Algal Research 27 (2017) 383–391

Contents lists available at ScienceDirect

Algal Research

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

A cascading biorefinery process targeting sulfated polysaccharides (ulvan) from *Ulva ohnoi*

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ARTICLE INFO

Keywords:
Seaweed
Bioremediation
Chlorophyta
Ulva
Ulvans
Biorefinery

ABSTRACT

We evaluated eight biorefinery processes targeting the extraction of ulvan from *Ulva ohnoi*. Using a factorial design the effect of three sequential treatments (aqueous extraction of salt; ethanol extraction of pigments; and $Na_2C_2O_4$ or HCl (0.05 M) extraction of ulvan) were evaluated based on the yield (% dry weight of biomass) and quality (uronic acid, sulfate, protein and ash content, constituent sugar and molecular weight analysis) of ulvan extracted. The aqueous extraction of salt followed by HCl extraction of ulvan gave higher yields (8.2 ± 1.1% *w*/w) and purity of ulvan than equivalent $Na_2C_2O_4$ extracts (4.0 ± 1.0% *w*/w). The total sugar content of HCl extracts (624–670 μg/mg) was higher than $Na_2C_2O_4$ extracts (365–426 μg/mg) as determined by constituent sugar with ulvan specific monosaccharides contributing 94.7–96.2% and 70.1–84.0%, respectively. Ulvan extracted from *U. ohnoi* was 53.1 mol% rhamnose, 27.8 mol% glucuronic acid, 10.1 mol% iduronic acid, and 5.3 mol% xylose with molecular weights ranging from 10.5–312 kDa depending on the biorefinery process employed. Therefore, the extraction of high quality ulvan from *U. ohnoi* is facilitated by an aqueous pre-treatment and subsequent HCl-extraction of ulvan as part of a cascading biorefinery model delivering salt, ulvan, and a protein enriched residual biomass.

1. Introduction

The intensive and targeted cultivation of macroalgae, both marine and freshwater, has been implemented as a mechanism to mitigate impacts from anthropogenic wastewaters. This process has the benefit of remediating contaminants from wastewaters, in particular nitrogen and phosphorous, through incorporation within the macroalgal biomass, which is then harvested and can be used as a bio-resource. Marine macroalgae have been the focus of this process because of their robustness, high productivities, novel biochemical profiles and metabolites, and ability to be cultivated at scale [1]. Species of the macroalgal genus *Ulva* (chlorophyta) are particularly suitable because of their high productivity and resilience to diverse growing conditions. These characteristics specifically facilitate the culture of species of the genus for the bioremediation of wastewaters produced from intensive land-based aquaculture of marine and brackish water fish and invertebrates in temperate and tropical regions [2–4]. Importantly, the algal biomass from this process can be used for applications ranging from animal feed supplements [5,6], fertilisers [7], composts [8,9], foods [10] and dietary supplements and nutraceuticals [11,12]. However, for this process to be cost effective it is essential to obtain the optimum value from the biomass. This has resulted in a focus on biorefinery processes where biomass is used as a feedstock for the production of high-value and other value-added products [1,13].

One product of specific interest in *Ulva* is the soluble fibre ulvan, which is a significant component of the cell wall of the alga [14,15]. Ulvans constitute between 8 and 29% of the dry weight (dw) of *Ulva* depending on species and growth conditions [16]. These complex sulfated polysaccharides are of biomedical interest for applications in tissue engineering, drug delivery and biofilm prevention [17,18]. Ulvans also have antiviral, antioxidant, anticoagulant, antihyperlipidemic and anticancer activity, in addition to immunostimulatory effects [14,15]. Structurally, ulvans are unique with mostly repeating disaccharide units composed of sulfated rhamnose with glucuronic acid, iduronic acid or xylose [15]. The two major disaccharides are designated as aldobiuronic acids; type A: ulvanobiuronic acid 3-sulfate (A_{3s}), a 1,4-linked glucuronic acid with *O*-3-sulfated rhamnose, and type B: ulvanobiuronic acid 3-sulfate (B_{3s}), a 1,4-linked iduronic acid with *O*-3-sulfated rhamnose (Fig. 1). Partially *O*-2-sulfated xylose can also occur in place of uronic acids affording aldobioses, U_{3s} and $U_{2's,3s}$ [15]. Both

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http://dx.doi.org/10.1016/j.algal.2017.07.001
Received 14 March 2017; Received in revised form 30 May 2017; Accepted 2 July 2017
Available online 26 July 2017

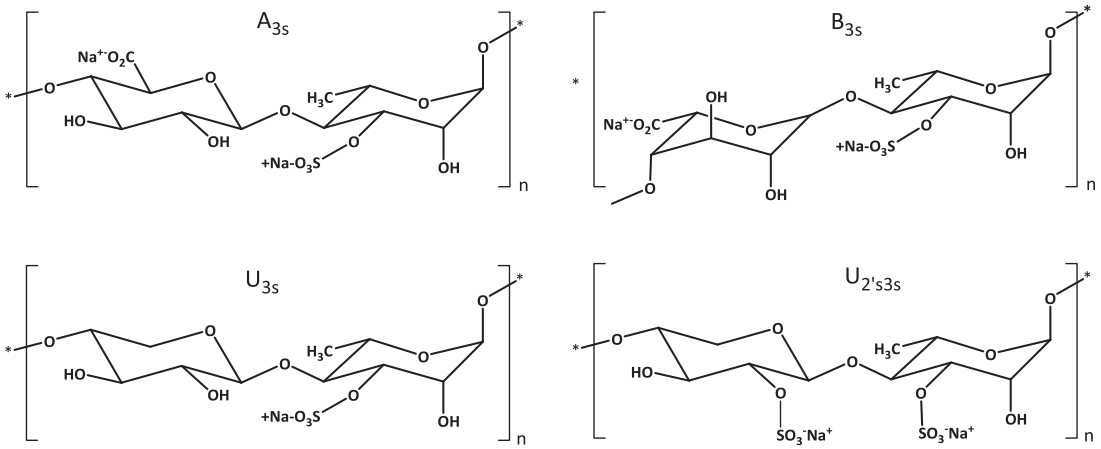

Fig. 1. Main repeating disaccharides in ulvans from species of *Ulva* include aldobiuronic acids (A_{3s} and B_{3s}) and aldobioses (U_{3s} and $U_{2's3s}$) (adapted from Robic et al. [19]).

species and season have demonstrated effects on the chemical structure, macromolecular characteristics, and rheological properties of ulvan extracts [16] and the physical and chemical properties of ulvan are also dependent on extraction methods [19] and stabilisation procedures [20].

