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Development and application of a high resolution liquid chromatographic method for the analysis of complex pigment distributions

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Abstract

Ternary and binary gradient systems have been developed for the high-performance liquid chromatographic analysis of complex pigment distributions typical of natural samples. Improved chromatographic resolution reveals significantly more pigment components in extracts from a sediment (Priest Pot, Cumbria, UK), a microbial mat (les Salines de la Trinital, South Catalonia, Spain) and a culture (*C. phaeobacteroides*) including novel bacteriochlorophyll derivatives. The methods developed are directly suited to LC–MS analysis and the automated acquisition of MS/MS data for pigments.

Keywords: *Chlorobium phaeobacteroides*; Pigments

1. Introduction

Because they are biosynthesised exclusively by photoautotrophic organisms, chlorophyll pigments in the natural environment provide a direct link to the primary producer community. The distributions of chlorophylls, related tetrapyrroles and accessory pigments can provide information on community composition [1–3] and also on the abundance of primary producers [2,4,5]. In addition, the presence of phaeophorbides and steryl chlorin esters (SCE's) is often taken as evidence of grazing by zooplankton [6–8]. SCE's are highly apolar components which have been identified in marine and lacustrine sediments [9–12] and in marine water columns [10,13]. Their formation in grazing experiments has led to their use as markers of zooplankton herbivory [7,14,15], and their sterol distributions are suggested to provide an accurate reflection of the algal community [16,17]. Sedimentary tetrapyrrole distributions can also provide evidence of contributions from specific groups of photoautotrophs at the time of sediment formation [18,19] and estimates of changes in palaeoproductivity [20].

Accordingly, analysis of photosynthetic pigments and their degradation products is of widespread application across a range of disciplines including oceanography [21,22], limnology [23,24], geochemistry [25] and plant biochemistry [26]. The complex pigment distributions found in natural waters and sediments arise due to the wide diversity of pigments occurring in nature and the large number of possible degradation and transformation products. A number

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of high-performance liquid chromatography (HPLC) methods have been developed for the separation of natural mixtures of pigments (for a review see Roy [27]). Methods widely cited in the literature include those of Mantoura and Llewellyn [28] and the Scientific Committee of Oceanographic Research (SCOR) [22]. Both methods use reversed-phase columns with gradient elution to achieve separation, in short run times, of carotenoids, chlorophylls and degradation products derived from phytoplankton and water column samples. Notably, however, coelutions are evident in published work [23,24,27] rendering identification and quantification problematic. This will be particularly problematic for extracts from sediment cores as these often exhibit distributions that are highly complex and display a broad range in polarity [29]. Improved separation of apolar components permitted the identification of purpurin-18 phytyl ester [30], a transformation product of chlorophyll *a*, which had been masked previously by co-elution and provides evidence of chlorophyll oxidation reactions in the natural environment. Clearly, HPLC method development is necessary if the information available from pigment distributions is to be used to its full potential.

Because of the distinctive nature of the electronic absorption spectra of pigments and their transformation products, photodiode array (PDA) detection is widely used with HPLC. However, a significant number of components show indistinguishable UV–Vis spectra [31]. Thus, liquid chromatography mass spectrometry (LC–MS) is often required and is used increasingly as a complement to, or substitute for, PDA detection [29,32]. We present here an improved HPLC method for the analysis of complex pigment distributions. The method is directly applicable to LC–MS and can be operated with reduced analysis time for examination of simple distributions.

2. Experimental

2.1. Sample extraction

Samples (50–55 cm, obtained from a 1 m core) from Priest Pot (Cumbria, UK), from a calcite/gypsum microbial mat (7–11 mm depth; les Salines de la Trinital, South Catalonia, Spain) and a culture of *C. phaeobacteroides* were analysed. Thawed samples were extracted by sonication in acetone. Following centrifugation (5 min at 2000 *g*, Jouan B3.11 centrifuge) the supernatant was filtered through a solvent extracted cotton wool plug. The extraction procedure was repeated and the extracts were combined and reduced to dryness by rotary evaporation. Before analysis by HPLC, free acids were methylated using diazomethane. The routine method for preparation of diazomethane and appropriate safety precautions have been described in detail [33].

2.2. HPLC and LC–MS

Reversed-phase HPLC was accomplished using a Waters (Milford, MA USA) system comprising a 717 autosampler, 600MS system controller and 996 photodiode array detector. Instrument control, data processing and analysis were performed using Waters Millennium 2010 software. All solvents were degassed using helium. Separations were performed in the reversed-phase mode using two Waters Spherisorb ODS2 3 μm columns (150 mm×4.6 mm I.D.) in-line with a pre-column containing the same phase (10 mm×5 mm I.D.). An additional low-cost pre-column (Phenomenex Security Guard, C_{18} ODS Octadecyl, 4 mm×3 mm I.D.) was used as an on-line filter to prevent rapid deterioration of the pre-column. A number of 5 μm stationary phases (Spherisorb ODS1, ODS2; Phenomenex Luna, Prodigy; Jones Apex octadecyl) were evaluated prior to the selection of Spherisorb ODS2. Elution was carried out using a mobile phase gradient comprising acetonitrile, methanol, 0.01 *M* ammonium acetate and ethyl acetate (Table 1) at a flow-rate of 0.7 ml min^{-1}.

