ALTERNATIVE PROTOCOLS FOR THE PRODUCTION OF MORE SUSTAINABLE AGAR-BASED EXTRACTS FROM *Gelidium sesquipedale*

Marta Martínez-Sanz[1], Laura Pilar Gomez-Barrio[2], Ming Zhao[2], Brijesh Tiwari[2], Svein Halvor Knutsen[3], Simon Ballance[3], Hanne Kristine Zobel[3], Anna Ekman Nilsson[4], Christoffer Krewer[5], Karin Östergren[4] and Amparo López-Rubio[1*]

[1]Food Safety and Preservation Department, IATA-CSIC, Avda. Agustin Escardino 7, 46980 Paterna, Valencia, Spain

[2]The Department of Food Chemistry and Technology, Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland

[3]Nofima AS, Norwegian Institute of Food, Fisheries and Aquaculture Research, Ås, Norway

[4]RISE Research Institutes of Sweden, Agriculture and Food, Ideon Science Park, Scheelevägen 17, SE-223 70 Lund, Sweden

[5]RISE Research Institutes of Sweden, Agriculture and Food, Frans Perssons väg 6, SE-402 29 Göteborg, Sweden

*Corresponding author: Tel.: +34 963200022; fax: +34 963636301

E-mail address: mmartinez@iata.csic.es

Abstract

Agar-based extracts from *Gelidium sesquipedale* were obtained by applying a conventional hot water treatment and alternative ultrasound- and microwave-assisted methods, with and without the application of an alkaline pre-treatment. The alkaline pre-treatment produced refined extracts with higher purity; however, extraction yields increased from 2-5% to 7-19% by omitting this step. In particular, the ultrasound-assisted extraction allowed reducing 4-fold the extraction time, while keeping constant or even increasing the yield (up to 19% for the 1h extraction) with respect to the conventional protocol. Interestingly, the presence of proteins and polyphenols conferred the semi-refined extracts a relatively high antioxidant capacity (19-24 μmol TE/g extract). The refined extract produced by the standard protocol formed the strongest hydrogels (>1000 g/cm^2). On the other hand, the semi-refined extracts produced by the alternative protocols formed slightly stronger hydrogels (337-438 g/cm^2) than the refined counterparts (224-311 g/cm^2), due to their greater molecular weights of the former ones. LCA assessment showed lower global warming potential for the semi-refined extracts, especially the ultrasound-assisted extraction, hence highlighting the potential of this method to produce more sustainable agar-based extracts for food-related applications.

Keywords: seaweed; *Gelidium sesquipedale*; ultrasounds**;** microwaves; phycocolloids; antioxidant capacity

1. Introduction

Agar is a complex mixture of polysaccharides extracted from red marine seaweeds (*Rhodophyta*) which has been used by humans in the far East for more than 300 years [1]. Industrially, agar is mainly extracted from *Gracilaria spp.* (53%) and *Gelidium spp.* (44%) [2]. Because of its unique physicochemical properties, such as gelling, thickening, stabilizing, cryoprotective effects, high biodegradability and large water holding capacity, agar is broadly used in food, pharmaceutical, cosmetic, biotechnological and even biomedical applications [3]. In fact, it is a high-value phycocolloid with a market size of USD 173 million in 2019 [2].

Agarose is the idealized structure of agar [4], which consists of repeating units of agarobiose or $(LA\text{-}G)_n$ [5], alternating β-D-galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl groups. However, this backbone is in general masked by substituent groups such as sulphate esters, methyl ethers or pyruvate acid ketals [6], which most often reduce the gelling ability and influence gelling temperature and melting behaviour. Agar gel properties, in terms of gel strength and gelling temperature are influenced by the chemical substituents, as well as by the molecular weight of the polysaccharides, which all depend on the seaweed species, environmental conditions, physiological factors and extraction and isolation methods [7]. The extraction procedure for agar is somewhat dependent on the specific seaweed species, but generally consists of an alkali pre-treatment followed by hot-water extraction at temperatures between 90–120 °C. In order to maximise agar yield, for commercial production pressurized vessels are used. These traditional multi-step procedures involve long extraction times, high energy and water consumption and the use of simple chemicals like acid and alkali. Specifically, the alkali treatment leads to the elimination of alkali labile 6 sulphate in the native agar, leading to an increased content of 3,6 anhydro L-galactose

(LA) and gel strength. The final agar yield and chemical characteristics will depend on the pre-treatment (alkali concentration, temperature and duration) and the subsequent extraction of the agar (time temperature and mixing). High alkali concentration may lead to severe depolymerisation of the polysaccharide chains and affect the extractability, yield and properties of the agar [7], but in general promote a more pure product. Many research studies have been focused on optimising agar extraction procedures from different seaweed sources, such as the works from Al-Nahdi and co-workers [8] or that of Meena et al. [9].

Recently, novel eco-friendly extraction technologies, such as ultrasound-assisted extraction [10,11], microwave-assisted extraction [12] , surfactant-induced coagulation [13] or enzyme-assisted extraction [14], have been explored with terrestrial and marine biomass, which may have economical, operational and environmental advantages (potentially reducing chemicals usage and improving extraction yields), but that need to be selected as a function of the target compound to be obtained and also taking into consideration the nature of the raw material [15]. However, their commercial use for obtaining seaweed phycocolloids is still limited [16] and there are only a few laboratory studies related with agar extraction [17–20]. In a recent study [18], simplified procedures for obtaining agar-based extracts, based in the combination of heat and sonication were reported, significantly reducing extraction time without affecting yield or extract properties compared to a hot water extraction. Moreover, omission of the alkali pre-treatment gave rise to agar-rich extracts containing more sulphate, proteins, polyphenols and minerals which, although resulted in weaker gels, had additional functionalities like antioxidant capacity, thus opening new application possibilities of these new materials.

Despite the potential of these new alternative extraction protocols, their sustainability (among other factors such as economic viability) needs to be studied before they can be industrially up-scaled. Sustainability is a key driver for the development of new products and processes, thus it needs to be evaluated at early development stages through environmental assessments such as life cycle assessment (LCA). LCA provides a better understanding of factors with environmental impact and avoid sub-optimization and shifting of environmental burdens either between inputs or life-cycle stages. Also, by having this understanding at early development stages, lock-in effects that can occur at later stages are avoided [21,22].

In this work, a comparison between water extraction at the lower temperature of 90 °C, combined with novel extraction techniques (ultrasound- and microwave-assisted extraction) for obtaining agar-based extracts with different purification degrees, has been carried out. The composition, thermal properties, molecular weight and rheological properties (viscosity and hydrogel strength) of the different extracts obtained were analysed. Additionally, an environmental assessment based on life-cycle methodology was carried out for the different extraction methods. The aim of it was to assess the environmental performance of the different extraction methods and identify environmental hot-spots that will be important to address when methodologies are further developed.

2. Materials and methods

2.1 Materials

Dried *Gelidium sesquipedale* was kindly donated by Hispanagar (Burgos, Spain). The dry seaweed was washed using tap water to remove sand and contaminants and was then placed in an oven at 60°C overnight. The dry seaweed was then ground into a fine powder using a

Christy and Norris Hammer Mill (Chelmsford, UK) and passed through a sieve to obtain particles of diameter <1 mm. The obtained material was vacuum-packed and stored at 4°C.

