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Characterization of high value products in the side-stream of *Laminaria hyperborea* alginate production - Targeting the phenolic content

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ABSTRACT

Sustainable production based on renewable biomass and efficient bioprocesses are important elements in the growing blue bioeconomy. The traditional *Laminaria hyperborea* alginate production disposes approximately 80 % of the raw material, ignoring large amounts of potential high-value products from the alga. Particularly, the leaf fraction of the seaweed is often disregarded. This study aimed to characterize high value products from the alginate production side-stream – focusing on the leaf biomass and particularly targeting the phenolic content. After extraction and solvent optimization, 60 % methanol was used for the extraction. The extract was further purified with PuriFlash and semi-preparative chromatography and increasing phenolic selectivity and purity was observed with TPC and qNMR, as well as antioxidant activity (ORAC). In the purified fractions, the LR LC-MS analyses displayed several masses, where 96 % (n = 1376) were of lower molecular weights (< 800 Da). Fifteen high value compounds were further identified using HR LC-MS (MS/MS) and/or NMR. This also included non-phenolics such as fucoxanthin, aliphatic acids and mannitol. Nonetheless, most compounds were identified as the targeted phenolics, consisting of lower molecular weight phenolic acids (salicylic acid, veratric acid, 5-caboxyvanillic acid, sinapic acid, 5-sulfosalicylic acid, vanillic acid 4-sulfate, and dihydrocaffeic acid 3-sulfate) and phlorotannins (trimer, tetramer, hexamer, and a sulfated dimer). None of the identified phenolics have previously been reported in *L. hyperborea*. In general, a high occurrence of sulfated phenolic compounds was observed and a sulfated diphlorethol/difucol was characterized for the first time. The isolation and characterization of high value components in the leaf biomass of L. *hyperborea* strongly supports the development of a total utilization of commercial alginate production. The characterization also adds information on the phenolic content of seaweeds at a molecular level, valuable to research on seaweed biosynthesis and development, chemical ecology, and ocean monitoring.

1. Introduction

Seaweeds are a natural source of a diverse chemical matrix with potential uses in food-, feed-, cosmetical-, and pharmaceutical industries [1,2]. This includes alginates, fucoidan, laminarin, cellulose, mannitol, polyphenols, and other pigments. Brown algae are one of the most promising marine biomasses as a renewable source contributing to the growing blue bioeconomy [3]. Human consumption of seaweed includes direct consumption as food, as food supplements, or as thickening agents such as alginate [4]. Additionally, natural antioxidants with multi-functional potential from seaweeds are of increasing interest. The brown alga *Laminaria hyperborea* is predominantly found in the subtidal zone of the Northern Atlantic Ocean. Due to its large content of structural and functional polysaccharides, specifically its unique alginate composition, *L. hyperborea* has long been exploited for alginate production. However, the traditional alginate production disposes approximately 80 % of the raw material either as liquid or solid waste streams, ignoring large amounts of potential high-value products from the alga [3,5]. Phenolics have been found to have several bioactivities including antioxidant, anti-cancer, antidiabetic, and antimicrobial activity, making them sought after by several industries [6–11]. Phenolics are a large and heterogenous group with structures ranging from simple monomer units to complex polymerized structures. They are synthesized in both marine- and terrestrial plants via either the acetate-malonate- or the

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shikimic pathway [12–16]. Terrestrial plant phenolics have been largely studied, whereas studies on seaweed phenolics are fewer. The occurrence of sulfated polyphenols seen in several marine plants and some algae species, is suggested to be an ecological adaptation to the marine environment [17–26]. However, the phenomenon is also observed for phenolics in some terrestrial species [27,28]. Sulfation increases the hydrophilicity and solubility of a molecule which increases the bioavailability of the compound [22,29,30]. It is also believed to influence the inactivation of toxic products and play a role in plant growth regulation [22,31–33]. The increased water solubility of sulfated polyphenols may also contribute to the colour of the seaweed biomass, as it can form stable complexes with other pigments [34]. However, the sulfate binding is susceptible to hydrolyzation, and this may create challenges with respect to degradation during extraction and isolation [27,31]. Characterization of polyphenols in seaweed is challenging, as they often occur at lower concentrations and are similar in structure. Particularly phlorotannins have been reported for several brown algae species. They are polymers of phloroglucinol and include several structural and conformational isomers [35]. Phlorotannins can be divided into groups based on the linkage of phloroglucinol units. Compounds with only ether linkages (C-O-C) are called fuhalols, compounds with only phenyl linkages (C-C) are called fucols, and compounds having both ether- and phenyl linkages are called fucophlorethols [36–40]. Due to the challenge of separation and isolation, phlorotannins are primarily characterized using LC-MS and classified according to their linkage type (C-C vs. C-O-C bond) and polymerization degree [18,39,41–44].

The extraction of phenolics, pigments and other high-value products from L. *hyperborea* as a side-stream of alginate production would provide access to several previously discarded bioactive compounds. Particularly the leaves are regarded as lower value compared to the stipe fraction, due to their alginate composition being lower in guluronic acid content [45]. By evaluating the by-products of the commercial alginate production and characterizing potential high-value products, the raw material utilization as well as the sustainability of the full seaweed biorefinery is increased [3,4,46]. This study aimed to evaluate the chemical composition of high-value products in *Laminaria hyperborea* to explore the possibility of producing high-value products from side streams of the alginate production and seaweed biorefinery.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade. The Folin-Ciocalteu (FC) reagent, gallic acid, phloroglucinol, sinapic acid (≥ 98 %), salicylic acid (≥ 99 %), fucoxanthin (≥ 95 %), methanol (≥ 99.9 %), ethanol (absolute), ethyl acetate (≥ 99.5 %), acetone (≥ 99.5 %), formic acid (≥ 98 %), $DMSO_2$ (TraceCERT®), and DMSO-d_6 (0.03 % TMS) were acquired from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Hexane (mixture of isomers) was acquired from Honeywell (Honeywell Inc., Charlotte, NC, USA). Deionized water was deionized at the University of Bergen (Bergen, Norway). Fluorescein sodium salt, AAPH, and Trolox used for the ORAC assay was acquired from Merck (Oslo, Norway). All reference standards were acquired from Merck.

