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Occurrence of high molecular weight lipids (C_{80+}) in the trilaminar outer cell walls of some freshwater microalgae. A reappraisal of algaenan structure

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Abstract

The purified cell walls of mother cells (CWM) were isolated from three strains of trilaminar sheath (TLS)- and algaenan-containing freshwater microalgae *Chlorella emersonii, Tetraedron minimum* and *Scenedesmus communis*. The chemical structures of CWM and algaenans were investigated by means of tetramethylammonium hydroxide (TMAH) hydrolysis and tetramethylammonium hydroxide thermochemolysis. The compounds released were characterised by ^{1}H and ^{13}C-NMR, gel permeation chromatography and desorption chemical ionisation mass spectrometry. The results show that the outer cell walls of the microalgae are constituted, at least in part, of linear (poly)esters containing extremely long chain alcohol and acid moieties (up to C_{80}) and that algaenans are mainly composed of extremely long chain (di)carboxylic acids up to C_{120}. The present results which are in direct contrast to the previous three-dimensional architecture proposed for algaenans, led us to re-interpret the algaenan structure.

1. Introduction

Insoluble, bacterially-resistant, highly aliphatic biopolymers have been detected in plant cuticles (cutan, suberan) (Nip et al., 1986; Tegelaar et al., 1995) and in cell walls of several freshwater and marine microalgae (algaenans) (de Leeuw and Largeau, 1993; Gelin et al., 1999). During the last decade it has become clear that, because of their high resistance to microbial and chemical degradation, these biopolymers are selectively preserved upon sedimentation, and diagenesis, and make significant contributions to kerogen. (Hatcher et al., 1983; Goth et al., 1988; Tegelaar et al., 1989; de Leeuw et al., 1991; van Bergen et al., 1995). Until now, algaenans of microalgae have been described as having a three-dimensional network composed of ether-linked long alky chain units (de Leeuw and Largeau, 1993). More recently, an algaenan structure composed of linear chains of esterified monomers (up to C_{34}) which are ether cross-linked has been proposed (Blokker et al., 1998a,b). However conventional approaches based on pyrolysis-gas chromatography combined with mass spectrometry and chemical degradations have limitations in depicting the structure of algaenans due to thermal rearrangement reactions and to the very low yield of products released upon chemical depolymerisation. Moreover, algaenans located in the trilaminar outer walls (TLS, for trilaminar sheath) were usually isolated from algal biomass and consequently the formation of melanoidin-like polymer artifacts via condensation reactions of sugars and proteins could occur during the isolation process (Allard et al., 1998). Isolation of algaenan from the resulting cell walls of mother cells (CWM) shed into the culture medium should minimize this latter drawback, since, during autospore formation

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the inner polysaccharide layer of complete walls is degraded by autolytic enzymes (Pickett-Heaps and Staehelin, 1975; Burczyk et al., 1999). This paper reports the isolation of algaenans from the CWM of three freshwater microalgae *Chlorella emersonii, Tetraedron minimum* and *Scenedesmus communis*. Algaenans and CWM from the different algal strains were analysed using TMAH hydrolysis and TMAH thermochemolysis. This latter technique introduced by Challinor (1989, 1991) has proved to be particularly effective in transforming macromolecular material containing polar functionalities to monomers by hydrolysis and subsequent alkylation (Hatcher and Clifford, 1994; Clifford et al., 1995; McKinney et al., 1996; del Rio and Hatcher, 1998).

2. Experimental

2.1. General

Enzymes, heptafluorobutyric anhydride and tetramethylammonium hydroxide (25% w/v in methanol) were obtained from Sigma; tetramethylammonium hydroxide (25% w/w in water) was obtained from Fluka and benzyltrimethylammonium hydroxide (40% w/w in water) was obtained from Aldrich.

Elemental compositions were determined by the Service Central d'Analyse of the CNRS by combustion at 1050 °C in a stream of oxygen.

Electron microscopy : The samples were fixed for TEM and observed as previously described (Berkaloff et al., 1983).

FTIR and GC-MS analyses were carried out as previously described (Allard and Templier, 2001).

NMR spectra were recorded on a Bruker Avance 400 at 298 K. ^{1}H NMR spectra (400.13 MHz) were obtained in 128 scans in 32 K data points with a recycle delay of 2.55 s. ^{13}C NMR spectra (100.61 MHz) were obtained from 12,000 to 72,000 scans, depending on the sample concentration, in 64 K data points with a recycle delay of 3.31 s. NMR spectra of the esterified mixtures of TMAH hydrolysates were measured in C_5D_5N. NMR spectra of the TMAH thermochemolysates were measured in $CDCl_3$.

2.2. Culture conditions

Axenic strains of *C. emersonii* (CCAP 211/8P), *S. communis* (CCAP 276/4b) and *T. minimum* (supplied by the Pflanzenphysiologische Institut und Botanischer Garten der Universitat Göttingen) were cultured by aeration and continuous illumination as previously described (Allard and Templier, 2000).

The successive isolation processes and the different analyses carried out are shown in Fig. 1.

2.3. Isolation of the cell walls of mother cells (CWM)

Cultures were harvested 30 days after inoculation. After centrifugation at 1000 g for 10 min the green sedimented algal cells were removed. The supernatant, containing the CWM, was centrifuged at 13700 g for 20 min. The CWM was collected as a pink pellet and washed several times with H_2O. To remove the soluble materials, the pellet was resuspended in H_2O and sodium dodecyl sulfate (SDS) was added (final concentration 1%) followed by stirring (18 h) at room temperature and centrifugation (13700 g for 20 min). The pellet was thoroughly washed with H_2O and freeze-dried.

2.4. Isolation of algaenans

The Lyophilised CWM was freed from lipids by extraction with $CHCl_3/CH_3OH$ 2/1 (v/v). Due to the low polysaccharide content in CWM, the isolation of algaenans was modified from a procedure reported earlier (Allard et al., 1998). The lipid-free CWM was hydrolysed with 6N HCl (110 °C, 18 h), filtered (millipore FH 0.5 μm filter) and washed until neutral. The protein-free CWM residue was then saponified [reflux 1 h in 5% KOH in 2-methoxy ethanol/H_2O 88/12 (v/v)]. The residue was filtered (millipore FH 0.5 μm filter) and washed with H_2O and 0.1 N HCl.

2.5. Proteolytic digestion of the CWM

About 5 mg of CWM were separately incubated in buffer (5 ml) with each of the following enzymes: papaïn (1 mg; pH 6.2; at 25 °C for 24 h), proteinase K (1 mg; pH 7.5; at 37 °C for 24 h), α-chymotrypsin (1 mg; pH 7.8; at 25 °C for 24 h), protease (1 mg; pH 7.5; at 30 °C for 24 h), pepsin (1 mg; pH 2; at 30 °C for 24 h), pancreatine (10 mg; pH 8.05; at 30 °C for 24 h). Digestion by a cocktail of proteases was carried out as follows : the CWM (ca. 10 mg in 5 ml buffer, pH 7.5) was incubated for 18 h with trypsin (1 mg), then with α-chymotrypsin (1 mg), then with proteinase K (1 mg) and finally with protease (1 mg). The mixture was then filtered (millipore FH 0.5 μm filter) and the residue analysed by FTIR spectrometry.

