Phytoplankton pigment biomarkers: HPLC separation using a pentafluorophenyloctadecyl silica column

Noelia Sanz, Ana García-Blanco, Antonio Gavalás-Olea, Patricia Loures and José L. Garrido*

Instituto de Investigaciones Marinas (CSIC). Av. Eduardo Cabello, 6. 36208 Vigo (Spain).

Summary

1. The use of pigment data to map microalgal populations in natural waters has become an established and convenient way of studying phytoplankton. However, the chromatographic analysis of algal pigments is a major challenge due to the diversity of molecules spanning a wide range of polarities, but also including many that have closely similar chemical structures. Among these sets of compounds of similar structure, the separation of mono- and divinylic pairs of chlorophylls is of particular importance due to their relevance as chemotaxonomical biomarkers.

2. In this work we have taken advantage of the special type of solute-stationary phase interactions provided by pentafluorophenyl phases together with the high retention values resulting from an octadecyl spacer in the column to develop a method for the joined

22 analysis of chlorophylls and carotenoids. The mobile phase contains organic solvents of low
23 toxicity, methanol and ethanol and ammonium acetate buffer, in a simple binary elution
24 gradient.
25 **3.** More than seventy photosynthetic pigments (chlorophylls, carotenes and xanthophylls)
26 can be determined in the same chromatographic analysis employing the method here
27 presented. The complete resolution of mono- and divinylic forms of chlorophylls *a*, *b* and *c*
28 is achieved in less than 42 minutes. The same analysis allows the separation of most
29 chemotaxonomically important carotenoids, including positional isomers.
30 **4.** The method can successfully be applied to the characterization of the pigment
31 composition of members of different classes in the main chloroplast lineages (red and
32 green) of the evolution of photosynthetic eukaryotes, in the study of biosynthetic processes
33 of chlorophylls and especially in the description of plankton populations in natural waters. It
34 is particularly suited for the simultaneous detection of green algae and the cyanobacteria of
35 outstanding global importance *Phrochlorococcus marinus*.
36
37 **Key words:** carotenoid, chlorophyll, divinylchlorophyll, liquid chromatography,
38 microalgae, phytoplankton diversity
39

40 Introduction

41
42 The analysis of pigments is a major task in studies on the evolution, biochemistry,
43 physiology and ecology of photosynthetic organisms (Scheer 2006; Keeling 2010;
44 Jeffrey *et al.* 2011; Takaichi 2011). In aquatic sciences, the detailed chromatographic

analysis of pigments is used, among other applications, for the characterization of plankton populations, to trace the trophic transfer from primary producers, to study the photosynthetic responses to changing light environments and for the ground-truthing of satellite derived biomass estimations (Jeffrey 1997; Jeffrey *et al.* 1997; Jeffrey & Wright 2006). The analysis of pigments by liquid chromatography is complementary to traditional microscopy of plankton samples. HPLC analysis is fast, reproducible and suitable for screening large numbers of environmental samples (Roy & Garrido 2013).

Such a great number of applications is only possible because of the selective distribution across different taxonomical categories of the variety of pigments that aquatic photosynthetic organisms have evolved after successive endosymbiotic events (Chan & Bhattacharya 2010; Keeling 2010). Photosynthetic pigments can be either water-soluble (phycobiliproteins) or soluble in organic solvents (carotenoids and chlorophylls (chls)). The joint analysis of chls and carotenoids is especially useful for measuring phytoplankton diversity, because they occur in all algal taxa with variable degrees of specificity (Jeffrey *et al.* 2011), and many of them are limited to particular classes or even genera. Moreover, they are easily extracted and can be detected with high sensitivity (Wright & Jeffrey 2006).

The molecular diversity of chls (Zapata *et al.* 2006; Scheer 2006) and carotenoids (Guedes *et al.* 2011; Takaichi 2011) that underlies their utility as biomarkers, explains also why the analysis of algal pigments is still a challenge for chromatographic methods (Garrido *et al.* 2011). Phytoplankton pigments can span a wide range of polarities, from

69 the very non-polar phytylated chlorophylls to the acidic chl *c* forms and
70 chlorophyllides; and from the hydrocarbon carotenes to very oxygenated xanthophylls
71 (Wright & Jeffrey 2006). In addition, many of them have closely related structures
72 (some differ only in a double bond or even are positional, *cis-trans* or stereo isomers).
73
74 Algal chls may occur in natural samples as pairs of MV and DV analogues of the same
75 basic structures (e.g. chl c_2/chl c_1, chl c_3/MVchl c_3, DVchl *b*/chl *b*, DVchl *a*/chl *a*). DV chl
76 *a* and DV chl *b* are marker pigments for the marine prokaryote *Prochlorococcus*
77 *marinus*, a species that contributes significantly to primary production in the
78 oligotrophic oceans (Partensky *et al.* 1999). Their separation is crucial for a correct
79 estimation of the contribution of *Prochlorococcus* to the planktonic biomass and to
80 avoid the overestimation of chls *a* and *b*, and thus of the abundance of other
81 photosynthetic organisms.
82
83 The goals of an ideal method for the analysis of phytoplankton pigments in natural
84 populations should be i) to obtain good chromatographic resolution of both DVchls *a*
85 and *b* from their MV analogues, ii) to resolve chl *c* pigments, iii) to separate the polar
86 derivatives of chls *a* and *b* and iv) to resolve taxon-specific carotenoids in a single
87 chromatographic run. Other cases of study (for example, the physiological studies of
88 the pigment composition of isolated species) are usually less demanding.
89
90 Different stationary phases have been used for the separation of phytoplankton
91 pigment signatures (Garrido *et al.* 2011; Roy & Garrido 2013). The methods based on
92 C8 columns are currently the most commonly used (Zapata *et al.* 2000; Van Heukelem

93 & Thomas 2001). Such methods achieve good resolution of the pairs chl c_1/chl c_2 and
94 chl *a*/DVchl *a* but fail in the separation of other important pairs as chl *b*/DVchl *b* (for a
95 critical comparison between both methods see Wright & Jeffrey 2006). Recently
96 Jayaraman *et al.* (2011) developed a method using a C16-amide phase that
97 demonstrated good resolution for MV/DV pairs of chls *a* and *b* and isomeric
98 carotenoids (Lutein/Zeaxantin) at the cost of the coelutions of chl c_3 with MV chl c_3 and
99 of DV Protochlorophyllide *a* (frequently designated as Mg-DVP in the marine sciences)
100 with chl c_2.
101
102 Pentafluorophenyl (PFP) stationary phases for liquid chromatography show alternative
103 selectivity compared to alkyl-bonded reversed phases through additional mechanisms
104 such as dipole-dipole, hydrogen bond and π,π interactions (Ashu-Arrah *et al.* 2013).
105 These phases contain a PFP group that is bound to the silica surface *via* an
106 unfluorinated alkyl chain spacer (frequently a propyl group, insufficient to achieve
107 proper retentions of photosynthetic pigments in their whole range of polarity).
108 Recently available, a PFP phase with a longer (C18) spacer conjugates unique
109 properties of hydrophobicity and selectivity. The aromatic character of chlorophyll
110 macrocycle and the conjugated π system of the carotenoid polyenic chain, combined
111 with different polarities and differences in oxygenated functional groups of chls and
112 carotenoids, led us to explore the use of a PFP-octadecyl silica column in the analysis
113 of photosynthetic pigments, with special emphasis in marine phytoplankton.
114
115 The growing concern in environmental and health risks and laboratory safety underlies
116 the efforts in the development of new methods in liquid chromatography focused to

minimize the amount of organic solvent in the mobile phases and to propose alternatives to the use of toxic solvents (Plotka *et al.* 2013). The method here proposed employs a binary gradient system, with components of lower toxicity than in other methods -no acetonitrile, acetone or pyridine-, for the analysis of biomarker pigments including the complete resolution of MV/DV pairs of chls *a*, *b* and *c*.

Materials and methods

ALGAL CULTURES, PIGMENT STANDARDS AND FIELD SAMPLES

Algal cultures were selected to be representative of algal classes found in marine phytoplankton, which together contain most diagnostic chls and carotenoids: *Emiliana huxley* CCMP 370, *Pavlova gyrans* CCMP 608 and *Tetraselmis marina* CCMP 898 (Provasoli-Guillard National Center for Marine Algae and Microbiota, West Boothbay Harbour, Maine, USA); *Rhodomonas baltica, Dunaliella salina* and *Nanochloropsis gaditana* (Culture Collection of Marine Microalgae from the Instituto de Ciencias Marinas de Andalucia, Cádiz, Spain); *Micromonas pusilla* CCAP 1965/4 and *Isochrysis galbana* CCAP927/1 (Culture Collection of Algae and Protozoa, Oban, UK)*; Alexandrium minutum* AL-1V-IEO (Instituto Español de Oceanografia, Vigo, Spain); *Lepidodinium chlorophorum* RCC1489, *Ostreococcus sp.* RCC788 and *Pelagomonas calceolata* RCC853 (Roscoff Culture Collection, Roscoff, France)*; Chrysocromulina throndsenii* (Department of Ecology, University of the Basque Country); *Exanthemachrysis gayraliae* PLY 488 (Plymouth Culture Collection of Marine Microalgae, Plymouth UK). Frozen filters of cultures of the dinoflagellate *Karlodinium*

veneficum VGO870 (isolated in Boughrara, Tunisia, Mediterranean Sea; Culture Collection of the Instituto Español de Oceanografía, Vigo, Spain) were kindly provided by Dr. Francisco Rodríguez.

All cultures were grown in L1 enriched seawater medium (Guillard & Hargraves 1993) and maintained at light irradiances of 100 μmol photons $m^{-2}s^{-1}$ (12:12 light-dark cycles) at 15 ºC. The macrophytic green alga *Codium tomentosum* (a source of free and esterified Siphonoxanthin) was collected in a local beach. Cucumber (*Cucumis sativus*) seeds were obtained from a local market and germinated in darkness at 25 ºC to obtain etiolated tissue as a source of MV and DV protochlorophyllide *a*. Chlorophyllides *a* and *b* were obtained from *Dunaliella salina* after promoting chlorophyllase activity (Jeffrey & Barret 1971). Pheophytins *a* and *b* were prepared by acidification of the corresponding chlorophylls with 0.1 N HCl, transferred to hexane and the hexane layer washed several times with water until the aqueous phase was neutral.

Standards of β-Cryptoxanthin and Capsanthin and a mixture of pigments (used to confirm the correct identification of a wide range of pigments and as source of DV chl *a* and DV chl *b*) were purchased from DHI (DHI Laboratory Products, Hoersholm, Denmark). Standards of bacteriochlorophyll *a* (from *Rhodopseudomonas sphaeroides*) and *trans*-β-Apo-8'-carotenal were obtained from Sigma-Aldrich Química (Tres Cantos, Madrid, Spain).

Natural samples from the Mediterranean Sea (obtained during the HOTMIX cruise along May 2014) and from a fixed station in the Ría de Vigo (kindly provided by Dr. Francisco Rodríguez, Instituto Español de Oceanografía) were used as examples of oligotrophic and estuarine waters. 5 ml culture aliquots and seawater samples (2 L) were filtered under low

vacuum through 25 mm diameter glass fibre filters (0.7 μm nominal pore size), excess water blotted out from folded filters between several layers of filter paper, and immediately frozen.

PIGMENT EXTRACTION

Frozen filters obtained from algal cultures and natural samples were extracted with 3 mL of 90% acetone in screw cap glass tubes, with polytetra-fluoroethylene (PTFE) lined caps, placed in an ice-water bath (HPLC grade acetone, from Panreac, Barcelona, Spain and Milli-Q water were employed for the preparation of the extraction solvent). After 15 minutes, filters were grinded using a stainless steel spatula and placed in an ultrasonic bath with water and ice for 5 minutes. The slurries were then centrifuged 5 minutes at 3940 g and supernatants filtered through 13 mm diameter polypropylene syringe filters (MS PTFE, 0.22 μm pore size) to remove cell and filter debris. All sample preparations were done under subdued light.

Fronds of *C. tomentosum* were cleaned under the tap with a smooth brush to detach epiphytic organisms, blotted between several layers of filter paper and then quickly immersed in 90% acetone. Cucumber etiolated tissues were directly immersed in cold 90% acetone. Subsequent grinding, centrifugation and filtration steps were as described above for microalgal filters.

