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## Integrated biorefinery approach to valorise *Saccharina latissima* biomass: Combined sustainable processing to produce biologically active fucoxanthin, mannitol, fucoidans and alginates



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### ABSTRACT

The feasibility of European seaweed farming depends on the valorisation of algal biomass harvested. In the present work we have combined sequential extraction processes from *Saccharina latissima* to produce a range of products, focusing on the extraction of fucoxanthin using supercritical CO<sub>2</sub> followed by different valorisation routes. We optimised the conditions for extraction of fucoxanthin (40 MPa, temperature has little impact on extraction) and the extracts obtained were tested on cancer cell cultures to determine the antiproliferative effects of this pigment. We established that the supercritical CO<sub>2</sub> extracts have an antiproliferative effect similar to that of commercial fucoxanthin (concentrations 0.1–0.4 mg/mL) and showed that the active compound in the extracts is fucoxanthin. In order to integrate this process with a holistic valorisation of the algal biomass, we explored the extraction of mannitol using a microwave-assisted protocol (4.15 wt % yield). We also evaluated the potential extraction of fucoidans and alginates from the solids remaining after supercritical CO<sub>2</sub> extraction (67.27 to 69.38 % of alginates). A life cycle analysis of the supercritical CO<sub>2</sub> extraction proposed shows that the drying process of algal biomass and the energy used to compress the CO<sub>2</sub> are the elements with the highest environmental impact (over 90% of CO<sub>2</sub> eq/g of extract) in this the process, indicating routes for reducing the environmental footprint. Combining supercritical CO<sub>2</sub> extraction and microwave-assisted extraction methods would enable European seaweed producers to obtain multiple marketable products from algal biomass.

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## 1. Introduction

Seaweed farming has grown in importance as an activity to produce sustainable feedstock for food, pharmaceutical products and materials. Although in Asia, seaweed farming is a well-established activity, the interest in developing a

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mature industrial scenario for seaweed farming in Western countries, and particularly in Europe, is quite recent. One of the main challenges faced by seaweed farming in Western countries is high costs of offshore seaweed production, particularly from the initial investment and seedling purchase (van den Burg et al., 2016). These high costs can be offset by developing products with higher market values that compensate for the investment required for offshore seaweed farming. Biorefining has been defined as the sustainable processing of biomass into a spectrum of marketable products and energy (Cherubini, 2010). Biorefining can add value to biomass by producing multiple products from the same feedstock, combining low volume/high value with high volume/low value products (van den Burg et al., 2021).

The bioactivity of seaweed-derived fractions has opened new markets in personal care, nutraceuticals and pharmaceuticals (Ganesan et al., 2019; Kang et al., 2020; Morais et al., 2021). These applications are particularly relevant for brown algae. Most research efforts have concentrated on studies of *Saccharina latissima*, commonly known as sugar kelp, owing to its promising performance in aquaculture systems.

Fucoxanthin and mannitol are two relatively high value products that can be extracted from seaweed and can increase the value of algal biomass. This opens the possibility of establishing additional sustainable value chains around seaweed. This can be further strengthened if more traditional products, such as alginates and fucoidans, are produced in the same process. Fucoxanthin, a carotenoid that is part of the light harvesting complex in the photosynthetic apparatus of brown algae (Wang et al., 2019), has attracted a substantial interest due to its therapeutic potential. Besides being proposed as a general antioxidant compound (Maeda et al., 2018), fucoxanthin has shown potential as an inhibitor of cell proliferation in glioblastoma (Liu et al., 2016). The mechanism of action of fucoxanthin on glioblastoma cells has shown synergy with synthetic pharmaceuticals, demonstrating potential for combined treatment of glioblastoma (Pruteanu et al., 2020). Mannitol is a sugar alcohol that constitutes a significant proportion of seaweed biomass and has various applications in confectionery, oral care, pharmaceuticals, food, industrial, surfactants, and cosmetics (Ghoreishi and Shahrestani, 2009). Although commercial production of mannitol is achieved by catalytic hydrogenation of fructose (Dai et al., 2017), in Asia mannitol is extracted from seaweed by different methods (Chen et al., 2020).

Novel extraction approaches enable selective fractionation of algal biomass, allowing targeting towards multiple products. Supercritical carbon dioxide (scCO<sub>2</sub>) has been shown as an effective solvent for the extraction of non-polar compounds and offers a number of advantages, such as selectivity by fine-tuning the temperature and pressure during extraction (Attard et al., 2015b). The economic analysis in the present work brings an environmental perspective while the method is still at low technological readiness level. Indeed, scCO<sub>2</sub> has been proven to be an effective method for fucoxanthin and xanthophyll extraction from *Fucus* and *Laminaria* (Heffernan et al., 2016). Microwave heating is also a fast and efficient method for biomass processing. Microwave heating performs well in biomass extraction, hydrolysis and pyrolysis processes. Yuan and Macquarrie (2015) applied a sequential extraction protocol to *Ascophyllum* to obtain alginate, fucoidan and biochar (Yuan and Macquarrie, 2015).

The main objective of this study is to develop a biorefinery process for the extraction of seaweed to produce biologically active fucoxanthin and mannitol. In the present work, we use scCO<sub>2</sub> extraction as a first step in a biorefinery approach to fractionate *Saccharina latissima* biomass. A visual representation of the subsequent extraction and analysis is presented in Fig. 1. We establish that the extracts obtained by scCO<sub>2</sub> extraction have biological activity, inhibiting cancer cell proliferation. We use microwave fractionation as a follow-up step for mannitol extraction, while demonstrating that the solids after scCO<sub>2</sub> extraction can be used for fucoidan and/or alginate extraction. An initial life cycle analysis was applied to the scCO<sub>2</sub> extraction of fucoxanthin to identify the most relevant approaches that can make the process more sustainable.

## 2. Material and methods

### 2.1. Materials

*Saccharina latissima* (Linnaeus) biomass from Norway used in this work was kindly provided by one of the industrial partners of project GENIALG, Seaweed Solutions. *Saccharina latissima* was farmed in Taraskjæra (N63.4228, E8.5223, Frøya, Norway) and harvested on the 28th of April 2017. Upon harvesting, seaweeds were hung for a few minutes to remove as much as seawater as possible, and then stored at  $-20^{\circ}\text{C}$ . Whole seaweed portions were freeze-dried, ground with a mortar and pestle, and kept at  $-80^{\circ}\text{C}$  before analysis.

