perkin-Elmer 2000 fluorimeter	436 436 (ex.)	30
13	Cucumber mono- and divinyl pChlids	LiChrosorb Si 60, 10 μm + Whatman HC Pellosil guard column	250 × 4.6 mm	90% hexane–10% acetone	F = 7 ml/min, P = 900 p.s.i.	Waters 440 spectrophotometer	436	25
10	*Chlorella* sp. Chl and Phe a, a′, b and b′ (preparative isolation)	Preparative, Nucleosil 50–5, 5 μm; analytical, id.	250 × 20 mm 150 × 4.6 mm	Preparative, 3% 2-propanol in hexane; analytical, 1–2% 2-propanol in hexane	P = 38 kg/cm² F = 1 ml/min	Uvilog-7 variable-wavelength detector	430	40

2. NORMAL-PHASE HPLC TECHNIQUES

The earliest HPLC method described for pigment analysis is that of Evans *et al.*[8], who used two long (90 × 0.22 cm I.D.) columns of stainless steel packed with pellicular silica (Corasil II) or porous silica (Merckosorb SI 60 or Partisil) to separate natural porphyrins and chlorophyll derivatives in a short time (10–30 min) by eluting with various proportions of ethyl acetate in light petroleum. Flow programming and gradient elution were tested. Results were given only for pheophytin *a* and *b* among the major chloropigments.

In the field of plant biochemistry, other examples of normal-phase separations of chloropigments are given in Table 1. Stransky[9] and Watanabe *et al.*[10] used Nucleosil columns, but with different mobile phases (see Table 1). Iriyama *et al.*[11] used a micro-column made of a fine PTFE tubing (65 × 0.5 mm I.D.) packed with silica gel powder (particle size 0.5 μm) (Japan Spectroscopic) and eluted pigments with a 75 min step gradient of isopropyl alcohol in hexane. Rebeiz *et al.*[12] used a 25 cm Spherisorb column (particle size 5 μm) with a step gradient of benzene, hexane and acetone. Hanamoto and Castelfranco[13] developed conditions for separating the more polar chlorophyllides (Chlid) of higher plants using a LiChrosorb silica column and a hexane–acetone mobile phase.

Geochemists have also shown interest in chloropigments, especially related to the phenomenon of diagenesis in marine sediments. A few studies have used NP HPLC[14] (see Table 1 for details).

The earliest description of an NP HPLC method for the analysis of phytoplankton pigments in oceanography was made by Jacobsen[15], who obtained a good separation of chlorophyll (Chl) *a* and *b* and pheophytin (Phe) *a* and *b* in 20 min using a silica-packed column and isocratic elution with a mixture of acetone and ligroin. Abaychi and Riley[16] were able to separate pheophorbide (Pheid) *a* and Chl *c* (these more polar chloropigments require different conditions than the phytolated chloropigments) using NP HPLC with a Partisil 10 column. They changed their solvent mixture to a more polar composition after about 40 min. Lorenzen[17] used an NP HPLC technique to determine the importance in the oceans of Chl *b*, a pigment that interferes significantly with the popular fluorimetric technique. He used a LiChrosorb silica column and eluted isocratically with a mobile phase composed of 2,2,4-trimethylpentane and acetone. Finally, Gieskes and Kraay[18] used an NP HPLC method similar to that of Abaychi and Riley[16] in their work in the North Sea and central Atlantic ocean. They identified unknown Chl *a* derivatives, which accounted for a large part of the difference between HPLC and conventional measurements of Chl *a*.

Thus, as can be seen in Table 1, a great variety of stationary and mobile phases exist for the NP HPLC determination of chloropigments. For relatively complete separations of phytolated chloropigments and carotenoids (although not discussed in this review, the conditions used for chloropigments also separate most carotenoids), the techniques of Jacobsen[15], Iriyama *et al.*[11], Stransky[9] and Abaychi and Riley[16] seem to be the most appropriate. Of these, Stransky's[9] method is the fastest (15 min) and gives a good resolution of all the major low-polarity chloropigments. In addition, it uses a simple isocratic elution. However, all of these separations encounter problems with the more polar (dephytolated) chloropigments (Chl *c*, Chlid and Pheid).

