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An assessment of physical and chemical conditions in alginate extraction from two cultivated brown algal species in Norway: *Alaria esculenta* and *Saccharina latissima*

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ABSTRACT

Alginates are linear anionic polysaccharides originating from brown algae. Their properties target several application areas in in food-, technical- and pharmaceutical industries, and thus they are highly valued. Norway is currently the largest producer of cultivated seaweed in Europe, where the predominant species are *Alaria esculenta* and *Saccharina latissima*. So far, the utilisation of these two species have mainly been targeting food and feed applications. However, utilisation of these species for much broader application areas, including biopolymer and biomaterial markets, is of great interest. Both species are interesting candidates for future alginate production, but protocols for efficient extraction of high-quality alginate are lacking. In the present study, protocols for alginate extraction from fresh *A. esculenta* and *S. latissima* have been established. This has been accomplished by the identification and variation of key parameters in the extraction protocol, including pH, temperature and incubation times, and study of their effects on alginate quality and purity. Optimal conditions in the present study were found to be pH 9 in the alkaline extraction, and short extraction time (1–5 hours). The alginates extracted at pH 9 had a yield of 185 ± 7 and 229 ± 12 mg/g dry weight seaweed, and a weight average molecular weight (M_w) of 537 ± 12 and 503 ± 24 kDa in *A. esculenta* and *S. latissima*, respectively. The purity of the extracted alginates was evaluated based on the content of coextracted impurities and was found to be comparable with high-quality commercial alginates.

1. Introduction

Alginates are a family of linear anionic polysaccharides, composed of 1→4 linked β-D-mannuronic acid (M) and its C5 epimer α-L-guluronic acid (G) residues [1]. They are arranged in G- and M-blocks (homopolymeric regions with M- and G-residues), interspersed by MG-blocks (regions where the two residues are alternating). The length and relative abundance of these blocks depend on the source of the alginate [2]. Their natural abundance in nature, high intrinsic viscosity, and ability to form hydrogels with divalent cations [3], have resulted in a wide range of industrial applications. Brown algae (Phaeophyceae) are the main source of commercial alginates, although alginates are also produced by some bacteria in the formation of extracellular cysts and biofilms [4].

Considering the long coastline and cold, nutrient rich water with high salinity, Norway has perfect growth conditions for brown algae [5]. This is evident in an 80-year-old alginate production industry predominantly from the wild harvested *Laminaria hyperborea* (Gunnerus) Foslie [6]. However, a large fraction of the natural kelp forests is unavailable for harvest due to large topographical variations in the seabed, complicating trawling by conventional methods. There is also a limit to how much biomass that can be harvested sustainably without a negative impact on the natural population and associated ecosystems [7].

Seaweed cultivation is a small, but rapidly growing sector within the marine industry in Norway. With a production of near 350 tons biomass in 2020 [8], the volumes are still low on a global scale. However, with an expansion of the seaweed market, the present technology could support a Norwegian seaweed production of millions of tons in the future [9]. Considering the low requirements for biomass growth (CO_2, seawater,

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sunlight) and low environmental impact on natural habitats [10], seaweed cultivation could be a significant contributor to achieving UN's sustainable development goals [11]. Continued future growth of the seaweed aquaculture industry in Europe will depend on increased diversity of applications, including production of alginates and other high-value compounds from large-scale biorefineries.

Alaria esculenta (Linnaeus) Greville (winged kelp) and *Saccharina latissima* (Linnaeus) C.E.Lane, C. Mayes, Druehl & G.W. Saunders (sugar kelp) are the predominant cultivated brown algae species in Europe (Fig. 1). Alginate from *L. hyperborea* (stipes) has more favourable gelling properties compared with alginate from other brown algal species, due to the high content of long G-blocks [12]. Over 50 % of the G-blocks in *L. hyperborea* has been shown to have a degree of polymerization (DP) >20 [13], and the fraction of G (F_G) usually range between 0.6 and 0.85 in alginate from the *L. hyperborea* stipe [14]. In G-blocks, G-residues are oriented diaxial to each other, which enables divalent ions to cross-link the alginate in the so-called "egg box-model", forming strong hydrogels [15]. Alginates from *A. esculenta* and *S. latissima* have a lower G-content compared with *L. hyperborea*. The fraction of G residues (F_G) has previously been measured to be 0.35–0.45 in *A. esculenta*, and 0.4–0.45 in *S. latissima* [2], and have also exhibited greater variations based on own unpublished results. It is, however, of interest to investigate these species as candidates for alginate production as they can provide a large future supply to complement wild harvest. The species can also provide high-molecular weight alginates suitable for thickening applications, which can further be enzymatically upgraded to enhance gelling properties [16,17].

Fig. 1. Thallus structures in Saccharina latissima and *Alaria esculenta*.

The general approach for extracting alginate from brown algae was first described and patented by Stanford in 1881 (British Patent 142 [18]). Although several changes have been done to this protocol, the main principles of Stanford's methods are still followed [2]: A pretreatment with acid, an alkaline extraction with sodium carbonate or sodium hydroxide, followed by precipitation of alginate with alcohol, acid, or divalent cations. However, due to a lack of standardisation, commercial alginates are derived from variable processing conditions (temperatures, pH, extraction times and subsequent purification steps), resulting in variable composition, purity, and final properties [19]. The seaweed cell walls are complex matrixes of different water-soluble compounds such as fucoidans, proteins, phenols, and minerals. In addition, brown seaweeds produce and store photosynthetic reserve products, such as water soluble laminarins and mannitol [20]. The prevalence of these compounds can have large interspecific and seasonal variations [21,22]. Thus, the probability of co-extracting other seaweed compounds in the alginate extraction is high and will depend on the processing conditions.

