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Composition and structure of cell wall ulvans recovered from *Ulva spp.* along the Swedish west coast

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ARTICLE INFO

ABSTRACT

The cell wall polysaccharide ulvan was isolated from two species of the seaweed *Ulva* collected along the Swedish west coast. Acidic extraction was benchmarked against hot water extraction with enzymatic purification and against commercial ulvan. Extracted ulvan contained 11–18 % g/g of ash, some protein (up to 1.3 % g N/g) but minimal colored impurities. The ulvans had high molecular weights (660,000–760,000 g/mol) and were composed of 77–79 % g/g carbohydrates, mainly rhamnose, xylose, glucose, glucuronic acid, and iduronic acid. The extraction protocol and the ulvan source strongly impact the molecular weight and the chemical composition. Acidic extraction caused almost complete desulfation of the isolated ulvan while the other method preserved a significant degree of SO_3 substituents. Elemental analysis of ash remaining after thermal degradation showed presence of common mineral elements such as Na, Ca, Mg, Al, and K, but none of the heavy metals Pb, Hg, or As.

1. Introduction

Seaweed farming is a fast-growing sector of biomass production with a potential of high areal productivities. Such farming has been done for centuries in Asia. To this point, it has been largely undeveloped in Sweden even though its long coast line indicates that there is great potential for this industry. Farming of seaweed offers several benefits compared to land-based biomass production, such as no competition for valuable land areas and no need for irrigation. Additionally, seaweed farming does not require the use of fertilizers and contributes to the avoidance of coastal eutrophication (Kraan, 2013).

Species of the green macroalgae genus *Ulva* (of the Chlorophyta family, some species previously named *Enteromorpha*) are commercially maricultured though this occurs to a lesser extent than other groups of seaweeds, and substantial amounts are also wild-harvested (White & Wilson, 2015). *Ulva* spp. are widely distributed along the coastline in oceans across the world, which makes these species a low-cost and abundant source of biomass. *Ulva* spp. are among the most popular edible seaweeds worldwide, e.g. *U. lactuca* is also known as "sea lettuce", with a high nutritional value due to its high levels of polysaccharides, proteins, vitamins and trace minerals (Taboada, Millan, & Miguez, 2010). *Ulva* spp. are particularly suitable for sea-based biomass

production due to their high productivity and ability to thrive under different growing conditions worldwide (Ye et al., 2011).

Earlier studies show that the main polysaccharides in *Ulva* spp. are cellulose, ulvan, xyloglucan, and starch. The cellulose, xyloglucan, and ulvan are located in the cell walls whereas starch serves as a dynamic storage source of glucose (Liquori, Pizzoferrato, Roveri, & Bigi, 1991). The molecular structure of the water-soluble sulfated polysaccharide ulvan is quite complex and has been studied in detail during the past decade (Lahaye & Robic, 2007; Lahaye, 1998; Quemener, Lahaye, & Bobin-Dubigeon, 1997; Robic, Gaillard, Sassi, Lerat, & Lahaye, 2009; Robic, Rondeau-Mouro, Sassi, Lerat, & Lahaye, 2009). Ulvan is mainly built up by repeating units of sulfated disaccharides with rhamnose, xylose, glucuronic acid and iduronic acid as the main building blocks. The main repeating units are β-D-glucuronic acid (1 → 4)-α-L-rhamnose-3-sulfate, and α-L-iduronic acid (1 → 4)-α-L-rhamnose-3-sulfate. The iduronic acid or glucuronic acid components may instead be a xylose unit (sulfated or non-sulfated) forming the characteristic monomers β-D-xylose (1 → 4)-α-L-rhamnose-3-sulfate and β-D-xylose-2-sulfate(1 → 4)-α-L-rhamnose-3-sulfate. Trace amounts of galactose and glucose can be found in ulvan (Quemener et al., 1997). The chemical structures of the characteristic disaccharides of ulvan are shown in Fig. 1.

The amount of ulvan in *Ulva* spp. varies between different species

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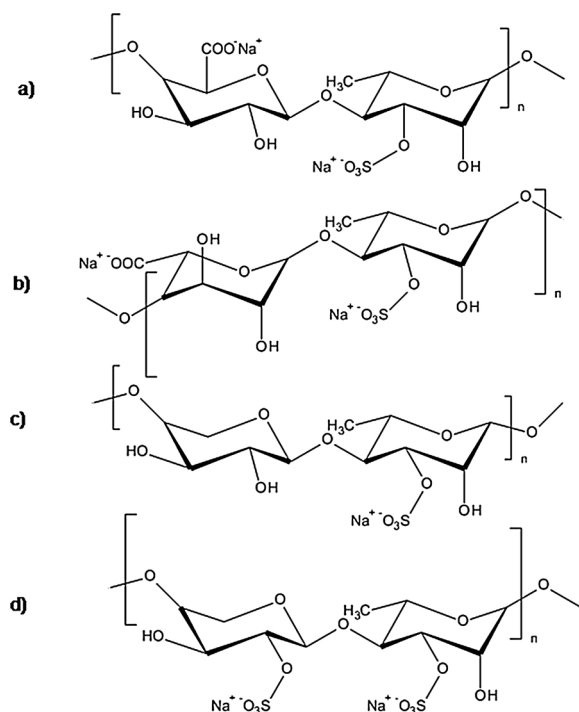[*] Corresponding author.
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Fig. 1. The characteristic disaccharide motifs in ulvan, a) β-D-glucuronic acid (1 → 4)-α-L-rhamnose-3-sulfate; b) α-L-iduronic acid (1 → 4)-α-L-rhamnose-3-sulfate; c) β-D-xylose (1 → 4)-α-L-rhamnose-3-sulfate; and d) β-D-xylose-2-sulfate (1 → 4)-α-L-rhamnose-3-sulfate.

and is also dependent on the growth conditions, and thus constitutes 8–29 % of the dry weight of biomass (Kaeffer, Benard, Lahaye, Blottiere, & Cherbut, 1999; Lahaye & Robic, 2007; Robic, Sassi, Dion, Lerat, & Lahaye, 2009). Several applications of ulvan have been reported. Ulvan has anticoagulant (Zhang et al., 2008) and antioxidant (Morelli & Chiellini, 2010) properties and may serve as an antitumor and immune modulator (Kaeffer et al., 1999). In addition, it was shown that ulvan has a strong affinity for heavy metal ions (Lahaye & Robic, 2007). The gelling properties of ulvan in the presence of calcium and borate ions has been investigated (Haug, 1976; Lahaye & Axelos, 1993) as well as its potential use as a key component in nanofibers (Toskas et al., 2011), hydrogels (Morelli, Betti, Puppi, & Chiellini, 2016), scaffolds (Dash et al., 2018) or as a factor with a potential skin anti-aging effect (Adrien et al., 2017).