These pigments are found to accumulate on the columns, causing deterioration[16,17], and their separation, attempted by only a few workers[16,18], requires a relatively large increase in polarity, achieved by changing to a different solvent under isocratic conditions.

Some workers have also mentioned another problem, that of the relatively high reactivity of the silica stationary phases, in some instances causing degradation of pigments[12]. However, others have not encountered this problem[16].

3. REVERSED-PHASE HPLC TECHNIQUES

Because most HPLC methods used for pigment analysis are in this category, a sub-division will be used: isocratic elution will be treated first, followed by gradient elution.

3.1. Isocratic elution

This form of HPLC has the advantage that simple equipment is required, as no change in the eluent composition takes place during the chromatographic run.

The first report of chloropigment separation using isocratic RP techniques is that by Staub[19], who studied the stratigraphy of diatoms and chlorophyll derivatives in the uppermost sediment of a lake in Switzerland. The chloropigments were analysed by RP HPLC on a C_{18} column eluted with methanol for about 30 min. Again, the resolution of the polar pigments was poor.

Other reports in aquatic sciences include that of Shoaf[20], who presented a simple RP HPLC separation of algal Chl *a* and *b* using a C_{18} column and eluting with a mixture of methanol and water. This was followed by the work of Liebezeit[21] and Bessière and Montiel[22], who separated Chl *a* and *b* and Phe *a* and *b* using Spherisorb ODS columns and similar mobile phases to that of Shoaf[20]. Bessière and Montiel[22] also compared various columns for the determination of Chl *a* and *b* and found Spherisorb S5W ODS C_{18} to be the best. They also noted that some filtration membranes made of cellulose derivatives interfered with the RP HPLC separation, and that the dimethyl sulphoxide extractant caused a large increase in viscosity and affected the resolution if injected directly into the column. Finally, Goeyens *et al.*[23] reported an isocratic RP HPLC separation of chloropigments, of which only Chl *a* was positively identified. Table 2 summarizes the technical details.

A number of plant biochemists also used RP HPLC isocratically for pigment separations: Schoch *et al.*[24] and Schoch[25] separated complex mixtures of pheophytins esterified with different C_{20} diterpene alcohols with the help of a LiChrosorb RP-8 or Bondapak C_{18} column eluted with methanol and acetone. Burke and Aronoff[26] separated Chl *a*,*a'*, *b* and *b'* on a Varian CH10 column. A change of mobile phase composition was necessary to elute the unwanted pheophytins. Rebeiz *et al.*[12], Scholz and Ballschmiter[27,28,29], Gleixner *et al.*[30], Shioi and Sasa[31] and Shioi *et al.*[32] used C_{18} columns for separations of various Chl *a* and *b* derivatives and bacteriochlorophylls (bChl)[28,29,32]. Various mobile phases were used but they were generally composed of methanol, acetone, water and acetonitrile.

A general conclusion of these studies is that isocratic elution is well suited for the rapid and good resolution of the low-polarity photosynthetic pigments (Chl and Phe). However, the more polar pigments are not well resolved with the techniques