2.2 Agar extraction

Agar-based extracts were produced from ground *Gelidium sesquipedale* seaweed and only partly based on the industrial process, which consists of an alkaline pre-treatment of whole dried seaweed followed by a hot water extraction at 120 °C. Our standard protocol is based on an extraction of the dry and milled seaweed at 90°C with and without an alkali pre-treatment (see below), yielding refined and semi-refined agar extracts respectively. Alternative protocols based on ultrasound-assisted and microwave-assisted extractions were also tested to compare their performances with our standard protocol.

For the alkaline pre-treatment, 40g of dry ground *Gelidium sesquipedale* was soaked in 1 L of 5% (w/v) NaOH at 80°C for 1.5h. After that, the mixture was filtered using muslin cloth to separate the solid fraction from the supernatant solution. The solid fraction was repeatedly washed using tap water to remove the residual NaOH, until the pH value of the filtrate reached a value of 7-8. This washed algal material was then used for extraction following the corresponding agar extraction protocol. The alkali extracts and the following washing liquid was not collected.

For the extraction of agar, 40 g of the seaweed material (the solid fraction obtained after the alkaline pre-treatment in the case of the refined extracts, and dry ground *Gelidium sesquipedale* in the case of the semi-refined extracts) were soaked in 1 L of distilled water at 90°C. The extraction step consisted of: (1) heating the mixture at 90°C in an oil bath for 4h with stirring at 240 rpm (samples coded as “control refined” and “control semi-refined”); (2) an ultrasound-assisted extraction was carried out in a jacket-wall container with

temperature control at 90°C using an ultrasound probe (UIP500hd transducer, Hielscher ultrasound technology, Germany) with 100% power at 25 kHz for 30 min (samples coded as "refined+US 30min" and "semi-refined+US 30min") or 1 h (samples coded as "refined+US 1h" and "semi-refined+US 1h"); (3) a microwave-assisted extraction using a lab-designed microwave system (cf. Figure S1) with 100% power of 480 W for 12 min of treatment which was divided in 8 sections, with every 1.5 min in one treatment section. The treatment included 2 sections (3 min) of pre-heating time to reach >90°C and 6 sections (9 min) of extraction time at ca. 95-100°C (samples coded as "refined+MW" and "semi-refined+MW").

After the extraction processes, the agar-containing solutions were separated from the solid residues by filtration using muslin cloth when the solutions were still hot. The filtrates were collected in aluminium trays and cooled down to room temperature (~ 20°C) until gels were formed, and subsequently frozen overnight at -21ºC. Then, the materials were subjected to freeze-thaw (-21ºC/25ºC) to remove water-soluble impurities and then the obtained gels were frozen once more and dried using a freeze-dryer (FD 80 model, Cuddon Engineering, New Zealand) for 36 h.

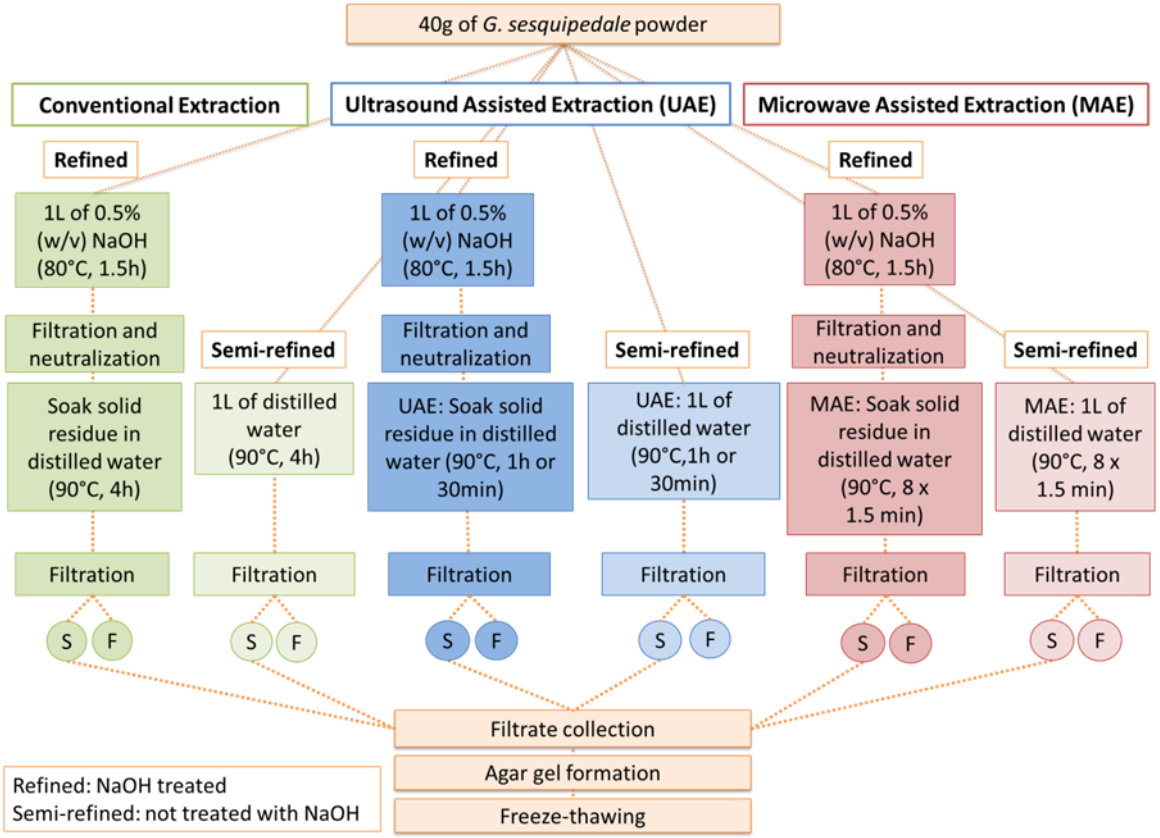


Figure 1. Schematic workflow of the different extraction protocols applied to obtain the *Gelidium sesquipedale* agar-based extracts (S: solid algal residue; F: filtrate).

2.3 Compositional analysis of the agar-based extracts

Carbohydrate composition via reductive hydrolysis

A procedure adapted from Quemener and Lahaye [23] and previously described [24] was followed using reductive hydrolysis followed by analysis of corresponding alditol acetates by GC-FID-MS. Typically, 10 mg of sample were dissolved in 5 mL of aqueous myo-inositol (0.5 mg/mL) by heating at 95 °C for 45 min. After cooling to 50 °C, 500 µL aliquots were evaporated to dryness at 50 °C in a stream of nitrogen. A pre-hydrolysis was initiated by adding 50 µL of morpholine borane (MMB) (80 mg/ml) and 200 µL of 3 M trifluoroacetic acid (TFA) and heating at 80 °C for 30 min. After cooling, 50 µL of MMB was added and the solution evaporated to dryness again. The main hydrolysis was then performed by adding

200 μL of 2 M TFA at incubating at 120 °C for 1 h. Samples were then cooled, 100 μL of MMB added, and evaporated to dryness again. After adding 500 μL of acetonitrile, the samples were evaporated to dryness and acetylation was performed. The detailed description of the protocol can be found in previous work [24].

