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Impact of stabilization treatments of the green seaweed *Ulva rotundata* (Chlorophyta) on the extraction yield, the physico-chemical and rheological properties of ulvan

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ABSTRACT

The impact of freezing, freeze-drying, hot-air drying, brining and dry salting stabilization methods of *Ulva rotundata* has been studied on the yield, physico-chemical characteristics and rheological properties of ulvan. Frozen (−30 and −80 °C) and freeze-dried algae yielded ulvan extracts mainly composed of high molecular weight polysaccharides. These had the highest intrinsic viscosity and conservation modulus (G') as copper/borate gels compared to ulvan from fresh algae (taken as a reference) or ulvan extracted from algae stabilized by other means. Cold stabilizations of algae yielded the lowest amounts of ulvan. High ulvan yields were obtained from *Ulva* air-dried at 50 and 70 °C but that at 50 °C was lower than that at 70 °C. Gelling properties and intrinsic viscosities from these ulvans were better than that of fresh algae but lower than from cold treated seaweeds. Brining induced ulvan degradation with poor rheological properties of the extracts compared to ulvan from fresh algae. However, this process appears to ease extraction of polysaccharides. Compared to fresh algae, salted seaweeds stored at room temperature yielded degraded ulvan. Decreasing the salting process temperature to 4 °C preserved ulvan composition, structure and properties. However, *Ulva* salting hinders drastically ulvan extraction. The different stabilization processes affected the chemical composition of ulvan extracts and in particular, sulphate and protein contents. The results show that different stabilization conditions of *Ulva* biomass can be selected according to their cost-effectiveness and the required ulvan chemical and macromolecular characteristics.

1. Introduction

Green algae belonging to Ulvales (*Ulva* and *Enteromorpha* sp.) are distributed worldwide and are very common in coastal areas. These seaweeds are frequently involved in algal proliferation in eutrophicated coastal and lagoon waters in the form of "green tides" (Fletcher, 1996). Up to now, this biomass has very low added-value and ways to use it besides compost (Cuomo, Perretti, Palomba, Verde, & Cuomo, 1995; Mazé, Morand, & Potoky, 1993), methane production (Briand & Morand, 1997) could be taking advantage of specific properties of their cell wall polysaccharides. The latter are essentially represented by ulvan, which is a complex sulphated polysaccharide drawing increasing interest as a potential source of new functional biopolymer (Lahaye & Robic, 2007).

Ulvan represents about 8–29% of the algae dry weight. It is composed of different repeating chemical sequences mostly based on disaccharides made of rhamnose, glucuronic acid, iduronic acid, xylose and sulphate (Percival & McDowell, 1967; Quemener, Lahaye, & Bobin-Dubigeon, 1997). The two major repeating disaccharides are aldobiuronic acids designated as type A: ulvanobiuronic acid 3-sulphate (A_{3s}) and type B: ulvanobiuronic acid 3-sulphate (B_{3s}) (Fig. 1). Xylose partially sulphated residues at O-2 can also occur as in place of uronic acids. In addition, glucuronic acid can branch at O-2 of rhamnose 3-sulphate (Ray & Lahaye, 1995a; Ray & Lahaye, 1995b). Among functional properties, ulvan yields viscous aqueous solutions able to form thermoreversible gels in the presence of multivalent cations and borate (Haug, 1976; Lahaye & Robic, 2007). Ulvan has also been claimed to form intercalated and exfoliated complexes with clays, which can be at the basis of new nanostructured materials (Laza et al., 2007). Ulvan also presents several potentially valuable biological properties for agricultural, food and pharmaceutical applications (Barcelo et al., 2000; Bobin-Dubigeon, Lahaye, & Barry, 1997; Cluzet et al., 2004; Ivanova et al., 1994; Kaeffer, Bénard, Lahaye, Blottière, & Cherbut, 1999; Lahaye & Kaeffer, 1997; Mao, Zang, Li, & Zhang, 2006).

Little is known about quantitative and qualitative variability of ulvan depending on *Ulva* sp. pretreatment. One important cause of

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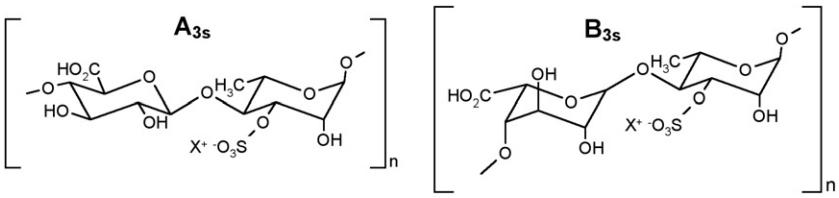

Fig. 1. Structure of the main repeating disaccharides in *Ulva* ulvan: ulvanobiuronic acids.

variability may lie in the post-collect itinerary of the biomass. "Green tides" seaweeds collected after stranding on beaches, are most often rapidly composted or spread on agricultural lands in order to cope with their fast decay. For industrial ulvan production, stabilization of the raw material would therefore be required if not treated at once. Usually, seaweeds for colloid extraction are treated as fresh material or stored after addition of preservatives or after sun drying (McHugh, 2003). In the case of ulvan producing green seaweeds, a critical assessment of stabilization methods is required to select appropriate and cost effective conditions that optimize yield, physico-chemical characteristics and rheological properties of the polysaccharide. In this work, we assessed the impact of freezing, freeze-drying, hot-air drying, brining and dry salting of *Ulva rotundata* on its ulvan yield and characteristics.