2.2. Seaweed material

Laminaria hyperborea (Laminariaceae, Phaeophyceae) leaves (lamina) were acquired from Alginor ASA. The biomass was harvested in September 2020 along the coast of Haugesund, Norway (Rogaland field 55E; N 59°11′ E 005°06′). All samples were rinsed thoroughly with fresh water and air-dried. The dried plant material was stored at -20 °C when not used.

2.3. Sample preparation

Crude extracts were obtained using traditional maceration. 20 g dried material was dissolved in 500 mL 60 % methanol and left stirring for 24 h. Two parallels were extracted simultaneously, and all extractions of the same material were pooled and dried for analysis. When not used, samples were dried and stored at -20 °C.

2.4. Flash chromatography

The crude extract was fractioned with flash chromatography using an Interchim puriFlash® with a Biotage Sfär C18 D column. The solvents used were water (A), methanol (B), and ethyl acetate (C), with initial conditions of 95 % A and 5 % B. The gradient followed as; 0–6 min: 95 % A + 5 % B, 6–12 min: 75 % A + 25 % B, 12–18 min: 50 % A + 50 % B, 18–24 min: 25 % A + 75 % B, 24–36 min: 100 % B, 36–42 min: 50 % B + 50 % C, 42–48 min: 100 % C. Flowrate was 12 mL/min and 15 mL sample were added to the column. Detection was done at 280 nm and using a spectral scan between 200 and 800 nm. Eight fractions were obtained using flash chromatography.

2.5. Preparative HPLC

Further, the first eluting fraction from the flash chromatography was further purified using preparative HPLC. The preparative HPLC system consisted of a Dionex Ultimate 3000 pump (Thermo Fisher Scientific, Sunnyvale, CA, USA), the Dionex 3000 variable wavelength detector (Thermo Fisher Scientific, Sunnyvale, CA, USA), and a 250 × 22 mm (10 μm) Econosphere C18 column (Dr. Maisch, Ammerbuch, Germany). The solvents were (A) super distilled water (0.5 % TFA) and (B) acetonitrile (0.5 % TFA). Initial conditions were 95 % A and 5 % B. Gradient followed as; 0–10 min: 95 % A + 5 % B, 10–20 min: 85 % A + 15 % B, 20–34 min: 60 % A + 40 % B. 34–35 min: 95 % A + 5 % B. The flowrate was 18 mL/min and aliquots of 2 mL were injected. Twelve fractions were obtained using preparative HPLC.

2.6. Total polyphenolic content (TPC) assay

Folin-Ciocalteu TPC assay was performed using a method optimized for seaweeds, as described in Wekre et al. (2022) [47]. Briefly, the method used 0.2 mL sample, blank, or standard, 1.59 mL Folin-Ciocalteu-reagent, 4.0 mL 20 % (*w/v*) Na_2CO_3 and made to a total volume of 20 mL with water. The mixture was incubated for 2 h in the dark, and absorbance was measured at 760 nm using a Biochrom Libra S32 UV instrument (Biochrom, Cambridge, United Kingdom). Gallic acid and phloroglucinol calibration curves were used to validate the linearity, sensitivity, precision, and accuracy of the TPC method (Table 1). Three parallels (n = 3) of each sample or standard were analyzed to ensure statistically significant results.

2.7. NMR analyses

Dried samples were dissolved in 0.6 mL DMSO-d_6 (0.03 % TMS) containing the internal standard $DMSO_2$ (C = 10 mM). Quantification using ^{1}H NMR analyses were performed employing a Bruker 600 MHz AVANCE NEO instrument with a QCI cryoprobe (Bruker BioSpin, Zürich, Switzerland). All spectra were recorded at 298 K. For accurate

Table 1
Calibration curve, limit of detection (LOD) and limit of quantification (LOQ) for gallic acid at 760 nm using the optimal TPC conditions.

Standard	Calibration curve	r^2	Range [ug/mL]	LOD [ug/mL]	LOQ [ug/mL]
Gallic acid	y = 0.00115× – 0.00101	0.999	1000–30	18.610	56.391

quantification, the T_1 value of each sample was measured to ensure complete relaxation between scans. The T_1 measurements were performed by applying the *t1ir* pulse sequence with a sweep width of 19.8 ppm, 16 k data points, 8 scans, 4 dummy scans, and 9 different inversion recovery delays between 1 ms and 5 s. To ensure complete relaxation, the d1-value was set to 5 x T_1 for all ^{1}H spectra obtained for qNMR analysis [48,49].

The one-dimensional (1D) ^{1}H NMR spectra used for quantifications were recorded using the *zg30* pulse sequence with a sweep width of 19.8 ppm, 64 k data points, 128 scans, aq = 2.75 s, fidres = 0.36 Hz, and the relaxation delay (d1) was 5 x T_1 for the selected sample. The spectra were processed using a line broadening of 0.3 Hz.

Selective qNMR was performed as described in previous publications [47]. All quantifications were performed based on Eq. (1) with $DMSO_2$ (10 mM, No. H = 6) as the internal standard.

$$C_{sample}\ [M] = \frac{I_{sample} \times n_{DMSO2} \times C_{DMSO2}}{I_{DMSO2} \times n_{sample}} \quad (1)$$

where C = molar concentration [M], I = signal integral, and n = number of protons yielding the signal.

2.8. LC-electrospray mass spectrometry (ESI-/TOF)

Low-resolution liquid chromatography mass spectrometry (LC-ESI MS(Q); LR LC-MS) was performed using an Agilent Technologies 1260 Infinity Series system and an Agilent Technologies 6420A triple quadrupole mass spectrometry detector. The following conditions were applied: ionization mode: negative, capillary voltage = 3000 *V*, gas temperature = 300 °C, gas flow rate = 3.0 L/min, acquisition range = 100–2000 *m/z*. The elution profile for HPLC consisted of the following gradient: 0–4 min: 1 % B, 4–5 min: 5–10 % B, 5–7 min: 60 % B, 7–9 min: 70 % B, 9–11 min: 75 % B, 11–13 min: 75–1 % B, 13–15 min: 1 % B, at a flowrate = 0.3 mL/min, where solvent A was super distilled water (0.5 % formic acid), and solvent B was acetonitrile (0.5 % formic acid). A 50 × 2.1 mm internal diameter, 1.8 μm Agilent Zorbax SB-C18 column was used for separation. UV–Vis spectral data for all peaks were accumulated in the range of 200–800 nm and the chromatographic profiles were recorded at 280 nm, 330 nm, and 360 nm.