Sulphur and nitrogen analysis

Sulphur and nitrogen contents were measured by elemental analysis via total combustion using a Vario-EL-cube elemental analyser. Values for sulphur are reported directly as % of dry weight or as degree of sulphate substitution of the idealized neoagarobiose backbone $(LA\text{-}G)_n$, using the formula DS= 4.5 x (S%/C%) as proposed by Melo et al. (Melo, Feitosa, Freitas, & De Paula, 2002). The measured sulphur content for $(DA\text{-}G4S)_2$ was 7.76 % with a corresponding calculated DS of 1.00 which correlates to one sulphate group per neocarribiose dimer. Crude protein was estimated by the formula %N x 6.25.

Phenolic content

Total phenolic content was estimated by the Folin-Ciocalteau colorimetric assay [25]. Briefly, Folin-Ciocalteau reagent was diluted 1:10 with distilled water and 1 mL of the final dilution was mixed with 0.2 mL of the sample (extract suspensions in distilled water at a concentration of 5 mg/mL, previously prepared by heating the samples to 95ºC) at room temperature. Finally, 0.8 mL of sodium carbonate (75 mg/mL) were added and the samples were heated up to 50ºC during 30 minutes. Absorbance values were read at 750 nm. A calibration curve was built using gallic acid as the standard, and the total phenolic content was expressed as mg of gallic acid (GA)/g extract. All determinations were carried out in triplicate.

2.4 $ABTS^{\cdot+}$ radical cation scavenging activity

The $ABTS^{\cdot+}$ radical cation scavenging activity of the agar-based extracts was determined according to [26]. Briefly, 0.192 g of ABTS were dissolved in 50 mL of PBS at pH 7.4 and mixed with 0.033 g of potassium persulfate overnight in the dark to yield the $ABTS^{\cdot+}$ radical cation. Prior to use in the assay, the $ABTS^{\cdot+}$ was diluted with PBS for an initial absorbance of ~0.700 ± 0.02 (1:50 ratio) at 734 nm, at room temperature. Free radical scavenging activity was assessed by mixing 1.0 mL diluted $ABTS^{\cdot+}$ with 10 µL of agar-based extracts (suspensions of 5 mg extract/mL water) and monitoring the change in absorbance at 6 minutes. A calibration curve was developed by using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox). The antioxidant capacity was expressed as mg Trolox equivalents (TE)/g extract. All determinations were carried out in triplicate.

2.5 Weight average molecular weight and intrinsic viscosity

A similar procedure to that reported by Rochas & Lahaye [27], with some modifications, as described in [24], was followed using size-exclusion chromatography followed by multi-angle light scattering, viscometry and refractive index detection. To 10-20 mg samples, 0.1 M $NaNO_3$ containing 0.02 % NaN_3 was added to make a concentration of 1 mg/mL. Tubes were kept in a boiling water bath for 20-30 min, until tube contents visually dissolved. Aliquots of the samples were then centrifuged while still hot at 13300 rpm for 10 min. 1 mL of supernatant was transferred to new tubes and again left in a boiling water bath for 1-2 min. Samples were then transferred to pre-heated vials and the sample tray kept at 95 °C. 100 µL of the unfiltered sample was injected via an autosampler into the chromatography equipment. A complete description of the experimental setup can be found elsewhere [24].

2.6 Thermogravimetric analyses (TGA)

The thermal stability of the agar-based extracts was studied by means of TGA analyses using a Perkin Elmer Pyris 1 thermogravimetric analyser (USA). Ca. 2 mg of each sample were placed in a platinum pan and heated from 30 °C to 800 °C at a rate of 10 °C/min under nitrogen atmosphere with a nitrogen flow of 20 ml/min.

2.7 Differential Scanning Calorimetry (DSC)

The thermal transitions of the agar-based extracts upon heating were studied by DSC analyses. The freeze-dried agar samples were analysed using a Perkin Elmer Pyris 4000 (USA) operating under a flowing nitrogen atmosphere. The machine was calibrated using an indium standard. The samples (~ 3 mg) were sealed in aluminium pans and heated from −50 to 200 °C at a rate of 10 °C/min. An empty aluminium pan with lid was used as the reference sample.

2.8 Hydrogel strength

Dispersions from the agar-based extracts were prepared at a concentration of 1.5% (w/w) in distilled water. To solubilize the agar-based extracts, the required amount of freeze-dried sample was added to distilled water and heated to ca. 95 ºC for 45 min. The hot solutions were transferred to methacrylate moulds (18 mm diameter and 10 mm depth) and were cooled to 25 ºC overnight to obtain disk-like hydrogel specimens. These were removed from the moulds and strength was measured at room temperature (20–25 °C) through penetration tests in a texture analyser (Stable Micro Systems model TA-XT2, Surrey, UK) equipped with a cylindrical Teflon plunger (1 cm diameter) and operating at a penetration rate of 1 mm/s to a depth of 5 mm. All measurements were performed, at least, in triplicate.

2.9 Statistics

All data have been represented as the average ± standard deviation. Different letters show significant differences both in tables and graphs ($p \leq 0.05$). Analysis of variance (ANOVA) followed by a Tukey-test were used.

2.10 Life cycle analysis (LCA)

The goal of the environmental assessment is to assess and compare the environmental performance of the extraction methods developed and described in this paper and to identify environmental hot-spots and possibilities for optimization. At this early development stage, the processes can only be compared among each other, not with commercial agar or other processes for agar extraction that are currently being developed. The assessment is based on the methodology for life cycle assessment that is described by ISO ("Environmental management – Life Cycle Assessment – Principles and framework," 2006; "Environmental management – Life Cycle Assessment – Requirements and guidelines," 2006). The functional unit is 1kg of pure and dry agar powder. The only environmental impact category included is Global Warming Potential (GWP) modelled according to IPCC 2013 [28] and expressed in CO_2-equivalents/FU.

The environmental assessment focuses on the extraction of agar. It is a gate-to-gate assessment in which environmental impacts caused by the production of raw material (*Gelidium sesquipedale* biomass) as well as the use of the agar are excluded. In the base case extraction is assumed to take place in Spain. A sensitivity analysis was carried out in which production in Ireland and Norway was also considered. *Gelidium* is assumed to be landed in southern Spain and then transported by lorry to the respective locations. The studied system is shown in Figure S2.

The extraction processes are modelled according to the methods described in section 2.2. Yields are based on the data given in Tables 1 and 2. All environmental impact is allocated to the agar and none to the biomass residue even though this could potentially be converted into additional products or bioenergy. Materials and energy used in extraction processes are included but waste-water treatment and solid waste management were not included at this early stage. Background data is extracted from the Ecoinvent database (version 3) [29]. Average electricity mixes for Spain, Norway and Ireland were used in the respective cases.