Several extraction methods for isolating ulvan from *Ulva* have been suggested, including hot water extraction (Costa et al., 2012; Paradossi, Cavalieri, Pizzoferrato, & Liquori, 1999), hot water with sodium oxalate (Lahaye & Robic, 2007), acidified ammonium oxalate (Robic, Rondeau-Mouro et al., 2009), and hydrochloric acid (Glasson, Sims, Carnachan, de Nys, & Magnusson, 2017; Yaich et al., 2013). The dissolved ulvan is precipitated by adding ethanol to the supernatant followed by freeze-drying of the precipitated fraction. Other components such as starch, proteins, pigments and salts are usually co-extracted with the ulvan, which reduces its purity (Costa et al., 2012; Glasson et al., 2017). Enzymatic treatment of the extracted ulvan with α-amylase and proteinase followed by dialysis is necessary to obtain an ulvan fraction free from proteins, starch and salts (Costa et al., 2012). Pigments can be removed by an organic solvent such as ethanol (Kitada et al., 2009) or activated charcoal (Alves, Caridade, Mano, Sousa, & Reis, 2010; Costa et al., 2012).

Previous studies indicate that the composition of the extracted ulvan depends on the extraction conditions in addition to the documented influence of growth conditions. In light of the interesting properties of ulvan and the escalating, sustainability-driven search for bio-based resources to replace fossil-based counterparts, *Ulva* spp. form an

attractive but largely unexplored biomass base for producing food/feed, fine chemicals and bio-based materials in a future biorefinery industry. Developing methods for extracting ulvan from *Ulva* is hence of great importance. However, the presence of contaminants such as minerals and proteins (the latter strongly associated with ulvan in the cell wall (Yaich et al., 2013)) may impair the applicability of the extracted ulvan. Moreover, a deeper understanding of the relationships between processing conditions and resulting composition is needed to propel the development of future biorefining intiatives.

For the above reasons, our aim was to develop protocols to extract and to provide a detailed structural and compositional map of ulvan from *Ulva* spp. grown along the Swedish west coast. We compare different extractions protocols and explore several different *Ulva* species to shed light on the differences in the isolated ulvan structure and composition. Such a map is important knowledge for the development of a production platform for ulvan-depolymerizing and modifying enzymes. With the aid of these tools, we are one step closer toward development of a green seaweed biorefinery.

2. Experimental

2.1. Sampling of Ulva spp. biomass

Seaweed biomass was collected at the Swedish West coast; *Ulva lactuca* in the Koster archipelago 18 July 2015 at the island Saltö (N 58°52.744′ E 11°07.146′) and 10 June 2015 at the island Inre Vattenholmen (N 58°52.6′, E 11°06.9′) and *Ulva* sp. with "intestinalis-like" morphology (identified as *U. compressa*) 21 September 2016 close to Skärhamn at the island Tjörn (N 57°59.059′ E 11°32.314′). The species were identified according to morphology (Burrows, 1991). Recent studies have pointed at the difficulty to distinguish *U. intestinalis* and *U. compressa* from Swedish waters based on morphology and thus species identity of *U. compressa* was verified by evaluation of the *tuf*A gene sequence as described (Steinhagen, Karez, & Weinberger, 2019). Recent studies have suggested that *U. lactuca* from the Northern hemisphere should be named *U. fenestrata* (Hughey et al., 2019), but in this paper *U. lactuca* will consistently be used. The biomass samples were stored at −80 °C, then freeze-dried, ground and again stored at −80 °C until use.

2.2. Reference ulvan, chemicals and materials

Reference ulvan from *Ulva armoricana*, type winter-heavy, purity min 90 % (≥80 % at a weight-average molecular weight (Mw) of 90 000–980 000 g/mol, CAS nr: 164252-34-0) was purchased from CarboSynth. It was according to information provided from the producer obtained from collected wild stock *U. armoricana* at St Michel en Grève, France (N 48°40.567′ W 3°34.467) at low tide and extracted in 0.05 M sodium oxalate solution, followed by ultrafiltration (concentration), diafiltration (demineralization) and freeze-drying based on the method previously described (Robic, Sassi et al., 2009).

Sodium acetate (99 %, CAS nr: 127-09-3), pyridine, (99.8 %, CAS nr: 110-86-1), deuterium oxide (99 atom % D, CAS nr: 7789-20-0), L-(+)-arabinose (99 %, CAS nr: 5328-37-0), D-(+)-glucose (99 %, CAS nr: 50-99-7), D-(+)-mannose (99 %, CAS nr: 3458-28-4), D-(+)-galactose (99 %, CAS nr: 59-23-4), D-(+)-xylose (99 %, CAS nr: 58-86-6), L-(+)-rhamnose (99 %, CAS nr: 10030-85-0), D-(+)-glucoronic acid (98 %, CAS nr: 6556-12-3), NaH$_2$PO$_4$·(≥99 %, CAS nr: 7558-80-7) and the enzymes α-amylase (*A. oryzae*) and proteinase K (*T. album*) were all purchased from Sigma Aldrich. Hydrochloric acid (37 %, CAS nr: 7647.01-0), trifluoroacetic acid (99 %, CAS nr: 76-05-1), and sodium hydroxide (99 %, CAS nr: 1310-73-2), were used from either Sigma or Merck.

