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Chlorophyll c_2 monogalactosyldiacylglyceride ester (chl c_2-MGDG). A novel marker pigment for *Chrysochromulina* species (Haptophyta)

Manuel Zapata[1,2,*], Bente Edvardsen[3], Francisco Rodríguez[1], Miguel A. Maestro[4], José L. Garrido[5]

[1]CIMA, Consellería de Pesca, Xunta de Galicia, Apdo. 13, 36620 Vilanova de Arousa, Spain
[2]Departamento de Bioloxía Vexetal, Universidade de Vigo, 36200 Vigo, Spain
[3]Section of Marine Botany, Department of Biology, University of Oslo, PO Box 1069, 0316 Oslo, Norway
[4]Servicios Xerais de Apoio a Investigación, Universidade da Coruña, 15071 A Coruña, Spain
[5]Instituto de Investigacións Mariñas, CSIC, Av. Eduardo Cabello 6, 36208 Vigo, Spain

ABSTRACT: The pigment composition of the haptophyte *Chrysochromulina polylepis* (Strain CCMP 286) was analysed by high-performance liquid chromatography (HPLC) using pyridine-containing mobile phases and polymeric C_{18}, or monomeric C_8 columns. The polar chlorophyll (chl) *c* pigment composition included chl c_2 and chl c_3 as major fractions, and divinyl protochlorophyllide *a* (DV pchlide or MgDVP) as a minor component. Several non-polar fluorescent peaks sharing a common chl *c*-type absorption spectrum were also detected. The main component of these peaks was isolated and characterised by chromatographic behaviour, UV-visible (UV-VIS) and fluorescence spectroscopy. Although spectral properties were similar to a high molecular weight non-polar chl *c* (1313 Da) recently characterised from *Emiliania huxleyi*, both chlorophylls showed different chromatographic behaviour. Fast atom bombardment-mass spectrometry (FAB-MS) analysis showed a high mass molecular ion (*m/z* 1265), and a fragmentation pattern compatible with a molecular structure consisting of a chl c_2 pigment linked by an ester bond to the sugar moiety of a monogalactosyl diacylglyceride (MGDG), which included 2 myristic acid (14:0) residues. To study the distribution pattern of the novel pigment, different strains of *C. polylepis* and other species of the genus *Chrysochromulina* were analysed. A similar chl c_2 ester was also present in 2 *C. polylepis* strains (K, B11) and in *C.* aff. *polylepis* (PLY 200), as well as in *C. acantha*, *C. camella*, *C. leadbeateri*, *C. strobilus*, *C. throndsenii*, and *Chrysochromulina* sp. (CS-410). Three species, *C. fragaria*, *C. hirta* (2 strains) and *C. kappa*, lacked the new chl *c*, but they contained detectable amounts of other non-polar chl *c*-like pigments. The feasibility of using chls *c* and carotenoids as marker pigments to detect *Chrysochromulina* species in field samples is discussed.

KEY WORDS: Chlorophyll c_2-galactolipid esters · *Chrysochromulina polylepis* · *Chrysochromulina* spp. · Fast atom bombardment-mass spectrometry · HPLC pigment analysis · Marker pigments · Chemotaxonomy

INTRODUCTION

Photosynthetic pigments are useful chemotaxonomic markers for identifying phytoplankton at different taxonomic levels (see Jeffrey & Vesk 1997). This approach is specially useful for nano- and picoplankton-sized species and other algal groups poorly preserved in standard fixatives or lacking useful morphological features (Gieskes & Kraay 1986, Simon et al. 1994, Jeffrey et al. 1999).

New developments in chromatographic techniques have enlarged our knowledge of pigment composition and blurred some well-established pigment distribu-

*E-mail: mzapata@cimacoron.org

tion patterns. Relevant advances in the separation of members of the chl *c* pigment family have resulted from the incorporation of pyridine-containing mobile phases to the HPLC pigment analysis, either in combination with polymeric C_{18} columns (Garrido & Zapata 1997), or monomeric C_8 columns (Zapata et al. 2000). The C_{18} method was used to study the pigment composition of the haptophyte *Emiliania huxleyi* (Garrido & Zapata 1998), allowing detection of a new chl c_3, the monovinyl (MV) compound, in addition to the original previously detected and characterised chl c_3 (the divinyl compound; Jeffrey & Wright 1987, Fookes & Jeffrey 1989).

During a survey of pigment composition from harmful algal species (Zapata et al. 1998), a culture of the haptophyte *Chrysochromulina polylepis* (CCMP 286) was analysed using the polymeric C_{18} HPLC method. A group of fluorescent peaks with retention times longer than chl *a*, but having chl *c*-type spectral properties, was detected. This type of pigment was not observed in an earlier study performed on *C. polylepis* (Johnsen et al. 1992), the absence of non-polar chl *c* was considered by these authors as the main difference between pigment composition of *C. polylepis* and *Emiliania huxleyi* (another bloom-forming haptophyte).

Since the *Chrysochromulina polylepis* harmful bloom in Skagerrak and Kattegat during May-June 1988 (Dahl et al. 1989), the detection of the genus *Chrysochromulina* has been included as a routine task in phytoplankton monitoring programs in coastal waters with fish or shellfish aquaculture farms (Thomsen et al. 1994, Dahl et al. 1998). Bloom dynamics and physiology of *Chrysochromulina* species have been exhaustively reviewed by Edvardsen & Paasche (1998). To date, more than 50 *Chrysochromulina* species have been described from both coastal and oceanic waters, often co-occurring in mixed phytoplankton assemblages. Light microscopy is not sufficient to confirm species identity, and transmission electron microscopy (TEM) must be used to examine taxonomically relevant patterns of the body scale. Any potential biochemical markers would speed detection of *C. polylepis* and other *Chrysochromulina* species in phytoplankton samples.

A recent study has described the chemical structure of a novel chl *c* pigment from *Emiliania huxleyi* (Garrido et al. 2000), previously misidentified as a phytylated chl *c*-derivative (Nelson & Wakeham 1989, Zapata & Garrido 1997). The pigment consists of a chl c_2-moiety esterified to a monogalactosyldiacylglyceride (chl c_2-MGDG) with myristic (14:0) and octadecatetraenoic (18:4 n-3) fatty acid residues (Garrido et al. 2000). It was postulated that other molecular species might occur due to changes in the fatty acid composition of the cells. Here we describe a new high-molecular weight (1.265 Da) chl c_2-galactolipid isolated from *Chrysochromulina polylepis* containing 2 myristic acid (14:0) residues. The use of marker pigments to detect *Chrysochromulina* species in field samples is discussed.

MATERIALS AND METHODS

Algae cultures. *Chrysochromulina polylepis* Manton et Parke strains and other *Chrysochromulina* species were obtained from various culture collections (Table 1). Species and strains analysed, and culture conditions used are also given in Table 1. Mass cultures of *C. polylepis* (CCMP 286) were grown in CHRY medium (Andersen et al. 1991) in two 20 l flasks at 15 ± 1°C under a 12:12 h light:dark cycle (ca 150 µmol photons m^{-2} s^{-1}) with aeration during the light period.

Natural sample. Seawater samples (2 l) from the Bay of Biscay (45°16.8′ N, 6°10.2′ W) were collected during the GIGOVI 98 cruise, August 1998, on board RV 'Professor Shtokman'. Water samples were filtered onto a Whatman GF/F filter. An estuarine sample was obtained from Ría de Arousa (Galician coast, NW Spain, May 2000). Seawater (1.5 l) was size-fractionated by sequential filtration through a 47 mm diameter Whatman GF/D filter (nominal pore size 2.7 µm) and Whatman GF/F filter (nominal pore size 0.7 µm). HPLC pigment analysis was performed by the C_8 HPLC method as indicated for algal cultures.