The aim of the present study has been to assess the effects of selected conditions (pH, extraction times, and temperature) on alginate extraction from fresh cultivated *A. esculenta* and *S. latissima*. The extracted alginates have been evaluated with respect to yield and quality (molecular weight, viscosity, purity, and chemical composition). This is to our knowledge the first paper exploring different conditions for alginate extraction from Norwegian cultivated *A. esculenta* (*AE*) and *S. latissima* (*SL*).

2. Materials and methods

2.1. Preliminary studies

Prior to evaluating processing conditions on fresh biomass, preliminary studies were carried out on frozen and thawed material from a previously harvested seaweed batch, to establish a standard alginate extraction protocol as a baseline for subsequent comparisons. All chemicals used in this study are of analytical grade and supplied by Sigma-Aldrich, Norway unless otherwise stated. Pre-treatment was performed with milled (mesh size = 6 mm) *AE* and *SL* in 0.2 M hydrochloric acid (HCl) overnight (pH 1), followed by alkaline extraction with 0.2 M sodium bicarbonate ($NaHCO_3$) overnight (pH 7.5). The alkaline extraction step was repeated twice to assess alginate yield in each fraction. In addition, different ratios between biomass and liquid were used to assess alginate yield and ease of processing. Results from these preliminary studies were used to establish the standard alginate extraction protocol described in Fig. 2. The pre-treatment with HCl is referred to as the "acid step", and the alkaline extraction with $NaHCO_3$ is referred to as the "alkaline step".

2.2. Seaweed collection

The cultivated seaweed used in this study is collected at the Seaweed Solutions (SES) farm located at Frøya (N 63°44.6720437′, E 8°53.1976789′), Norway. The seaweed is cultivated on ropes 2 m below the sea surface, and the water here is classified as being euhaline (>30 PSU) and highly wave exposed [23]. *AE* and *SL* were not harvested the same time due to harvesting logistics. The *AE* sporelings were deployed in the sea January 2021, and harvested 24th of May 2021, whereas the *SL* sporelings were deployed in January 2021 and harvested on the 27th of April 2021. The two species are deployed at different ropes, and a correct species identification was guaranteed by the presence of a midrib in *AE* (Fig. 1). Following harvest of each species, the seaweed was milled and stored cold (4 °C) overnight prior to extraction experiments.

2.3. Alginate extraction

The general alginate extraction procedure is shown in Fig. 2, and the

Seaweed harvest
Stored cold (4°C)

Seaweed milling
Mesh size = 6 mm

ACID STEP
5 g seaweed + 40 mL 0.2 M HCl
50 mL tube
Shaking incubation
RPM: 200, orbital movement 2.5 cm amplitude
Time: 20 hours
Temperature:

Ex. 1	Ex. 2	Ex. 3	Ex. 4	Ex. 5	Ex. 6	Ex. 7
20°C	20°C	20°C	20°C	20°C	**50°C**	**50°C**

Centrifugation
3220×g, 10 min

Washing
Ion free water, 3220×g, 5 min

ALKALINE STEP
Acid washed seaweed + 35 mL 0.2 M $NaHCO_3$
50 mL tube
Shaking incubation
RPM: 200, orbital movement 2.5 cm amplitude
pH:
Time:
Temperature:

Ex. 1	Ex. 2	Ex. 3	Ex. 4	Ex. 5	Ex. 6	Ex. 7
pH=7.5	**pH=9**	pH=7.5	pH=7.5	pH=7.5	pH=7.5	pH=7.5
20 hours	20 hours	**1 hour**	**5 hours**	20 hours	20 hours	20 hours
20°C	20°C	20°C	20°C	**50°C**	20°C	**50°C**

Centrifugation
3220×g, 10 min

Separation
Pellet and alginate solution

Alginate solution filtration
Mesh size = 100 µm

Alginate precipitation
Ethanol (50%) + NaCl (2 g/L)

Alginate washing
Ethanol (70%)
Ethanol (100%)

Lyophilisation and weighing

CHARACTERISATION

Fig. 2. Alginate extractions in the present study. Extraction (Ex.) 1 represents the "standard extraction protocol", and the changes done to the protocol are highlighted in the figure under extractions (Ex.) 2–7 and are also described in Table 1. The pre-treatment with 0.2 M HCl is referred to as the "acid step", and the alkaline extraction with 0.2 M $NaHCO_3$ is referred to as the "alkaline step".

different parameters applied are shown in Table 1. In total, seven different extractions were carried out for both species in parallel. The conditions were chosen based on preliminary screening trials and previous studies on various species [24,25]. For each sample, 5 g fresh milled biomass was added to a 50 mL centrifuge tube with triplicates for each extraction condition. For the acid step, 0.2 M HCl was added to the tubes (40 mL) and incubated overnight under shaking (200 rpm, orbital movement 2.5 cm amplitude) at 20 or 50 °C, followed by centrifugation (3220 ×*g*, 10 min) and washing of the biomass with ion-free water. The supernatant was discarded, whereas the insoluble alginic acid was kept within the biomass. For the alkaline step, 0.2 M $NaHCO_3$ was added (35 mL) followed by pH adjustment to 9 with 3 M NaOH of selected samples (Table 1) and incubation (1, 5 or 20 hours) under the same conditions as the acid step described above. After incubation, the alkaline alginate extract was separated from the residual biomass by centrifugation (3220 ×*g*, 10 min), followed by filtering (mesh size = 100 µm). Then, 20 mL of the alkaline extract was precipitated by adding NaCl (2 g/L) and an equal volume of ethanol, and the precipitate was washed twice in ethanol (70 and 100 %) before lyophilisation.

Table 1
Description of the different chemical and physical parameters tested in the extractions. The treatment names correspond to extraction (Ex.) 1–7 described in Fig. 2.