### 2.2. Optimisation of supercritical CO<sub>2</sub> extraction in *Saccharina*

Supercritical CO<sub>2</sub> extractions were carried out using a THAR SFE 500 system according to the methodology previously described (Attard et al., 2015b; Sin, 2012). Freeze-dried algae biomass (60 g) was loaded into the extraction system, which was heated to the desired temperature, and supercritical fluid grade (99.99%) CO<sub>2</sub> was introduced into the system until the desired pressure was achieved (exact temperatures and pressures are provided in Supplementary Tables 1 and 2). The system was maintained at a flow rate of  $35\text{ g min}^{-1}$  for 4 h, at which point the flow was stopped and the system depressurised. The extract was collected by washing the separating flask with  $3 \times 25\text{ cm}^3$  of 3:1 ethyl acetate:ethanol, which was then removed *via* rotary evaporation until constant mass of the sample was achieved. Carotenoids were determined by colorimetry after extraction in acetone and liquid/liquid extraction with hexane. Carotenoids were measured in the hexane fraction and chlorophylls in the acetone fraction. Carotenoids were monitored at 438–450 nm using a UV–Vis detector, while chlorophylls were monitored at 650 nm.

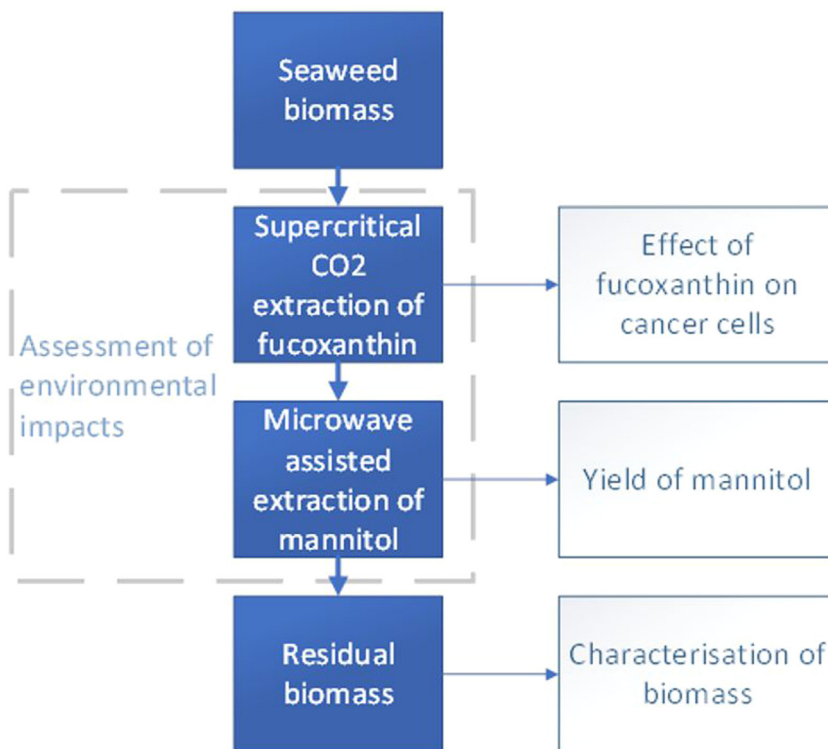


Fig. 1. Schematic representation of the process followed in the present work.

### 2.3. Microwave-assisted extraction of mannitol

The microwave-assisted extraction of seaweed samples before and after scCO<sub>2</sub> was performed in a Milestone FlexiWave microwave reactor. For each experiment, a 1:10 solid:liquid ratio was used, 4.0 g of seaweed and 40 mL of distilled water in a 100 mL polytetrafluoroethylene (PTFE) vessel that was sealed ahead of extraction. The mixture was heated at a constant rate over 15 min from ambient temperature to the final temperature (150–230 °C) under continuous stirring and a maximum microwave power input of 1800 W (varied power during the ramping). After the reaction was complete, the vessel was cooled to ambient temperatures and then liquid and solid residues were separated. The solid residue was dried in an oven at 105 °C for a minimum of 24 h until constant weight was achieved, and the solid residue weight determined. A liquid fraction (5 mL) was dried in an oven at 105 °C for a minimum of 24 h until a constant weight was achieved to give the solid content of material extracted into the liquid fraction. Mannitol was determined using an enzyme-based mannitol assay kit (Megazyme, Wicklow, Ireland). All microwave extraction experimental conditions were performed in triplicate.

### 2.4. Determination of fucoxanthin effects on cell proliferation

The human glioblastoma astrocytoma cell lines U87MG (ECACC 89081402) and A172 (ECACC 88062428) were obtained from the European Collection of Authenticated Cell Cultures. The T98G cell line was obtained from the American Type Culture Collection (ATCC<sup>®</sup> CRL1690<sup>™</sup>). All cell lines were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco, Thermo Fisher Scientific, Paisley, UK) supplemented with 10% foetal bovine serum (Merck Life Science, Gillingham, UK) and 5% antibiotic antimycotic solution (10,000 units penicillin, 10 mg streptomycin and 25 µg/mL amphotericin B; Merck Life Science, Gillingham, UK) at 37 °C in the humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

Both commercial and extracted fucoxanthin were used in the experiments. Commercial fucoxanthin was purchased from Sigma (F6932, Gillingham, UK). Both fucoxanthin preparations were dissolved in dimethyl sulfoxide (DMSO) to obtain 10–100 mM stock solutions and tested at 1–100 µM.

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) assay (Sigma, Gillingham, UK). U87MG, A172 and T98G cells were seeded at a density of 8,000 cells/well in 96-well plates and allowed to adhere overnight at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. No cells were seeded in the perimeter wells to ensure measurement accuracy and those were filled with 100 µL of sterile water. Then, culture medium was removed from the plates, and fresh medium containing fucoxanthin at different dilutions was added. Control cells were treated with vehicle solution

containing 1% DMSO. Blank controls without cells were also prepared. At 72 h after treatment, 5  $\mu\text{L}$  of the CCK-8 solution was added to every well containing 100  $\mu\text{L}$  of tested compounds, controls or blank. After 3 h of incubation at 37 °C in the dark, the plates were read using a Mithras LB940 multimode microplate reader (Berthold Technologies, Harpenden, UK), and the absorbance values were determined at 490 nm. The percentage of viable cells was calculated for each well as follows: % cell survival =  $\{(At-Ab)/(Ac-Ab)\} \times 100$ , where At is absorbance of the medium with tested compound; Ac is absorbance of control medium; and Ab is absorbance of blank medium. Concentration–effect relationships for proliferation inhibition assays were analysed by using Prism 8 (GraphPad, Inc., San Diego, CA, USA).