Notably, the fractionation of ulvans from other cell wall components, for example glucuronans, xyloglucans, cellulose and proteins, represents a significant challenge. Conventionally ulvans are extracted at 80–90 °C in aqueous solutions of sodium oxalate or ammonium oxalate to chelate the Ca^{2+} that crosslinks ulvan strands in the cell wall [15,19]. In a seminal study ulvan was extracted from *U. rotunda* (stabilised using a variety of methods prior to extraction), at 85 °C in 0.05 M sodium oxalate, with 25–60% recovery [20]. However, the extracts also contained significant content of proteins (up to 35%) and salts (up to 30%).

Our objective is to develop and assess a cascading biorefinery process for the species *Ulva ohnoi* Hiraoka et Shimada, used for the bioremediation of nutrients (N and P) from intensive land-based aquaculture [2], to extract ulvan while minimizing the content of salts and proteins. To do this we examine the effects of pre-washing the biomass, the pre-extraction of pigments, and alternative methods for the extraction of ulvan, using a factorial design. The initial pre-washing of biomass is targeted to extract salts with a low Na: high K ratio as an initial product for the functional food market while facilitating the improved yield and quality of ulvan [21]. The subsequent pre-extraction of pigments is also targeted to improve quality. Finally, comparison of the extraction of ulvan using sodium oxalate and hydrochloric acid is targeted to optimise both yield and quality. Extracts obtained from these processes are subsequently assessed for product quality in terms of purity and chemical composition while the structure of ulvan extracts are determined using constituent sugar and molecular weight analysis and NMR spectroscopy.

2. General methods

2.1. Cultivation of biomass

Ulva ohnoi Hiraoka et Shimada (Genbank accession number KF195501, strain JCU 1 [2]) is domesticated and was collected from a land-based aquaculture facility near Ayr (19°29′S, 147°28′E), Queensland, Australia, where it is cultivated commercially. Biomass was harvested weekly over three consecutive production cycles ($n = 3$) of 7-days in April 2016. Harvested biomass samples (8 × 100 g fresh weight [fw]) were collected and stored (− 20 °C) in separate zip-lock bags until extraction of ulvan as described in Sections 2.3–2.5. Samples (100 g fw) were also taken from each harvest and dried (60 °C, 24 h) to determine the fresh weight to dry weight ratio (fw:dw) and composition of the starting material (untreated biomass) as described in Section 2.6. Harvest specific fw:dw ratios were used to calculate crude extract yields as g extract per g dw biomass.

2.2. Experimental design

This study tests the assumption that the sequential removal of salts and pigments in a cascading biorefinery process will improve the yield and quality of ulvan extracted in a subsequent step. We use a factorial experimental design to quantify the effect of three extraction treatments; treatment 1 targets the extraction of salts; treatment 2 targets the extraction of pigments; and treatment 3 targets the extraction of ulvan (Fig. 2). During treatment 1, biomass was either subjected to a warm aqueous extraction (Salt Reduced Biomass = SRB) or left untreated (Control Biomass = CB). During treatment 2, biomass was extracted with ethanol (Pigment Reduced Biomass = PRB, and Salt and Pigment Reduced Biomass = SPRB) or left untreated (CB and SRB). During treatment 3, biomass was extracted with 0.05 M $Na_2C_2O_4$ (EP1, EP3, EP5, EP7) or 0.05 M HCl (EP2, EP4, EP6, EP8). To quantify variation between harvests the experiment was conducted in triplicate from three harvests over a 3 week period.

2.3. Treatment 1: extraction of salts

Ulva ohnoi biomass was treated as per Magnusson et al. [21]. Briefly, whole *U. ohnoi* biomass (100 g fw) was submerged in distilled water (1 L; biomass to water ratio of 1:10) at 40 °C for 30 min using a temperature controlled water-bath. Following this the biomass was recovered by filtration through a 200 µm mesh filter.

2.4. Treatment 2: extraction of pigments

Ulva ohnoi biomass was treated using a modification of the methodology by Robic et al. [19]. Residual *U. ohnoi* biomass from previous treatments was suspended in absolute ethanol (1 L) at room temperature for 1 h. The biomass was isolated by filtration (200 µm mesh) and the extraction procedure described above repeated 2 more times.

2.5. Treatment 3: extraction of ulvan

Sodium oxalate extraction method [19]. Residual *U. ohnoi* biomass from previous treatments was suspended in 0.05 M $Na_2C_2O_4$ (1 L) and heated at 85 °C for 1 h. The suspension was filtered (200 µm mesh) prior to vacuum filtration through diatomaceous earth (Celatom®). The extract was then filtered (Whatman® GF/F), concentrated (10 ×) by ultrafiltration (ÄKTA flux 6 fitted with a Xampler 10 kDa NMWC Cartridge), diafiltered (5 volumes of deionised water) and freeze dried.

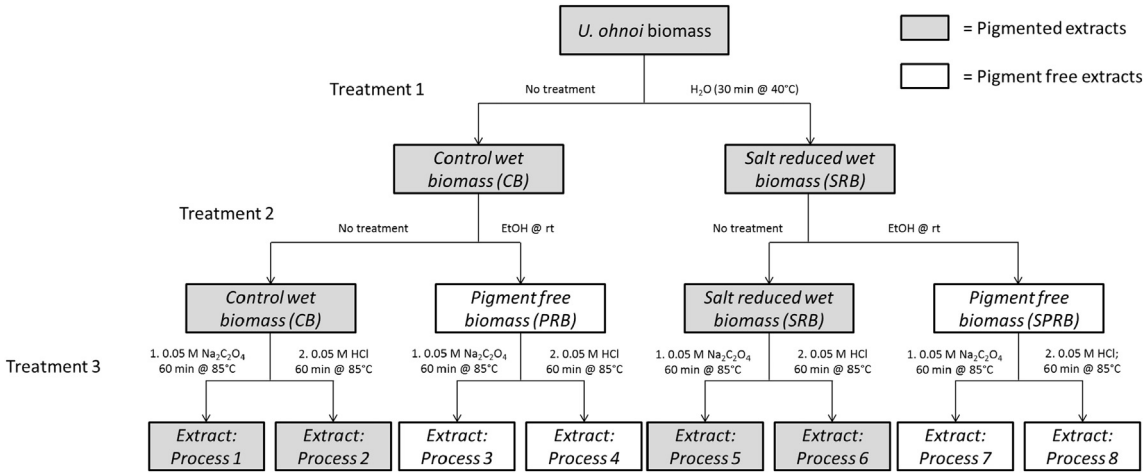

Fig. 2. Schematic representation of the eight processes used to investigate the yield and quality of ulvan.

Hydrochloric acid extraction method [19]. Residual *U. ohnoi* biomass from previous treatments was suspended in 0.05 M HCl (1 L) and heated at 85 °C for 1 h. The suspension was filtered (200 μm mesh) prior to vacuum filtration through diatomaceous earth (Celatom®). The extract was then filtered (Whatman® GF/F) prior to adjusting the pH to 7 by the addition of 1 M NaOH. The filtrate was concentrated, diafiltered and freeze-dried as above.