3. Results and Discussion

3.1 Extraction yield and composition of the agar-based extracts

Different extraction protocols, partially based on the industrial process and alternative ultrasound- and microwave-assisted extractions, with and without the application of an alkaline pre-treatment, were applied for the production of agar-based extracts from *Gelidium sesquipedale* and the composition and extraction yields were determined (cf. Table 1). As observed, the extraction yields for the refined extracts (those obtained with the application of the alkaline pre-treatment) were within the range of 2-5% for all the tested methods. These yields are quite low as compared to the values reported in the literature for several agarophytes, which can be as high as 35% for certain *Gelidium* species [30–35]. However, a direct comparison of the yields reported in this work with those available in the literature would not be appropriate due to the large variability in the extraction parameters (extraction time, temperature and water-to-seaweed ratio, particle size of seaweed) and the equipment used to perform the extractions and washing/filtration steps. On the other hand, compositional differences due to the seaweed species and growth/harvesting conditions should be less pronounced. A previous work reported even lower extraction yields (2-3%) for the agar extracted from the same *Gelidium sesquipedale* seaweed using similar extraction

and recovery conditions [18]. In that case a more concentrated alkaline pre-treatment was performed (1 g seaweed/10 mL of 10% (w/v) NaOH solution at 90ºC for 2h) and the extraction time was shorter (2h). Despite the differences in the extraction protocols, the low yields here reported indicate that the conditions for the alkaline pre-treatment and the extraction step should be optimized prior to their potential industrial application. In particular, the conditions for the alkaline pre-treatment have been suggested to be critical for maximizing the recovery of agar [18,36,37]. The application of an alkaline pre-treatment is typically done in the industry to soften the seaweed cell walls, aiding the extraction of agar in the subsequent extraction step, to remove non-agar components and, most crucially, to remove alkali labile sulphate with a corresponding increase of 3,6 anhydro L-galactose content, hence, considerably enhancing the gel strength and quality of the agar. However, if too harsh conditions are applied, chain scission occurs in parallel with the formation of 3,6-anhydro-L-galactose [38,39] and mechanically losses in the alkali solution, as well as during the recovery of the agar gel in the thawing proces, can take place. The degraded agar fraction diffuses towards the aqueous medium (which is discarded after the alkaline pre-treatment) thus significantly reducing the extraction yield [24,40,41]. The optimal conditions for the alkaline pre-treatment are very different depending on the seaweed species. For instance, while the application of a 1N NaOH pre-treatment reduced the extraction yield ca. 13% for *Pterocladia capillace* seaweed, a marked reduction of ca. 50-70% was reported for *Gelidium serrulatu* [31]. Similarly, our results indicate that partial degradation or loss of agar must had taken place during the alkaline pre-treatment, markedly reducing the extraction yields of the refined extracts with respect to the semi-refined extracts. It should be noted that amongst the semi-refined extracts, the microwave-assisted extraction provided the lowest yields; on the contrary, the ultrasound-assisted extraction showed promising results, achieving even higher yields (ca. 19% for the 1h extraction) than the standard extraction

protocol (ca. 14%) with a 4-fold reduction in the extraction time. This result is in line with previous works which already highlighted the potential of the ultrasound-assisted extraction of agar-based fractions [24]. Notably, the yield seems to increase when increasing the extraction time, suggesting that the productivity could be further enhanced if the extraction conditions are optimised, including an increased stirring and extraction temperature.

As deduced from Table 1, all the extracts were mainly composed of polysaccharides, ranging from ca. 52% for the semi-refined+US 1h extract to ca. 70% for the control refined extract. Although the differences between the extracts were not statistically significant, there was an apparent trend of increased total carbohydrate content in the refined extracts as compared with the semi-refined ones. The protein content was consistently greater in the semi-refined extracts, which was not unexpected since omission of the alkaline pre-treatment is known to increase the amount of non-agar components in the extracts [18,24]. In particular, ultrasound seems to promote the extraction of proteins, which is also in agreement with previous works [18,24]. Note that the conversion factor of 6.25, used to estimate the protein content from the nitrogen analyses, may lead to overestimated values [42]. To obtain more accurate numbers, the protein conversion factor for the *Gelidium sesquipedale* species should be determined. Previous work has demonstrated the presence of polyphenols in non-purified agar-based extracts [18], which may confer them interesting antioxidant properties [43,44]. The extracts obtained in this work presented low contents of polyphenols, which were slightly higher in the semi-refined samples. Even though the polyphenol content was not dramatically affected by the extraction method, the antioxidant capacity of the extracts was clearly higher in the semi-refined extracts. It has been previously suggested that the antioxidant capacity of water-soluble extracts from red seaweeds is not only determined by their polyphenol content [18,45], but it seems to be mostly related to the presence of other

bioactive components such as sulphated polysaccharides, peptides and amino acids [46,47]. In fact, lower antioxidant capacities have been previously reported for the aqueous extracts from *Gelidium pusillum* and *Gelidium corneum* seaweed (< 4 μmol TE/g extract) [48], which in turn contained greater amounts of phenolic compounds (3.5- 6.5 mg GA/g extract) than the extracts characterized in this work. These results evidence the potential of the semi-refined extracts to be used as bioactive ingredients, which could be of interest within the food and pharma industries.

Table 1. Extraction yields, basic composition and antioxidant capacity of the *Gelidium sesquipedale* agar-based extracts. Data shown as mean +/-SD, n=3.

	Extraction yield (%)	Carbohydrate* (%)	Protein (%)	Polyphenols (mg GAE/g extract)	Antioxidant capacity ABTS (μmol TE/g extract)
Control Refined	4.8 ± 0.9 ^{a}	69.7 ± 7.5 ^{b}	2.6 ± 0.0 ^{e}	0.6 ± 0.2 ^{a}	7.9 ± 0.6 ^{a}
Control Semi-refined	13.5 ± 2.5 ^{bc}	63.3 ± 0.1 ^{ab}	8.7 ± 0.4 ^{d}	3.4 ± 0.4 ^{c}	23.2 ± 1.9 ^{d}
Refined+US 30min	2.1 ± 0.2 ^{a}	68.4 ± 0.8 ^{b}	5.0 ± 0.0 ^{de}	2.9 ± 0.3 ^{bc}	15.0 ± 1.8 ^{b}
Semi-refined+US 30min	10.9 ± 0.5 ^{b}	59.4 ± 0.2 ^{ab}	14.5 ± 1.7 ^{b}	2.9 ± 0.5 ^{bc}	19.4 ± 1.0 ^{c}
Refined+US 1h	4.5 ± 1.0 ^{a}	57.8 ± 0.6 ^{ab}	9.8 ± 0.3 ^{cd}	2.8 ± 0.2 ^{bc}	17.5 ± 1.0 ^{bc}
Semi-refined+US 1h	18.5 ± 2.4 ^{c}	52.2 ± 2.6 ^{a}	17.8 ± 0.1 ^{a}	3.8 ± 0.5 ^{c}	20.4 ± 1.7 ^{cd}
Refined+MW	1.7 ± 0.2 ^{a}	63.4 ± 3.8 ^{ab}	7.3 ± 1.1 ^{d}	1.6 ± 0.4 ^{ab}	8.6 ± 0.3 ^{a}
Semi-refined+MW	6.8 ± 1.6 ^{ab}	59.3 ± 3.0 ^{ab}	7.8 ± 0.4 ^{d}	4.1 ± 0.0 ^{c}	23.5 ± 1.3 ^{d}

Values with different letters are significantly different ($p \leq 0.05$). Data were analyzed by ANOVA followed by a Tukey-test.

*The total gross carbohydrates were estimated as the sum of the agar, glucose and xylose contents (cf. Table 2).

A detailed analysis of the carbohydrate composition in the different extracts was carried out by means of monosaccharide analyses and the results are compiled in Table 2. As expected,

the agar content in the refined extracts (representing ca. 92-95% of the total carbohydrate fraction) was significantly higher than in the corresponding semi-refined extracts (86-91% of the carbohydrate fraction). Interestingly, the semi-refined extracts obtained by ultrasound-assited extraction contained greater amounts of glucose, which can be ascribed to the presence of floridean starch [49]. Furthermore, the semi-refined extracts were slightly richer in xylose. The consistently greater sulphur content in the semi-refined extracts can be attributed not only to the sulphate substitution in the agar component, but also to the presence of other sulphated components such as proteins, which is indicated by a higher nitrogen content.