TABLE 2

REVERSED-PHASE CHROMATOGRAPHY: ISOCRATIC ELUTION

Ref.	*Analysis*	*Column*		*Mobile phase*		*Detection*		*Time (min)*
		Stationary phase	*Dimensions*	*Composition*	*F and/or P*	*Instrument*	*Wavelength (nm)*	
19	Sedimentary chloropigments	μBondapak C_{18}	300 × 6.4 mm	100% methanol	F = 2 ml/min, P = 400 p.s.i.	Spectrophotometer	665	30
20	Algal Chl a and b	Whatman Partisil PXS 1025, ODS-2	25 cm	95% methanol–5% water	F = 4 ml/min	Variable-wavelength spectrophotometer	654	30
25	Phe esterified with different alcohols	LiChrosorb RP-8, 5–10 μm	250 × 5 mm	95% methanol–5% water	F = 1.5 ml/min	LDC 1202 variable-wavelength detector	667	20
24	Phe esterified with different alcohols	μBondapak C_{18}	100 × 4 mm	90% methanol–10% acetone	F = 1.5 ml/min	LDC 1202 variable-wavelength detector	410	15
26	Chl, a, a′, b and b′	Varian CH10	n.a.	95% methanol–5% water	F = 4 ml/min	n.a.	648 nm (Chl b), 665 nm (Chl a)	40
21	Algal Chl a	Spherisorb ODS, 5 μm	230 × 4 mm	94.5% methanol–5.5% water	F = 2 ml/min	LDC-UVIII or Gilson Spectrochrom M or Schoeffel FS970 spectrofluorimeter	254 or 650; 390 (ex.), 580 (em.)	50

12	Higher plant Chls	Spherisorb ODS, 10 μm	25 cm	75% methanol–20% acetone–5% water	F = 1 ml/min	Schoeffel FS970 fluorimeter	425 (ex.)	10
27	Spinach Chl a, a′, b, and b′	LiChrosorb RP-18, 5 μm	120 × 4.5 mm	94% acetonitrile–6% water	F = 3 ml/min, P = 2000 p.s.i.	Perkin-Elmer LC55 spectrophotometer	436	30
28	Photosynthetic bacteria bChls	LiChrosorb RP-18, 5 μm	n.a.	80% acetonitrile–20% water	n.a.	n.a.	365	15
29	Photosynthetic bacteria bChls	LiChrosorb RP-18, 5 μm	120 × 4.5 or 250 × 8 mm	For bChl a: 85% acetonitrile–15% water For bChl b: 90% acetonitrile–10% water	F = 1–4 ml/min, P = 50–250 atm	Alltex 153 spectrophotometer	365	80
30	Cyanobacteria Chl a and derivatives	μBondapak C_{18}	300 × 3.9 mm	95% acetonitrile–5% tetrahydrofuran	F = 1 ml/min	n.a.	380	30
22	Algal Chl a and b	Spherisorb S5W, ODS C_{18}	250 × 3 mm	97% methanol–3% water	F = 1 ml/min	Schoeffel FS970 fluorimeter	427 (ex.), >470 (em.)	15
23	Algal Chl a	Alltex RP-18, 5 μm	250 × 4 mm and 250 × 10 mm	75% methanol–22% acetone–3% water 75% methanol–25% acetone	F = 1 ml/min F = 3 ml/min	UV–visible Micrometrics 785 and Gilson Spectra GLO fluorimeter	428, 5–60 + 2–60 filters	45
31	Higher plant protoChls	DuPont Zorbax ODS	250 × 4.6 mm	100% methanol	F = 1.5 ml/min	Hitachi 650-60 spectrofluorimeter	440 (ex.) 640 (em.)	22
32	Higher plant protoChls + bChl a	DuPont Zorbax ODS	250 × 4.6 mm	100% methanol	F = 1–1.5 ml/min	Hitachi 650–60 spectrofluorimeter	Variable	20

described above. In most studies reported here a C_{18} column was used and the elution solvent most often consisted of high proportions (>95%) of methanol in water. Acetonitrile could also replace methanol and give good results. In fact, it was recommended over methanol in some instances[27] because of the possibility of allomerization occurring with methanol.

3.2. Gradient elution

In gradient elution, the eluent composition is generally continuously changing (unless stepwise elution is used). This requires more sophisticated HPLC instrumentation, normally including a mixing chamber where two or more solvents can be mixed in different proportions according to a pre-selected program before being pumped into the column. It is often preferable to use gradient elution when the separation involves a series of compounds of greatly varying polarities, as in pigment analysis.