Sample preparation. Cells were harvested during the exponential phase of growth by filtering the cultures onto Whatman GF/F filters under reduced pressure (<10 cm Hg). Pigments were extracted with cold (5°C) 90% acetone (C_{18} HPLC method) or 95% methanol (C_8 HPLC method). Extracts were filtered again through GF/F filters to remove cell and filter debris. To avoid peak distortion, which affects early eluting pigments, 0.4 or 0.2 ml of Milli-Q water was added to each millilitre of acetone or methanol extracts, respectively, immediately before injection (Zapata & Garrido 1991). Samples were prepared under subdued light.

HPLC. Aliquots of sample extracts were analysed using a Waters Alliance HPLC System consisting of a 2690 separations module, a Waters 996 diode-array detector (1.2 nm optical resolution) interfaced with a Waters 474 scanning fluorescence detector by means of a Sat/In analog interface. Analytical separations were performed either using a polymeric C_{18} Vydac 201 TP54 column (Garrido & Zapata 1997) or a monomeric C_8 Waters Symmetry (Zapata et al. 2000). For semi-preparative pigment separation and purification, a polymeric C_{18} Vydac 201 TP510 column was

Table 1. Culture conditions and origin of the algal species and strains used in this study. CCMP: Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, USA; UIO: University of Oslo Algal Culture Collection, Norway; CS:. CSIRO Algal Culture Collection, Hobart, Tasmania, Australia

Species (strain code)	Collection site	Culture conditions		
		Medium	Temp. (°C)	Light intensity (µmol photons m^{-2} s^{-1})
Chrysochromulina polylepis Manton et Parke (CCMP 286)	Kristineberg, Norway	CHRY	15	47
C. polylepis Manton et Parke (UIO B11)	59° 00′ N, 10° 45′ E, Torbjørnskjær, Norway	IMR1/2 + 10nM selenite	16	130
C. polylepis Manton et Parke (UIO K)	58° 30′ N, 9° 30′ E, Risør, Norway	IMR1/2 + 10nM selenite	16	130
C. aff. *polylepis* Manton et Parke (PLY PML, PLY200)[a]	50° 02′ N, 4° 22′ W, Plymouth, UK	IMR1/2 + 10nM selenite	16	130
C. acantha Leadbeater et Manton (UIO, T20)	58° 25′ N, 8° 45′ E, Flødevigen, Norway	IMR1/2 + 10nM selenite	16	130
C. camella Leadbeater et Manton (CSIRO, CS-268)	49° 10′ N, 6° 10′ W	GSe	15	60
C. fragaria Eikrem et Edvardsen (UIO S19)	58° 25′ N, 8° 45′ E, Flødevigen Norway	IMR1/2 + 10nM selenite	16	130
C. hirta Manton (UIO C. hi-1)	60° 04′ N, 5° 23′ E, Austevoll Norway	IMR1/2 + 10nM selenite	16	130
C. hirta Manton, CSIRO CS-228	Polar Inst. Japan[b]	GSe	15	60
C. kappa Parke et Manton (UIO EN3)	59° 20′ N, 10° 35′ E, Oslofjorden Norway	IMR1/2 + 10nM selenite	16	130
C. leadbeateri Estep et al. (UIO ERIK)	68° 24′ N, 15° 51′ E, Erikstad Norway	IMR1/2 + 10nM selenite	16	130
C. strobilus Parke et Manton (CSIRO CS-231)	Polar Inst. Japan[b]	GSe	15	60
C. throndsenii Eikrem (UIO K11)	58° 24′ N, 8° 46′ E Ærøy Norway	IMR1/2 + 10nM selenite	16	130
Chrysochromulina sp. (CSIRO CS-410)	Corner Inlet. Vic. Australia	GSe	16	60
Emiliania huxleyi (Lohmann) Hay et Mohler (CCMP 370)	Oslo fjord, Norway	f/2	15	47
Prymnesium parvum f. *patelliferum* (Green, Hibberd et Pienaar) Larsen (UIO P. patRY89)	59° 33′ N, 6° 24′ E Hylsfjord, Norway	IMR1/2 + 10nM selenite	16	130

[a]UIO, initially from Plymouth Marine Laboratory, UK
[b]No data on collection site available

used. Column characteristics, mobile phases, elution gradients and other experimental conditions applied for analytical and preparative HPLC analyses are shown in Table 2. For quantitative analysis, chlorophylls and fucoxanthin and its acyloxy derivatives were isolated as described by Zapata et al. (2000) and employed as standard solutions for external standard calibration. Extinction coefficients, when available, were obtained from data compiled by Jeffrey (1997). Novel pigments were quantified considering the most similar chromophore whose extinction coefficient had been documented (e.g. non-polar chl *c* and chl c_3 were measured as chl c_2-equivalents, whereas acyloxy-fucoxanthin derivatives were estimated as fucoxanthin-equivalents). Pigment ratios relative to chl *a* were calculated for each strain studied.

Pigment identification. Visible absorption spectra obtained by the diode-array detector (350 to 750 nm) were used for routine pigment identification. After checking for peak homogeneity, spectral information was compared with a library of chlorophyll and carotenoid standard spectra from pigments prepared from standard phytoplankton cultures (Jeffrey & LeRoi 1997). For standards of recently isolated new pigments, please consult Egeland et al. (2000), Garrido et al. (2000), and Zapata et al. (2000). The scanning fluorescence detector was used as a selective detector for chlorophylls. Excitation (440 nm) and emission (650 nm) wavelengths were selected for the detection of chlorophylls.

Isolation and purification of the non-polar chl *c* fraction. Mass cultures (35 l) of *Chrysochromulina polylepis* (CCMP 286) were harvested by continuous centrifugation using a Beckman Avanti J-25 centrifuge with a JCF-Z rotor. The freshly packed biomass was extracted with 9 times its volume of cold acetone (5°C); after filtration (GF/F), a clarified aliquot was injected into the C_{18} HPLC system under semi-preparative conditions (Table 2). The major unknown non-polar chl *c* fraction was isolated by collecting the chromatographic peak from several runs at the detector outlet. This fraction was re-chromatographed by isocratic elution with an eluent consisting of a mixture of Mobile Phases A and B (20:80, v:v; see Table 2), and a single peak was collected from several injections. Once 25 ml of the purified fraction were recovered, an equal volume of diethyl ether, and 15 vol cold 10%

Table 2. *Chrysochromulina* spp. Chromatographic conditions of the HPLC methods employed in the study of pigment composition

	Chromatographic method	
	Garrido & Zapata (1997)	Zapata et al. (2000)
Stationary phases		
Column	Vydac 201 TP 54/Vydac 210 TP 54[a]	Waters Symmetry
Dimensions	250 × 4.6 mm/250 × 10 mm[a]	150 × 4.6 mm
Bonding chemistry	Polymeric C_{18}	Monomeric C_8
Particle size	5 µm	3.5 µm
Pore size	300 Å	100 Å
Mobile phases		
A	Methanol:acetonitrile: 0.25 M aqueous pyridine[b] (45:35:20 v:v:v)	Methanol:acetonitrile: 0.25 M aqueous pyridine[b] (50:25:25 v:v:v)
B	Acetone	Methanol:acetonitrile:acetone (20:60:20 v:v:v)
Elution gradient	Min: %B; t_0: 0%, t_{28}: 60%, t_{32}: 100%, t_{37}: 100%, t_{40}: 0%	Min: %B; t_0: 0%, t_{22}:40%, t_{28}: 95%, t_{37}: 95% , t_{40}: 0%
Flow rate	1.2 ml min^{-1}, 4.0 ml min^{-1} [a]	1.0 ml min^{-1}
Temperature	27°C	25°C

[a]Semi-preparative HPLC conditions
[b]The aqueous pyridine solution was adjusted to pH 5.0 with acetic acid addition

NaCl solution were added to transfer the pigment immediately to the ether phase for spectral characterisation.