Ex.	Treatment name	Description
1	Ctrl	Standard extraction protocol.
2	pH=9	pH increased from 7.5 to 9 in the alkaline step.
3	1 hour	Incubation time in the alkaline step reduced from 20 to 1 hour.
4	5 hours	Incubation time in the alkaline step reduced from 20 to 5 hours.
5	50 °C base (B)	Incubation temperature in the alkaline step increased from 20 to 50 °C.
6	50 °C acid (A)	Incubation temperature in the acid step increased from 20 to 50 °C.
7	50 °C acid+base (A+B)	Incubation temperature in both the acid and alkaline step increased from 20 to 50 °C.

2.4. Characterization

2.4.1. Element analysis and estimation of contaminants

The content of carbon (C), nitrogen (N) and sulfur (S) in the raw material and the extracted alginate was determined by weighing approximately 5 mg lyophilized sample in tin capsules. The samples were oxidised at 1150 °C and analysed on a Vario-El-Cube CNS element analyser (Elementar).

Concentration of the following elements Na, Ca, K, Mg and P was measured by inductively coupled plasma mass spectrometry (ICP-MS) in both the raw material and the extracted alginate. Further, Cl, I, Br, Fe, As, Zn, Ba, Cu, Cd, Se and Pb was measured in the raw material. All solutions and dilutions were prepared using ultra-purified water 18.2 MΩ from an OmniaTap 10 UV system (Stakpure, Germany) and concentrated nitric acid (65 % HNO_3) purified by a Savillex DST-100 Acid Purification System (Savillex, USA) or 25 % tetramethylammoniumhydroxide (TMAH, Acros). Standards for calibration curves were prepared from single element and mixed standard solutions from Inorganic Ventures, USA. Na, Mg, P, S, K, Ca, Fe, Cu, Zn, As, Se, Cd, Ba and Pb were diluted in 5 % HNO_3 (v/v), while 1 % (v/v) TMAH were used for chlorine (Cl), bromine (Br) and iodine (I). Indium (In) and tellur (Te) were used as internal standards. 200 mg lyophilized seaweed samples

were digested with 5 ml 50 % (v/v) nitric acid (HNO_3) at 250 °C in an UltraWAVE microwave oven (Milestone, Italy) and diluted to 5 % (v/v) upon analysis. Samples for Cl, Br and I analysis were extracted in parallel with 5 ml 20 % (v/v) TMAH at 80 °C in a bead bath overnight and diluted to 1 % (v/v) upon analysis.

The measured sulfur was used to estimate fucoidan levels in the precipitated alginate. The sulfur (S) and fucose content (fc) have been measured in an extracted fucoidan from *SL*, and was 11.8 and 33.6 %, respectively (Birgersson, unpublished data). Assuming that only the fucose residues in the fucoidan are being sulphated, the sulphation degree (DS) is 1.68, translating to 84 % of the free hydroxyl groups being sulphated. These numbers are in good accordance with those reported by Bilan et al. [26]. Based on fc and DS, the degree of fucoidan contamination in the *AE*- and *SL*-alginates was calculated based on Eqs. 1, 2 and 3 [27]:

$$w(SO_4) = \frac{w(S) \times M(SO_3 + Na - H)}{M(S)} \quad (1)$$

$$w(fucose) = \frac{w(SO_4) \times M(fucose - H_2O)}{M(SO_3 + Na - H) \times DS} \quad (2)$$

$$w(fucoidan) = \frac{w(fucose)}{fc} \times (100 - fc) + w(fucose) \quad (3)$$

where w = mass fraction, M = molar mass (g/mol), DS = degree of sulfation and fc = estimated fucose content in the original fucoidan.

Nitrogen content was used to calculate protein content in the alginate samples, based on a nitrogen-protein factor (K_p) of 3.8, originating from work done by Forbord et al. [28].

The extracted alginate will be on sodium form (Na-alginate). Assuming one Na ion per alginate residue, the weight contribution of Na will be 11.62 % ($M_{Na}/M_{Na\text{-}alginate}$ = 23 g/mol/198 g/mol), meaning that in 1 g Na-alginate, 116.2 mg will be Na-ions. Na-contents exceeding this value are considered excess Na.

2.4.2. Dry matter and ash analysis

Dry matter (DM) and ash content was measured in the raw material of both *A. esculenta* and *S. latissima*. For both species, approximately 5 g fresh, milled material was weighed out in crucibles. Measurements were done in triplicates. The samples were dried at 105 °C in 22 hours, and then cooled down to room temperature in a desiccator. Each crucible was weighed, and the dry matter content calculated based on the seaweed wet weight (ww) and Eq. 4:

$$DM(\%of\ ww) = \frac{\text{Total weight after drying} - \text{weight crucible}}{ww} \times 100\% \quad (4)$$

The crucibles with dried material were burned in an ashing furnace at 550 °C for 12 hours. Ash content was calculated based on Eq. 5:

$$Ash(\%of\ DM) = \frac{\text{Total weight after combustion} - \text{weight crucible}}{DM} \times 100\% \quad (5)$$

2.4.3. Gel permeation chromatography (GPC)

GPC was performed on an Agilent 1260 Infinity II with a refractive index (RI) detector. The instrument was equipped with two columns from Agilent Technologies (PL aquagel-OH, 8 µm, Mixed-C, 300 × 7.5 mm, 6000–10,000,000 g/mol range and PL aquagel-OH, 8 µm, Mixed-C, 300 × 7.5 mm, 1000–500,000 g/mol range) and a guard column (PL aquagel-OH, 8 µm, 50 × 7.5 mm). The columns and detector were maintained at 60 °C. The analysis was run at 1 mL/min with an isocratic mobile phase (0.2 M $NaNO_3$, 0.01 M NaH_2PO_4, pH 7). The system peak occurred at approximately 19 minutes and the sequence was run for 40 min (for chromatograms, see supplementary material, Fig. S1). The columns were calibrated against polyethylene oxide/glycol (PEG-PEO) standards (7 standards between 1,370,000 and 615 g/mol, Agilent Technologies, PL2080-0201).