LC–MS was performed using a Finnigan (San Jose, CA, USA) system comprising a Thermo Separations AS3000 auto-sampler, P4000 gradient pump, UV2000 UV–Vis detector (Thermo Quest, Hemel Hempstead, UK) and a Finnigan MAT LCQ ion trap mass spectrometer equipped with an APCI source operated in the positive ion mode. The HPLC conditions used were as described above. LC–MS settings were as follows: capillary temperature 150°C, APCI vapouriser temperature 450°C, discharge current 5 μA, sheath gas flow 60 (arbitrary

Table 1
HPLC gradient programmes employed in the study

Time (min)	% Ammonium acetate (0.01 *M*)	% Methanol	% Acetonitrile	% Ethyl acetate
Method A[a]				
0	5	80	15	0
5	5	80	15	0
100	0	20	15	65
105	0	1	1	98
110	0	1	1	98
115	5	80	15	0
Method B[b]				
0	5	80	15	0
5	5	80	15	0
81	1	32	15	52
85	5	80	15	0
Method C[c]				
0	5	80	15	0
5	5	80	15	0
100	0	19	1	80
105	5	80	15	0
110	5	80	15	0

[a] Quarternary solvent delivery system. For ternary system: bottle A, starting composition; bottle B, composition at 100 min; bottle C, composition at 105 min.
[b] Quarternary solvent delivery system. For binary system: bottle A, starting composition; bottle B, composition at 81 min.
[c] Quarternary solvent delivery system. For binary system: bottle A, starting composition; bottle B, composition at 100 min.

units). On-line demetallation of chlorophylls in LC–MS was achieved as described previously [34].

3. Results and discussion

3.1. Choice of stationary phase

Resolution of bacteriochlorophyll *e* homologues from an extract of *Chlorobium phaeobacteroides* was compared for a number of stationary phases (see Experimental section) using the same mobile phase [30]. The Apex octadecyl and 5 μm Spherisorb ODS2 stationary phases produced the sharpest, most symmetrical peaks with separation of a greater number of homologues being achieved on the Spherisorb ODS2. Doubling the column length improved the separation markedly, and further improvement in resolution was achieved using 3 μm Spherisorb ODS2 at a column length of 30 cm.

3.2. Choice of mobile phase components

A four solvent elution program was devised to achieve optimal separation of the complex pigment distributions found in natural samples. Acetonitrile and ammonium acetate (0.01 *M*) accompanied methanol in the polar composition. A high concentration of ammonium acetate (cf. [21]) was not required as we routinely methylate free acids using diazomethane to prevent peak tailing and stabilise the pigments, allowing storage for future analysis. The low concentration of ammonium acetate in the mobile phase provides readily available cations to bind to active silanol sites (cf. [35]), and neutralises acidity in natural extracts, resulting in improved peak shape. Acetonitrile has a beneficial effect on the resolution of components in the polar region of the chromatogram. Changes in the relative proportions of acetonitrile and methanol affect the retention order of carotenoids with respect to chlorophylls (see later).

In previous methods, a variety of apolar modifiers

have been employed. We used ethyl acetate in preference to acetone because the latter absorbs strongly at the blue end of the spectrum and obscures pigment absorptions in this region. In particular, the acetone absorptions overlap with low intensity carotenoid absorption bands that depend on the stereochemistry of double bonds in the carotenoid and thus, are useful diagnostically.

3.3. Eluent composition

The aqueous content of the initial eluent was selected to be as high as possible without causing an undesirable increase in back pressure and broadening of peaks early in the chromatogram due to decreased solubility of the pigments in the mobile phase. Lower aqueous content caused all peaks to elute earlier and gave poorer separation. The relative compositions of acetonitrile and methanol were selected to optimise the separation of early eluting tetrapyrroles and carotenoids. The initial 5 min isocratic period (Method A, Table 1) retains a number of early eluting components on the column, enabling their separation. After the initial isocratic period, the proportions of water and methanol were reduced gradually, with a concomitant increase in ethyl acetate, to effect elution and improved separation of polar and medium polar components. Although a high percentage of ethyl acetate gave narrow and highly symmetrical peaks in the apolar region of the chromatogram, it also caused early elution with consequent poor separation of polar components. A final percentage of 65% was found to be optimal. In order to ensure the elution of all apolar components, an isocratic period (ethyl acetate 98%) was introduced in the final stages of the gradient programme prior to a reconditioning period.