Ethanol (70 and 96 %, CAS nr: 64-17-5) and methanol (99.98 %, CAS nr: 67-56-1) were purchased from VWR Chemicals. Acetyl chloride (99 %, CAS nr: 75-36-5) was purchased from Acros Organics. L-(+)
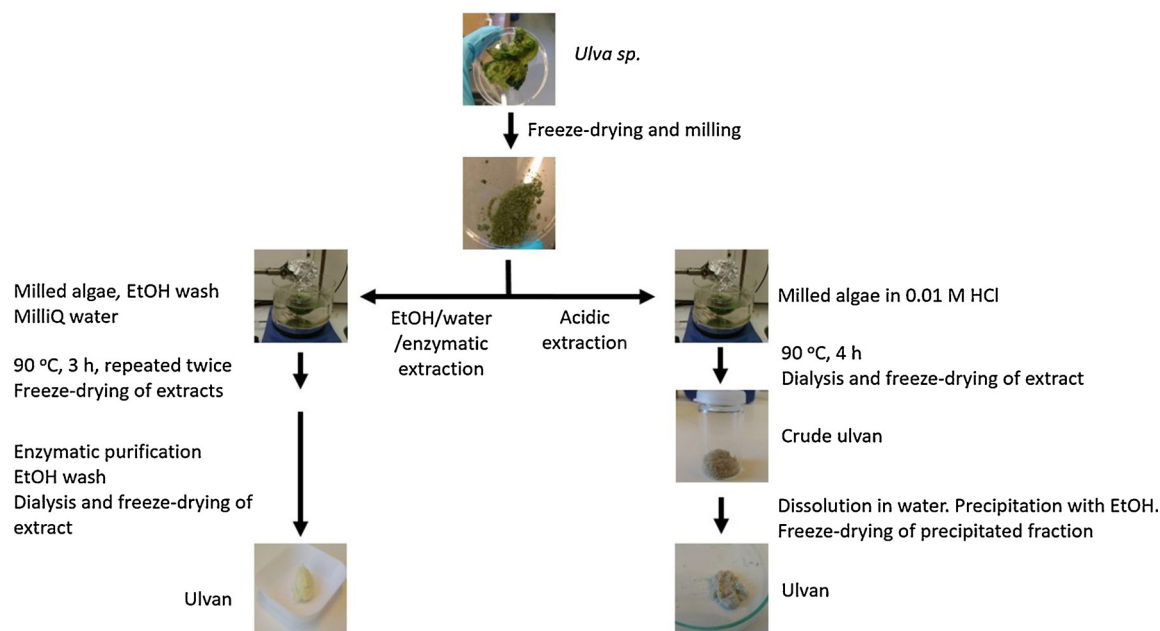
Fig. 2. Schematic illustration of the two different extraction protocols for extracting ulvan from *Ulva* spp. grown along the Swedish west coast.

iduronic acid sodium salt ($\geq$ 99 %, CAS nr: 2073-35-0,61199-83-5) was purchased from CarboSynth. Pullulan references for the SEC measurements (M_w = 342, 1080, 6100, 8600, 22 000, 47 100, 107 000, 194 000, 337 000, and 708 000 g/mol) were purchased from Polymer Standard Service, Germany.

Dialysis membranes used in the acidic extraction (molecular weight cut off 1 000 g/mol, 6.4 mL/cm) were purchased from Spectrum Laboratories Inc and dialysis tubing cellulose membranes (molecular weight cut off 14 000 g/mol, average flat width 10 mm, 10 mL/ft) used in the hot water extraction were purchased from Sigma Aldrich.

2.3. Extraction of ulvan

Ulvan was extracted using two different protocols as described below in Sections 2.3.1–2.3.2 and outlined schematically in Fig. 2.

2.3.1. Hot water extraction with ethanol wash and enzymatic purification of ulvan from Ulva lactuca and Ulva compressa

The extraction protocol was a combination of individual steps described previously as follows: ethanol wash (Collén, Sassi, Rogniaux, Marfaing, & Helbert, 2011), hot water extraction (Costa et al., 2012), enzymatic hydrolysis (Costa et al., 2012; Pankiewicz et al., 2016) and dialysis (Paradossi et al., 1999). For the initial wash, 2.5 g of freeze-dried biomass (*U. lactuca* and *U. compressa*) was suspended in 25 mL 70 % ethanol and incubated at room temperature for 8 h and 300 rpm shaking. The samples were centrifuged (5000 xg, 4 °C, 5 min), supernatant was discarded, and the wash was repeated 3 times. In the last wash, 96 % ethanol was used, and remaining ethanol was evaporated overnight. The pellet was suspended in 40 mL MilliQ water and incubated at 90 °C, for 3 h and 750 rpm shaking in a Thermomixer. The supernatant was collected in a new tube and the extraction was repeated twice with 35 mL water for 2 h. The supernatants were freeze-dried and combined by dissolving in 45 mL sodium phosphate buffer (0.1 M, pH 6.9) during gentle heating. For hydrolysis of starch, 160 μL of α-amylase (approx. 50 U) was added and the sample incubated at 20 °C for 1 h. For protein hydrolysis, pH was adjusted to 7.5 with NaOH, 20 μL of proteinase K (approx. 16 U) was added and the sample was incubated at 37 °C for 24 h. The enzymatic digestion was terminated by heating to 100 °C for 5 min. The sample was dialyzed with cellulose membrane using 3 L water for 32 h with exchange of water 5 times

before lyophilization. The freeze-dried fraction was washed with 25 mL of 96 % ethanol, centrifuged, and then the supernatant was discarded and dried overnight. The residue was freeze-dried and stored at −20 °C. The ulvan samples extracted with this protocol are herein denoted "ulvan La water" and "ulvan Co water", when isolated from *U. lactuca* and *U. compressa*, respectively.

2.3.2. Acidic extraction of ulvan from Ulva lactuca

To evaluate what benefits a simpler extraction protocol based on fewer steps might have, only *U. lactuca* was taken through the acidic extraction. Freeze-dried *U. lactuca* (8 g) was suspended in 200 mL of 0.01 M HCl (pH = 2) and heated to 90 °C for 4 h. After cooling to room temperature, the mixture was centrifuged at 3850 xg for 10 min. The supernatant was collected, dialyzed with Spectrum membrane for 48 h and then freeze-dried. The freeze-dried fraction was re-dissolved in 15 mL of water and then precipitated with 60 mL of cold EtOH. The mixture was centrifuged at 3850 xg for 10 min and the precipitated fraction was collected and freeze-dried. The ulvan sample extracted with this protocol is herein denoted "ulvan La acid".

2.4. Characterization of the ulvan chemical composition

The color of the extracted ulvans was measured with a Chroma Meter CR-400 (Konica Minolta) using Spectra Magic NX software to collect data and calculate the L* (brightness), a* (greenness/redness) and b* (blueness/yellowness) components.

2.4.1. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of the dried ulvan fractions were recorded between $4000 - 600$ cm^{-1} at room temperature using a Perkin-Eimer Spectrum 2000 FTIR with an attenuated total reflectance crystal accessory (ATR Golden Gate). Corrections were made for atmospheric water and carbon dioxide. The obtained spectra were calculated as an average of 64 scans. The data was analyzed using the PerkinElmer Spectrum software.

2.4.2. Elemental composition and ash analyses

Composition of C, H, N and S elements was determined by a Vario MICRO Cube (Elementar) analyzer using the manufacturer's recommendations on at least two replicates with $2 - 8$ mg of sample weighted to 0.001 mg accuracy.

Ash was determined on $50-60$ mg sample by combusting dried samples (done overnight at 105 °C) in a furnace at 550 °C for at least 5 h until constant weight was recorded. The crucibles used were cleaned beforehand by running the furnace at 550 °C for 3 h.

