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Multiple response optimisation of the aqueous extraction of high quality ulvan from *Ulva ohnoi*

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ABSTRACT

Response surface methodology was used to determine the effects of the solvent pH, the temperature of extraction, and the duration of extraction on the yield, purity, molecular weight, viscosity, and total metal content of ulvan extracted from *U. ohnoi*. Quadratic models identified the optimised responses for yield (72.6%) purity (68.2% *w*/w), molecular weight (92.9 kDa), viscosity (491.1 s), and total metal content (~0 mg/kg). These responses occurred between a solvent pH of 2.2–4.0, an extraction temperature of 61.3–90.0 °C, and an extraction duration of 55.0–90.0 min. The overall desirability of the ulvan product was determined using a Derringer's desirability function, which identified a solvent pH of 2.92, an extraction temperature of 90 °C, and an extraction duration of 90 min. These extraction conditions minimise the requirement for downstream purification and are suitable for upscaling the extraction of a high quality ulvan product.

1. Introduction

Green tide forming species of the genus *Ulva* (chlorophyta) are well suited for the bioremediation of nutrients (nitrogen [N] and phosphorus [P]) from intensive land-based aquaculture and other nutrient rich marine wastewater streams (Nardelli et al., 2019; Lawton et al., 2013; Mata et al., 2016). A major benefit of this approach is that the cost of the bioremediation process is offset by exploiting the unique biochemical profile of the biomass produced. For example, biomass from *Ulva* species has been used to produce plant growth stimulants (Michalak et al., 2015; Hernández-Herrera et al., 2016), biomaterials (Alves et al., 2012; Vlachou et al., 2018; Morreli and Chiellini, 2010), cosmetics (Adrien et al., 2017), therapeutics and health products (Kidgell et al., 2019), and protein for animal and human food (Kazir et al., 2019; Angell et al., 2017). Therefore, there is potential to target multiple products from a single biomass harvest in a cascading biorefinery. However, to be successful each step of the cascading biorefinery should be high-yielding, selective and non-destructive, and result in a high-quality residual biomass that can be further exploited.

A cascading biorefinery was developed for *Ulva ohnoi* that targets a seaweed salt, ulvan, and a protein and energy rich residual biomass (Magnusson et al., 2016). In that cascading biorefinery, seaweed extracted with warm water (30 min at 40 °C) resulted in a salt product yielding up to 29% dry weight (dw) of the initial biomass, which subsequently resulted in an increase in protein (up to 13%) and energy content (50% increase) in the residual biomass. A further study used a cascading biorefinery process to produce seaweed salt, soluble fibre (ulvan), and protein products from the residual biomass (Magnusson et al., 2019). The content of protein increased from 22.2 ± 0.5% dw in the unprocessed biomass to between 39.5 ± 2.6% in protein enriched biomass and 45.5 ± 1.1% in a protein isolate. This cascading biorefinery scheme was then extended to include a sulfated polysaccharide (ulvan) product (Glasson et al., 2017). The seaweed salt extraction process enhanced the yield of ulvan from 6.7 to 8.2% dw biomass when an acid extraction process was used. Furthermore, the purity of the ulvan extracted using the acid extraction process was superior to that extracted with sodium oxalate. However, under the acid extraction conditions there was significant depolymerisation of the ulvan, potentially affecting its efficacy in some applications. In this regard, the structural features of ulvan, including molecular weight and sulfate content, influence its rheological properties (Robic et al., 2008; Yaich et al., 2014) and biological activities (e.g. antioxidant (Qi et al., 2005), antihyperlipidemic (Pengzhan et al., 2003), anticoagulant (Mao et al., 2006; Qi et al., 2012; Qi et al., 2013a, 2013b), and immunomodulation (Adrien et al., 2017)). Therefore, an optimised acid extraction process that affords ulvan in high yield and purity, while retaining the native

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structural features of molecular weight and sulfate content, is required.

The key parameters that influence the degree of polysaccharide depolymerisation and desulfation in aqueous solutions are pH (Yaich et al., 2014; Robic et al., 2009a), temperature (Yaich et al., 2014; Tsubaki et al., 2016), and time (Robic et al., 2009a). Manipulation of these parameters provides a broad capacity to fine-tune biorefinery processes to optimise the acid extraction of ulvan in high yield and purity, while minimising the degree of depolymerisation. Therefore, the aim of this study was to optimise the acid extraction process to deliver a high molecular weight ulvan while retaining high purity (selectivity) and yield. The chemical properties of uronic acid and sulfate content, molecular weight, and rheological properties were used as defining parameters.

2. General methods

2.1. Cultivation of biomass

Ulva ohnoi Hiraoka et Shimada (Genbank accession number KF195501, strain JCU (Lawton et al., 2013)) is domesticated and is currently used to bioremediate N and P from the discharge waters from land-based aquaculture. In this study, *U. ohnoi* was cultivated at the Marine and Aquaculture Research Facility at James Cook University, Townsville, Australia (latitude: 19.33 S; longitude: 146.76 E) as described in (Mata et al., 2016). Biomass was kiln-dried at 60 °C, homogenised, and milled using a 1 mm screen. This material was then subdivided into 40 g samples for extraction. Data from the elemental analysis and proximate data for the biomass is presented in the supplementary material (presented in the e-supplementary) using methods described below.

2.2. Experimental design and statistical analysis

To optimise the acid extraction of ulvan, a Box-Behnken design (BBD) with a replicated central point ($n = 3$) was used to examine the combined effect of the independent variables of the solvent pH, the temperature during extraction, and the duration of the extraction on ulvan yield, purity, molecular weight, viscosity, and the concentration of total metals (Table 2). Preliminary, single factor experiments (unpublished data) and previously-published data (Glasson et al., 2017; Robic et al., 2009a; Hernandez-Garibay et al., 2011) were used to determine the ranges for the independent variables.