3.4. Priest pot pigment composition

The analysis time has been extended compared to other HPLC methods routinely used for pigment analysis (e.g. [22,28]). To demonstrate the need for better resolution, an extract from the bottom sediment of Priest Pot (Cumbria, UK) was analysed using the method recommended by SCOR [22] and our method. The chromatograms obtained (Fig. 1) reveal dramatic differences. The SCOR method (Fig. 1a) generally shows poor resolution. Between 10 and 18 min, the on-line UV–Vis spectra show the peaks to comprise carotenoids and free base chlorophyll derivatives, with evidence of significant coelution. Between 18 and 24 min other carotenoids, chlorophyll *a* and apolar phaeopigments are evident. Analysis of the extract using the extended method (Fig. 1b) yields a far more complex distribution, demonstrating the significant improvement in resolution. In most cases, the extended method resolves components to baseline, which will permit more reliable quantification. The sediment core from Priest Pot has been examined previously [9] using gel permeation chromatography (GPC) fractionation followed by HPLC and LC–MS. The HPLC gradient elution employed in that analysis comprised acetone, methanol and water with a run time of 75 min. Due to the rapid decrease in the polarity of the initial eluent composition, the method was more suited to the separation of apolar components such as SCE's than polar components. Thus, considerable coelution among the early eluting carotenoids and phaeophorbides, and good separation of the apolar SCE's was evident. The improved separation across a wide polarity range in a single chromatographic run (Fig. 1b) has allowed spectral information to be obtained from many more components and identification of significantly more compounds than were identified previously by GPC fractionation followed by LC–MS analysis. Components were identified using LC–MS/MS combined with data from PDA detection (Table 2). The improved separation afforded by our method also permits automated acquisition of MS/MS data which necessitates sufficient chromatographic separation to ensure the molecular ion of an eluting peak is predominant in the full mass spectrum.

The application of Method A in the analysis of pigments from Priest Pot reveals seven bacteriochlorophyll *c*/*d* derivatives where only one was tentatively identified in an earlier study [9]. The bacteriochlorophylls comprise series' of homologues in which differences exist in the alkylation at C-8 and/or C-12 and the esterifying alcohols. Notably, the range of alcohols includes two (C_{17} and hexahydrofarnesol) that have not previously been reported in Chlorobiaceae. The latter is most likely a product of microbial reduction of the double bonds in