In addition to testing pure fucoxanthin, we also studied effects of three  $\text{scCO}_2$  extracts in the cell proliferation assay. The extracts were dissolved in ethanol to prepare the stock solution at a concentration of 0.1  $\text{mg}/\mu\text{L}$  (or 100  $\text{mg}/\text{mL}$ ). We made further dilutions of each stock solution to test the effect of extracts at concentrations of 1  $\mu\text{g}/\mu\text{L}$ , 0.5  $\mu\text{g}/\mu\text{L}$ , 0.25  $\mu\text{g}/\mu\text{L}$ , 0.1  $\mu\text{g}/\mu\text{L}$ , 0.05  $\mu\text{g}/\mu\text{L}$  and 0.025  $\mu\text{g}/\mu\text{L}$ .

Samples of the blank (ethanol), freeze-dried  $\text{scCO}_2$  extract, and air-dried  $\text{scCO}_2$  extract (40 each) were dried in a freeze-drier. Extract samples were dissolved in pure ethanol to give the highest concentration possible for testing on cells

### 2.5. Analysis of the polysaccharide fraction after $\text{scCO}_2$ extraction

Approximately 5 mg of each sample was weighed in triplicate in screw capped tubes. Samples were partially hydrolysed by adding 500  $\mu\text{L}$  of 2 M trifluoroacetic acid (TFA). The vials were flushed with dry argon, mixed and heated at 100 °C for four hours, being mixed periodically. The vials were then cooled to room temperature and dried in a centrifugal evaporator with fume extraction. 500  $\mu\text{L}$  of 2-propanol was added to the samples, and vortexed before drying in centrifugal evaporator. This process was repeated once. Finally, the samples were resuspended in 200  $\mu\text{L}$  of deionised water, mixed, centrifuged at 11,600 rpm for 5 min. The supernatant was filtered through 0.45  $\mu\text{m}$  PTFE filters into HPLC vials, and analysed by high-performance anion-exchange chromatography on a Dionex Carbopac PA-10 column using integrated amperometry detection (Jones et al., 2003). To enable quantification, a standard sugar mixture containing arabinose, fucose, galactose, glucose, mannose, rhamnose, xylose, galacturonic acid, glucuronic acid, guluronic acid, mannuronic acid, and mannitol, was prepared and treated as indicated above for algal samples.

### 2.6. Life cycle assessment scope and data sources

The environmental impact of  $\text{scCO}_2$  extraction was mapped in order to demonstrate the basic LCA approach and to identify improvement opportunities at early stage of technology development. The required drying and extraction are included in the system boundaries. The preceding seaweed cultivation and downstream processing are excluded. Isolation of pure fucoxanthin is not considered and not included in the analysis. The production of capital goods (buildings, machinery and equipment) was excluded since their contributions would be minimal.  $\text{CO}_2$  losses are negligible so that the initial consumption of  $\text{CO}_2$  should be distributed over a very large number of extraction cycles, hence overall consumption is negligible. The solvent used at lab scale would not be necessary present in a commercial process, with the extract removed using residual pressure and/or mechanically. For the goal of this study, the environmental impact of the process is of interest, so the functional unit is one extraction, on the scale that provides 6 g of extract. This corresponds to treating 1 kg of freeze dried seaweed, while 1.05 kg hot air dried and air dried seaweed are required due to slightly lower dry matter contents.

The  $\text{scCO}_2$  extraction process itself can be scaled up by installing more capacity and by continuously conducting extraction cycles over a 24 h (Attard et al., 2015b), so scale-up effects will be very small. This process requires 2.41 MJ heat and 0.57 and 7.05 MJ electricity for cooling and pressurising. The grinding before drying requires 0.0033 MJ per kg of seaweed. Since it was anticipated that the drying method influences the environmental impact, three scenarios with different drying options were considered: (a) Freeze drying (current case), requiring 112 MJ electricity per kg of dried seaweed (Pérez-López et al., 2014; Prosapio et al., 2017); (b) Hot air drying (van Oirschot et al., 2017; Wernet et al., 2016), requiring 0.842 MJ electricity and 25.8 MJ heat per kg of dried seaweed. (c) Air drying (Slegers et al., 2021) requiring 4.10 MJ electricity per kg of dried seaweed. The emissions and resource usages were translated to environmental impacts with the characterisation method “ReCiPe 2016 Midpoint (H) V1.04/ World (2010) H” (Huijbregts et al., 2017). SimaPro 9.1 was used for modelling.

## 3. Results and discussion

### 3.1. Optimisation of $\text{scCO}_2$ extraction

Factorial experimental design was used to allow rapid optimisation of conditions using the minimal number of experiments. As there are two controlled variables, this is referred to as two-level factorial design. Other variables, such as flow rate and biomass loading were kept constant. A variety of temperatures and pressures were utilised in an experimental 2  $\times$  2 plot. A pressure range between 80–400 bar (8–40 MPa) was applied at temperatures between 35 and 65 °C.  $\text{CO}_2$  becomes supercritical at 31 °C and 72.8 bar (7.3 MPa), as such the low temperature and pressure points were



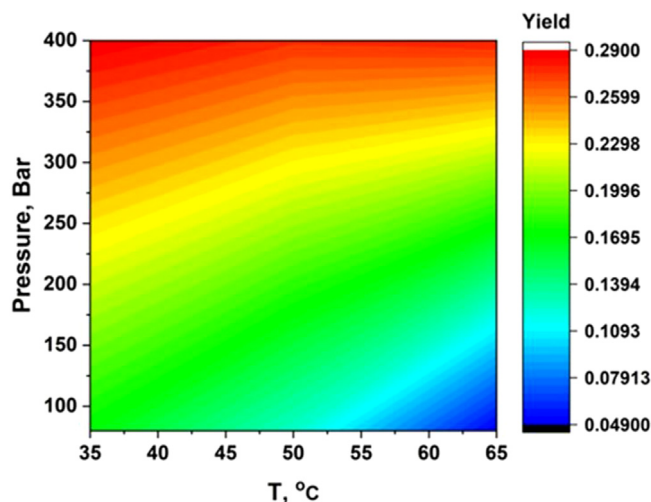


Fig. 2. Crude yield of *Saccharina* extract as a function of temperature and pressure, as percentage of the initial biomass.

Table 1

% yields of extracts from *Saccharina lattissima* for each experiment point.