2.6. Characterisation of the untreated biomass and crude ulvan extracts

Chemical compositions of the untreated biomass ($n = 3$) and ulvan extracts (8 processes, $n = 3$ per process unless otherwise stated) were quantified as follows. Elemental analysis (% C, H, N, S; $n = 1$ per process, where sub-samples of extracts from the 3 replicates were pooled and analysed in duplicate) and determination of ash content (% dw) were conducted by OEA labs (http://www.oealabs.com, Callington, UK), where samples were combusted in pure oxygen and separated and quantified using GC-TCD. Percent O was calculated as % O = 100 − Σ(C, H, N, S, ash) where C, H, N, S, and ash are expressed as a percentage of the total mass. Sulfate content was measured using the turbidimetric assay reported by Craigie et al. [22]. Protein content of the untreated biomass was calculated as Protein = $\%N_{biomass} * k$, where $N_{biomass}$ is the N content (% dw) of the biomass and k is the species specific nitrogen-to-protein conversion factor, which is 5.0 for *U. ohnoi* [23]. For statistical analysis, the protein content of extracts was quantified by the Bradford method using a Total Protein Kit, Micro (Sigma Aldrich, Castle Hill, Australia) following the manufacturer's instructions. Dietary fibre content of the untreated biomass was determined using AOAC official methods (AOAC 985.29 for total dietary fibre (TDF); AOAC 991.42 for insoluble dietary fibre (IDF); soluble dietary fibre (SDF) = TDF − IDF); procedures were conducted by the Australian Export Grains Innovation Centre. Uronic acid (UA) was measured colorimetrically using the *m*-phenyl-phenol method with glucuronic acid as the standard [24]. The ratio of sulfate: uronic acid was used as a proxy for changes in the composition of ulvan.

2.7. Constituent sugar composition, molecular weight and structure

The constituent sugar composition, molecular weight and structure of the ulvan were determined after further purification to remove salts and low molecular weight components. Samples of the freeze-dried extracts (150–180 mg) were dissolved in deionised water (~8 mL) by heating at 50 °C for 5 min and dialysed (MWCO 6–8 kDa, Spectrapor) against deionised water. The water was changed (3–4 times) until there was no further increase in conductivity (~2.5 μS/cm) and the samples were freeze dried.

2.7.1. Constituent sugar analysis

Constituent sugar composition was determined by high-performance anion-exchange chromatography (HPAEC) after hydrolysis of the polysaccharides present to their component monosaccharides [25]. Samples were hydrolysed with methanolic HCl (3 N, 80 °C, 18 h), followed by aqueous TFA (2.5 M, 120 °C, 1 h). The resulting hydrolysates were analysed on a CarboPac PA-1 (4 × 250 mm) column equilibrated in 30 mM NaOH and eluted with simultaneous gradients of NaOH (30–10 mM from 0 to 25 min, then 10–100 mM from 25 to 30 min and held to 50 min) and sodium acetate (0–500 mM from 30 to 50 min) at 30 °C and a flow rate of 1 mL min^{-1}. The sugars were identified from their elution times relative to standard sugar mixtures treated under the same conditions as the samples, and quantified from response calibration curves of different concentrations of each sugar.

2.7.2. Molecular weight profiles

Molecular weight profiles were determined using size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). Samples (5 mg/mL) were dissolved in 0.1 M $NaNO_3$, allowed to fully hydrate by standing at room temperature overnight and centrifuged (14,000 × *g*, 10 min) to clarify. The soluble material was injected (100 μL) and eluted from two columns (TSK-Gel G5000PW_{XL} and G4000PW_{XL}, 300 × 7.8 mm, Tosoh Corp., Tokyo, Japan), connected in series, with 0.1 M $NaNO_3$ (0.7 mL min^{-1}, 60 °C) using a SECcurity GPC system (PSS Polymer Standards Service GmbH, Mainz, Germany). The eluted material was detected using a UV spectrophotometer (280 nm), a SDL7000 MALLS detector (PSS Polymer Standards Service GmbH, Mainz, Germany) and a refractive index monitor (Agilent Technologies, Santa Clara, USA). The data for molecular weight determination was analysed using WinGPC Unichrom software (v8.2.1, PSS Polymer Standards Service) using the Zimm procedure (first-order fit) and a refractive index increment, dn/dc, of 0.146 mL g^{-1} [20]).

2.7.3. Nuclear magnetic resonance (NMR) analysis

Ulvan was exchanged with deuterium by freeze-drying with D_2O (99.9 atom%) three times. Samples were dissolved in D_2O and ^1H and ^{13}C (both ^1H coupled and decoupled) spectra were recorded on a Bruker Avance DPX-500 spectrometer at 30 °C. Assignments were made from heteronuclear single quantum coherence (HSQC) COSY experiment and by comparing the spectra with published data for ulvans [26–28].

2.8. Data analysis

The effect of extraction treatment on the yield (% of dw biomass extracted) and overall quality of the extracts (as % of uronic acid and sulfate, sulfate: uronic acid ratio, and % of protein (Bradford assay) and

ash in the extract) was analysed using four-factor permutational analyses of variance (PERMANOVA) [29], with extraction treatments (salt, pigment, and ulvan extraction method) as fixed factors and harvest as a blocked (random) factor. The effect of extraction treatment on the quality of the extracts was also analysed individually for each quality parameter. All analyses were conducted in Primer v6 (Primer-E Ltd., UK) using Bray-Curtis dissimilarities on fourth root transformed data and 9999 unrestricted permutations of raw data. Tukey's multiple comparison was used to determine any differences between treatments. When there were significant interactions between terms the variance component (% variance explained, η^2) was calculated to interpret the relative importance of the significant terms in the model [29]. Pearson's correlations were taken between the content of uronic acid and sulfate (% of extract) (Statistica 13, StatSoft Inc.).

3. Results

3.1. Chemical composition of the untreated biomass

The untreated biomass contained protein (18.5 ± 1.5%), fibre (29.0 ± 0.7%; comprising insoluble fibre, 16.8 ± 0.4% and soluble fibre, 12.3 ± 1.0%) and ash (28.8 ± 1.6%) (Table 1). The uronic acid content was 3.6 ± 0.8% of biomass by dry weight, and the content of sulfur was 5.3 ± 0.3%.