2.6. Amino acid analysis

The 6N HCl hydrolysis mixture (see 2.4.) was evaporated to dryness and amino acids were analysed by GC-MS as their N-heptafluorobutyryl isobutyl esters (Mackenzie and Tenaschuk, 1974).

2.7. Tetramethylammonium hydroxide (TMAH) hydrolysis

Native and lipid-free CWM (50–100 mg) were hydrolysed with aqueous TMAH 25% (w/w) at 100 °C for

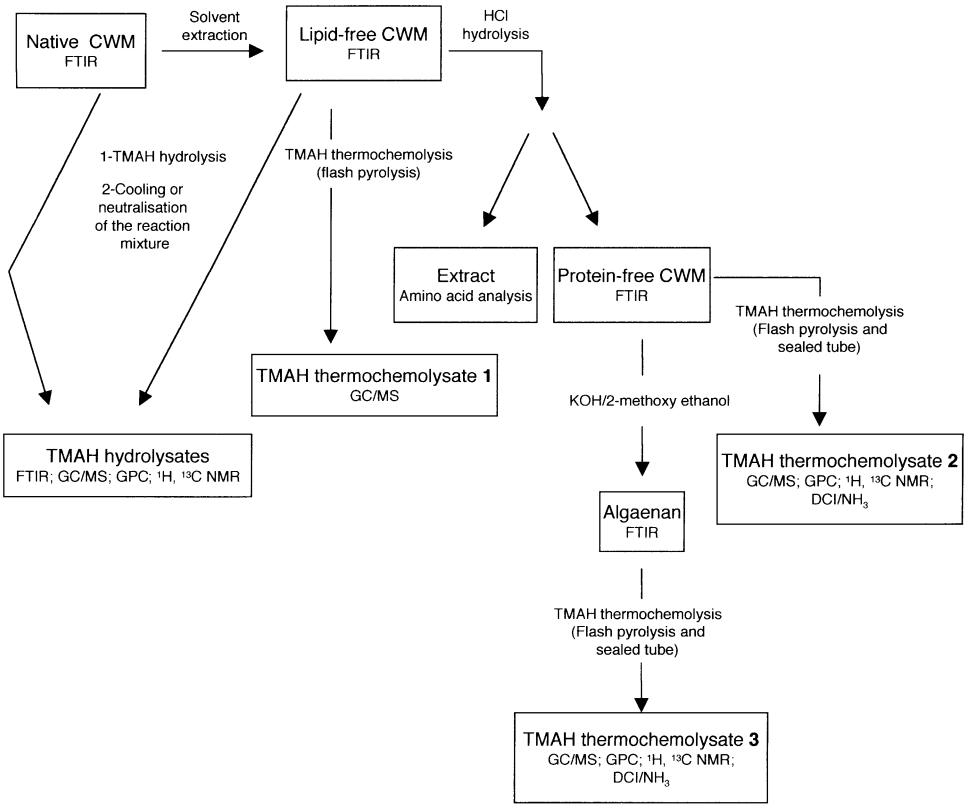

Fig. 1. Schematic isolation processes of lipid-free CWM, protein-free CWM and algaenan indicating the analyses carried out.

18 h. The reaction mixture was neutralised with 6N HCl, the precipitate formed upon neutralisation was recovered by centrifugation and washed thoroughly with H_2O then with CH_3OH. The wet precipitate was treated with 2-methyl-1-propanol/HCl for 1 h at 100 °C, the esterified precipitate was analysed by gel permeation chromatography and NMR. For GC-MS analysis the esterified precipitate was acetylated with a mixture of acetic anhydride/pyridine (1/1 v/v) at 100 °C for 1 h. After addition of H_2O the solvents were evaporated and the residue dissolved in pyridine.

2.8. Gel permeation chromatography (GPC)

Analyses were performed as previously described (Allard and Templier, 2001).

2.9. Flash pyrolysis with in situ methylation using tetramethylammonium hydroxide (TMAH)

Samples (0.5–2 mg) were loaded into a ferromagnetic tube (Curie temperature 445 °C). Five microliters of 25% TMAH in methanol were added four times in 2 h. The sample was pyrolysed (Fisher 031 6M) at the Curie temperature for 10 s. The pyrolysis unit was directly coupled to a HP5890 gas chromatograph equipped with a fused silica RSL-300 capillary column (25 m × 0.25 mm i.d.; 0.2 μm film thickness) programmed from 50 to 100 °C at 2 °C min^{-1} then from 100 to 300 °C at 4 °C min^{-1} and held at this temperature for 30 min. The gas chromatograph was coupled to a HP5989 mass spectrometer operated at 70 eV.

2.10. Pyrolysis with in situ methylation using tetramethylammonium hydroxide (TMAH) in sealed tube

Samples (50–100 mg) were placed in a stainless steel tube with 1 ml of TMAH (25% w/v in methanol). The methanol was evaporated under vacuum. The tube was sealed (Swagelok) and heated at 445 °C for 15 min. After cooling, the tube contents were extracted with CH_2Cl_2 (5 × 5 ml) and the combined extracts filtered (millipore FH 0.5 μm filter) and reduced to dryness under a stream of nitrogen. The samples were dissolved in $CDCl_3$ for NMR analysis, desorption chemical ionisation mass spectrometry and gel permeation chromatography.

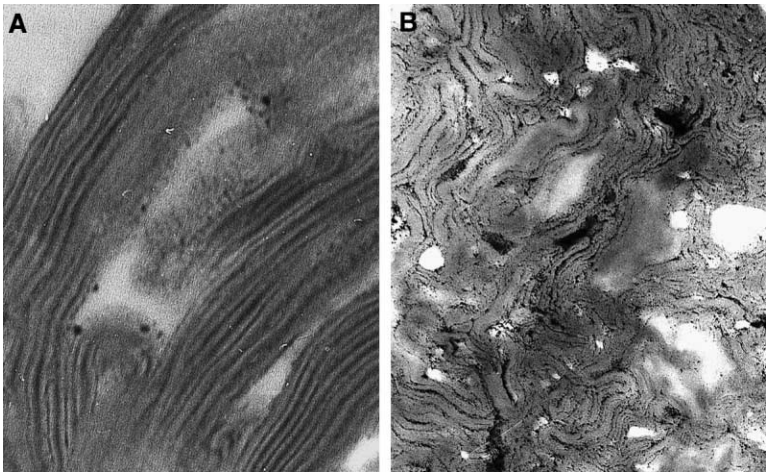

Fig. 2. Transmission electron microphotographs (TEM) of (**A**) lipid-free CWM from *C. emersonii* (× 60,000), (**B**) protein-free CWM from *T. minimum* (× 30,000).