The agar yield for each of the extraction protocols was estimated based on the agar content and the overall extraction yield values. From the results it is clear that even though the application of the alkaline pre-treatment produces extracts with higher purity, the semi-refined methods lead to greater agar extraction yields for the same extraction time. In particular, the ultrasound-assisted extraction with a prolonged time (1h) produced the highest agar yield. These results suggest that it would be worth exploring further this route for the production of agar, since it is capable of producing greater yields with significantly reduced extraction times as compared to the standard protocol.

Table 2. Carbohydrate composition and degree of sulphate substitution (DS) of the *Gelidium sesquipedale* agar-based extracts. The results from the sugar constituents are expressed as g polysaccharide per 100 g dry weight sample. Data shown as mean +/-SD, n=3.

	LA	G	LA2M	G6M	Agar	Glc	Xyl	DS (%)	Agar extraction yield (%)
Control Refined	27.9 ± 3.1 ^{c}	34.9 ± 4.1 ^{b}	3.0 ± 0.4 ^{d}	0.4 ± 0.0 ^{a}	66.2 ± 7.6 ^{d}	2.7 ± 0.0 ^{a}	0.8 ± 0.1 ^{ab}	0.06 ± 0.00 ^{a}	3.2
Control Semi-refined	23.3 ± 0.4 ^{bc}	32.0 ± 0.3 ^{ab}	2.1 ± 0.1 ^{b}	0.3 ± 0.0 ^{a}	57.7 ± 0.2 ^{abcd}	4.5 ± 0.1 ^{b}	1.2 ± 0.0 ^{c}	0.19 ± 0.02 ^{bc}	7.8
Refined+US 30min	28.0 ± 0.6 ^{c}	33.9 ± 0.1 ^{b}	2.9 ± 0.1 ^{d}	0.5 ± 0.0 ^{a}	65.2 ± 0.4 ^{cd}	2.5 ± 0.4 ^{a}	0.7 ± 0.0 ^{a}	0.07 ± 0.01 ^{a}	1.4
Semi-refined+US 30min	20.9 ± 0.2 ^{ab}	28.9 ± 0.1 ^{ab}	1.8 ± 0.1 ^{ab}	0.5 ± 0.1 ^{a}	52.0 ± 0.3 ^{ab}	6.1 ± 0.4 ^{c}	1.3 ± 0.1 ^{c}	0.20 ± 0.02 ^{bc}	5.7
Refined+US 1h	21.4 ± 0.4 ^{ab}	29.5 ± 0.0 ^{ab}	2.2 ± 0.0 ^{bc}	0.3 ± 0.0 ^{a}	53.4 ± 0.4 ^{abc}	3.4 ± 0.1 ^{ab}	1.0 ± 0.1 ^{abc}	0.17 ± 0.00 ^{b}	2.4
Semi-refined+US 1h	17.6 ± 1.3 ^{a}	25.7 ± 0.5 ^{a}	1.4 ± 0.1 ^{a}	0.3 ± 0.0 ^{a}	45.0 ± 1.9 ^{a}	6.2 ± 0.8 ^{c}	1.1 ± 0.1 ^{bc}	0.23 ± 0.00 ^{c}	8.3
Refined+MW	25.4 ± 1.4 ^{bc}	31.4 ± 2.2 ^{ab}	2.8 ± 0.1 ^{cd}	0.3 ± 0.0 ^{a}	59.8 ± 3.5 ^{bcd}	2.8 ± 0.2 ^{a}	0.8 ± 0.0 ^{ab}	0.08 ± 0.02 ^{a}	1.0
Semi-refined+MW	20.8 ± 0.6 ^{ab}	30.9 ± 2.1 ^{ab}	1.7 ± 0.1 ^{ab}	0.3 ± 0.1 ^{a}	53.6 ± 3.0 ^{abcd}	4.3 ± 0.1 ^{b}	1.4 ± 0.1 ^{c}	0.24 ± 0.01 ^{c}	3.6

Values with different letters are significantly different ($p \leq 0.05$). Data were analyzed by ANOVA followed by a Tukey-test.

LA=3,6-anhydro-L-galactose; G=D-galactose; LA2M=2-O-methyl-3,6-anhydro-L-galactose; G6M=6-O-methyl-D-galactose; Glc=D-glucose; Xyl=xylose. Nomenclature is according to [6].

3.2 Molecular weight and hydrogel strength

The molecular weight of the different agar-based extracts, as well as their intrinsic vicosities are summarised in Table 3. Interestingly, the semi-refined extracts presented, in general, higher weight average molecular weight than the corresponding refined extracts. This effect was more obvious for the extracts produced by the ultrasound-assisted method. A previous work showed similar results for semi-refined agar-based extracts, which presented higher molecular weights than commercial and purified agars, as determined by SEC [18]. From the composition analyses (cf. Table 1) it can be hypothesised that the greater weight average molecular weight of the semi-refined extracts, especially those obtained from the ultrasound-assisted extractions, may be originated from a significant amount proteins being linked to the agar fraction. It should also be noted that the extracts obtained by the ultrasound-assisted protocols presented in general much greater polydispersity indexes than the other samples, indicating a great variability in the chain length of the molecules composing the extracts. Although the extraction protocol and the characteristics of the raw seaweed are determinant for the molecular weight of the extracted agar, as a reference, Mw of 265 kDa, significantly lower than that of the control refined extract, have been reported for the agar extracted from *Gelidium pulchellum* [50], while higher values of 1112 kDa and 781 kDa have been reported for the semi-refined and refined agar-based extracts from *Gelidium sesquipedale* produced by means of extraction protocols similar to the standard method applied in this work [18].

The intrinsic viscosity is known to be related to the molecular weight by the Mark-Houwink-Sakurada equation ($[\eta] \sim K \cdot M^{\alpha}$, where K and α are constants depending on the solvent, type of polymer and temperature of the system). Rochas & Lahaye estimated this relationship for agarose and agarose-type polysaccharides, obtaining the following equation $[\eta] = 0.07 \cdot M^{0.72}$ [27]. Figure 2 shows the log-log plot for the intrinsic viscosity versus the molecular

weight of the agar samples produced in this work. For comparison purposes, the data previously reported by Rochas & Lahaye, as well as the data corresponding to previous samples produced by our research group [24], were also plotted. As observed, while the samples produced by the different extraction procols without the application of sonication showed a similar behaviour to that reported by Rochas & Lahaye, the samples produced by the ultrasound-assisted protocols clearly showed a very different behaviour. The reason for this different behaviour is unknown, but it certainly points out towards the fact that the ultrasound treatment may be producing structural changes in the agar-based extracts.