The yield, purity, molecular weight, viscosity, and the concentration of total metals were treated as univariate responses to fit separate predictive models. Ordinary least squares were used on the BBD to estimate the parameters of linear and second-order regression models with the following equations:

$$\hat{y} = \beta_0 + \beta_1 X_{pH} + \beta_2 X_{temp} + \beta_3 X_{time} \quad (1)$$

$$\hat{y} = \beta_0 + \beta_1 X_{pH} + \beta_2 X_{temp} + \beta_3 X_{time} + \beta_4 X_{pH} X_{temp} + \beta_5 X_{pH} X_{time} + \beta_6 X_{pH} X_{time} + \beta_7 X_{pH}^2 + \beta_8 X_{temp}^2 + \beta_9 X_{time}^2 \quad (2)$$

where $\hat{y}$ is the predicted response, β_i are the coefficients of the model, X_{pH} is the extraction pH in coded units (Eq. (3)), X_{temp} is the extraction temperature in coded units (Eq. (4)), and X_{time} is the duration of the extraction in coded units (Eq. (5)). The pH of the solvent, the temperature during extraction, and the duration of the extraction were converted into coded units using the following equations:

$$X_{pH} = \frac{(Z_{pH} - 2.5)}{1.5} \quad (3)$$

$$X_{temp} = \frac{(Z_{temp} - 65.0)}{25.0} \quad (4)$$

$$X_{time} = \frac{(Z_{time} - 50)}{40.0} \quad (5)$$

where Z_{pH} is the pH of the solvent on the pH scale, Z_{temp} is the temperature of the extraction in degrees Celsius (°C) and Z_{time} is the duration of the extraction in min.

Regression models were evaluated with analysis of variance (ANOVA) and the fit of the models to the experimental data were evaluated using a lack-of-fit test. A histogram of the studentised residuals and scatter plots of independent variables and the residuals were used to determine that the residuals of each model had a normal distribution, with an expected value of 0 and a random distribution of errors that was un-correlated to the independent variables. For each response variable, the linear and second-order models were compared using Akaike's information criteria (AIC) and the model with the lower AIC value was selected. Models were fit and evaluated using Python's Statsmodels library (Seabold and Perktold, 2010).

The combination of independent variables that resulted in the maximum (ulvan yield, ulvan purity, molecular weight, and viscosity) or minimum (total metals) response was determined by respectively maximising and minimising the second-order models within the bounds of the experimental space ($-1 \leq X_{pH}, X_{temp}, X_{time} \leq 1$, where X_{pH}, X_{temp} and X_{time} are the values of solvent pH, temperature during extraction, and duration of extraction in coded units, respectively). This was done using sequential least squares programming with Python's SciPy library (Jones et al., 2001).

Derringer's desirability function was sequentially used to find the experimental conditions (levels of each of three independent factors) that simultaneously optimised each of the five measured response variables (Derringer and Suich, 1980). This was done by transforming predicted responses $\hat{y}_i$ into desirability scores $d_i(\hat{y}_i)$ between 0 and 1 using the following equations:

$$d_i(\hat{y}_i) = \begin{bmatrix} 0 & if\ \hat{y}_i < L_i \\ \left(\frac{\hat{y}_i - L_i}{T_i - L_i}\right)^{s_i} & if\ L_i \leq \hat{y}_i \leq T_i \\ 1 & if\ \hat{y}_i > T_i \end{bmatrix} \text{ if the objective is to maximise } \hat{y}_i \quad (6)$$

$$d_i(\hat{y}_i) = \begin{bmatrix} 1 & if\ \hat{y}_i < T_i \\ \left(\frac{\hat{y}_i - T_i}{U_i - T_i}\right)^{s_i} & if\ T_i \leq \hat{y}_i \leq U_i \\ 0 & if\ \hat{y}_i > U_i \end{bmatrix} \text{ if the objective is to minimise } \hat{y}_i \quad (7)$$

where T_i is the target value, L_i is the lower acceptable value for the ith response (for responses where the objective is to maximise), U_i is the upper acceptable value for the ith response (for responses where the objective is to minimise), and s_i is the weight allocated to the ith response based on the relative importance of this response reaching the target (Table 2). The lower acceptable value and target for yield and purity were based on those measured for extraction process 2 (EP2) and extraction process 6 (EP6) in (Glasson et al., 2017). These samples represent the lowest and highest yielding acid extraction processes and correspond to the lowest and second highest extract purities recorded. The lower acceptable value and target for molecular weight (number average molecular weight) is based on the capacity for the ulvan extracted to form a gel (presented in the e-supplementary) using the method described below. The lower and acceptable value and target for viscosity are also based on the capacity for the ulvan to form gels (presented in detail in the e-supplementary). Finally, the upper acceptable value and target for the concentration of total metals is based on achieving < 2 mg/kg Cd, which is approximately equal to the median total metals value (presented in the e-supplementary). The purity of ulvan and the molecular weight were assigned a weight (s) of 5 due to increased purification requirements and the limited versatility of a low

molecular weight product, respectively.

An overall desirability score D was determined for the individual desirability score $d_i(\hat{y}_i)$ of each response parameter by calculating their geometric mean as follows:

$$D = \left[\prod_{i=1}^{n} d_i(\hat{y}_i)\right]^{1/n} \quad (8)$$

where n is the number of response variables. The combination of independent variables that maximised the overall desirability score D was determined by maximising this function (Eq. (8)) within the bounds of the experimental space ($-1 \leq X_{pH}, X_{temp} \leq 1$, $X_{time} \leq 1$, where X_{pH}, X_{temp}, and X_{time} are the values of solvent pH, temperature during extraction, and duration of extraction in coded units, respectively). This was done using sequential least squares programming with Python's SciPy library (Jones et al., 2001).

The combination of independent variables (solvent pH, temperature during extraction, and the duration of extraction) that resulted in the maximised overall desirability score was validated experimentally using the methods described below (Section 2.3). For each response variable, experimental data ($n = 3$) were statistically compared to predicted values with one-sample t-tests, and differences were considered significant if $p < 0.05$.

2.3. Extraction and purification protocol

A simple extraction and purification procedure was used to target ulvan from dried milled *U. ohnoi* biomass. Briefly, a stirred (400 rpm) suspension of *U. ohnoi* biomass (40 g) in 1 l of H_2SO_4 (pH 1.0, 2.5, and 4.0) was heated (40, 65, and 90 °C) over varying durations (10, 50, and 90 min). Note, to avoid solvent loss over the duration of these experiments, a 2 l conical flask covered with aluminium foil was used as the extraction vessel. On completion, the extract was separated from the biomass by filtration through a 200 µm bag filter. The extract was centrifuged (3000*g*) for 20 min prior to sequential vacuum filtration through diatomaceous earth (Celatom®) and Whatman® GF/F filters. The extract was then concentrated (10×) by ultrafiltration (ÄKTA flux 6 fitted with a Xampler 10 kDa NMWC Cartridge filter), diafiltered with five volumes of deionised water, and freeze dried. Samples were powderised and analysed as described below.