2.4.3. Thermal gravimetric analysis (TGA)

The char and ash content of freeze-dried *U. lactuca, U. compressa* and extracted ulvans were estimated using a Mettler Toledo TGA/DSC. Approximately 4 mg of freeze-dried ulvan was heated in alumina cups from 40 °C to 800 °C at a heating rate of 10 °C/min under N_2 atmosphere. The flow rate was set to 50 mL/min. After being heated to 800 °C, O_2 was introduced and the temperature was kept at 800 °C for 15 min. The moisture content was calculated as the weight loss sustained when the samples were heated from 40 °C to 110 °C. The char content was calculated as the weight that remained after the samples were heated to 800 °C and the ash content was calculated as the mass that remained after the heating program.

2.4.4. Scanning electron microscopy (SEM) and elemental analysis by energy dispersive x-ray spectroscopy (EDS) of ash after TGA

The residuals remaining after TGA analysis were pooled and observed by ultra-high-resolution field emission scanning electron microscopy (FE-SEM) using a Hitachi S-4800. The samples were air-dried at room temperature overnight and then attached to the sample supports using double-sided adhesive carbon tape and sputter-coated with a 7 nm Pt/Pd layer using a Cressington 208HR under an inert atmosphere. The elemental composition of each sample was assessed via SEM-EDS (Energy Dispersive X-ray Spectrometry) using an X-Max[N] from Oxford instruments. All samples were analyzed at three different areas and the average weight percent (% g/g) was calculated for each element. AZtec 3.0 software was used for imaging SEM-EDS data and calculations.

2.4.5. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The composition of monosaccharides and uronic acids of the extracted ulvans were measured by HPAEC-PAD. Ulvan was hydrolyzed using the method suggested by De Ruiter, Schols, Voragen, and Rombouts (1992) with some modifications. A dried ulvan sample (1.0 mg) was placed in an oven-dried Pyrex tube and 1 mL of 2 M HCl solution in methanol (dried with Na_2SO_4) was added to the tube. The tube was sealed and heated to 100 °C for 5 h in a heating block. The solution was neutralized by adding 200 μL of pyridine to the reaction tube, cooled to room temperature and the solvent was evaporated under flowing N_2 gas. Hydrolysis was performed by adding 1 mL of 2 M trifluoroacetic acid (TFA) to the dried sample, and the solution was heated to 120 °C for 1 h in a heating block followed by cooling to room temperature.

The composition of monosaccharides and uronic acids was determined using a HPAEC-PAD (HPAEC-PAD, ICS-3000 Dionex) with a CarboPac PA1 (4×250 mm) column. The eluent was pumped at 1.5 mL/min using a gradient program starting with 0.10 M NaOH and increasing to 0.16 M NaOH and 0.19 M sodium acetate during the run. A mixture of arabinose, rhamnose, galactose, glucose, xylose, mannose, glucuronic acid and iduronic acid was used as the reference. The data were processed and analyzed using Chromeleon 7.1.

2.4.6. Sulfate content

Sulfate groups on the ulvan backbones were released by hydrolysis in 2 M TFA at $3.3-4$ mg/mL for 3 h at 100 °C according to (Costa et al., 2012). Released sulfate was measured by a turbidimetric sulfate assay kit (product number MAK132, Sigma-Aldrich) with 0.2 M TFA in the standard curve to fit the dilution of samples. Absorbances at 600 nm were measured with a SPECTROstar[Nano] plate reader (BMG LABTECH). Free sulfate in the solid material was determined by dissolving $3.3-4$ mg ulvan in 1 mL milliQ water and heating as for samples, 3 h at

100 °C. Aliquots of the samples were centrifuged at 20 000 xg for 10 min and sulfate was analyzed on the supernatants by the Sigma kit above (standards without TFA). The degree of sulfation was calculated by adjusting the sulfate determined after TFA hydrolysis with the free sulfate level.

2.5. Characterization of polymer characteristics and structure

2.5.1. Size-exclusion chromatography (SEC)

Molecular weight averages and dispersity of the extracted ulvan fractions were estimated using a Dionex Ultimate-3000 HPLC system. Dried ulvan samples (0.5 mg each) were completely dissolved in 1 mL of 10 mM NaOH. Before injection, the samples were filtered through 0.20 μm PTFE nylon filters (Fisher Scientific). The HPLC system was equipped with an WPS-3000SL auto-sampler, an LPG-3400SD gradient pump, three PSS Suprema columns (Polymer Standard Service) in series (300×8 mm, 10 μm particle size) with 30 Å, 1000 Å and 1000 Å pore size, respectively, together with a guard column (50×8 mm, 10 μm particle size) and a Waters-410 refractive index detector. The mobile phase was 10 mM NaOH and the temperature was kept at 40 °C. Pullulan samples with a molecular weight ranging from 342 to 708 000 g/mol were used as references.

2.5.2. Zeta-potential

The zeta-potential was determined on triplicates using a Zetasizer Nano ZS (Malvern Instruments) with a 50 mW diode-pumped solid-state laser (wavelength 532 nm) as light source. The measurements were performed at 25 °C using DTS1070 disposable folded capillary cells. The samples were stabilized for 120 s inside the instrument and five measurements per sample were done with five runs per measurement. Samples were prepared by dilution to 0.05 % g/g, pH was adjusted to 3.5 and run overnight through ion exchange (Dowex® Marathon™ MR-3 hydrogen and hydroxide form) with subsequent removal of resin beads by filtration.

2.5.3. Nuclear magnetic resonance spectrometry (NMR)

The chemical structure of the ulvans was investigated by ^{13}C-NMR on 7 mg samples that were dissolved in 0.7 mL D_2O. The NMR measurements were recorded at 25 °C on an Oxford 800 magnet, Bruker Avance III HD spectrometer equipped with a 5 mm TXO cryoprobe. Standard Bruker pulse programs were applied in the experiments (´zg30´ for ^{13}C-NMR). All spectra were processed and analyzed using MestreNova software.

3. Results and discussion

We developed two protocols for extraction of ulvan from the green macroalgae *U. lactuca* and *U. compressa* collected along the Swedish west coast. The acidic extraction protocol involved fractionation in dilute HCl at elevated temperature and subsequent purification by precipitation into ethanol. The hot water extraction protocol involves an ethanol pre-treatment step, followed by extraction in water at elevated temperature and subsequent purification by enzymatic treatment with α-amylase and proteinase K. Structural and compositional data were compared to commercially available ulvan from *U. armoricana* analyzed in parallel. Such data will give a deeper understanding of the exact composition and structural elements of the extracted ulvans and how ulvans vary between different species as well as how the extraction technique affects the composition.