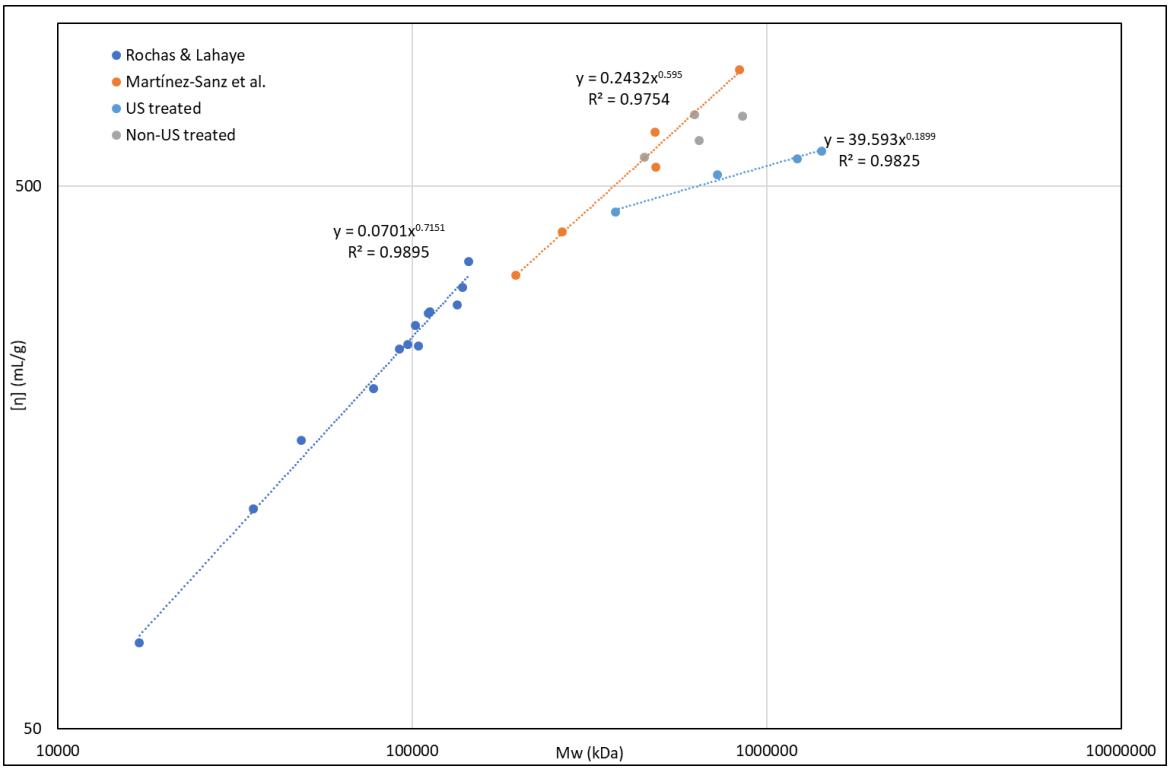


Figure 2. Double logarithmic plots of intrinsic viscosity versus molecular weight for the *Gelidium sesquipedale* agar-based extracts. Reference data from [27] and previous data from [24] have also been plotted for comparison purposes.

The strength of hydrogels from the different agar-based extracts was also determined to evaluate the effect of the composition and structure on their mechanical performance (cf. Table 3). As observed, the refined extract produced by the standard extraction protocol clearly produced much stronger hydrogels than the rest of the extracts. On the other hand, although the large standard deviations preclude from drawing definite conclusions, the hydrogels from the semi-refined extracts produced by the alternative protocols seemed to present slightly greater strength values than their corresponding refined extracts. The marked difference in the strength of the refined extract produced by the standard protocol with respect to the other refined extracts can only be explained by a combination of factors. In first place, the refined extracts produced by the alternative methods presented slightly lower agar contents than the control refined extract. The agar content has been shown to be a critical factor controlling the mechanical properties of agar-based hydrogels [24]. Fewer or smaller agar aggregates, responsible for holding the hydrogel network structure, are typically formed from extracts with lower agar contents, thus leading to softer hydrogels. Furthermore, these extracts presented either lower molecular weights than the control refined extracts or higher polydispersity indexes. Greater molecular weights have been seen to promote the formation of stronger and stiffer networks of agar double-helix aggregates [24]. The lower gel strength of the agar-based extracts produced by the alternative protocols would make them suitable for a different range of applications. Agar is mainly used within the microbiology and food industry sectors. For microbiology applications agars with high gelling strength are required to form a solid phase upon cooling; however, for food applications the requirements are broader depending on the final use. Softer gelling agars may be more suitable for their use as texturizing agents, thickening agents, stabilizing agents or even as matrices for the protection of bioactive components. Thus, the agar-based extracts produced by the alternative methods would be desirable as cheaper food ingredients, with

additional functionalities, for food related applications where high gel strength is not a constraint.

Interestingly, although marked differences were detected in the DS from the refined vs the semi-refined extracts produced by the alternative protocols (cf. Table 2), this did not have an impact on the hydrogel strength. This is in contrast with previous studies which claimed that stronger gels can be obtained from agars with lower sulphate content [51]. This is substantiated on the assumption that the gel strength of an agar depends on the three equatorial hydrogen atoms on the 3,6-anhydro-L-galactose residues, since these are critical for the formation of the double helices that initiate the formation of hydrogel networks [52]. Moreover, the reduced agar content of the semi-refined extracts did not have a negative effect either. Our results indicate that the greater molecular weight of the semi-refined extracts produced by the alternative methods was the controlling factor determining their slightly higher hydrogel strength as compared to the refined extracts. It should be highlighted that, despite the high variability of the samples, the semi-refined+US 1h extract presented the highest hydrogel strength amongst all the semi-refined extracts.

Table 3. Weight average molecular weight (Mw), number-average molecular weight (Mn), polydispersity index (PI), intrinsic viscosity ([η]) and gel strength of the *Gelidium sesquipedale* agar-based extracts. Data shown as mean +/-SD, n=3.

	Mw (kDa)	Mn (kDa)	PI (Mw/Mn)	[η] (mL/g)	Gel strength (g/cm^2)
Control Refined	645	244	2.6	607.2	1216 ± 341 [a]
Control Semi-refined	853	362	2.4	672.9	377 ± 136 [b]
Refined+US 30min	726	168	4.3	524.5	290 ± 115 [b]
Semi-refined+US 30min	1428	309	4.6	581.2	337 ± 85 [b]

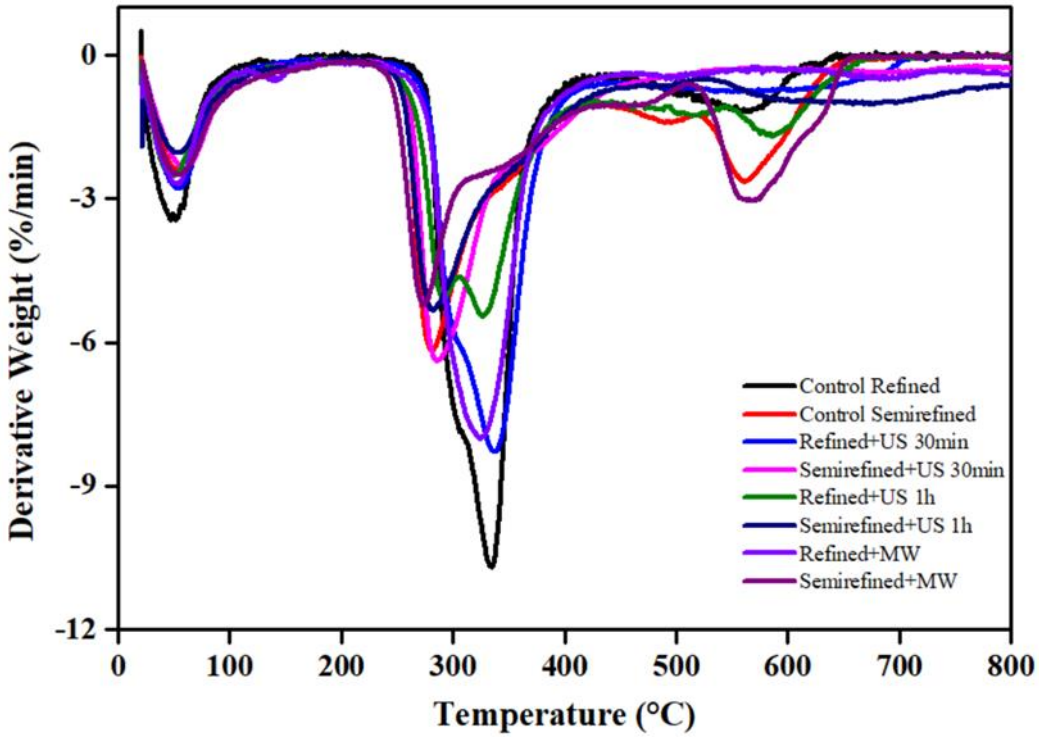
Refined+US 1h	375	266	1.4	447.9	311 ± 66 [b]
Semi-refined+US 1h	1219	287	4.2	562.2	438 ± 47 [b]
Refined+MW	627	219	2.9	678.5	224 ± 35 [b]
Semi-refined+MW	451	277	1.6	565.9	372 ± 158 [b]