To obtain high purity ulvan samples, each crude extract was subject to a further ultrafiltration process such that the conductivity of the permeate reached < 10 µS/cm. These samples were then reanalysed as described below.

2.4. Characterisation of biomass and ulvan extracts

Chemical composition of the untreated biomass and ulvan extracts (15 processes) were quantified as follows. Elemental analysis (% C, H, N, S; $n = 1$ per process) and ash content (% dw) were quantified externally (OEA Laboratories Ltd., Callington, Cornwall, UK). Protein content was calculated as $Protein = \%N_{(sample)} {}^{*}k$, where $N_{(sample)}$ is the N content of the biomass or extract, and $k = 5.13$, which is the species-specific N to protein conversion factor for *U. ohnoi* (Angell et al., 2016). Uronic acid content was measured colourimetrically using the *m*-phenyl-phenol method with glucuronic acid as the standard (Craigie et al., 1984). Sulfate content of hydrolysed extract samples (10 mg of sample in 0.5 ml of 2 M HCl heated at 100 °C for 2 h) was determined by ion chromatography (Metrohm Compact IC Flex fitted with a Metrosep A Supp 5150/4.0 anion exchange column) by interpolation of sulfate peak area to a K_2SO_4 (SO_4^{2-} concentrations range of 0–50 µg/ml) calibration curve. Metals analysis (Cr, Cd, Cu, Pb, Zn, Se, As, and Hg) was commercially determined using ICP-MS by the Advanced Analytical Centre at James Cook University.

Extraction efficiency was estimated based on the ulvan content of the *U. ohnoi* biomass. The ulvan content (7.2% dw biomass) was quantified by measuring the uronic acid content of extracts ($n = 3$) from an exhaustive extraction (pH 1; 90 °C; 120 min), and estimated on the basis of the stoichiometry of the content of rhamnose, xylose, and uronic acids in EP6 from (Glasson et al., 2017).

2.5. Molecular weight determination

Ulvan samples (4 mg) were dissolved in eluent (50 mM $NaNO_3$ and 0.02% NaN_3) and filtered (0.22 µm syringe filter). Sample solutions were analysed with a high-performance size exclusion chromatography system (1260 Infinity II LC GPC/SEC system, Agilent Technologies) fitted with a 50 × 7.5 mm PL aquagel-OH Guard coupled to a 7.5 × 300 mm PL aquagel-OH Mixed-H column. Each sample (5 µL) was injected at 45 °C at a flow rate of 0.7 ml/min. The eluted material was detected by refractive index and UV spectrophotometer (280 nm). Molecular weight (M_n = 1.4–1194 g/mol) was calculated using Agilent GPC/SEC software against polyethylene glycol standards.

2.6. Rheological measurements

Viscosity was measured on 0.5% *w/v* ulvan solutions in deionised water at 37 °C using a capillary viscometer (140 mm × 0.5 mm) and recorded as flow time through the viscometer in seconds.

Ulvan hydrogels were prepared using a modified method based on (Lahaye et al., 1996). Briefly, ulvan (50 mg) was suspended in dimethyl sulfoxide (0.5 ml) and heated at 60 °C for 1 h, after which, 14 mM $CaCl_2$ (0.4 ml) was added and the mixture was heated for 1 h, and finally, 66 mM $B(OH)_3$ (0.1 ml) was added and the mixture was heated for a further 2 h. The gels were cooled to room temperature overnight prior to compression testing with a computer controlled universal mechanical tester (Mecmesin MultiTest 2.5-dV test stand fitted with a Mecmesin Advanced force gauge 5 N). Compression testing was done according to a previously reported method (Obata et al., 2017) by using a 10 mm compression plate at a rate of 1 mm/min with the force (N) at 0.5 mm of compression reported. Each gel was prepared and tested in triplicate. Data and discussion presented in the e-supplementary.

3. Results and discussion

Response surface methodology was used to determine the effects of the solvent pH, the temperature during extraction, and the duration of extraction on the yield, purity, molecular weight, viscosity, and total metal content of ulvan extracted from *U. ohnoi*. For all response variables examined, quadratic models (Table 3) fit the data (Table 4) well and were better approximations than linear models (lower AIC values). Optimised responses for yield, purity, molecular weight, viscosity, and total metal content were 72.6%, 68.2% *w/w*, 92.9 kDa, 491.1 s, and ~0 mg/kg, respectively, and occurred between a solvent pH of 2.2–4.0, an extraction temperature of 61.3–90.0 °C, and an extraction duration of 55.0–90.0 min (Table 5). However, the maximum overall desirability occurred at a solvent pH of 2.92, an extraction temperature of 90 °C, and an extraction duration of 90 min, demonstrating that there are trade-offs among these five response variables. At these experimental conditions the crude ulvan extract was predicted to have a yield of 72.1%, a purity of 59.7%, a molecular weight of 79.3 kDa, a viscosity of 453.7 s, and a total metal concentration of 72.1 mg/kg. This study optimised an acid extraction process targeting high quality ulvan with high molecular weight.