Visible spectroscopy. Absorption spectra were measured with a Beckman DU-70 UV-VIS spectrophotometer (resolution 2 nm). Fluorescence spectra were recorded using a Perkin-Elmer LS 50 B luminescence spectrometer. Excitation spectra (400 to 500 nm) and emission spectra (600 to 800 nm) were recorded at the respective emission (632 nm) and excitation (452 nm) maxima wavelengths, calculated by means of the pre-scan facility. Emission and excitation band widths were 5 nm.

Fast atom bombardment-mass spectrometry (FAB-MS). Spectrometric analysis was performed using a VG Quattro double quadrupole mass spectrometer (Fison). Ions were produced using a Cesium ion gun operating at 22 kV. An aliquot of the ether solution was checked and cleaned for lipids (see Garrido et al. 2000), dried under N_2, and re-dissolved in acetone. The spectrally pure pigment was dissolved in the matrix by dropping small volumes of the acetone solution onto a layer of the matrix previously placed on the probe of the mass spectrometer. Positive-ion mass spectra were obtained using 3-nitrobenzyl alcohol (3-NBA) as sample matrix. When needed, HCl was applied to the sample dissolved in 3-NBA on the spectrometer probe to induce the demetalation of the pigment. Negative-ion mass spectra were obtained using triethanolamine (TEA) as sample matrix. Tandem mass spectra (MS/MS) were obtained by collision-induced dissociation (CID) of the molecular ion, employing argon as the collision gas.

RESULTS

Pigment composition of *Chrysochromulina polylepis*

Chlorophyll composition

The fluorescence chromatogram of an acetone extract from *Chrysochromulina polylepis* (CCMP 286) obtained by the polymeric C_{18} HPLC method (Fig. 1A, Table 3) shows the presence of MgDVP (Peak 8), chl c_3 (Peak 4) and chl c_2 (Peak 9), chl *a* (Peak 29), and trace amounts of chl *a* allomer (Peak 28) and chl *a* epimer (chl *a*′, Peak 30). The main non-polar chl *c*-like pigment from *C. polylepis* (Fig. 1A, Peak 31) was the most retained fluorescent peak, eluting after the non-polar chl c_2-MGDG from *Emiliania huxleyi* (Fig. 1B, Peak 26).

The chromatogram obtained by the C_8 HPLC method (Fig. 1C, Table 3) shows an inversion in the eluting order for the pigment pair MgDVP (Peak 8) and chl c_3 (Peak 4) compared to the C_{18} HPLC method, as well as a clear change in the elution pattern of the non-polar chl *c*-like pigments relative to chl *a*. So, the major non-polar chl *c*-like pigment from *Chrysochromulina polylepis* (CCMP 286) (Fig. 1C, Peak 31) elutes after

chl *a* (Peak 29), while the chl c_2-MGDG from *Emiliania huxleyi* (Fig. 1D, Peak 26) elutes just ahead of chl *a*.

The on-line diode-array spectra show similar characteristics for both non-polar chl *c*-like pigments (Table 3). The relative proportion of non-polar chl *c* from *Chrysochromulina polylepis* (CCMP 286) to total chl *c* pigments, measured at exponential growth phase and calculated by using the extinction coefficients of chl c_2 at 450 nm, was around 15%. The absorption spectrum, and the excitation and emission fluorescence spectra of the novel pigment, both in diethyl ether, are shown in Fig. 2A, B and C, respectively.

Carotenoid composition

The absorbance chromatogram obtained using the C_{18} HPLC method (Fig. 3A) shows the following carotenoids (Table 3): 19′-butanoyloxyfucoxanthin (But-fuco, Peak 11), fucoxanthin (Fuco, Peak 12), the novel carotenoid 4-keto-19′-hexanoyloxyfucoxanthin (4-k-Hex-fuco, Peak 14), 19′-hexanoyloxyfucoxanthin (Hex-fuco, Peak 15), diadinoxanthin (Diadino, Peak 16), diatoxanthin (Diato, Peak 17) and β,β-carotene (ββ-Car, Peak 33).

The C_8 HPLC method allows greater resolution of carotenoids than the C_{18} method (Fig. 3). Increased resolution occurred between two pigment pairs: Fuco (Peak 12) and 4-k-Hex-fuco (Peak 14) ($R_s > 2.5$), and 4-k-Hex-fuco (Peak 14) and Hex-fuco (Peak 15) ($R_s > 2.0$). In addition, the C_8 method allows detection of several unknown carotenoids (Peaks 13, 18 and 21). Table 3 shows the retention time and spectral properties of the separated pigments using the monomeric C_8 column.

FAB-MS analysis of main non-polar chl *c* from *Chrysochromulina polylepis*

The positive-ion mass spectrum of the isolated chlorophyll (Fig. 4A) showed a peak profile closely resembling that recently obtained for the chl c_2-MGDG from *Emiliania huxleyi* (Garrido et al. 2000): a prominent peak in the high mass zone of the spectrum (*m/z* 1265), that could be attributed to the molecular ion ($[M]^+$), a zone at intermediate mass range lacking prominent ions (except *m/z* 693; see below), and a cluster of fragment ions (608, 591, 577, 563, 549, 532, 503, 476, 461 and 447) derived from chl c_2. The ions in the region 400 to 610 *m/z* can be explained considering