HPLC SYSTEM

188 The chromatographic equipment was a Waters 2690 Alliance separations module with
189 a 996 photodiode array detector. Analytical separations were performed using an ACE
190 C18 PFP column, 150 mm x 4.6 mm, 3 μm particle size (Advanced Chromatography
191 Technologies, Aberdeen, Scotland) at 33 ºC. The mobile phase gradient, at a constant
192 flow rate of 1 mL min^{-1}, consisted of a mixture methanol: 225mM ammonium acetate
193 (82:18, v:v) as solvent A and ethanol as solvent B (Table 1). A re-equilibration time of 2
194 minutes in initial conditions was allowed between injections. Methanol and ethanol,
195 both HPLC gradient grade, were obtained from Panreac (Barcelona, Spain) and Merck
196 (Darmstad, Germany) respectively. Ammonium acetate was analytical grade from Carlo
197 Erba (Milano, Italy).
198 Samples were mixed with Milli-Q water to avoid peak distortion (Zapata & Garrido
199 1991; Latasa *et al.* 2001) by either manually adding 0.4ml of Milli-Q to 1 ml of each
200 sample extract immediately before injection (200 μl) or by employing an automatic
201 injection sequence in which 74 μl water, 130 μl sample and 37 μl water were
202 consecutively placed in the injection loop.
203
204 PIGMENT IDENTIFICATION
205
206 Chlorophylls and carotenoids were detected by diode-array spectroscopy (300-720
207 nm). Identification was based on the comparison of the retention times and spectral
208 characteristic of peaks with those of standards or extracts from reference
209 phytoplankton cultures. Peak purity was checked by testing its spectral homogeneity.
210 The resolution between two given peaks (a and b) was quantified using the equation
211 $Rs(a/b)= 2\ (Rt_b-Rt_a)\ (W_a+W_b)^{-1}$, where Rt_b and Rt_a refer to the retention times and W_a

212 and W_b are the baseline bandwidths (Snyder *et al.* 1988).

213

214 # Results

215

216 MOBILE PHASE COMPOSITION AND COLUMN TEMPERATURE

217

218 Several organic solvents (methanol, acetonitrile, acetone, ethanol, ethyl acetate, 2-
219 propanol and tetrahydrofuran) were tested to modify the selectivity of the system and
220 its effects on resolution (data not shown). As best results were obtained with alcoholic
221 solvents, methanol was chosen as the component of solvent A due to its relatively low
222 eluotropic strength in reversed phase. Ethanol was preferred as solvent B due to its
223 adequate values of solvent strength and viscosity (providing assumable pressures in
224 the system) and its low toxicity.

225

226 Initial gradient conditions consisting of 82% of methanol and 18% ammonium acetate
227 solutions provided the initial aqueous phase needed to modulate the retention of
228 acidic chlorophylls and polar carotenoids. The sequential separation of progressively
229 less polar pigments required the gradual substitution of solvent A by ethanol, ending
230 with a 100% ethanol phase in order to achieve the elution of pheophytins *b* and *a*
231 within 42 min.

232

233 The presence of ammonium acetate in eluent A has a drastic effect on the retention of
234 acidic chlorophylls (Fig. 1), especially at low concentrations (5 to 200 mM). Above 200

mM ammonium acetate, only small increments of retention are produced with increasing concentration. Polar (but neutral) carotenoids decrease slightly their retention times with increasing ammonium acetate. In this scenario, some coelutions are produced at different salt concentrations: Peridinin coelutes with chl c_2 at 50 mM ammonium acetate and with chlorophyllide *a* at 500 mM. Several concentrations allowed the separation of these compounds, but a value of 225 mM ammonium acetate was finally chosen as it is the smaller salt concentration that achieves the overall resolution of the polar compounds. These conditions allow the separation of DV and MV pairs such as MVchl c_3/chl c_3 or chl c_2/chl c_1.

Column temperature affected the resolution of certain critical groups whose separation could be somewhat improved. Even in the narrow margin between 27 and 35 °C (lower temperatures increase the overall system pressure and higher ones could compromise the stability of the analytes) temperature changes affected the retention of various pigments, conditioning its resolution (Table 2). A column temperature of 33 °C was selected.

ELUTION PROFILE OF ALGAL PIGMENTS AND THE SEPARATION OF MV-/DV- chl PAIRS

The mix of pigment standards from DHI supplemented with chlorophyllides and pheophytins (maximum and minimum polarities that determine the length of analysis) and different mixtures of algal extracts were used in order to select the eluent composition and to develop the gradient profile. The three mixtures encompass a set of pigments representative of the structural diversity expected in natural samples. The

overall chromatographic performance of the method can be appreciated from the resulting chromatograms in the chosen conditions (Fig. 2 a-c), together with the application of the method to cultures of known composition (Figs. 3 and 4), and data in Table 3 that lists the pigments in increasing elution order with their respective UV-VIS spectral characteristics in the HPLC eluent.

The xanthophylls Vaucheriaxanthin, Siphonaxanthin and Peridinin eluted before chls c_2 and c_1 in the first part of the chromatogram. In the central zone, many chemotaxonomically significant carotenoids are eluted, including Fucoxanthin-related Haptophyte and Pelagophyte markers (peaks 16, 19, 22 and 23); Prasinophyte markers (specially Loroxanthin, peak 18 and Prasinoxanthin, peak 24) and the xanthophylls Alloxanthin (a marker for cryptophytes, peak 31) and Gyroxanthin (taxonomically significant for certain dinoflagellates, peak 41). Good separation is also achieved for the photoprotective xanthophylls implied in the epoxidation/de-epoxidation cycles of green algae (Violaxanthin, Antheraxanthin and Zeaxanthin; peaks 25, 30 and 34) or chromophytes (Diadinoxanthin and Diatoxanthin; peaks 27 and 32). The separation of isomeric carotenoids is somewhat compromised, especially when components of the pair occur in very different proportions (Fig. 2 c), with only partial separations of Zeaxanthin/Lutein (peaks 34 and 35, Rs= 0.98) and β,ε-Carotene/β,β-carotene (peaks 66 and 67, Rs= 0.55). The synthetic carotenoid *trans*-β-Apo-8'-carotenal (proposed as internal standard for the chromatographic analysis of pigments), coelutes with Canthaxanthin (peaks 44 and 45).

The ability to separate MV- from DV- forms of the same chl basic structure is

283 maintained along the chromatogram: the resolutions of chl c_3/ MV-chl c_3 (peaks 2 and
284 3, Rs>1.5) and chl c_2/chl c_1 (peaks 9 and 10, Rs=1.28) are achieved in the first part of
285 the chromatogram together with the simultaneous separation of the isomeric pairs chl
286 c_1/DV-Protochlorophyllide *a* (Mg DVP), both of molecular mass 610.94, differing in the
287 position of a double bond, (peaks 10 and 12, baseline resolution) and MV chl c_3 and chl
288 c_{CS170} (molecular mass 654.96, also positional isomers; peaks 3 and 4, Rs= 1.09). The
289 separation of the chl biosynthetic intermediates Protochlorophyllide *a* and DV-
290 Protochlorophyllide *a* (Mg-DVP), isolated from cucumber etiolated tissue (peaks 12
291 and 13, Rs= 0.78, Table 3), is also achieved. At higher retention times, two chlorophylls
292 of unknown structure from *E. gayraliae* elute with baseline resolution (peaks 14 and
293 17). The first of these pigments and the polar chl *c* from *P. gyrans* have been claimed
294 to be the same compound (Zapata *et al.* 2004), and in fact they coelute also in this
295 method. Their on-line spectra closely resemble those of chls c_2 and c_1, respectively,
296 suggesting a DV-/MV- nature of the chromophore of these pigments. At higher
297 retention times, the pair DV-chl *b*/chl *b*, a major challenge for many HPLC methods is
298 completely resolved (peaks 49 and 50, Rs>1.5). DV-chl *a* and chl *a* also elute with
299 baseline separation (peaks 60 and 61 Rs=1.5).
300
301 PIGMENT ANALYSIS OF UNIALGAL CULTURES
302
303 Extracts of species representative of different pigment types in the green algal lineage
304 (Jeffrey et al 2011) and containing, as a whole, 22 from their 24 characteristic
305 xanthophylls (Jeffrey *et al.* 2011), were analysed (Fig 3a-d). Chls *a* and *b* are always
306 constitutive of their pigment profiles.

307

308 The chromatographic trace of *T. marina* (Fig. 3a) shows the complete separation of
309 typical xanthophylls from prasinophycean pigment-type 2A (*sensu* Jeffrey et al. 2011)
310 that encompasses the common carotenoids in Chlorophyta (i.e., those corresponding
311 to the Chlorophyte 1 pigment type: 9'-*cis*-Neoxanthin, Violaxanthin, Antheraxanthin,
312 Zeaxanthin and Lutein) plus Loroxanthin and its two esters. A partial resolution of β,β-,
313 β,ε- and β,ψ carotenes was also obtained.

314

315 The distinctive pigment profile of *Ostreoccocus sp.* (Prasinophyte type 3A,) is well
316 characterised with the proposed method (Fig. 3b): the polar chls, Chl $c_{CS\ 170}$ and DV-
317 Protochlorophyllide a (Mg-DVP), a group of carotenoids in the central zone with
318 Uriolide, Prasinoxanthin (pigment markers for Mamiellales), 9'-*cis*-Neoxanthin and
319 Violaxanthin, and at higher retention times the additional pigments Dihydrolutein and
320 an unknown non polar carotenoid (eluting later than chl *a*).

321

322 The dinoflagellate *L. chlorophorum* shows the typical profile of a chlorophycean
323 chloroplast (Fig. 3c), plus an unknown carotenoid eluting well differentiated from
324 Lutein.

325

326 *Codium tomentosum* extracts render a chromatogram (Fig. 3d) of a typical Chlorophyte
327 pigment type 2 (Jeffrey *et al.* 2011), with the xanthophyll siphonoxanthin and its ester
328 with dodecenoic acid as biomarkers.

329

330 Eight representative species of the red algal lineage (always without chl *b* and most

331 times with some pigment of the chl *c* family) are shown in Fig. 4.

332

333 The prymnesiophycean *Isochrysis galbana* (Haptophyte pigment type 1) with a
334 pigment composition shared by members of other classes (several pigment types in
335 diatoms, haptophytes, and dinophytes) (Jeffrey *et al.* 2011) serves to illustrate (Fig. 4a)
336 the separation of chls c_2 and c_1 and the complete resolution of Fucoxanthin,
337 Diadinoxanthin and Diatoxanthin. Two non-polar chlorophylls were detected, both
338 with visible spectrum corresponding to a chl c_2 chromophore (probably corresponding
339 to two molecular species of a chlorophyll c_2 linked to galactosyl diglycerides with
340 different fatty acid moieties).

341

342 The chromatogram of *Emiliania huxleyi* (Haptophyte pigment type 6) shows (Fig. 4b)
343 the baseline resolution of its characteristic DV-/MV- pair of acidic chlorophylls (chl c_3
344 and MV-Chl c_3). The separation of xanthophylls related to Fucoxanthin (Fucoxanthin,
345 19'-Hexanoyloxyfucoxanthin and 19'-Hexanoyloxy-4-keto-fucoxanthin) is also
346 achieved. A change in the elution order of 19'-hexanoyloxyfucoxanthin and 19'-
347 Hexanoyloxy-4-keto-fucoxanthin compared to methods employing octylsilica
348 stationary phases is observed. The method allows also the differentiation of two esters
349 of chlorophyll c_2 with monogalactosyl diacylglycerides eluting before chl *a*.