3.1. Yield

High yield has multiple benefits for a biorefinery process, primarily increased product combined with high selectivity leads to dilution of contaminants in the product (i.e. increase product purity), which reduces the need for downstream processing. In this study, the yield of ulvan ranged from 15.1 to 73.2% with the lowest recovery at pH 2.5;

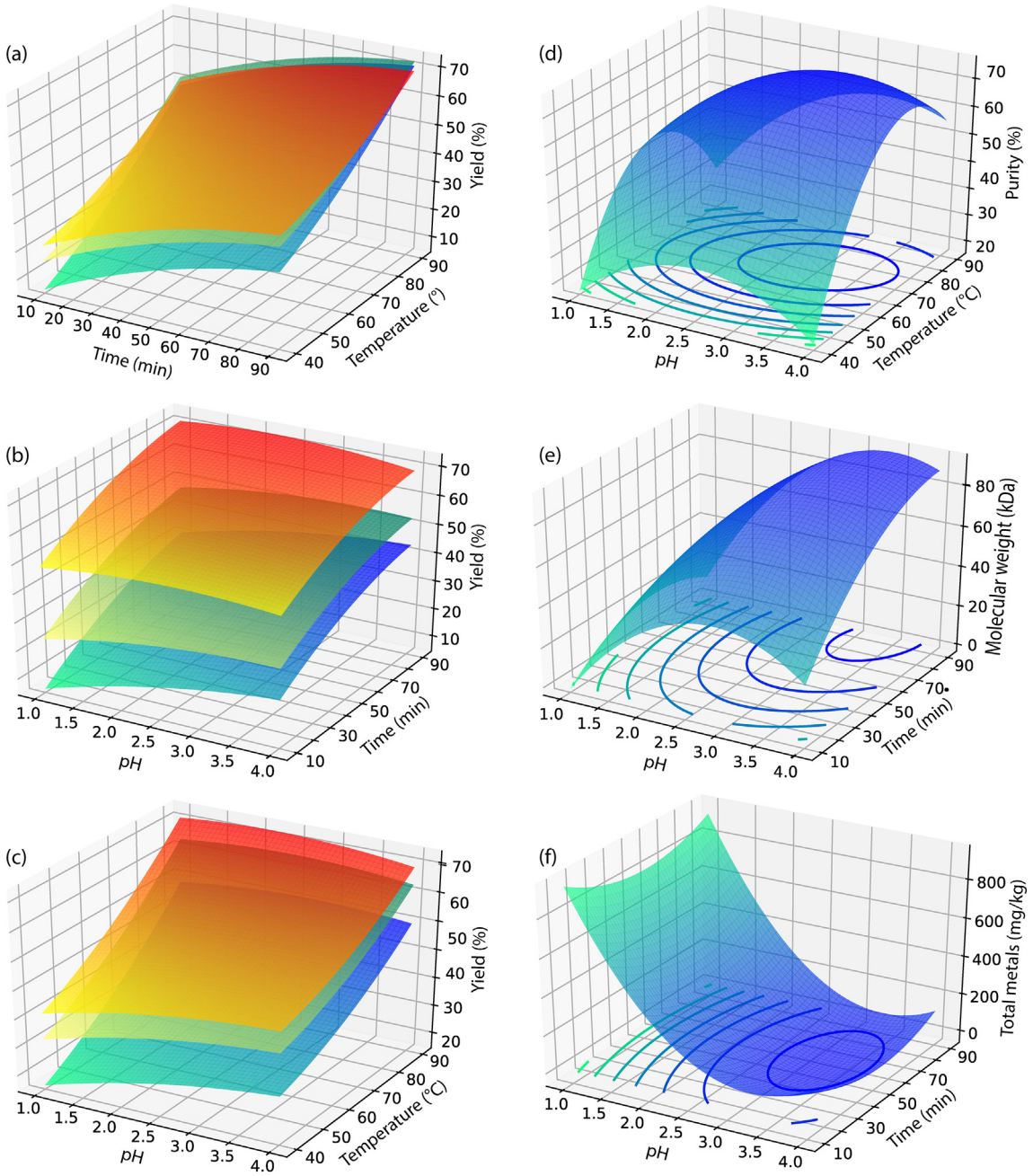

Fig. 1. The second-order response surface model that predicts the yield of ulvans from *U. ohnoi* are presented in (a) the pH of the solvent; (b) the temperature during extraction; and (c) the duration of extraction. Separate surfaces in these plots are representative of the different levels within each independent variable (blue = low; yellow = medium; orange = high). The second-order response models for (d) ulvan purity; (e) molecular weight; and (f) total metals are presented at 90 min, 73.9 °C and 90 °C, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

40 °C; 10 min (Exp. 8) and the highest recovery at pH 2.5; 90 °C; 90 min (Exp. 11) (Table 4). While all independent variables significantly influenced yield, extraction temperature and duration had the greatest effects (Table 3; Fig. 1) in the form of strong positive correlations. The solvent pH had a smaller positive effect on yield and was dependent on temperature (Fig. 1c). At the lowest temperature, the effect of solvent pH on yield was highest, but decreased with increasing temperature with no effect of pH at the highest temperatures examined ($\beta_4 = -3.55$ $\{p = 0.1\}$) (Fig. 1c). Importantly, the yield of ulvan from *U. ohnoi* is influenced by its high rhamnose content (Glasson et al., 2017), which limits its solubility in aqueous extractants (Robic et al., 2009b). Therefore, extractions that employ higher temperatures assist the solubilisation of ulvan. The solubility of ulvan is reduced at low pH, below the pK_a of both the carboxylates (pK_a ~3.28) and the sulfate ester groups (pK_a ~2. 0) due to a reduction in the charge density of ulvan. Consequently, a combination of low pH and low temperature results in low yields (Fig. 1b). However, higher temperatures overcome the decreased solubility at low pH (e.g. Exp. 6, pH 1; 90 °C; 50 min, yield = 65.8%). Under the latter conditions there is also an increase in the depolymerisation of ulvan (see molecular weight discussion), which leads to higher solubilities (Hu and Goff, 2018). The yield was optimised at a solvent pH of 2.21 when the temperature and duration of extraction were at the highest levels examined in this study (Table 5). This suggests that higher yields are likely to be predicted at higher extraction temperatures and longer extraction times than those tested here. Although the optimum yield is predicted when solvent pH is 2.21,