High-resolution mass spectrometry (LC-ESI MS(QqTOF); HR LC-MS) was performed using the same gradient and solvent system as for LR-MS, using a similar column (Agilent Zorbax Eclipse HD, 2.1 × 50 mm, 1.8 μm) and flowrate = 0.250 mL/min, capillary voltage = 4000 *V*, gas temperature = 280 °C, gas flow rate = 8.0 L/min. MS/MS experiments (LC-ESI MS(QqQ)) were acquired for precursor ions using helium as collision gas with a collision energy of 15–35 eV.

2.9. High performance liquid chromatography (HPLC-DAD)

HPLC-DAD analyses were performed on an Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 1260 diode array detector (DAD) and a 200 × 4.6 mm, 5 μm ODS Hypersil column (Agilent Technologies, Santa Clara, CA, USA). HPLC analysis was performed using two solvents, (A) super distilled water (0.5 % TFA) and (B) acetonitrile (0.5 % TFA), in a gradient (0–10 min: 95 % A + 5 % B, 10–20 min: 85 % A + 15 % B, 20–34 min: 60 % A + 40 % B. 34–35 min: 95 % A + 5 % B). The flow rate was 1.0 mL/min, and aliquots of 20 μL were injected with an Agilent 1260 vial sampler. UV–Vis absorption spectra were recorded during the HPLC analysis over the wavelength range of 200–600 nm in steps of 2 nm.

2.10. Free radical scavenging by oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed in black 96-well microtiter plates. Fluorescein sodium salt AAPH (2,2′-azobis(2-methylpropionamide) dihydrochloride) and Trolox (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were dissolved in 60 mM phosphate buffer (PB). Trolox was used as a calibration curve (Table 2). Standards and samples were pipetted into a microtiter plate in duplicate with fluorescein and incubated at 37 °C for 15 min. Ice cold AAPH was added, and the plate was immediately moved to the Tecan Spark multimode reader. Fluorescence (Ex 485 nm ± 20 and Ex 525 nm ± 20) was recorded 25 times in intervals of 90 s. The area under the curve (AUC) was calculated and the AUC for 0 μM Trolox was subtracted to find the area between curves (ABC). ABC for samples were compared to the Trolox standard curve to find Trolox equivalents (TE). Results are reported as μmol TE/mg DW. All ORAC tests were performed at MARBIO (Tromsø, Norway).

2.11. Vibrational spectroscopy - infrared and Raman

Selected purified L. *hyperborea* samples (PuriFlash and preparative HPLC) were scanned with FTIR and Raman micro spectroscopy. Infrared spectroscopy was performed using a Bruker Alpha II, equipped with a Platinum ATR module, a Rocksolid™ interferometer and a deuterated triglycine sulphate (DTGS) detector. Samples were analyzed both as solids and in liquid form. Liquid samples were dissolved in water or methanol and scanned from 400 to 4000 cm^{-1} (integration time of 360 s). The OPUS software was used to process the data.

Raman micro spectroscopy was performed at room temperature using a Bruker Senterra II Spectrometer equipped with a 785 nm laser and scanned from 100 to 4000 cm^{-1} (integration time 30 s, 3 accumulations). A sample size of <0.5 mg gave sufficient resolution due to the microscope array. No further sample preparation was necessary.

3. Results and discussion

3.1. Extraction and quantification of polyphenols

Several methods were tested on dried L. *hyperborea* biomass for extraction of potential high-value side stream products of alginate production, primary targeting polyphenols. The extraction methods included accelerated solvent extraction (ASE), ultrasound assisted extraction (UAE), and maceration. Different ratios of aqueous methanol and aqueous ethanol were tested as extraction solvents [50–53]. The ASE method yielded slightly higher phenolic contents; however, the method has limitations with respect to low sample size, application, and clogging of extraction cells due to the polysaccharides in the biomass. The UAE and maceration extractions showed similar phenolic extraction yields – making the traditional maceration preferable. Maximum and similar extraction yields were observed when using 60 % aqueous methanol or ethanol.

Additional purification of the maceration crude extract was performed to improve characterization accuracy of the phenolics [35]. Flash chromatography and preparative HPLC were used for pre-fractionation and purification, the target being high polyphenol recovery (Sections 2.3-2.5). The effect of increased purification was observed with increased quantitative phenolic content (TPC and qNMR), as well as with increased antioxidant values (ORAC) (Fig. 1).

3.1.1. Selective extraction of phlorotannins

The use of liquid-liquid extraction for increased selectivity is particularly seen for phlorotannins, often succeeding maceration, either

Table 2
Calibration curve, limit of detection (LOD) and limit of quantification (LOQ) for Trolox used in ORAC assay.

Standard	Calibration curve	r^2	Range [μM]	LOD [μM]	LOQ [μM]
Trolox	y = 10,454,393.2× + 12,880,433	0.952	12.5–0.78	3.899	11.82

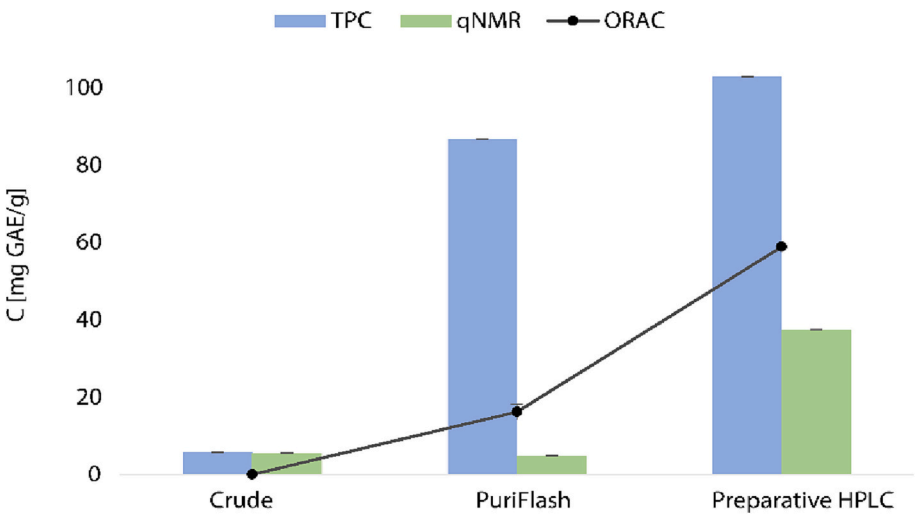

Fig. 1. Total phenolic content (TPC and qNMR reported as gallic acid equivalents (GAE)) in crude methanolic L. *hyperborea* extract, and in selected purified samples (PuriFlash and preparative HPLC) targeting the phenolics. A trendline displays the antioxidant activity for the different samples measured with ORAC reported as Trolox equivalents (TE) [μmol TE/mg] (crude; 0 μmol TE/mg, PuriFlash; 16.20 ± 2.04 μmol TE/mg, preparative HPLC; 58.85 ± 0.30 μmol TE/mg). All values are reported relative to the total mass of the individual sample.