3.2. Yields of crude extracts

Yields (% dw biomass) of crude ulvan extracts ranged between 3.7 ± 0.9% (EP1) and 8.2 ± 1.1% (EP6) (Table 2) and were significantly influenced by the process used. The major driver was treatment 3 (ulvan extraction method, $Na_2C_2O_4$ or HCl) (Pseudo-$F_{1, 14}$ = 101.88, $P < 0.01$), explaining 73.7% of the variance, with HCl extracts (EP2, EP4, EP6, EP8) consistently having higher yields (ranging between 6.7 and 8.2%) than $Na_2C_2O_4$ extracts (EP1, EP3, EP5, EP7, ranging between 3.7 and 4.3%). There was an effect of harvest (replicates) (pseudo-$F_{2, 14}$ = 27.834, $P < 0.01$) on the yields, where the absolute content of ulvan in the biomass varied between weeks, however, the relative pattern of extraction efficiency between the processes remained consistent (Fig. 3). Treatment 1 (mineral extraction) increased the yield of HCl extracts by 15–20% (EP6 and EP8 compared to EP2 and EP4), however, this effect was not apparent in $Na_2C_2O_4$ extracts and there was no statistically significant effect of treatment 1 overall (pseudo-$F_{1, 14}$ = 1.029, $P = 0.067$). Treatment 2 (pigment extraction) also had no statistically significant effect on yields (pseudo-$F_{1, 14}$ = 0.521, $P > 0.05$).

3.3. Quality of crude extracts

The content of uronic acid and sulfate, the ratio of sulfate: uronic acid, the content of protein and ash, all parameters in combination (overall quality) and individually, and the elemental composition (CHNS) of the crude extracts were used to determine quality. Extraction process had a significant effect on the overall quality of the extracts (Table 2) with treatment 3 (ulvan extraction method, $Na_2C_2O_4$ or HCl) explaining over 82% of the variance (pseudo-$F_{1,14}$ = 103.44, $P < 0.01$, η^2 = 82.3%). Additionally, treatment 1 (mineral extraction, pseudo-$F_{1,14}$ = 10.149, $P < 0.01$, η^2 = 8%) and harvest (pseudo-$F_{1,14}$ = 3.835, $P < 0.05$, η^2 = 3.1%) both accounted for minor components of the variance. There was a weak interaction between treatments 1 and 3, however, this was not statistically significant (pseudo-$F_{1,14}$ = 3.76, $P = 0.052$).

The effect of extraction process differed between the individual parameters investigated. Specifically, the uronic acid content was significantly affected by treatment 3 (pseudo-$F_{1,14}$ = 55.575, $P < 0.01$, η^2 = 76.3%) and HCl extracts consistently contained higher amounts of uronic acid (20–24% dw extract) than $Na_2C_2O_4$ extracts (9–13% dw extract). Treatment 1 (pseudo-$F_{1,14}$ = 4.688, $P < 0.05$, η^2 = 6.4%) and harvest (pseudo-$F_{1,14}$ = 5.385, $P < 0.05$, η^2 = 7.4%) also accounted for minor components of the variance associated with the uronic acid content, with processes that included a water treatment generally resulting in extracts with 15–30% more uronic acid compared with processes that did not. For the content of sulfate, there was an interactive effect of treatments 1 and 3 (pseudo-$F_{1,14}$ = 34.772, $P < 0.01$, η^2 = 22.85%). Again, treatment 3 explained most of the variance (pseudo-$F_{1,14}$ = 7.66, $P < 0.01$, η^2 = 50.35%) regardless of the interaction, and the content of sulfate varied less and was higher in HCl extracts (12.3–12.5% dw extract) than in $Na_2C_2O_4$ extracts (7.1–11.5% dw extract). There was a weak but significant correlation between the contents (%) of uronic acid and sulfate (Pearson correlation, r^2 = 0.568, $p < 0.01$) in the ulvan extracts, and there was an interactive effect of treatments 1 and 3 (pseudo-$F_{1,14}$ = 5.622, $P < 0.05$, η^2 = 14.7%) on the ratio of the contents of sulfate and uronic acid. However, treatment 1 (pseudo-$F_{1,14}$ = 15.627, $P < 0.01$, η^2 = 40.7%) explained the largest amount of the variance, and $Na_2C_2O_4$ extracts without a water treatment (EP1 = 1.45:1; EP 3 = 0.97:1) had a ratio 1.7–2.6 times higher than those with a water wash (EP5 = 0.58:1; EP7 = 0.57:1), with a similar but less pronounced pattern for HCl extracts.

The content of protein in ulvan extracts was significantly affected by treatment 3 (pseudo-$F_{1,14}$ = 64.956, $P < 0.001$, η^2 = 90.4%), with HCl extracts (0.4–0.7% *w*/w) being >5 times lower in protein than $Na_2C_2O_4$ extracts (4.1–5.9% *w*/w). Finally, the content of ash was also significantly affected by treatment 3 (pseudo-$F_{1,14}$ = 7.4551, $P < 0.01$, η^2 = 47.8%) with HCl extracts consistently having a 10–20% lower ash contents relative to those of the $Na_2C_2O_4$ extracts within each process (i.e. with the same treatment 1 and 2 prior to treatment 3). Processes that included a freshwater extraction (EP5–8) had 8–30% less ash in the final ulvan extracts compared with processes that did not (EP1-4), however, these differences were not statistically significant under the chosen ($P = 0.05$) significance level at this level of replication (pseudo-$F_{1,14}$ = 4.3877, $P = 0.059$).

3.4. Constituent sugar composition, molecular weight and structure

The yields of freeze-dried material following dialysis of the extracts were 52–65% for the $Na_2C_2O_4$ extracts and 75–86% for the HCl extracts. In general, the yields were higher for extracts from salt-reduced biomass than the equivalent extracts from control biomass.

3.4.1. Total sugar content determined by constituent sugar analysis

The process used to extract ulvan from *Ulva ohnoi* had an effect on the total sugar content and the sugar composition of the extracts

Table 1
Composition (% of dw biomass) of untreated *Ulva ohnoi* biomass. Average ± S.E. (n = 3).

Elemental					Proximate					
					Fibre					
C	H	O	N	S	Total	Insoluble	Soluble	UA	Protein	Ash
27.0 ± 1.2	5.5 ± 0.1	44.4 ± 1.8	3.7 ± 0.3	5.3 ± 0.1	29.0 ± 0.7	16.8 ± 0.4	12.3 ± 1.0	3.6 ± 0.8	18.5 ± 1.5	28.8 ± 1.6

Table 2
Yield and composition of crude ulvan extracts. Average ± S.E. (n = 3).