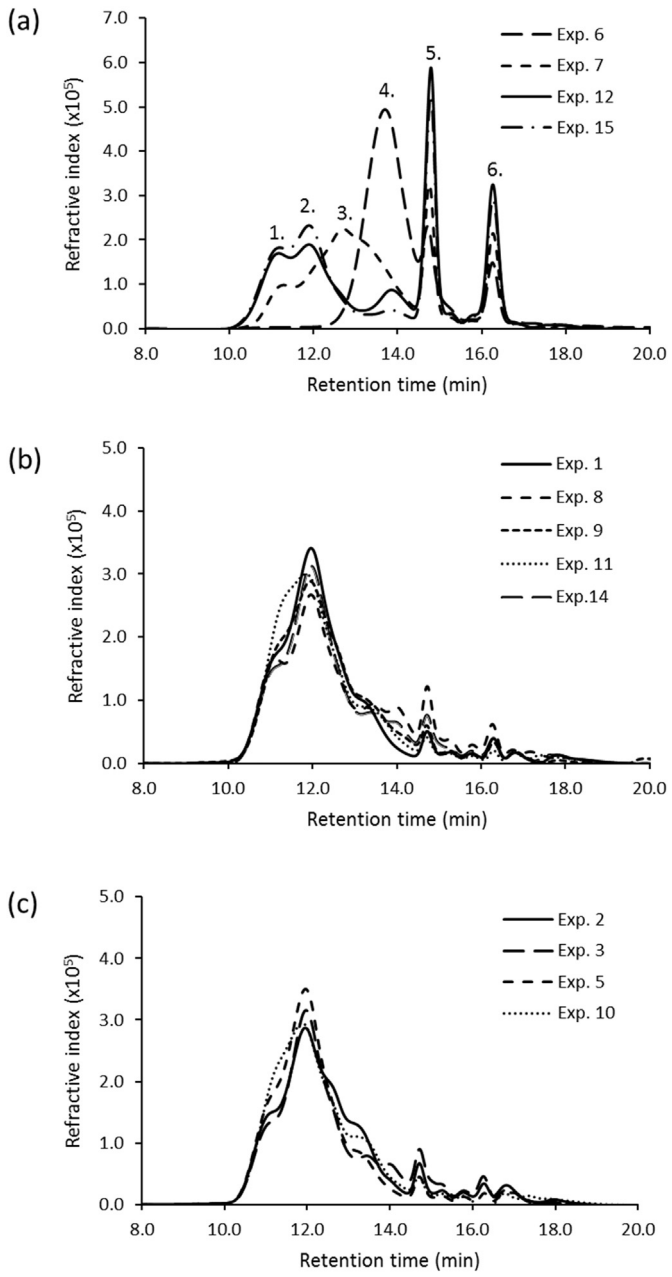

Fig. 2. Size exclusion chromatography traces for crude ulvan extracts from (a) pH 1; (b) pH 2.5; and (c) pH 4. Exp. 1 is representative of the replicated centre points of the BBD design (Exp. 1, 4, and 13; Table 4).

the overall effect of solvent pH on yield is low (Table 3, Fig. 1) and small deviations from this optimum solvent pH will result in little change in yield. Therefore, other response variables will play a stronger role in determining the optimal solvent pH (e.g. purity, molecular weight, and physical properties).

3.2. Purity

High extract purity reduces downstream purification requirements and is a significant consideration for registration of new supplements and therapeutics. The purity of extracts ranged from 31.20 to 65.26% *w*/w and was strongly affected by the solvent pH, the temperature during extraction, and duration of extraction (Table 3). The pH and the temperature both had large positive linear effects and large negative quadratic effects (albeit non-significant at $\alpha = 0.05$ for temperature of extraction), suggesting that purity was at a maximum at intermediate values within the extraction parameter ranges used. In contrast, only a strong positive linear effect of extraction duration on purity was detected, meaning that purity was highest at the longest extraction times tested. However, there was also a strong interaction between extraction temperature and extraction duration, such that the effect of extraction duration was reduced at high extraction temperatures and vice-versa. The predicted optimal purity was 68.17% and occurred when the solvent pH was 2.99, the temperature of extraction was 61.3 °C, and the extraction duration was 90 min (Table 5). Notably, the predicted purity of ulvan was resistant to changes in the temperature of extraction and solvent pH between pH 2 and 4, while extractions with a solvent pH $<$ 2 had a reduction in purity.

The purity of ulvan extracts is a function of the relative yield of ulvan compared to impurities, which were predominantly ash and, to a lesser extent, other macromolecules such as protein (Table 5). The ash content, which varied from 11.2 to 41.2% with the lowest level for Exp. 10 (pH 4; 90 °C; 50 min; Table 4) and the highest level for Exp. 12 (pH 1; 65 °C; 10 min; Table 4), was predominantly influenced by the differences in the quantity of salt generated during the neutralisation of extracts from treatments at different pH. Salt associated with the biomass also contributed to the ash content of the ulvan extracts and played a significant role for extractions done at pH 2.5 and 4.0 (Magnusson et al., 2016). The protein content varied from 4.1 to 15.0% with the lowest recorded for Exp. 15 (pH 1; 40 °C; 50 min; Table 4) and the highest recorded for Exp. 3 (pH 4; 40 °C; 50 min; Table 4). This is indicative of the solubility of proteins above and below the isoelectric point (pI) of protein. In this regard, the pI for macroalgal aqueous and alkaline soluble proteins is generally between pH 3–4 (Harnedy and FitzGerald, 2015). However, lower pI values for soluble proteins from *Ulva* (pI = 2.25) have been reported (Angell et al., 2017). The temperature and duration of the extraction influenced the purity of the ulvan extract through their relative effects on the yield of salts, ulvan, and protein. As discussed above, higher yields of salts from seaweed biomass can occur at moderate extraction temperatures and extraction times (Magnusson et al., 2016). Conversely, higher yields of ulvan occurred at longer extraction times and at higher temperatures resulting in the dilution of impurities. However, given purity is resilient to changes in solvent pH, other response variables such as molecular weight and physical properties play a stronger role in determining the optimal solvent pH.

3.3. Molecular weight

Molecular weight has a significant influence over the physical properties and biological activities of ulvan and it is important that this structural feature is preserved in order to maintain the versatility of the ulvan produced (Kidgell et al., 2019). In particular, number average molecular weight is a useful measure of the degree of polymerisation and, in this study, was used to obtain a relative measure of depolymerisation. The number average molecular weight of the ulvan extract ranged from 2.0 to 84.1 kDa and was strongly affected by the solvent pH, the temperature of extraction, and extraction duration (Table 3). Notably, all three extraction parameters had strong positive linear effects and strong negative quadratic effects (Table 3), demonstrating that the maximum predicted molecular weight of the extract occurred within the experimental space of all three extraction parameters (pH 3.4; 73.92 °C; 86.21 min; Table 5).