with ethyl acetate, hexane, or both [17,48]. An alternative extraction method was therefore tested, using 70 % aqueous acetone followed by liquid-liquid purification using hexane prior to ethyl acetate (EtOAc) [17,39,54–58]. The resulting extracts were analyzed using TPC, qNMR, and LC-MS.

The LR LC-MS analyses revealed that the 70 % aqueous acetone extract (TPC: 6.51 ± 0.02 mg GAE/g, qNMR: 3.40 ± 0.00 mg GAE/g) and its liquid-liquid purification phases (hexane, EtOAc, H_2O) did not contain any additional phenolic or phlorotannin masses compared to the 60 % aqueous methanol extract (TPC: 6.55 ± 0.08 mg GAE/g, qNMR: 4.51 ± 0.00 mg GAE/g) and its corresponding fractions, based on mass hits in the bibliographic database. Thus, no increased selectivity of phenolics or phlorotannins was observed with this algae biomass using a liquid-liquid extraction operation. Moreover, both crude sample extracts exhibited similar mass distribution trends in the LC-MS analyses, with most of their detected compounds falling within the 200–400 Da range. Therefore, based on the comparable polyphenolic yields obtained by both extraction methods, the methanolic extract and its purified fractions were selected for further analysis.

3.2. Characterization of the alga extract and purified samples

The methanolic crude extract and its purified fractions were analyzed using both low - and high resolution (LR/HR) LC-MS, TPC, ORAC and NMR. The identified polyphenols (Table 3) were detected in the purified fractions from the preparative HPLC, which originated from an early-eluting, polar fraction from PuriFlash (Sections 2.4 and 2.5). The selection of fractions with high polyphenolic content was based on HPLC-DAD analyses. Due to their relatively high concentration in the alga, fucoxanthin and mannitol could be identified by NMR analysis (Section 3.2.5).

3.2.1. Observed mass distribution

The purified samples of L. *hyperborea* were first analyzed with LR LC-MS and the observed masses were compared with a prepared bibliographic database containing about 600 compounds including polyphenols, pigments and aliphatic acids. MS spectra indicated about 1400 different masses in the acquisition area (m/z 100–2000). Around 45 % of these masses were found between 200 and 400 Da (Fig. 2) and of these, 15 % gave a result matching a compound in the bibliographic database. The majority of the tentatively identified compounds (database matches) were also found in the 200–400 Da range (68 %). A limited number of masses were detected above 800 Da (4 %), and of these 1.5 % gave a match with compounds in the database. Of the masses matching the bibliographic database, 6 % (n = 15) were further characterized using LR LC-MS/MS, and HR LC-MS (Tables 5 and 6). Additionally, four non-phenolic compounds were identified using HR LC-MS and NMR (Section 3.2.5).

Table 3
Overview of all identified compounds in side stream methanolic extract and its corresponding fractions.

Compound	Molecular formula	Molecular weight [g/mol]
Hydroxybenzoic acids (HBA)		
Salicylic acid	$C_7H_6O_3$	138.1
Veratric acid	$C_9H_{10}O_4$	182.2
5-Carboxyvanillic acid	$C_9H_8O_6$	212.1
5-Sulfosalicylic acid	$C_7H_6O_6S$	218.2
Vanillic acid 4-sulfate	$C_8H_8O_7S$	247.2
Hydroxycinnamic acids (HCA)		
Sinapic acid	$C_{11}H_{12}O_5$	224.2
Dihydrocaffeic acid 3-sulfate	$C_9H_{10}O_7S$	262.2
Phlorotannins		
Trimer [39,42,59]	$C_{18}H_{14}O_9$	374
Tetramer [39,41,42]	$C_{24}H_{18}O_{12}$	498
Hexamer [41]	$C_{36}H_{26}O_{18}$	746
Sulfated dimer	$C_{12}H_{10}O_9S$	330
Aliphatic acids		
Citric acid	$C_6H_8O_7$	192.1
Ascorbic acid	$C_6H_8O_6$	176.1
Carotenoids		
Fucoxanthin	$C_{42}H_{58}O_6$	658.9
Carbohydrates		
Mannitol	$C_6H_{14}O_6$	182.2

3.2.2. Sulfated phenolics

Several compounds detected in the MS spectra of the purified L. *hyperborea* fractions showed masses just below whole numbers (example: 217.9885), normally seen for sulfur containing compounds (Sulfur: MW = 31.9721 g/mol) [60]. Sulfated conjugates of both phenolic acids and phlorotannins were found in the fractions, either through fragmentation patterns or exact masses (Tables 5 and 6).

Selected purified fractions of L. *hyperborea* were also analyzed with Raman and IR spectroscopy to explore the occurrence of sulfation. The Raman spectrum shows a very strong and characteristic band at 1049 cm^{-1} assigned to symmetric sulphate group stretching, further