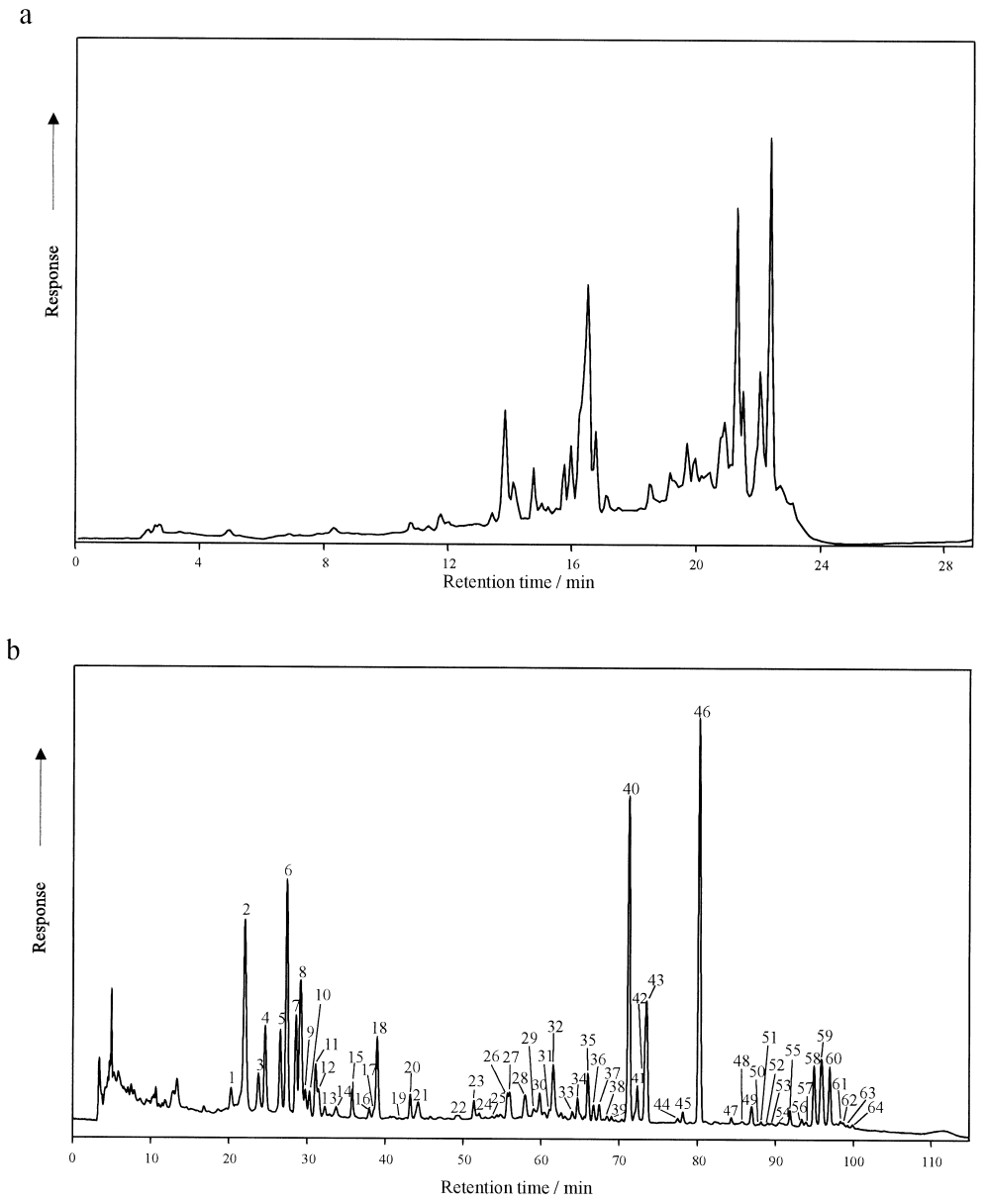

Fig. 1. HPLC–PDA (300–800 nm) chromatograms of Priest Pot sediment extract obtained using: (a) Method recommended by SCOR; and (b) Method A (Table 1). For peak assignments see Table 2.

farnesol. Furthermore, the high relative abundances of the homologues possessing alcohols other than farnesol is unusual in Chlorobiaceae, but has been observed in cultures grown at low light intensity [36].

Most carotenoids give poor yields on ionisation using APCI–MS, further demonstrating the importance of good chromatographic separation in order to obtain PDA data of sufficiently high quality to facilitate assignments. Even with our extended method, chromatograms extracted at specific wavelengths demonstrated a small number of coelutions. There-

Table 2
Assignment of components in Priest Pot sediment and calcite/gypsum microbial mat

Peak no.	Main UV–Vis absorption bands (nm)	$[M+H]^{+}$ [b] (m/z)	Prominent fragment ions[c] (m/z)	Assignment	Esterifying alcohol
a[a]	388	545		Scytonemin[e]	
b[a]	450, 470	601		Siphonaxanthin[e]	
c[a]	388	545	528, 489	Scytonemin (stereoisomer of a)[e]	
d	333, 421, 441, 460	573		Unidentified carotenoid	
e	474, 449, 505	567		Unidentified carotenoid	
f	448, 474, 500	567		Unidentified carotenoid	
1	425, 452, 475			Unidentified carotenoid	
1a	441, 467, 501	567		Unidentified carotenoid	
1b	447, 475, 507	567	549	Unidentified carotenoid	
2[a]	410, 664	533	505, 461	Chlorophyllone	
3[a]	402, 669	533	505, 461	Chlorophyllone	
3a[a]	420, 450, 477	567	549	Diatoxanthin[e]	
4	429, 452, 481	565	547, 461	Unidentified carotenoid	
4a	472	567	524	Unidentified carotenoid	
5	427, 453, 481	567	533, 508	Unidentified carotenoid	
5a[a]	420, 445, 473	569	551	Lutein[e]	
6	420, 445, 473	551	533	Unidentified carotenoid	
6a	421, 452, 479	569	551, 481	Zeaxanthin[e]	
7	411, 665	607	547, 461	Phaeophorbide *a* methyl ester	Methanol[d]
7[a]	453, 480	565		Alloxanthin[e]	
7a	420, 442, 471	573	555, 461	Unidentified carotenoid	
8	421, 443, 473			Unidentified carotenoid	
9	412, 663	607	547, 461	Phaeophorbide *a* methyl ester[e]	Methanol[d]
10	418, 441, 469	551	523, 495	Unidentified carotenoid	
11[a]	420, 447, 475	567	549	Monadoxanthin[e]	
11a[a]	420, 448, 473	569	552, 496	Carotene diol[e]	
12	421, 440, 468			Unidentified carotenoid	
12a	463, 486	561	543, 445	Unidentified carotenoid	
13	438, 660	563	531, 503, 488	Pyrophaeophorbide *b* methyl ester	Methanol[d]
13a	339, 420, 445, 470	528		Unidentified carotenoid	
13b[a]	479	581		Adonirubin[e]	
14	389, 415, 673	563		Unidentified chlorin	
15	481	565	537, 505	Canthaxanthin[e]	
16	386, 419, 676	563		Unidentified chlorin	
17	410, 673	579		Unidentified chlorin	
18	410, 665	549	521, 435	Pyrophaeophorbide *a* methyl ester	Methanol[d]
18a	320, 339, 357	512	494, 476/543, 483	Unidentified carotenoid	
18b[a]	312, 395, 440, 683	887		Unidentified chlorin	Phytol
18c	460, 491, 520	563	545	Rhodoxanthin[e]	
19		771	567, 523	Bacteriophaeophytin *c*/*d*[f]	Farnesol
19a	303, 394, 461, 687	887		Unidentified chlorin	Phytol
20	422, 446, 475	551	533, 494	Crocoxanthin[e]	
20a	381, 460, 483, 514	623	563	Unidentified carotenoid	
20b	381, 458, 484, 510	623	595	Unidentified carotenoid	
21[a]	428, 650	827	549, 521	Pyrochlorophyll *b*	Phytol
21a	383, 460, 487, 510	579		Unidentified carotenoid	
22		847		Unidentified chlorin	Phytol
22a[a]	418, 460, 487, 518	579	547	Okenone[e]	
23[a]	433, 665	871	593, 533	Chlorophyll *a*	Phytol
23a	377, 480	551	533	Echinenone[e]	
24		789	567, 549	Bacteriophaeophytin[f]	$C_{16:1}$
24a[a]	406, 660	763	567, 549	Bacteriophaeophytin *c*[f]	C_{14}
24b	417, 467, 676	867	595, 577	Unidentified bacteriophaeophytin	Geranyl geraniol
24c[a]	376, 476	569		Unidentified carotenoid	