350

351 The chromatogram of *Chrysochromulina throndsenii* (Fig. 4c) illustrates the
352 simultaneous separation of four acidic chl pigments: chl c_3, and the consecutively
353 eluting triplet formed by the DV/MV- analogues chl c_2 and chl c_1 and the
354 Protochlorophyllide Mg-DVP. The photoprotective carotenoids Diadinoxanthin and

355 Diatoxanthin, completely resolved, are also detected. Two distinctive esters of chl c_2
356 with galactosyl diglycerides are also eluting before chl *a*.
357
358 The chromatographic profile of *Pelagomonas calceolata* (Fig. 4d) shows the complete
359 separation of 19'-Butanoyloxyfucoxanthin (a pigment marker for Pelagophyta) from
360 Fucoxanthin, and traces of β,ε– and β,β–carotene.
361
362 The xanthophyll Alloxanthin, diagnostic for Cryptophyta, dominates the chromatogram
363 of *Rhodomonas baltica* (Fig. 4e). The characteristic carotenoids Monadoxanthin and
364 Crocoxanthin are also completely separated. As expected, only chl c_2 is detected.
365
366 *Nannochloropsis gaditana*, lacking chls *c*, reveals the resolution of Vaucheriaxanthin
367 and its esters (key pigments for Eustigmatophyta) besides the major carotenoids
368 Violaxanthin and Astaxanthin (Fig. 4f).
369
370 Two dinoflagellates representative of characteristic pigment patterns were analyzed.
371 The toxic dinoflagellate *Alexandrium minutum* (dinoflagellate pigment type 1, (*sensu*
372 Zapata *et al.* 2012) shows (Fig. 4g) the biomarker carotenoid Peridinin completely
373 resolved from chl c_2, and the separation of a characteristic peridinin-like pigment
374 (whose structure is still unknown) from Mg-DVP. The coelution of Dinoxanthin is
375 observed as a deformation in the Diadinoxanthin peak and the simultaneous
376 occurrence of both pigments had to be checked by the spectral characteristics at
377 different zones of the peak. *Karlodinium veneficum*, a representative of dinoflagellate
378 pigment type 3 (Zapata *et al.* 2012), shows the elution of the marker xanthophyll

Gyroxanthin as an isolated peak, besides 19'-Butanoyloxyfucoxanthin, Fucoxanthin, 19'-Hexanoyloxyfucoxanthin, Diadinoxanthin and Diatoxanthin, all of them completely resolved (Fig. 4h).

NATURAL SAMPLES

The capability of the method in determining the pigment composition of natural plankton populations was tested in water samples from the Mediterranean Sea. Fig. 5 shows the carotenoids and chlorophylls present in a water sample taken from the deep chlorophyll maximum (DCM) (36º15.27'N, 18º17.85'E; 120 m depth). The chromatogram shows the co-dominance of DVchl *a* and chl *a* (peaks 60 and 61) and the occurrence of DVchl *b*, Mg-DVP, Zeaxanthin and β,ε-carotene, a pigment pattern characteristic of the low light ecotypes of the cyanobacteria *Prochlorococcus*. The importance of 19'-Butanoyloxyfucoxanthin (peak 16) reveals the key contribution of pelagophytes. The dominance of 19'-Hexanoyloxyfucoxanthin (peak 22) over Fucoxanthin (peak 19), together with chl c_3 and the characteristic chl c_2-MGDG suggests the abundance of haptophytes related to the genus *Chrysochromulina* (pigment type 7, Jeffrey *et al.* 2011). The small amounts of chl *b*, Dihydrolutein and traces of Violaxanthin and Neoxanthin indicate the presence of low concentration of green algae (prasinophytes, chlorophytes).

The chromatographic trace of the pigment extract of a seawater sample collected from the Ria de Vigo (Galician Coast, NW Spain) in April 2015 shows the dominance of pigment associated with diatoms that can be related to a typical spring bloom

403 situation. Fucoxanthin is the predominant carotenoid (peak 19), together with a
404 variety of acidic chls (peaks 2, 9, 10, 12 and 14) that have been described to occur in
405 certain diatoms (Zapata *et al*. 2011). However, the presence of 19'-
406 Hexanoyloxyfucoxanthin (peak 22) can be related to the occurrence of haptophytes,
407 that could also contribute part of the polar chls. The presence of Peridinin (peak 8) is
408 associated with dinoflagellates (pigment type 1, Zapata *et al.* 2012), whereas
409 Alloxanthin (peak 31) might come from algae from the class Cryptophyceae or be
410 explained by the occurrence of members of the genus *Dinophysis*, a pigment type 5
411 dinoflagellate (Zapata *et al.* 2012; Rial *et al.* 2013), frecuent in the galician coasts.
412

413 ## Discussion

414

415 The method here proposed is able to separate MV/DV pairs of acidic and esterified
416 chls in a single run together with most taxonomically important carotenoids (Table 3).
417 The simultaneous separation of MV/DV pairs of polar (chls *c*, protochlorophyllides) and
418 non-polar (chls *a* and *b*) pigments had been only achieved in the past by the use of
419 polymeric octadecylsilica columns at low temperatures (Garrido & Zapata 1997), in a
420 method not intended for the separation of carotenoids. Any other methodology
421 available until now suffered from the coelution of one or another chl pair: coleutions
422 of chl c_2/Mg-DVP and chl c_1/chlorophyllide *a* (Van Heukelem & Thomas 2001),
423 coelution of chl *b*/DVchl *b* (Zapata *et al.* 2000), coelutions of chl c_3/MVchl c_3 and Mg-
424 DVP/chl c_2 (Jayaraman *et al.* 2011). The ability of the method for the baseline
425 separation of the pair chl *b*/DVchl *b* can help to discriminate the contributions of
426 prochlorophytes and green algae in oligotrophic waters.

The special selectivity of the method towards chlorophyll with subtle structural differences is made clear in the case of the isomeric MV chl c_3 and chl c_{CS170} (Rs= 1.09). Their similarity in chromatographic properties, UV-vis spectra and mass spectra, led some authors to claim that both pigments were actually the same compound (Goericke 2000) and explains why their structures have been unknown until very recently (Álvarez *et al.* 2012; Álvarez *et al.* 2013).

Most marker carotenoids were separated with resolution values comparable to other general purpose methods (Zapata *et al.* 2000; Van Heukelem & Thomas 2001; Jayaraman *et al.* 2011), but the isomeric pair Zeaxanthin/Lutein was not completely resolved (Rs=0.98). The separation of Zeaxanthin and Lutein can be substantially enlarged by increasing the ethanol content, at the cost of losses in the resolution of acidic chlorophylls (data not shown).

The chromatographic method here described results especially useful to separate the pigments of type 2A prasinophytes (Jeffrey *et al.* 2011). In a survey on pigment composition of the prasinophytes, Latasa *et al.* (2004) could not detect the pigment Loroxanthin that coeluted with Neoxanthin in the method employed (Zapata *et al.* 2000). To overcome this problem, Garrido *et al.* (2009) proposed a new method and a double analysis procedure that has been recently employed to characterize the composition of scaly green flagellates (Alonso-González *et al.* 2014). The HPLC method here proposed, with all common pigments completely separated (Loroxanthin and Neoxanthin Rs >1.5), is a good option for a re-examination of the still doubtful

xanthophyll distribution in the prasinophytes.

As pigments appear in very low concentrations in samples of natural waters, techniques of structural identification such as MS and specially NMR are very difficult to apply. To overcome this problem, several criteria of identification have been proposed (Egeland 2011) including the identity of the chromatographic properties of the unknown and the reference standard in different chromatographic systems. So, a new system based on a different type of chromatographic interactions offers an additional tool to confirm or discard the identity of certain pigments. The case of the unknown carotenoid of *Lepidodinium chlorophorum* is paradigmatic in this aspect. Dinoflagellates from the genus *Lepidodinium* (*L. chlorophorum* and *L. viride*) are known to harbour chloroplasts of chlorophyte origin, and a major carotenoid in these species was identified as Lutein based on the identity of both retention times in the Zapata *et al*. (2000) method (Matsumoto *et al.* 2012). However, our method demonstrates that this carotenoid is not Lutein, as it elutes well separated (Rs> 1.5) and shows a markedly different on-line UV-Vis spectrum.

The synthetic carotenoid *trans*-β-Apo-8'-carotenal has been proposed as internal standard for the chromatographic analysis of plankton pigments, but its coelution with Canthaxanthin (peaks 44 and 45) precludes its use in the method here developed. As an alternative we propose the use of Capsanthin, a pigment from red pepper fruits that is not expected to occur in plankton extracts (peak 26, Rt= 17.2), that elutes baseline separated between Violaxanthin (peak 25, Rt= 17.2) and Diadinoxanthin (peak 27, Rt= 19.0).

475

476 Several chromatographic and instrumental advantages of the method deserve being
477 highlighted:

478

479 While other methods for pigment analysis employ ternary gradients (Wright *et al.*
480 1991; Jayaraman *et al.* 2011), so they cannot be implemented in high-pressure binary
481 systems, this one is based in a binary elution gradient that can be reproduced with
482 instruments equipped either with high- or low- pressure gradient mixing systems.

483

484 The eluents employed are transparent in the near-ultraviolet region of the spectrum,
485 thus allowing the detection of the characteristic peaks of *cis*-carotenoids. The quest for
486 bacteriochlorophyll *a* (whose absorption is very low in the range of 420-500 nm) in
487 natural waters led to other authors to propose alternative methods devoted only to
488 this pigment (Goericke, 2002). The method here proposed requires only the extraction
489 of an additional channel at 364 nm from the set of diode array data. The UV cut-off
490 values of solvents in other methods (e.g. acetone and pyridine in Zapata *et al.* 2000)
491 preclude their use for these purposes.

492

493 The use of the volatile ion-pairing reagent ammonium acetate instead of
494 tetrabutylammonium (Van Heukelem & Thomas 2001) or pyridinium salts (Zapata *et al.*
495 2000) makes the method compatible with mass spectrometry detection.

496

497 The organic solvents are restricted to methanol and ethanol, excluding some
498 components of higher toxicity like acetonitrile (Wright *et al.* 1991; Zapata *et al.* 2000;

Jayaraman *et al.* 2011) or pyridine (Zapata *et al.* 2000). Ethanol is a particularly desirable solvent because it is less volatile, less toxic and has lower disposal costs than methanol and specially than acetonitrile (Sadek 2002; Plotka *et al.* 2013).

Acknowledgments

We thank Francisco Rodríguez (IEO, Vigo) for his critical review of the manuscript. This work was co-financed by Spanish Ministerio de Economía y Competitividad (MINECO) and FEDER through project CTM2012-32181. A.G.O. is funded by a MINECO doctoral grant (BES-2013-065752).

Data accessibility

All data used are present in the article.

References

Alonso-González, A., Orive, E., David, H., García-Etxebarria, K., Garrido, J.L., Laza-Martínez, A. & Seoane, S. (2014). Scaly green flagellates from Spanish Atlantic coastal waters: molecular, ultrastructural and pigment analyses. *Botanica Marina*, **57**, 379–402.

Álvarez, S., Rodríguez, F., Riobó, P., Garrido, J.L. & Vaz, B. (2013). Chlorophyll *c* CS 170 isolated from *Ostreococcus* sp. is [7-Methoxycarbonyl- 8-vinyl] protochlorophyllide *a*. *Organic Letters*, **20**.

Álvarez, S., Zapata, M., Garrido, J.L. & Vaz, B. (2012). Characterization of [8-ethyl]-chlorophyll *c3* from *Emiliania huxleyi*. *Chemical Communications*, **48**, 5500–5502.

Ashu-Arrah, B.A., Glennon, J.D. & Albert, K. (2013). Synthesis, characterisation and chromatographic evaluation of pentafluorophenyl and phenyl bonded silica

525 phases prepared using supercritical carbon dioxide as a reaction solvent. *Journal*
526 *of Chromatography A*, **1273**, 34–43.

527 Chan, C.X. & Bhattacharya, D. (2010). The Origin of Plastids. *Nature Education*, **3**, 1–5.

528 Egeland, E.S. (2011). Appendix C. Minimun identification criteria for phytoplankton
529 pigments. *Phytoplankton Pigments. Characterization, Chemotaxonomy and*
530 *Applications in Oceanography.* (eds S. Roy, C.A. Llewellyn, E.S. Egeland & G.
531 Johnsen), pp. 650–652. Cambridge University Press., Cambridge, U.K.

532 Garrido, J.L., Airs, R.L., Rodriguez, F., Van Heukelem, L. & Zapata, M. (2011). New HPLC
533 separation techniques. *Phytoplankton Pigments. Characterization,*
534 *Chemotaxonomy and Applications in Oceanography.* (eds S. Roy, C.A. Llewellyn,
535 E.S. Egeland & G. Johnsen), pp. 165–179. Cambridge University Press., Cambridge,
536 U.K.

537 Garrido, J.L., Rodríguez, F. & Zapata, M. (2009). Occurrence of loroxanthin, loroxanthin
538 decenoate, and loroxanthin dodecenoate in *Tetraselmis* species (Prasinophyceae,
539 Chlorophyta). *Journal of Phycology*, **45**, 366–374.

540 Garrido, J.L. & Zapata, M. (1997). Reversed-phase high-performance liquid
541 chromatographic separation of mono- and divinyl chlorophyll forms using
542 pyridine-containing mobile phases and a polymeric octadecylsilica column.
543 *Chromatographia*, **44**, 43–49.

544 Goericke, R., Olson, R.J. & Shalapyonok A. (2000). A novel niche for *Prochlorococcus* sp.
545 in low-light suboxic environments in the Arabian Sea and the Eastern Tropical
546 North Pacific. *Deep-Sea Research I*, **47**, 1183-1205.

