Carbohydrate Polymers 88 (2012) 537–546

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Characterization of ulvan extracts to assess the effect of different steps in the extraction procedure

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ARTICLE INFO

Article history:
Received 30 September 2011
Received in revised form
22 December 2011
Accepted 24 December 2011
Available online 3 January 2012

Keywords:
Ulvan
Green algae
Ulva lactuca
Extraction
Purification
Polysaccharide characterization

ABSTRACT

An effective application development of the polysaccharide ulvan requires a comprehensive knowledge about the influence of the extraction process on composition of the extracts and in ulvan itself. In this context, the two main objectives of the present work are (1) the establishment of an efficient extraction process for ulvan and (2) development of an accurate characterization methodology to evaluate the extract composition and ulvan content.

Three ulvan-rich extracts obtained by different schemes of extraction were studied. The methodology for the analysis was improved and a detailed analysis of extracted ulvan was provided. The polysaccharide is rich in ulvanobiuronic acid 3-sulfate type A [→4)-β-D-GlcA*p*-(1 → 4)-α-L-Rha*p* 3S-(1→], with minor amounts of ulvanobiuronic acid 3-sulfate type B [→4)-α-L-IdoA*p*-(1 → 4)-α-L-Rha*p* 3S-(1→]. The extract with the higher degree of purification is a high molecular weight polysaccharide (790 kDa) composed of rhamnose (22.4%), glucuronic acid (22.5%), xylose (3.7%), iduronic acid (3.1%) and glucose (1.0%). It is highly sulfated (32.2%) and contains 1.3% of proteins and 10.3% of inorganic material. Applying simple extraction scheme it was possible to obtain an extract from green algae with high content of ulvan without affecting the overall chemical structure of the polysaccharide.

1. Introduction

The emergent attention on renewable resources for chemicals and polymers has been leading to a special focus on marine biomass, in particular on green algae. Ulvan is one of the main polysaccharides composing the algae belonging to *Ulvaceae* genera, representing a potential source to be explored. Ulvan has already proven to be a remarkable polysaccharide with different attractive properties that make it suitable for a wide range of applications (Castro et al., 2006; Castro, Zarra, & Lamas, 2004; Cluzet et al., 2004; Harada & Maeda, 1998; Kaeffer, Benard, Lahaye, Blottiere, & Cherbut, 1999; Mao, Zang, Li, & Zhang, 2006; Pengzhan, Ning, et al., 2003; Pengzhan, Quanbin, et al., 2003; Qi, Zhang, et al., 2005; Qi, Zhao, et al., 2005; Warrand, 2006). In fact, the relevance of natural polymers for different technological and industrial-related applications is increasing. These are believed to be a better alternative to the use of fully synthetic materials in various contexts (Mano et al., 2007).

The major repeating structure (designated as aldobiuronic acid) of ulvan is the ulvanobiuronic acid 3-sulfate composed by *O*-3-sulfate rhamnose and glucuronic acid (type A, A3S). In other typical repeating structure, iduronic acid replaces glucuronic acid (type B, B3S) (Lahaye, Brunel, & Bonnin, 1997; Lahaye, Inizan, & Vigouroux, 1998):

$$\text{A3S}: \quad \rightarrow 4)\beta\text{-D-Glc}Ap(1 \rightarrow 4)\text{-}\alpha\text{-L-Rha}p\ 3S(1 \rightarrow \quad (1)$$

$$\text{B3S}: \quad \rightarrow 4)\alpha\text{-L-Ido}Ap(1 \rightarrow 4)\text{-}\alpha\text{-L-Rha}p\ 3S(1 \rightarrow \quad (2)$$

where *p* is a indicative of a pyranoside form of the compound. Xylose (*O*-2 sulfated or not) can also occur linked to sulfated rhamnose. The designation are ulvanobioses U3S and U2′S,3S (Lahaye, 1998):

$$\text{U3S}: \quad \rightarrow 4)\beta\text{-D-Xyl}p(1 \rightarrow 4)\alpha\text{-L-Rha}p\ 3S(1 \rightarrow \quad (3)$$

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doi:10.1016/j.carbpol.2011.12.041

U2′S,3S : → 4)β-D-Xylp 2S(1 → 4)α-L-Rhap 3S(1 → (4)

Other repeating structures (involving the same residues), some of them branched with GlcA, are reported in the literature as belonging to ulvan structure (Lahaye, 1998; Lahaye & Robic, 2007). Glucuronans, xyloglucans, cellulose and proteins are cell-wall components found in close association with ulvan (Lahaye, Jegou, & Buleon, 1994; Lahaye & Ray, 1996; Lahaye & Robic, 2007). In the extractive process of ulvan, these associations would lead to co-extraction of a fraction of these components, affecting the purity in ulvan and the biological responses of the extract (Cannell et al., 1998; Hernández-Garibay, Zertuche-González, & Pacheco-Ruíz, 2011). In this context, accurate analysis of the carbohydrate composition of the extracts is required.

Concerning the chemical characterization, the depolymerization of polysaccharides is often performed by acid-catalyzed hydrolysis of glycosidic bonds, as Saeman hydrolysis (Kamerling & Gerwig, 2007; Saeman, 1945). However, this procedure is not effective for linkages such as aldobiuronic (Conrad, 1980; McKinnell & Percival, 1962) due to the high stability of glycosidic linkages involving uronic acids (Dahlman, Jacobs, Liljenberg, & Olsson, 2000; De Ruiter, Schols, Voragen, & Rombouts, 1992) and monosaccharides, once released, are highly susceptible to further degradation (Biermann, 1988; Willför et al., 2009). An alternative is the depolymerization by acid methanolysis (HCl in anhydrous methanol) (De Ruiter et al., 1992; Sundberg, Sundberg, Lillandt, & Holmbom, 1996). In this process, both neutral and acid monosaccharides are released and converted to methyl glycosides, while the carboxyl groups of uronic acids are converted to methyl esters. One advantage of this method is their good performance in the cleavage of glucuronosyl bonds and the improvement of the stability of resulting monosaccharides (Willför et al., 2009). Besides this benefit, it is still possible to detect and quantify some resistant disaccharide. The improvement of conditions (time, temperature and HCl concentration) in order to obtain the maximum yield has been discussed in the literature concerning commercial gelling algae (Quemener, Lahaye, & Metro, 1998) and lignocellulosic polysaccharides (Bertaud, Sundberg, & Holmbom, 2002; Sundberg et al., 1996).

In general, ulvan is extracted with hot aqueous solutions (Lahaye & Robic, 2007); however, the final step for removing contaminants may vary considerably. One of the objectives of the present work is the validation of a previously developed extraction methodology to obtain ulvan from green algae (Alves, Caridade, Mano, Sousa, & Reis, 2010). For this, the composition of the extracts produced by the three schemes of extract preparation is assessed, as well as the structural characteristics of the polysaccharide fraction. The method of acid methanolysis was improved and tailored for the maximum yield on typical monosaccharides for the analysis of ulvan-rich extracts and results were compared with those from enzymatic hydrolysis. Complementary HPSEC and ^{13}C NMR spectroscopic data on the extracts were provided.

2. Materials and methods

2.1. Extraction of ulvan polysaccharide from green algae Ulva lactuca

A general procedure was employed to extract ulvan, based on the procedure developed by Alves et al. (2010).

All reagents were supplied by Sigma–Aldrich. Green algae *U. lactuca* were supplied by Setalg (France). General procedure: Soxhlet extraction of dried *U. lactuca* with dichloromethane and ethanol removed most of the lipids and coloring matter which otherwise would contaminate the water extract. The residual off-white weed was dried and subjected to three hot-water extractions, typically

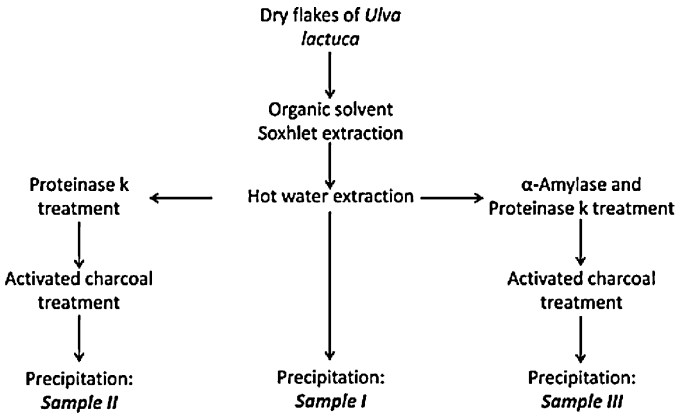

Fig. 1. Scheme of the ulvan extraction from green algae *Ulva lactuca*.