2. Materials and methods

2.1. Materials

Ulva rotundata (55 kg fresh weight) was collected on the coast at Pleubian (France), in November 2005. After thoroughly washing with seawater and manual sorting to remove epiphytes, the seaweeds were ground to pieces of about 1 mm with Urschel mill (Comitrol 3600, Urschel International Ltd., Lisses, France) equipped with a 1 mm impeller and divided into 11 samples (5 kg each) that were processed separately. Two samples were dried in ventilated ovens for 48 h at 50 °C (France ETUVES, type XLVH 1400) and at 70 °C (Thirode Pulsair Polycuiseur). They are referred to as S5 and S7, respectively. The seaweed thickness was between 2 and 3 cm on each tray. The dried samples were kept in polyethylene pouches at room temperature in a dry place. Dry salted samples were mixed manually to homogeneity with sodium chloride (25% w/w) and stored in high-density polyethylene buckets with clipped lids. Salted seaweeds were stored either at 4 °C for 33 weeks (sample N4) or at room temperature for 26 weeks (sample NA). Cold treated samples were frozen at −30 °C and kept at −30 °C (F3), or stored at −80 °C (F8), or freeze-dried (FD) (Lyophilisateur CRYO RIVOIRE) and stored in a polyethylene bag. Brined samples were bathed in a mixture of acetic acid (100 g/L), citric acid (10 g/L) and sodium chloride (50 g/L; in a ratio 1/5 w/v algae/brine) and stored at 4 °C for 2, 7 and 22 weeks. These samples are referred to as B2, B7 and BN, respectively. Brined and salted seaweeds were rinsed with deionised water to remove acid and/or salt prior to ulvan extraction. Fresh seaweeds, referred to as OO, were stored 36 h at 4 °C prior to ulvan extraction.

2.2. Extraction of ulvan

A wet suspension of ground seaweeds (dry solid content: 6.8% w/w) in 0.05 M sodium oxalate in deionized water was refluxed for 2 h at 85 °C under constant stirring. The suspension was diluted 1-fold with 0.05 M sodium oxalate. Two percent w/v diatomaceous earth was added prior to filtration (Filter plates Orion® C40, Klockner Holstein Seitz). The residues were re-extracted with water (dry initial solid content: 1% w/w) for 3 h at room temperature. Resulting slurry was filtered as previously without further dilution or addition of filter aid. The combined extracts were concentrated (100×) by ultrafiltration (Mw 10 kDa, Amicon). The retained solution was diafiltered with five volumes of deionized water and then freeze-dried.

2.3. Chemical analysis

Dry solid content corresponded to the weight of samples determined after 24 h at 103 °C. Organic matter was quantified gravimetrically after 12 h at 550 °C. Sulphate content was measured according to Tabatabai (1974). Protein content was estimated as *N* Kjelhdahl × 6.25. Total uronic acid content was analyzed colorimetrically by the *m*-phenyl phenol method using glucuronic acid as standard (Thibault, 1979). Neutral sugar and uronic acid composition of each extract was determined after methanolysis in MeOH–HCl and HPLC analysis (Absorbosphere RP18, 5 μm, 4 × 250 mm; Quemener, Marot, Mouillet, Da Riz, & Diris, 2000). Chromatographic peaks were attributed by comparison with reference sugars. Identification and quantification of neutral sugars was also performed by gas–liquid chromatography (GC) after sulphuric acid degradation (Englyst & Cummings, 1988; Hoebler, Barry, David, & Delort-Laval, 1989). Insoluble samples were dispersed in 13 M sulphuric acid for 30 min at 25 °C, prior to dilution to 1 M and hydrolysis (2 h, 100 °C). For calibration, standard monosaccharide solutions and inositol (internal standard) were used. All measurements were done in triplicate and statistical differences were evaluated by variance analysis using StatGraphics.

2.4. Structural analysis

^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 400 spectrometer. For ^{1}H NMR, 10 mg samples were dissolved in 1 mL of D_2O (99.9%), freeze-dried twice to remove exchangeable protons and then dissolved in 0.5 mL of D_2O (100%) with a trace of acetone as an internal reference (^{1}H δ = 2.22 ppm). The experiment was carried out at 333 K. For ^{13}C NMR, about 80 mg sample was dissolved in 1 mL of D_2O (99.9%) and 1 mL of H_2O with trace of acetone as internal reference (31.45 ppm).

2.5. Molecular weight determinations

Ulvan (4 mg/mL) was dissolved in 50 mM $NaNO_3$ containing NaN_3, passed through 0.1 μm membrane filter and injected at 25 °C on a high-performance size exclusion chromatography (HPSEC) system constituted of two Shodex OH-pack SB HQ 804 and 805 columns (Shodex, Showa Denko KK, Miniato, Japan) eluted at 0.7 mL/min with 50 mM $NaNO_3$ containing 0.02% NaN_3. On-line molar mass and intrinsic viscosity ([η]) determinations were performed at room temperature using a multi-angle laser light scattering (MALLS) detector (mini-Dawn®, Wyatt, USA, operating at three angles: 41°, 90° and 138°), a differential refractometer (ERC 7517 A) (dn/dc = 0.146 mL/g), a differential viscometer (T-50A, Viscotek, USA) and a UV detector. Average molecular weight was calculated using ASTRA 1.4 software (Wyatt, USA) and [η] using a

trisec software (Viscotek, USA). These determinations were made on material eluted from 16.5 to 20.7 min and from 20.7 to 25.7 min. Protein elution was followed by UV absorbance at 280 nm.

2.6. Rheological measurements

Dynamic viscosity analysis was performed with a Carri-med CS-50 stress-controlled rheometer (TA Instrument, France) with a temperature-regulated cone-plate device (radius 2 cm, cone-plate angle 4°1′22″). The polysaccharide concentration in buffer solutions (Tris–HCl, pH 7) and salt solutions ($CuSO_4$, 7 mM and H_3BO_3, 30 mM) was 1.6% (w/v) and experiments were carried out at 20 °C. Copper ions and boric acid were added simultaneously just before pouring the polysaccharide solution onto the Carri-med plate. Paraffin oil was layered onto the cone and sample to prevent evaporation. The kinetics of gel formation was monitored for 45 min by measuring the complex modulus at 0.159 Hz (1 rad s^{-1}) with a low deformation of 3%.