2.11. Desorption chemical ionisation mass spectrometry (DCI-MS)

Positive ion desorption chemical ionisation measurements were carried out on a JEOL MS700. The wire (tungsten) heating current was programmed from 0 to 800 mA at 20 mA s^{-1}. The chemical ionisation reagent gas was NH_3 at a pressure of 2×10^{-4} Torr. The electron energy was set to 200 eV and the emission current was 350 μA. The mass ranges scanned were m/z 100–2000.

3. Results and discussion

3.1. Spectroscopic features of purified cell walls of mother cells (CWM)

The sporangial walls of mother cells (CWM) released in the culture medium consist mostly of the trilaminar outer walls (TLS). Transmission electron microscopy (TEM) showed that the appearance of the maternal cell walls does not differ significantly between the different microalgae. TLS appeared as a bright band sandwiched between two dark bands which likely represent the polar surface of the TLS (Fig. 2A). FTIR spectra of the CWM of the three microalgae were similar and showed that TLS consist predominantly of protein and lipid moieties. FTIR spectra of lipid-free CWM (Fig. 3A) were characterised by strong absorption bands at ca. 1650 and 1550 cm^{-1} (amide I and amide II), 2920 and 2850 cm^{-1} (ν_{as} CH_2 and ν_s CH_2), 1735 cm^{-1} (ν C=O), 1475 cm^{-1} (δCH of methylene and methyl) and 720 cm^{-1} (long CH_2 chains). According to the FTIR spectra, and in agreement with the elemental analyses (C, 67.5, 58.9, 62.3; H, 10.6, 9.0, 9.2; O, 17.9, 25.3, 23.6; N, 4.0, 6.8, 4.9

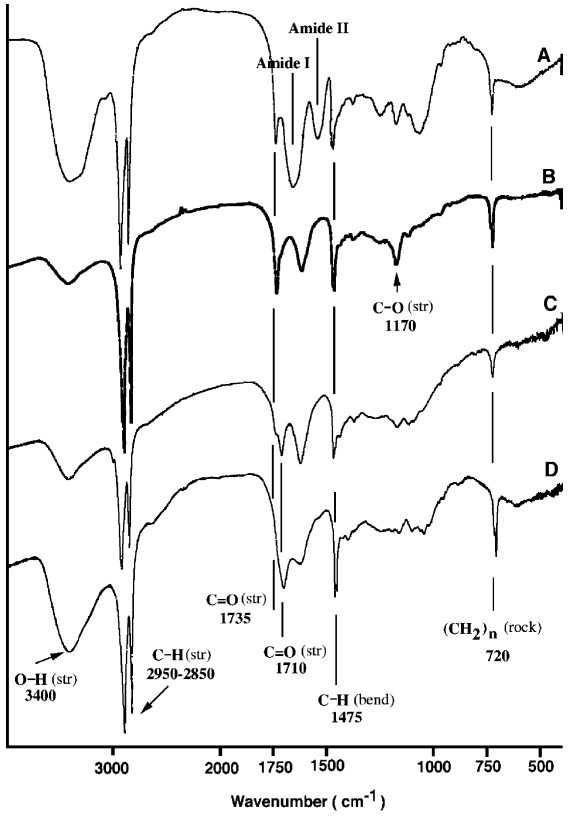

Fig. 3. Fourier-transform infrared spectroscopy (FTIR) (KBr) from *C. emersonii* CWM (**A**) lipid-free CWM. (**B**) protein-free CWM obtained after 6 N HCl hydrolysis of the lipid-free CWM, (**C**) algaenan isolated after saponification of the protein-free CWM (KOH in 2-methoxy ethanol/H_2O). (**D**) precipitate obtained upon neutralisation of the aqueous TMAH hydrolysate of the lipid-free CWM [25% (w/w) aqueous].

for the lipid-free CWM isolated from *C. emersonii*, *S. communis* and *T. minimum*, respectively), the polysaccharide content of CWM is rather low. In contrast TEM observations showed that the complete cell walls isolated from the homogenate of the disrupted algal cells (CWH) exhibit a trilaminar structure which forms an outer layer adjacent to a polysaccharide inner layer. The FTIR spectra of CWH are dominated by polysaccharide absorptions (1000–1200 cm^{-1}) and by absorption bands of proteins (data not shown).

3.2. Acid hydrolysis of CWM. Amino acid analysis

Acid hydrolysis of lipid-free CWM with 6N HCl yielded protein-free CWM which accounts for 35 to 60% of the lipid-free CWM. TEM observations showed that only the bright layer is retained after acid hydrolysis (Fig. 2B). The FTIR data leads us to postulate that these bright bands represent the lipid envelope of the CWM. Indeed the FTIR spectra of the protein-free CWM are dominated by absorption bands at ca. 1735 cm^{-1}, 1475 and 720 cm^{-1} (Fig. 3B) and suggest that ester functions predominate in the protein-free CWM. The low branching level of the alkyl moieties is indicated by the high ratio of the intensities of absorption bands at 1475 cm^{-1} (CH_3 and CH_2 asymmetric bending) and 1375 cm^{-1} (CH_3 symmetric bending). Unsaturation in the protein-free CWM isolated from *S. communis* is indicated by absorption bands at ca. 3010 and 985 cm^{-1}. In all FTIR spectra of protein-free CWM a broad absorption band centred at ca. 1620 cm^{-1} was observed. The assignment of this band is still unclear. In contrast, proteolytic digestions of native or lipid-free CWM, by using different proteases or a cocktail of them, with different incubation times failed to remove large amounts of proteins. After extensive proteolytic digestion only ca. 10% of proteins were removed. This suggests that a large fraction of CWM proteins is embedded in the lipid structure that protects them from proteolytic degradation.

Amino acid analyses of the acid hydrolysates indicated that the CWM of all the strains contain typical protein amino acids. This result, corroborated by the FTIR data, and in agreement with previous studies (Loos and Meindl, 1982; Burczyk et al., 1999), supports the participation of proteins in the formation of the cell walls of these microalgae. The most abundant amino acids are leucine (17%), alanine (15%) threonine (14%) and aspartic acid (12%) for the CWM of *C. emersonii*, glycine (17%), aspartic acid (16%) and glutamic acid (9%) for the CWM of *S. communis* and serine (15%), aspartic acid (12%) and leucine (12%) for the CWM of *T. minimum*. The high content of polar residues, accounting for ca. 30% of the total amino acids, suggests that the lipids that constitute the TLS are attached to the surface proteins through ester bonds.