The weight yield of ulvan extracted from *U. lactuca* using acidic extraction (ulvan La acid) was 18 ± 2 % (g/g dry starting material) and 11 ± 3 % when using water extraction (ulvan La water) (mean from triplicate extractions with standard deviation given after ± sign). These yields could potentially be improved since no optimization was done in this work. The yield of extraction from *U. compressa* was not recorded but assumed to have similar efficiency as for *U. lactuca*.

Table 1

The color of extracted ulvans expressed as L*-brightness, a*greenness/redness and b*-blueness/yellowness. At least 7 measurements were taken on the samples; means and standard deviations are shown.

Sample	L*	a*	b*
ulvan La acid	84.4 ± 0.0	0.08 ± 0.01	7.59 ± 0.01
ulvan La water	84.9 ± 0.3	− 2.45 ± 0.06	7.38 ± 0.15
ulvan Co water	82.9 ± 0.3	− 0.97 ± 0.03	6.72 ± 0.06

3.1. Characterization of ulvan chemical composition

An impurity that can be present in ulvans is pigments (Costa et al., 2012; Glasson et al., 2017). The extracted ulvans were less pigmented than the reference ulvan (that was darker and with a more brown/yellow color). From the color measurements it can be concluded that they were all very light (L* = 83–85 with rather low values of the two other color components), indicating that our methods reduced pigments quite well (Table 1). The largest component of the color was the yellowness (positive b*), with the highest value for ulvan La acid and the lowest for ulvan Co water. The ulvan La water also had a fairly large negative a* value indicating a more greenish color. Thus, the water extraction methods appear to be slightly better than the acid method in removing pigments.

3.1.1. General chemical composition by FTIR and elemental analysis

FTIR spectra of the extracted ulvans showed many of the characteristic peaks for polysaccharides. Lists of identified peaks and peaks designations are available in supplementary data, Tables S1–S4. The broad band at $3500 - 3200\ cm^{-1}$ corresponds to the O−H stretching for the hydroxy groups and the bands from $3000 - 2800\ cm^{-1}$ correspond to C–H stretching. The sharp peak around $1020 - 1050\ cm^{-1}$ corresponds to symmetric stretching of C−O−C linkages. The carboxyl groups in the uronic acid moieties show two characteristic peaks: one symmetric stretching around $1620 - 1600\ cm^{-1}$ and one asymmetric stretching around $1420\ cm^{-1}$. The small peak around $895\ cm^{-1}$ is characteristic of the β-glycoside bond, which is an indication of the presence of polysaccharides. The peak at $1220\ cm^{-1}$ corresponds to S=O stretching of sulfate groups and the peak at $840\ cm^{-1}$ is characteristic of C−O−S stretching, suggesting the presence of sulfate groups, which are usually abundant in ulvan. All the extracted ulvans showed the same characteristics and were very similar to the reference ulvan (Fig. 3) and previously published spectra (Robic, Bertrand, Sassi, Lerat, & Lahaye, 2009).

Protein is one component that has been indicated as an important impurity in extracted ulvan (Costa et al., 2012; Glasson et al., 2017).
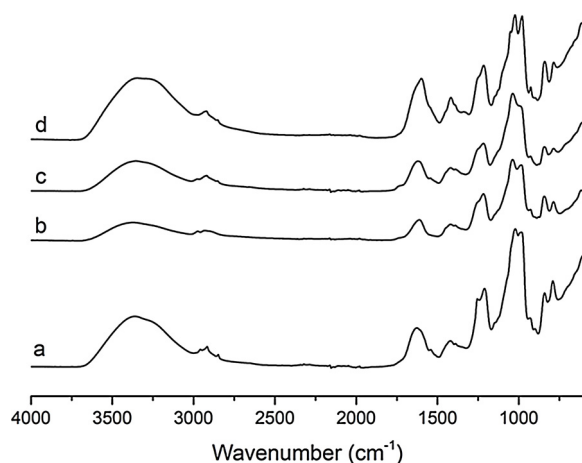

Fig. 3. FTIR spectra of extracted and reference ulvans: a) ulvan La acid, b) ulvan La water, c) ulvan Co water, and d) reference ulvan.

Table 2

Elemental composition (% g/g ulvan) of extracted and reference ulvans. Data are given as the mean values with ± standard deviations from triplicate measurements.

Sample	Carbon	Hydrogen	Nitrogen	Sulfur
ulvan La acid	27.32 ± 0.28	5.63 ± 0.14	1.34 ± 0.03	5.62 ± 0.27
ulvan La water	30.39 ± 0.95	5.47 ± 0.08	0.66 ± 0.02	6.01 ± 0.60
ulvan Co water	33.79 ± 0.38	6.02 ± 0.03	1.30 ± 0.01	4.29 ± 0.18
reference ulvan[a]	30.44 ± 0.31	5.68 ± 0.05	1.47 ± 0.03	4.33 ± 0.48

[a] Duplicates.

Proteins are characterized by amide bonds, which results in peaks around $1600\ cm^{-1}$ in the FTIR spectra and particularly, N−H bending at $1545\ cm^{-1}$ (Mayers, Flynn, & Shields, 2013). The intensity of this peak in the spectra was compared to the peak at $1030\ cm^{-1}$ specific for polysaccharides (Supplementary Table S5). Interestingly, the *U. lactuca* ulvan extracted by the water method (ulvan La water), which employs protein degrading enzymes, showed the lowest ratio of 0.06. The highest ratio of 0.26 was seen for the reference ulvan. This is consistent with the finding that oxalate-based extraction methods result in higher protein impurities than acid-based methods (Glasson et al., 2017). To verify the indication of protein content, the elemental composition of the ulvans was determined (Table 2). It was confirmed that the ulvan La water contained the lowest amount of nitrogen (0.66 %), whereas the reference ulvan contained the highest amount of nitrogen (1.47 %). This is consistent with previously reported data for the reference ulvan recovered from *U. armoricana* harvested at different seasons (1.06–2.22 % g N/g) (Robic, Sassi et al., 2009). From the nitrogen data, the protein content can be calculated using the universal seaweed nitrogen conversion factor of 5 (Angell, Mata, de Nys, & Paul, 2016). Thus, the protein content was 6.7 ± 0.2 % in ulvan La acid, 3.3 ± 0.4 % in ulvan La water, 6.5 ± 0.1 % in ulvan Co water and 7.4 ± 0.2 % in reference ulvan. These data indicate that the extraction for *U. compressa* was not fully satisfactorily, particularly that the treatment with proteinase did not degrade protein impurities adequately.

The other elements, C, H, S, were found at similar levels in all the ulvans. The sulfur contents determined, 4–6 %, were in agreement with previously reported data, 3.6–4.7 % (Glasson et al., 2017) and 8.4 % (Costa et al., 2012). Sulfur is found in the sulfate groups of ulvan and possibly also in sulfur-containing amino acids (such as cysteine and/or methionine) in the proteins.