			Composition (% w/w)							
EP	Yield (% of algal dw)	Uronic acid	Sulfate	Protein	Ash	C	H	O	N	S
1	3.7 ± 0.9	9.2 ± 2.5	10.9 ± 1.1	4.1 ± 0.3	32.0 ± 3.3	26.65	4.28	29.18	3.65	4.24
2	6.7 ± 1.4	19.6 ± 1.9	12.3 ± 0.6	0.5 ± 0.2	26.4 ± 1.1	25.85	5.38	36.23	1.08	5.02
3	4.3 ± 0.5	13.2 ± 2.5	11.5 ± 0.7	5.1 ± 0.5	28.5 ± 2.6	27.50	4.73	31.38	3.27	4.61
4	7.0 ± 1.2	20.3 ± 1.3	12.4 ± 0.6	0.7 ± 0.0	24.4 ± 0.7	26.74	5.14	37.49	1.00	5.26
5	4.0 ± 1.0	12.7 ± 0.5	7.4 ± 0.6	4.6 ± 0.8	25.9 ± 2.9	29.52	4.75	33.94	3.45	2.40
6	8.2 ± 1.1	23.3 ± 1.4	12.5 ± 0.3	0.4 ± 0.1	23.3 ± 0.5	26.49	5.29	39.83	0.67	4.40
7	3.8 ± 1.0	12.7 ± 1.2	7.1 ± 0.7	5.9 ± 0.7	27.2 ± 2.7	28.92	4.66	32.20	3.43	3.60
8	8.1 ± 1.0	23.8 ± 0.4	12.5 ± 0.6	0.4 ± 0.0	22.5 ± 0.1	30.62	5.82	35.62	0.71	4.74

(Table 3). The total sugar content for HCl extracts (624.2–670.4 μg/mg extract) was > 1.5 times that of $Na_2C_2O_4$ extracts (365.2–426.3 μg/mg). The treatments used to remove salts (treatment 1) and pigments (treatment 2) also effected the total sugar content of extracts. For the $Na_2C_2O_4$ treatment, extracts obtained following treatment 2 (EP3 and EP7; 422.9 and 426.3 μg/mg) had a higher total sugar content than those obtained without treatment 2 (EP1 and EP5; 365.2 and 380.3 μg/mg). For the HCl treatment, extracts obtained following treatment 1 (EP6 and EP8; 670.1 and 670.4 μg/mg) had a higher total sugar content than those obtained without this treatment (EP2 and EP4; 635.1 and 624.2 μg/mg). Processes 6 and 8 yielded ulvan extracts (EP6 and EP8) with the highest total sugar content of all the samples analysed.

3.4.2. Constituent sugar composition

Extracts from all the processes were composed predominantly of rhamnose, glucuronic acid, iduronic acid and xylose, sugars that are typical constituents of ulvans. Smaller amounts of other sugars including galactose, glucose, mannose, fucose, arabinose and ribose were also present, though in larger amounts in the $Na_2C_2O_4$ extracts (Tables 3 and 4). Extracts EP6 and EP8, which had the highest total sugar content (~670 μg/mg), both had very similar constituent sugar compositions containing about 53 mol% rhamnose, 27–28 mol% glucuronic acid, 10 mol% iduronic acid and 5 mol% xylose, accounting for 95–96 mol% of the total sugar content (Table 4). While the remaining HCl extracts (EP2 and EP4) had similar constituent sugar compositions, the $Na_2C_2O_4$ extracts had notably different compositions containing 33–41 mol% rhamnose, 24–29 mol% glucuronic acid, 6–7 mol% iduronic acid and 6–7 mol% xylose, with these four monosaccharides only accounting for 70–84 mol% of the total sugar content. With the exception of EP1 and EP5, which had lower proportions of acidic sugars (29 and 33 mol%, respectively) relative to neutral sugars (71 and 67 mol%, respectively), the proportion of acidic sugars (35–38 mol%) to neutral sugars (62–65 mol%) remained relatively constant regardless of the extraction process.

3.4.3. Size-exclusion chromatography-multi-angle laser light scattering (SEC-MALLS)

The molecular weight profiles for HCl extracts were distinct from those for $Na_2C_2O_4$ extracts, as shown by typical traces from EP6 and EP5 (Fig. 4, Table S1). The HCl extracts showed a single peak in the refractive index trace eluting between 21 and 30 min, with a small peak in the UV (280 nm) trace eluting between 27 and 31 min. The light scattering data showed that the weight average molecular weights of the HCl-extracted ulvan ranged from 10.5–16.3 kDa, with extracts from EP6 and EP8 having a lower molecular weight (10.5 and 10.8 kDa, respectively) than those from EP2 and EP4 (13.5 and 16.3 kDa, respectively). In contrast, the refractive index traces for the $Na_2C_2O_4$ extracts were complex and showed multiple peaks eluting across the entire eluting range of the columns (15–32 min). The UV traces were similarly complex, but showed greatest intensity for a peak eluting from 24 to 32 min. The major peak in the refractive index traces, eluting from 18.6–24.3 min, had a weight average molecular weight ranging from 219 to 312 kDa, with the extract from EP1 having the largest molecular weight (312 kDa) and the extract from EP7 the smallest (219 kDa).

3.4.4. NMR spectroscopy

The NMR spectra for extracts from all of the processes were

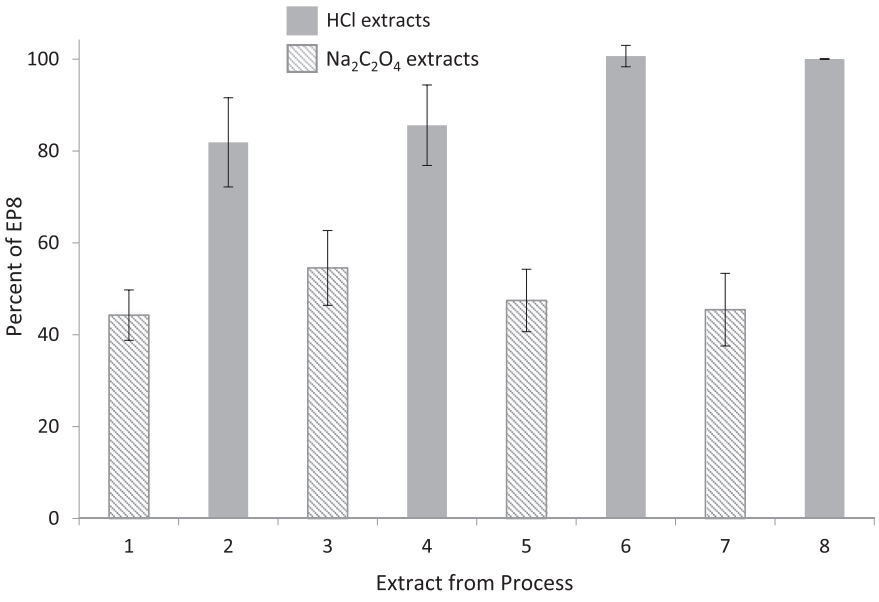

Fig. 3. Mean yield of crude extracts from process 1–7 (EP1-EP7) relative to the yield of crude extract from process 8 (EP8) (average ± S.E %; n = 3) emphasising extract yields for ulvan extraction processes using HCl (solid fill) compared to $Na_2C_2O_4$ (hashed fill).