3.3. Alkaline hydrolysis of protein-free CWM. Algaenan isolation

Alkaline hydrolysis of protein-free CWM yielded an insoluble residue (i.e. algaenan) which accounts for 10–20% of the lipid-free CWM. FTIR spectra of acid washed algaenans isolated from the CWM of the three strains did not show significant differences and were similar to those of algaenans isolated from the lipid-free algal biomass (Allard and Templier, 2000) (Fig. 3C). They were characterised by absorptions at ca. 3400, 2920, 2850, 1710, 1475 and 720 cm^{-1}. Alkaline hydrolysis results in a strong decrease in the intensity of the band at ca. 1735 cm^{-1} and the FTIR spectra were dominated by a band at ca. 1710 cm^{-1}. This latter was completely shifted by alkaline treatment in favour of typical carboxylate bands and was therefore assigned to carbonyl stretching of carboxylic acid groups. However, no significant change in the intensity of the broad band centred at ca. 1620 cm^{-1} was observed. In the FTIR spectra of the algaenans isolated from the CWM of *S. communis* and *T. minimum*, unsaturation is indicated by bands at ca. 3010 and 985 cm^{-1}. In agreement with previous studies (de Leeuw and Largeau, 1993; Blokker et al., 1998a,b; Gelin et al., 1999) FTIR data reveal the highly aliphatic nature of algaenans. However, in contrast to previous studies (Blokker et al., 1998a), there was no evidence for C-O absorption of ether groups in the range 1050–1150 cm^{-1}. This indicates that the contribution of ether type linkages to the algaenan structure is minor. The absence of significant ether absorption together with the high acid carbonyl/ester carbonyl absorption ratio in the FTIR spectra of all algaenans studied seems to contradict the polymeric structure containing polyester chains cross-linked by ether linkages as proposed for *S. communis* and *T. minimum* algaenan (Blokker et al., 1998a). Furthermore, it was possible to achieve complete disaggregation of native or lipid-free CWM when they were hydrolysed with aqueous tetramethylammonium hydroxide (TMAH) i.e. no algaenan was recovered after filtration of the hot mixture. In contrast, similar alkaline treatment of protein-free CWM failed to produce complete disaggregation and invariably yielded an insoluble residue. Even the use of methanolic TMAH or benzyltrimethylammonium hydroxide, a more lipophilic base, failed to disaggregate the protein-free CWM. This result suggests that the removal of proteins promotes an irreversible cohesion of the lipid moieties leading to a compact structure which can no longer be disaggregated by alkaline hydrolysis. The low reactivity of the CWM proteins to proteolytic enzymes leads us to infer that expulsion of the protein fraction, buried in the hydrophobic core of lipids, maximises the attraction between the lipid chains provided by van der Waals forces and result in an irreversible cohesion of the protein-free CWM. On the other hand

this result shows that the obtention of algaenan strongly depends on the experimental conditions. It is likely that the partition of the base from the hydrophilic region to the lipid domain plays an important role in the disaggregation of the cell walls.

3.4. Analysis of the TMAH hydrolysates of the native and lipid-free CWM

3.4.1. Fourier transform infrared spectroscopy

Upon cooling or neutralisation, the TMAH hydrolysis mixtures of native or lipid-free CWM yielded a precipitate that, once dried, became insoluble in organic solvents. Very low amounts of products were recovered upon extraction of the TMAH hydrolysates and a layer appeared between the aqueous and organic phases. FTIR spectra of the precipitates and of this layer were similar, and had no absorption bands assignable to proteins; they showed the same absorption bands as the algaenans (Fig. 3D). It is likely that these precipitates are composed of the partly, or completely, hydrolysed lipid moiety of the CWM. However, the acid carbonyl/ester carbonyl absorption ratio is markedly higher than in the algaenans (Fig. 3C). More obviously than in the case of algaenans, the FTIR data (Fig. 3D) preclude a polymeric structure for the TMAH hydrolysis precipitates. Indeed, FTIR data show that carboxyl and hydroxyl groups mainly predominate in the TMAH hydrolysis precipitates and cannot act as cross-links in a polymeric network. Based on the quantitative yield of algaenan (ca. 30–60% of the protein-free CWM), we consider that it accounts for at least 30% of the precipitate, thus increasing our uncertainty regarding the polymeric structure of algaenans.

3.4.2.. ^{1}H and ^{13}C-nuclear magnetic resonance spectroscopy

The damp precipitates, isolated from the TMAH hydrolysis, were soluble in 2-methyl-1-propanol at temperatures above 90 °C and could be esterified. The esterified precipitates were soluble in pyridine.

^{1}H and ^{13}C NMR spectra of the esterified TMAH hydrolysis mixtures from the native or lipid-free CWM of *C. emersonii* (Figs. 4 and 5) and *S. communis* (spectra not shown) are similar. They show the presence of hydroxyl and 2-methyl-1-propyl ester groups (Table 1). The peak intensity of the bulk-methylene protons (δ 1.10–1.80 p.p.m.) supports the presence of long alkyl chains in these hydrolysates. The ^{1}H and ^{13}C NMR spectra of the TMAH hydrolysate of CWM from *T. minimum* showed additional signals characteristic of carbon-carbon double bonds (Table 1) (inset of Fig. 4). NMR analyses of the esterified TMAH hydrolysates clearly shows the absence of proton and/or carbon signals corresponding to mid-chain esters (^{1}H NMR spectrum of docosanyl docosanoate displayed a triplet

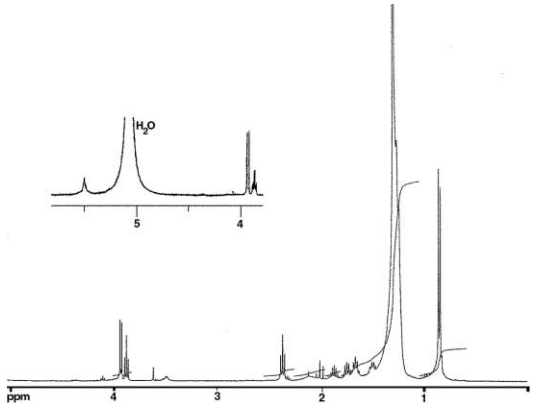

Fig. 4. ^{1}H NMR spectrum (C_5D_5N) of the esterified TMAH hydrolysate of the lipid-free CWM from *C. emersonii*. Inset: Partial ^{1}H NMR spectrum of the TMAH hydrolysate of the lipid-free CWM from *T. minimum*.

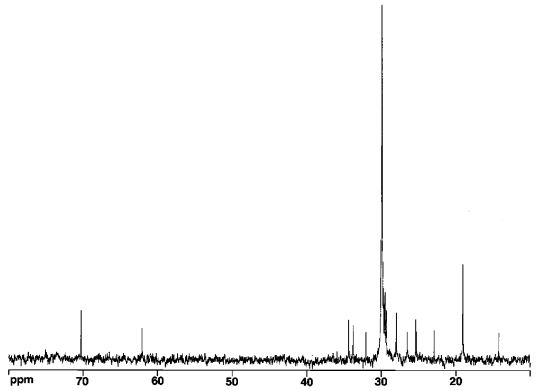

Fig. 5. ^{13}C NMR spectrum (C_5D_5N) of the esterified TMAH hydrolysate of the lipid-free CWM from *C. emersonii*.