2.4.4. Rheological characterization

The viscosity of the alginates was measured using a Kinexus rheometer (Netzsch, Germany) equipped with a CP1/40 L1495 SS upper geometry and a PLS61 S4410 SS lower geometry. The alginate was dissolved in water to a concentration of 1 % (w/v) and the viscosity was measured over a shear rate of 0.1–100 s^{-1} with 10 measurements per decade, at 25 °C.

2.4.5. ^1H NMR

The alginates were analysed by following the ASTM (American Society for Testing and Materials) standard test method for determining the chemical composition of alginate by NMR spectroscopy [29] and as described before [30–32]. In summary, the alginates were partially degraded by stepwise acid hydrolysis to an approximate degree of polymerization (DP) of 50 for ^1H NMR analysis. 15 mg alginate were dissolved in ion-free water, pH adjusted to 5.6 with 0.1 M HCl, and hydrolysed at 95 °C for 1 hour. The solutions were immediately cooled to room temperature, pH adjusted to 3.8, followed by hydrolysis at 95 °C for 50 minutes. The solutions were cooled, neutralised with 0.1 NaOH (pH 6.8–7.5) and lyophilised.

Approximately 10 mg of the degraded samples were dissolved in 600 µl D_2O (d-99.9 %) and dissolved overnight. Triethylenetetramine-hexaacetic acid (TTHA) in D_2O (0.3 M, 20 µL) was added as chelator. The samples were centrifuged, and the supernatants were transferred to NMR tubes. 3-(Trimethylsilyl)-propionic-acid sodium salt (TSP) (Aldrich, Milwaukee, WI) in D_2O (1 %, 5 µL) was added for internal chemical shift reference. ^1H NMR spectra were recorded at 82 °C on a BRUKER NEO 600 MHz spectrometer (Bruker BioSpin AG, Fälladen, Switzerland) equipped with 5 mm iProbe and 2D heteronuclear single quantum coherence (HSQC) were recorded on a Bruker AV-IIIHD 800 MHz spectrometer (Bruker BioSpin AG, Fälladen, Switzerland) equipped with a 5 mm cryogenic CP-TCI z-gradient probe. For NMR characterization of the alginate samples the following spectra were recorded: 1D proton with 30° flip angle (spectral width 12 ppm, spectral resolution 64 k points, number of scans 64, interscan delay 1 s), 2D {^1H–^{13}C} heteronuclear single quantum coherence (HSQC) with multiplicity editing (spectral width C 140 ppm/H 12 ppm, spectral resolution H 2k/C 256k points, number of scans 32, interscan delay 1.8 s). The resonance assignment spectra are based previously published assignments for alginate and fucoidan [30].

The spectra were recorded using TopSpin 4.0.8 software (Bruker BioSpin) and processed and analysed with TopSpin 4.0.7 software (Bruker BioSpin).

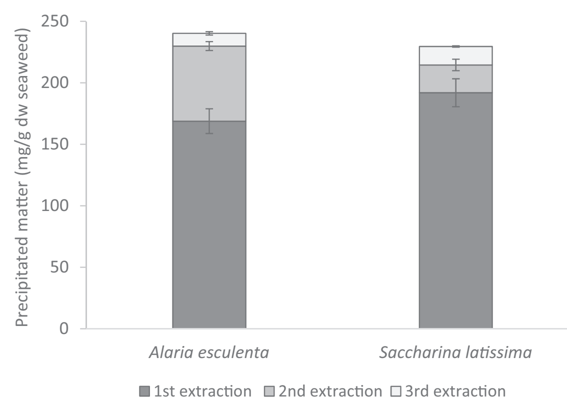

Fig. 3. Yield of precipitated matter from preliminary studies. Data are presented as means ± standard deviation, n = 3. Following a pre-treatment with HCl, alkali extraction with $NaHCO_3$ was performed in three rounds, each for 20 hours at room temperature.

3. Results

3.1. Establishment of standard alginate extraction protocol

Initial studies (results not presented) showed that alginate can be extracted under relatively mild conditions compared with previous experiences and literature values for other species. These mild conditions were thus employed as the standard extraction protocol (Fig. 2). Further preliminary investigations showed that three consecutive alkaline extractions, each for 20 h at room temperature with $NaHCO_3$, resulted in a cumulative yield of 240 ± 15 and 230 ± 17 mg precipitated matter/g dw seaweed for *A. esculenta* and *S. latissima*, respectively (Fig. 3). The results further showed that approximately 29.7 ± 0.5 % and 16.4 ± 1.7 % of the extractable matter is retained in the biomass after one extraction round for *AE* and *SL*, respectively. The apparent lower extractability of *AE* alginate has also been observed in other experiments (data not shown). This result may indicate different structural organization of the two species but can also arise from seasonal variations in the biomasses. Based on these observations it was reasonable to assume that the chemical and physical conditions can be optimized to maximize alginate yield and quality in a one-step extraction procedure, to ensure a time- and energy-efficient process. Furthermore, as the viscosity increased rapidly upon exposure to alkali, it was hypothesized that the extracted alginate was saturated in solution at an earlier time point than 20 h.

In a separate experiment, alginate was extracted under standard conditions (Fig. 2) using different volume ratios of fresh *AE* biomass and alkali to determine an optimal ratio for subsequent experiments. To maximize yield and minimize alkali and water use, extraction at 143 g fresh biomass/L alkali (5 g fresh seaweed + 35 mL $NaHCO_3$) was chosen as the standard fresh seaweed/alkali ratio for subsequent experiments (Supplementary material, Fig. S2).