3.3 Thermal properties of the agar-based extracts

The thermal properties of the agar-based extracts were studied by means of TGA and DSC analyses. All the extracts showed similar degradation patterns (cf. Figure 3A) characterized by a three-step degradation mechanism. The first weight loss occurred at temperatures close to 100ºC and corresponds to the desorption of water. The second degradation step, at 200-400ºC, corresponds to the thermal degradation of agar and other components such as proteins. In all of the refined extracts two different peaks, most likely indicating the presence of agar fractions with different molecular weights, were clearly discerned. On the contrary, the semi-refined extracts showed only one degradation peak. The maximum of the peaks for this degradation step (T_d) appeared at temperatures ranging from 259ºC to 295ºC (cf. Table 4). From the results compiled in Table 2, it seems that thermal stability was highly correlated to the agar content and DS of the extracts, i.e. those samples with greater agar content and lower DS presented greater thermal stability (as indicated by the higher T_d values). Thus, the refined extracts were more thermally stable than the semi-refined ones. A third degradation step occurred at temperatures higher than 400ºC, similarly to what has been reported for other agars extracted from *Gelidium sesquipedale* [53].

DSC analyses (cf. Figure 3B) evidenced an endothermic transition corresponding to the melting of agar, with the peak maximum (i.e. melting point (T_m)) located at 70-80ºC (cf. Table 4). Although the differences were not dramatic, the semi-refined extracts showed, in general, greater T_m than the corresponding refined extracts. This may be explained by the

higher molecular weight of the semi-refined extracts. On the contrary, the associated melting enthalpy (cf. Table 4) was higher for the refined extracts. This suggests that the lower agar content and greater molecular chain heterogeneity in the semi-refined extracts led to the formation of less crystalline or ordered structures, i.e. a reduced number of aggregates of agar double helices was originated. This is in line with a previous study which demonstrated that the presence of other components such as proteins in semi-refined agar-based extracts did not distort the formation of agar double helices and bundles, but it simply reduced the amount of these ordered structures [24]. In any case, it should be noted that all the extracts did not undergo thermal degradation at the temperatures required for their melting transition, which is crucial for their application to obtain gel-like products.



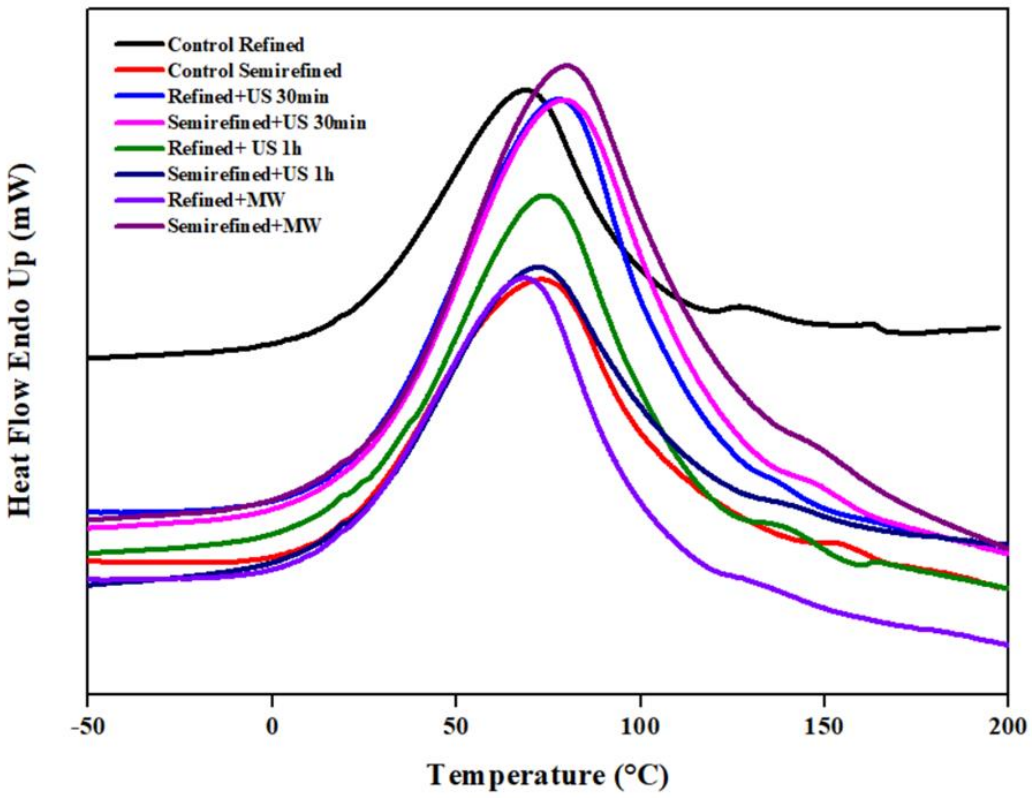


Figure 3. (A) Derivative thermogravimetric curves and (B) DSC thermograms of the *Gelidium sesquipedale* agar-based extracts.

Table 4. Thermal properties of the *Gelidium sesquipedale* agar-based extracts. T_d: temperature corresponding to the maximum of the second degradation step; T_m: Melting temperature; ΔH_m: Melting enthalpy. Data shown as mean +/-SD, n=2.