Table 3
Constituent sugar composition and total sugar content (μg/mg extract) of purified ulvan extracts from processes 1–8.

EP	Sugars (μg/mg extract)												
	Rha	Xyl	GlcA	IdoA	Fuc	Ara	Gal	Glc	Man	Rib	GalN	GlcN	Total
1	114.3	22.9	96.3	22.4	11.6	3.0	24.3	26.2	20.1	19.3	1.5	3.3	365.2
2	314.9	33.0	185.2	74.4	1.7	0.6	9.2	8.3	2.6	4.9	0.1	0.2	635.1
3	162.6	20.7	137.3	34.6	6.3	1.6	17.4	15.1	11.3	13.3	0.7	2.0	422.9
4	306.2	28.5	188.5	67.8	1.5	0.4	9.7	15.2	2.6	3.8	0.0	0.0	624.2
5	133.2	20.1	111.9	26.4	7.7	2.3	19.8	20.2	19.5	15.9	1.0	2.3	380.3
6	333.3	30.2	206.4	74.8	1.1	0.3	8.2	10.3	2.1	3.4	0.0	0.0	670.1
7	155.3	20.4	132.6	33.1	6.3	1.9	18.4	24.2	16.5	14.7	0.8	2.1	426.3
8	332.2	30.1	202.7	73.2	1.0	0.3	8.3	16.3	1.9	4.4	0.0	0.0	670.4

Rha = rhamnose; Xyl = xylose; GlcA = glucuronic acid; IdoA = iduronic acid; Fuc = fucose; Ara = arabinose; Gal = galactose; Glc = glucose; Man = mannose; Rib = ribose; GalN = galactosamine; GlcN = glucosamine.

Table 4
Constituent sugar composition (normalised mol %) of purified ulvan extracts from processes 1–8.

EP	Sugars (normalised mol %)											
	Rha	Xyl	GlcA	IdoA	Fuc	Ara	Gal	Glc	Man	Rib	GalN	GlcN
1	33.2	7.3	23.6	5.5	3.4	1.0	6.4	6.9	5.3	6.1	0.4	0.9
2	52.8	6.1	26.3	10.5	0.3	0.1	1.3	1.3	0.4	0.9	0	0
3	41.1	5.7	29.4	7.4	1.6	0.4	4.0	3.5	2.6	3.7	0.2	0.5
4	52.3	5.3	27.2	9.8	0.3	0.1	1.5	2.4	0.4	0.7	0	0
5	37.3	6.2	26.5	6.3	2.2	0.7	5.1	5.1	5.0	4.9	0.3	0.6
6	53.1	5.3	27.8	10.1	0.2	0	1.2	1.5	0.3	0.6	0	0
7	39.0	5.6	28.1	7.0	1.6	0.5	4.2	5.5	3.8	4.0	0.2	0.5
8	52.8	5.2	27.2	9.8	0.2	0.1	1.2	2.4	0.3	0.8	0	0

Rha = rhamnose; Xyl = xylose; GlcA = glucuronic acid; IdoA = iduronic acid; Fuc = fucose; Ara = arabinose; Gal = galactose; Glc = glucose; Man = mannose; Rib = ribose; GalN = galactosamine; GlcN = glucosamine.

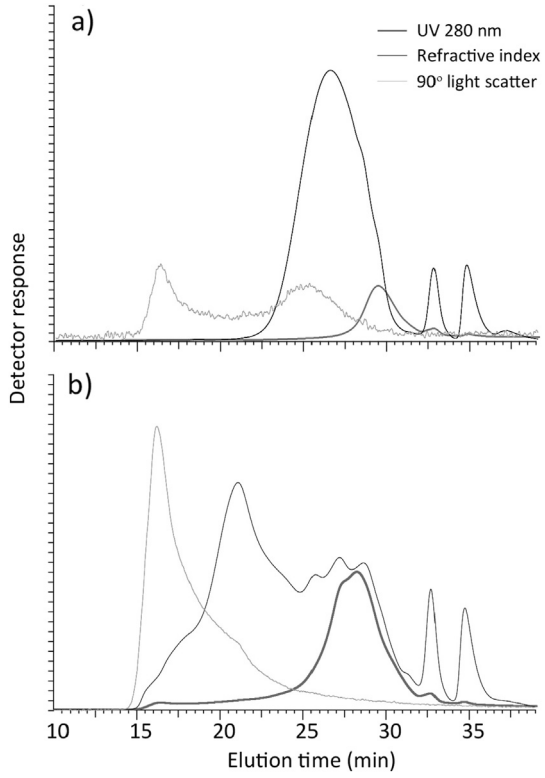

Fig. 4. Typical chromatograms of HCl (top) and $Na_2C_2O_4$ (bottom) extracts showing refractive index, UV 280 nm and 90° light scattering traces.

consistent with published spectra for ulvans [26–28] and examples of spectra for ulvan extracted with either HCl or $Na_2C_2O_4$ are shown in Fig. 5. The signals in the spectra for the HCl extracts were noticeably sharper and showed greater signal to noise than those for the $Na_2C_2O_4$ extracts, which was consistent with the lower molecular weight observed for the HCl extracts. Signals assigned to protein in the proton spectra (Fig. 5A) were less intense in the HCl extracts than in the $Na_2C_2O_4$ extracts, consistent with a higher total sugar and lower protein content of these extracts.

The carbon (Fig. 5B) and 2D (Fig. S1) spectra show the major anomeric signals for GlcA (G1, 103.7 ppm) and Rha (R1, 100.4 ppm) of the major disaccharide component of ulvan, -4-GlcA-1,4-Rha(3*S*)-1-, along with minor signals consistent with the presence of Rha linked to IdoA (R1′, 101.6 ppm) and Rha linked to xylose or xylose-2-sulfate (R1x (s), 97.9 ppm) [28].

4. Discussion

The yield and quality of ulvan extracted from cultivated *U. ohnoi* by alternative processes were investigated with the aim of identifying a cascading biorefinery process suitable for the selective and efficient extraction of ulvan. The best extraction process incorporated a warm water treatment to remove salt and subsequent extraction of ulvan with hot dilute hydrochloric acid (EP6). An additional treatment to remove pigments (EP8) was found to be unnecessary, having little effect on the yield or quality of extracted ulvan. Extracts from the EP6 process had the lowest protein and ash content, with the highest content of uronic acid and sulfate. EP6 provided the highest purity ulvan extract having a total sugar content of 670 mg/g of extract and comprising 53.1% Rha, 5.3% Xyl, 27.8% GlcA and 10.1% IdoA.