3.2. Compositional analysis of biomasses

The two biomasses employed in the present study differ from each other in composition and were characterised following harvest. Dry matter and ash analysis showed that the water content was higher in *SL* than in *AE* (Fig. 4). Here, it must be noted that while the seaweed was treated similarly after harvest, the water content analysis did not account for eventual differences in surface water. Ash constituted 50 % of the dry matter in *SL*, whereas 65 % of the dry matter in *AE* was organic content, and 35 % was ash. The higher organic content in *AE* is also reflected in the higher carbon content shown in Table 2. For the other elements measured by CNS and ICP-MS analyses, there are some clear interspecific differences (Table 2), such as the higher salt and iodine content in *SL* compared to *AE*. There was also observed a twofold higher sulfur content in *SL* compared with *AE*, which can be associated with a higher fucoidan content in the biomass. As the biomass was harvested before any growth of epiphytes, the calcium content was presumably mainly associated with the alginate and did not differ significantly between the two species and can be an indication of similar alginate levels.

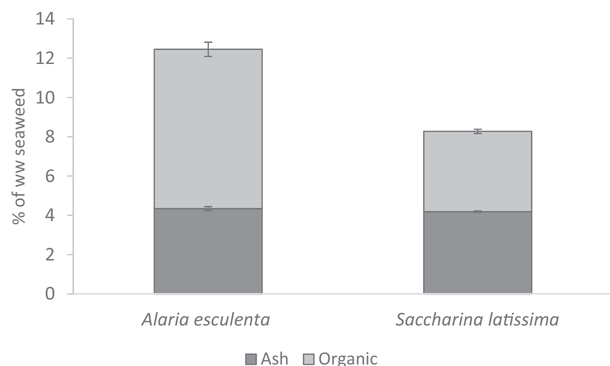

Fig. 4. Ash and organic content (% of wet weight) in the seaweed biomass utilized in the following experiments. Data are presented as means ± standard deviation, n = 3.

Table 2
Elements in the untreated seaweed biomass utilized in the following experiments. The content of C, N and S are data from the Vario-El-Cube CNS element analyser, whereas the remaining elements are measured by ICP-MS analysis. Data are presented as means ± standard deviation, n = 3.

	Alaria esculenta	*Saccharina latissima*
	mg/kg dw seaweed	
C	299433 ± 14651	240467 ± 14137
N	16600 ± 2272	26900 ± 1114
S	8443 ± 918	16140 ± 1396
Cl	88312 ± 6965	146285 ± 4106
K	65545 ± 9480	131182 ± 11580
Na	49816 ± 6671	63059 ± 2135
Ca	9489 ± 309	8739 ± 170
Mg	8126 ± 697	7738 ± 117
P	2524 ± 266	3623 ± 145
I	670 ± 83.1	3264 ± 58.0
Br	599 ± 276	779 ± 40.6
Fe	74.1 ± 9.73	226 ± 102
As	50.7 ± 6.76	66.0 ± 1.74
Zn	40.7 ± 2.92	25.0 ± 1.24
Ba	11.2 ± 1.12	10.0 ± 0.36
Cu	1.73 ± 0.19	1.73 ± 1.23
Cd	1.53 ± 0.18	0.69 ± 0.02
Se	0.13 ± 0.02	0.10 ± 0.01
Pb	0.17 ± 0.03	0.23 ± 0.11

3.3. Possible to extract alginate with high yield and quality from AE and SL

Seven different extraction conditions were evaluated; the effect of increased pH (from 7.5 to 9), the effect of shorter incubation time in the alkaline step (1 and 5 hours compared to 20), and the effect of increased temperature (50 °C compared to 20 °C). The yields of precipitated matter (alginate and co-extracted impurities) are summarized in Fig. 5A–C, and the weight average molecular weights (M_w) are shown in Fig. 5D–F. See supplementary material, Table S1, for number average molecular weight (M_n) and polydispersity (M_w/M_n), and Fig. S3 for viscosity measurements.

Increasing the pH from 7.5 (Control) to 9 resulted in an increased yield of precipitated matter for both species accompanied by no significant changes to the M_w, nor a decrease in shear viscosity (Fig. S3). Decreasing the incubation time in the alkaline step from 20 hours to 1 and 5 hours, was found to have no effect on the yield of precipitated matter nor the M_w and shear viscosity of the alginate.

Increasing the temperature was found to have a different impact on the two biomasses studied. For both species, increasing the temperature from 20 °C to 50 °C in only the alkaline, or both alkaline and acid step, resulted in a significantly increased yield of precipitated matter. This effect was also observed in *SL* when increasing temperature from 20 °C to 50 °C in the acid step, but not for *AE*. Increasing the temperature in the acid step resulted in a substantial drop in the M_w and shear viscosity of the alginate for both species, whereas only a minor decrease in M_w was observed after performing the alkali extraction at 50 °C (Fig. 5F).

^{1}H NMR analysis allowed characterization of the extracted alginate structural composition based on published assignment in the literature [29,30]. Table 3 (and supplementary material, Fig. S6) summarises the fractions (F) of β-D-mannuronic acid (M) and α-L-guluronic acid (G), and the dyads GG, GM, MG and MM in the alginates. The *AE* samples were found to have a higher F_G compared with *SL*, and no apparent structural changes were observed between the different extraction conditions. The ^{1}H NMR spectra of the analysed precipitated matter revealed traces of fucoidan in all samples, identified by the characteristic signal of the methyl group in fucose ~1.3 ppm for proton and ~17 ppm for carbon