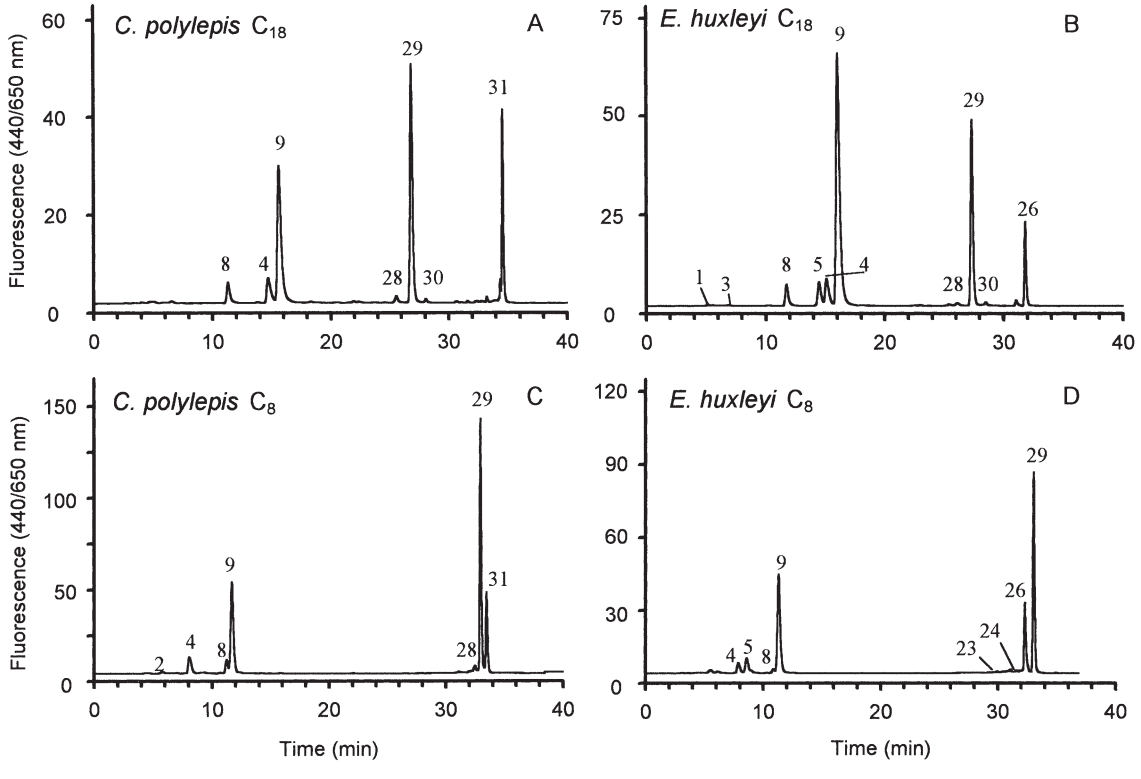

Fig. 1. (A,C) *Chrysochromulina polylepis* CCMP 286, and (B,D) *Emiliania huxleyi* CCMP 370. HPLC chromatograms of pigment extracts. (A,B) polymeric C_{18} column, and (C,D) monomeric C_8 column. Detection is by fluorescence (excitation = 440 nm, emission = 650 nm). Peak identifications as in Table 3

Table 3. *Chrysochromulina* spp. Identification of pigments detected using HPLC methods based on monomeric C_8 and polymeric C_{18} columns. The C_{18} HPLC method was used for *C. polylepis* CCMP 286 strain. Pigments from *Emiliania huxleyi* and *Prymnesium parvum* f. *patelliferum* are listed for comparative purposes. Wavelengths in parentheses denote shoulders. MGDG: monogalactosyldiacylglyceride

Peak no.	Pigment	Retention time (min)		Absorption maxima in mobile phase
		C_8	C_{18}	C_8 HPLC method
1	Chlorophyll *c*-like	5.06		450, 583, 631
2	Chlorophyll *c*-like	5.68		450, 583, 631
3	Chlorophyll *c*-like	6.22	6.19	450, 583, 631
4	Chlorophyll c_3	7.97	14.44	459, 591, (628)
5	Monovinyl-chlorophyll c_3	8.74	13.53	452, 586, 628
6	Chlorophyll *c*-like	9.23		450, 583, 631
7	Chlorophyllide *a*	10.61		430, 581, 663
8	MgDVP (divinyl protochlorophyllide *a*)	11.10	10.89	439, 577, 628
9	Chlorophyll c_2	11.61	15.36	453, 586, 633
10	Chlorophyll c_1	12.25	13.57	449, 583, 631
11	19′-butanoyloxyfucoxanthin	17.98	8.15	446, 469
12	Fucoxanthin	18.91	9.54	450
13	Unknown carotenoid fucoxanthin-related	19.94		446, 469
14	4-keto-19′-hexanoyloxyfucoxanthin	21.17	9.15	447, 470
15	19′-hexanoyloxyfucoxanthin	21.98	10.22	446, 469
16	Diadinoxanthin	24.18	17.46	(422), 446, 476
17	Diatoxanthin	26.21	21.64	(426), 453, 481
18	Unknown carotenoid	27.01	17.96	(426), 452, 477
19	Non-polar chlorophyll *c* from *C. hirta*	27.51		455, 584, 633
20	Non-polar chlorophyll *c* from *C. hirta*	27.93		455, 584, 633
21	Unknown carotenoid	28.85		(419), 445, 472
22	Non-polar chlorophyll *c* from *C. hirta*	30.06		455, 584, 633
23	Non-polar chlorophyll *c* from *E. huxleyi*	30.35		455, 584, 633
24	Non-polar chlorophyll *c* from *E. huxleyi*	31.72		455, 584, 633
25	Non-polar chlorophyll *c* from *C. fragaria*	32.10		455, 584, 633
26	Chlorophyll c_2-MGDG (*E. huxleyi*)	32.41	31.10	455, 584, 633
27	Non-polar chlorophyll *c* from *P. parvum* f. *patelliferum*	32.54	30.41	451, 581, 631
28	Chlorophyll *a* allomer	32.78	25.40	430, 615, 664
29	Chlorophyll *a*	32.98	26.63	431, 616, 664
30	Chlorophyll *a* epimer	33.21	27.85	430, 615, 664
31	Chlorophyll c_2-MGDG (*C. polylepis*)	33.55	33.55	455, 584, 633
32	β,ε-carotene	35.47	31.62	(422), 448, 476
33	β,β-carotene	35.67	32.03	(426), 454, 481

the general fragmentation pattern proposed for chlorophylls, and results from combined losses of C-17[3] groups and polar groups at position C-13[2], followed by further methyl losses (Hunt et al. 1981, Chait & Field 1982, 1984, Grese et al. 1990, Hunt & Michalski 1991, Goericke et al. 2000). Most of the fragments were previously reported in the MS spectra of non-esterified chl c_2 obtained either by FAB-MS (Saitoh et al. 1993) or by electrospray-MS/MS (Goericke et al. 2000).

The confirmation of the molecular ion of a chlorophyll can be achieved when the spectrum is obtained in the presence of an acid (Grese & Gross 1992), which allows the substitution of the Mg atom by 2 H atoms, giving the corresponding pheoporphyrin, whose protonated molecular ion is expected to appear at an *m/z* value of 21 mass units below the molecular mass of the parental chlorophyll ($[(M-Mg+2H)+H]^+$). When such a spectrum was obtained for the pigment under study, a new ion at *m/z* 1244 was generated, confirming the ion at *m/z* 1265 as the molecular ion of the non-polar chlorophyll.

A subsequent CID-MS/MS experiment was performed, employing the suspected molecular ion as precursor ion. The spectrum contained several product fragments that included ions characteristic of the tetrapyrrolic macrocycle (*m/z* 590, 532, 503). Especially interesting were 3 peaks in the intermediate mass range (*m/z* 1037, 809 and 693), which were interpreted as the consecutive losses of 1 (*m/z* 1037, $[M-228]^+$) and 2 (*m/z* 809, $[M-228-228]^+$) myristic acid residues and the combined loss of dimyristylglycerol and the $-COOCH_3$ group from the fifth ring (*m/z* 693, $[M-513-59]^+$). The presence of myristic acid in the molecule was confirmed in a subsequent negative-ion

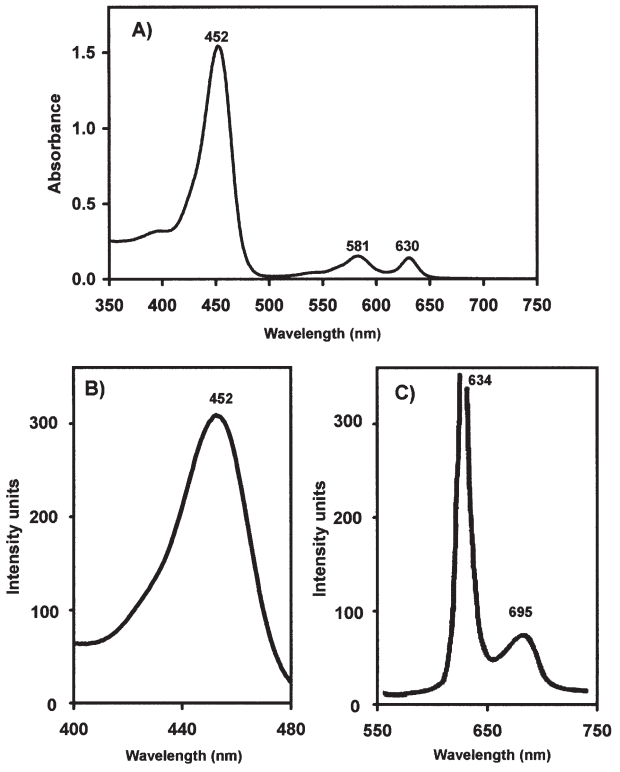

Fig. 2. *Chrysochromulina polylepis* CCMP 286. Visible spectrum of non-polar chl *c*. (A) Absorption spectrum; (B) fluorescence excitation spectrum (emission = 632 nm), and (C) fluorescence emission spectrum (excitation = 452 nm). Solvent was diethyl ether

spectrum that showed, besides a peak corresponding to the deprotonated molecular ion (*m/z* 1264, $[M–H]^-$), a very prominent ion at *m/z* 227 that was attributed to the carboxylate anion of this fatty acid. All this evidence led us to propose a tentative structure for the pigment in which a chl c_2 is linked, via an ester bond, to a monogalactosyldimyristylglycerol (Fig. 4B).