100 g dry algae per 3000 mL of water, for a total of 7 h (3 h the first extraction and 2 h the following two extractions) at 75–85 °C, on a boiling-water bath, under continuous stirring. After filtration through a cotton cloth, the aqueous extracts were centrifuged, and the liquid supernatant was filtered. The water extract was concentrated until 10–20% of its initial volume, in a rotary evaporator. The derived solution was then subjected to enzymatic hydrolysis to remove contaminating starch and proteins. Afterwards, the solution was decolorized and deodorized by adsorption on activated charcoal. The water extract was centrifuged, filtered and precipitated with 4 vol. of absolute ethanol. Finally, the recovered precipitate was freeze dried. Some deviations to this general method are introduced to obtain different ulvan extracts (referred to as samples) to be further characterized. A minimum of three extractions were performed for each sample.

Sample I: crude ulvan is obtained by a reduction in the general method, i.e., without further purification. In this sense, after Soxhlet extraction of dried *U. lactuca* followed by hot-water extraction, the water extract was concentrated until 10–20% of its initial value, in a rotary evaporator, precipitated and freeze dried.

Sample II: obtained by applying the general procedure without the use of an enzyme to remove starch.

Sample III: obtained by applying the general procedure. Enzymatic hydrolysis is achieved by applying both α-amylase and proteinase K to remove starch and proteins, respectively.

A representative scheme of the ulvan extraction to obtain the three samples is shown in Fig. 1. Algae used on this study belong to *U. lactuca* species originated from the same batch, in order to eliminate any variability associated with the biomass source.

2.2. Ash content

Ash content was determined by thermogravimetric analysis (TGA). For TGA, a small amount (10–15 mg) of powder was taken for analysis in a Perkin Elmer TGA7. The samples were heated from 30 to 900 °C at a rate of 20 °C/min in air atmosphere.

2.3. Quantification of sulfates

Sulfate groups were released after hydrolysis of 10–12 mg of polysaccharide ulvan with 3 mL trifluoroacetic acid (TFA, 99% Prolabo), 2 M, for 3 h at 100 °C, according to the method described by Ray and Lahaye (1995). For sulfate groups in extract ashes, a sample of 10 mg was dissolved in 10 mL of deionized water with 0.1 mL of TFA. Sulfate quantification was performed by using a Dionex ion chromatograph (Dionex DX-120) equipped with a suppressed conductivity detector. The column was a DionexIonpac AS9-HC

(250 mm × 4 mm I.D.) operated at room temperature. The eluent was 9 mM Na_2CO_3 aqueous solution at a flow rate of 1.0 mL/min.

2.4. Protein quantification

Protein present in the extracts was quantified by a colorimetric protein assay, using BCATM Protein Assay Kit (Pierce), following manufacturer instructions. Briefly, polysaccharide solution was exposed to a working solution containing bicinchoninic acid (BCA) as detection reagent for Cu^{1+} and optical density, at 562 nm, was measured using a microplate reader (Redinbaugh & Turley, 1986). A standard curve was created and sample protein values were read off from the standard graph. Albumin solutions were used as standards (0–2000 μg/mL).

2.5. Elemental analysis

Carbon (C), hydrogen (H), sulfur (S), nitrogen (N) and oxygen (O) contents were quantified using a Thermo Scientific Flash 2000 Organic Elemental Analyser.

2.6. High pressure size exclusion chromatography (HPSEC)

Ulvan extracts, about 4 mg, were dissolved in 1 mL of eluent solution −50 mM $NaNO_3$ (99%, Panreac) containing 0.02% NaN_3 (99%, Sigma–Aldrich). The solutions (filtered by membrane 0.22 μm) were analyzed on a HPLC (Gilson, France) equipped with a Shodex OH-pack SB HQ 804 (Shodex, Japan) and a refractive index (RI) detector (model 131, Gilson). Elution was performed at 25 °C with a solution of 50 mM $NaNO_3$ (0.02% NaN_3) at a flow rate of 0.7 mL/min. Pullulans (PSS, Germany) with molecular weight (Mw) values between 342 Da and 805 kDa were the standards used for calibration of RI signal. A linear relationship between retention time of standards with the logarithmic function of Mw was obtained (r^2 = 0.982). Protein elution was followed in parallel by ultra-violet (UV) detector (model 117, Gilson) at 280 nm.

2.7. Assessing to the carbohydrate fraction of the ulvan extracts

2.7.1. Enzymatic approach

Two different approaches using enzymatic preparations were tested aiming to obtain the maximum yield of released monosaccharides: 1. Combining partial acid-hydrolysis with and β-glucuronidase from *Helix pomatia* (G-0751, Sigma–Aldrich) – chemical-enzymatic method; 2. Applying a pool of enzymes, kindly supplied by Novozymes (North America), containing: 25% cellulase complex (NS22074, 1.0 EGU/g), 25% xylanase (NS22036, 1.0 FXU/g), 25% β-glucosidase (NS50010, 250 CBU/g) and 25% β-glucanase and xylanase mixture (NS22002, 45 FBG/g). The activities are expressed as EGU – Endo-Glucanase Unit, FXU – Fungal Xylanase Unit, CBU – Cellobiase Unit and FBG – Fungal β-Glucanase Unit. The enzymatic mixture is hereafter designated as EM.

Acid pre-hydrolysis and enzymatic hydrolysis with β-glucuronidase was performed as described in the literature (Quemener, Lahaye, & Bobin-Dubigeon, 1997) with some modifications. Partial hydrolysis of ulvan extracts, about 10 mg, was carried out with 3 mL trifluoroacetic acid, 2 M, for 45 min at 120 °C. The pre-hydrolysate was evaporated at low pressure and temperature, resuspended in 5 mL of acetate buffer pH 5 containing 500–600 U of β-glucuronidase and incubated at 37 °C for 16 h. The reaction was stopped by immersing the reaction tubes in boiling water for 5 min.

For the second approach, the EM supplied by Novozymes was used as received. Each sample (20 mg) and the same weight of the EM were dissolved in sodium acetate buffer pH 5 (4 mL). The hydrolysis was carried out at 50 °C and after 5 h the enzymes were thermally deactivated as above described. Control experiments were performed to evaluate the presence of monosaccharides as contaminant in enzymatic formulations.

The resulting hydrolysates were freeze-dried and analyzed by GC-FID and GC–MS after trimethylsilylation as describe in Section 2.7.3. Myo-inositol (≥99.0% Sigma–Aldrich) was used as internal standard (1 mL of a methanolic solution 0.1 mg/mL).

2.7.2. Acid methanolysis: kinetic and improvement of the method

The ulvan extracts (10–12 mg, dried in vacuum over phosphoric pentoxide (≥98.5%, Sigma–Aldrich) were submitted to acid methanolysis as described in the literature (Sundberg et al., 1996) in 2 mL of HCl 2 M prepared by dilution with anhydrous methanol (>99%, Sigma–Aldrich) of a commercial solution 3 M HCl in methanol (Supelco). Methanolysis reaction was performed at 100 °C, initially for 4 h. For kinetic of acid methanolysis of ulvans in two HCl concentrations, 2 M and 3 M, the reaction was carried out for 3, 4, 6, 24, 48 and 72 h at 100 °C. The final goal was to establish the parameters leading to the maximum released of monosaccharides with minimal degradation. The analyses were performed as described in Section 2.7.3.

2.7.3. Analysis of the partially methylated monosaccharide: identification and quantification

After acid methanolysis, pyridine (≥99.0% Sigma–Aldrich) was added to methanolysates to neutralize the remaining HCl, followed by addition of 1 mL of sorbitol (>98.0%, Sigma–Aldrich) methanolic solution (0.1 mg/mL) and evaporation to dryness in a rotavapor. Then, the dried methanolysates were dissolved in 150 μL of pyridine, 150 μL of bis(trimethylsilyl)-trifluoroacetamide (≥99% Fluka) and 50 μL of chlorotrimethylsilane (≥99.0% Fluka), maintaining the reaction mixture at 80 °C for 30 min.