Table 1
^{1}H NMR (400.13 MHz) and ^{13}C NMR (100.61 MHz) data for the TMAH hydrolysates of the lipid-free CWM from *C. emersonii*, *S. communis* and *T. minimum* (C_5D_5N)

	δ ^{1}H (p.p.m.)	δ ^{13}C (p.p.m.)
-$CH_2\underline{CH_3}$	0.8–0.9	14.3
-$COO\underline{CH_2}CH(\underline{CH_3})_2$	0.85 (*d*, J = 6.7 Hz)	19.0
-$CH_2(\underline{CH_2})_nCH_2$-	1.10–1.80	22.9–32.1
-$COOCH_2\underline{CH}(CH_3)_2$	1.87 (*m*)	28.0
-$\underline{CH_2}COO$-	2.37 (*t*, J = 7.4 Hz)	34.4
-$\underline{CH_2}OH$	3.88 (*t*, J = 6.6 Hz)	62.1
-$COO\underline{CH_2}CH(CH_3)_2$	3.93 (*d*, J = 6.6 Hz)	70.3
-COO-		173.5
-CH = CH-$CH_2\underline{CH_2}OH$[a]	3.87 (*t*, J = 6.6 Hz)	62.1
-$CH_2\underline{CH}=\underline{CH}CH_2$-[a]	5.50 (*b*)	130.2, 130.7

[a] Proton and carbon signals observed for the TMAH hydrolysate of the lipid-free CWM from *T. minimum*.

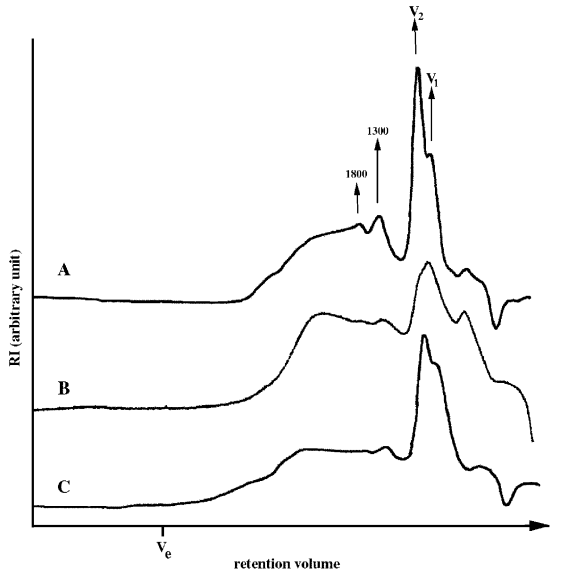

Fig. 6. Gel permeation chromatograms (GPC) of esterified precipitates (HCl/2-methyl-1-propanol) isolated from aqueous TMAH hydrolysis of the lipid-free CWM of (**A**) *C. emersonii*, (**B**) *S. communis*, and (**C**) *T. minimum*. V_e: exclusion volume, V_1: elution volume of octacosanoic acid methyl ester, V_2: elution volume of docosanyl docosanoate.

characteristic of oxymethylene protons at δ 4.22 p.p.m.) and to mid-chain ether linkages since ^{13}C NMR experiments did not show resonances of oxygen-bearing methine carbons in the range expected (δ 70–80 p.p.m.) (Metzger and Casadevall, 1992).

3.4.3. Gas chromatography-mass spectrometry and gel permeation chromatography of the TMAH hydrolysates

GC-MS analysis of the acetoxy 2-methyl-1-propyl ester-derivatised TMAH hydrolysates showed they have the same composition as the fractions extracted from TMAH hydrolysis mixtures. They comprise even-carbon- length saturated alcohols ranging from *n*-C_{26} to *n*-C_{30} (25% and 6% of the GC amenable products of *C. emersonii* and *S. communis*, respectively), saturated acids from *n*-C_{24} to *n*-C_{30} (65%, 60% and 80% of the GC amenable products of *C. emersonii*, *S. communis* and *T. minimum*, respectively), with *n*-C_{28} as the major component. Significant amounts of ω-hydroxy acids were identified in the hydrolysates from *C. emersonii* (saturated *n*-C_{30} and *n*-C_{32}, 10%), *S. communis* (saturated *n*-C_{32}, 10%) and *T. minimum* (saturated and mono-unsaturated *n*-C_{30} to *n*-C_{36}, 10%). Saturated *n*-C_{32} α,ω-diol was present in the hydrolysates from *C. emersonii* (1%) and *S. communis* (24%). Note that neither alcohol nor diol were identified in the TMAH hydrolysate from the CWM of *T. minimum*. However, in contrast to the extracted fractions, only ca. 10% of the TMAH hydrolysis product was GC amenable, indicating the possible presence of high molecular weight products not detected by the GC system used. This was corroborated by gel permeation chromatography (GPC) of the hydrolysates of the lipid-free CWM. For all species the gel permeation chromatograms (Fig. 6) showed two prominent narrow peaks corresponding to M_rs of ca. 450 and 650 Da and a broad distribution ranging from ca. 1,000 to 20,000 Da revealing the presence of high molecular weight compounds in these hydrolysates.

The collected results from FTIR, NMR, GC-MS and GPC analyses of the TMAH hydrolysates of the native and lipid-free CWM of all three algae suggest the following:

(i) The ester functions present in the native and lipid-free CWM have been entirely cleaved during TMAH hydrolysis yielding moieties containing only primary hydroxyl groups and/or carboxyl groups. This suggests that the TMAH hydrolysates are composed of the building blocks that constitute the lipids of the CWM.
(ii) FTIR and NMR analyses clearly demonstrate the absence of ether groups in the lipids that constitutes the CWM.
(iii) Besides the compounds identified by GC-MS, the lipids of the CWM are composed of extremely long chain alcohols, acids, ω-hydroxy acids and/or α,ω-diols ($>C_{36}$). Similar GC amenable components have been identified by Blokker et al. (1998a,b) in the saponification products of the cell walls of *S. communis* and *T. minimum*.

Thus protein-free CWM could be constituted, in part, of linear (poly)esters formed by self esterification of ω-hydroxy acids or by esterification of acids (or ω-hydroxy acids) and alcohols (or diols). This is in direct contrast to the conclusion of Blokker et al. (1998a) who suggest that only unsaturated ω-hydroxy acids are involved in the formation of the cell walls of *S. communis* and *T. minimum*, the unsaturations acting as the starting position of ether cross-linking. Despite the likely absence of cross-links, the structure of the CWM may be similar to land plant cuticular membranes. However molecular weights ranging up to 20,000 Da is surprising and whether aggregates of the hydrolysis compounds are formed is still an open question.