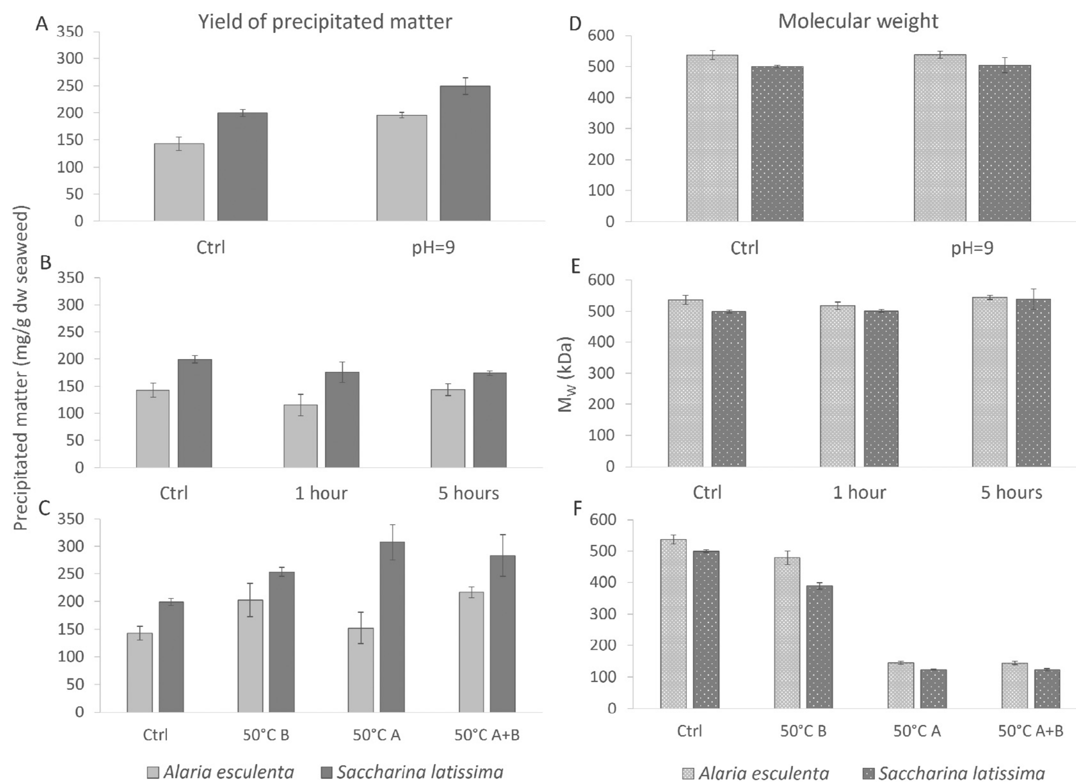

Fig. 5. Yield of precipitated matter (A–C) and molecular weight of alginate (D–F) with different extraction parameters: pH increased from 7.5 to 9 (A and D), incubation time in the alkaline step reduced from 20 to 1 and 5 hours (B and E), temperature increased from 20 to 50 °C in the alkaline and/or acid step (C and F). Data are presented as means ± standard deviation, n = 3.

Table 3
Chemical composition and some sequential parameters of the extracted alginates based on the characteristic signals for M and G residues obtained from the anomeric region (see supplementary material, Fig. S6).

	Alaria esculenta					*Saccharina latissima*				
	F_G	F_M	F_{GG}	$F_{GM,\ MG}$	F_{MM}	F_G	F_M	F_{GG}	$F_{GM,\ MG}$	F_{MM}
Ctrl	0.61	0.39	0.45	0.17	0.22	0.49	0.51	0.28	0.20	0.31
pH=9	0.58	0.42	0.41	0.17	0.25	0.49	0.51	0.29	0.20	0.31
1 hour	0.60	0.40	0.43	0.17	0.23	0.51	0.49	0.31	0.20	0.29
5 hours	0.59	0.41	0.42	0.17	0.24	0.51	0.49	0.30	0.20	0.29
50 °C B	0.59	0.41	0.42	0.17	0.24	0.48	0.52	0.28	0.20	0.32
50 °C A	0.60	0.40	0.45	0.15	0.25	0.49	0.51	0.30	0.20	0.31
50 °C A+B	0.62	0.38	0.45	0.16	0.22	0.48	0.52	0.28	0.20	0.32

Table 4
Carbon, nitrogen and sulfur in alginate extracted from cultivated *Alaria esculenta* and *Saccharina latissima* (mg/g dw precipitated matter). Data are presented as means ± standard deviation.

mg/g dw precipitated matter	*Alaria esculenta*			*Saccharina latissima*		
	C	N	S	C	N	S
Ctrl	303 ± 35.6	4.65 ± 1.63	5.28 ± 0.54	302 ± 12.5	5.75 ± 1.48	4.15 ± 0.28
pH=9	279 ± 2.29	4.27 ± 1.18	3.51 ± 1.61	254 ± 4.01	5.40 ± 1.23	7.15 ± 1.39
1 hour	283 ± 4.99	5.10 ± 0.61	6.23 ± 2.04	282 ± 1.87	6.50 ± 1.87	2.44 ± 0.42
5 hours	289 ± 1.31	4.67 ± 0.35	4.78 ± 1.34	276 ± 1.39	5.63 ± 0.42	2.30 ± 0.41
50 °C B	274 ± 3.87	5.63 ± 0.55	4.39 ± 0.95	269 ± 4.70	5.10 ± 0.61	3.68 ± 0.30
50 °C A	284 ± 6.39	3.50 ± 0.17	2.88 ± 1.17	208 ± 17.9	4.50 ± 0.70	5.20 ± 1.06
50 °C A+B	285 ± 2.57	4.93 ± 0.32	4.03 ± 0.25	237 ± 16.6	5.00 ± 0.35	3.92 ± 0.41

(Supplementary material, Figs. S4 and S5) [26,27]. Laminarin and mannitol were not detected in the alginate samples based on NMR analysis (Supplementary material, Fig. S5).