3. Results

The impact of drying, freezing, freeze-drying, dry salting and brining processes on *U. rotundata* ulvan was first assessed on the basis of organic matter content of the treated algae and on the yield of ulvan. The composition of the extraction residues was also characterized in order to evaluate the impact of the stabilization process on ulvan recovery. The chemical composition, the macromolecular properties (molecular weight distribution, intrinsic viscosity) and gelling ability of each extract were then characterized.

3.1. Effect of stabilization conditions on seaweed organic matter content and extraction yield of ulvan

The organic matter content of seaweeds following stabilization conditions is reported in Table 1. Fresh *Ulva* dry weight contained 54.5% organic matter. Freezing, freeze-drying and drying had no marked impact on the organic matter content. Brining and salting increased organic matter probably as a consequence of the deionised water rinsing of algae performed to remove acid and/or salt prior to ulvan extraction. Sand particles were observed to settle at the bottom of the bucket during brining and their removal also contributed to the increase in organic matter of the samples. The reduction in the organic matter content observed over time for brined algae may indicate loss of ulvan into the brine solution. The decrease in the algal salt content due to the water washes of the brined material may have induced ulvan leaching. Overall, organic matter contents were close to or higher than that of the fresh algae sample.

Table 1
Nomenclature and effect of stabilization treatments on *Ulva* organic matter (% of dry matter) and yield of ulvan extracted (expressed as % rhamnose content in the raw seaweed dried at 50 °C)

Samples	Nomenclature	Organic matter (%)	Ulvan yield (%)
Fresh *Ulva*	OO	54.5	58.6
Frozen *Ulva* (−30 °C)	F3	55.0	34.8
Frozen *Ulva* (−80 °C)	F8	58.5	38.0
Freeze-dried *Ulva*	FD	55.7	47.6
Dried *Ulva* (70 °C)	S7	57.5	58.2
Dried *Ulva* (50 °C)	S5	55.3	44.8
Salted *Ulva* (rt)	NA	72.0	25.8
Salted *Ulva* (4 °C)	N4	72.0	33.2
Brined *Ulva* (2 w)	B2	74.3	55.4
Brined *Ulva* (7 w)	B7	67.1	59.4
Brined *Ulva* (22 w)	BN	57.9	44.6

Since rhamnose is the main neutral sugar constituent in ulvan, ulvan yield was expressed as the percentage recovery of the initial rhamnose content in the fresh algae (Table 1). Rhamnose yield varied between 25% and 60% of the initial rhamnose according to the stabilization and storage conditions. The best yields were obtained with fresh (OO), dried (S7) and brined (B2, B7) seaweeds. The lowest yields were obtained with salted and frozen seaweeds.

3.2. Composition of U. rotundata residues after ulvan extraction

The total sugars, sulphate and protein contents of the algal residues after ulvan extraction are given in Table 2. To take into account for the diatomaceous earth added as a filter aid, all data are expressed on the basis of the organic matter content of the residues.

The extraction residues contained from 22.3% to 35.9% sugars. The poorest residues were from fresh algae and the richest were from salted (N4), brined (B7) and frozen (F8) algae.

Protein content in the residues ranged from 19.1% to 35.5% of the organic matter weight. The highest protein content was found in salted seaweeds extraction residues. Extraction residues from brined algae showed decreasing protein contents with time of storage. It varied from 35% after 2 weeks (B2) to 31% after 7 weeks (B7) and represented only 19% after 22 weeks (BN) of storage. All other residues showed similar protein contents at around 28% of organic matter.

The sum of total sugars and proteins did not add up to 100% of the organic matter indicating incomplete quantification of sugars and/or proteins. The discrepancy can also reflect the presence of other macromolecular components such as those coming from the algal cuticle.

Sulphate content was expressed on the basis of organic matter content. Sulphate in the fresh algae prior extraction accounted for 15.7% of the organic matter. It ranged from 2.4% to 8.7% of the organic matter in the extraction residues. The lowest content was observed for residues from fresh algae and the highest from residues of frozen algae.

The sugar composition of extraction residues is given in Table 2. Glucose was the major sugar (11.9–20.0%) with smaller amounts of rhamnose (3.4–8.6%), xylose (2.0–4.8%), uronic acids (4.0–7.5%), galactose (0.5–1.2%) and traces of mannose.

Residues from fresh algae contained less glucose than other residues. The highest glucose content was found in the brined (B7) algae residues. Extraction residues from frozen (F8) *U. rotundata* were the richest in rhamnose (8.6%) while those of brined (B2, BN) and fresh algae were the poorest (3.4–3.6%). No major difference in rhamnose content was observed for the other extraction residues. Fresh *U. rotundata* extraction residues contained the lowest amount of xylose while residues from salted algae (N4) were the richest. All other residues ranged between 3.0% to 4.0% xylose. The lowest uronic acids content was obtained with fresh and brined residues (4.0–4.4%). The other residues contained similar levels in uronic acids at around 5–6% of the organic matter dry weight with a slightly higher content for frozen (F8) extraction residues (7.5%).

Glucose and xylose contents were positively correlated as were sulphate, rhamnose and uronic acid contents (Table 3). Glucose and xylose were attributed to cellulose and hemicelluloses while sulphate, rhamnose and uronic acids were attributed to ulvan. Residual ulvan proportion in the residues was estimated from the molar ratio of rhamnose to glucose. The ratio in fresh *U. rotundata* prior to ulvan extraction was 0.8. The highest ulvan extraction efficiencies were observed for brined, fresh and salted *Ulva* (Rha/Glc: 0.2–0.3). The lowest ulvan extraction efficiencies were obtained with dried, freeze-dried and frozen *Ulva* (0.4–0.6).