3.5. Tetramethylammonium hydroxide (TMAH) thermochemolysis of the lipid-free CWM, protein-free CWM and algaenan

3.5.1. Flash pyrolysis with in situ methylation

The total ion current (TIC) of the products released upon flash pyrolysis using tetramethylammonium hydroxide of the lipid-free CWM (TMAH thermochemolysate **1** in Fig. 1), protein-free CWM (TMAH

thermochemolysate **2** in Fig. 1), and algaenans (TMAH thermochemolysate **3** in Fig. 1) are shown in Figs. 7–9. The compounds, identified in the TMAH thermochemolysate of lipid-free and protein-free CWM of the three strains do not differ significantly. In contrast to the recent studies of Knicker et al. (2001), proteinaceous moieties were not detected as amino acid derivatives and were present only in very small amounts in the thermochemolysate of the lipid-free CWM. The discrepancies between our data and that of Knicker et al. (2001) may be due to (1) our TMAH thermochemolyses have been carried out at a far higher temperature, and degradation of the released amino acids might have occurred or (2) the isolation procedure of algaenan from *S. communis* used by these authors is known to lead to the significant formation of melanoidin-like polymer artifacts (Allard et al. 1998), and it was not possible to ensure that the amino acid derivatives identified in their TMAH thermochemolysate originate from the algaenan or from melanoidin-like polymers. The predominant compounds were even-carbon-length acid methyl esters (n-C_{16} to n-C_{32}), alcohol methyl ethers (n-C_{26} to n-C_{32}), α,ω-diol dimethyl ethers (n-C_{30} and n-C_{32}) and α,ω-methoxy acid methyl esters (n-C_{30} to n-C_{36}). Thermochemolysis of lipid-free and protein-free CWM generated the same products as those released upon TMAH hydrolysis. Similar compounds have been also identified in the thermochemolysate of high molecular weight mono- and polyesters isolated from the algal biomass (Allard and Templier, 2001). As in this latter case small amounts of methoxylated aromatic structures were identified in the thermochemolysate of lipid-free CWM from *S. communis* and *T. minimum*. No trifunctional compound was identified and all the products can be rationalised as being derived from alkaline hydrolysis of linear (poly)esters and/or from quantitative methylation of free fatty acids, alcohols, α,ω-diols and/or ω-hydroxy acids.

In contrast, the GC fingerprints of the products generated upon TMAH thermochemolysis of the algaenans was quite different (Figs. 7C, 8C, 9B). The contribution of ω-methoxy acid methyl esters is relatively small and neither alcohols nor diols were detected. The chromatograms were dominated by acid methyl esters ranging from n-C_{16} to n-C_{32} and α,ω-dicarboxytic acid dimethyl esters in the ranges n-C_7 to n-C_{11}, n-C_{19} to n-C_{21} and n-C_{29} to n-C_{36} with no odd/even carbon number predominance. Coelutions obscured the two first series in *T. minimum* but they were detected by selective ion detection at m/z 98. These products cannot be derived from a polyester structure or from a three-dimensional polymer network. It is noteworthy that similar α,ω-dicarboxylic acids were identified by Blokker et al. (1998a, 2000) in the RuO_4 oxidation product mixtures of the algaenans from *T. minimum* and *S. communis*. This could indicate that these α,ω-dicarboxylic acids are present as such in the algaenans and do not derive from an oxidative cleavage of ether bridges as they postulate. However, the differences between the chromatograms of TMAH thermochemolysis of protein-free CWM and algaenan remain difficult to explain. Indeed, if protein-free

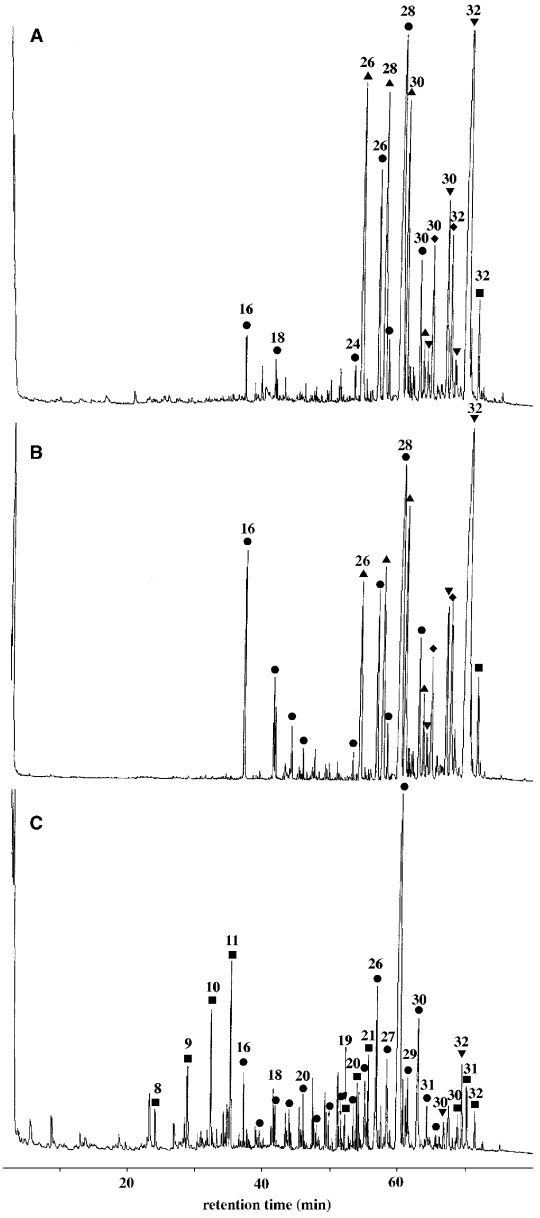

Fig. 7. Gas chromatography-mass spectrometry (GC-MS) chromatograms for the flash pyrolysis in the presence of TMAH of CWM from *C. emersonii* (**A**) lipid-free CWM, (**B**) protein-free CWM, (**C**) algaenan. • carboxylic acid methyl ester, ▲ alcohol methyl ether, ◆ α,ω-diol dimethyl ether, ▼ ω-methyl ether acid methyl ester, ■ α,ω-dicarboxylic acid dimethyl ester, + phytol, ∗ hydrocarbon, ■ aromatic compound. Numbers indicate chain length.

CWM are constituted of linear (poly)esters, significant amounts of alcohols, diols and α,ω-hydroxy acids would be expected in the TMAH thermochemolysate of algaenans since these latter result from the more or less complete saponification of the protein-free CWM. Oxidation reactions occurring during TMAH thermochemolysis, as observed by Hatcher and Minard (1995) can be ruled out since similar reactions would occur