The content of carbon, nitrogen, and sulfur in the alginate samples from the seven extractions are summarized in Table 4. Based on these values, protein and fucoidan in the alginate samples were calculated and are shown in Fig. 6 alongside the content of phosphor, potassium, magnesium, calcium, and excess sodium (not associated with alginate as counterions), which together are expected to be the most abundant contaminants in the extracted alginate. For a full overview of the

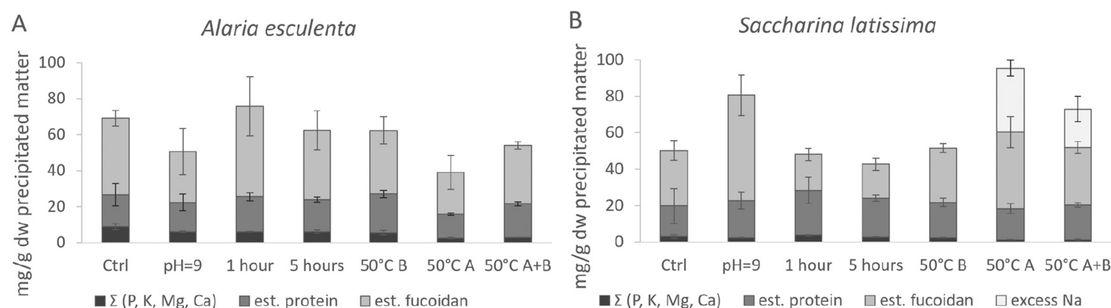

Fig. 6. The sum of P, K, Mg and Ca content, calculated protein and fucoidan content, and excess Na (not associated as counterions on the polymer chain) in the extracted fractions (mg/g dw precipitated matter). A: *Alaria esculenta*, B: *Saccharina latissima*. Data are presented as means ± standard deviation, n = 3.

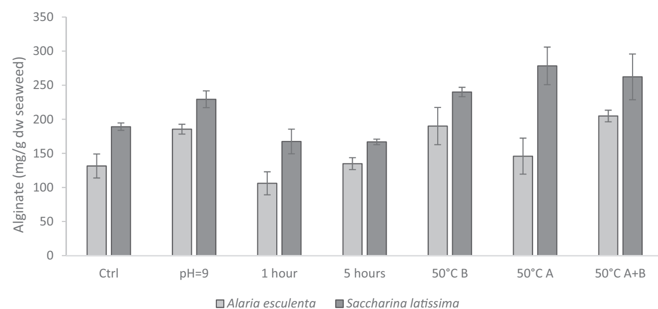

Fig. 7. An estimation of alginate yields from the different extractions. Yield of precipitated matter is subtracted with calculated protein and fucoidan contamination, excess Na (free Na-ions not acting as counterions for alginate polymer), and the P, K, Mg and Ca content. Data are presented as means ± standard deviation, n = 3.

elements measured in the alginate samples by ICP-MS (Supplementary material, Tables S2 and S3). While analyses of the native biomass showed a higher sulfur content in *SL* presumably associated with higher levels of fucoidan, this difference was not observed in the precipitated extracts. The calculated impurities comprise between 3 and 8 % of the precipitated matters, depending on extraction method and species. The excess sodium observed in *SL* in the two extractions including an acid step at 50 °C, could originate from the precipitation process where sodium is added (Fig. 2). Subtracting these impurities from the yields of precipitated matter provides an estimate of the alginate extracted under each condition (Fig. 7). Here, the estimated alginate yields showed the same trend as the initially measured precipitated matter (Fig. 5).

4. Discussion

The results from the present study indicated that higher alkali and temperature can increase the yield of extracted alginate from *A. esculenta* and *S. latissima* but can also have a negative impact on the molecular weight, viscosity and/or purity of the alginate. This is evident for the increased temperature of the acid step, which accelerates acidic hydrolysis of the alginate glycosidic bonds. The resulting depolymerization can in turn break structures in the seaweed matrix and enhance extraction. Alginate can also depolymerise at alkaline conditions through a β-elimination mechanism. However, increasing the pH to 9 was in the present study not found to negatively impact the M_w but increased the yield of alginate. Noteworthy, decreasing the extraction time from 20 to 1 or 5 hours did not have an impact on the yield. This can have a positive impact on the economics of the process and allow stronger alkaline conditions if shown to be favourable. Considering the conditions applied in the present study, it would be beneficial to carry out alkaline extraction of alginate at pH around 9 for 1 to 5 hours.

However, the validation of biopolymer quality is not only based on M_w, but also chemical composition and the content of co-extracted impurities [33]. ^{1}H NMR analysis of the extracted alginates showed that the chemical composition has gone through very little or no change throughout the different extraction methods compared to the control. The higher F_G in *A. esculenta* compared to *S. latissima* could be explained by interspecific differences, in addition to the different harvesting times, since chemical composition and sequential structure of alginates may vary with season [2].

The calculated contents of fucoidan, protein and minerals/salts in the alginates varied between the different extraction conditions. At pH 9, which was favourable for alginate yield and M_w, there was observed a higher fucoidan content in the *S. latissima* alginate compared to the control. To account for these impurities, the weight contribution of the co-extracted compounds was subtracted from the yield of precipitated matters. After these calculations, the pH 9 extraction was still shown to be more favourable considering alginate yield. The same high fucoidan content in the pH 9 extraction was not observed for *A. esculenta*. These fucoidan contents are estimations and must be interpreted with care. Impurities can be further reduced by additional post-precipitation washing steps, although the added time and costs of this must be considered against the improved quality of the alginates.

The consistently higher alginate yields in *S. latissima* compared to *A. esculenta* throughout all the extractions, could be explained by the different harvesting times of the two biomasses. Brown algae, together with most seaweeds, are photoautotrophic organisms that store photosynthetic reserve products in cytoplasmic vacuoles, to prepare themselves for periods with less day light and photosynthetic activity [34]. Members of Phaeophyceae build up their winter reserves with laminarins, a class of storage β-glucans with a molecular size of about 5 kDa [35,36] and mannitol, a 6-carbon alcohol [37]. These storage products have been shown to accumulate in the seaweed biomass during spring and early summer, and will consequently constitute more of the seaweed biomass later in the season [20,38]. Thus, the lower alginate yield from *A. esculenta* could be due to the biomass being harvested one month later than *S. latissima*. Interestingly, no traces of these storage products were found in the extracted alginates. Both mannitol and laminarin are water soluble and will consequently be removed during the acid step in this alginate extraction protocol.