Chls *c* in *Chrysochromulina polylepis* and *Chrysochromulina* spp. strains

Table 4 lists the distribution pattern of polar and non-polar chls *c* into *Chrysochromulina polylepis* strains and several *Chrysochromulina* species. Non-polar chl *c* patterns from *Emiliania huxleyi* and *Prymnesium parvum* f. *patelliferum* are also included. While all *C. polylepis* strains contained the new pigment chl c_2-MGDG [14:0/14:0], it was only detected in trace amounts in *C. hirta*, *C. fragaria* and *C. kappa* (Fig. 5). In these species, fluorescent peaks showing similar spectral properties were detected at shorter retention times (Peaks 19, 20, 22 and 25; Table 3). These species also contained a compound showing a UV-VIS spectrum and chromatographic behaviour (in both chromatographic systems) very similar to that of chl c_2-MGDG [14:0/18:4] from *Emiliania huxleyi*. Table 6 lists pigment ratios of different chlorophyll *c* pigments to chl *a* for the *C. polylepis* strains and the *Chrysochromulina* species.

Distribution of carotenoids in *Chrysochromulina polylepis* strains and *Chrysochromulina* spp.

Table 5 summarises the distribution of the major carotenoids of the *C. polylepis* strains and *Chrysochromulina* species studied. Even though 19′-hexanoyloxy-fucoxanthin is the dominant carotenoid in all *C. polylepis* strains analysed, *C. camella*, *C. hirta*, *C. kappa* and C. *strobilus* showed fucoxanthin as the major carotenoid (Fig. 5). The ratios of fucoxanthin and related pigments to chl *a* listed in Table 6 show the variability of Hex-fuco:chl *a* ratios for the *C. polylepis* strains (0.334 to 0.652), and the *Chrysochromulina* species (0.039 to 0.652).

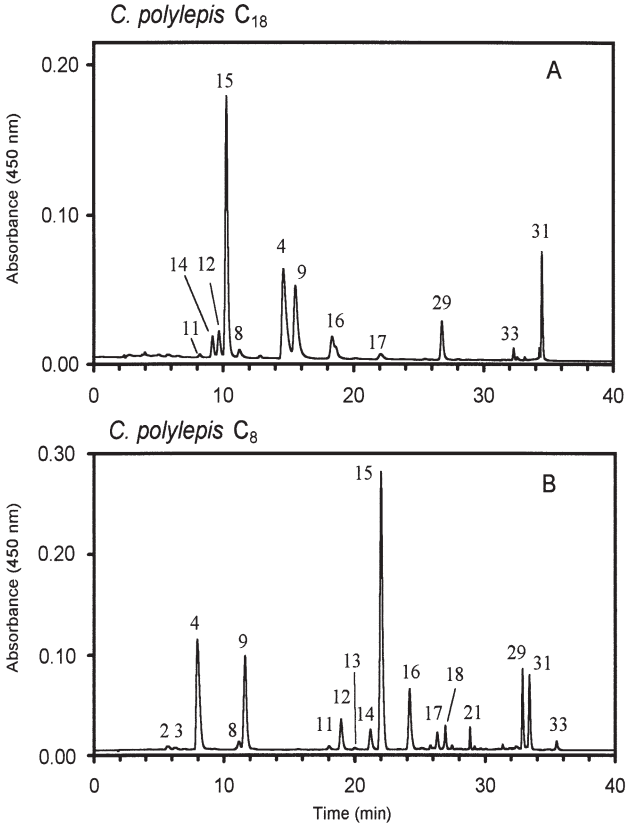

Fig. 3. *Chrysochromulina polylepis* CCMP 286. HPLC chromatograms. (A) Polymeric C_{18} column; (B) monomeric C_8 column. Detection by absorbance at 450 nm. Peak identifications as in Table 3