Table 2. Continued

Peak no.	Main UV–Vis absorption bands (nm)	$[M+H]^{+}$ [b] (m/z)	Prominent fragment ions[c] (m/z)	Assignment	Esterifying alcohol
25	432, 662	871	593, 533	Chlorophyll *a* epimer	Phytol
25a[a]	406, 660	777	581	Bacteriophaeophytin *c*[f]	C_{14}
25a	360, 515, 749	887			
25b	380, 405, 681	903		Unidentified chlorin	Phytol
26[a]	433, 665	813	535, 507	Pyrochlorophyll *a*	Phytol
27	405, 662	777	567, 549	Bacteriophaeophytin *c*[f]	Hexahydrofarnesol
27a	377, 401, 678	903		Unidentified chlorin	Phytol
28[a]	409, 660	791	581, 563	Bacteriophaeophytin *c*[f]	Hexahydrofarnesol
29[a]	404, 660	791	567, 549	Bacteriophaeophytin *c*[f]	C_{16}
30[a]	358, 526, 749	889	611, 551	Bacteriophaeophytin *a*	Phytol
30a	418, 446, 475			Unidentified carotenoid	
31[a]	413, 650	805	567, 549	Bacteriophaeophytin *d*[f]	C_{17}
31a[a]	381, 412, 511, 543, 680	887		Unidentified chlorin	Phytol
32	400, 665	845		Unidentified chlorin	
32a[a]	406, 649	819	595, 577	Bacteriophaeophytin *d*[f]	C_{16}
32b[a]	357, 527, 747	889	611, 551	Bacteriophaeophytin *a* epimer	Phytol
32c[a]	381, 411, 514, 547, 681	887		Unidentified chlorin	Phytol
33[a]	407, 660	819	581, 563	Bacteriophaeophytin *c*[f]	C_{17}
34	436, 650	885	607, 547	Phaeophytin *b*	Phytol
34a[a]	406, 660	819	567, 549	Bacteriophaeophytin *c*[f]	C_{18}
35	407, 667	887	609, 591	Hydroxyphaeophytin *a*	Phytol
36	416, 436, 667	829		Unidentified chlorin	
37	411, 666	887	609, 591	Hydroxyphaeophytin *a* epimer	Phytol
37a[a]	357, 531, 749	831	552, 523	Pyrobacteriophaeophytin *a*	Phytol
38		685	666, 648	Unidentified carotenoid	
39[a]		917	639, 551	Purpurin-7 phytyl ester[g]	Phytol
40	409, 665	871	593, 533	Phaeophytin *a*	Phytol
41	453, 482	537		Carotene[f]	
42	412, 665	871	593, 533	Phaeophytin *a* epimer	Phytol
42a	448, 475	537		Carotene[f]	
43	436, 660	827	549, 507	Pyrophaeophytin *b*	Phytol
43a	339, 425, 447, 475	537		Carotene[f]	
44		857		Unidentified chlorin	Phytol
44a	358, 533, 745				
45[a]	409, 544, 696	843	565, 503	Purpurin-18 phytyl ester	Phytol
46	411, 665	813	535, 507	Pyrophaeophytin *a*	Phytol
46a	380, 395			Unidentified carotenoid	
46b	377, 396			Unidentified carotenoid	
47[a]	413, 667	961	593, 533	Phaeophorbide *a* ester	C_{27} sterol[h]
47[a]	443, 475	1067	535, 507	Pyrophaeophorbide *a* ester	Carotenoid (532 daltons)
48[a]		987	593, 533	Phaeophorbide *a* ester	C_{29} sterol[i]
49	398, 499, 660	961		Unidentified chlorin ester	
50[a]		977	593, 533	Phaeophorbide *a* ester	C_{28} sterol
51		963		Unidentified chlorin ester	
52[a]		917	549, 521	Pyrophaeophorbide *b* ester	C_{27} sterol[h]
53[a]		931	549, 521	Pyrophaeophorbide *b* ester	C_{28} sterol[h]
54[a]		943	549, 521	Pyrophaeophorbide *b* ester	C_{29} sterol[i]
55	410, 664	901	535, 507	Pyrophaeophorbide *a* ester	C_{27} sterol[i]
56		915	535, 507	Pyrophaeophorbide *a* ester	C_{28} sterol[i]
57		929	535, 507	Pyrophaeophorbide *a* ester	C_{29} sterol[i]
58	411, 665	903	535, 507	Pyrophaeophorbide *a* ester	C_{27} sterol[h]
59	410, 667	929	535, 507	Pyrophaeophorbide *a* ester	C_{29} sterol[i]
59		917	535, 507	Pyrophaeophorbide *a* ester	C_{28} sterol[h]
60	410, 667	931	535, 507	Pyrophaeophorbide *a* ester	C_{29} sterol[h]
61[a]		919	535, 507	Pyrophaeophorbide *a* ester	C_{28} sterol
62[a]		945	535, 507	Pyrophaeophorbide *a* ester	C_{30} sterol[h]
63[a]		933	535, 507	Pyrophaeophorbide *a* ester	C_{29} sterol
64		948	535, 507	Pyrophaeophorbide *a* ester	C_{30} sterol