Experiment Point	Temperature (°C)	Pressure (bar)	Overall % Yield	Carotenoid yield (mg g <sup>-1</sup> )	Chlorophyll yield (mg g <sup>-1</sup> )
A	35	400	0.29	0.95	4.43
B	65	400	0.28	0.98	17.53
C	35	80	0.12	0.45	0.45
D	65	80	0.05	1.14	1.00
E	50	200	0.17	1.26	5.05

selected just above this point (Brogle, 1982). The maximum pressure at which the system is rated is 600 bar (60 MPa) although for capital expenditure and operational expenditure at scale, this is not an industrially viable operating pressure. As such 400 bar (40 MPa) was chosen as the upper pressure limit. The range of temperatures and pressures were chosen to show the impact of these variables on extraction yield and composition. Four experimental points were selected at maximum and minimum temperatures and pressures. A centre point was also introduced in order to ensure there was no risk of missing a non-linear relationship within the experimental range. The impact of pressure and temperature was modelled by means of a dimensionless factor coordinate system, whereby “-1” was assigned for the low level and “+1” was given to the high level for each parameter. The centre point was assigned a coordinate value of “0” (coincides with the origin of the system) as shown in Supplementary Table 1.

From the yields obtained, 2-D conformational plots of pressure and temperature (Fig. 2) were obtained in order to see the effects of the different variables (P and T) on the percentage of extraction yield. Multiple linear regression was employed in order to analyse the relationship between % crude yield and temperature and pressure. This was carried out using the first order polynomial function, shown in Eq. (1). The % yields of crude extract obtained at the different pressures and temperatures are summarised in Table 1.

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 \quad (1)$$

**Equation 1:** First Order polynomial function. Y represents the total % crude yield,  $b_0$  is the centre point yield (i.e. the response at “zero” level, labelled E in Supplementary Table 2),  $b_1$  and  $b_2$  are the dominant effects of the coordinate values  $x_1$ (temperature) and  $x_2$  (pressure) and  $b_{12}$  refers to the second order interaction term.

These % yields were used to carry out the multiple linear regression, using Eqs. (2)–(5) to derive a 1st order polynomial function in order to model the supercritical extraction of *Saccharina lattissima*.

$$b_0 = \frac{1}{4}(y_1 + y_2 + y_3 + y_4) \quad (2)$$

$$b_1 = \frac{1}{4}(-y_1 + y_2 - y_3 + y_4) \quad (3)$$

$$b_2 = \frac{1}{4}(-y_1 - y_2 + y_3 + y_4) \quad (4)$$

$$b_{12} = \frac{1}{4}(y_1 - y_2 - y_3 + y_4) \quad (5)$$

**Table 2**

Crude yield of large scale extractions. The CO<sub>2</sub> extractions were performed for 2 h at 35 g min<sup>-1</sup>, while the ethanol as co-solvent run was for 2 h at 10 ml min<sup>-1</sup>.

Extraction conditions	% Yield	Total mass of biomass processed
CO <sub>2</sub> 65 °C, 400 bar	0.27	602 g
CO <sub>2</sub> 35 °C, 400 bar	0.27	258 g
CO <sub>2</sub> 50 °C, 350 bar	0.21	152 g
CO <sub>2</sub> 50 °C, 350 bar	0.40 <sup>c</sup>	122 g
Ethanol co-solvent		

### Equations 2–5: Coefficient calculations for 1st order polynomial function

$$Y = 0.185 + 0.02x_1 + 0.1x_2 + 0.015x_1x_2 \quad (6)$$

### Equation 6: 1st order polynomial function for the supercritical extraction of carotenoids from *Saccharina latissima*.

Eq. (6) shows the coefficients of temperature, pressure as well as the second order interaction term. These coefficients may be utilised to aid in understanding how temperature, pressure and the combined effect of the two parameters affects the extraction process. There is good correlation between the theoretical % yield for centre point coordinate E (0.185%) with the experimental value obtained (0.17%) – a 0.015% difference with 8.8% error, demonstrating that the MLR model behaves well for this scCO<sub>2</sub> extraction. The model shows that in this extraction, pressure is the dominant factor on the extraction yield with the value of  $x_2$  for pressure significantly higher than that of  $x_1$  for temperature. In fact, the value of  $x_1$  is negligible, showing that temperature has little to no effect on the extraction process. This indicates that the density of CO<sub>2</sub> has a significant influence on the extraction of carotenoids from *Saccharina latissima* since increasing the pressure at constant temperature results in a corresponding increase in CO<sub>2</sub> density (Salgin et al., 2006). Temperature decreases density, but can impact on yield by increasing the solubility of lipids and also melting waxes (Bulushi et al., 2018). This suggests that the lipids within *Saccharina* are readily soluble at lower temperatures and that there are few or no high melting point waxes. The maximum yield of carotenoids was obtained at 50 °C and 200 bar (20 MPa). Interestingly, the optimum condition for chlorophyll extraction is different (65 °C and 400 bar (40 MPa)) showing the different extractability of these two pigments (Table 1).

Once the best extraction conditions for scCO<sub>2</sub> were established, three conditions were selected for scaling from 60 g of freeze dried *Saccharina* to multiple runs of 120 g of biomass per extraction. This was carried out in order to obtain enough material for cancer cell proliferation tests and subsequent fractionation. Table 2 shows the conditions selected and crude yield obtained. These results are in broad agreement with those obtained from the factorial modelling work. Yields for entry 1 and 2 (Table 2) are almost identical, which highlights the limited impact that temperature plays in these extractions. Reducing the pressure by 50 bar (5 Mpa) results in a significant drop in yield (Table 2). It should be noted that there is an energy penalty in operating extractions at higher pressures. Previous work has shown that the energy requirements for pumping CO<sub>2</sub> account for over 90% of the cost of utilities ( $C_{UT}$ ) when extracting at 65 °C and 400 bar (40 MPa) (Attard et al., 2015a). Increasing the pressure, increases the density of CO<sub>2</sub> which in turn increases the energy requirements of the pump.

Most striking is the impact of the use of a co-solvent, such as EtOH (Table 2). This extraction was carried out on a fraction of biomass residue that had already been subjected to CO<sub>2</sub> 65 °C, 400 bar (40 MPa), thus did not contain any non-polar extractives. Post scCO<sub>2</sub> extraction, the samples were analysed by HPLC to determine the carotenoid profile and concentration of each carotenoid. The average yield of fucoxanthin in these extractions was 13 mg/g.