Eskins *et al.*[33] were the first to use gradient elution RP HPLC for chloropigments. They separated and identified chlorophylls and carotenoids from spinach and the marine alga *Nitzschia closterium* using two 610 × 7 mm I.D. C_{18} columns in series and a mobile phase consisting of various mixtures of methanol, water and diethyl ether. A total time of 290 min was needed for the complete separation of the major pigments. Of the polar pigments, only Chl *c* was identified.

Other studies in plant physiology and biochemistry include the resolution of various forms of bChl *c* from the green sulfur bacteria *Chlorobium limicola,* using polyethylene powder as the stationary phase[34]. Disposable Sep-Pak cartridges (Waters Assoc.) were used by Eskins and Dutton[35] in their sample preparation procedure for the RP HPLC of chloropigments. Gugliemelli *et al.*[36] separated Chl *a*, c_1 and c_2 from pigment–protein complexes of *Phaeodactylum tricornutum* using a μBondapak C_{18} column and gradient elution with various proportions of methanol, water and ethyl acetate. Burke and Aronoff[37] were able to separate the chlorophyllides, pheophorbides and methyl pheophorbides of higher plants using semi-preparative columns and mixtures of methanol and water. This type of column was also utilized by Eskins and Harris[38] for the separation of protochlorophyllide, chlorophyllide and various forms of Chl *a*. Braumann and Grimme[39] compared the separation of low-polarity chloropigments from the green alga *Chlorella fusca* using a C_{18} and a C_8 column. They found that the separation took longer with the C_{18} column but that the resolution was better than with the C_8 column. The separation of Chl *a* and *a'* could only be achieved with the C_{18} column. They also demonstrated the negative effects of silica gel stationary phases by passing a spinach chloroplast extract through a low-pressure silica gel column and re-chromatographing the chlorophyll fraction on their RP HPLC system. Several artificialy produced chlorophyll isomers were reported, proving the reactivity of the silica gel.

Brown *et al.*[40] were the first to report the use of a gradient elution C_{18} RP HPLC technique for chloropigments in oceanography. They used it to analyse sedimentary pigments. However, separation was restricted to the low-polarity (phytolated) chloropigments. Falkowski and Sucher[41] reported a good resolution of all major chloropigments (including the more polar ones but excluding allomers and isomers) using a C_8 column and a step gradient consisting of methanol and water. Liebezeit and Bartel[42] used a gradient composed of four solvents (methanol, water,

acetone and acetonitrile) to resolve, not always completely, sedimentary chlorophylls, pheophytins, chlorophyllides, pheophorbides and Chl *c*. Gieskes and Kraay[43] analysed phytoplankton chloropigments from the North Sea by gradient elution RP HPLC. They reported the separation of Chl *a* and two of its derivatives, of Chl *b* and *c* and many carotenoids on a LiChrosorb RP-18 column with a mobile phase of methanol, ethyl acetate and water. Wright and Shearer[44] also used RP HPLC for the analysis of algal chloropigments and carotenoids. Their chromatographic system was modified from that of Eskins and Dutton[35]; they also utilized a Sep-Pak cartridge clean-up procedure, but replaced methanol with acetonitrile in the mobile phase to obtain an excellent resolution for both high- and low-polarity chloropigments. They noted, as did Gieskes and Kraay[18], the presence of a number of Chl *a* derivatives, including Chl *a'* and allomers of Chl *a* in extracts of healthy algal cultures. They also analysed salp faeces and krill gut contents and found three or four unknown pigments thought to be Pheid *a* derivatives. Sartory[45] described a gradient elution RP HPLC method by means of which all the major chloropigments were separated, but with better resolution for the non-polar chloropigments. Again, based on Eskins and Dutton[35], a clean-up sample preparation step using Sep-Pak C_{18} cartridges preceded the HPLC proper. Pigment separation was realized on a C_{18} column eluted with a gradient mixture of methanol, acetone and water.