3.1.2. Char and ash content and elemental composition of the ash

The char and ash content in freeze-dried *U. lactuca* and *U. compressa* both before fractionation as well as from extracted ulvans were calculated from the TGA thermogram (Table 3). The TGA thermograms and the weight loss diagrams are shown in Supplementary Figs. S1-S6. Samples exhibited a small mass loss (5–10 %) between 25–150 °C

Table 3

The char and ash content (% g/g ulvan) in freeze-dried *U. lactuca* and *U. compressa* before fractionation, in reference ulvan and in extracted ulvans. Data are given as the mean with ± standard deviations of triplicates for TGA-based data and as the mean of duplicates for weights after combustion.

Sample	Char	Ash (TGA)	Ash (combustion)
ulvan La acid	25.2 ± 2.6	14.5 ± 0.8	12.1 ± 0.1
ulvan La water	28.6 ± 1.9	20.3 ± 2.6	17.8 ± 0.4
ulvan Co water	26.2 ± 1.9	15.8 ± 0.2	10.9 ± 1.3
reference ulvan	27.1 ± 1.7	19.0 ± 1.0	14.0[a]
U. lactuca before ulvan extraction	37.0 ± 4.2	21.5 ± 2.1	NM
U. compressa before ulvan extraction	37.5 ± 1.4	25.8 ± 1.6	NM

NM-not measured.
[a] Single measurement.

Table 4
Mineral elements (% g/g of total detected elements as determined by SEM-EDS) in pooled ashes remaining after TGA analysis of whole *U. lactuca* and *U. compressa* biomass, reference ulvan, and extracted samples. Data are given as the mean values of three different areas for each sample.

Element	ulvan La acid	ulvan La water	ulvan Co water	reference ulvan	*U. lactuca* before ulvan extraction	*U. compressa* before ulvan extraction
Na	9.7	27.7	10.0	13.1	4.0	13.9
Mg	23.3	–	22.8	15.1	31.3	7.9
Al	–	0.5	–	–	–	0.1
P	0.7	0.6	2.3	0.6	0.3	1.2
S	6.4	14.7	8.1	8.7	2.1	3.5
Cl	10.0	1.4	–	30.4	8.6	38.5
Ca	1.2	–	–	–	–	3.6
K	7.6	2.1	–	12.5	2.6	8.1

- below detection limit.

corresponding to evaporation of water. All ulvan samples undergo major weight loss between 200 °C and 300 °C, after which a char residue remains. This weight loss, with a peak disintegration temperature around 230 °C, is typical for polysaccharides and corresponds to the cleavage of glycosidic linkages. The ash content of *U. lactuca,* 21.5 %, corresponds well with the ash content reported in previous studies (Yaich et al., 2011). The extracted ulvan samples had an ash content ranging from 14 to 20 % based on TGA. However, the ash content after combustion was lower, 11–18 %, but showed the same trends. These values are on the lower end of the range of published data (Adrien et al., 2017; Alves et al., 2010; Costa et al., 2012; Glasson et al., 2017; Robic, Sassi et al., 2009), indicating that our protocols resulted in high quality ulvan with low levels of impurities. These data indicate that both the source of the extracted ulvan and the extraction technique has a strong influence on the total ash content.

The ash remaining after TGA analysis was further assessed with SEM-EDS to detect the elemental composition of the ash, above all the inorganic content (Table 4). Some carbon was detected in these samples (data not shown) supporting the fact that the ash determination by combustion gave lower values than using TGA. It is important to note that the instrument used is not equipped to analyze light elements (atom number < 10), generates highly approximate measurements for atom numbers up to 16 and cannot detect hydrogen at all. Thus, the results are more informative for heavier elements. The exact composition can vary from one point in the sample to the next and thus calculated averages are given in Table 4. Approximately 9.7–27.7 % sodium (Na) was found in the ulvan fractions and this result is likely due to sodium acting as the counterion to ulvan. Al, Mg, Cl, P, K and Ca were also found, and their presence may be due to the composition of the seawater from which the seaweeds were collected. The high content of Mg in ulvan La acid and the reference ulvan can be explained by the strong affinity of ulvan to metal ions (Lahaye & Robic, 2007).

Importantly, no heavy metals or other toxic elements, such as Pb, Hg, or As were detected.

3.1.3. Monosaccharide and uronic acid composition and degree of sulfation

The monosaccharide and uronic acid composition of extracted ulvans where measured by HPAEC-PAD. However, an accurate determination of monosaccharides and uronic acids in ulvan is difficult. Previous studies have shown that hydrolysis of ulvan with strong acids such as sulfuric acid leads to partial degradation of iduronic acid (Conrad, 1980). Additionally, the glycosidic linkages between rhamnose and uronic acid exhibit resistance to acid hydrolysis (Quemener et al., 1997). However, a combination of methanolysis and a mild acid hydrolysis leads to efficient cleaving of all glycosidic bonds and complete release of the monosaccharides and uronic acids (De Ruiter et al., 1992).

The estimated carbohydrate content in the ulvan samples ranges from 76.8–79.9 % g/g (Table 5). The chromatograms with assigned peaks from the HPAEC-PAD analysis are shown in Supplementary Fig. S7. As expected, all samples contained rhamnose, xylose, glucuronic acid and iduronic acid, which are typical building blocks in ulvan. Glucose as well as trace amounts of mannose were also present in all samples, which is in agreement with previous studies (Glasson et al., 2017; Quemener et al., 1997; Yaich et al., 2013). No arabinose or galactose were found in any of the samples.

A difference in carbohydrate composition was observed between ulvan La water and ulvan La acid, which indicates that the composition of the extracted ulvan fractions from *U. lactuca* depends on the extraction conditions. Ulvan La acid had a higher content of glucose than ulvan La water (Table 5). On the other hand, ulvan La water showed a higher content of rhamnose and glucuronic acid. The higher content of glucose in ulvan La acid may be explained by the fact that the acid-based extraction method used did not include any treatment with α-amylase to remove the starch that is co-extracted with the ulvan. Therefore, ulvan La acid is likely to contain a higher level of starch than ulvan La water, which would lead to a higher content of glucose in ulvan La acid as found. The carbohydrate composition also varied between different species of *Ulva* as evident by comparison of monosaccharide composition of ulvan Co water and ulvan La water. Ulvan Co water exhibited a higher content of iduronic acid, glucose and a lower content of xylose than ulvan La water, but the contents of rhamnose and glucuronic acid were similar for both samples. This is an indication that ulvan extracted from *U. compressa* has a higher content of iduronic acid than ulvan extracted from *U. lactuca*. Another interesting observation is that the peak for iduronic acid in the HPAEC-PAD chromatograms (Fig. S7) appears as a double-peak in the extracted ulvan samples but appears as a single peak for the reference ulvan. Iduronic acid is a hexapyranose sugar, which typically occurs in a pyranose configuration. However, previous NMR-studies on ulvan extracted from *U. lactuca* suggest that a minor part of the iduronic acid might exist in the less

Table 5
The monosaccharide and uronic acid composition (% g/g ulvan), with the relative % of total mass carbohydrates in brackets, and degree of sulfation (% g SO₃/g ulvan). The abbreviations for the carbohydrates are as follows: Rha = Rhamnose, Ara = Arabinose, Gal = Galactose, Glc = Glucose, Xyl = Xylose, Man = Mannose, GlcA = Glucuronic acid, and IduA = Iduronic acid. The total carbohydrate content was calculated as the sum of all monosaccharides and uronic acids in each sample.