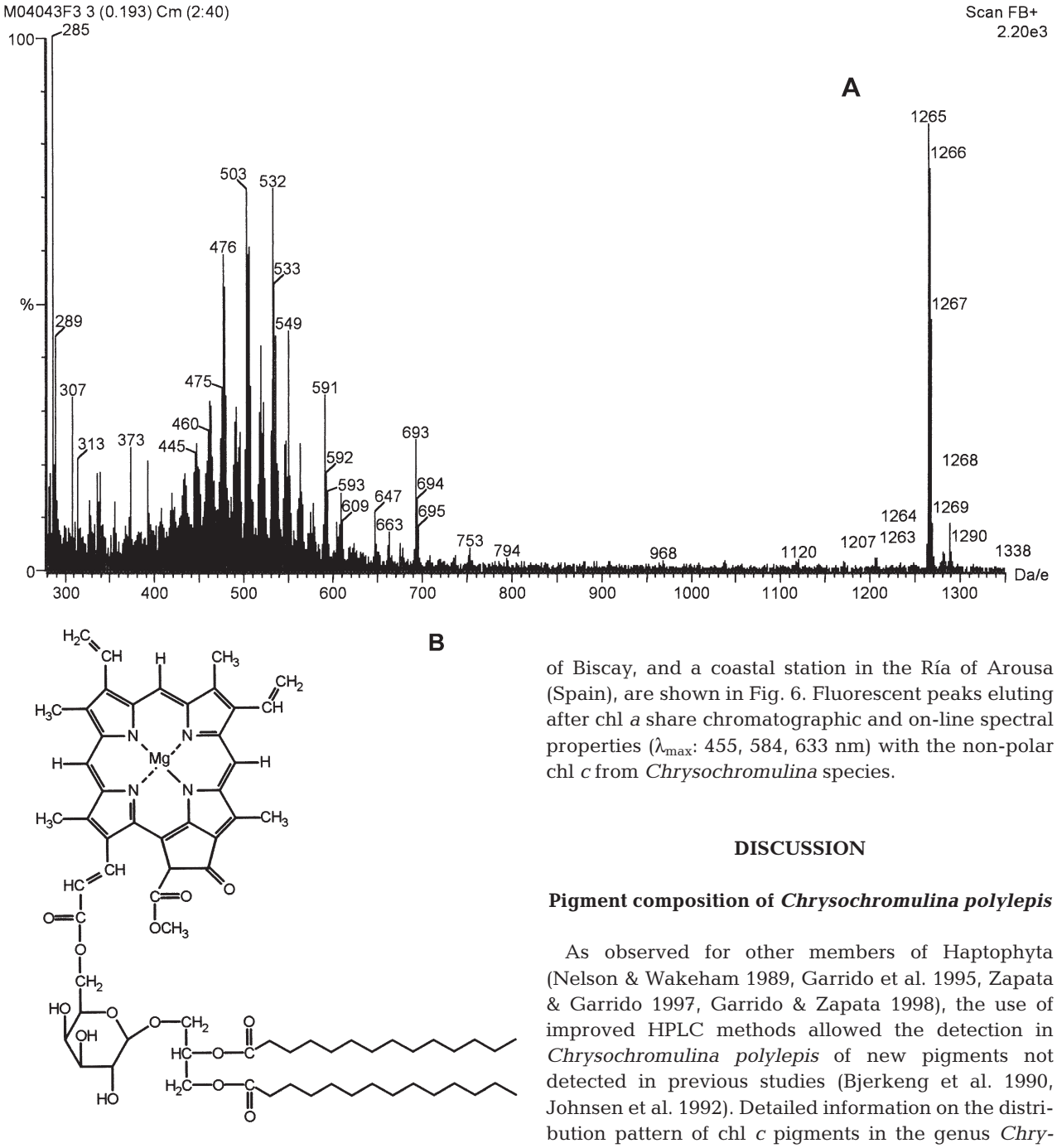

Fig. 4. *Chrysochromulina polylepis*. (A) Fast atom bombardment mass spectrum of novel non-polar chl *c* isolated from Strain CCMP286. Positive ion spectrum using 3-nitrobenzyl alcohol as matrix. (B) Tentative molecular structure proposed for the chl c_2-MGDG isolated from Strain CCMP 286

Non-polar chl c from *Chrysochromulina polylepis* in natural samples

The chromatographic traces of seawater samples collected from a station in the continental slope of Bay of Biscay, and a coastal station in the Ría of Arousa (Spain), are shown in Fig. 6. Fluorescent peaks eluting after chl *a* share chromatographic and on-line spectral properties (λ_{max}: 455, 584, 633 nm) with the non-polar chl *c* from *Chrysochromulina* species.

DISCUSSION

Pigment composition of *Chrysochromulina polylepis*

As observed for other members of Haptophyta (Nelson & Wakeham 1989, Garrido et al. 1995, Zapata & Garrido 1997, Garrido & Zapata 1998), the use of improved HPLC methods allowed the detection in *Chrysochromulina polylepis* of new pigments not detected in previous studies (Bjerkeng et al. 1990, Johnsen et al. 1992). Detailed information on the distribution pattern of chl *c* pigments in the genus *Chrysochromulina* had been difficult due to analytical limitations. The use of pyridine-containing HPLC methods either on polymeric C_{18} or monomeric C_8 columns allowed detection of MgDVP (Peak 8, C_8 HPLC system), in addition to chl c_2 and chl c_3, in all *Chrysochromulina* species studied.

Divinyl protochlorophyllide *a* (DV pchlide *a* or MgDVP), a chl *a* biosynthesis precursor implicated in photosynthesis (Larkum et al. 1994) whose chemical structure was recently confirmed (Helfrich et al. 1999), had previously been detected, together with chl *a* and

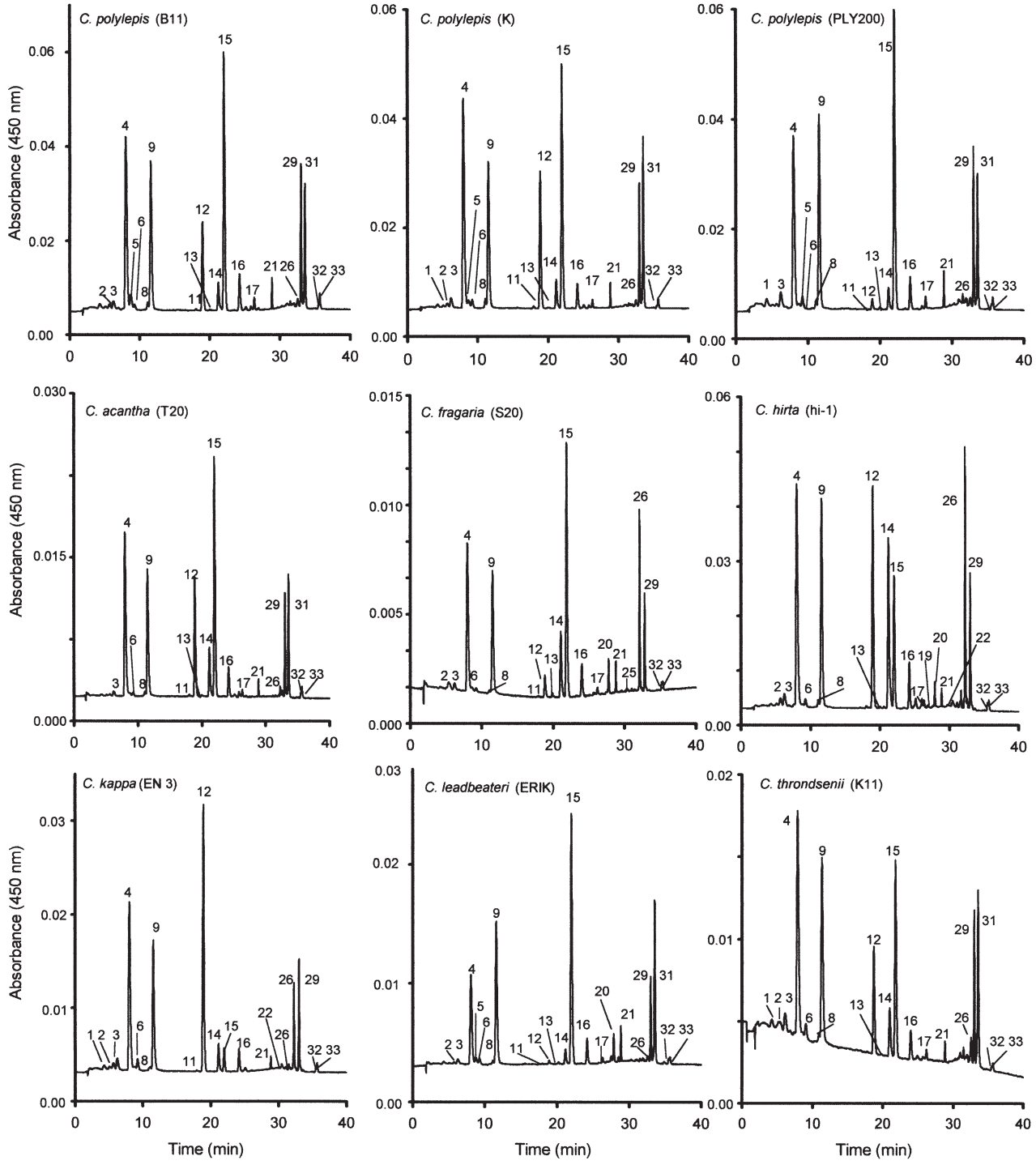

Fig. 5. *Chrysochromulina* spp. HPLC chromatograms of pigment extracts from selected species showing the diverse array of non-polar chl *c* pigments and carotenoids. Detection by absorbance at 450 nm. Peak identifications as in Table 3

b and prasinoxanthin, in some chlorophytes belonging to the class Prasinophyceae (see Jeffrey & Vesk 1997). Improved HPLC methods, able to separate MgDVP from chl c_1 and chl c_2, allowed MgDVP to be detected in chl *c*-containing algal classes (Garrido et al. 1995, Zapata et al. 1998, 2000). In consequence, MgDVP should no longer be considered as a marker pigment for members of the class Prasinophyceae (Chlorophyta).