For quantification, the products were analyzed by gas chromatography with flame ionization detector (GC-FID) on a DANI GC 1000 chromatograph, with a capillary column ValcoBond VB1 (30 m × 0.32 mm I.D., 0.25 μm film thickness), using the following temperature program: 100–175 °C at 3 °C/min and 175–290 °C at 8 °C/min. The temperature of the detector and the injector were kept at 290 °C and 260 °C, respectively. External calibration was performed for rhamnose (99%, Merk), xylose (99%, Merk), glucuronic acid (98%, Alfa Aesar), glucose (99.5%, Sigma–Aldrich) and iduronic acid (98%, Carbosynth, UK). The GC–MS analyses were performed in a Trace Gas Chromatograph 2000 Series equipped with a Finnigan Trace MS mass spectrometer (EI), using helium as carries gas (35 cm/s). The chromatographic conditions, including the column, were the same as described for GC-FID with a transfer-line temperature of 290 °C and split ratio of 1:100.

Methanolysis of polysaccharides leads to the cleavage of the glycoside bonds with the production of the α- and β-anomers and pyranose (*p*) and furanose (*f*) ring forms of the monosaccharides. The identification was performed based on the number of glycoside peaks, their relative retention time and signal intensity proportion (Bleton, Mejanelle, Sansoulet, Goursaud, & Tchapla, 1996; Doco, O'Neill, & Pellerin, 2001), and mass spectra of trimethylsilyl (TMS) methyl glycoside derivatives using spectral data reported in the literature (Bleton et al., 1996; Doco et al., 2001) and standards.

2.7.4. ^{13}C NMR

^{13}C NMR spectra were recorded on a BRUKER AVANCE III 400 spectrometer operating at 400 MHz. 30 mg of dried ulvan sample were dissolved in 1.0 mL of D_2O (99.9%) and data were acquired at 100.62 MHz for ^{13}C at 70 °C. Standard parameters for ^{13}C NMR measurements were used: simple 1D pulse sequence, recycling time of 2.0 s, spectral width of 250 ppm, and 1D sequence with power-gated decoupling using 30° flip angle.

Table 1
Non-carbohydrate components of ulvan-rich extracts (as % w/w dry extract material) and elemental analysis.

Parameter	Sample		
	I	II	III
Ashes			
Total	43.2 ± 0.6	38.3 ± 1.4	26.2 ± 0.3
Sulfate-free	17.5 ± 1.2	17.0 ± 1.2	10.3 ± 0.5
Sulfate (as SO_3^-)	29.9 ± 1.1	25.9 ± 1.5	32.2 ± 0.4
Proteins	2.4 ± 0.2	1.1 ± 0.1	1.3 ± 0.1
Elemental analysis			
%O	48.7	49.6	40.5
%N	1.2	0.4	1.0
%C	16.5	18.8	22.8
%H	3.5	4.0	4.3
%S	11.7	11.0	8.4

Data are presented as mean ± standard deviations (SD) of triplicate essays.
Values of elemental analysis: average of two essays.

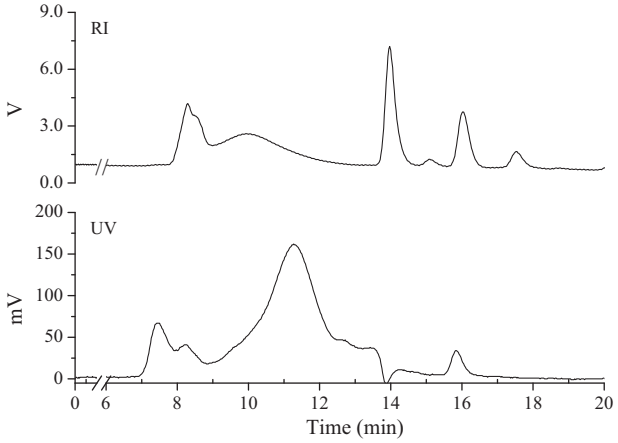

Fig. 2. Typical HPSEC profile of ulvan-rich extracts (presented for sample III). Refractive index (RI) and ultraviolet (280 nm) detection (UV).

3. Results and discussion

The main objective of the present research work was to better understand the influence of the different steps of the extraction methodology on composition and characteristics of ulvan. For this, different ulvan extracts were obtained and characterized, taking into consideration their non-carbohydrate and carbohydrate fractions.

3.1. Non-carbohydrate fraction of the extracts

As part of any characterization work on natural origin polysaccharides obtained by extraction process involving different steps, one has to be aware of the presence of different non-carbohydrate components of the polysaccharide, namely inorganic compounds and protein. Table 1 describes the percentages of non-carbohydrate fraction determined for the ulvan samples. The results of elemental analysis are also presented.

A common feature among all samples was the high content of inorganic material quantified as ash (26–43% of dry extract). The ash content clearly decreases with the increase of the number of steps in the extraction procedure: from sample I, with minimal or without purification (43%), to sample III, with two purification steps (26%). It is recurrently reported in the literature the high content of ash in polysaccharides of marine origin, namely for sulfated polysaccharides, including ulvan (Lahaye & Jegou, 1993; Pengzhan, Quanbin, et al., 2003; Quemener et al., 1997). Considering that ulvan is a sulfated polysaccharide, the sulfate content of ashes was quantified and the results shown that more than an half of ashes weight are composed by sulfates. As result, the sulfate-free ash content was found to be in range 10–18% of extract weight; therefore, the high content of inorganics can be attributed to the high content of sulfate in the polysaccharide, as demonstrate the values found directly from the extracts.

The sulfate content of the ulvan extracts (26–32%) is within the values published in the literature (Hernández-Garibay et al., 2011; Quemener et al., 1997). Sample III is that with the highest amount of sulfate groups in both dry material and ashes (and low value of sulfate-free ashes) confirming that the purification steps were effective in elimination of inorganic material other than sulfate; these results substantiate that sulfate groups are linked to the polysaccharide chain since they were not preferentially removed in the purification steps. Hence, the sulfate content found is closely related with the degree of substitution by sulfate in the polysaccharide chain. The remaining inorganic material in this sample is probably coming from counterions of sulfate groups or carboxylic groups (Lahaye, 1991; Ray & Lahaye, 1995). Counterions were identified by Scanning Electron Microscopy-Energy Dispersive Spectroscopy (SEM-EDS) and include sodium, magnesium and potassium (data not shown).

Proteins have already been described as potential contaminants of cell wall polysaccharides, mostly because they are part of the structure of cell walls and closely associated with polysaccharides (Robic, Rondeau-Mouro, Sassi, Lerat, & Lahaye, 2009; Robic, Sassi, & Lahaye, 2008). This is clear by the presented results. In sample I (minimal purification), 2.4% of proteins are co-extracted with ulvan. However, the extract was deproteinased to some extent by enzymatic hydrolysis with proteinase K, as can be seen for samples II and III (about 1% of protein).

Concerning the elemental analysis results shown in Table 1 and, in particular, the elements N and S, the values follow the same pattern of variation between samples as described for protein and sulfate, respectively.

3.2. Molecular weight distribution

Another important issue in polysaccharide characterization is its molecular weight (Mw) distribution. The effect of flow rate (0.5–1.0 mL/min), temperature (25–50 °C) and concentration of suppressor (0.05–0.5 M) in the chromatographic profiles of HPSEC were evaluated to guarantee the best resolution of ulvan chromatograms. The best resolution was found to flow rate 0.7 mL/min. Regarding temperature and $NaNO_3$ concentration, no effect on elution profiles was observed and, therefore, the chosen temperature was 25 °C and the lowest value of concentration in the range was used (50 mM) for HPSEC analysis.

Fig. 2 shows the typical HPSEC profile of the ulvan-rich extracts, represented by sample III. The three samples revealed similar elution profiles on RI with multimodal distribution. It should be emphasized that due to the charge density of ulvan, and eventual branching, the hydrodynamic volume may differ of those of pullulans (used as standards). Therefore, the estimated should be interpreted as comparative data between samples. For the three samples, in the region of high molecular weight (lower retention time) the peaks are wide and not well resolved. This may be due to the above referred tendency of ulvan to undergo aggregation. In general, samples are mostly composed of two broad macromolecular fractions, centered at 775 kDa (rt 8.3 min) and at 150 kDa (rt 9.8 min) and a third intense peak at 1.5 kDa (rt 14 min). This last could be attributed to an ulvan fraction or co-extracted saccharides with low polymerization degree.