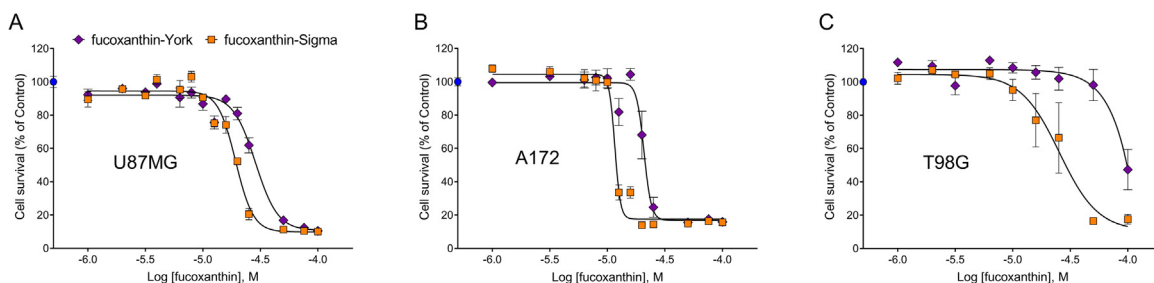
### 3.2. Mannitol extraction using MW

The valorisation of seaweed biomass requires, at least within Europe, to maximise the value through different products in a cascade of extractions (van den Burg et al., 2021). To test the compatibility of scCO<sub>2</sub> extraction of fucoxanthin with subsequent extraction of mannitol, we used microwave-assisted fractionation of the extracted biomass over different temperature ranges and evaluated the amount of mannitol extracted.

Following the scCO<sub>2</sub> extraction of the seaweed samples, microwave-assisted fractionation was performed using a temperature range between 150 °C and 230 °C and evaluated how the scCO<sub>2</sub> treatment affects the second extraction step. Table 3 shows that the microwave extraction removes between 61 and 65% of the solids, both with and without scCO<sub>2</sub> extraction of the biomass. Similarly, a yield between 4.15 and 3.62 wt % of mannitol could be extracted using microwave, with a maximum yield between 190 °C and 210 °C. The lack of correlation between increases in temperature indicates that the process can be performed at lower temperatures. Amounts of mannitol between 25 and 10 wt% have been reported for *Saccharina* during different seasons (Schiener et al., 2015). Even though the mannitol values obtained in this MW extraction are lower than those from the literature, the materials previously extracted with scCO<sub>2</sub> displayed little difference in mannitol yield to that of the raw seaweed. This indicates that the first extraction process had little

**Table 3**Mass balance and yield of mannitol from *Saccharina* biomass after under different conditions of microwave-assisted fractionation.

Sample	Average wt% of biomass in the pellet	Average wt% of biomass extracted	Mannitol (wt% of dry biomass)	
			Extracted	Standard deviation
Original Seaweed 150 °C	31.36	63.48	3.62	0.08
Original seaweed 170 °C	23.87	64.23	3.84	0.13
Original seaweed 190 °C	20.42	64.87	3.94	0.01
Original seaweed 210 °C	16.24	65.64	3.81	0.03
Original seaweed 230 °C	15.44	64.76	3.63	0.03
Seaweed after ScCO <sub>2</sub> 150 °C	26.43	61.91	3.50	0.15
Seaweed after ScCO <sub>2</sub> 170 °C	22.38	63.32	3.77	0.06
Seaweed after ScCO <sub>2</sub> 190 °C	20.05	61.43	3.79	0.30
Seaweed after ScCO <sub>2</sub> 210 °C	16.54	65.20	3.83	0.11
Seaweed after ScCO <sub>2</sub> 230 °C	14.34	67.23	3.68	0.53
Seaweed after ScCO <sub>2</sub> + EtOH 150 °C	25.46	57.62	3.72	0.39
Seaweed after ScCO <sub>2</sub> + EtOH 170 °C	24.10	59.81	3.80	0.11
Seaweed after ScCO <sub>2</sub> + EtOH 190 °C	17.66	63.22	4.15	0.17
Seaweed after ScCO <sub>2</sub> + EtOH 210 °C	16.01	62.34	3.86	0.06
Seaweed after ScCO <sub>2</sub> + EtOH 230 °C	15.25	65.01	3.66	0.38



**Fig. 3.** Effect of fucoxanthin on cell proliferation. Concentration–response relationships for the inhibitory action of fucoxanthin on the viability of U87MG, A172 and T98G cells were obtained using the CCK-8 assay. Purple dots show the effect of fucoxanthin purified from *Saccharina* (fucoxanthin-York) and orange dots represent the effects of commercial (fucoxanthin-Sigma) fucoxanthin. Data are presented as the mean  $\pm$  standard error of the mean ( $n = 6$ ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

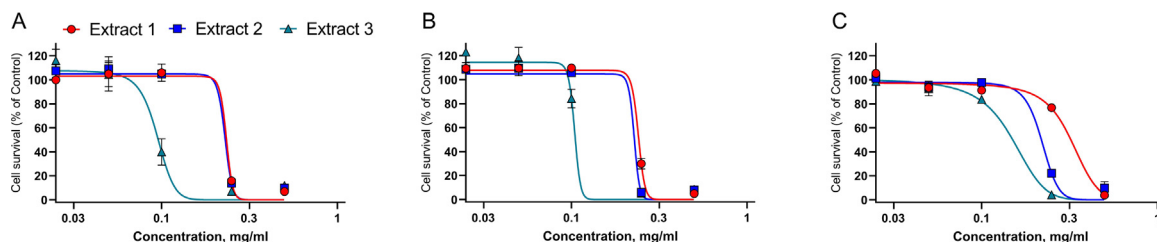
to no effect on mannitol yields. The temperature in the microwave extraction process or a combination of scCO<sub>2</sub> with ethanol extraction had negligible influence on yield across the temperature range examined. These results indicate that the process can be undertaken at lower temperatures, requiring less energy to attain similar yields, and that initial extraction of fucoxanthin using scCO<sub>2</sub> is compatible with mannitol extraction.