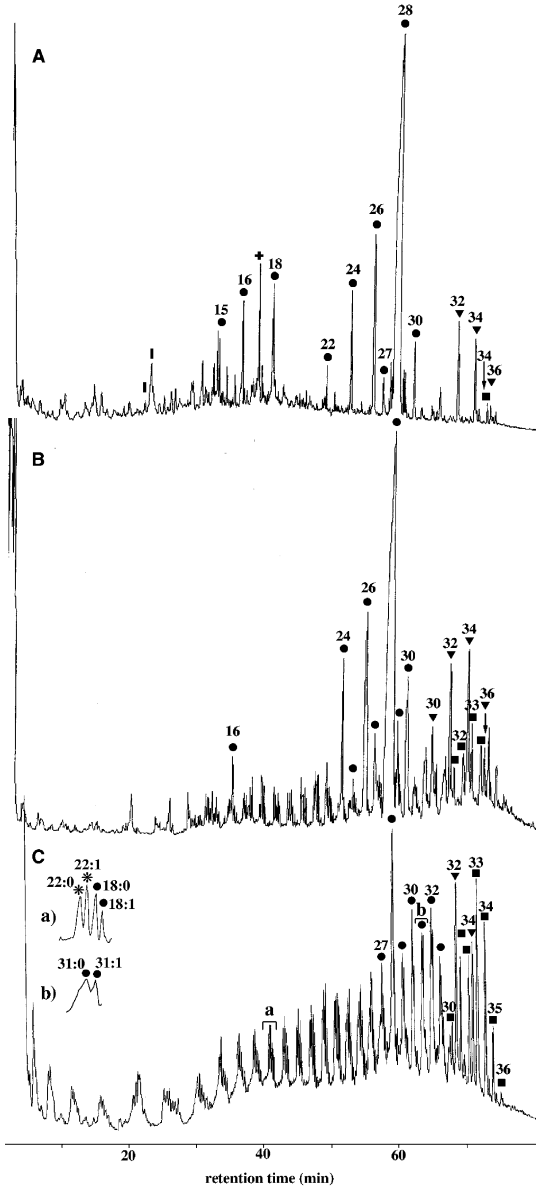

Fig. 8. Gas chromatography-mass spectrometry (GC-MS) chromatograms for the flash pyrolysis in the presence of TMAH of CWM from *T. minimum* (**A**) lipid-free CWM, (**B**) protein-free CWM, and (**C**) algaenan. The notation system is the same as in Fig. 7.

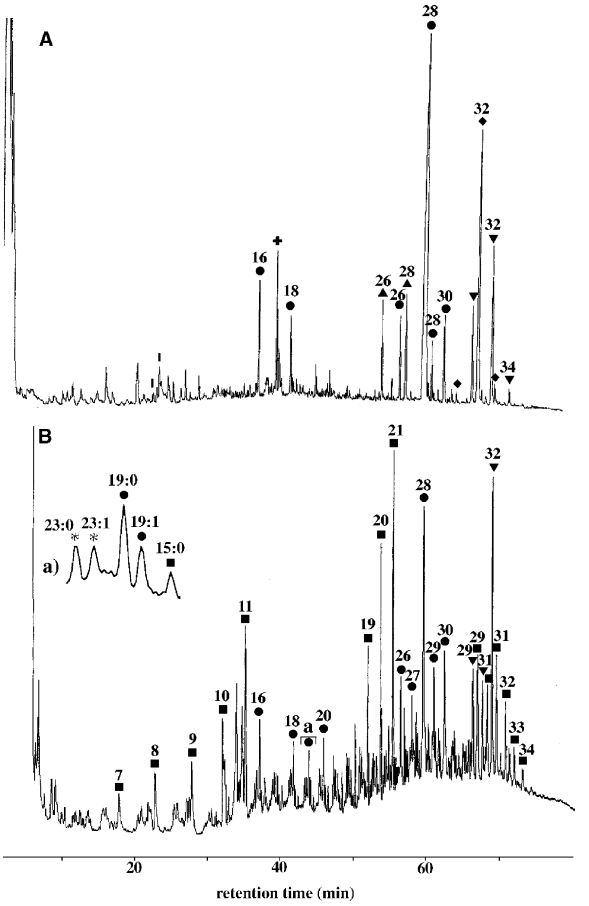

Fig. 9. Gas chromatography-mass spectrometry (GC-MS) chromatograms for the flash pyrolysis in the presence of TMAH of CWM from *S. communis* (**A**) lipid-free CWM, and (**B**) algaenan. The notation system is the same as in Fig. 7.

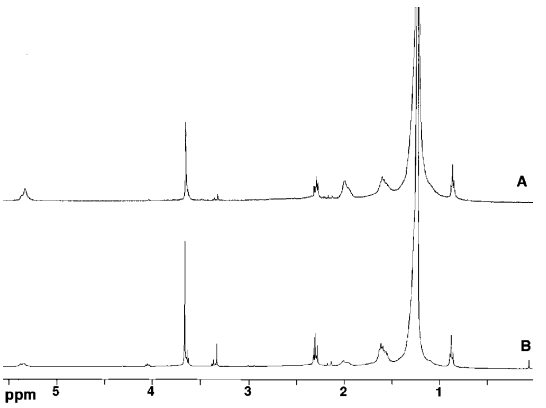

Fig. 10. ^{1}H NMR spectrum ($CDCl_3$) of the TMAH thermochemolysate from *C. emersonii* (**A**) algaenan, and (**B**) protein-free CWM.

Table 2
^{1}H NMR (400.13 MHz) and ^{13}C NMR (100.61 MHz) data for the TMAH thermochemolysates of the protein-free CWM and algaenan from *C. emersonii* ($CDCl_3$)

	δ ^{1}H (p.p.m.)	δ ^{13}C (p.p.m.)
-$CH_2\underline{CH_3}$	0.87 (*t*, $J=7.1$ Hz)	14.2
-$CH_2(\underline{CH_2})_nCH_2$-	1.10–1.70	22.8–29.8
-$\underline{CH_2}COOCH_3$	2.30 (*t*, $J=7.6$ Hz)	34.2
-$CH_2COO\underline{CH_3}$	3.66 (*s*)	51.5
-$CH_2O\underline{CH_3}$[a]	3.33 (*s*)	58.6
-$\underline{CH_2}OCH_3$[a]	3.36 (*t*, $J=6.6$ Hz)	73.1
-$\underline{CH_2}OH$	3.64 (*t*, $J=6.6$ Hz)	63.2
-$\underline{CH}=\underline{CH}$-	5.34 (*b*)	130.0
-$\underline{CH_2}CH=CH\underline{CH_2}$-	2.00 (*b*)	27.3, 32.7[b]
-COO-		174.4

[a] Proton and carbon signals observed only for the protein-free CWM thermochemolysate.

[b] Signals at δ 27.3 p.p.m. and 32.7 p.p.m. can be assigned to methylene carbons adjacent to a cis and to a trans carbon-carbon double bond respectively. (Batchelor et al., 1974).

during the TMAH thermochemolysis of protein-free CWM giving α,ω-dicarboxylic acids.

Whether these α,ω-dicarboxylic acids arise from oxidation reactions during the saponification of the protein-free CWM cannot be answered for the present.

3.5.2. Sealed tube TMAH thermochemolysis

TMAH thermochemolysis of protein-free CWM and of algaenan from *C. emersonii* in sealed tubes showed that ca. 80–90% of the initial material was released. However only 10% of the thermochemolysis products were GC amenable. As for TMAH hydrolysates, gel permeation chromatography of these thermochemolysates indicated the presence of high molecular weight compounds (450 to 20,000 Da) and the molecular weight heterogeneity of the samples.

^{1}H and ^{13}C NMR spectra of the thermochemolysate of protein-free CWM from *C. emersonii* (Figs. 10B and 11A) exhibited characteristic signals due to long (un)saturated alkyl chains, methyl ester and methyl ether groups (Table 2). However, the presence of hydroxyl group-bearing methylenes indicates that only partial alkylation of hydroxyl groups had occurred. Although ^{1}H and ^{13}C NMR spectra of the thermochemolysate of the algaenan (Figs. 10A and 11B) show similar functionalities to those of the thermochemolysate of protein-free CWM, they displayed significant quantitative differences. In particular, these NMR spectra reveal the lower proportion of hydroxyl groups, and the absence of methyl ether groups, in the algaenan thermochemolysate. This result indicates that the hydroxyl groups had not been methylated and supports the flash pyrolysis with in situ methylation data indicating the rather low proportion of hydroxyl and/or ester groups in the algaenan. A higher proportion of carbon-carbon double bonds in this thermochemolysate is also indicated by ^{1}H NMR. Furthermore, as for TMAH hydrolysates, NMR analysis of TMAH thermochemolysates indicates the complete cleavage of mid-chain esters of protein-free CWM and the absence of ether linkages in both protein-free CWM and algaenan.