The UV chromatograms (Fig. 2, typical chromatogram is shown for sample III) evidenced similar elution profiles, with several

fractions of material absorbing at 280 nm, probably proteins. Of the three samples, samples II and III showed lower relative intensity in UV which is in accordance with the lower protein contents already stated in Table 1. The elution profiles on RI and UV shows that these protein fractions are not co-eluting with polysaccharides suggesting that these components are not chemically bonded. This observation is important if further purification of ulvan is considered, since it contributes to understand the nature of contaminants association in the extracts. Different molecular weight distributions and molecular weights of polysaccharide ulvan have been found by other authors (Lahaye & Robic, 2007; Robic, Gaillard, Sassi, Lerat, & Lahaye, 2009; Robic et al., 2008). The aggregation phenomenon of ulvan polysaccharide as polyelectrolyte is the most probable reason for the large variation of Mw obtained by the different techniques in the literature.

3.3. Analysis of the carbohydrate composition of the ulvan extracts

The characterization of the polysaccharides in the extracts based on the identification and quantification of composing monosaccharides assumes a crucial role in the study of the effect of different steps in the extraction procedure. Monosaccharide in each sample was determined by (1) enzymatic hydrolysis following two approaches and (2) acid methanolysis with GC-FID and GC–MS analysis.

3.3.1. Enzymatic essays

The enzymatic hydrolysis approach using EM was performed to provide a wide range hydrolytic activity, since it is expected that other cell-wall polysaccharides could be co-extracted with ulvan. β-Glucuronidase approach over the TFA hydrolysates (chemical-enzymatic method) was also performed, in this case, aiming to improve the hydrolysis of aldo-glucuronosyl linkages (Lahaye & Robic, 2007; Quemener et al., 1997; Willför et al., 2009). The results are presented in Table 2.

In both approaches, the glucuronic acid and rhamnose are the main residues in the extracts. GlcA liberated by chemical-enzymatic method accounts for about 45% of the total of polysaccharides, almost the double of the value obtained by EM (see also % mol in Table 2). Confirming the effectiveness of chemical-enzymatic method toward aldo-glucuronosyl linkages in comparison to EM, the content of glucuronic acid found (12–19 wt%) is within the range reported in the literature (6–19%) (Lahaye & Robic, 2007; Quemener et al., 1997), while for EM the values for GlcA is considerable lower (5–9 wt%). In accordance, the disaccharide Rha-GlcA was not detected after the treatment with β-glucuronidase, confirming its effective activity toward glycosidic linkage of aldobiuronic acids that remain after TFA treatment. Rha content found by the two methods is similar (7–13 wt%) but considerable lower than the range of values of the literature (17–45%) (Lahaye & Robic, 2007).

The other residues are iduronic acid, xylose and glucose. Iduronic acid, a typical residue found in ulvan from *U. lactuca* (in ulvanobiuronic acid type B) accounts for 2–3%, close to values found in the literature for *U. lactuca* (Lahaye & Robic, 2007; Quemener et al., 1997). Quemener et al. (1997) have detected iduronic acid for the first time in algae polysaccharides based on hydrolysis improvement using the TFA hydrolysis and β-glucuronidase (chemical-enzymatic method), referring to it as the most favorable for the quantitative release of iduronic acid. In accordance, moderately higher content for iduronic acid was found for samples II and III by chemical-enzymatic method. Xylose is referred to as part of ulvan structure in ulvanobioses moieties and glucose is frequently associated with glucans or amorphous cellulose co-extracted with ulvan. As general trend, one point out that proportion of glucose and xylose obtained are higher for EM approach.

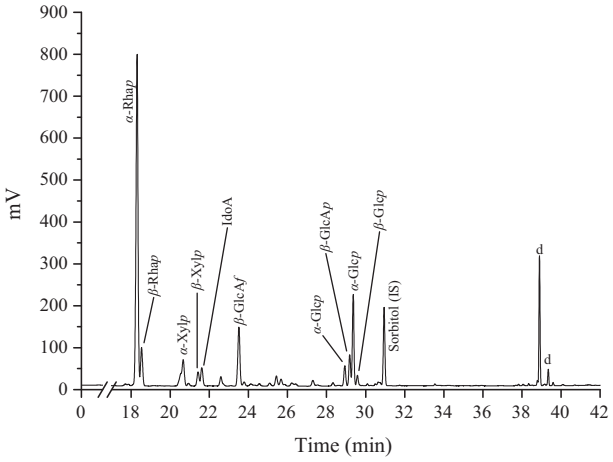

Fig. 3. Gas chromatogram (GC-FID) of TMS methyl glycosides of monosaccharides obtained by acid methanolysis of sample II. Conditions: 2 M HCl/MeOH, 4 h of reaction, 100 °C (α-Xyl*p*: α-xylopyranoside; β-Xyl*p*: β-xylopyranoside; α-Rha*p*: α-rhamnopyranoside; β-Rha*p*: β-rhamnopyranoside; α-Glc*p*: α-glucopyranoside; β-Glc*p*: β-glucopyranoside; α-GlcA*p*: α-glucuronic acid pyranoside; β-GlcA*p*: β-glucuronic acid pyranoside; β-GlcA*f*: β-glucuronic acid furanoside (6,3 lactone); IdoA: iduronic acid (lactone form); d: disaccharides; Sorbitol (IS – internal standard)).

The sum of the sulfate-free ashes, sulfate and protein contents of the three sample fails to complete the compositional analysis: for the chemical-enzymatic approach, the overall sum of the components is 75%, 72% and 82% for samples I, II, and III, respectively; EM hydrolysis gave overall sums of 78%, 64% and 78% for samples I, II and III, respectively. In spite of the observed improvement by the chemical-enzymatic method, these results prompt us to test different analysis approach to overcome the hydrolysis resistance demonstrated by enzymatic hydrolysis used in this work.

3.3.2. Conventional acid methanolysis 4 h, 2 M: results and identified limitations

Methanolysis of ulvan-rich extracts was carried out for 4 h at 100 °C, with 2 M HCl/MeOH, the conditions referred to as the best compromise between efficient cleavage of glucuronosyl linkages and low degradation of the monomers released (Bertaud et al., 2002; Rabemanolontsoa, Ayada, & Saka, 2011), although Quemener et al. (1997) have pointed out that this method were not efficient to quantitatively release uronic acids. Fig. 3 depicts a typical chromatogram of TMS methyl glycosides obtained by acid methanolysis. The quantification results are presented in Table 3. The total content of monosaccharides obtained by acid methanolysis (2 M, 4 h) is in the range 28–55%, which is a considerable high value comparatively to that found by other authors using the same conditions (19%) (Quemener et al., 1997). This could be attributed to differences of the extract composition rather than quantification issues. Notice that between samples I, II and III there are also significant differences, suggesting that the samples has different susceptibility to methanolysis conditions. GlcA and Rha are the main residues in the extracts, accounting for 70–85% of total weight of polysaccharide, in accordance with the results obtained by EM and chemical-enzymatic method.

Comparatively to the other two, sample III shows significantly higher values for both Rha and GlcA (about 22% of extract dry weight). Considering the results of Table 1 (total content of non-carbohydrate components – sulfate-free ashes, sulfate and proteins – 44–50%) and the total content of polysaccharide depicted in Table 3 (28–55%), the total quantified weighs are 77%, 73% and

Table 2
Composition of the polysaccharide fraction in the ulvan-rich extracts accessed by chemical-enzymatic method and enzymatic hydrolysis using EM.

	Sample I		Sample II		Sample III	
	% w/w	% mol	% w/w	% mol	% w/w	% mol
Chemical-enzymatic method						
Rha	7.8 ± 0.4	33	9.1 ± 0.4	35	11.9 ± 1.1	34
GlcA	12.4 ± 0.3	45	13.7 ± 1.0	45	19.3 ± 0.6	46
IdoA	2.1 ± 0.1	8	2.1 ± 0.1	7	3.2 ± 0.2	8
Glc	1.8 ± 0.0	7	1.6 ± 0.1	6	1.9 ± 0.1	5
Xyl	1.6 ± 0.1	7	1.6 ± 0.0	7	2.3 ± 0.1	7
Disaccharides	–	–	–	–	–	–
Total	25.6 ± 0.2	100	28.0 ± 1.4	100	38.5 ± 2.1	100
Hydrolysis using EM						
Rha	11.0 ± 0.6	42	7.3 ± 0.3	43	13.4 ± 0.8	45
GlcA	7.6 ± 0.6	25	4.6 ± 0.4	23	9.2 ± 0.7	26
IdoA	2.1 ± 0.2	7	1.5 ± 0.1	8	2.3 ± 0.0	7
Glc	2.6 ± 0.1	9	1.0 ± 0.1	5	1.5 ± 0.0	5
Xyl	2.3 ± 0.1	10	1.6 ± 0.1	10	2.7 ± 0.1	10
Disaccharides	4.3 ± 0.6	7	4.2 ± 0.2	11	5.2 ± 0.1	7
Total	29.9 ± 0.6	100	20.2 ± 1.1	100	34.3 ± 1.5	100

% w/w of monosaccharide calculated as regular anhydropolymers.
Data are presented as mean ± standard deviations (SD) of triplicate essays.