[a] Not identified in previous studies [9,25]. [b] All chlorophyll derivatives appear as Bphe MH^{+} due to post column demetallation prior to sequential mass scanning [34]. [c] Obtained by resonance excitation during LC–MS analysis. [d] Due to esterification prior to analysis. [e] Tentative assignment. [f] One of several possible structural isomers. [g] Ref. [41]. [h] One double bond. [i] Two double bonds.

fore, the analysis of pigment distributions using shorter run times combined with the extraction of selected wavelength chromatograms via PDA detection would be inadequate.

3.5. C. phaeobacteroides pigment composition

Borrego and Garcia-Gil [37] demonstrated the separation of homologues of bacteriochlorophyll *e* from *C. phaeobacteroides* as two sets of peaks. Using an extract from the same culture, a shortened version of our method (Method B: Table 1) was developed to permit more rapid analysis of less complex systems. The bacteriochlorophylls are annotated using the notation of Borrego and Garcia-Gil [37] (Fig. 2). Within the early eluting components e_1–e_4, the distribution shows no significant differences (cf. [37]). By contrast, the second cluster shows marked differences with resolution of twelve peaks where only eight were observed previously. In addition, three of the prominent peaks show pronounced shoulders demonstrating that our method affects partial separation of additional components. On-line PDA detection gave absorption spectra characteristic of bacteriochlorophyll *e* for all peaks between 15 and 45 min. (inset, Fig. 2). It is expected that the bacteriochlorophylls of the second cluster (I–XII) differ in their extent of alkylation at positions C-8 and C-12 as has been reported for bacteriochlorophylls [36,38] and in the nature of the esterifying alcohol at position C-17, as reported in some species of green sulphur bacteria [39,40]. More detailed analysis using LC–MS and MS/MS is required to identify individual components and will be the subject of future work. As would be expected, the esterifying alcohol at position C-17 and the degree of alkylation at positions C-8 and C-12 do not affect the chromophore of the bacteriochlorophylls in vitro. Therefore, even with PDA detection, co-elutions can be detected with HPLC only if they give rise to asymmetric peaks.