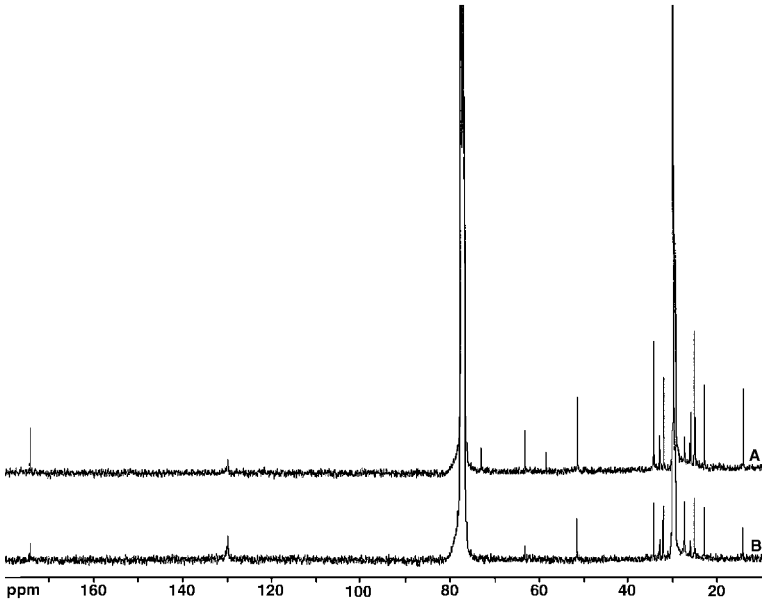

Fig. 11. ^{13}C NMR spectrum ($CDCl_3$) of the TMAH thermochemolysate from *C. emersonii* (**A**) protein-free CWM, and (**B**) algaenan.

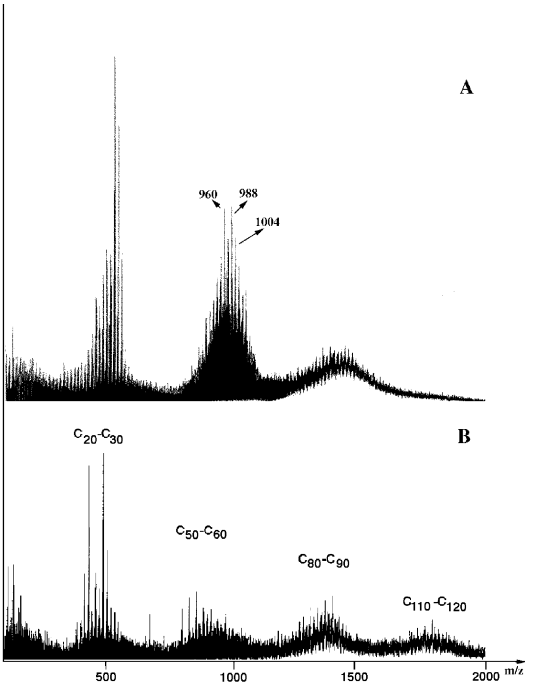

Fig. 12. Positive ion desorption chemical ionisation mass spectra (DCI-MS) of the products released from TMAH thermochemolysis in a sealed tube. (**A**) protein-free CWM of *C. emersonii*. The mass spectrum reported is the superimposition of two spectra recorded at the beginning and near the end of the desorption. m/z 960 [M + NH_4^+ can be assigned to C_{64} carboxylic acid methyl ester, C_{61} mono-unsaturated α,ω-dicarboxylic acid dimethyl ester or C_{62} mono-unsaturated ω-methyl ether carboxylic acid methyl ester; m/z 1004 can correspond to C_{64} α,ω-dicarboxylic acid dimethyl ester or C_{65} ω-methyl ether carboxylic acid methyl ester (**B**) algaenan isolated from *C. emersonii*.

Comparison of GC-MS results from flash pyrolysis with in situ methylation and NMR analyses of the thermochemolysates suggests that the GC amenable products generated upon TMAH thermochemolysis of the protein-free CWM, and of the algaenan, are qualitatively representative of the overall thermochemolysis products. Indeed, the small proportion of hydroxyl groups in the thermochemolysate of algaenan, indicated by the NMR analysis, corroborates the small contribution of ω-methoxy acid methyl esters and the absence of alcohol and diol observed in the chromatogram of the flash pyrolysis with in situ methylation. On the other hand, the ratio [ether + hydroxyl]/[ester] determined from integrated peak intensities of the NMR spectrum of the thermochemolysate of protein-free CWM (ca. 0.7) is in good agreement with that estimated from peak areas of GC-MS flash pyrolysis chromatograms (ca. 1).

Desorption chemical ionisation mass spectra (DCI-MS) of the TMAH thermochemolysates of the protein-free CWM and algaenan from *C. emersonii* (Fig. 12) consist of a sequence of gaussian-like mass ion distributions ranging to near mass 2,000. In both cases the sequence is characterised by an approximately equal mass increment corresponding to ca. 30 CH_2 units from one maximum to the next. This latter result provides additional support for our hypothesis on the occurrence of extremely long chain compounds ($>> C_{30}$) in both algaenan and protein-free CWM of microalgae.

4. Conclusion

Our results constitute a strong piece of evidence that the lipid moiety of the outer cell walls of the studied TLS-containing microalgae is composed, at least in part, of (poly)esters constituted of extremely long chain alcohol and acid moieties reaching C_{80}. Upon alkaline hydrolysis these (poly)esters are partly or entirely cleaved depending on the hydrophilicity/hydrophobicity balance of the base. However, due to their very high molecular weight, most of the released products precipitate yielding an insoluble aggregate. This insoluble aggregate, which has been termed algaenan, is not a polymeric material. As yet only the algaenan isolated from *Botryococcus braunii* has been proved to be a polymer exhibiting an aliphatic polyaldehydic network as basic structure (Metzger and Largeau, 1999). Furthermore, it should be stressed that a series of hydrocarbons extending to over C_{110} have been identified in numerous geochemical materials including source rock bitumens (Mueller and Philp, 1998; Killops et al., 2000) and crude oils (del Rio et al., 1992; del Rio and Philp, 1999; Hsieh et al., 2000; Hsieh and Philp, 2001). They are thought to be very resistant to biodegradation (Health et al., 1997) and to originate from the same biological or diagenetic sources as low molecular weight hydrocarbons (Carlson et al., 1997). However, their exact origin remains unclear and several precursor-product relationships have been proposed (e.g. del Rio and Philp, 1992; Killops et al., 2000). Our results may shed light on a possible biological origin of these high molecular weight hydrocarbons.

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