Technical details of the various methods using gradient elution discussed in this section are given in Table 3.

In summary, gradient elution RP HPLC is better than isocratic elution for chloropigment analysis because of the range of polarities encountered among the various chloropigments. With gradient elution, separation of the more polar pigments can be carried out in the same chromatographic run as the other chloropigments. Of the work discussed here, this is best accomplished with the conditions developed by Falkowski and Sucher[41], Gieskes and Kraay[43], Wright and Shearer[44] and Sartory[45]. Some methods may be preferred to others, depending on the degree of resolution needed. However, for general oceanographic application, the methods of Falkowski and Sucher[41] and of Wright and Shearer[44] can be recommended for their short analysis times (20 min or less) and good separations of the major pigments. In general, C_{18} columns were preferred over others because of increased resolution. Many of the mobile phases include methanol and water, and polar pigments usually need 10–20% of water for elution, whereas the pheophytins will elute with 100% methanol. Acetonitrile may be favored over methanol as in isocratic elution[44], and acetone or ethyl acetate is often chosen as a third solvent to improve the selectivity further.

4. ION-PAIR HPLC TECHNIQUES

Ion-pair chromatography (IPC), as adapted to modern LC, was first applied in the mid-1970s. It finds its usefulness with difficult separations involving compounds that are very polar, multiply ionized and/or strongly basic. IPC can be carried out in either NP or RP modes, but the latter is more popular. Ion suppression will also be included in this section.

The first application of IPC to chloropigment analysis was described by Hanamoto and Castelfranco[13], who separated monovinyl and divinyl protochlorophyl-

TABLE 3

REVERSED-PHASE CHROMATOGRAPHY: GRADIENT ELUTION

Ref.	*Analysis*	*Column*		*Mobile phase*		*Detection*		*Time (min)*
		Stationary phase	*Dimensions*	*Composition*	*F and/or P*	*Instrument*	*Wavelength (nm)*	
33	Spinach and algal Chl and carotenoids	2 columns in series packed with 37–75 μm Bondapak C_{18}–Porasil B	610 × 7 mm	80% methanol, 0–20 min; 90%, 20–65 min; 95%, 65–110 min; 97.5%, 110–175 min; 100% 175–215 min; 10% ether in methanol 215–245 min; 50%, 245–270 min; 75%, 270–290 min	F = 2.5 ml/min, P = 600 p.s.i.	UV detector	440	290
34	Green sulfur bacteria bChl c	USI-Microthene FN 500 polyethylene powder	275 × 8.9 cm	65–75% aqueous acetone gradient	F = 14 ml/min, P = 2000 p.s.i.	Spectrophotometer	n.a.	2400
35	Soybean pigments	μ-C_{18}	n.a.	90% methanol in water at 0 min to 50% ethyl acetate–45% methanol–5% water at 20 min	n.a.	Spectrophotometer	436	30
36	Algal Chl–protein complexes	μBondapak C_{18}	300 × 3.9 mm	90% methanol in water at 0 min to 50% methanol–50% ethyl acetate at 20 min	F = 1 ml/min	Waters 440 absorbance detector	436	20
37	Higher plant polar pigments	Varian CH10	n.a.	95% methanol in water, 0–19 min; 96%, 19.1–40 min; 96% → 100%, 40.1 → 45 min; 100% methanol, 45–60 min	F = 4 ml/min	n.a.	445	60
	Chlids	Whatman PXS 1025 ODS-2, Magnum 9 series	n.a.	85% methanol in water, 0–25 min; 95% 25–60 min	F = 4 ml/min		445	60
	Pheids	Whatman PXS 1025 ODS-2, Magnum 9 series	n.a.	95% methanol in water	F = 4 ml/min		445	18