3.6. Calcite/gypsum mat pigment composition

The distribution of bacteriochlorophylls in cultures can be very complex, but the range of polarity of the pigments is not as challenging as that seen in sediment extracts, which can show very high levels of complexity. An extract from a microbial mat shown previously to contain bacteriochlorophylls as well as a range of pigments from eukaryotes [25] was examined using our extended Method A. The

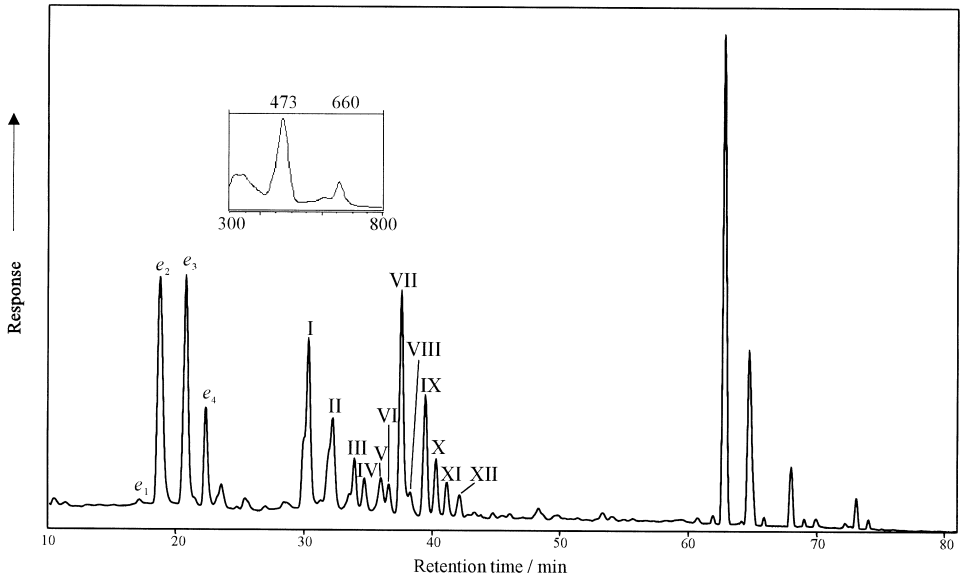

Fig. 2. HPLC–PDA (300–800 nm) chromatogram of *C. phaeobacteroides* extract obtained using Method B (Table 1). For peak assignments see text.

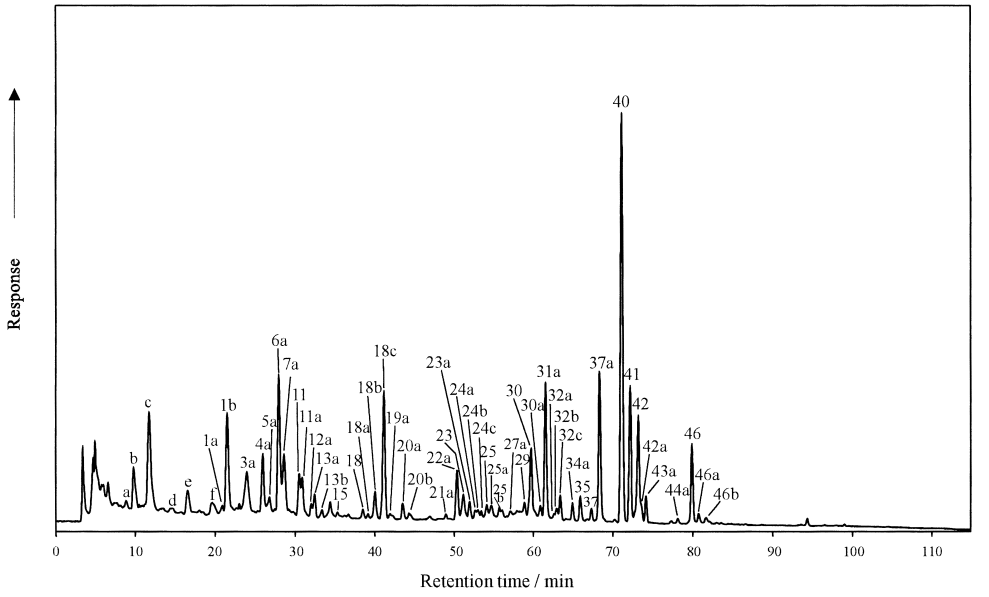

Fig. 3. HPLC–PDA (300–800 nm) chromatogram of microbial mat extract obtained using Method A (Table 1). For peak assignments see Table 2.

chromatogram obtained (Fig. 3) shows a complex distribution, with components eluting over the entire polarity range except between 80 and 90 min where SCE's would elute if present (cf. Fig. 1b). The improved conditions permitted separation of 57 components and identification of 33 of these (Table 2) compared to 19 identified previously [25]. These include a greater number of bacteriochlorophyll *c*/*d* derived components, none of which possess farnesol as an esterifying alcohol. This observation is consistent with, and lends support to, a previous study in which the Chlorobiineae in the mat were described as Chloroflexus-like [25]. In addition, a C_{14} esterifying alcohol has not previously been reported in the bacteriochlorophylls of Chloroflexus.