Table 2
Mean chemical composition of extraction residues (% dry weight of organic matter, $n = 3$)

Samples residues[a]	Total sugars	Rha[b]	Xyl	Gal	Glc	UA	Total	Rha/Glc	Sulphate	Protein
OO	22.3	3.4	2.0	0.7	11.9	4.4	22.3	0.3	2.4	28.2
F3	31.4	6.9	3.2	0.8	14.5	6.1	31.4	0.5	8.0	27.7
F8	35.9	8.6	3.4	0.8	15.5	7.5	35.9	0.6	8.7	27.4
FD	33.6	6.9	3.3	0.7	16.3	6.4	33.6	0.5	5.2	28.1
S7	29.3	6.4	3.0	0.9	12.9	6.0	29.3	0.5	6.7	29.2
S5	27.9	5.1	3.1	1.0	13.3	5.4	27.9	0.4	3.9	28.0
NA	29.3	4.8	4.0	0.5	14.6	5.4	29.3	0.4	3.5	34.2
N4	35.2	6.3	4.8	0.8	16.6	6.7	35.2	0.4	3.8	34.1
B2	30.0	3.6	3.9	1.2	17.2	4.1	30.0	0.2	2.6	35.5
B7	35.2	4.7	4.1	1.2	20.0	5.2	35.2	0.3	3.9	31.0
BN	29.3	3.4	3.3	1.0	17.6	4.0	29.3	0.2	2.9	19.1

Total sugars represent the sum of sugar contents determined by GC and colorimetry; Rha/Glc: estimation of ulvan extraction efficiency based on the molar ratio of rhamnose to glucose in the extraction residues.
[a] see Table 1 for nomenclature.
[b] Rha, rhamnose; Xyl, xylose; Gal, galactose; Glc, glucose; AU, uronic acid.

Table 3
Correlation between chemical constituents in extraction residues

	Rha[a]	Xyl	Gal	Glc	UA	Sulphate
Rha	1.0					
Xyl	0.1	1.0				
Gal	−0.3	0.2	1.0			
Glc	−0.1	0.7	0.5	1.0		
UA	1.0	0.2	−0.4	−0.1	1.0	
Sulphate	0.9	0.0	−0.2	0.0	0.8	1.0

[a] Rha, rhamnose; Xyl, xylose; Gal, galactose; Glc, glucose; UA, uronic acid.

3.3. Composition of ulvan extracts

The chemical composition of each extract is given in Fig. 2. The proportion of organic matter in the ulvan extracts ranged from 70.1% to 77.2%.

The proportions in total sugars ranged from 50.2% to 58.5%. The extracts with the highest sugar contents were obtained with salted (N4), brined (B2, B7), dried (S7) and frozen (F8) *U. rotundata*. Ulvans consisted mainly of rhamnose and glucuronic acid with variable contents of xylose, iduronic acid, glucose and galactose. Rhamnose content ranged from 23.6% to 29.3% of the extract dry weight. Extracts from algae brined for 2 weeks (B2), freeze-dried, and heat dried at 50 °C were not significantly different from that of fresh algae. The highest rhamnose content was obtained from frozen (F8) and salted (N4) samples and the lowest contents, from the salted (NA) and brined (B7 and BN) extracts. Glucuronic acid content ranged from 13.6% to 19.8% of the extract dry weight. Except for the highest value measured for brined (B7) extract and for the lowest value measured for frozen (F3) extract, all the other extracts did not significantly differ from that of fresh algae. Xylose content ranged between 2.9% and 6.5%. Only values for brined (B7), frozen (F3 and F8) and salted (N4) extracts were significantly higher than that of fresh seaweed. The highest content in iduronic acid was measured in brined (BN) seaweeds extract at 4.6% of dry weight. For other samples, values were not significantly different from that of fresh algae. The proportion of galactose and glucose in the extract ranged from 1.1% to 2.8% and from 0.6% to 4.6%, respectively. The highest galactose content was measured for dried S5 U. *rotundata* extracts. Other values were not significantly different from that of fresh algae. A high value of 4.6% of glucose was repeatedly obtained for the extract from brined B7 seaweeds. Except for this last sample and for salted NA and N4 extracts, values were not significantly different from that of fresh algae.

Sulphate content varied between 9.3% and 16.7% of extract dry weight (Fig. 2). Sulphate contents of ulvans extracted from brined *Ulva* were not significantly altered by storage time with a mean value of 9.7%. Ulvans extracted from heat dried *Ulva* also show no variation in sulphate content according to drying temperature with a mean value of 12%. However, brining and drying significantly lowered the sulphate content in ulvan extract compared to fresh algae extraction. The sulphate contents of ulvans from frozen *Ulva* or from dry-salted algae stored at 4 °C (N4) were not significantly different from that of fresh *Ulva*. In contrast, ulvan from freeze-dried *Ulva* and dry-salted algae stored at room temperature (NA) was markedly and significantly lower than that of fresh *Ulva*.

The protein content ranged from 3.8% to 7.4% of extract dry weight (Fig. 2). It was significantly lower for ulvans from all treated algae compared to fresh *Ulva* at about 4.6%. Only the protein contents of ulvan extract from brined *Ulva* for 7 and 22 weeks (B7, BN) and freeze-dried *Ulva* (FD) were significantly different from the other treatments at about 5.3%.

The sum of total sugars, sulphate and protein contents account for 66–79% of the dry weight of the extracts. The difference may be due to the contribution of counter-ions of sulphate and uronic acids, residual salts and metabolites as well as to an incomplete quantification of proteins and sugars.

3.4. Structural analysis of ulvan

NMR (^{1}H and ^{13}C) spectra were recorded for all the extracts. Typical ulvan proton and carbon chemical shifts were observed (Table 4). Peak attributions were done by comparison with published data for ulvan and ulvan oligosaccharides (Lahaye et al., 1999; Lahaye, Brunel, & Bonnin, 1997; Lahaye, Inizan, & Vigouroux, 1998; Lahaye & Ray, 1996).

In all spectra, the signals for the ulvanobiuronic acid 3-sulphate type A and B were observed. Several minor signals likely reflected other linkages between rhamnose, uronic acids and sulphate as well as the presence of the other sugars residues like xylose, galactose and glucose. The resonance at 83 ppm for contiguous 1,4-linked β-D glucuronic acids (Gg4 for GlcA C-4) in ulvan or from contaminating glucuronan was observed on each spectra with variable intensities.