98% for samples I, II and III, respectively. This verification revealed an underestimation of the polysaccharide content of ulvan-rich extracts (except for sample III) which could be attributed to the chemical stability of glucuronosyl linkages (Biermann, 1988; Conrad, 1980; McKinnell & Percival, 1962; Willför et al., 2009). As an example (1 → 4)-β-D-glucuronan have been reported as very resistant toward hydrolysis with sulfuric acid (De Ruiter et al., 1992). The underestimation of the quantification also can be also related with other non-detected oligosaccharides present in the mixture. This underestimation was already stated for the enzymatic methodologies referred in Section 3.3.1.

3.3.3. Kinetics of methanolysis and effect of HCl concentration: method improvement

A study concerning the release of residues along reaction time (3–72 h), with two HCL concentrations (2 M and 3 M) was developed aiming to achieve maximum values for monosaccharide content. According to Chambers and Clamp (1971), methanolic 4 M and 6 M HCl lead to the considerable destruction of the monosaccharides units released and therefore higher acid concentrations were not attempted. The variation of monosaccharides percentages released from ulvan-rich extracts with reaction time and acid concentration are shown Fig. 4A and B for sample I, C and D for sample II and E and F for sample III.

The results show that the lower content of total released monosaccharides was achieved after 4 h of reaction time for sample I and sample II due to the loss of GlcA. This behavior can be related to degradation of GlcA during the initial reaction time due to its particular instability in acid. Some authors claims that HCl is mostly consumed in the first hours providing the advantage of a reasonable stability of released methyl glycosides, up to 24 h (Bertaud et al., 2002; Sundberg et al., 1996). Other authors (Quemener & Lahaye, 1998) have proven that the maximum yields for studied sugars were obtained when methanolysis was performed with pH values of 6–7, which is in accordance with the high stability of released sugars when the acidity of the solution drops rather quickly (Willför et al., 2009). All these assumptions could explain the release of more labile monosaccharides until 3 h of reaction and their degradation

Table 3
Monosaccharides composition of ulvan-rich extracts released after acid methanolysis, as % w/w dry material and % mol.

	Sample I		Sample II		Sample III	
	% w/w	% mol	% w/w	% mol	% w/w	% mol
Acid methanolysis using 2 M HCl/MeOH, 4 h, 100 °C						
Rha	8.6 ± 0.1	35	9.2 ± 0.3	36	22.4 ± 0.9	45
GlcA	10.6 ± 1.0	36	12.1 ± 1.3	40	22.5 ± 1.1	38
IdoA	2.6 ± 0.1	10	2.5 ± 0.6	8	3.1 ± 0.2	5
Glc	1.8 ± 0.0	7	1.4 ± 0.1	5	1.0 ± 0.0	2
Xyl	1.8 ± 0.1	8	1.9 ± 0.1	8	3.7 ± 0.1	8
Disaccharides	2.2 ± 0.5	4	1.5 ± 0.1	3	1.9 ± 0.0	2
Total	27.6 ± 1.7	100	28.6 ± 1.4	100	54.6 ± 2.3	100
Acid methanolysis using 3 M HCl/MeOH, 48 h, 100 °C						
Rha	11.1 ± 0.3	32	16.1 ± 0.7	40	13.5 ± 1.1	45
GlcA	21.7 ± 0.1	52	20.4 ± 0.8	43	24.2 ± 1.7	38
IdoA	2.6 ± 0.2	6	3.0 ± 0.1	6	2.6 ± 0.1	5
Glc	1.7 ± 0.0	4	1.6 ± 0.0	4	0.9 ± 0.0	2
Xyl	2.1 ± 0.1	6	2.6 ± 0.1	7	1.6 ± 0.1	8
Disaccharides	–	–	–	–	–	2
Total	39.3 ± 0.6	100	43.7 ± 0.1	100	42.8 ± 2.9	100

% w/w of monosaccharide calculated as regular anhydropolymers.
Data are presented as mean ± standard deviations (SD) of triplicate essays.

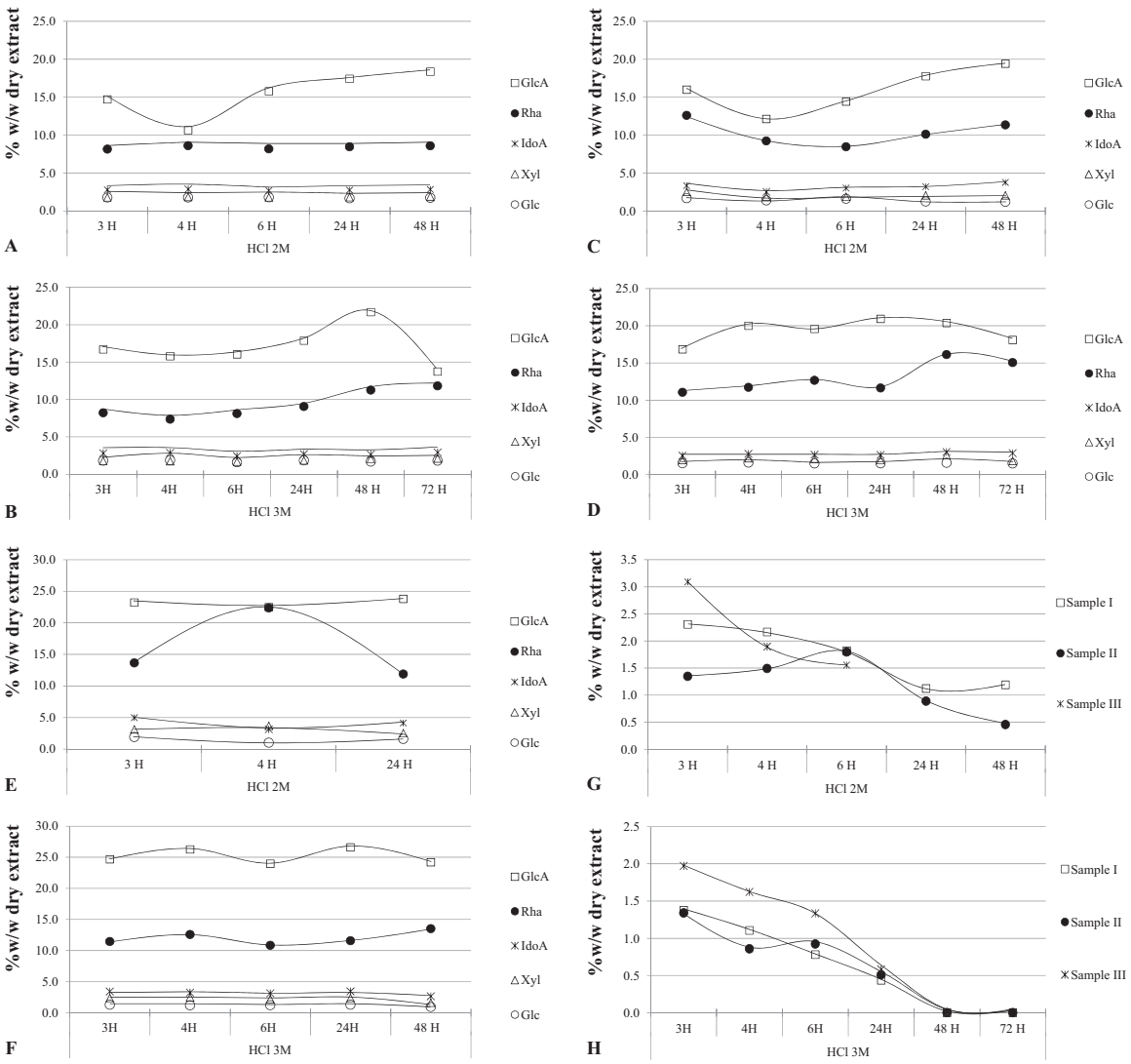

Fig. 4. Quantification of monosaccharides released by acid methanolysis for different reaction times and HCl concentrations in sample I (A, B), sample II (C, D) and sample III (E, F), and disaccharides content in the three samples (G, H). A, C, E, G refer to HCl 2 M and B, D, F, H refer to HCl 3 M.

after this time. After 6 h, the cleavage proceeds and more resistant/protected linkages are cleaved releasing the monosaccharides of the ulvan structure and increasing the quantified value. However, the destroyed monosaccharides are no longer recovered and does not account for the total. Hence, the total content is always underestimates. The increase of Rha and GlcA percentage is accompanied by a decrease of disaccharides (GlcA-Rha) content, as demonstrate Fig. 4G and H. This behavior is in accordance with the well-known aldo-glucuronosyl linkage resistance assigned to ulvanobiuronic acid, D-GlcA-(1,4)-L-Rha (Quemener et al., 1997).