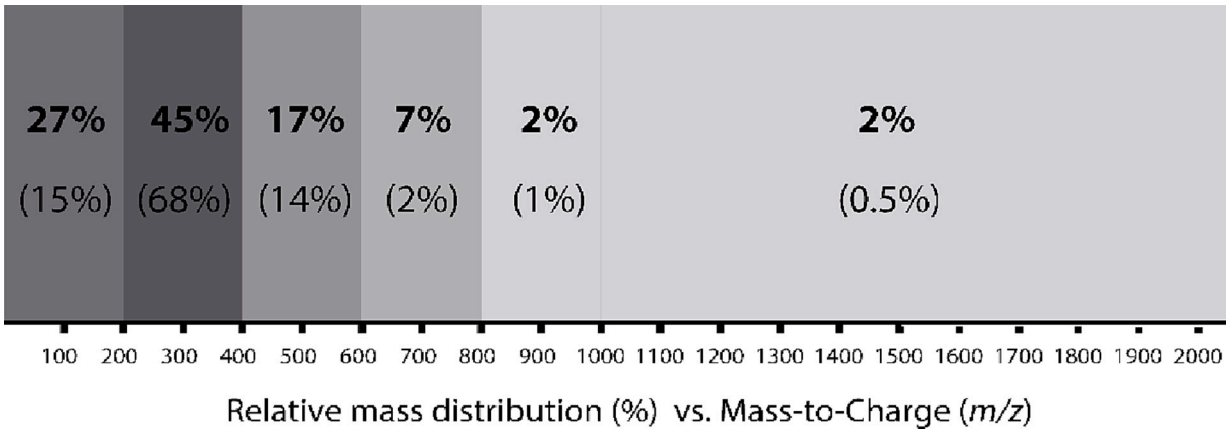

Fig. 2. Relative mass distribution (bold) for the observed masses ($n = 1376$) in the TIC scans of the LR LC-MS analyzed L. *hyperborea* extracts. The mass distribution of masses matching the bibliographic database ($n = 211$) are shown in brackets.

indicating the presence of sulfated polyphenols in L. *hyperborea* (Fig. 3, Table 4) [61–63]. The Raman band at 1456 cm^{-1} strongly suggests the presence of methylation in the compounds [63,64]. IR bands at 1671 cm^{-1} and 1728 cm^{-1} are linked to C═O stretching modes, likely stemming from carboxylic acid groups in phenolic acids.

3.2.3. Phenolic acids

Seven phenolic acids, including three sulfated phenolic acids, were identified in L. *hyperborea*, making up 44 % of the identified compounds. Identification was performed using HR LC-MS and LR LC-MS/MS (Table 5). Phenolic acids exhibit several biological activities, including anti-inflammatory, antioxidant, anti-tumor, and anti-diabetic activities, making them high-value products that can be extracted from *Laminaria hyperborea* [16,69–72].

Salicylic acid (syn.: *p*-hydroxybenzoic acid) was identified in the purified fractions of *L. hyperborea*, with a mass deviation of −0.52 ppm in HR LC-MS (Δppm ≤5). Additionally, its fragmentation pattern showed a peak at *m/z* 93, corresponding to a loss of 44 Da (-CO_2), a common observed fragment loss for acids [73]. By using HPLC and reference standards, Chakraborty et al. (2017) identified phenolic acids

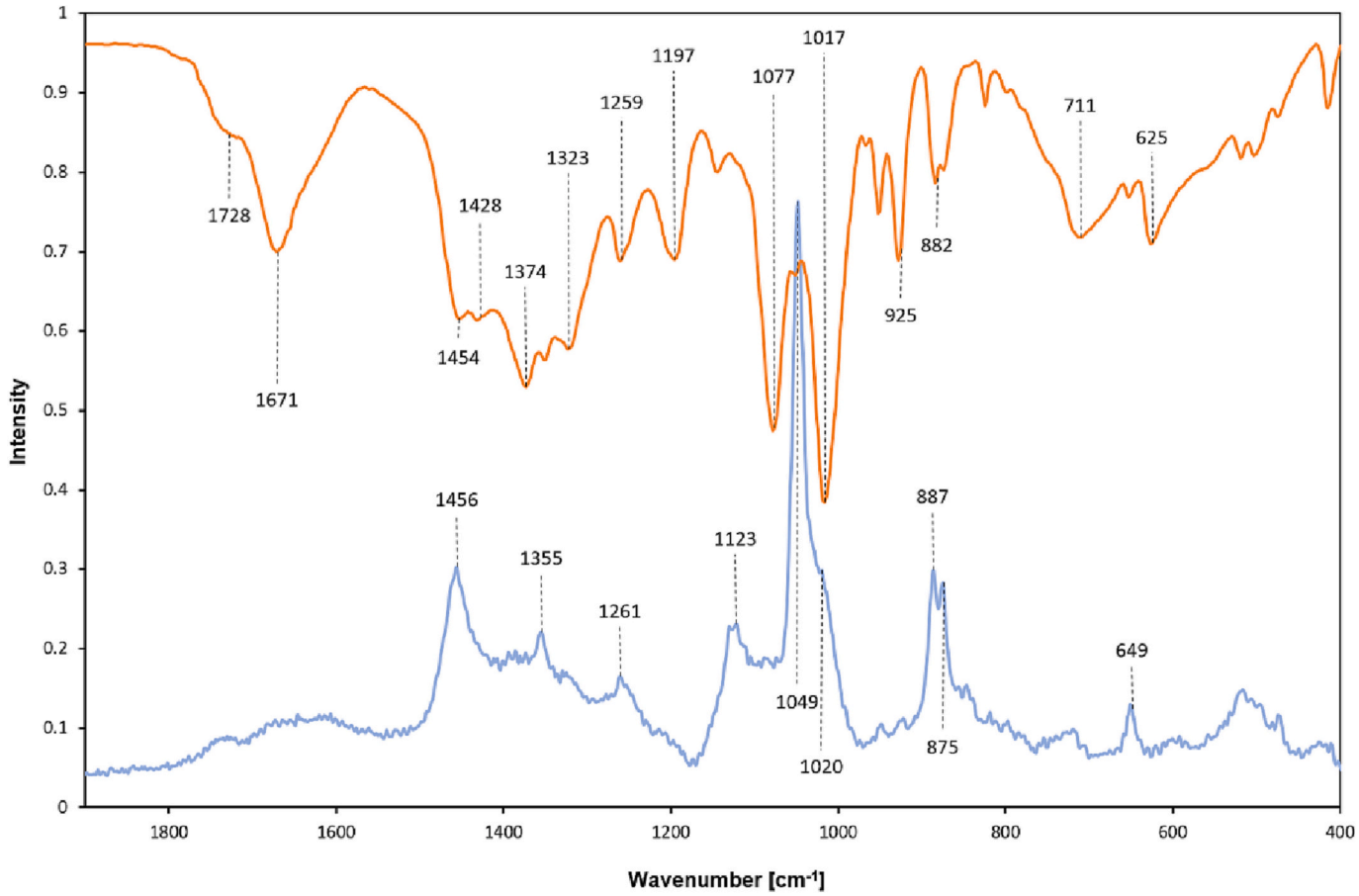

Fig. 3. Overlaid Raman (blue)- and IR- spectra (orange) of a purified fraction of the L. *hyperborea* extract showing indications of sulfated polyphenols. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Vibrational bands assigned in the purified fractions of L. *hyperborea* extracts; δ and ν refers to bending and stretching vibrations, s to symmetrical vibration modes.