547 Goericke, R. (2002). Bacteriochlorophyll *a* in the ocean: Is anoxygenic bacterial
548 photosynthesis important? *Limnology and Oceanography*, **47**, 290–295.

549 Guedes, A.C., Amaro, H.M. & Malcata, F.X. (2011). Microalgae as sources of
550 carotenoids. *Marine Drugs*, **9**, 625–44.

551 Guillard, R.R.L. & Hargraves, P.E. (1993). *Stichochrysis immobilis* is a diatom, not a
552 chrysophyte. *Phycologia*, **32**, 234–236.

553 Jayaraman, S., Knuth, M.L., Cantwell, M. & Santos, A. (2011). High performance liquid
554 chromatographic analysis of phytoplankton pigments using a C16-Amide column.
555 *Journal of chromatography A*, **1218**, 3432–3438.

556 Jeffrey, S.W. (1997). Application of pigment methods to oceanography. *Phytoplakton*
557 *pigments in oceanography.* (eds S.W. Jeffrey, R.F.C. Mantoura & S.W. Wright), pp.
558 127–166. Unesco Publishing, Paris, France.

559 Jeffrey, S.W. & Barret, J. (1971). A note on the occurrence of chlorophyllase in marine
560 algae. *Journal of Experimental Marine Biology and Ecology.*, **7**, 255–262.

561 Jeffrey, S.W., Llewellyn, C.A., Barlow, R.G. & Mantoura, R.F.C. (1997). Pigment
562 processes in the sea: a selected bibliography. *Phytoplankton pigments in*
563 *oceanography.* (eds S.W. Jeffrey, R.F.C. Mantoura & S.W. Wright), pp. 167–178.
564 Unesco Publishing, Paris, France.

565 Jeffrey, S.W. & Wright, S.W. (2006). Photosynthetic pigments in marine microalgae:
566 Insights from cultures and the sea. *Algal cultures. Analogues of blooms and*
567 *applications* (ed D. V. Subba Rao), pp. 33–90. Science Publishers, Enfield, New
568 Hampshire, U.S.A.

569 Jeffrey, S.W., Wright, S.W. & Zapata, M. (2011). Microalgal classes and their signature
570 pigments. *Phytoplankton pigments. Characterization, Chemotaxonomy, and*
571 *Applications in Oceanography.* (eds S. Roy, C.A. Llewellyn, E.S. Egeland & G.
572 Johnsen), pp. 1–77. Cambridge University Press., Cambridge, U.K.

573 Keeling, P.J. (2010). The endosymbiotic origin, diversification and fate of plastids.
574 *Philosophical Transactions of the Royal Society of London. Series B, Biological*
575 *Sciences*, **365**, 729–48.

576 Latasa, M., Van Lenning, K., Garrido, J.L., Scharek, R., Estrada, M., Rodríguez, F. &
577 Zapata, M. (2001). Losses of chlorophylls and carotenoids in aqueous acetone and
578 methanol extracts prepared for RP-HPLC analysis of pigments. *Chromatographia*,
579 **53**, 385–391.

580 Latasa, M., Scharek, R., Le Gall, F. & Guillou, L. (2004). Pigment suites and taxonomic
581 groups in Prasinophyceae. *Journal of Phycology*, **40**, 1149–1155.

582 Matsumoto, T., Kawachi, M., Miyashita, H. & Inagaki, Y. (2012). Prasinoxanthin is
583 absent in the green-colored dinoflagellate *Lepidodinium chlorophorum* strain
584 NIES-1868: Pigment composition and 18S rRNA phylogeny. *Journal of Plant*
585 *Research*, **125**, 705–711.

586 Partensky, F., Hess, W.R. & Vaulot, D. (1999). *Prochlorococcus*, a marine
587 photosynthetic prokaryote of global significance. *Microbiolgy and Molecular*
588 *Biology Reviews*, **63**, 106–127.

589 Plotka, J., Tobiszewski, M., Sulej, A.M., Kupska, M., Górecki, T. & Namieśnik, J. (2013).
590 Green chromatography. *Journal of Chromatography A*, **1307**, 1–20.

591 Rial, P., Garrido, J.L., Jaen, D. & Rodríguez, F. (2013) Pigment composition in three
592 *Dinophysis* species (Dinophyceae) and the associated cultures of *Mesodinium*
593 *rubrum* and *Teleaulax amphioxeia*. *Journal of Plankton Research*, **35**, 433-437.

594 Roy, S. & Garrido, J.L. (2013). Pigments. Liquid chromatography. *Reference Module in*
595 *Chemistry, Molecular Sciences and Chemical Engineering* pp. 1–10. Elsevier,
596 Waltham, Massachusetts, U.S.A.

Sadek, P.C. (2002). *The HPLC Solvent Guide.*, 2nd edn. Wiley-Interscience, New York, U.S.A.

Scheer, H. (2006). An overview of chlorophylls and bacteriochlorophylls: biochemistry, biophysics, functions and applications. *Chlorophylls and Bacteriochlorophylls. Biochemistry, Biophysics, Functions and Applications. Advances in Photosynthesis and Respiration. Vol. 25* (eds B. Grimm, R.J. Porra, W. Rudiger & H. Scheer), pp. 1–26. Springer, Dordrecht, The Netherlands.

Snyder, L.R., Glajch, J.L. & Kirkland, J.J. (1988). *Practical HPLC method development.* Wiley-Interscience, New York, U.S.A.

Takaichi, S. (2011). Carotenoids in algae: distributions, biosyntheses and functions. *Marine Drugs*, **9**, 1101–1118.

Van Heukelem, L. & Thomas, C.S. (2001). Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *Journal of Chromatography A*, **910**, 31–49.

Wright, S.W. & Jeffrey, S.W. (2006). Pigment markers for phytoplankton production. *Marine Organic Matter: Biomarkers, Isotopes and DNA* (ed J.K. Volkman), pp. 71–104. The Handbook of Environmental Chemistry. Springer-Verlag, Berlin, Heidelberg, Germany.

Wright, S.W., Jeffrey, S.W., Mantoura, R.F.C., Jewellyn, C.A., Bjorland, T., Repeta, D. & Welschmeyer, N. (1991). Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Marine Ecology Progress Series*, **77**, 183–196.

Zapata, M., Fraga, S., Rodríguez, F. & Garrido, J.L. (2012). Pigment-based chloroplast types in dinoflagellates. *Marine Ecology Progress Series*, **465**, 33–52.

Zapata, M. & Garrido, J.L. (1991). Influence of injection conditions in reversed-phase high-performance liquid of chromatography of chlorophylls and carotenoids. *Chromatographia*, **31**, 589–594.

Zapata, M., Garrido, J.L. & Jeffrey, S.W. (2006). Chlorophyll *c* pigments: Current status. *Chlorophylls and Bacteriochlorophylls. Biochemistry, Biophysics, Functions and Applications. Advances in Photosynthesis and Respiration. Vol. 25* (eds B. Grimm, R.J. Porra, W. Rudiger & H. Scheer), pp. 39–53. Springer, Dordrecht, The Netherlands.

Zapata, M., Rodríguez, F., Fraga, S., Barra, L. & Ruggiero, M.V. (2012). Chlorophyll *c* pigment patterns in 18 species (51 strains) of the genus *Pseudo-nitzschia* (Bacillariophyceae). *Journal of Phycology*, **47**, 1274–1280.

Zapata, M., Rodríguez, F. & Garrido, J.L. (2000). Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed

634 phase C8 column and pyridine-containing mobile phases. *Marine Ecology Progress*
635 *Series*, **195**, 29–45.

636

637

Table 1. Gradient profile of the HPLC method. Solvents: A= methanol : 225mM ammonium acetate (82:18 v:v); B= ethanol

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
20	61,8	38,2
22	25	75
33	20	80
36	10	90
37	0	100
40	0	100
42	100	0

651 **Table 2.** Effect of column temperature on the chromatographic retention and

652 resolution of selected pigments

	Temperature (ºC)							
	27		30		33		35	
Peak (Pigment)	Rt (min)	*Rs* (peaks)	Rt (min)	*Rs* (peaks)	Rt (min)	*Rs* (peaks)	Rt (min)	*Rs* (peaks)
10 (Chlorophyll c_1)	12,72	*0,64* (10/11)	12,18	*0,83* (10/11)	11,52	*0,94* (10/11)	11,24	*0,96* (10/11)
11 (Peridinin-*like)*	12,94	*1,13* (11/12)	12,44	*0,95* (11/12)	11,80	*0,85* (11/12)	11,52	*0,72* (11/12)
12 (DV Protochlorophyllide *a* (Mg-DVP))	13,37		12,78		12,07		11,76	
22 (19´-Hexanoyloxyfucoxanthin)	17,01	*0,78* (22/23)	16,46	*0,88* (22/23)	15,78	*0,93* (22/23)	15,46	*1,01* (22/23)
23 (19´-Hexanoyloxy-4-ketofucoxanthin)	17,32	*0,95* (23/24)	16,78	*0,81* (23/24)	16,11	*0,71* (23/24)	15,81	*0,53* (23/24)
24 (Prasinoxanthin)	17,69		17,07		16,35		15,98	
34 (Zeaxanthin)	23,56	*0,45* (34/35)	23,11	*0,51* (34/35)	22,37	*0,56* (34/35)	21,90	*0,78* (34/35)
35 (Lutein)	23,70		23,31		22,66		22,20	

653

654

655

656

657

658

659

660

661

662

663 **Table 3.** Retention times, on-line Vis spectral maxima and resolution of selected pairs

664 of chlorophylls and carotenoids

Peak	Pigment	Rt (min)	Absorption max (nm)			Rs (peak number)	
1	Chlorophyllide *b*	5.40	471	605	653		
2	Chlorophyll c_3	7.76	460	592	638	> 1.5	(2/3)
3	MV Chlorophyll *c*3	8.44	450	586	657	1.09	(3/4)
4	Chlorophyll $c_{cs\text{-}170}$	8.80	456	586	626		
5	Chlorophyllide *a*	9.63	433	620	668		
6	Vaucheriaxanthin	10.22	418	442	471		
7	Siphonaxanthin	10.37		463			
8	Peridinin	10.39		477			
9	Chlorophyll c_2	11.09	449	583	633	1.28	(9/10)
10	Chlorophyll c_1	11.50	444	582	633	0.80	(10/11)
11	Peridinin-*like*	11.86		479		0.75	(11/12)
12	DV Protochlorophyllide *a* (Mg-DVP)	12.05	442	580	632	0.78	(12/13)
13	MV Protochlorophyllide *a*	12.38	435	577	631		
14	Chlorophyll c_2 type from *E. gayraliae*	12.70	460	591	654		
15	Uriolide	13.10		456	477		
16	19´-Butanoyloxyfucoxanthin	13.24		449	471		
17	Chlorophyll c_1 type from *E. gayraliae*	13.33	456	585	643		
18	Loroxanthin	13.84		448	474		
19	Fucoxanthin	14.18		454		> 1.5	(19/20)
20	9´-*cis*-neoxanthin	14.75	414	438	467		
21	Micromonol	14.90		428	454		
22	19´-Hexanoyloxyfucoxanthin	15.77		449	471	0.96	(22/23)
23	19´-Hexanoyloxy-4-ketofucoxanthin	16.11		450	471	0.64	(23/24)
24	Prasinoxanthin	16.32		460			
25	Violaxanthin	17.22	417	442	471		
26	Capsanthin	18.42		480			
27	Diadinoxanthin	19.00		448	478	NR	(27/28)
28	Dinoxanthin	19.20		443	472		
29	Astaxanthin	19.46		483			
30	Antheraxanthin	19.97	423	448	475		
31	Alloxanthin	20.45		454	483		
32	Diatoxanthin	21.41		454	481		
33	Monadoxanthin	21.58		448	477		
34	Zeaxanthin	22.43		452	480	0.98	(34/35)
35	Lutein	22.73		446	475	>1,5	(34/36)
36	Unknown carotenoid from *L. chlorophorum*	23.25		444	473		
37	Vaucheriaxanthin ester (1) from *N. gaditana*	23.37		446	474		
38	Dihydrolutein	23.49		429	455		
39	Vaucheriaxanthin ester (2) from *N. gaditana*	24.33		446	474		
40	Siphonaxanthin dodecenoate	24.37		463			
41	Gyroxanthin	24.63		446	473		
42	Loroxanthin 19-(2-decenoate)	24.78		449	477		
43	Loroxanthin 19-(2-dodecenoate)	25.26		449	477		
44	*Trans*-β-apo-8'-carotenal	25.42		467			
45	Canthaxanthin	25.42		480			
46	Bacteriochlorophyll *a*	25.59	364		608		
47	Crocoxanthin	26.75		448	478		
48	Cryptoxanthin	27.28		451	483		
49	DV Chlorophyll *b*	27.32	477	605	654	> 1.5	(49/50)
50	Chlorophyll *b*	27.76	466	602	651	0.85	(50/51)
51	Chl c_2-MGDG from *Emiliania huxleyi*	27.94	460	590	635		
52	Chl c_2-MGDG from *Chrysocromulina throndseni*	28.11	460	588	637		
53	Chl c_2-MGDG from *Emiliania huxleyi*	28.26	460	588	637		
54	Chl c_2-MGDG from *Chrysocromulina throndseni*	28.36	460	588	637		
55	Chl c_2-MGDG from *Isochrysis galbana*	28.39	460	588	637		
56	DV chlorophyll *b* epimer	28.55	475	608	657		
57	Chl c_1-MGDG from *Isochrysis galbana*	28.58	457	587	637		
58	Echinenone	28.93		467			
59	Chlorophyll *b* epimer	29.27	464	601	649		
60	DV Chlorophyll *a*	29.86	442	620	666	> 1.5	(60/61)
61	Chlorophyll *a*	30.37	433	619	665	> 1.5	(61/62)
62	Unknown carotenoid from *Ostreococcus sp.*	31.08		445	473		
63	DV Chlorophyll *a* epimer	31.97	442	619	666		
64	Chlorophyll *a* epimer	32.76	432	619	666		
65	e,e-carotene	33.29	419	442	472		
66	β,e-carotene	33.33		448	475	0.55	(66/67)
67	β,β-carotene	33.58		454	480		
68	β,ψ-carotene	34.30	440	463	494		
69	Lycopene	35.25	448	474	504		
70	Pheophytin *b*	38.62	438		655		
71	Pheophytin *a*	40.79	410		667		