Sample	Carbohydrates									Degree sulfation [a]
	Rha	Ara	Gal	Glc	Xyl	Man	GlcA	IduA	CH Total	
ulvan La acid	26.6 (34.6)	- (-)	- (-)	12.3 (16.0)	11.5 (15.0)	0.1 (0.1)	22.1 (28.8)	4.3 (5.6)	76.8	< 0.5
ulvan La water	31.8 (41.4)	- (-)	- (-)	2.7 (3.5)	11.6 (15.1)	0.1 (0.1)	26.8 (34.9)	3.8 (4.9)	76.8	14.3 ± 0.6
ulvan Co water	32.5 (40.7)	- (-)	- (-)	6.9 (8.6)	8.2 (10.3)	0.1 (0.1)	26.2 (32.8)	6.0 (7.5)	79.9	9.3 ± 0.3
reference ulvan	32.0 (40.8)	- (-)	- (-)	7.5 (9.6)	5.8 (7.4)	0.1 (0.1)	20.8 (26.6)	12.1 (15.5)	78.3	8.7 ± 0.5

- below detection limit.
[a] Free sulfate for ulvan La acid was 17.8 ± 0.6 % g SO₄/g ulvan and for ulvan La water, ulvan Co water and reference ulvan was 0.01-0.03 % g SO₄/g ulvan (given as mean and standard deviation of triplicates).

stable hexafuranose form (Quemener et al., 1997). The hexapyranose and hexafuranose forms of iduronic acid possibly have slightly different elution times and therefore the iduronic acid appears as a double-peak, where one of the peaks corresponds to the pyranose form and the other peak corresponds to the furanose form of iduronic acid. As no double peak was observed for the iduronic acid in the reference ulvan, possibly all the iduronic acid was in the hexapyranose form rather than a mixture of the two possible configurations. Another possible explanation is that the extracted ulvan samples were contaminated with a compound that was co-extracted with the ulvan. If the peak for this compound appears very close to the peak for iduronic acid, it might result in a double-peak. However, when the total amount of iduronic acid in the extracted ulvan was calculated, it was assumed that both peaks correlate with iduronic acid.

Sulfation is another feature of ulvan and it was found that the extraction protocol influenced the amount of sulfate groups bound (Table 5). The ulvan La water sample showed the highest degree of sulfation, 14.3 %. On the other hand, the acid-based extraction protocol resulted in removal of the sulfate groups, which were present as free sulfate in the sample. The degree of sulfation for the ulvan Co water and reference ulvan was rather similar at 9.3 and 8.7 %, respectively. The different location of sulfate in the samples—bound to the carbohydrate chain in water-extracted samples and reference ulvan or found as free sulfate in ulvan La acid—could be indicated in the FTIR spectra. The S=O stretch may be affected if the sulfate is bound or not; indeed, this peak was shifted to $1228\ cm^{-1}$ for the ulvan La acid as compared to the similar wavenumber $(1215-1218\ cm^{-1})$ found for the other ulvans (Tables S1–4). The sulfur present in sulfate comprised 80–97 % of the total sulfur determined in the elemental analysis (reference ulvan at lower end and both ulvan La water and La acid at higher end). Our data on degree of sulfation were found to be in agreement with previously reported data of ulvans extracted from the same species: 9.2–12.5 % for the reference ulvan (Robic, Sassi et al., 2009), 13.45 % for *U. lactuca* by acid/enzymatic extraction (Yaich et al., 2014), 15.88 % for *U. lactuca* by acid extraction (Yaich et al., 2014), and 26–32 % for *U. lactuca* by hot water extraction (Costa et al., 2012).

With the knowledge of that ulvan La acid has no bound sulfate groups, the C-molar formula of the ulvans can be calculated from the elemental and ash contents. The residual of the ash-free weight was assumed to be oxygen. The formulas were: for ulvan La acid $CH_{2.45}O_{1.01}N_{0.04}$, for ulvan La water $CH_{2.14}O_{0.76}N_{0.02}(SO3)_{0.07}$, ulvan Co water $CH_{2.12}O_{0.83}N_{0.04}(SO3)_{0.03}$ and for reference ulvan $CH_{2.22}O_{0.93}N_{0.04}(SO3)_{0.05}$.

3.2. Characterization of polymeric characteristics and structure

3.2.1. Molecular weight and zeta-potential

The average molecular weights of the ulvans varied between different species of *Ulva* (Table 6). It is worth noting that SEC is a blunt method for assessing the molecular weight of ulvan in the sense that the molecular weights of samples are obtained in relation to a reference compound of known molecular weight. The chosen reference may differ in terms of hydrodynamic volume and solvent interactions (Yamamoto, Tadokoro, Imai, & Mita, 1980). Ulvan is an anionic polysaccharide while the calibration standards used in the measurements (pullulan) is a neutral polysaccharide. Pullulan and ulvan are likely to have different affinities to the eluent so their hydrodynamic volumes are not fully comparable. The obtained values are therefore estimates. Still, internal comparisons are possible. The molecular weight of the reference ulvan, extracted from *U. armoricana*, was almost twice as high as that of the ulvans extracted from *U. lactuca* and *U. compressa*, respectively. Nevertheless, the data for *U. lactuca* are in the same range as molecular weights reported at 775 kDa (Costa et al., 2012) and the molecular weight of the reference ulvan is in agreement with information from the producer (molecular weights up to 980 000 g/mol).