Higher values were achieved, for samples I and II, using more severe conditions: 48 h at 100 °C with 3 M HCl/MeOH (Table 3). In these conditions, the GlcA percentage was remarkably high; Rha also increase while for IdoA, Glc and Xyl no changes were noticeable. These conditions represented the best compromise to achieve the maximum quantity of released monosaccharides. After that time, a decreased of the amount of monosaccharides was observed.

For sample III the highest yield on total monosaccharides, in particularly rhamnose, was achieved for 4 h, with 2 M HCl/MeOH. For 3 h of reaction, the cleavage of linkages involving Rha and GlcA was greater than for the other samples; this probably led to higher acid consumption in the first hours (protecting the liberated monosaccharides from further degradation), however without affecting the subsequent development of methanolysis since high yields were achieved. This behavior could be attributed to the high content of Rha in this sample (22% released in 4 h) comparatively to the others samples, 8.6% for sample I and 9.2% for sample II using the same conditions. The decrease in total sugar content in sample III for more than 4 h of reaction time is almost exclusively due to it degradation as depicted in Fig. 4E and F. In accordance, rhamnose is referred to as a labile monosaccharide (Choy & Dutton, 1973).

In the optimized methanolysis conditions for each sample, the percentage of saccharide fraction found was between 39% and 55%. Rha and GlcA are main monosaccharides, but minor amounts of glucose, iduronic acid and xylose were also detected in the range of 1.0–3.7%. Sample III revealed the lowest amount of glucose, which is in accordance with the enzymatic treatment applied as an additional step in the extraction procedure to remove starch as a contaminant. Considering the sum of all the identified components of ulvan-rich extracts (carbohydrate fraction, ashes, protein and sulfates) a total content close to 90% was obtained for samples I and II while for sample III this value reached to 98%. These results confirmed acid methanolysis as highly effective for ulvan, leading to very good total yields of carbohydrates; but, more important, this study reveals also that the conditions should be studied for each polysaccharide extract.

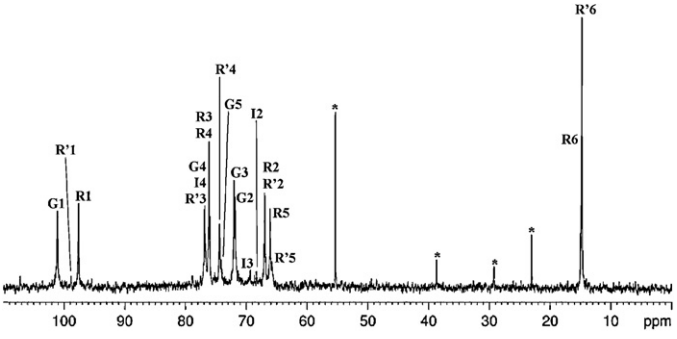

Fig. 5. Typical ^{13}C NMR spectrum of ulvan-rich extracts (presented for sample III) dissolved in D_2O, acquired at 70 °C for a minimum of 48 h at 100.62 MHz. G and I correspond to the carbon signals of the glucuronic acid and iduronic acid, respectively. R and R′ refers to rhamnose linked to glucuronic acid and iduronic acid, respectively. Number refers to the carbon number in the structure of each residue. Peaks noted by * are unattributed signals, most probably corresponding to carbon signals of the protein (contaminant).

The total content of carbohydrate obtained from the two different approaches of enzymatic hydrolysis was lower than the obtained after acid methanolysis (at optimized conditions for each ulvan-rich extracts). The hydrolytic activity is limited by the type of enzymes in each approach and, consequently, the acid methanolysis had led to a broader action toward the polysaccharides.

3.3.4. ^{13}C NMR spectroscopy

The typical ^{13}C NMR spectrum of the ulvan-rich extracts is depicted in Fig. 5 for sample III. The spectra of the three samples do not present significant differences, confirming that the overall composition of ulvan structure was close whatever the extraction procedure. The chemical shifts were attributed based on references reporting assignments for ulvan and oligosaccharides (Lahaye et al., 1997, 1998, 1999; Lahaye & Ray, 1996; Lahaye, Ray, Baumberger, Quemener, & Axelos, 1996; Lahaye & Robic, 2007).

The signals assignments corresponding to carbons of rhamnose (**R1** – 97.6 ppm, **R2** – 67.0 ppm, **R3**, **R4** – 76.1 ppm, **R5** – 66.1 ppm, **R6** – 14.9 ppm) and glucuronic acid (**G1** – 101.1 ppm, **G2** – 71.8 ppm, **G3** – 72.0 ppm, **G4** – 76.8 ppm, **G5** – 74.1 ppm) that constitute ulvanobiuronic acid 3-sulfate – type A were identified, as depicted in Fig. 5. The chemical shifts attributed to iduronic acid (**I2** – 68.3 ppm, **I3** – 69.3 ppm and **I4** – 76.8 ppm) and rhamnose 3-sulfate linked to iduronic acid (**R′1** – 98.8 ppm, **R′2** – 67.0 ppm, **R′3** – 76.8 ppm, **R′4** – 74.6 ppm, **R′5** – 65.8 ppm, **R′6** – 14.8 ppm) in ulvanobiuronic acid 3-sulfate – type B, were also detected allowing to confirm the presence of this sequence. The C-6 of GlcA (and IdoA) was assigned at 173.0 ppm (not shown in the spectra).

In previous studies, some authors performed spectroscopic characterization of ulvan using ^{13}C NMR analysis and noticed the presence of carbon signals representing contiguous 1,4-linked β-D-glucuronic acid units in polysaccharide structure and/or a separate 1,4-linked β-D-glucuronan (seaweed glucuronans) co-extracted with ulvan, and occurring as a contaminant (Lahaye et al., 1998, 1999; Lahaye & Robic, 2007). However, the typical chemical shifts of carbon in these structures were not detected in any of the samples.

3.3.5. Effect of the extraction steps in the composition of the extracts

The uncertainty related with the identification of the residues belonging to the ulvan and those from to co-extracted polysaccharides has been frequently referred in the literature (Elboutachfaiti et al., 2009) representing a challenge in the evaluation of the real content of ulvan in the extracts. Overcome this limitation is essential regarding application studies where the starting material should be carefully controlled. For this, our proposal is to establish a criterion for ulvan content in the algae extracts (obtained by variants of the same methodologies of extraction) based on the molar percentage of the major repeating units: the ulvanobiuronic acids and ulvanobioses. Other basic repeating sequence referenced, →4)-β-D-Glc*p*A-(1 → 4)-α-L-Rha*p*-(1 → 4)-β-D-Xyl*p* (Lahaye & Ray, 1996) was also considered in this approach. Gathered together the data of acid methanolysis at the optimized conditions, repeating sequences were established as follow resulting the empirical formulas that represent distribution of residues and sulfate groups in each extract:

Sample I – $[GlcA\text{-}Rha]_{20}\ [IdoA\text{-}Rha]_6\ [GlcA\text{-}Rha\text{-}Xyl]_6\ GlcA_{26}\ Glc_4\ [SO_3^-]_{30}$
Sample II – $[GlcA\text{-}Rha]_{27}\ [IdoA\text{-}Rha]_6\ [GlcA\text{-}Rha\text{-}Xyl]_7\ GlcA_9\ Glc_4\ [SO_3^-]_{26}$
Sample III – $[GlcA\text{-}Rha]_{25}\ [IdoA\text{-}Rha]_6\ [GlcA\text{-}Rha\text{-}Xyl]_4\ GlcA_{24}\ Glc_2\ [SO_3^-]_{32}$

Considering the total sulfate groups in each ulvan-rich extract, one can suggest that sulfated sequences represented more than 2/3 of the total that constitutes the polysaccharide chain. Glucose can be associated with co-eluting contaminants, the glucans. Sample III showed the lowest proportion of glucose in accordance with the treatment preformed in the extraction procedure. Considering that no signal in ^{13}C NMR spectra were assigned to 1,4-linked β-D-glucuronan one can suggest that the additional GlcA reported in the empirical formulas could be present linked to the chain, in accordance with already reported branching at *O*-2 of rhamnose by glucuronic acid unit (Lahaye & Ray, 1996).