Wavenumber [cm^{-1}]	Activity	Vibrational mode	Reference
822	Infrared	ν(C—H)	Grasel et al. (2016) [65]
1017	Infrared	ν(C—O)	Grasel et al. (2016) [65]
1049	Raman	ν_s(O=S=O)	Buzgar et al. (2009), Pereira et al. (2009), Dörschmann et al. (2023) [61–63]
1077	Infrared	ν(C—O)	Grasel et al. (2016) [65]
1259	Infrared	ν(S=O), ν(C—O)	Mathlouthi & Koenig (1987), Glombitza & Knöss (1992) [66,67]
1454	Infrared	ν(C-C)$_{aromatic}$	Nogales-Bueno et al. (2017) [64]
1456	Raman	$\delta_s(CH_3)$	Dörschmann et al. (2023), Nogales-Bueno et al. (2017), da Silva et al. (2008) [63,64,68]
1671	Infrared	ν(C=C), ν(C=O)	Grasel et al. (2016) [65]
1728	Infrared	ν(C=O)	da Silva et al. (2008) [68]
2845	Raman	$\nu_s(CH_2)_{shoulder}$	da Silva et al. (2008) [68]
2939	Raman	ν(C—H)	da Silva et al. (2008) [68]

Table 5
Phenolic acids in *Laminaria hyperborea* identified using HR LC-MS and LR LC-MS/MS.

Compound	Molecular ion [M-H]-	Mass deviation [ppm]	MS/MS ions[a]
Salicylic acid	137.0245	−0.52	**93**
Veratric acid	181.0510	1.93	–
5-Carboxyvanillic acid	211.0252	1.70	151, 107, 83, **65**, 63
5-Sulfosalicylic acid	216.9815	0.72	137, **93**
Vanillic acid 4-sulfate	246.9923	2.33	121, **108**, 93, 80
Sinapic acid	223.0616	1.58	208, 193, **149**, 93
Dihydrocaffeic acid 3-sulfate	261.0082	−1.53	**181**, 166, 122, 81

[a] MS/MS ion marked in bold represent the base peak.

in the brown algae *Anthophycus longifolius*, *Sargassum plagiophyllum*, and *Sargassum polycystum* (formerly *Sargassum myriocystum*), in which salicylic acid was found in the latter [58]. Agregán et al. (2017) identified salicylic acid as a "hydroxybenzoic acid derivative" in the common brown algae *Ascophyllum nodosum,* using LR LC-MS/MS fragmentation patterns. To the author's knowledge this is the first-time salicylic acid has been identified in L. *hyperborea*. Salicylic acid has broad applications and is extensively used in industry, in the production of aspirin, as food preservatives, and as antiseptics [74–79]. Sinapic acid, a hydroxycinnamic acid, was identified with a small mass deviation of 1.58 ppm and a characteristic fragmentation pattern [80]. A fragment at *m/z* 208 represents a loss of one CH_3 group ([M-15-H]$^-$), and a fragment at *m/z* 193 represents a double CH_3 loss (− 2 x CH_3). The base peak at *m/z* 149 (M-74), is most likely the loss of the two methyl groups in addition to the CO_2 loss from the acid group (2 x CH_3 + CO_2). Sinapic acid has previously been identified using LC-MS/MS in the analysis of the green algae Ulva sp., Caulerpa sp., and Grateloupia sp. [81] In the red alga *Gracilaria dura,* Sumayya et al. (2020) also reports the presence of sinapic acid - based on HPLC analyses [82]. To the authors knowledge, sinapic acid has not been identified in L. *hyperborea* prior to this study. 5-Carboxyvanillic acid was identified showing an expected loss of 60 Da ([M-60-H]$^-$), representing the acid group and a methyl group (COOH + CH_3) followed by the typical loss of 44 Da (CO_2). No fragmentation pattern was observed for veratric acid; however, the high-resolution mass deviation was found to be below 5 ppm. Dihydrocaffeic acid 3-sulfate and 5-sulfosalicylic acid both showed the typical loss of a SO_3 group ([M-80-H]$^-$). Vanillic acid 4-sulfate did not show a fragment representing the loss of a SO_3 group; however, a fragment was observed at *m/z* 80 which supports the presence of a sulfate group.

3.2.4. Phlorotannins

Four phlorotannins were identified in L. *hyperborea*, making up 27 % of the identified compounds. All four phlorotannins had different polymerization degrees and one was sulfated. (Table 6). Further three phlorotannins could be identified by their linkages (phlorotannin type) due to characteristic fragmentation patterns.