The molecular weight profiles recorded were generally multimodal demonstrating the presence of several distinct polysaccharide and oligosaccharide size classes (Fig. 2). Fractions with retention times at ~11 min (peak 1) and ~12 min (peak 2) are predominant in treatments at pH 2.5 (Fig. 1b) and pH 4.0 (Fig. 1c), while these are offset with fractions at 14 min, ~14.5 min, and ~16.25 min to varying degrees for pH 1.0 treatments (Fig. 1a). The latter is indicative of a continuum between the lower solubility of high molecular weight ulvan at pH 1.0

and 40 °C (e.g. Exp. 15) and a higher degree of depolymerisation at 90 °C (Exp. 6) (Hu and Goff, 2018). Indeed, peak 1 and peak 2 are completely absent in the Exp. 6 extract, suggesting significant depolymerisation. The latter is consistent with the literature reporting the effects of solvent pH on molecular weight (Glasson et al., 2017; Yaich et al., 2014; Robic et al., 2009a). Importantly, the results from this study demonstrate that high molecular weight ulvan can be isolated using milder acid extraction processes than previously reported, with only small variations in other desirable responses (e.g. yield and purity), including sulfate ester content (presented in detail in the e-supplementary), being detected.

3.4. Viscosity

Viscosity measurements are often a first step in the assessment of the rheological properties of hydrocolloids because viscosity is correlated with the structural features of the polysaccharide (e.g. molecular weight, branching, and sulfate content) and other rheological characteristics (Lahaye and Robic, 2007). Therefore, viscosity measurements were made on ulvan solutions to find extraction parameter settings that enhance this response. The viscosity of 0.5% *w*/w solutions of extracts measured by flow time ranged from 208 to 455 s and was predominantly influenced by the solvent pH and the temperature of extraction (Table 3). These two factors interacted such that at low temperatures the effect of solvent pH on viscosity was small but increased with increasing temperature. Overall, this meant that viscosity of the ulvan extract was highest at the maximum levels tested for all three factors (pH 4, 90 °C, and 90 min; Fig. 3). The viscosity of crude extracts was also positively correlated with the molecular weight ($r = 0.80$, $p < 0.05$) suggesting that ulvan was the major driver of the rheological properties in most extracts. However, at low pH, two factors influenced the viscosity of the ulvan extract solutions: depolymerisation of ulvan and high ash content. At low solvent pH and high extraction temperatures depolymerisation was significant, while at low solvent pH and low extraction temperatures high contents of ash reduced the effective concentration of the ulvan solutions. Although maximum viscosity was predicted at the highest levels of all extraction parameters, solid gels were formed by most extracts at viscosities as low as 305 s (presented in detail in the e-supplementary). This meant that the rheological properties of the ulvan in the extracts were conserved within acceptable limits except under the harshest extraction conditions (e.g. low pH, and high extraction temperature and duration).

3.5. Content of metals

The accumulation of metals by green macroalgae is well known (Deng et al., 2007; Suzuki et al., 2005; Bulgariu and Bulgariu, 2012; Areco et al., 2012) and an important consideration for the application of its biomass (Circuncisao et al., 2018). Thus, a selection of metals relevant to food and supplements including Cd, Cr, As, Hg, Pb, Cu, Zn, and Se were measured in the untreated biomass and in the extracts (presented in the e-supplementary). The content of metals in extracts ranged from 92 to 1367 mg/kg and was predominantly influence by Zn (62.13–1269.27 mg/kg), Cu (11.45–94.75 mg/kg), and Cr (1.06–26.60 mg/kg), with smaller contributions from Cd (1.10–5.75 mg/kg), Pb (0.04–3.42 mg/kg), Se (1.00–2.40 mg/kg), As (< 1 mg/kg), and Hg (< 0.1 mg/kg) (presented in the e-supplementary). Metal content was significantly affected by the solvent pH, the temperature of extraction, and extraction duration (Table 3). The solvent pH had the greatest effect on the concentration of metals with a large negative linear effect and a large positive quadratic effect (Table 3). Shifting to intermediate solvent pH from low solvent pH resulted in large decreases in the metal content of extracts, while further increases in the solvent pH had little to no effect on the metal content of extracts (Fig. 1f). This pH dependence is consistent with the increased mobility of metals at lower pH due to proton interactions with the chemical moieties that bind metals in the biomass (Deng et al., 2007). This effect was reduced at higher temperatures with an interaction between the solvent pH and the temperature of extraction (Table 3). This is due to the higher yields of ulvan achieved at higher extraction temperatures, which lead to the dilution of the content of metals in the ulvan extracts. Although temperature and duration generally had less of an effect on the metal content of extracts than pH, temperature had a relatively strong negative linear effect that reduced at high pH due to its interaction with pH. Therefore, the concentration of metals decreased with increasing temperature but this rate of decrease reduced with increasing pH. Extraction duration only affected the concentration of metals through a large positive quadratic term, meaning that the concentration of metals increased with longer extraction times but plateaued at the longest extraction duration. The optimised extraction conditions for metals (pH 3.3, temperature 90 °C, and duration 55.1 min) predicted a metal content of −45.88 mg/kg (Table 5), which is indicative of error in the model. However, the high extraction temperature and moderate solvent pH are consistent with extraction conditions that favour a high yield of ulvan, and the reduced mobility of metals in the biomass, respectively.