3.7. Binary HPLC method

The HPLC eluent composition is such that it can be run directly on a ternary solvent delivery system. To permit the method to be used on binary systems, we developed a variant of the method (Method C, Table 1). The essential difference in the method is in the composition of ethyl acetate, which increases to 80% over 95 min, excluding the need for an apolar isocratic period (cf. Method A, Table 1). The higher percentage of ethyl acetate used in Method C is mainly compensated for by a reduction in the percentage of acetonitrile. The Priest Pot extract analysed using the binary method (Fig. 4) gives a very similar chromatogram to that obtained using the ternary method (Fig. 1b), although some differences in resolution and elution order are apparent. For example, carotenoids 4 and 5 elute earlier than chlorin 2 (cf. Fig. 1b) due to the lower relative content of acetonitrile in the eluent composition, and the change in retention order caused carotenoid 5 to mask chlorin 3. Towards the end of the chromatogram, the shorter retention using the binary method results in poorer resolution of SCE's. A beneficial effect is the separation of previously coeluting carotenoid and chlorin components in peaks 7 and 8. Also, the reduction in acetonitrile content caused the earlier elution of carotene 43.

In certain situations, such as shipboard analysis, users may wish to avoid the use of diazomethane. This can be achieved in all of the methods (A–C) by increasing the concentration of ammonium acetate from 0.01 *M* to 0.5 *M*. Under these conditions the free acid components (e.g. phaeophorbides) elute c. 15 min earlier using Method A whereas the t_R of most other components is relatively unchanged.

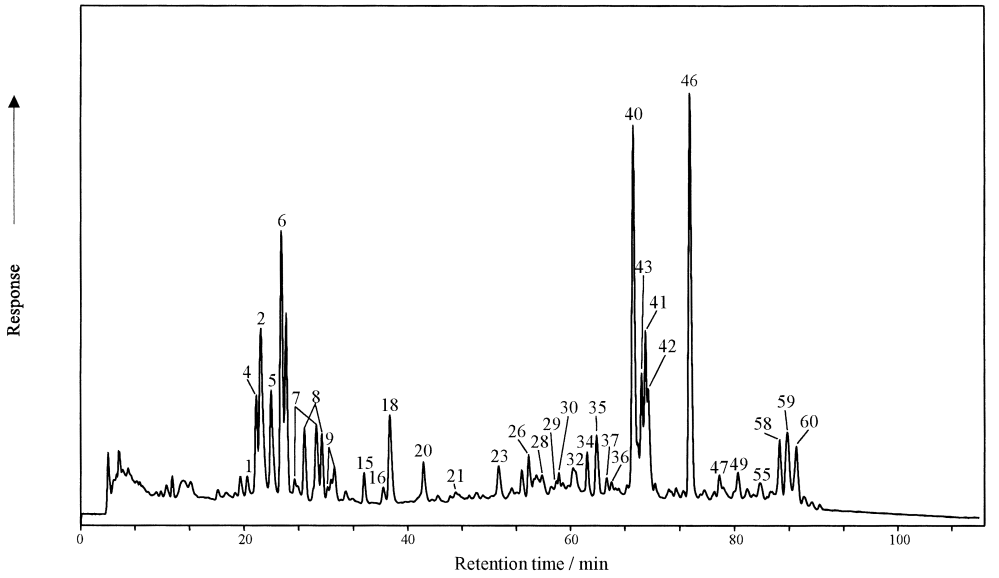

Fig. 4. HPLC–PDA (300–800 nm) chromatogram of Priest Pot sediment extract obtained using Method C (Table 1). For peak assignments, see Table 2.

There are, however, disadvantages to this approach: the extracts exhibit poor stability in storage even in the short term, certain transformation products such as purpurin-7 phytyl ester [41] are not observed due to poor peak shape, and the APCI–LC/MS response of the free acid components is about two orders of magnitude lower than their corresponding methyl esters.

4. Conclusions

The improvements in HPLC methodology reported herein have revealed a much greater degree of complexity in the pigment distributions of extracts from two sediment cores and a culture of *C. phaeobacteroides* compared to published methods. The improvement in resolution facilitates automation of LC–MS/MS, leading to faster and more routine LC–MS analysis and the identification of a far greater number of components. Application of the method has allowed identification in sediments of novel bacteriochlorophyll derived pigments that have not previously been identified in Chlorobiaceae.

In the light of the complexity of the pigment distributions shown here, we strongly recommend that where very short run times are required to permit high sample throughput, selected samples are analysed using an extended method to ensure that serious co-elutions do not occur. The binary and ternary methods described herein are directly applicable to LC–MS, and the considerable improvements in chromatographic separation of pigments makes them suitable for studies of their distributions in geochemistry, limnology and oceanography.

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