38	Bean leaf pChlid, and carotenoids	Analytical or semi-preparative μ-C_{18}	n.a.	80% methanol in water at 0 min to 50% ethyl acetate–40% methanol–10% water at 20 min	F = 1 ml/min (analytical) F = 2 ml/min (preparative)	n.a.	436	45
40	Sediment Chl and Phe	Brownlee RP-18, 10 μm + Waters Bondapak C_{18} (37–50 μm) guard column	250 × 4.6 mm, 20 × 4.6 mm	95% methanol in water at 0 min to 75% methanol–20% acetone–5% water at 6 min	F = 2 ml/min	Schoeffel FS970 spectrofluorimeter	412 (ex.) >550 (em.)	12
41	Algal Chls	LiChrosorb RP-8, 5 μm + LiChrosorb RP-8 guard column	150 × 4.6 mm, 50 × 4.6 mm	90% methanol in water, 0–4 min; 98%, 4–16 min	F = 1.5 ml/min	Farrand Optical A-4 filter fluorimeter	420 (ex.)	16
39	Higher plant Chls	LiChrosorb RP-8, 10 μm or Sil60 RP-18, 10 μm	250 × 4.6 mm, 300 × 3 mm	60% acetonitrile–20% methanol–20% water at 0 min to 71% acetonitrile–24% methanol–5% water at 10 min; 75% acetonitrile–25% methanol, 10-20 min or 75% (75% acetonitrile–25% methanol) in water to 100% in 20 min	F = 1.7 ml/min F = 1.5 ml/min	Perkin-Elmer LC-55 variable-wavelength UV–visible detector	445	20
42	Fossil chlorins	Spherisorb ODS, 5 μm	230 × 4 mm	60% methanol in water to 50% acetone–50% acetonitrile in 30 min	F = 1.5 ml/min	Schoeffel FS970 spectrofluorimeter	390 (ex.), >580 (em.)	40
43	Algal pigments	LiChrosorb RP18, 10 μm	300 × 3.9 mm	25% (20% ethyl acetate–80% methanol)–75% (70% methanol–30% water) to 95:5 in 40 min	F = 1.5 ml/min	Waters 440 absorbance detector + Perkin-Elmer 2000 spectrofluorimeter	436 436 (ex.), 660 (em.)	40
44	Algal pigments	2 Rad-PakA cartridges (C_{18}, 5 μm) in series + RCSS Guard guard column	n.a.	90% acetonitrile in water to 100% ethyl acetate in 20 min. Also 90% methanol in water; 80% acetonitrile in water and 84% methanol–10% water–6% THF	F = 2 ml/min	Waters 440 2-channel absorbance detector	405 and 436	20
45	Algal Chls and polar pigments	Waters Resolve RP-C_{18}, 5 μm + Sep-Pak cleaning	150 × 3.9 mm	100% (97% methanol–water), 0–15 min; gradient to 77% (97% methanol–water)–23% (97% acetone–water), 15 to 20 min, fixed to 40 min	F = 1 ml/min	Varian Fluorochrom filter	350–550 (ex.), >600 (em.)	40

TABLE 4

ION-PAIR CHROMATOGRAPHY

Ref.	*Analysis*	*Column*		*Mobile phase*		*Detection*		*Time (min)*
		Stationary phase	*Dimensions*	*Composition*	*F and/or P*	*Instrument*	*Wavelength (nm)*	
13	Higher plants mono- and divinyl pChlid	Beckman Ultrasphere ODS, 5 μm + Whatman CO:Pell ODS pre-column	250 × 4.6 mm	70% methanol–30% PicA reagent (5 m*M* soln.)–6% methyl ethyl ketone for 0–73 min; 70%:30%:12% for 73–105 min (N.B.: temp. = 0°C)	*F* = 0.8 ml/min, *P* = 3000 p.s.i.	Waters 440 absorbance detector	436	105
49	Algal pigments	ODS-Hypersil, 5 μm (tested Zorbax C3-TMS, C_8 OS and C_{18} ODS)	250 × 5 mm	80% methanol–10% water–10% ion-pairing soln. at 0 min to 80% methanol–20% acetone at 10 min; fixed to 22 min	*F* = 1.8–3.2 ml/min, *P* = 2000 p.s.i.	DuPont 836 fluorimeter + (in series) Perkin-Elmer LC-75 variable-wavelength stop-flow absorbance detector	430 (ex.), >600 (em.) 440	25
50	Algal pigments	Hypersil C_{18}, 3 μm	100 mm	80% methanol–10% water–10% ion-pairing soln. at 0 min to 60% methanol–40% acetone at 5 min; fixed to 10 min	*F* = 1.5 ml/min	Hewlett-Packard 1040A diode array spectrophotometer. DuPont 838 fluorescence detector	440 430–440 (ex.), >600 (em.)	10