Sample III had the highest content of total sugar and sulfate groups, with the lowest amounts of proteins (1.3%) and inorganic material (10%). This sample is composed by rhamnose (22%), glucuronic acid (23%), xylose (3.7%), iduronic acid (3.1%) and glucose (1.0%) and it is highly sulfated (32%). These findings are in accordance with previous reported features for ulvan of this alga species (Quemener et al., 1997; Siddhanta, Goswami, Ramavat, Mody, & Mairh, 2001). However, this particular ulvan is rich in glucuronic acid and the ratio Rha/GlcA is approximately 1 for the three samples. This peculiarity may be an influence of different factors, such as seasonality or extraction methodology (Hernández-Garibay et al., 2011; Lahaye et al., 1999; Robic et al., 2008; Siddhanta et al., 2001; Wong & Cheung, 2000). Ulvan obtained through the developed extraction procedure revealed a high molecular weight, with apparent Mw in the range 770–790 kDa. Concerning the SEC profiles, sample III showed superior intensity of high molecular weight fractions and lower contribution of protein, in accordance with chemical characterization results.

4. Conclusion

The chemical composition and structural characteristics of ulvan-rich extracts were assessed by complementary methodologies. Sugar analysis by acid methanolysis was improved and proved to be the best mean for quantifying sugar content in ulvan extracts. This is an important tool to assess the effect of the different steps in the extraction procedures.

The three modes of extraction and purification of ulvan proved to impact the content of contaminants and the sugar analysis of the extracts without affecting the overall chemical structure.

The extraction allowed obtaining a final extract with 10% of inorganic compounds, and the enzymatic treatment steps were effective on the reduction of both protein and glucose, to a maximum of 2% on the final extracts. There were no evidences for the presence of glucuronans in the extracts and, therefore, all the carbohydrates should be related with the polysaccharide ulvan. In

this sense, it is possible to extract a purified polysaccharide from green algae employing a simple methodology. The ulvan obtained is a high molecular weight and highly sulfated polysaccharide, rich in rhamnose and glucuronic acid (ulvanobiuronic acid A), with minor amounts of iduronic acid, and xylose (ulvanobiuronic acid B). Considering the overall results, the final assessment of ulvan percentage in the sample III was 88%.

Acknowledgments

This work was supported by the project IBEROMARE ('Centro Multipolar de Valorização de Recursos e ResíduosMarinhos'), approved by Operational Programme for Cross-border Cooperation: Spain – Portugal, 2007–2013 (POCTEP), with funding contribution through the European Regional Development Fund (ERDF co-funding) and POCTEP.

Anabela Alves is grateful for financial support from Fundaçãopara a Ciência e Tecnologia (FCT) through the SFRH/BD/39359/2007 grant.

The authors thank Novozymes for kindly providing the enzymatic mixture. Authors are also grateful to Drª. Mariana Andrade and Materials Center of the University of Porto (CEMUP) for the acquisition of ^{13}C NMR spectra (NMR spectrometer is part of the National NMR Network and was purchased in the framework of the National Program for Scientific Re-equipment, contract REDE/1517/RMN/2005, with funds from POCI 2010 (FEDER) and FCT).

References

Alves, A., Caridade, S. G., Mano, J. F., Sousa, R. A., & Reis, R. L. (2010). Extraction and physico-chemical characterization of a versatile biodegradable polysaccharide obtained from green algae. *Carbohydrate Research*, *345*, 2194–2200.

Bertaud, F., Sundberg, A., & Holmbom, B. (2002). Evaluation of acid methanolysis for analysis of wood hemicelluloses and pectins. *Carbohydrate Polymers*, *48*, 319–324.

Biermann, C. J. (1988). Hydrolysis and other cleavages of glycosidic linkages in polysaccharides. In R. S. Tipson, & H. Derek (Eds.), *Advances in carbohydrate chemistry and biochemistry* (pp. 251–271). San Diego, CA: Academic Press.

Bleton, J., Mejanelle, P., Sansoulet, J., Goursaud, S., & Tchapla, A. (1996). Characterization of neutral sugars and uronic acids after methanolysis and trimethylsilylation for recognition of plant gums. *Journal of Chromatography A*, *720*, 27–49.

Cannell, R. J. P., Dufresne, C., Florence, A. J., Gailliot, F. P., Gibbons, S., Gray, A. I., et al. (1998). Natural products isolation. In R. J. P. Cannell (Ed.), *Methods in biotechnology* (pp. 343–408). Totowa, NJ: Humana Press.

Castro, R., Piazzon, M. C., Zarra, I., Leiro, J., Noya, M., & Lamas, J. (2006). Stimulation of turbot phagocytes by *Ulva rigida* C. Agardh polysaccharides. *Aquaculture*, *254*, 9–20.

Castro, R., Zarra, I., & Lamas, J. (2004). Water-soluble seaweed extracts modulate the respiratory burst activity of turbot phagocytes. *Aquaculture*, *229*, 67–78.

Chambers, R. E., & Clamp, J. R. (1971). An assessment of methanolysis and other factors used in the analysis of carbohydrate-containing materials. *Biochemical Journal*, *125*, 1009–1018.

Choy, Y.-M., & Dutton, G. G. S. (1973). Structure of the capsular polysaccharide of *Klebsiella* K-type 56. *Canadian Journal of Chemistry*, *51*, 3021–3026.

Cluzet, S., Torregrosa, C., Jacquet, C., Lafitte, C., Fournier, J., Mercier, L., et al. (2004). Gene expression profiling and protection of Medicago truncatula against a fungal infection in response to an elicitor from green algae *Ulva* spp. *Plant, Cell & Environment*, *27*, 917–928.

Conrad, H. E. (1980). The acid lability of the glycosidic bonds of L-iduronic acid residues in glycosaminoglycan. *Biochemical Journal*, *191*, 355–363.

Dahlman, O., Jacobs, A., Liljenberg, A., & Olsson, A. I. (2000). Analysis of carbohydrates in wood and pulps employing enzymatic hydrolysis and subsequent capillary zone electrophoresis. *Journal of Chromatography A*, *891*, 157–174.

De Ruiter, G. A., Schols, H. A., Voragen, A. G. J., & Rombouts, F. M. (1992). Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Analytical Biochemistry*, *207*, 176–185.

Doco, T., O'Neill, M. A., & Pellerin, P. (2001). Determination of the neutral and acidic glycosyl-residue compositions of plant polysaccharides by GC–EI-MS analysis of the trimethylsilyl methyl glycoside derivatives. *Carbohydrate Polymers*, *46*, 249–259.

Elboutachfaiti, R., Delattre, C., Petit, E., El Gadda, M., Courtois, B., Michaud, P., et al. (2009). Improved isolation of glucuronan from algae and the production of glucuronic acid oligosaccharides using a glucuronanlyase. *Carbohydrate Research*, *344*, 1670–1675.

Harada, N., & Maeda, M. (1998). Chemical structure of antithrombin-active rhamnan sulfate from *Monostrom nitidum*. *Bioscience Biotechnology and Biochemistry*, *62*, 1647–1652.

Hernández-Garibay, E., Zertuche-González, J., & Pacheco-Ruíz, I. (2011). Isolation and chemical characterization of algal polysaccharides from the green seaweed *Ulva clathrata* (Roth) C. Agardh. *Journal of Applied Phycology*, *23*, 537–542.

Kaeffer, B., Benard, C., Lahaye, M., Blottiere, H. M., & Cherbut, C. (1999). Biological properties of ulvan, a new source of green seaweed sulfated polysaccharides, on cultured normal and cancerous colonic epithelial tells. *Planta Medica*, *65*, 527–531.