### 3.3. Effect of fucoxanthin on cancer cell proliferation

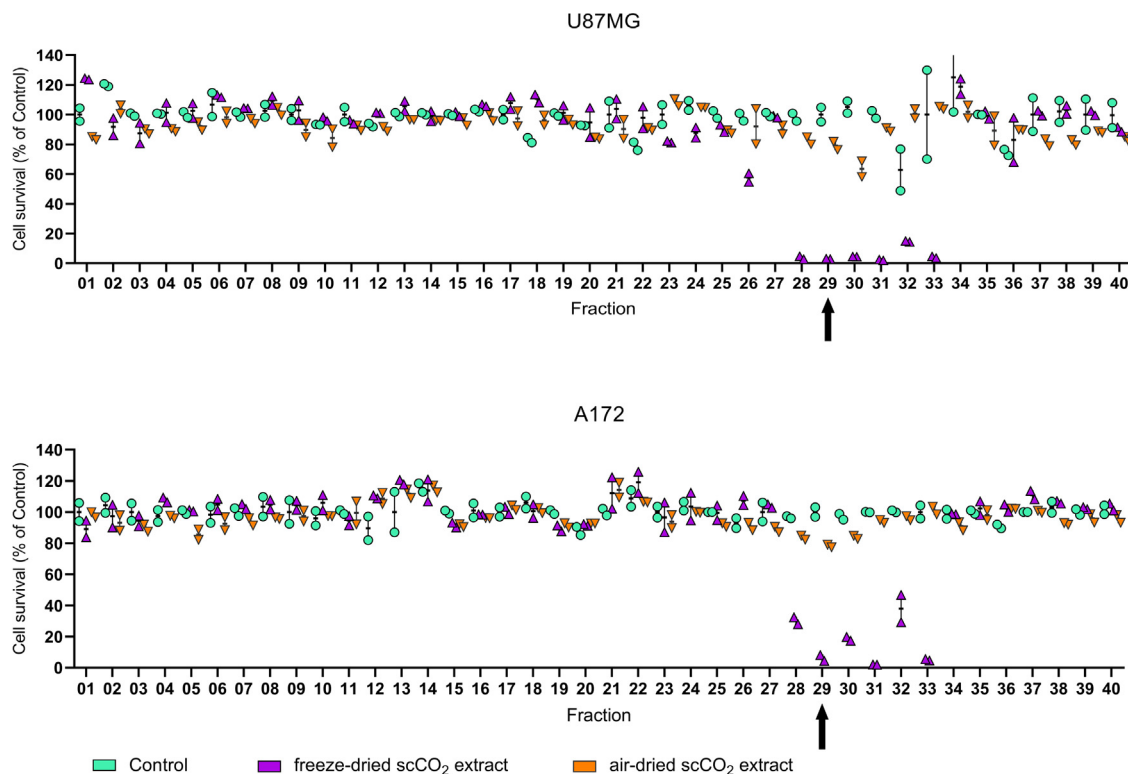
Seaweeds are a source of several biologically active compounds that are characterised by cytotoxic activity towards cancer cells (Sharma et al., 2020). In particular, fucoxanthin has been previously demonstrated to inhibit viability and proliferation of glioblastoma cells (Liu et al., 2016; Lopes et al., 2020; Pruteanu et al., 2020). Therefore, it was of interest to examine whether fucoxanthin obtained from *S. latissima*, as well as fractions extracted using scCO<sub>2</sub>, would inhibit the survival of glioblastoma cells. In our experiments, we demonstrated that both commercial and extracted fucoxanthin decreased the proliferation of U87MG, A172 and T98G glioblastoma cells with IC<sub>50</sub> values ranging from 11.6 to 28.7  $\mu$ M, except for the effect of extracted fucoxanthin on T98G cells, which was about an order of magnitude weaker (Fig. 3). These IC<sub>50</sub> values were broadly similar to those reported by us in U87MG cells previously (Pruteanu et al., 2020) and in A172 and other glioblastoma cells as previously described (Lopes et al., 2020).

Once we established the inhibitory effect on cell proliferation of fucoxanthin, we studied the effect of scCO<sub>2</sub> extract optimised for carotenoid yields on the proliferation of the three above-mentioned glioblastoma cell lines. We observed that the extracts inhibited the viability of glioblastoma cells at concentrations 0.1–0.4 mg/mL, showing that scCO<sub>2</sub> extracted fractions of *Saccharina* retain the inhibitory effect on cell proliferation exerted by pure fucoxanthin. Extracts carried out at 50 °C and 350 bar (35 MPa) had a slightly stronger inhibitory effect compared to the other two conditions selected for scale up (Fig. 4). scCO<sub>2</sub> extractions in the conditions optimised in our work remove a range of compounds with low polarity from the seaweed material. In order to exclude the possibility that the inhibitory effect observed in the scCO<sub>2</sub> fractions extracted from *Saccharina* biomass on cancer cell proliferation is due to compounds other than fucoxanthin, we collected fractions after HPLC separation and tested their effects on cancer cell cultures. Fig. 5 shows the effect of individual





**Fig. 4.** Effect of supercritical CO<sub>2</sub> extracts on human cancer cell lines. Concentration–response relationships for the inhibitory action of three scCO<sub>2</sub> extracts on the viability of U87MG (A), A172 (B) and T98G (C) cells in the CCK-8 assay. Extract conditions were as follows: 35 °C, 400 bar (Extract 1); 50 °C, 350 bar + 10 g min EtOH (Extract 2); 50 °C, 350 bar (Extract 3). Data are presented as the mean ± standard deviation ( $n = 6$ ).

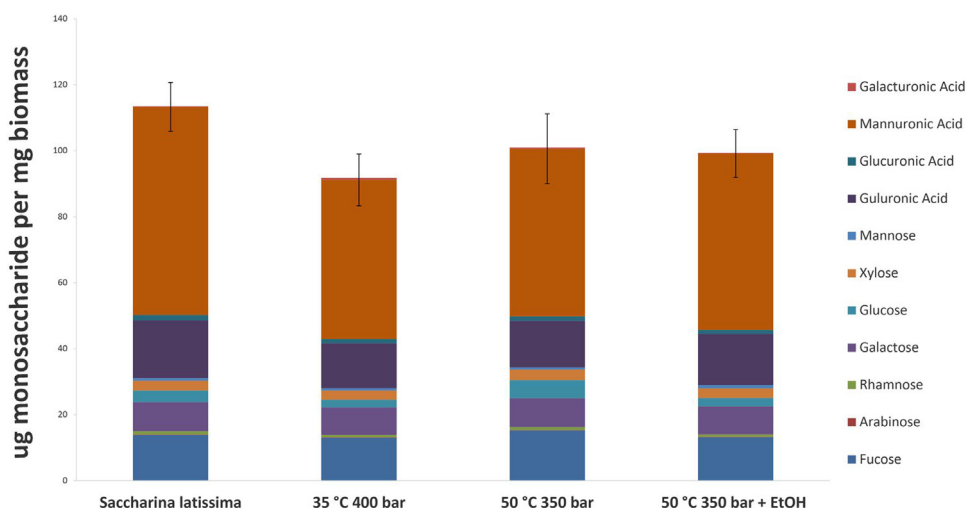


**Fig. 5.** Effect of freeze-dried and air-dried scCO<sub>2</sub> fractions after HPLC separation on the proliferation of U87MG (top panel) and A172 (bottom panel) cell lines determined in the CCK-8 assay. Fraction number indicates each fraction collected at 1 min intervals. Turquoise, purple and orange dots indicate the effects of fractions collected without injection of scCO<sub>2</sub> extract, freeze dried immediately after collection, and air dried after collection, respectively. The arrows indicate fraction 29 that contains the fucoxanthin peak (see Fig. 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