51	Algal chloropigments	Radial-Pak C_{18}, 10 μm	100 × 8 mm	80% methanol–10% water–10% ion-pairing soln. from 0 to 3 min; 100% methanol from 3 to 15 min	F = 9.9 ml/min	Perkin-Elmer 650-40 spectrofluorimeter	434 (ex.), 670 (em.)	15
46, 47	Photosynthetic bacteria bChl b	Knauer RP-8	300 × 4 mm	89% methanol–11% sodium ascorbate solution (1%)	F = 1.5 ml/min	LDC II variable-wavelength detector	600	10
48	Higher plant Chlid and Pheid	Whatman Partisil-10 ODS-2 or DuPont Zorbax ODS	250 × 4.6 mm	95% methanol–5% (13 mM acetic acid in water, pH 4.2) or gradient 80 → 100% methanol in the acetic acid solution	F = 1 ml/min	Hitachi Model 650-60 fluorimeter	430 (ex.), 650 (em.)	30
52	Algal chloropigments	Hypersil ODS-5	250 × 4.6 mm	100% (80% methanol–10% water–10% soln. P) at 0 min to 80% methanol–20% acetone at 10 min; fixed to 24 min	F = 1.5–2.5 ml/min	Schoeffel FS970 fluorimeter	430 (ex.), >580 (em.)	30
53	Algal pigments	Waters Novapack C_{18} + Bondapak C_{18} Corasil	150 mm	100% (70% methanol–30% phosphate buffer) at 0 min to 100% (80% methanol–20% ethyl-acetate) at 20 min	F = 0.8 ml/min	Waters 440 absorbance detector Perkin-Elmer 2000 spectrofluorimeter	436 or 658 407 (ex.), 670 (em.)	30

lides and chlorophyllides from cucumber cotyledons. They used Waters Assoc. Pic A reagent (5 m*M* solution of tetrabutylammonium phosphate, pH 7) as the ion-pairing substance. This reagent was used to stabilize the carboxylate ions of the chlorophyllides which have no phytol side-chain. Other reports of the use of IPC for pigment separation in the field of plant biochemistry include the work of Steiner and co-workers[46,47], who used a form of ion suppression in their separation of bChl *b* to prevent this pigment from reacting with the stationary phase during the chromatographic separation. This was done by adding 1% sodium ascorbate to the aqueous phase (Table 4). Shioi *et al.*[48] used a 13 m*M* solution of acetic acid in the aqueous phase to prevent interaction between sample components and the stationary phase for measurement of chlorophyllides and pheophorbides. The stationary phase used (Partisil ODS-2) contained 25% residual silanol groups, which caused high and selective adsorption of ionized solutes. Under acidic conditions, the ionization was suppressed and separation could take place.