Kamerling, J. P., & Gerwig, G. J. (2007). Strategies for the structural analysis of carbohydrates. In P. K. Johannis (Ed.), *Comprehensive glycoscience* (pp. 1–68). Oxford: Elsevier.

Lahaye, M. (1991). Marine algae as sources of fibres: Determination of soluble and insoluble dietary fibre contents in some sea vegetables. *Journal of the Science of Food and Agriculture*, *54*, 587–594.

Lahaye, M. (1998). NMR spectroscopic characterisation of oligosaccharides from two *Ulva rigida* ulvan samples (Ulvales, Chlorophyta) degraded by a lyase. *Carbohydrate Research*, *314*, 1–12.

Lahaye, M., Alvarez-Cabal Cimadevilla, E., Kuhlenkamp, R., Quemener, B., Lognoné, V., & Dion, P. (1999). Chemical composition and ^{13}C NMR spectroscopic characterisation of ulvans from *Ulva* (Ulvales, Chlorophyta). *Journal of Applied Phycology*, *11*, 1–7.

Lahaye, M., Brunel, M., & Bonnin, E. (1997). Fine chemical structure analysis of oligosaccharides produced by an ulvan-lyase degradation of the water-soluble cell-wall polysaccharides from *Ulva* sp. (Ulvales, Chlorophyta). *Carbohydrate Research*, *304*, 325–333.

Lahaye, M., Inizan, F., & Vigouroux, J. (1998). NMR analysis of the chemical structure of ulvan and of ulvan–boron complex formation. *Carbohydrate Polymers*, *36*, 239–249.

Lahaye, M., & Jegou, D. (1993). Chemical and physical–chemical characteristics of dietary fibres from *Ulva lactuca* (L.) Thuret and *Enteromorpha compressa* (L.) Grev. *Journal of Applied Phycology*, *5*, 195–200.

Lahaye, M., Jegou, D., & Buleon, A. (1994). Chemical characteristics of insoluble glucans from the cell wall of the marine green alga *Ulva lactuca* (L.) Thuret. *Carbohydrate Research*, *262*, 115–125.

Lahaye, M., & Ray, B. (1996). Cell-wall polysaccharides from the marine green alga *Ulva rigida* (Ulvales, Chlorophyta) – NMR analysis of ulvan oligosaccharides. *Carbohydrate Research*, *283*, 161–173.

Lahaye, M., Ray, B., Baumberger, S., Quemener, B., & Axelos, M. A. V. (1996). Chemical characterisation and gelling properties of cell wall polysaccharides from species of *Ulva* (Ulvales, Chlorophyta). *Hydrobiologia*, *326–327*, 473–480.

Lahaye, M., & Robic, A. (2007). Structure and functional properties of ulvan, a polysaccharide from green seaweeds. *Biomacromolecules*, *8*, 1765–1774.

Mano, J. F., Silva, G. A., Azevedo, H. S., Malafaya, P. B., Sousa, R. A., Silva, S. S., et al. (2007). Natural origin biodegradable systems in tissue engineering and regenerative medicine: Present status and some moving trends. *Journal of the Royal Society Interface*, *4*, 999–1030.

Mao, W., Zang, X., Li, Y., & Zhang, H. (2006). Sulfated polysaccharides from marine green algae *Ulva conglobata* and their anticoagulant activity. *Journal of Applied Phycology*, *18*, 9–14.

McKinnell, J. P., & Percival, E. (1962). Structural investigations on the water-soluble polysaccharide of the green seaweed *Enteromorpha compressa*. *Journal of the Chemical Society (Resumed)*, 3141–3148.

Pengzhan, Y., Ning, L., Xiguang, L., Gefei, Z., Quanbin, Z., & Pengcheng, L. (2003). Anti-hyperlipidemic effects of different molecular weight sulfated polysaccharides from *Ulva pertusa* (Chlorophyta). *Pharmacological Research*, *48*, 543–549.

Pengzhan, Y., Quanbin, Z., Ning, L., Zuhong, X., Yanmei, W., & Zhi'en, L. (2003). Polysaccharides from *Ulva pertusa* (Chlorophyta) and preliminary studies on their antihyperlipidemia activity. *Journal of Applied Phycology*, *15*, 21–27.

Qi, H., Zhang, Q., Zhao, T., Chen, R., Zhang, H., Niu, X., et al. (2005). Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) in vitro. *International Journal of Biological Macromolecules*, *37*, 195–199.

Qi, H., Zhao, T., Zhang, Q., Li, Z., Zhao, Z., & Xing, R. (2005). Antioxidant activity of different molecular weight sulfated polysaccharides from *Ulva pertusa* Kjellm (Chlorophyta). *Journal of Applied Phycology*, *17*, 527–534.

Quemener, B., & Lahaye, M. (1998). Comparative analysis of sulfated galactans from red algae by reductive hydrolysis and mild methanolysis coupled to two different HPLC techniques. *Journal of Applied Phycology*, *10*, 75–81.

Quemener, B., Lahaye, M., & Bobin-Dubigeon, C. (1997). Sugar determination in ulvans by a chemical-enzymatic method coupled to high performance anion exchange chromatography. *Journal of Applied Phycology*, *9*, 179–188.

Quemener, B., Lahaye, M., & Metro, F. (1998). Assessment of methanolysis for the determination of composite sugars of gelling carrageenans and agarose by HPLC. *Carbohydrate Research*, *266*, 53–64.

Rabemanolontsoa, H., Ayada, S., & Saka, S. (2011). Evaluation of different methods to determine monosaccharides in biomass. In T. Yao (Ed.), *Zero-carbon energy Kyoto 2010* (pp. 123–128). Tokyo: Springer.

Ray, B., & Lahaye, M. (1995). Cell-wall polysaccharides from the marine green alga *Ulva 'rigida'* (Ulvales, Chlorophyta). Chemical structure of ulvan. *Carbohydrate Research*, *274*, 313–318.

Robic, A., Gaillard, C., Sassi, J. F., Lerat, Y., & Lahaye, M. (2009). Ultrastructure of ulvan: A polysaccharide from green seaweeds. *Biopolymers*, *91*, 652–664.

Robic, A., Rondeau-Mouro, C., Sassi, J. F., Lerat, Y., & Lahaye, M. (2009). Structure and interactions of ulvan in the cell wall of the marine green algae *Ulva rotundata* (Ulvales, Chlorophyceae). *Carbohydrate Polymers*, *77*, 206–216.

Robic, A., Sassi, J. F., & Lahaye, M. (2008). Impact of stabilization treatments of the green seaweed *Ulva rotundata* (Chlorophyta) on the extraction yield, the physico-chemical and rheological properties of ulvan. *Carbohydrate Polymers*, *74*, 344–352.

Redinbaugh, M. G., & Turley, R. B. (1986). Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. *Analytical Biochemistry*, *153*, 267–271.

Saeman, J. F. (1945). Kinetics of wood saccharification – Hydrolysis of cellulose and decomposition of sugars in dilute acid at high temperature. *Industrial & Engineering Chemistry*, *37*, 43–52.

Siddhanta, A. K., Goswami, A. M., Ramavat, B. K., Mody, K. H., & Mairh, O. P. (2001). Water soluble polysaccharides of marine algal species of *Ulva* (Ulvales, Chlorophyta) of Indian waters. *Indian Journal of Marine Sciences*, *30*, 166–172.

Sundberg, A., Sundberg, K., Lillandt, C., & Holmbom, B. (1996). Determination of hemicelluloses and pectins in wood and pulp fibres by acid methanolysis and gas chromatography. *Nordic Pulp and Paper Research Journal*, *11*, 216–219.

Warrand, J. (2006). Healthy polysaccharides – The next chapter in food products. *Food Technology and Biotechnology*, *44*, 355–370.

Willför, S., Pranovich, A., Tamminen, T., Puls, J., Laine, C., Suurnäkki, A., et al. (2009). Carbohydrate analysis of plant materials with uronic acid-containing polysaccharides – A comparison between different hydrolysis and subsequent chromatographic analytical techniques. *Industrial Crops and Products*, *29*, 571–580.

Wong, K. H., & Cheung, P. C. K. (2000). Nutritional evaluation of some subtropical red and green seaweeds. Part I. Proximate composition, amino acid profiles and some physico-chemical properties. *Food Chemistry*, *71*, 475–482.