A quasi-molecular ion peak at *m/z* 373.1196 ([M – H]$^-$) was observed, indicating a trimer phlorotannin. Fragmentation indicated that the trimer may only be connected through ether linkages (C-O-C) due to the observation of a fragment at *m/z* 265 ([M – 108 – H]$^-$), representing a dimer phlorotannin with ether linkages. Alternatively, one of the two ether linkage oxygens might be an extra hydroxyl group, and the bond broken could be a phenyl linkage (C-C). Still, this is rarely observed for smaller phlorotannins, and thus less likely. Lastly, a fragment representing a simple phloroglucinol unit (*m/z* 126) was observed, further indicating that the compound is a phlorotannin. Based to the overall observed fragmentation pattern, the compound was concluded to be a fuhalol type phlorotannin. A tetramer phlorotannin was identified with a quasi-molecular ion peak at *m/z* 497.1867 ([M – H]$^-$). The tetramer mass has also been reported in *Laminaria digitata* and *Ascophyllum nodosum*, but different fragmentation patterns are reported in these analyses [39,41]. An additionally different fragmentation pattern was observed for the same mass in our study of L. *hyperborea,* highlighting the difficulty of identifying phlorotannins. A loss of 44 Da is described as a typical loss for cross-ring cleavage of phlorotannins [17,35]. For the tetramer phlorotannin, two such losses were observed. First between the fragments *m/z* 298 and *m/z* 254 (−44 Da), and between fragments a *m/z* 241 and *m/z* 197 (−44 Da). The fragment at *m/z* 371 represents a loss of a phloroglucinol unit ([M-126-H]$^-$), and the resulting phloroglucinol fragment was also observed at *m/z* 126. A similar fragment at *m/z* 371 was reported for tetramers in both *Laminaria digitata* and *Ascophyllum nodosum* by Vissers et al. (2017) and Sardari et al. (2021), respectively. Different fragmentation patterns will be seen for phlorotannin isomers; however, the HPLC-MS methodology can also influence the fragmentation patterns observed. Therefore, MS/MS experiments were performed at two fragmentation energies (25 eV and 35 eV), and the fragmentation at 35 eV gave some additional unidentified lower-mass ions (*m/z* 130 and *m/z* 83). Based on the fragmentation data obtained, the compound was identified as a phlorotannin tetramer, without further phlorotannin subclass characteristics. A hexamer phlorotannin mass was observed at *m/z* 745.9562 ([M – H]$^-$). This hexamer mass has been reported to be present in four brown algae; *Laminaria digitata*, *Sargassum vulgare*, *Sargassum fusiforme* and *Ascophyllum nodosum* [17,39,41,44]. However, similar to the tetramer phlorotannin, various fragmentation patterns are suggested, as expected for "higher order" phlorotannins [17,39,41,42,44]. From the MS/MS spectrum at 25 eV, a fragment at *m/z* 249 was observed which is typically reported as a dimer (ether linkage), easily lost from a larger

Table 6
Fragmentation pattern observed for phlorotannins in *Laminaria hyperborea* using HPLC-HRMS/MS (fragmentation energy = 25 eV).

Compound	Phlorotannin type	[M – H]$^-$	MS/MS ions[a]
Trimer	Fuhalol	373.1196	265, 229, **126**
Tetramer	Unknown	497.1867	371, 298, 254, 241, 197, **155**, 126
Hexamer	Fucophlorethol	745.9562	**681**, 461, 331, 281, 249, 229
Sulfated dimer	Fuhalol/fucol	328.9978	**249**

[a] MS/MS ion marked in bold represent the base peak.

hexamer structure. Another fragment observed at 25 eV was *m/z* 229, which could represent a different dimer with fucol linkage. Additionally, when using 35 eV as the fragmentation energy, a fragment at *m/z* 461 was observed, also reported by Lopes et al. (2018) for a hexamer structure [35]. Thus, it was concluded that the mass represents a hexamer phlorotannin with both ether- and phenyl linkages; a fucophlorethol.

One early eluting (t_R = 1.46 min) sulfated phlorotannin was identified with a quasi-molecular ion peak at *m/z* 328.9978 $[M - H]^-$. The compound showed indications of being a sulfated phlorotannin due to the mass being just below a whole number (Section 3.2.2). The fragmentation spectrum showed a fragment at *m/z* 249, yielding a loss of −80 Da ($[M\text{-}80\text{-}H]^-$), typical for a SO_3 group. A fragment at *m/z* 249 is often seen for tetramer phlorotannins, representing a dimer with two phloroglucinol units [17,36,41,83]. Therefore, the compound was concluded to be a sulfated dimer phlorotannin, possibly sulfated difucol (C-C) or sulfated diphlorethol (C-O-C) (Fig. 4). The two possible compounds have the same exact mass and therefore also yield the same mass deviation of 3.31 ppm from the observed mass. Accounting for previous results from ^{13}C NMR for L. *hyperborea*, which indicated the presence of more ether linkages than phenyl linkages, it seems most likely that the compound is sulfated diphlorethol [47]. A similar compound was reported by Glombitza and Knöss (1992) in the brown alga *Pleurophycus gardneri* using TLC, IR, and NMR [67]. Additionally, Chouh et al. (2022) report sulfated phlorotannins in *Sargassum vulgare* [17]. To the authors knowledge, sulfated phlorotannins have not previously been reported for *Laminaria hyperborea*.

3.2.5. Fucoxanthin, mannitol, and aliphatic acids

Carotenoids are often extracted alongside phenolics in aqueous alcohols, and fucoxanthin was found in the crude extract of L. *hyperborea* [84]. The pigment was further quantified (qNMR: 16.5 mg/g; ORAC: 40.6 ± 0.9 μmol TE/mg) and identified with ^1H NMR data and a reference standard (Fig. 5) in a late-eluting and less polar PuriFlash fraction. Fucoxanthin is a well-known pigment found to be present in L. *hyperborea* [46,85,86].

^1H NMR spectra of the early-eluting and highly polar fractions from the PuriFlash, revealed the presence of characteristic mannitol chemical shift values alongside the phenolic content. Mannitol is known to be present in brown algae, making up part of the carbohydrate content of L. *hyperborea,* along with alginate [87,88]. The semi-preparative HPLC purification was able to separate between the phenolic content and mannitol.

Two other non-phenolic compounds were identified, both aliphatic acids. Citric acid (MW = 192.12 g/mol) and ascorbic acid (MW = 176.12 g/mol) were observed in HR LC-MS spectra and had mass defects of 2.27 ppm and 1.93 ppm, respectively. Aliphatic acids have been widely reported in brown algae [17,18,46,89].

4. Conclusion

Aqueous alcoholic (60 % methanol/ethanol) maceration of L. *hyperborea* leaves gave similar phenolic extraction yields as UAE and ASE facilitated extractions (TPC: 5.72 ± 0.07 mg GAE/g; qNMR: 5.51 ± 0.013 mg GAE/g). No antioxidant activity was observed for the crude extract. However, for polar preparative-HPLC purified fractions, a high phenolic recovery was obtained (TPC: 102.34 ± 0.09 mg GAE/g; qNMR: 37.34 ± 0.017 mg GAE/g), and the increased purity was confirmed with higher antioxidant activity (ORAC: 58.85 ± 0.30 μmol TE/mg). Alternative methods were applied to increase the phenolic recovery, including phlorotannin selectivity. This included manipulation of extraction solvent polarity and purification with liquid-liquid partitioning with solvents of decreasing polarity. However, no high molecular weight phlorotannins were observed. This may be due to the natural polyphenolic composition of L. *hyperborea* leaves, primarily consisting of soluble low molecular weight compounds (< 800 Da). Several hydroxybenzoic- and hydroxycinnamic acids were identified, and the phenolic acids make up the majority of the phenolic contents. A general high occurrence of sulfated phenolic compounds was observed in LC-MS and a dimeric sulfated difucol/diphloroethol was characterized for the first time. To the author's knowledge, this is the first study reporting characterization of individual polyphenols in *Laminaria hyperborea*.