4.1. Chemical composition of the untreated biomass

The total fibre content of the untreated *U. ohnoi* biomass was 29.0% of the dry weight, with soluble fibre ranging between 12.1 and 15.1%. These values are at the lower end of the ranges reported for fibre (29–41% total fibre; 12–18% soluble fibre) from *U. ohnoi* [11,21,30] and other species of *Ulva* (8–29% soluble fibre) [16]. The relatively low content of soluble fibre in the *U. ohnoi* biomass used in this study may be partly attributed to its content of protein (18.5 ± 1.5%) and ash (28.8 ± 1.6%). However, this protein content is typical for *U. ohnoi* grown under non-nitrogen-limiting conditions [31], with protein and ash ranging from 13 to 23% and 25–33%, respectively [21,30].

4.2. Yields of crude extract

Yields of crude ulvan extracts from *U. ohnoi* ranged from 3.7–8.2% with the highest yields obtained using HCl as the extractant. Yields from HCl extractions were approximately twice those using $Na_2C_2O_4$ (a chelator). Similar results have been reported, with HCl resulting in a

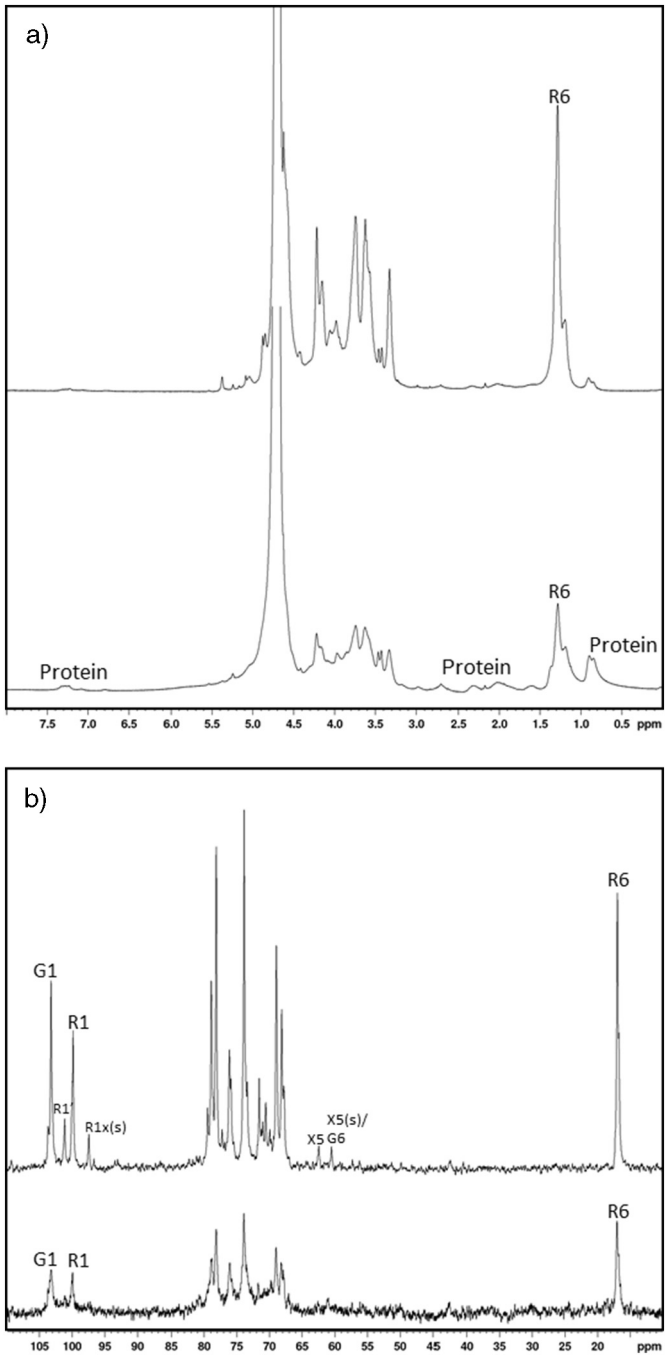

Fig. 5. Typical examples of 1D proton (A) and carbon (B) NMR spectra of HCl (top line) and $Na_2C_2O_4$ (bottom line) extracts.

42% increase in the extraction efficiency of ulvan (87% extraction efficiency) compared with $Na_2C_2O_4$ (45% extraction efficiency) [19]. Hernandez-Garibay et al. [32] also demonstrated a 36% increase in ulvan yields when HCl was used as the extractant compared with the chelator EDTA. The increased yields from HCl extraction processes can be explained by the physicochemical properties of ulvans and their solution properties. Firstly, ulvans are stabilised structurally within the cell wall by ionic interactions with divalent cations, such as Ca^{2+}, thus at pH values below the pK_a of the uronic acids (~3.28) [33] the carboxylic acids are protonated, destabilising the interactions of ulvans with other cell wall components (including positively charged amino acids in cell wall proteins). Secondly, the behaviour of ulvan solutions is influenced by pH, with cross-linked aggregates formed under alkaline conditions and discrete water-soluble microbeads formed at pH values below the pK_a of uronic acids [34]. Therefore lower pH facilitates higher extraction efficiencies of ulvans.

Aqueous extraction of salts (treatment 1) prior to ulvan extraction with HCl resulted in moderate (15–20%) increases in yields. It is proposed that the aqueous extraction increases ulvan yields in two ways, related to their interactions and distribution within the cell wall [19]. Firstly, the biomass is osmotically shocked rupturing cells and increasing the accessibility of the extractant to the cell wall components. Secondly, through the reduction of divalent ions, for example Ca^{2+}, known to stabilise the integrity of cell wall components including ulvan [14,19]. Aqueous extraction of salts (treatment 1) prior to extraction with $Na_2C_2O_4$ did not result in higher yields of ulvan, however, it is worth noting that there was no decrease in the yield.

4.3. Extract quality

The quality of extracted ulvan is critical in terms of its application and any further processing requirements. For applications requiring high purity ulvans, further processing to remove contaminants, such as other carbohydrates, proteins and small molecules, can be significant. In this regard, extraction of ulvan with HCl yielded the highest purity extracts containing less protein, ash and contaminating carbohydrates (see Section 4.4). Previous studies have also demonstrated that the extraction of ulvan under acidic conditions results in a lower protein content relative to the more commonly employed chelation methodologies [19,32]. The lower protein content is likely caused by chemical properties relating to protein, soluble fibre and their interactions. This is primarily, a combination of protonated aspartic acid (pK_a = 3.71) and glutamic acid (pK_a = 4.15), that respectively represent 12.3% and 12.5% of the total amino acids in *U. ohnoi* biomass [30], and a high ionic strength affording an excess of Cl^- ions to counter the positively charged amino acids, leading to lower solubility than expected for proteins at pH less than their isoelectric points [35–37]. Furthermore, extraction of ulvans at pH values < pK_a of uronic acids (3.28) and sulfate groups (~2.0) reduces the total charge on ulvans, which limits ulvans electrostatic interactions with the positively charged amino acids in cell wall proteins reducing the tendency for protein co-extraction. Conversely, protein solubility is high at neutral pH leading to its extraction with $Na_2C_2O_4$. Furthermore, extractions at pH above the pK_a of uronic acids maximise electrostatic interactions that facilitate the co-extraction of protein.