Signals attributed to R1x(s) at around 98 ppm for C-1 in rhamnose 3-sulphate linked to xylose or xylose 2-sulphate, to X5 at 65 ppm for C-5 of xylose and to X5s/C6 for C-5 of xylose 2-sulphate and for C6 of different hexoses at around 60 ppm were present to variable extents on all the spectra. These last signals were mainly found for ulvans from brined seaweeds. The strongest intensity of X5s/C6 was observed with ulvan from brined *Ulva* during 7 weeks. This result is in accordance with the high glucose content of this extract. The other extracts do not show major variations for these resonances.

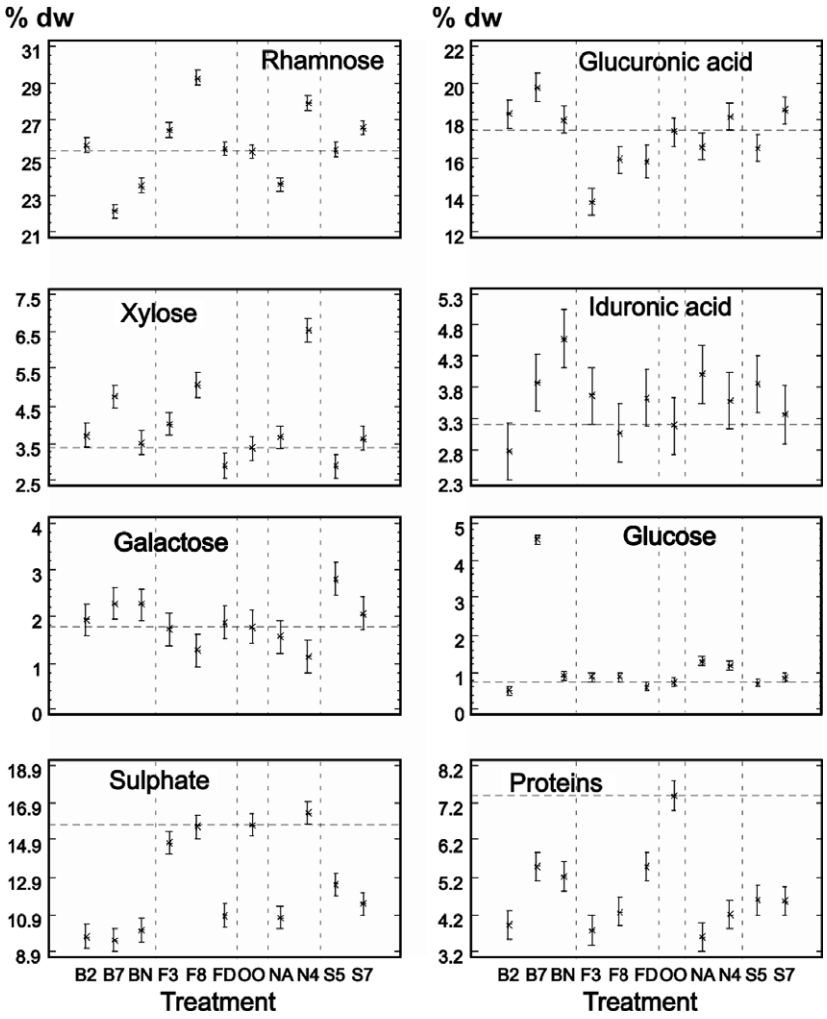

Fig. 2. Chemical composition of ulvan extracts (% dry weight basis; mean ± 95% confidence interval, $n = 3$).

Table 4
Chemical shifts (ppm) of ulvan obtained from fresh *U. rotundata*

	Nucleus	1	2	3	4	5	6
R	^{1}H	4.8	4.2	4.5	3.6	4.2	1.3
	^{13}C	101	70.2	79.8	79.8	70.0	17.7
R'	^{1}H	4.9	4.2	4.5	3.6	4.0	1.2
	^{13}C	102.2	70.0	80.0	80.0	70.4	17.9
G	^{1}H	4.6	3.3	3.6	3.6	3.7	
	^{13}C	104.4	75.2	75.4	80.2	77.6	175.9
I	^{1}H	5.9	3.7	3.7	4.0	4.6	
	^{13}C	104.2	72.0	73.0	80.4	72.2	175.1

R refers to non-reducing end rhamnose 3-sulphate linked to glucuronic acid, R′ refers to non-reducing end rhamnose 3-sulphate linked to iduronic acid, G and I refer to glucuronic and iduronic acids, respectively.

3.5. Macromolecular and rheological characteristics of extracts

The macromolecular characteristics of the extracts were obtained by size exclusion chromatography coupled to refractive index (RI) and UV detections as well as with multi-angle laser light scattering and on-line viscosimetry detections. Based on the RI profiles, extracts contained one to two major macromolecular populations (Fig. 3). All of them gave a small UV response, which was indicative of proteins co-eluting with ulvan. Extracts from brined (B7) and from salted *Ulva* (NA) yielded a strong UV response in the small molecular weight region, probably as the result of increased protein co-extraction enrichment and/or protein degradations. The macromolecular characteristics of material eluted from 16.5 to 20.7 min (peak A) and from 20.7 to 25.7 min (peak B) were analyzed separately and are given in Table 5.

The weight average molecular weights of ulvan from population A ranged from 300 to 500 $\times 10^3$ g mol^{-1} whereas that from population B was between 85 and 180 $\times 10^3$ g mol^{-1}. Most ulvan extracts were composed of the two broad macromolecular populations in similar proportions (Fig. 3). Only ulvans from frozen and freeze-dried seaweeds were mainly composed of the high molecular weight population (about 75%). Drying temperature affected ulvan distribution between population A and B. The proportion of the lower molecular weight component (B) increased in the ulvan extracted from algae dried at lower temperature (50 °C). Cold storage of salted algae preserved better the high molecular weight ulvan compared to storage at room temperature. Brining of algae contributed to decrease the molecular weight of ulvan. For peak B, the highest average molecular weight was obtained with ulvan from frozen *U. rotundata* (170 and 180 $\times 10^3$ g mol^{-1} for F3 and F8, respectively). A series of values ranging between 100 and 140 $\times 10^3$ g mol^{-1} was obtained with ulvans from fresh (OO), freeze-dried, dried (S7), salted (N4) and brined (B7 and BN) *U. rotundata*. Lower values were obtained with ulvan from dried (S5) and brined (B2) seaweeds.