Safety levels for toxic heavy metals in seaweeds in most countries are not set and generally fall under broader food and supplement regulations (Circuncisao et al., 2018). However, France has regulated safe toxic metal contents for 21 edible seaweeds including *Ulva spp* (e.g. inorganic As 3 mg/kg, Hg 0.1 mg/kg, Pb 5 mg/kg, and Cd 0.5 mg/kg) (Circuncisao et al., 2018; French Regulation, 2014). Concerning these guidelines, extraction conditions were identified that produced ulvan extracts with acceptable contents of As, Hg and Pb, while the Cd

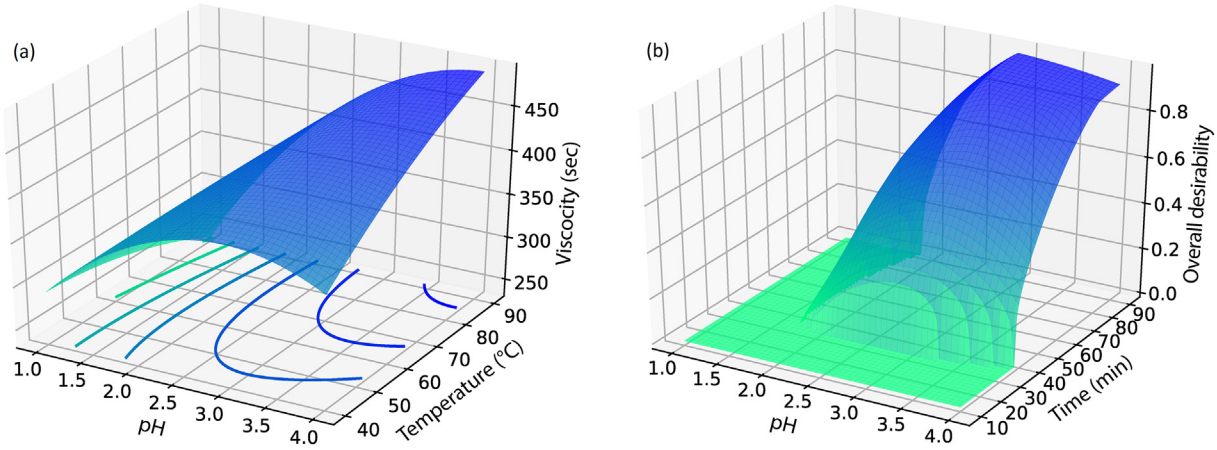

Fig. 3. Second-order response models for (a) viscosity (sec) presented at 90 min, and (b) overall desirability presented at an extraction temperature of 90 °C.

content (1.10–5.75 mg/kg) exceeded the guidelines. With regards to the latter, based on a 1 g ulvan supplement given to a 60 kg adult daily, and given the provisional tolerable monthly intake (PTMI) of Cd recommend by the Food and Agricultural Organisation and World Health Organisation (25 μg/kg bw), this represents 2.3–11.9% of the recommended limit (WHO, 2011). Under extraction conditions that minimise the content of toxic metal contamination, the Cd contribution of a 1 g daily supplement to the PTMI falls to the lower end of this range. Further reductions in Cd content in ulvan extracts may be achieved using a pre-wash step of the biomass prior to ulvan extraction to remove labile metal ions (Magnusson et al., 2016; Stevant et al., 2018).

3.6. Overall desirability

The predicted optimised responses for yield, purity, molecular weight, viscosity, and total metals occurred over a moderate to large range of extraction conditions (Table 5), demonstrating that these conditions cannot be optimised simultaneously. Under such circumstances, desirability functions can be employed to predict the best overall compromise between multiple responses, as has been reported for the biorefinery production of fucoidan and alginate from brown algae (Lorbeer et al., 2015). Here we directed the functions according to unacceptable levels, desirable targets, and the relative importance of each response (Table 2) in order to identify the best overall set of extraction parameters (Table 1). Maximal desirability occurred at a solvent pH of 2.92, an extraction temperature of 90 °C, and an extraction duration of 90 min. Under these extraction conditions, the crude ulvan extract was predicted to have a yield of 72.1%, a purity of 59.7%, a molecular weight of 79.3 kDa, a viscosity of 453.7 s, and a total metal concentration of 72.1 mg/kg. Experimental validation ($n = 3$) for these conditions resulted in similar values for yield (76.0 ± 3.0%; t-value = 2.82, $p > 0.05$), purity (63.6 ± 4.2%; t-value = 2.00, $p > 0.05$), and total metal content (78.8 ± 3.6 mg/kg; t-value = 3.86, $p > 0.05$), but significantly different values for molecular weight (51.8 ± 2.1 kDa; t-value = −28.35, $p < 0.05$) and viscosity (432.0 ± 8.7 s; t-value = −5.30, $p < 0.05$). The discrepancy between the measured and predicted values for molecular weight and viscosity are likely due to the strong positive correlation ($r = 0.80$, $p < 0.05$) between these responses. It is difficult to ascertain the exact cause of these discrepancies. One possibility may be that degradation of the dry biomass during storage (~6 months) between the model experiments and the validation experiments led to a reduction in molecular weight and thus viscosity. In this regard, only minor concurrent degradation (depolymerisation) of ulvan would result in this departure from the modelled responses. No data exists in the literature to directly validate this hypothesis, although stabilisation methods have been demonstrated to have a significant effect on the extractability and molecular weight of ulvan (Robic et al., 2008). It was noted that the measured values for both molecular weight and viscosity still fell within the ranges deemed acceptable (Table 2).

The optimised extraction conditions (pH 2.92, 90 °C, 90 min) are at the midpoint of solvent pH used in the literature and consistent with both extraction temperature and duration (Kidgell et al., 2019). The extraction of ulvan has been reported under a range of hot aqueous conditions using a number of extractants (e.g. sodium oxalate, sodium EDTA, and various acids). Thus, solvent pH varies widely (pH ~1–7) and is used without consideration of its selectivity for the extraction of ulvan over other macromolecules (e.g. proteins and other carbohydrates). Extracts derived from bioprocesses that employ solvent pH at the high end of the discussed pH range (e.g. pH 5–7) have high levels of macromolecular contaminants (e.g. proteins and other polysaccharides) and require further downstream purification. Extractions that use lower solvent pH (e.g. pH 1–2) yield ulvan extracts with higher purity, however, the ulvan is significantly affected by depolymerisation (Glasson et al., 2017; Yaich et al., 2014; Robic et al., 2009a). The optimised extraction temperature (90 °C) was constrained by the practicality of working below the boiling point of water and the susceptibility of the hydrolysis of the polysaccharide beyond these temperatures (Tsubaki et al., 2016). The optimised extraction duration (90 min) is likely to be adjusted on the basis of other practical limitations within a bioprocessing plant (e.g. heating and cooling).

Table 1
Coded and un-coded independent variables for the three-level three-factor BBD.