Spectral properties of the chl c_3 in *Chrysochromulina polylepis* strains were similar to chl c_3 from *Emiliania*

Table 4. *Chrysochromulina* spp. Polar and non-polar chl *c* pigments in different species. Data from *Emiliania huxleyi* and *Prymnesium parvum* f. *patelliferum* are shown for comparison. Strain codes as in Table 1. t: trace

Species	Strain code	MgDVP	MV chl c_3	Chl c_3	Chl c_2	Non-polar chl *c*-type		
						C. hirta	*E. huxleyi*[a]	*C. polylepis*
C. polylepis	CCMP 286	+	t	+	+	–	+	+
C. polylepis	UIO B11	+	t	+	+	–	+	+
C. polylepis	UIO K	+	t	+	+	–	+	+
C. aff. *polylepis*	PLY200	+	t	+	+	–	+	+
C. acantha	UIO T20	+	–	+	+	–	+	+
C. camella	CSIRO CS-268	+	–	+	+	+	+	+
C. fragaria	UIO S19	+	–	+	+	–	+	–
C. hirta	UIO C. hi-1	+	–	+	+	+	+	–
C. hirta	CSIRO CS-228	+	–	+	+	+	+	–
C. kappa	UIO EN3	+	–	+	+	+	+	–
C. leadbeateri	UIO ERIK	+	–	+	+	–	+	+
C. strobilus	CSIRO CS-231	+	–	+	+	–	+	+
C. throndsenii	UIO K11	+	–	+	+	–	+	+
Chrysochromulina sp.	CSIRO CS-410	+	–	+	+	–	+	+
Emiliania huxleyi	CCMP 370	+	+	+	+	–	+	–
Prymnesium parvum f. *patelliferum* (chl c_1-containing)	UIO P. patRY89	+	–	+	+	–	+	–

[a]Non-chromatographically different from non-polar chl c_2-MGDG [14:0/18:4] from *E. huxleyi*

Table 5. *Chrysochromulina* spp. Fucoxanthin and acyloxy-derivatives in the species studied. Superscripts indicate the 3 most abundant fucoxanthin pigments. Strain codes as in Table 1; full pigment names in Table 3. t: trace

Species	Strain code	But-fuco	Fuco	Unknown-fuco	4-k-Hex-fuco	Hex-fuco
C. polylepis	CCMP 268	+	$+^2$	+	$+^3$	$+^1$
C. polylepis	UIO B11	+	$+^2$	t	$+^3$	$+^1$
C. polylepis	UIO K	+	$+^2$	+	$+^3$	$+^1$
C. aff. *polylepis*	PLY200	+	$+^3$	+	$+^2$	$+^1$
C. acantha	UIO T20	+	$+^2$	+	$+^3$	$+^1$
C. camella	CSIRO CS-268	–	$+^1$	+	$+^2$	$+^3$
C. fragaria	UIO S19	+	$+^3$	+	$+^2$	$+^1$
C. hirta	UIO C. hi-1	+	$+^1$	t	$+^2$	$+^3$
C. hirta,	CSIRO CS-228	t	$+^1$	t	$+^2$	$+^3$
C. kappa	UIO EN3	+	$+^1$	–	$+^2$	$+^3$
C. leadbeateri	UIO ERIK	+	$+^3$	+	$+^2$	$+^1$
C. strobilus	CSIRO CS-231	t	$+^1$	–	$+^2$	t
C. throndsenii	UIO K11	+	$+^2$	t	$+^3$	$+^1$
Chrysochromulina sp.	CSIRO CS-410	+	$+^2$	+	$+^3$	$+^1$

huxleyi (Jeffrey & Wright 1987), whose chemical structure was established as 3,8 divinyl, 7-methoxycarbonyl chl c_2 (Fookes & Jeffrey 1989). Trace amounts of MV chl c_3 (Peak 7), a pigment first detected and characterised in *E. huxleyi* (Garrido et al. 1995, Garrido & Zapata 1998), were also found in 2 strains of *C. polylepis* (B11 and K). Several non-polar chl *c*-like pigments were also found as minor peaks eluting well ahead of the main non-polar compound present in *Chrysochromulina* species (Table 3).

The identity of the non-polar chls *c* present in *Chrysochromulina hirta* and *C. fragaria* remains open. Even though these pigments coeluted with the major non-polar chl c_2 from *Emiliania huxleyi* (chl c_2-MGDG [14:0/18:4]), a slight difference in their respective retention time was observed by the C_8 HPLC method. Further studies employing HPLC-MS will be needed to confirm the presence of chl c_2-MGDG [14:0/18:4] in *Chrysochromulina* species.

Chromatographic behaviour of the non-polar chl *c*

When extracts of *Chrysochromulina polylepis* and *Emiliania huxley* were analysed using the polymeric C_{18} method (Fig. 1A,B), the non-polar chl *c*-like pig-

Table 6. *Chrysochromulina* spp. Ratios of pigments to chl *a* for *C. polylepis* strains and several *Chrysochromulina* species studied. Full pigment names in Table 3

Chrysochromulina species	Strain code	Carotenoids:chl *a*					Chlorophylls *c*:chl *a*			
		But-fuco	Fuco	Unknown-fuco	4-k-Hex-fuco	Hex-fuco	Chl c_3	Chl c_2	Non-polar chl *c* *E. huxleyi*-type	Non-polar chl c_2 *C. polylepis*-type
C. polylepis	CCMP 286	0.008	0.072	0.006	0.048	0.652	0.151	0.139	0.000	0.125
C. polylepis	UIO B11	0.001	0.112	0.001	0.041	0.334	0.130	0.127	0.000	0.120
C. polylepis	UIO K	0.003	0.205	0.000	0.048	0.371	0.181	0.147	0.000	0.192
C. aff. *polylepis*	PLY 200	0.001	0.010	0.003	0.027	0.385	0.112	0.146	0.000	0.118
C. acantha	UIO T20	0.002	0.207	0.001	0.092	0.427	0.164	0.149	0.010	0.164
C. fragaria	UIO S19	0.000	0.043	0.006	0.129	0.494	0.155	0.157	0.211	0.000
C. hirta	UIO C. hi-1	0.002	0.296	0.002	0.253	0.178	0.177	0.185	0.223	0.000
C. kappa	UIO EN3	0.000	0.442	0.000	0.045	0.039	0.160	0.149	0.084	0.000
C. leadbeateri	UIO ERIK	0.000	0.007	0.004	0.030	0.543	0.111	0.203	0.000	0.269
C. throndsenii	UIO K11	0.000	0.131	0.000	0.063	0.244	0.152	0.155	0.000	0.172

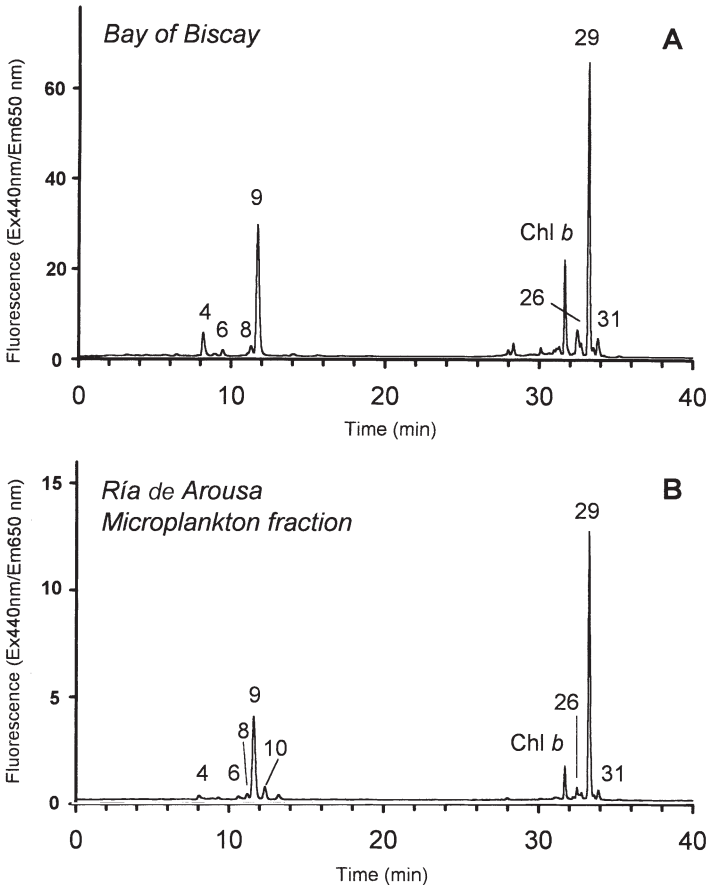

Fig. 6. Chromatograms of field samples of seawater from (A) Bay of Biscay (45° 16.8′ N, 6° 10.2′ W, GIGOVI 98 cruise), and (B) Ría de Arousa, NW Spain. C_8 HPLC method. Detection by fluorescence (excitation = 440 nm, emission = 650 nm). Peak 31 is non-polar chl *c Chrysochromulina*-type. Peak identifications as in Table 3