The intrinsic viscosity of ulvan populations A and B ranged from 120 to 460 mL g^{-1}. It increased linearly with weight average

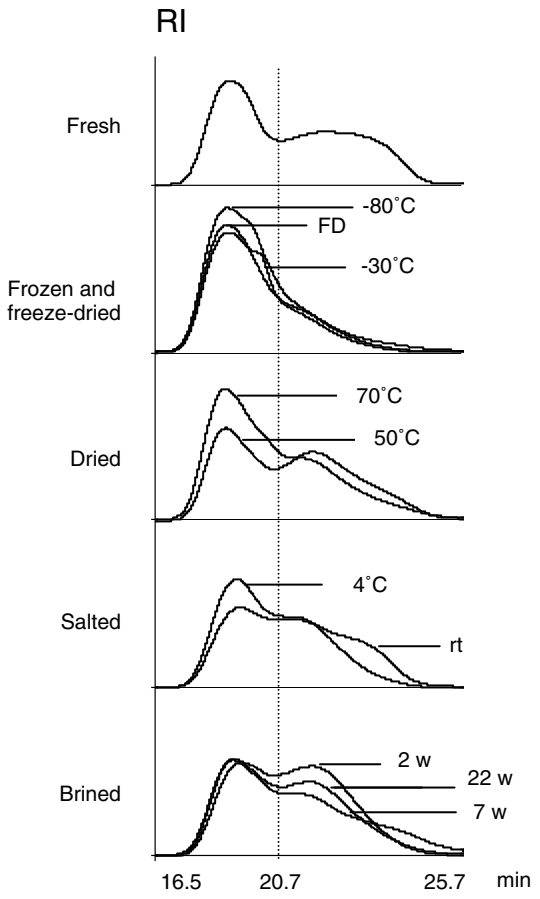

Fig. 3. High pressure size exclusion chromatographic profiles of ulvan extracts coupled to RI detections.

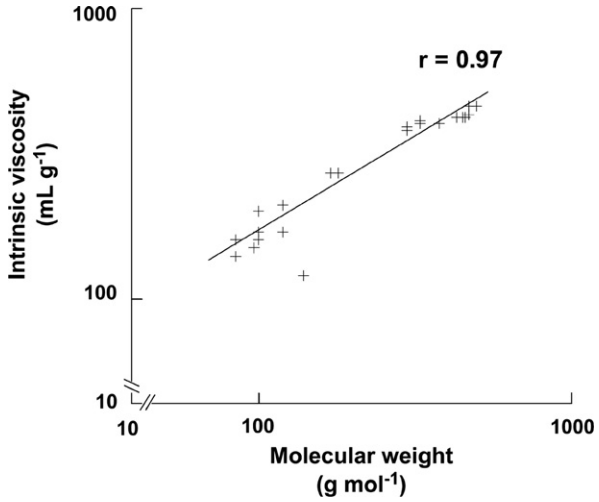

Fig. 4. Relation between weight average molecular weights and intrinsic viscosities of the ulvan populations measured by HPSEC–MALLS and HPSEC–viscosimetry.

molecular weight of ulvan population (Fig. 4). Only the ulvan population B from fresh *U. rotundata* departed from this linear relation and was not taken into consideration in the determination of the regression line.

Conservation modulus (G') of the gels made with the extract in presence of copper (7 mM) and borate (30 mM) at pH 7 are given in Table 4. It ranged from 1 to 179 N m^2. A linear relationship was observed between the proportion of high molecular weight ulvan (A) and G' (Fig. 5). This regression fitted most of the ulvan extracts ($r = 0.90$) except for ulvan from salted algae stored at room temperature (NA) and ulvan from brined conditions (B2, B7, BN).

4. Discussion

Several stabilization processes are used in the seaweed industry. For colloid productions (agar, carrageenan or alginate), red and brown seaweeds are generally sun-dried or stabilized fresh with a preservative (McHugh, 2003). For other seaweed-based products, algae are dried by hot-air at moderate temperatures (40–50 °C) (Hamdy & Dawes, 1988), frozen, freeze-dried, stored wet after pickling (acidic brine) (Mabeau, Cavaloc, Fleurence, & Lahaye, 1992) or salted with dry salt (Moen, Larsen, Ostgaard, & Jensen, 1999). Understanding the impact of the stabilization conditions of Ulvale biomass on ulvan chemical and rheological properties is of key importance for industrial use of ulvan as a colloid. Being a polyelectrolyte, ulvan likely networks in the algae through ionic interactions with itself and other cell wall polymers, such as proteins or other charged polysaccharides. Any process modifying its molecular integrity or its interactions in the cell wall may affect yield and functional properties of the polysaccharide. The present work tested several simple stabilization methods on a common green seaweed species in Brittany, *U. rotundata*. The ef-

Table 5
Molecular weight (Mw, g mol^{-1}), fraction proportion (%) and intrinsic viscosity (η, mL g^{-1}) of high pressure size exclusion chromatography (HPSEC) fractions A and B and conservation modulus (G', N m^{-2}) of ulvan gels in the presence of copper and borate