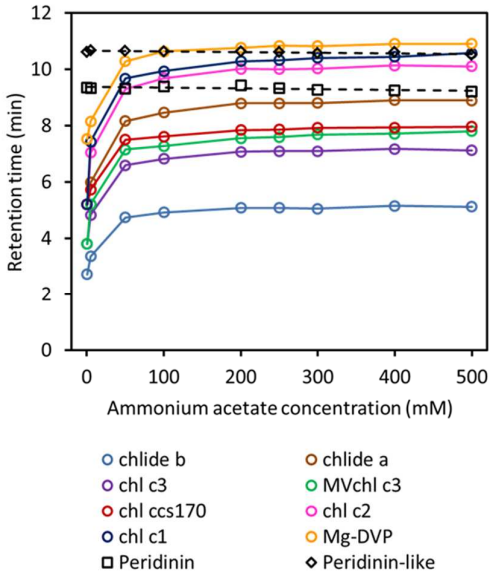
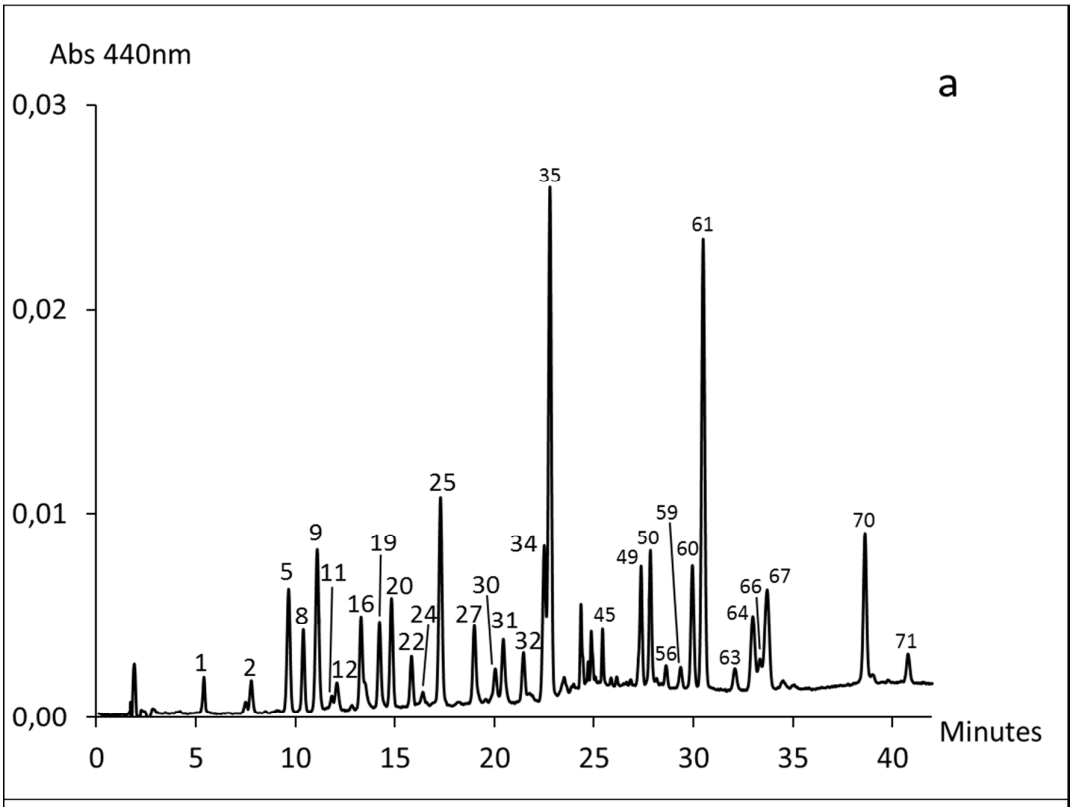
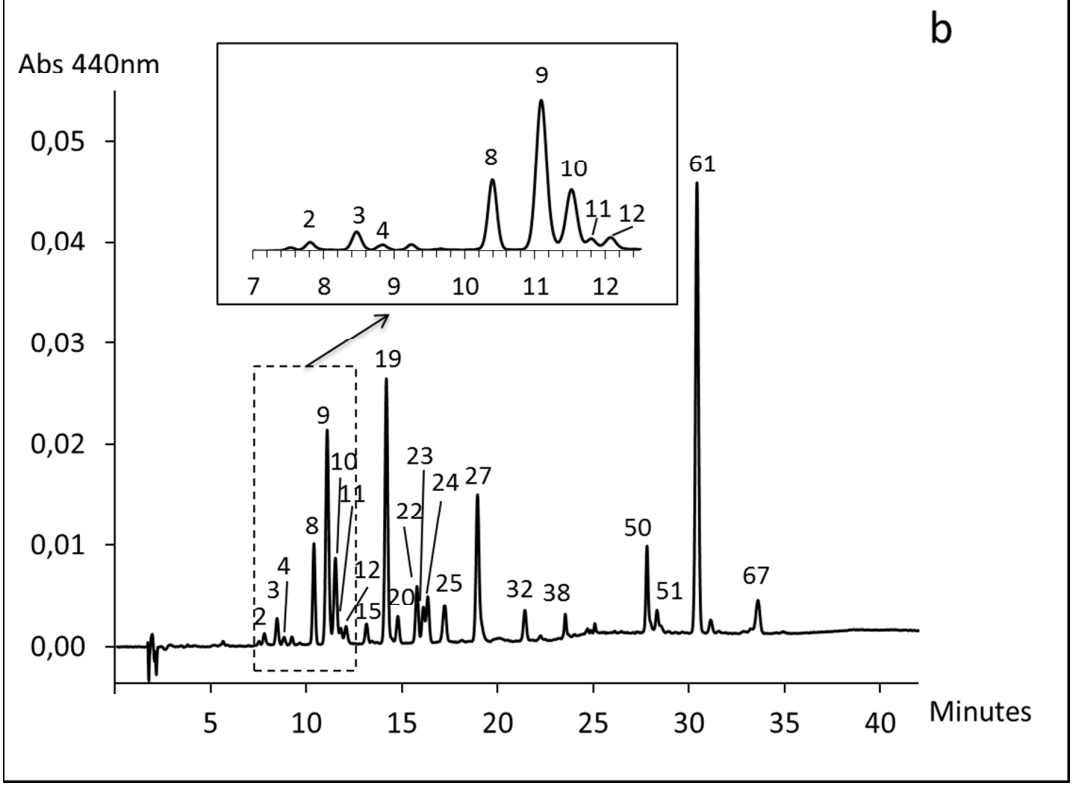


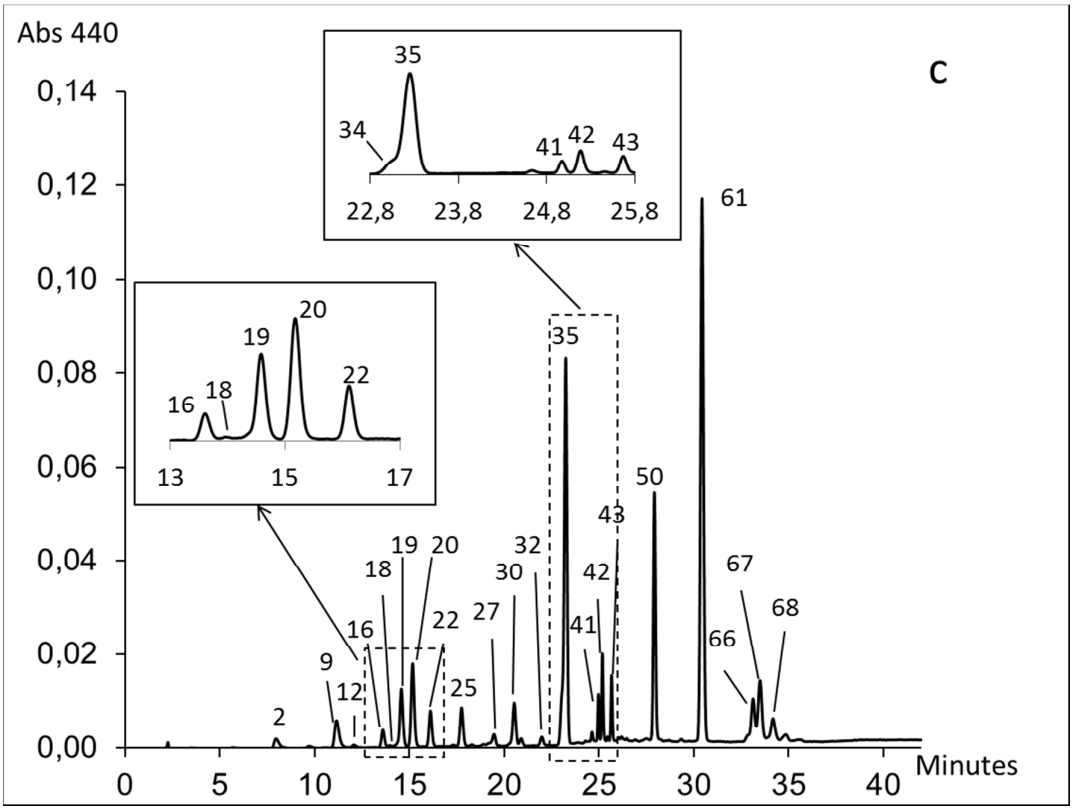
Fig. 1. Influence of ammonium acetate in the aqueous component of the eluent on the retention of acidic chlorophylls and two polar carotenoids


Abs 440nm
a
0,03
0,02
0,01
0,00
0
5
10
15
20
25
30
35
40
Minutes
1
2
5
8
9
11
12
16
19
20
22
24
25
27
30
31
32
34
35
45
49
50
56
59
60
61
63
64
66
67
70
71

677


b
Abs 440nm
0,05
0,04
0,03
0,02
0,01
0,00
5
10
15
20
25
30
35
40
Minutes
7
8
9
10
11
12
2
3
4
8
9
10
11
12
19
61
2
3
4
8
9
10
11
12
15
19
20
22
23
24
25
27
32
38
50
51
67

678



679

680

681 **Fig. 2.** HPLC chromatogram of pigment mixtures. a) DHI mixed pigments standard plus

682 chlorophyllides *b*and *a* and pheophytins *b* and *a*. b) Mixed pigment extracts of

683 *Emiliania huxleyi*, *Isochrysis galbana*, *Ostreococcus sp*. and *Alexandrium minutum*. c)