Run number	pH of solvent		Extraction temperature		Extraction duration	
	pH	Coded units	Temp. (°C)	Coded units	Time (min)	Coded units
1[a]	2.5	0	65	0	50	0
2	4.0	1	65	0	10	−1
3	4.0	1	40	−1	50	0
4[a]	2.5	0	65	0	50	0
5	4.0	1	65	0	90	1
6	1.0	−1	90	1	50	0
7	1.0	−1	65	0	90	1
8	2.5	0	40	−1	10	−1
9	2.5	0	90	1	10	−1
10	4.0	1	90	1	50	0
11	2.5	0	90	1	90	1
12	1.0	−1	65	0	10	−1
13[a]	2.5	0	65	0	50	0
14	2.5	0	40	−1	90	1
15	1.0	−1	40	−1	50	0

[a] Central point.

Table 2
The lower (L_i) and upper (U_i) limits, targets (T_i), and weightings (s) for response variables used in the Derringer's desirability function.

Response	Lower value (L_i)	Target (T_i)	Upper value (U_i)	s
Extraction efficiency (%)	60	74.0	–	1
Purity (% w/w)	44.2	52.8	–	5
Molecular weight (kDa)	25	79.3	–	5
Viscosity (sec)	300	380	–	1
Total metals (mg/kg)	–	200	374.1	1

Table 3
The model summary (ANOVA), lack-of-fit test results, and parameters of the selected model for each response variable.

	Yield	Purity	Molecular weight	Viscosity	Total metals
Model summary					
F-statistic	36.23	5.22	10.25	5.20	62.31
DF (residual, model)	(5, 9)	(5, 9)	(5, 9)	(5, 9)	(5, 9)
p-Value	< 0.001	0.042	< 0.001	0.042	< 0.001
R^2	0.98	0.90	0.95	0.90	0.99
R_{adj}^2	0.96	0.73	0.86	0.73	0.98
Lack of fit					
F-statistic	1.95	1.44	1.06	7.67	6.37
p-Value	0.357	0.436	0.518	0.118	0.139
Model parameters					
intercept	45.16[a]	60.58[a]	76.53[a]	397[a]	206.51[a]
X_{pH}	3.48[a]	7.53[a]	23.95[a]	56.54[a]	−512.95[a]
X_{time}	11.22[a]	5.64[a]	17.32[a]	15.63	−24.77
X_{temp}	17.11[a]	5.30	7.66	13.25	−181.81[a]
$X_{pH}X_{time}$	−0.83	−1.34	11.90	24.75	−9.30
$X_{pH}X_{temp}$	−3.55	2.71	7.42	46.83[a]	123.46[a]
$X_{time}X_{temp}$	−0.75	−8.04[a]	3.01	5.33	−0.48
X_{pH}^2	−2.36	−8.92[a]	−31.29[a]	−59.71[a]	364.12[a]
X_{time}^2	−3.73	0.74	−14.08	3.13	119.86[a]
X_{temp}^2	3.49	−6.31	−20.74[a]	−11.63	35.49

[a] Significant at α = 0.05.

Table 4
Experimental conditions for the three-level three-factor BBD with response values. Validation conditions and response values (R1-3), average and standard deviation ($n = 3$).

Exp.	pH	Temp. (°C)	Time (min)	Yield (%)	Purity (% w/w)	Molecular weight (kDa)	Viscosity (sec)	Total metals (mg/kg)	Protein (% w/w)	Ash (% w/w)
1	2.5	65	50	42.3	54.9	83.4	410	178.4	6.2	12.2
2	4.0	65	10	35.2	56.1	35.7	361	146.5	8.9	12.3
3	4.0	40	50	33.8	49.2	25.8	350	200.5	15.0	12.1
4	2.5	65	50	45.6	61.6	83.4	401	195.0	6.5	12.1
5	4.0	65	90	53.2	62.3	84.1	425	102.3	6.9	11.7
6	1.0	90	50	65.8	36.1	8.4	208	764.9	9.8	19.5
7	1.0	65	90	44.6	51.4	2.8	270	1253.1	6.4	30.4
8	2.5	40	10	15.1	31.2	17.5	333	584.1	12.5	14.7
9	2.5	90	10	49.5	65.1	21.2	397	213.1	5.9	12.5
10	4.0	90	50	62.3	58.0	61.6	422	92.1	7.6	11.2
11	2.5	90	90	73.2	62.7	71.9	455	138.7	6.5	11.4
12	1.0	65	10	23.3	39.9	2.0	305	1260.1	6.3	41.2
13	2.5	65	50	47.6	65.3	62.8	380	246.2	6.9	12.7
14	2.5	40	90	41.9	61.0	56.2	370	511.6	10.6	12.4
15	1.0	40	50	23.1	38.1	2.3	323	1367.0	4.1	39.9
R1	2.92	90	90	79.5	68.4	51.3	426	75.3	7.6	10.3
R2				73.9	60.7	54.1	442	82.5	7.9	10.7
R3				74.7	61.7	50.1	428	78.5	7.4	10.5
Av.				76.0	63.6	51.8	432	78.8	7.6	10.5
SD.				3.0	4.2	2.1	8.7	3.6	0.2	0.2

Table 5
The optimum values and corresponding independent variables for each response variable based on the selected models. Note: independent variables bounded by the experimental space examined (pH: 1–4, temperature: 40–90 °C, and duration: 10–90 min).

Response	Objective	Optimal value	pH	Temperature (°C)	Duration (min)
Purity	Maximise	68.17 (g/100 g)	2.99	61.31	90.00
Extraction efficiency	Maximise	72.59 (%)	2.21	90.00	90.00
Molecular weight	Maximise	92.89 (kDa)	3.40	73.92	86.21
Viscosity	Maximise	491.13 (sec)	4.00	90.00	90.00
Total metals	Minimise	−45.88 (mg/kg)	3.30	90.00	55.05

4. Conclusion

Given the broad range of desirable physicochemical properties and biological activities of ulvan, it is likely to find applications in a diverse range of products. Therefore, bioprocesses targeting intact ulvan that can be used as-is or further modified (e.g. controlled depolymerised) will help to streamline the commercialisation of this resource. This study demonstrated the capacity to fine-tune the acid extraction of ulvan in high yield and quality. Furthermore, control over the degree of contamination was also demonstrated suggesting that the quality of ulvan produced can be controlled to adhere to the guidelines set by food and supplement regulating bodies.

Declaration of Competing Interest

No conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2019.100262.

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