ments were completely resolved e.g. chl *a*, chl c_2-MGDG [14:0/18:4] from *E. huxleyi*, and lastly chl c_2-MGDG [14:0/14:0] from *C. polylepis*. The same extract analysed by the C_8 HPLC method showed a dramatic change in the elution order, with chl *a* eluting between the chl c_2-MGDG [14:0/18:4] from *E. huxleyi* and the chl c_2-MGDG [14:0/14:0] from *Chrysochromulina* (Fig. 1C,D).

The chromatographic behaviour of both non-polar chls *c* can be explained considering their equivalent carbon number (ECN). This parameter is defined as the number of carbon atoms in the fatty acid acyl residues less twice the number of total double bonds contained in such residues (Christie 1987). The ECN value for the *Emiliania huxleyi* non-polar chl c_2 [14:0/18:4] is 24, while the ECN for the *Chrysochromulina polylepis* non-polar chl c_2 [14:0/14:0] is 28. Although the latter pigment has a shorter carbon chain, it was retained more than the non-polar chl c_2 from *E. huxleyi*. In consequence, the ECN value

seems to control the retention order in both HPLC methods.

Based on this structure-retention relationship, the combination of HPLC methods with different chromatographic selectivity will provide a powerful tool to infer subtle differences in the molecular structure of this family of high molecular weight non-polar chl *c*-type pigments. However, other spectroscopic techniques (mass spectrometry, NMR) will be required for a correct structure elucidation.

Pigment pattern in *Chrysochromulina polylepis* and *Chrysochromulina* spp. strains

Chemotaxonomic implications: a qualitative approach

Non-polar chl *c* was not previously detected in *Chrysochromulina polylepis* even though its ratio to total chl *c* (ca 15% by moles) is higher than that in *Emiliania huxleyi* CCMP 370 (ca 11 to 13%), and *Isochrysis galbana* CCMP 1323 (ca 7%) (Garrido et al. 1995, 2000, Zapata & Garrido 1997).

This fact could be explained by the chromatographic behaviour of non-polar chls *c* pigments from *Emiliania huxleyi* and *Chrysochromulina polylepis* using HPLC methods based on monomeric C_{18} columns (Mantoura & Llewellyn 1983, Zapata et al. 1987, Wright et al. 1991). Such methods provide similar retention times for chl c_2-MGDG from *E. huxleyi* and a cluster of chl *a*-like pigments (e.g. chl *a* allomers, chl *a* and/or DV chl *a*), whereas chl c_2-MGDG from *C. polylepis* elutes very close to chl *a* and its epimer (chl *a*). As a consequence, the failure in resolution could mask the non-polar chl *c* identification.

A non-polar chl *c*-like pigment was detected in natural samples collected from the north-eastern Atlantic during the 1990 spring bloom (Barlow et al. 1993). In order to find which phytoplankton species was the origin of this pigment, the prominent flagellates *Chrysochromulina* sp., *Imantonia rotunda* and *Emiliania huxleyi* were isolated from the study area and cultures were established (Barlow et al. 1993). A non-polar chl *c*-like pigment, eluting just after chl *a*, was detected in *Chrysochromulina* sp. (Strain PCC-1901gA), but neither *E. huxleyi* (Strain PCC1779Ge) nor *Imantonia rotunda* (Strain PCC1779b) showed a similar pigment. The visible absorption spectrum of non-polar chl *c* from *Chrysochromulina* sp. was very similar to that reported for the non-polar chl *c* from *E. huxleyi* (Nelson & Wakeham 1989).

Barlow et al. (1993) failed to identify the pigment from *Chrysochromulina* sp. as a novel compound; they did not realise it was different from that found in *Emiliania huxleyi*, and they also claimed that non-polar chl *c* was not always present in *E. huxleyi* isolates.

In a recent study focused on *Chrysochromulina leadbeateri*, Johnsen et al. (1999) detected the presence of a non-polar chl *c* pigment characterised by absorption maxima at 457, 586 and 634 nm eluting after chl *a*. Considering both the retention time and spectral data, this pigment could correspond to the chl c_2-MGDG described here.

Chemotaxonomic implications: a quantitative approach

The very variable proportions of fucoxanthin, 19′-hexanoyloxyfucoxanthin and other acyloxy derivatives found in *Chrysochromulina polylepis* strains and *Chrysochromulina* species (Table 6) precludes the use of a simple pigment ratio (e.g. Hex-fuco:chl *a*) to estimate the contribution of these algal species to total chl *a* biomass.

This drawback affects the chemotaxonomic methods based on fixed pigment ratios (Letelier et al. 1993, Goericke & Montoya 1998). On the other hand, such methods claim fucoxanthin is contributed by diatoms. So, if fucoxanthin-containing *Chrysochromulina* species are present, fucoxanthin will be misinterpreted, and converted into chl *a* associated with diatoms. This problem could be partly solved by using the CHEMTAX program, which employs the full range of pigments of each algal class or chemotaxonomic category as defined by base to pigment composition (Mackey et al. 1996). The lower variability of chl *c* to chl *a* ratios compared to fucoxanthin and/or acyloxy-derivatives to chl *a* ratios (Table 6) support the utility of chlorophylls for chemotaxonomic reconstruction.

Are pigment markers useful for detecting *Chrysochromulina* species?

Since the detection of toxic and/or bloom-forming phytoplankton species in coastal waters is of importance for fish- and shellfish farmers, any simple method to detect and quantify harmful phytoplankton species should be encouraged.

The new chl c_2-MGDG [14:0/14:0] described here may be suitable as a marker for *Chrysochromulina* species, including *C. polylepis* and C. *leadbeateri*, both of which are considered potentially harmful species in natural waters (Edvardsen & Paasche 1998, Johnsen et al. 1999). The presence of this pigment in other *Chrysochromulina* species that (so far) have been considered non-harmful, implies a constraint to the chemotaxonomic approach in monitoring. However, as long

as our knowledge on ichthyotoxins, their biosynthetic pathways, and environmental factors inducing bloom development and toxicity in *Chrysochromulina* species is incomplete, all members of the genus *Chrysochromulina* should be regarded as potentially harmful species.

Several techniques, such as electron microscopy (Eikrem & Throndsen 1998), flow cytometry (Boddy et al. 2000), and molecular probes (Simon et al. 1997) have been employed for identifying *Chrysochromulina* species. However, the necessity for rapid diagnostic coupled with intensive sampling limits the routine use of some of these techniques. Marker pigments of harmful algal species offer one possible means of accomplishing the rapid detection and quantification of *Chrysochromulina* species in monitoring programs.

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