The low heterogeneity and seemingly higher solubility of the polyphenolic compounds found in the leaf fraction of L. *hyperborea* allows for

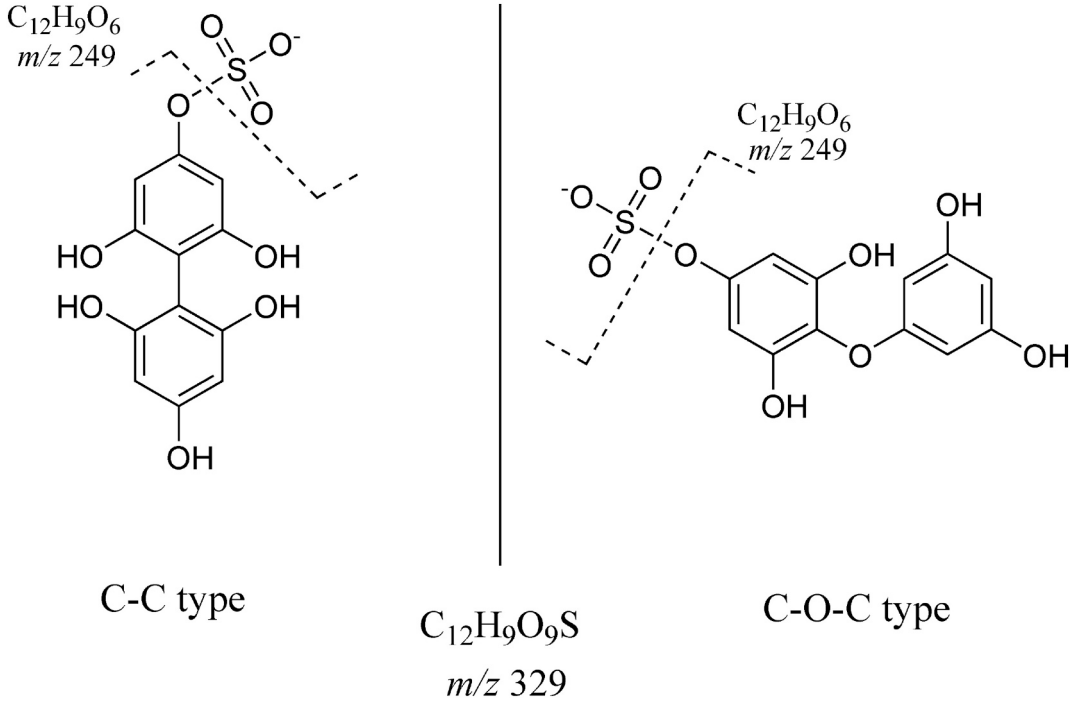

Fig. 4. Isomeric structures of sulfated difucol/diphlorethol at $[M - H]^-$ = 328.9978 (330 g/mol) with fragmentation (*m/z* 249) and neutral loss of the sulfate group ($O_3S^\bullet$, 80 Da). The position of the sulfate group substitution is unknown and arbitrary placed in the figure.

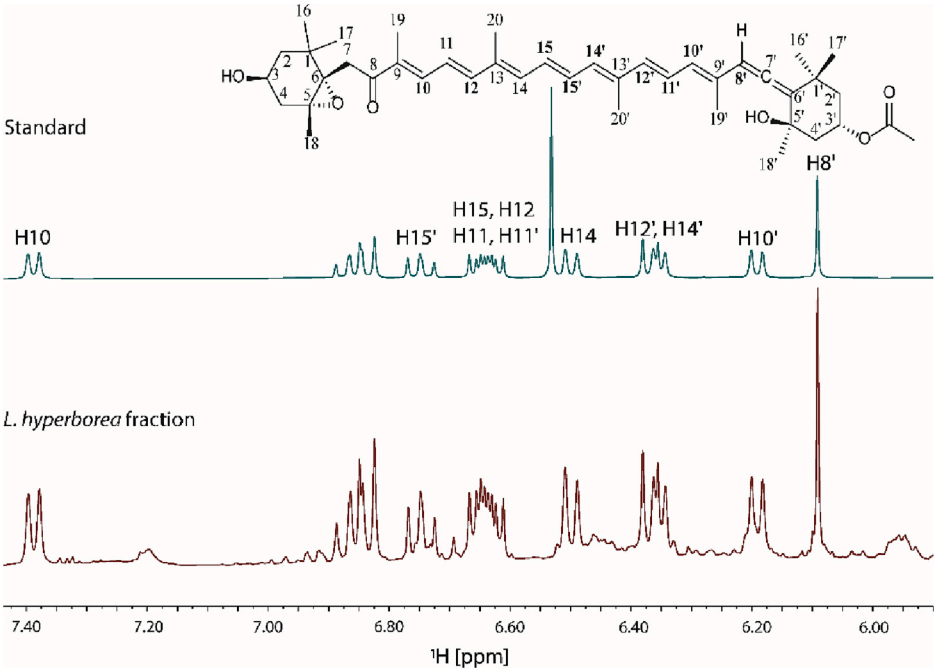

Fig. 5. Stacked ^{1}H NMR spectra of a fucoxanthin reference standard (blue) and isolated fucoxanthin in a late-eluting, less polar PuriFlash L. *hyperborea* fraction (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

extraction of high value products from both solid and liquid side streams of commercial alginate production. Additionally, other high value compounds and fucoxanthin and mannitol were successfully separated from the phenolic fractions. The results will contribute to increasing the value of the leaf fraction and further improving the sustainability of alginate production and seaweed biorefinery.

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CRediT authorship contribution statement

Marie Emilie Wekre: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization. **Bjarte Holmelid:** Investigation, Resources, Methodology. **Jarl Underhaug:** Methodology, Supervision, Resources. **Bjørn Pedersen:** Investigation. **Georg Kopplin:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Monica Jordheim:** Conceptualization, Methodology, Data curation, Formal analysis, Validation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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