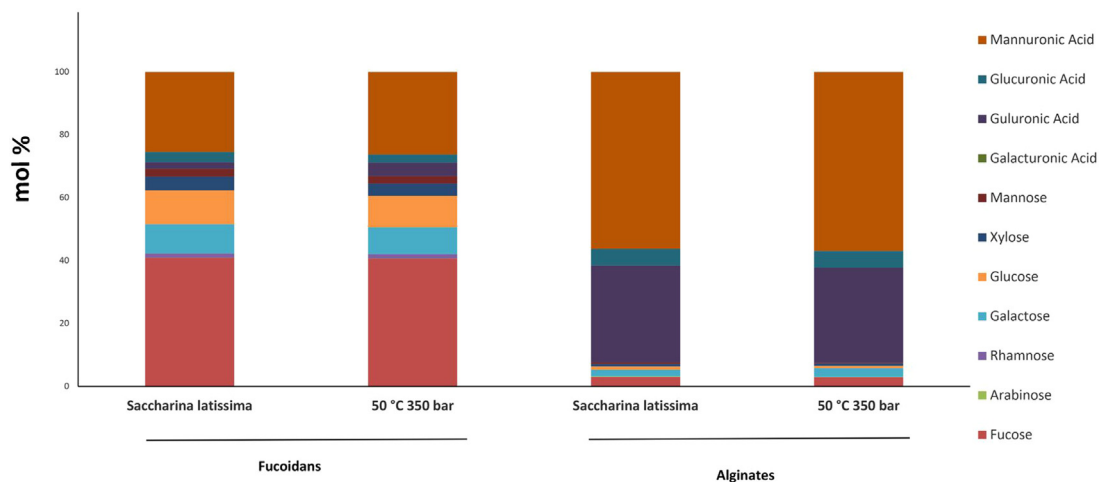
fractions collected after separation on U87MG and A172 and freeze drying and air drying. Fraction 29 corresponds to the peak of fucoxanthin observed in the HPLC profile (supplementary Figure 1) and shows a strong inhibitory effect on both cell lines when the fractions are immediately freeze dried after purification. The air dried fractions do not show this effect, indicating that a fast preservation of the samples is essential to maintain integrity of fucoxanthin and its properties.

### 3.4. Evaluation of the solids after scCO<sub>2</sub> extraction

Biorefining involves the sequential valorisation of biomass to produce a spectrum of products that together can add value to the original material. We have shown that after scCO<sub>2</sub> extraction, *Saccharina* biomass can be extracted using MW to efficiently produce mannitol. In order to explore further valorisation of *Saccharina*, we determined the monosaccharide profile of the solids after the extraction with the optimised conditions of scCO<sub>2</sub> to investigate if other valuable commercial components of the biomass are preserved after extraction. We determined the monosaccharide profile present in the solids after TFA hydrolysis as the resulting monosaccharide profile is an optimal indicator of the polysaccharides present.



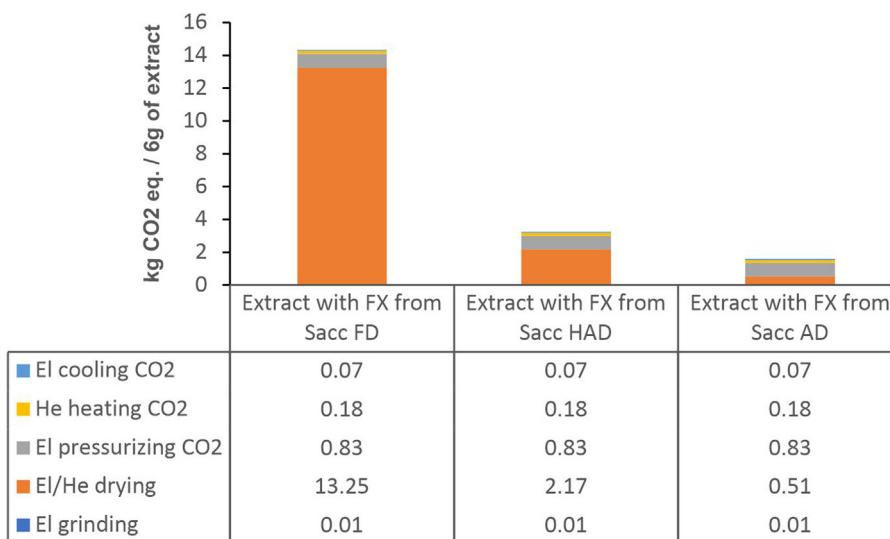
**Fig. 6.** Composition of the solids after  $\text{scCO}_2$  extraction. Monosaccharide profile of *Saccharina latissima* and solids after  $\text{ScCO}_2$  extraction under selected conditions and TFA hydrolysis.



**Fig. 7.** Composition of the alginate and fucoidan fractions of *Saccharina* after  $\text{scCO}_2$  extraction. Native *Saccharina* biomass and biomass extracted with  $\text{scCO}_2$  at 50 °C were used to extract fucoidans (left) and alginates (right). After TFA hydrolysis the mol% composition obtained was determined.

Fig. 6 shows that the total monosaccharide fraction in *Saccharina* biomass is 113 mg/g. This fraction is reduced after  $\text{scCO}_2$  extraction under all conditions, but this reduction is greater when the biomass is extracted under 400 bar (40 MPa) at 35 °C (19.5%). Under the other extraction conditions, the reduction in the monosaccharide fraction is lower than 10%. The most abundant monosaccharides in native and extracted biomass were mannuronic acid, guluronic acid and fucose. This indicates that during  $\text{scCO}_2$  extraction the vast majority of alginates and fucoidan remain in the biomass. The unextracted *Saccharina* biomass contains 71% of alginates (calculated by combining mannuronic and guluronic acids content). The biomass extracted under 400 bar (40 MPa) at 35 °C, 350 bar (35 MPa) 50 °C and 350 bar (35 MPa) 50 °C+EtOH contain 67.27, 64.33, and 69.38% of alginates respectively. The M/G ratio remained unchanged after  $\text{scCO}_2$  extraction under all conditions. Fucose, the main sugar component of fucoidans, represented 12.21% of the biomass without extraction. Interestingly, after  $\text{scCO}_2$  extraction the mol % of fucose increased slightly. Besides indicating that the biomass after  $\text{scCO}_2$  extraction can be used for conventional fucoidan extraction, these results indicate the selectivity of  $\text{scCO}_2$  for non-polar compounds. Fucoidans are highly sulphated and consequently polar compounds.