Samples[a]	HPSEC fractions[b]						
	A			B			
	Mw × 10^{-3}	%	[η]	Mw × 10^{-3}	%	[η]	G'
OO	460	54.1	420 ± 3	140	45.9	120 ± 3	22 ± 1.5
F3	470	79.3	460 ± 3	170	20.7	270 ± 8	100 ± 0.2
F8	500	79.5	460 ± 5	180	20.5	270 ± 3	179 ± 2.5
FD	470	75.1	430 ± 4	120	24.9	210 ± 11	120 ± 1.8
S7	450	65.5	420 ± 6	120	34.5	170 ± 7	78 ± 1.4
S5	430	51.0	420 ± 7	85	49.0	140 ± 7	15 ± 0.8
NA	300	49.5	380 ± 5	97	50.5	150 ± 4	1 ± 0.1
N4	330	62.1	400 ± 4	100	37.9	200 ± 2	25 ± 0.8
B2	300	48.3	390 ± 2	85	51.7	160 ± 2	1 ± 0.0
B7	330	54.6	410 ± 7	100	45.4	170 ± 5	3 ± 0.1
BN	380	54.0	400 ± 2	100	46.0	160 ± 3	2 ± 0.1

[a] For nomenclature of samples see Table 1 and 2.
[b] A and B: macromolecules eluted between 16.5 and 20.7 min and 20.7 and 25.7 min from the column, respectively.

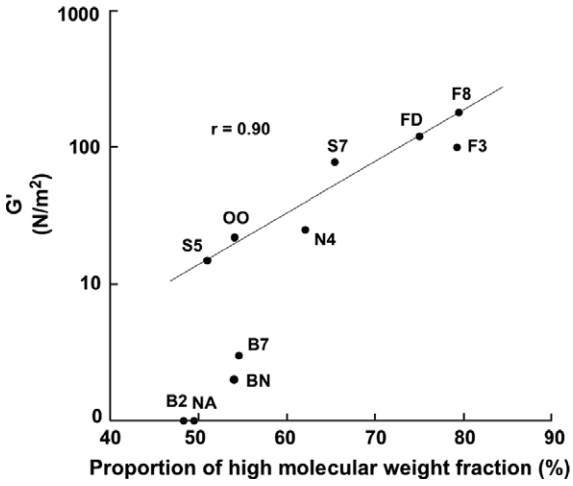

Fig. 5. Relation between the proportion of high molecular weight ulvan (population A) and conservation modulus (G') of the gel in the presence of copper and borate at pH 6.5. The lowest points correspond to ulvans extracted from salted (NA) and brined algae (B2, B7, BN).

fect of physical (freezing, freeze-drying, hot-air drying) and chemical (brining and salting) treatments on ulvan yields and characteristics was assessed with respect to fresh *Ulva* taken as a reference of raw material for ulvan extraction.

Fresh *U. rotundata* yielded one of the highest amount of ulvan compared to seaweeds stabilized according to different treatments (Table 1). Ulvan chemical structure, viscosity and gelling characteristics were in good agreement with those reported previously (Haug, 1976; Lahaye & Axelos, 1993; Lahaye & Jegou, 1993; Percival & Wold, 1963; Yamamoto, 1980). The ulvan extract obtained from fresh algae was basically composed of two broad macromolecular distributions of polysaccharides centered at about 460 and 140×10^3 g mol^{-1} (Fig. 3). The sulphate and protein contents in this ulvan extract were high (Fig. 2). This suggests that the raw material was not markedly modified between the collect and the processing. However, this extract is contaminated by non ulvan polymers and particularly proteins. This may explain the deviation of the medium molecular weight population of fresh *Ulva* (fraction B) from the linear relationship between viscosity and molecular weight (Fig. 4).

Freezing of food has been used for a long time because it generally does not perturb product quality (Persson & Londahl, 1993). However, different mechanisms can damage cells during the freezing process (Karlsson & Toner, 1996; Suzuki & Mittler, 2006). The main damage arises from the formation of ice crystals that rupture cell membranes and disorganize cells and cell walls (Meryman, 1966; Snell 1991). Freeze-drying is one of the mildest drying techniques. It preserves most of the original qualities of biological materials as it prevents fluids and solutes migration. When applied to *U. rotundata*, freezing or freeze-drying immediately after collect yielded a raw material suitable for producing high molecular weight ulvan (Fig. 3) with the highest texturizing properties (Table 5). Ulvans extracted from these stabilized seaweeds demonstrated even better characteristics than that of fresh algae. For the latter, the short storage period (36 h at 4 °C) prior to processing may have been sufficient to initiate partial degradation of ulvan. Fresh algae ulvan was richer in the medium molecular weight population (Fig. 3) and developed lower viscosity and gelling characteristics compared to ulvan from cold-treated algae (Table 4). However, freezing the seaweeds may have modified ulvan interactions in the cell wall and/or better preserved its molecular integrity compared to the fresh *Ulva* since the ulvan yield from cold-treated algae were among the lowest among the different treatments. Ulvan from frozen seaweeds was also richer in rhamnose and xylose than reference ulvan (Fig. 2). This may reflect higher proportions of ulvan in the extracts particularly for F8 ulvan. Ulvan from cold-treated algae had sulphate contents similar to that of the reference ulvan from fresh *Ulva*. Small chemical composition differences were found between ulvan from frozen *Ulva* depending on frozen storage temperature. This result may indicate a low level of metabolism at −30 °C that is better inhibited at −80 °C (Taylor & Fletcher, 1999). The lower sulphate content in freeze-dried extract may reflect an evolution of the dry algae with partial desulphation during storage in sealed polypropylene bag at room temperature before extraction.