684 Mixed pigment extracts of *Tetraselmis marina* and *Karlodinium veneficum*. Peak

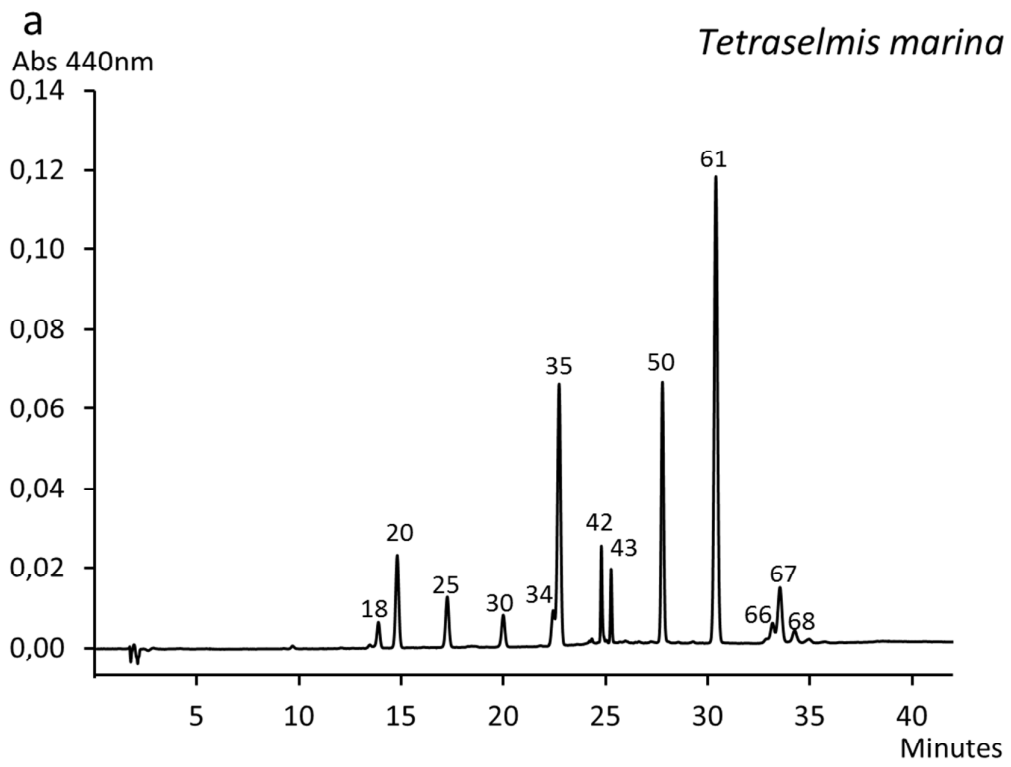
685 identification as in Table 3.

686

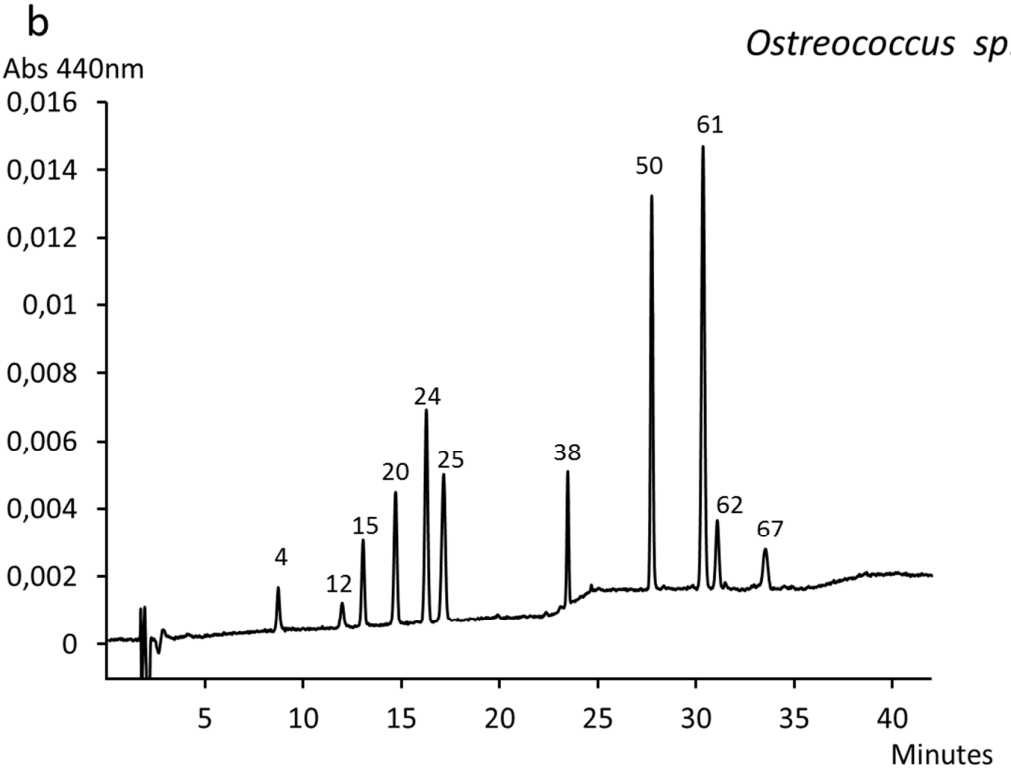
687

688

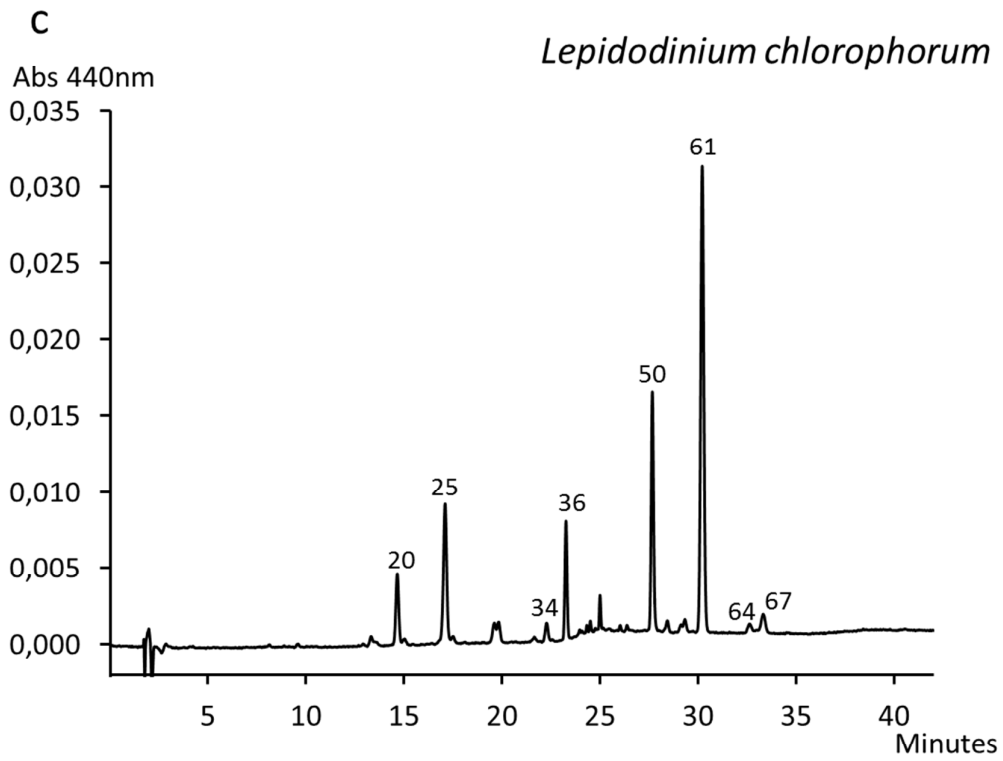
689


a
Tetraselmis marina
Abs 440nm
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
18
20
25
30
34
35
42
43
50
61
66
67
68
5
10
15
20
25
30
35
40
Minutes

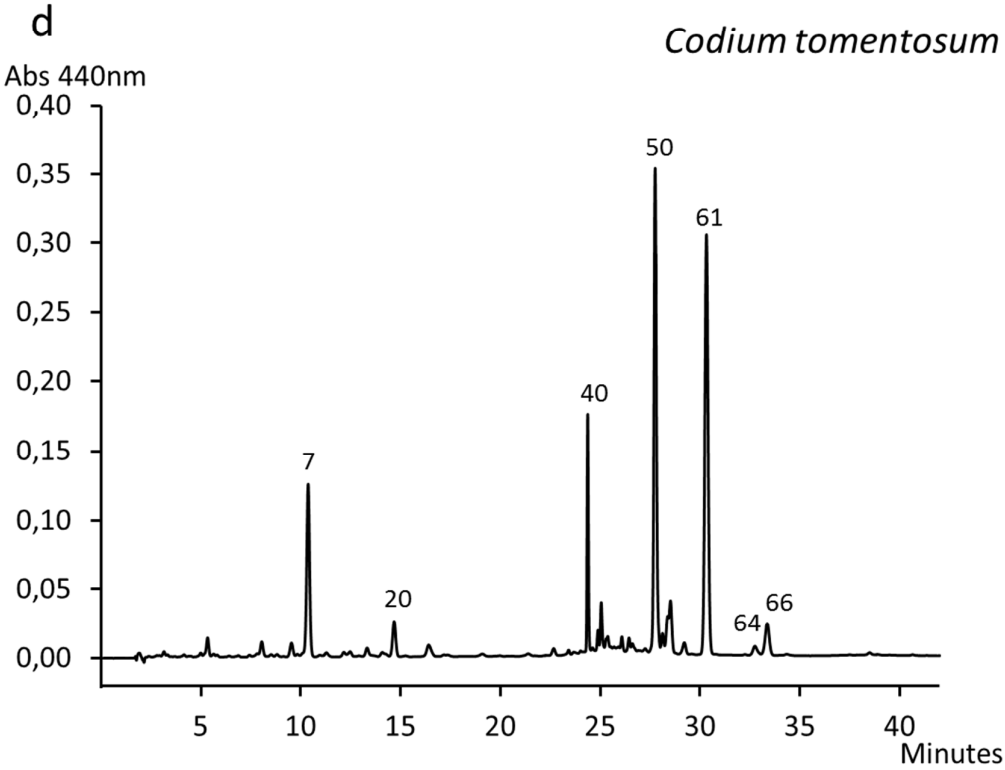
690


b
Ostreococcus sp.
Abs 440nm
0,016
0,014
0,012
0,01
0,008
0,006
0,004
0,002
0
4
12
15
20
24
25
38
50
61
62
67
5
10
15
20
25
30
35
40
Minutes