The use of colorimetric uronic acid assay data was an effective proxy for ulvan content in extracts, with ulvan extracts from HCl extractions having higher UA contents relative to $Na_2C_2O_4$ extractions (as verified by the total sugar content and constituent sugar compositions, see Section 4.4). Sulfate to UA ratios also reflected compositional changes between ulvan extracts from alternative extraction processes (EP1-8). The sulfate to UA ratios of extracts varied from 0.56–1.18 for $Na_2C_2O_4$ treatments and 0.53–0.63 for HCl treatments, with extracts which had undergone an initial treatment to remove salts (treatment 1) having the lowest values. This variation indicates that a more highly sulfated polysaccharide (e.g. ulvan) fraction is removed with treatment 1. This has interesting implications for the use of sequential extraction protocols to target distinct sulfated polysaccharide fractions for different applications.

4.4. Constituent sugar composition, molecular weight and structure

Rhamnose, xylose, glucuronic acid and iduronic acid are the major constituent sugars present in ulvans, with variation in their relative abundance determined on the basis of taxonomic, ecophysiological and methodological differences [15]. The accurate determination of the constituent sugar composition of ulvans is problematic given the predominance of aldobiouronic acids and the resistance of the uronic acid-neutral monosaccharide glycosyl linkage to acid hydrolysis [38]. The method used in this study, which involves methanolysis followed by

TFA hydrolysis, is effective at cleaving these acid resistant aldobiouronic acid linkages [39].

For the development of a biorefinery process targeting the extraction of ulvan from *U. ohnoi*, 0.05 M HCl is a more selective extractant than 0.05 M $Na_2C_2O_4$. The total sugar content of the HCl extracts was > 1.5 times that of the $Na_2C_2O_4$ extracts (62–67% of the extract dry weight compared to 37–43%). Notably, ulvan extracted from *Ulva rotundata* with HCl also had a higher sugar content than $Na_2C_2O_4$ extracts [19]. In addition, 95–96% of the sugars present in the HCl extracts were constituents of the ulvan component of the algal cell walls (i.e. rhamnose, xylose, glucuronic and iduronic acids). By comparison, 16–30% of sugars present in the $Na_2C_2O_4$ extracts are derived from other polysaccharide components of the algal cell.

In terms of product applications, constituent sugar compositions are linked to both physicochemical properties and biological activities of polysaccharides [40]. Ulvan from HCl-treated *U. ohnoi* have a high content of rhamnose (53.1 mol%) and uronic acid (37.9 mol%) and low xylose (5.3 mol%) relative to the reported sugar compositions for ulvans from other species of *Ulva* (rhamnose, 16.8–48.0 mol%; uronic acids, 6.5–46.0 mol%; xylose, 2.1–12.0 mol%) [15,41]. Ulvan from *U. rigida* with a similar composition (48 mol% rhamnose; 46 mol% uronic acids) to those isolated from *U. ohnoi* had pro-inflammatory activity and immunomodulatory behaviour through modification of macrophage activity [41]. In this regard, rhamnose-rich polysaccharides (50–60% rhamnose) also have a range of properties of cosmetic and dermatological interest, including anti-inflammatory activity, anti-adhesive properties, and protection against UV–induced erythema [42]. Ulvan from *U. ohnoi* may show similar activities in related applications and this will be a focus of future studies.

SEC-MALLS revealed considerable differences in the molecular weights of HCl- and $Na_2C_2O_4$-extracted ulvan. The RI chromatograms of HCl-extracted ulvan showed one main peak with light scattering data determining molecular weights ranging from 10.5–16.3 kDa. By comparison, the RI chromatograms of $Na_2C_2O_4$-extracted ulvan showed multimodal distributions and a considerable portion of these samples were high molecular weight molecules (219–312 kDa). To a lesser extent, the molecular weights of ulvan were also lower when extracted from biomass first subjected to the aqueous extraction of salts. In a previous study ulvan extracted from *U. ohnoi* with water at 100 °C was in the range of 200–800 kDa [43], with ulvan molecular weights from species of *Ulva* spanning 10^4–10^6 kDa [15,43,44]. Much of the variation in the reported molecular weights of ulvan can be attributed to ulvan extraction protocols, with similar differences in molecular weights between $Na_2C_2O_4$ and HCl extracts from *U. rotundata* [19] supporting significantly higher polysaccharide depolymerisation under acidic conditions.

The depolymerisation of ulvan is an important consideration as molecular weight is correlated with rheological properties [19,20,43] and antioxidant [45], antihyperlipidemic [46], anticoagulant [40] and anti-aging [47] activities. Variables in extraction procedures that can influence the degree of polysaccharide depolymerisation include extractant [19], pH [19,43], temperature [43,44], duration [19] and pretreatment [20]. This list of variables allows a broad capacity to fine-tune biorefinery processes with a focus on application. For example, Yaich et al. [43] demonstrated that ulvan extracted from *U. lactuca* at pH 1.5 resulted in significantly lower molecular weights than at pH 2, while at pH 1.5 a temperature decrease from 90 °C to 80 °C significantly reduced polysaccharide depolymerisation without sacrificing extract composition (e.g. protein, sulfate and ash content). Consequently, acidic extractions can be optimised to limit or enhance polysaccharide depolymerisation, while extractions using chelators such as sodium oxalate can be used to obtain higher molecular weight ulvan.

5. Conclusions

The acidic extraction of ulvan, following an initial aqueous treatment to remove salts (EP6), provided the highest yield of product having the best quality profiles in terms of monosaccharide composition and carbohydrate, protein, sulfate, and ash content. Although extraction of ulvan with sodium oxalate afforded significantly lower yield and quality profiles, the higher molecular weights obtained may be targeted for specific applications. Ulvan extracted from *U. ohnoi* have a high content of rhamnose (53.1 mol%) and uronic acid (37.9 mol%) and a low content of xylose (5.3 mol%) relative to other species of *Ulva*. By combining a pre-treatment to extract salts and subsequent HCl-treatment to extract ulvan, a cascading biorefinery process has been developed for *U. ohnoi* producing a salt-rich product, ulvan and a residual protein-rich biomass.

Acknowledgements

This research was funded by and is part of the MBD Energy Research and Development program. We thank Kial Griggs for the provision of *U. ohnoi* from the MBD Pacific Reef Fisheries Bioremediation Facility. We thank Andrew Lorbeer and Nicolas Neveux for assistance with the chemical analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2017.07.001.

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