In addition, the molecular weight depends on the extraction conditions as evident by comparison of the molecular weights of ulvan La acid and ulvan La water. This is in line with a previous study on ulvan extracts from *U. rotundata* using different extraction protocols (Robic, Rondeau-Mouro et al., 2009). The average molecular weight of ulvan La acid was somewhat higher than for ulvan La water. Ulvan La acid was extracted at a lower pH which may have led to the liberation of some high molecular weight ulvan chains. However, too low of a pH during acid extraction may also lead to depolymerization and molecular weight reduction of ulvan (Glasson et al., 2017). In contrast to the very low degree of sulfation in ulvan La acid, the ulvans extracted according to the hot water protocol and the reference ulvan are still substituted with sulfate groups to some extent (9–14 %) (Table 5). Hence, ulvan La acid has a much more neutral character than the other ulvans, and therefore should have a hydrodynamic volume in the SEC eluent that is more similar to that of the calibration standard pullulan. This has to be taken into account when comparing the water-extracted ulvans to the acid-extracted one.

Another possible explanation for the fact that ulvan La acid had a higher molecular weight than ulvan La water is that the elemental analysis of the ash (Table 4) shows that Mg is present in ulvan La acid, but not in ulvan La water. Previous studies have shown that the presence of metal ions causes aggregation of the ulvan molecules (Paradossi, Cavalieri, & Chiessi, 2002). The presence of Mg in ulvan La acid may lead to a higher degree of aggregation than for ulvan La water, so the estimated hydrodynamic volume of ulvan La acid is probably the hydrodynamic volume of a cluster of ulvan molecules rather than the individual chain coils. The aggregation of the ulvan chains in the presence of Mg might also explain why the estimated molecular weight of the reference ulvan was so high.

The dispersity of the extracted ulvan fractions ranges from 1.1 to 1.2 which indicates a narrow distribution of molecular weight. The low dispersity of the extracted ulvan is in line with previous studies (Yamamoto et al., 1980). Zeta-potential values provide an indirect measurement of the surface charge of the ulvans dispersed in water. The zeta-potential was substantially higher for ulvan La acid $(-44\ mV)$ compared to the other ulvans (-53 to $-59\ mV$) (Table 6). This finding is consistent with the observation that ulvan La acid is a less negatively charged molecule as a result of the lower degree of sulfation.

3.2.2. Structure by NMR

To get additional information on the ulvan structures, ^{13}C NMR analyses were performed on the two ulvans from *U. lactuca* and on the reference ulvan (Fig. S8). The samples appeared to be more complex as compared to other ulvans isolated and characterized (Lahaye & Robic, 2007; Lahaye, Inizan, & Vigouroux, 1998). Compared to previously published ulvans, such as ones from *U. armoricana*, *U. scandinavia*, *U. rotundata* and *U. rigida*, the ulvans in this paper have more signals in the acetal, 1–4 linkage area, $90-110$ ppm (Lahaye et al., 1999). From this acetal region, $90-110$ ppm, it can be seen that all samples have signals from rhamnose, xylose, glucuronic acid and iduronic acid. There was some difference between the samples with regards to chemical shift and

Table 6
Molecular weight averages and dispersities (Đ) of the extracted and reference ulvans, determined on single samples. The zeta potential was determined on duplicates with 5 measurements on each replicate and mean and standard deviation are given.

Sample	M_n (g/mol) [a]	M_w (g/mol) [b]	$Đ = (M_w / M_n)$	Zeta-potential (mV)
ulvan La acid	673 700	761 540	1.1	-44.0 ± 2.0
ulvan La water	562 590	657 025	1.2	-59.2 ± 2.8
ulvan Co water	634 360	685 910	1.1	NM
reference ulvan	1 117 100	1 172 170	1.1	-53.0 ± 2.0

[a] Number-average molecular weight.
[b] Weight-average molecular weight, NM-not measured.

the frequencies of the signals. This indicates heterogeneity between the samples even though they have rather similar carbohydrate content, as measured in the carbohydrate analysis. The carbonyl signal showed different features for the different ulvans. Sample ulvan La acid has two distinct carbonyl signals, $170-180$ ppm, indicating that the ulvan contains two different acids, which is in agreement with the carbohydrate analysis in which we identified glucuronic acid and iduronic acid. For sample ulvan La water there is only one distinct carbonyl signal, 175 ppm. The ^{13}C spectra of *U. armoricana* differed slightly compared to spectra published in the literature (Lahaye et al., 1999), which is due to the inherent heterogeneity of natural polysaccharides.

3.3. Comparison between extraction protocols

Two different extraction protocols for ulvan were used and compared in this study. The extraction with hydrochloric acid is a one-step process and is therefore significantly faster than the multi-step water-based extraction, which also has a higher cost due to the use of enzymatic purification. The extraction with hydrochloric acid also resulted in a higher yield of product than the water-based extraction. However, the water-based extraction leads to a purer and more unaltered ulvan fraction as evidenced by the lower starch content and higher degree of sulfation in the ulvan fractions. Whether the acid-based or water-based extraction protocol is preferred depends completely on the final application of the ulvan. For biomedical applications, the water-based extraction followed by enzymatic purification would be preferred since it leads to a purer ulvan fraction that is still sulfated. High purity is essential for biomedical applications because contaminants such as starch and proteins could potentially disrupt the biological function of ulvan. Additionally, sulfation of ulvan is important for maintaining its biological activity (Kidgell, Magnusson, de Nys, & Glasson, 2019). For other applications where the purity of the extracted ulvan is less important, the acidic extraction is preferred because it is faster, cheaper and gives higher yields than the water-based extraction. Several studies have shown that ulvan can be used in applications such as nanofibers (Toskas et al., 2011) and hydrogels (Morelli et al., 2016). In both these cited studies, the extracted ulvan did not undergo any enzymatic purification and was instead obtained using a one-step extraction procedure. Potentially, both our methods could be preferred over the traditional oxalate extraction used for the commercial reference ulvan since our ulvans generally contained less impurities.

4. Conclusions

The sulfated polysaccharide ulvan was successfully extracted and isolated from the green macroalgae *Ulva lactuca* and *Ulva compressa*. Two different extraction protocols were developed and compared: i) extraction with hot water followed by enzymatic purification, and ii) extraction with hydrochloric acid. The extraction protocol, as well as the seaweed species used in the extraction, had a strong impact on the purity, degree of sulfation, molecular weight, and monosaccharide composition. The total carbohydrate content of the extracted ulvan was in the range 76–79 % w/w. All samples had a low content of proteins and residual pigments. The extraction with hydrochloric acid removed almost all the sulfate groups from the ulvan. Monosaccharide analysis showed that the extracted ulvans contained rhamnose, glucuronic acid, iduronic acid, and xylose as the main carbohydrate building blocks. Ulvan extracted with hydrochloric acid had a significantly higher content of glucose than all the other samples, probably due to a higher content of starch that was co-extracted along with ulvan.

Declaration of Competing Interest

None.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.carbpol.2020.115852.

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