In order to verify the possibility of extracting alginates and fucoidans after  $\text{scCO}_2$  extraction of *Saccharina*, we applied conventional protocols for extraction of alginates and fucoidans. Fig. 7 shows that the molar composition of the alginates and fucoidans obtained from non-extracted and extracted *Saccharina* biomass present a very similar monosaccharide composition. This indicates that  $\text{scCO}_2$  pretreatment does not alter the subsequent products that can be obtained.



**Fig. 8.** Comparison of climate change impact of extractions of seaweed dried with different methods. The values corresponding to each individual step are presented below each column.

### 3.5. LCA analysis of the biorefining process

The different environmental impacts that were evaluated (global warming, ozone formation, terrestrial acidification, freshwater and marine eutrophication, and mineral and fossil resource), show the same distribution across the five energy contributions tracked. Global warming potential can serve as a lead indicator for the overall environmental impact. The absolute values for the three scenarios are shown in Fig. 8. The energy demand for freeze-drying is so high that the impact of the extraction becomes relatively small. For hot air drying, the energy demand is reduced but relatively large. Air drying requires the least energy of the drying options, so that the energy demands for extraction (specifically pressurising) become a relatively large contribution. Thus, the variable drying energy demands determine the overall environmental impact of drying and extraction. The variation in all three scenarios is substantial due to the uncertainty in drying energy requirements. There was no reason and no data to assume variability in the  $scCO_2$  process itself. A partial Monte Carlo analysis focussing on variations in all relevant drying variables (outgoing mass and energy requirements using uniform and triangular distributions based on minimums and maximums from literature) results in substantial coefficients of variations (of 10, 15 and 11 for the respective scenarios) but support the trend in the results.

The different seaweed drying methods before extraction determine the differences between the scenarios. The data sources for the three drying methods are very diverse and involve proxies to some extent: freeze drying energy demand was based on strawberry and microalgae drying, hot air drying was based on maize drying as supported by research work on macroalgae, air drying was based on data on macroalgae drying from a single industrial site. While such extrapolations are common in early-stage LCAs because of their explorative character, direct measurements on industrial and laboratory scale for different drying methods are recommended. The overall variability analysis shows that the trends are robust despite the limitations, because of the substantial differences between the scenarios.

Both drying and extraction activities are high energy investments, yielding a pharmaceutically active ingredient of high value. The residue of the treatment is employed in mannitol extraction, but does not benefit from the  $scCO_2$  process. An environmental assessment with a broader focus, including mannitol extraction and other products from a future macroalgae biorefinery could shed more light on the balance between environmental impact and functional value of the different products. However, this early environmental impact information can already inform the further scale-up of individual steps and the development of biorefinery designs.

In the future, more will be known about the applications of the seaweed derived products and this will aid optimising the process further. If slightly higher water contents in the starting material can be tolerated in the  $scCO_2$  process, less drying energy could be invested. The fucoxanthin and other pigments in the extract are sensitive to oxygen and light. They should be protected from these conditions in the production chain, as soon as the seaweed is harvested. While freeze drying is quick and oxygen free it requires a lot of energy, therefore other methods could be adapted to ensure the mild conditions. The more environmentally friendly forced air drying which already takes place in dark chambers and a forced nitrogen flow could be considered.

## 4. Conclusions

This study assessed the feasibility of an integrated biorefinery approach to valorise the seaweed *Saccharina latissima*. The combination of  $scCO_2$  extraction and microwave-assisted extraction of mannitol yielded various valuable products

from algal biomass. Economic value is added if multiple products are obtained from the same feedstock. An integrated biorefinery is, for these reasons, sought for by European seaweed producers for whom production for the global commodity market is financially not attractive.

Supercritical industrial processes at large scale/high pressure have been developed for multiple applications and at present is considered a mature technology with applications not only in the extraction of pharma and food products, but also in textiles, recycling of tyres, cleaning, and degreasing (Brunner, 2010). Our study illustrates that fucoxanthin extracted from *Saccharina latissima* using an optimised scCO<sub>2</sub> extraction method has antiproliferative effect on human cancer cell lines, showing effectiveness similar to commercial fucoxanthin. We have also demonstrated that the active compound in these extracts is fucoxanthin. scCO<sub>2</sub> extraction represents a simple and sustainable process to produce this active antiproliferative carotenoid.

Analysis of the solids after production of active scCO<sub>2</sub> extracts indicates that both fucoidans and alginates can be extracted after fucoxanthin without altering the composition of these polysaccharides. Furthermore, MW extraction after scCO<sub>2</sub> can increase the economic value of *Saccharina* biomass by including mannitol as a product in a sequential valorisation process.

In the LCA of scCO<sub>2</sub>, it was found that seaweed drying and electricity use for pressurising CO<sub>2</sub> during scCO<sub>2</sub> contribute the most to different environmental impacts. The energy required differs considerably for the three seaweed drying options studied here. Scale-up will not change this, but a trade-off between energy efficiency and mild conditions should be found for the drying method. The environmental considerations would not dominate choices during technical development, although at the current stage of development they coincide with economic considerations. Early stage LCA of these seaweed processing methods provide interesting recommendations for further technical development processes.

### CRedit authorship contribution statement

**Con Robert McElroy:** Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Liliya Kopanitsa:** Investigation, Methodology, Resources, Writing – original draft. **Roel Helmes:** Investigation, Methodology, Writing. **Jiajun Fan:** Investigation, Methodology, Resources, Writing – original draft. **Thomas M. Attard:** Investigation, Methodology, Resources, Writing – original draft. **Rachael Simister:** Investigation, Methodology, Resources, Writing – original draft. **Sander van den Burg:** Investigation, Methodology, Writing. **Graham Ladds:** Investigation, Methodology, Resources, Writing – original draft. **David S. Bailey:** Conceptualization, Funding acquisition, Writing – review & editing. **Leonardo D. Gomez:** Conceptualization, Writing – original draft, Writing – review & editing, 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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### Appendix A. Supplementary data

Supplementary material related to this article can be found online at <https://doi.org/10.1016/j.eti.2023.103014>.

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