Throughout this study, alginate yield and quality (M_w, viscosity, chemical composition and purity) from the different extractions have been evaluated against the alginate from the standard extraction protocol, illustrated in Fig. 2. To evaluate this alginate on a general basis can be a challenging task. The overall characterization of cultivated *A. esculenta* and *S. latissima* is in a relatively early stage, due to the only newly evolved industrial interest in these species. What constitutes a high quality of alginate will also inherently depend on the application areas.

The reported alginate yields of *A. esculenta* (20–42 %) and *S. latissima* (16–33 %) in the literature exhibit large variation based on both seasonal changes in the biomass and experimental methods used for extraction and quantitation [22,38–40]. Colorimetric methods can be

applied, and studies that have done this report alginate yields in *A. esculenta* and *S. latissima* that are higher compared to the yields obtained in this study [22,38]. However, these methods are not based on quantitative reactions, and will only with thorough standardization give reproducible results [41]. Acid hydrolysis with H_2SO_4 or trifluoroacetic acid (TFA) followed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) or high-performance liquid chromatography (HPLC), is another method to estimate the content of alginate in dried seaweed material. Studies employing this approach report alginate yields lower or similar compared to the yields obtained in this study [39,40]. However, results obtained with this method must be interpreted with care. The method could be misleading due to the varying degradation rate in the different uronic acids. Thus, gravimetric methods combined with NMR is suggested to give a better estimate of the alginate content [42].

The challenge with gravimetric determination of alginate yield, is that the method relies on complete extraction and precipitation of alginate, which is difficult to achieve in reality [41]. However, several rounds of extractions ensure lower alginate retention in the seaweed, as results from the preliminary studies show, and could give an indication of the total extractable alginate. Following three rounds of alkaline extractions in the present study, the total yield of precipitated matter was 240 ± 15 and 230 ± 17 mg/g dw seaweed for *A. esculenta* and *S. latissima*, respectively. Here it should be considered that the purity of the precipitated alginate was not characterized to the same extent as in the rest of the study. Furthermore, the biomasses used for the preliminary studies were of a different batch than what was used in the main study.

After evaluating the results from the three alkaline extractions in the preliminary studies, it was assumed that it is possible to enhance the yield of alginate from one round of alkaline extraction, without significantly compromising the alginate quality. As the results from this study demonstrates, a one round alkali extraction at pH 9 enables this, and more effective alginate extraction protocols are feasible.

When evaluating the alginate qualities in this study, the absence of a reference is still a challenge since the needed degree of purity and molecular weight depends on application area. In biomedical and pharmaceutical applications, such as wound healing, drug delivery and tissue engineering, the alginates should be ultrapure or sterile [43], whereas the purity requirement is lower for alginates in other application areas, such as biobased packaging [44,45]. For commercial alginates the final quality is primarily determined by the clean-up and purification procedures following extraction of the alginate, whereas improving the quality at earlier stages in the process can reduce costs and environmental impacts.

Protanal® LF 10/60 is a commercial alginate that has been thoroughly characterised in many papers due to its good gelling properties, high viscosity and resulting wide application range [46–48]. It originates from *L. hyperborea* stipes and is usually sold in bulk packages, with food, pharma, and research as main application areas [49]. Together with the alginate samples from this study, a triplicate of LF 10/60 was also analysed on the Vario-El-Cube CNS element analyser. The content of sulfur (3.4 ± 0.1 mg/g alginate) and nitrogen (1.3 ± 0.2 mg/g alginate) were lower in LF 10/60 compared to the alginates from the standard extraction protocol. However, compared to some of the other extracted alginates, such as the *A. esculenta* pH 9 alginate (Table 4), the sulfur content in LF 10/60 was not lower. In fact, considering traces of co-extracted fucoidan, more than half of the extracted alginates in this study match the quality of LF 10/60. However, the protein content should be lower to reach the quality of LF 10/60. Employing acid precipitation prior to ethanol washing could reduce co-precipitation of protein together with the alginate. Evidently, additional purification steps, such as filtration, dialysis and size exclusion chromatography could also be implemented in the protocol to achieve purer alginates [50].

Excluding the alginates depolymerised during acid hydrolysis at 50 °C, the alginates extracted in this study have a generally high weight average molecular weight (M_w) compared to commercial alginates, which usually range between 32 and 400 kDa [43]. Industrially produced alginates are often priced proportional to the molecular weight since higher molecular weight increases the intrinsic viscosity of the polymer [3], a property valued by the industry. However, high viscosity can also make the separation of alginate from seaweed residues more difficult [19]. The challenge is to find the right balance between depolymerisation, handling costs and alginate price. The high viscosity of the alginate solutions in this study did not cause any separation problems. However, it remains to be investigated how an upscaling of the extraction using more industry-relevant equipment and methodology would influence this matter.

5. Conclusion and future perspectives

To efficiently extract alginate with high yield and quality from cultivated *Alaria esculenta* and *Saccharina latissima*, this study suggests a short alkaline extraction (1–5 hours) with pH 9 carried out at 20 °C as the favourable conditions. These findings may serve as a baseline for expanded laboratory studies, pilot trials and eventually future industrial alginate production based on Norwegian cultivated *AE* and *SL*. It is advantageous for industrial stakeholders that the alginates have been extracted from fresh seaweed material, to avoid excessive costs connected to drying followed by addition of water prior to extraction [19]. However, to avoid decomposition of material before alginate extraction, processing of fresh seaweed requires biorefineries close to the cultivation facilities, or other seaweed preservation techniques, such as acid preservation, necessitating additional research and technology development.

CRediT authorship contribution statement

Katharina Nøkling-Eide: Conceptualization, Investigation, Analysis, Visualization, Writing – Original Draft Preparation. Anne-Mari Langeng: Investigation, Analysis. Andreas Åslund: Analysis. Finn Lillelund Aachmann: Analysis, Funding acquisition, Project administration, Supervision, Writing – review & editing. Håvard Sletta: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing, Øystein Arlov: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2022.102951.

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