691



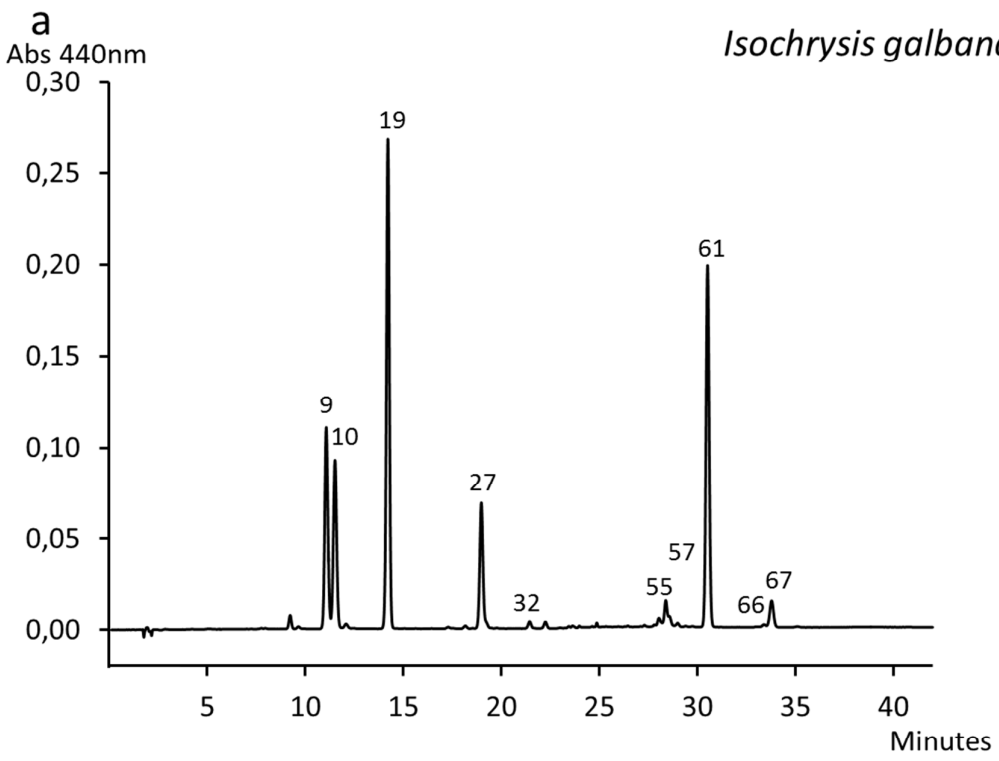
692



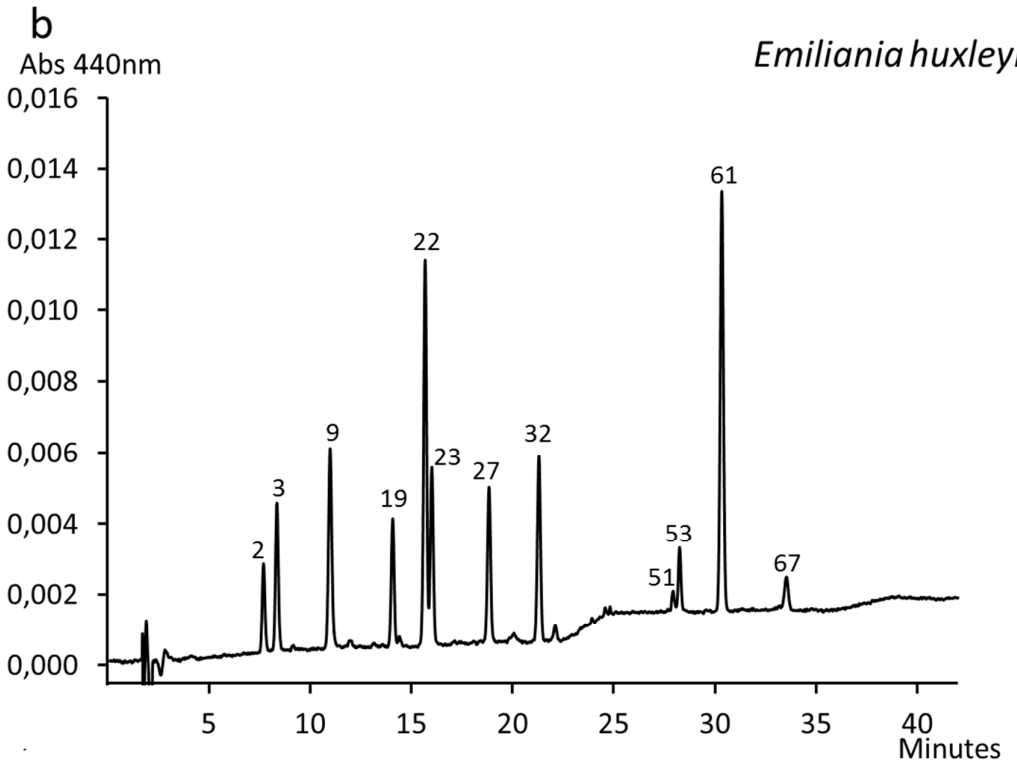
693

694 **Fig. 3.** HPLC chromatograms of pigment extracts of microalgal cultures representative

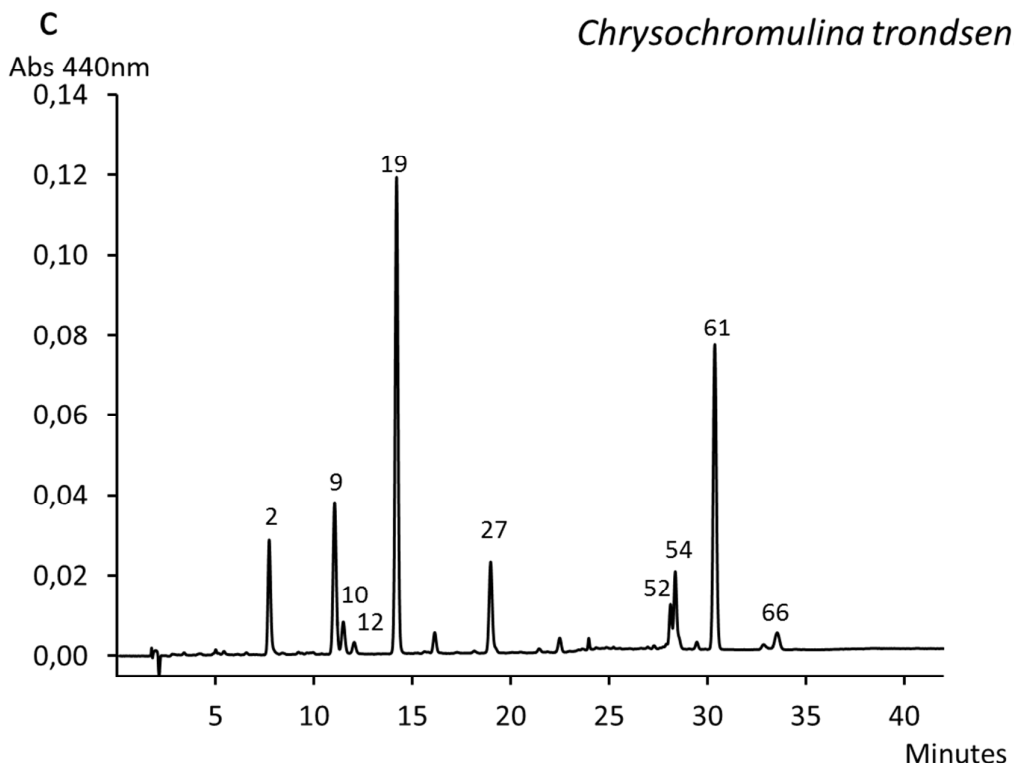
695 of chloroplasts in the green lineage. Peak identification as in Table 3.


a
Abs 440nm
Isochrysis galbana
0,30
0,25
0,20
0,15
0,10
0,05
0,00
19
61
9
10
27
57
55
67
66
32
5
10
15
20
25
30
35
40
Minutes

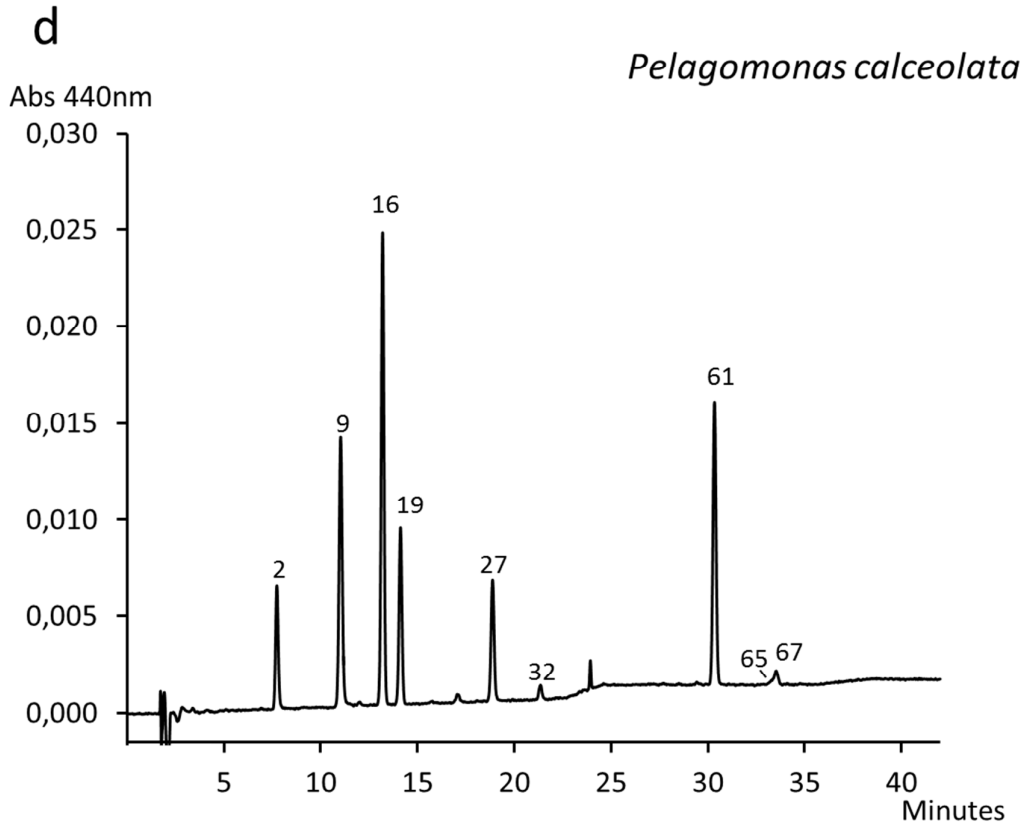
696


b
Abs 440nm
Emiliania huxleyi
0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
61
22
9
32
23
27
3
19
53
2
51
67
5
10
15
20
25
30
35
40
Minutes

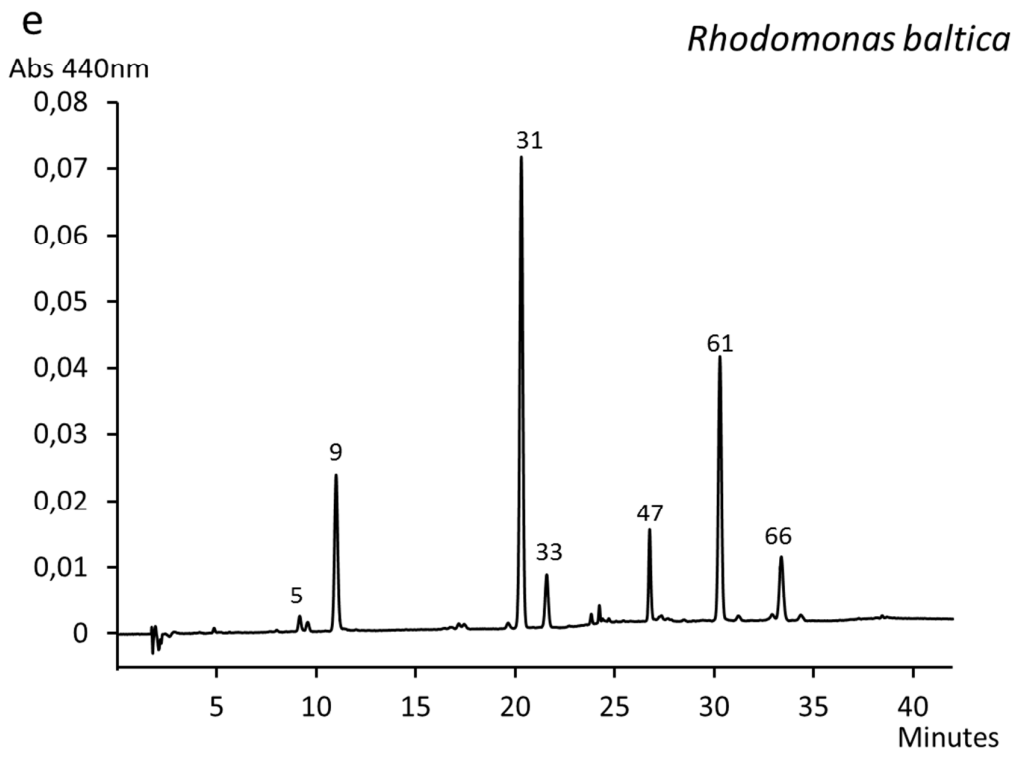
697


c
Chrysochromulina trondsenii
Abs 440nm
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
19
61
9
2
27
54
52
10
12
66
5
10
15
20
25
30
35
40
Minutes