Ion-pair chromatography has also been applied in oceanography. Mantoura and Llewellyn[49] first used it to obtain, in one run, the separation of all major polar and non-polar chloropigments and carotenoids. A Hypersil ODS column was chosen from a number of different RP columns tested because of the absence of residual silanol sites (fully end-capped), thus reducing the chance of adsorption on these sites or of interaction with ionized sample molecules. The notably difficult separation of the polar pigments was traced to the anionic character of the carboxylic acid group which replaces the phytol side-chain in these pigments. Dissociation of the carboxylic acid group, normally occurring at neutral pH, can be suppressed by ion pairing with a counter ion. Of the various compounds tested, Mantoura and Llewellyn[49] found buffered (pH 7.1) tetrabutylammonium acetate to be the best. In subsequent work[50], they reduced the analysis time to about 10 min by using a 5 min instead of a 10 min linear gradient and increasing the strength of the final solvent composition from 80:20 to 60:40 methanol–acetone. They also suggested the use of Hamilton Chrom Prep cartridges in a trace enrichment step for the concentration of algal pigments from oligotrophic waters. Bidigare *et al.*[51] based their work on Mantoura and Llewellyn's 1983 earlier work[49], but they chose a regular RP-18 column (see Table 4) and changed the eluent composition, starting with Mantoura and Llewellyn's[49] initial solvent phase and ending their gradient with 100% methanol after 3 min. The separation time was reduced to 15 min by using a much higher flow-rate (9.9 ml/min). Roy[52] also used Mantoura and Llewellyn's[49] technique (identical conditions) to separate successfully chloropigments from algal cultures and natural phytoplankton populations. Gieskes and Kraay[53] proposed a slightly different approach: they used a phosphate buffer instead of the ion-pairing reagent of Mantoura and Llewellyn[49], advocating the lower cost of the chemicals involved. In their system, a 15-cm long Waters Assoc. Novapak C_{18} column was eluted with a gradient mixture of methanol, ethyl acetate and phosphate buffer.

Table 4 summarizes the technical details of the methods using IPC for chloropigment analysis. IPC is the latest development in the HPLC analysis of these pigments; by this means a good separation of all the major chloropigments and carotenoids can be achieved in a single run within a short time.

5. SUMMARY AND CONCLUSIONS

The various HPLC methods that have been used for chloropigment analysis, especially in aquatic sciences, have been reviewed. NP HPLC is generally inadequate for this type of analysis because of the reactivity of the stationary phase material and because a complete separation of all chloropigments (and carotenoids) is generally impossible in a single run. Additionally, the extraction solvent normally used for chloropigments (acetone or methanol) is not compatible with NP HPLC, an extra step thus being required before the HPLC proper. Of the various RP HPLC techniques reported, isocratic elution can be recommended only for the analysis of selective pigments. For a complete separation of all major chloropigments, gradient elution is better because of the range of polarities encountered among these various compounds. Good results are obtained with C_{18} columns eluted with secondary or tertiary gradients of methanol, water and acetone or ethyl acetate mixtures. However, for the best reproducibility and separation of high-polarity pigments (dephytolated chloropigments), IPC or phase buffering is recommended. The analysis time is less than 30 min for many techniques described in this review; this will probably decrease with the use of the smaller particle size columns (3 μm) now available. New detectors such as diode array spectrophotometers increase the information output and help with the identification of unknown pigments, especially abundant in algal degradation products or sediment-related samples.

The range of pigments (both chloropigments and carotenoids) discovered up to now in marine and lake samples raises numerous questions concerning the origin and causes of the appearance of these compounds. Obviously, older and simpler techniques such as filter fluorimetry[54], which have been very popular in aquatic sciences, cannot answer these questions because pigments are not adequately separated and measured. The newer chemical techniques, such as HPLC, are the tools needed for this and also to substantiate values obtained with these simpler techniques still in vogue owing to their low cost, straightforwardness and in some instances the possibility of carrying out continuous monitoring (*in vivo* fluorimetry[55]).

It is hoped that this review of HPLC techniques devoted to pigment analysis, especially in the marine environment, will help potential and present users of the technique to select the most appropriate conditions for their analyses and to appreciate the diversity of HPLC methods used in this field. It is also hoped that this work will be a step towards a rationalization of the chromatographic measurement of pigments in the aquatic environment so that one or a few methods may be agreed upon as "standard" and allow easier comparisons between results from different laboratories.

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