Heat dehydration is another very common technique for stabilizing fresh plant materials. It can be achieved by several means: convection, conduction (by a heated surface) or by infrared or microwave energy (Mafart, 1996). These processes generally yield mid-quality products and require high amounts of energy. Hot-air convection drying is a standard process for seaweed-based intermediary products (Hamdy & Dawes, 1988). Two phases are observed during drying. The first one consists in the transfer by convection of heat from the external environment to the surface of the product. The second phase corresponds to the diffusion of heat in the product to its geometric center (Mafart, 1996). Controlling air temperature is very important in the second phase because it is proportionally related to the rate of water movement through the plant tissues. It is also generally associated with loss of volatile compound and hardening of the product by migration of solutes towards the surface (Roux, 1994). Two oven temperatures (50 and 70 °C) have been tested on *U. rotundata*. The yield of ulvan from *Ulva* dried at 50 °C was lower than from seaweed dried at 70 °C. Faster drying kinetic was probably more efficient in inhibiting endogenous enzymatic breakdown of ulvan. The lower proportions of high molecular weight ulvan and the lower viscosifying characteristics of the ulvan extracted from the seaweed dried at 50 °C support this hypothesis (Fig. 3, Table 5). On the other hand, drying temperature did not affect the sugar composition of ulvan. However, the sulphate content was lower than that for the ulvan extracted from fresh algae. Dehydration by hot-air may not be efficient in inhibiting rapidly desulphating mechanisms, carried out by putative endogenous sulphatases.

In wet stabilization treatments, the chemical aspect plays an essential role in stabilization. Immersion of fresh plant material in a hypertonic aqueous solution leads to loss of water from the material due to osmotic compensation (Sereno, Moreira, & Martinez, 2001). Such dewatering is known to inhibit endogenous enzymes (Pokharkar, Prasad, & Das, 1997). The combined choice of salt concentration and acidity used in the brine helps in selecting and maintaining microorganisms, which also contribute to preserving the material. Such a technique is used to stabilize some algae used for food (Roux, 1994). When applied to *U. rotundata*, it induced ulvan degradation as judged from the evolution of the molecular weight distribution profiles and from the poor rheological properties of the extracts compared to ulvan from fresh algae (Fig. 3, Table 5). The combined acidity of the medium and the presence of microorganisms originating in part from the seaweeds and feeding on *Ulva* cell wall material are likely to be at the origin of this degradation. However, brining appears to ease extraction of polysaccharides (Table 1). Nevertheless, the rhamnose content in the ulvan from seaweeds brined for 7 and 22 weeks was lower than that from fresh algae suggesting a contamination by other polymers. This is particularly true for the extract obtained after 7 weeks, which showed a high glucose content of unknown origin. As a consequence, the rheological properties of ulvan from brined seaweeds depart from those observed with physically treated algae (Fig. 5). Moreover, the decrease in yield of ulvan after 22 weeks is likely associated with the consumption of the organic matter by the microflora. The sulphate content of the brined-seaweed ex-

tracts was the lowest of all. It is likely that under the fermentation conditions of the brine with restricted oxygen supply, anaerobic bacteria use sulphate as source of energy (Roden & Tuttle 1992).

Dry salting of plant material with 5–10% inhibits growth of a majority of bacteria but some microorganisms are tolerant to higher NaCl concentrations. For example, *Halomonas* species can grow in up to 25% NaCl concentrations (Holt & Krieg, 1994). Salt penetrates easily in hydrated tissues and dewater them by osmosis. Dry salting of *U. rotundata* was realized at 4 °C and room temperature (18–22 °C) and the biomass was kept for 33 and 26 weeks, respectively, prior to extractions. Storage of the salted seaweeds at room temperature induced degradation of the ulvan compared to fresh algae as judged from the molecular weight profiles and the rheological properties (Fig. 3, Table 5). Decreasing the storage temperature to 4 °C preserved ulvan composition, structure and properties. The sugar and sulphate contents of ulvan from salted algae stored at 4 °C was very close to that from fresh *Ulva*. It showed an unexpected higher xylose content and was less contaminated by proteins. In contrast, endogenous cell wall degrading enzymes and residual microbial growth are likely to be less inhibited at room temperature. Ulvan from salted *Ulva* stored at room temperature showed a marked deviation from that of fresh algae with regard to the sulphate content, which may have been cleaved by endogenous sulphatases. Yield of ulvan was low from the salted *Ulva.* This effect was probably due to loss of material consecutive to two different mechanisms. For the sample stored at room temperature, ulvan degradation is suspected and supported by the chromatographic profile, viscosity and gelling characteristics (figure, Table 5). For the sample stored at 4 °C, the low yield probably reflect retention of ulvan in algal cell wall due to the high salt content. Ulvan is known to aggregate in the presence of sodium (Paradossi, Cavalieri, & Chiessi, 2002) and the extraction conditions may not have been optimal for breaking all these interactions. This hypothesis is supported by the high sulphate and rhamnose contents in the extraction residues (Table 2) attesting for residual ulvan particularly for the algae stored at 4 °C.

5. Conclusion

Post-collect itineraries of *U. rotundata* impact markedly ulvan extraction yield, chemistry and rheological properties. None of these extracts represent pure ulvan and the impact of the different stabilization treatments on the extraction of proteins and other polysaccharides has yet to be precised. It is likely that such contaminations impact on weight yield, composition and rheological properties of the extracts. Refining of ulvan may be necessary to better control the end use of the polysaccharide. The difference observed in ulvan characteristics between fresh algae stored for a short period at 4 °C and that from immediately frozen samples clearly shows the remarkably rapid evolution of the biomass and points to the necessity of applying fast stabilization conditions of the raw material if high molecular weight ulvans with the highest rheological potential are desired. The mechanisms of biomass modification by the different stabilization treatments applied are complex as they likely combine chemical and physical factors to biochemical reactions. Of interest is the impact of the different treatments on sulphate and protein contents in the extracts. These observations point to the need to better define the nature and mechanisms of endogenous enzymatic systems and/or from associated flora in order to understand and control these biochemical reactions. These results show that it is possible to select *Ulva* stabilization conditions with respect to cost-effectiveness and in accordance with ulvan characteristics needed to meet specific application criteria. Best conditions for high molecular weight ulvan and optimal rheological properties are provided by immediate cold treatment on collect. However, in these conditions, ulvan yields are reduced. *Ulva* dried under air at 70 °C is a good alternative and provides better ulvan yield. Effective treatments for reducing molecular weights and viscosifying properties were particularly provided by brining.

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