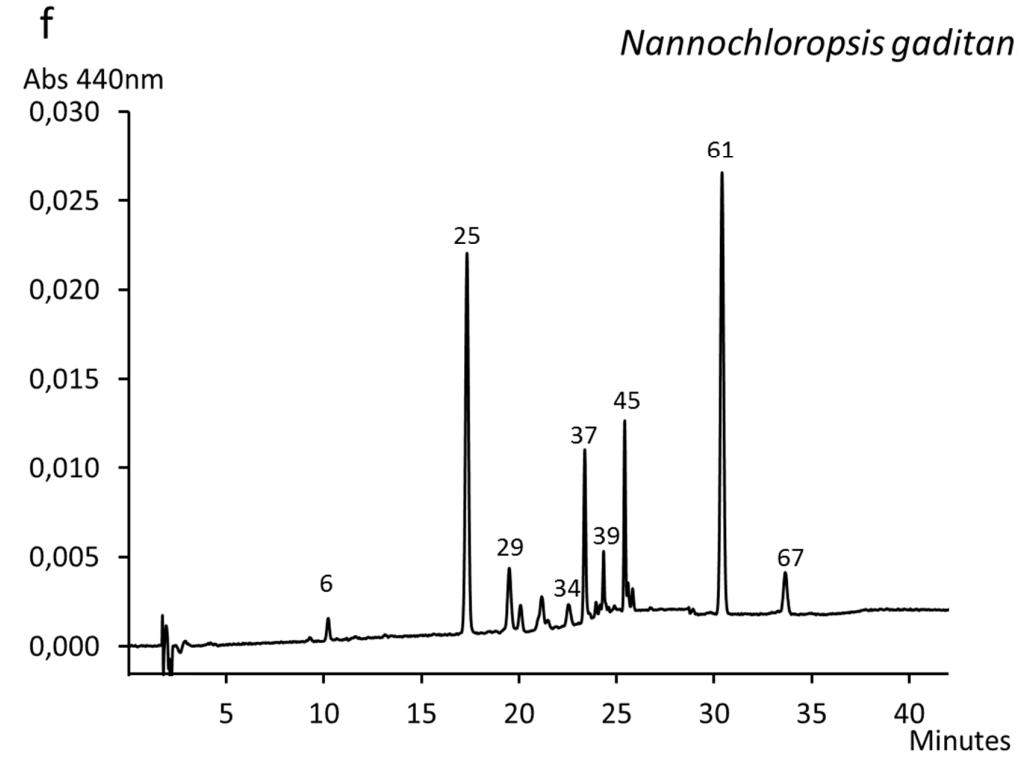
698


d
Pelagomonas calceolata
Abs 440nm
0,030
0,025
0,020
0,015
0,010
0,005
0,000
16
61
9
19
2
27
32
65
67
5
10
15
20
25
30
35
40
Minutes

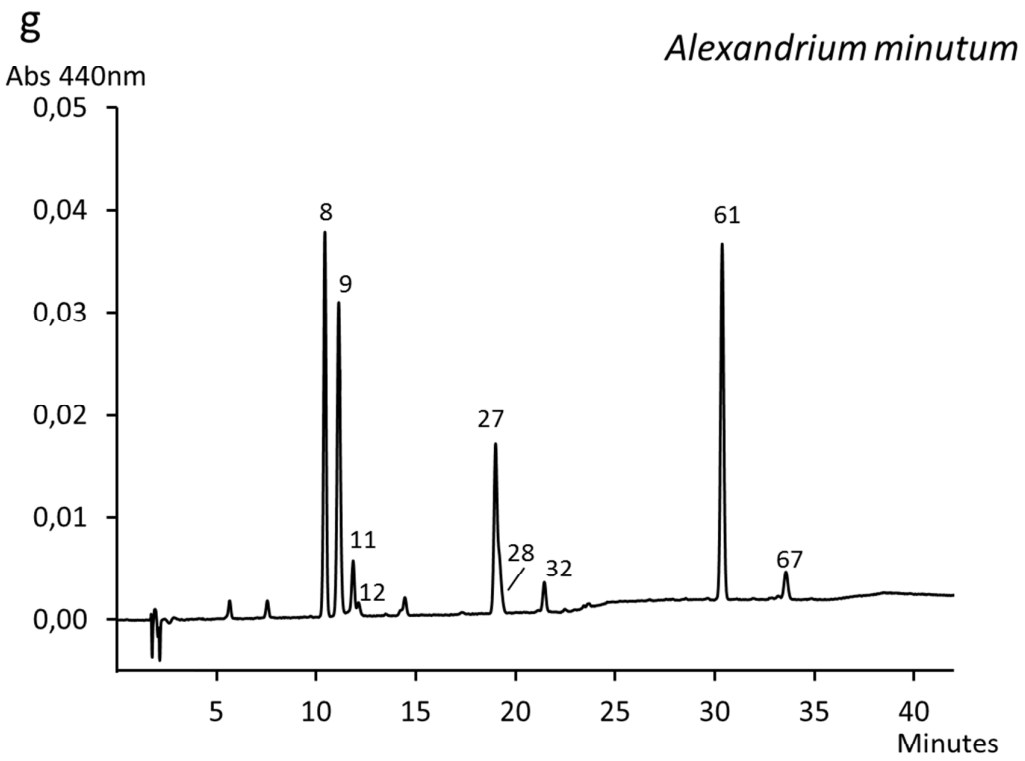
699


e
Rhodomonas baltica
Abs 440nm
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
5
9
31
33
47
61
66
5
10
15
20
25
30
35
40
Minutes

700


f
Nannochloropsis gaditana
Abs 440nm
0,030
0,025
0,020
0,015
0,010
0,005
0,000
6
25
29
34
37
39
45
61
67
5
10
15
20
25
30
35
40
Minutes

701



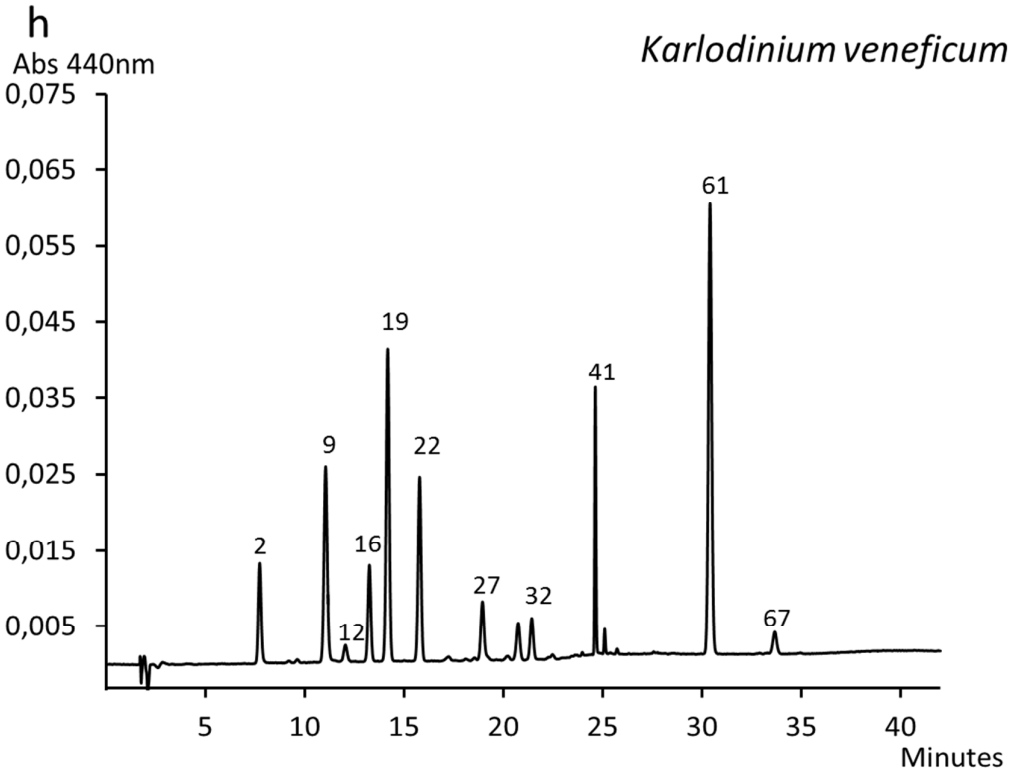
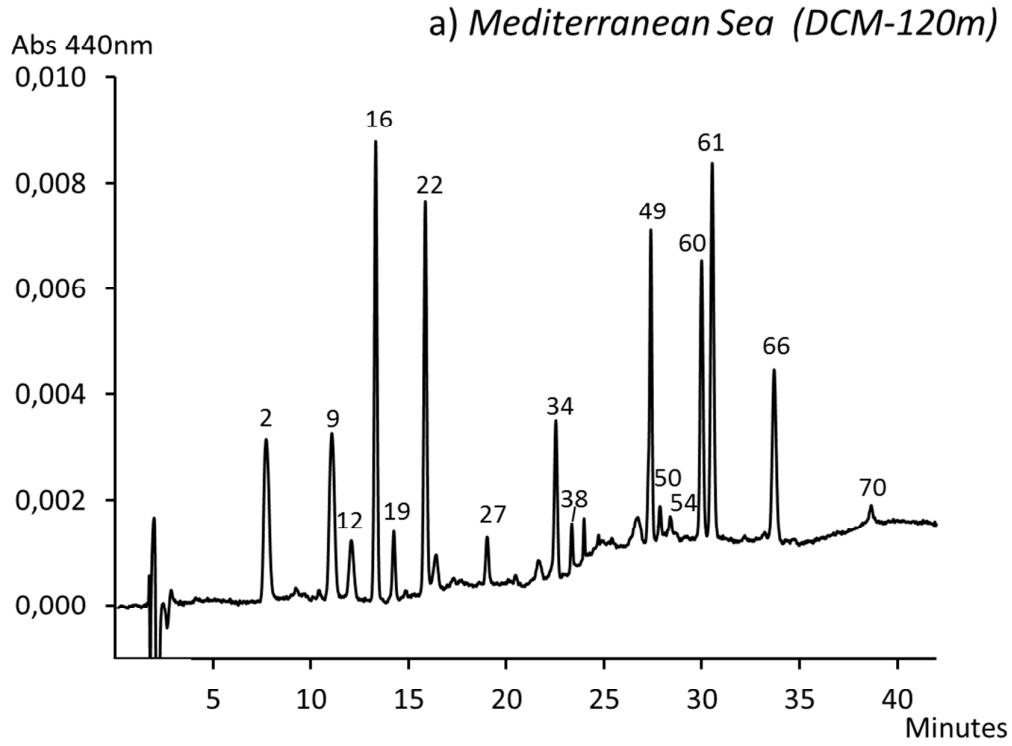
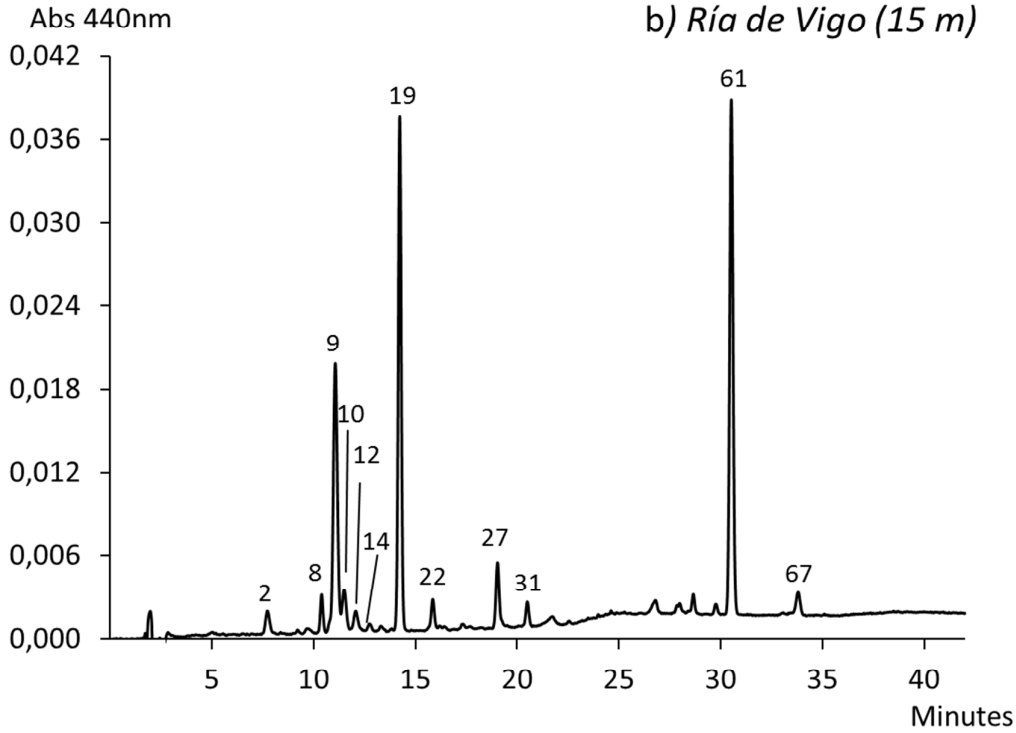


Fig. 4. HPLC chromatograms of pigment extracts of microalgal cultures representative of chloroplasts in the red lineage. Peak identification as in Table 3.



706



707

708 **Fig. 5.** HPLC pigment profile of natural samples. a) Mediterranean Sea

709 (36º15.27'N,18º17.85'E) at the deep chlorophyll maximum (120 m depth). b) Ría de

710 Vigo (Galician coast, NW Spain, 42º 13,3' N; 8º 47,7' W). Peak identification as in Table

711 3.