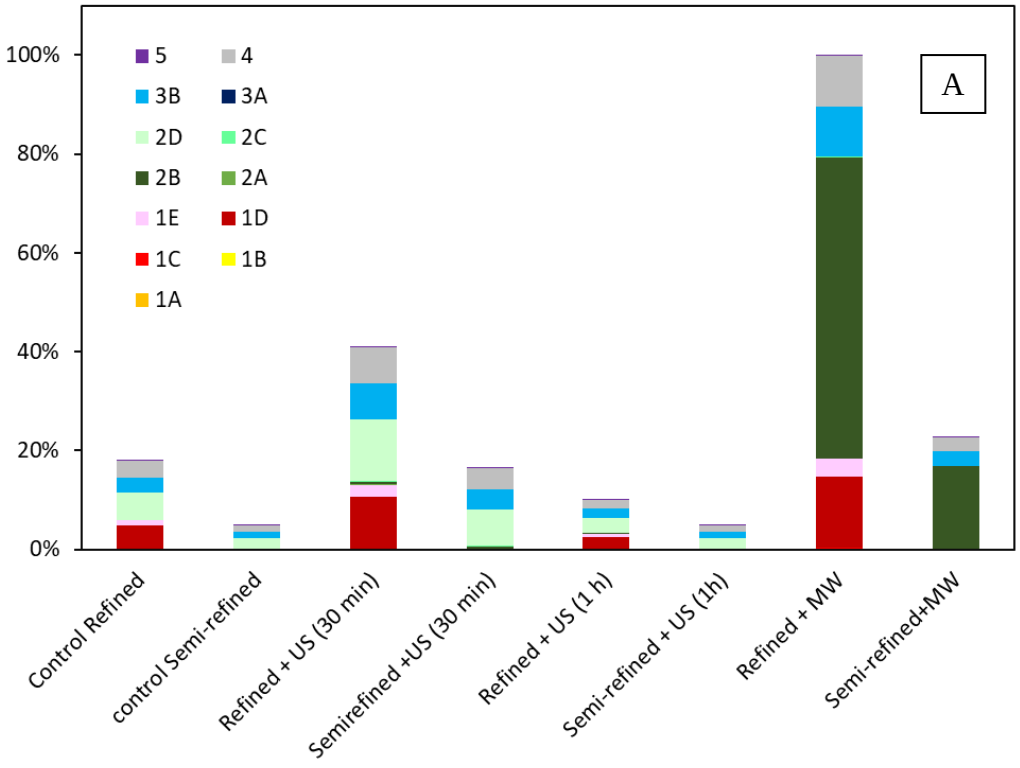
	T_d (ºC)	T_m (ºC)	ΔH_m (J/g)
Control Refined	294.7	68.8	562.5
Control Semi-refined	265.4	73.3	482.5
Refined+US 30min	295.1	77.7	469.8
Semi-refined+US 30min	267.1	79.3	463.4
Refined+US 1h	274.7	74.8	432.6
Semi-refined+US 1h	263.5	72.4	379.2
Refined+MW	288.3	69.0	486.1
Semi-refined+MW	259.0	79.8	427.1

3.4 Life Cycle Analysis (LCA)

To evaluate the environmental performance of the different extraction methods, a comparative life cycle assessment (LCA) was carried out. It is important to note that since the LCA results are based on data from research in lab-scale, they are not to be compared to the industrial-scale commercial agar production processes; instead, they are intended to be used as a guide to evaluate the potential of the alternative extraction methods to be used as more sustainable processes. As it is seen in Figure 4, there were large variations in the emissions caused by the different agar extraction methods. The most important factor was the yield of agar from the raw material. The yield of agar is directly correlated to the use of water, chemicals and energy required for the extraction. The semi-refined processes caused lower emissions of CO_2-eq than the refined processes. This was mainly due to avoiding one process step (i.e. the alkaline pre-treatment) while maintaining or even increasing the extraction yield. The electricity used in the processes was the input that gave the largest contribution to global warming. This was particularly important for the processes using the microwave technology, which consumes high amounts of electricity. Heat was also an important parameter, which in this study was produced from natural gas. Water, NaOH and transportation give minor contributions. However, despite not giving a large contribution to global warming, water use is an important issue to consider when developing processes for agar extraction. Availability of water may affect the decision for location of an agar-production facility.

We also studied the effect of location on the climate impact of the agar extraction processes. In addition to the base-case in Spain, production in Norway and Ireland were also considered in a sensitivity analysis (cf. Figure 4B). For all the cases it was determined to

be more beneficial to have production in Norway, despite the increased transport distance. The benefits were significantly larger for processes that consume large amounts of electricity, such as the microwave technology. For production in Norway, the transportation of the raw seaweed from Spain (which in this case was considered as the location where agar extraction takes place) would contribute to 20-30% of the total emissions of greenhouse gases. However, emissions from transportation could be reduced by switching from road transport to transport by boat or train.



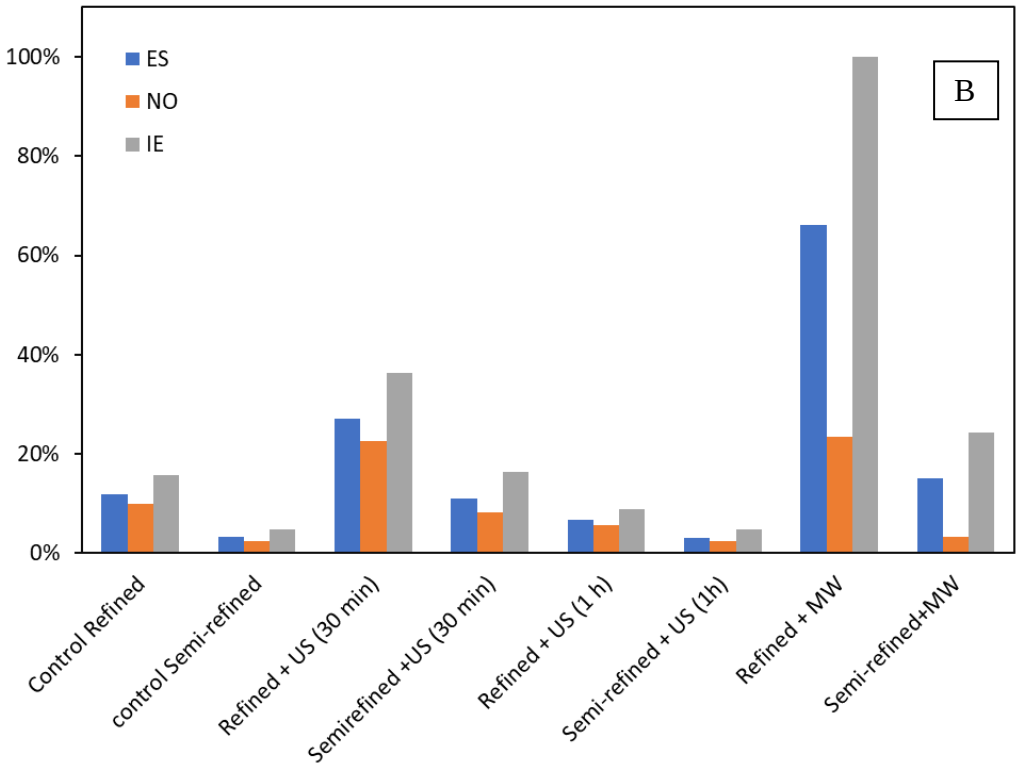


Figure 4. (A) Results from calculations of global warming potential for agar produced by the different extraction methods. Emissions are calculated as kg CO_2-eq/kg pure agar, normalized values are presented. 1A: Soaking and filtration - Filtration – Electricity; 1B: Soaking and filtration - Soaking – Electricity; 1C: Soaking and filtration - Soaking – Water; 1D: Soaking and filtration - Soaking – Heat; 1E: Soaking and filtration - Soaking - Sodium hydroxide; 2A: Extraction - Filtration – Electricity; 2B: Extraction - Soaking – Electricity; 2C: Extraction - Soaking – Water; 2D: Extraction - Soaking – Heat; 3A: Purification by freezing - Filtration/Pressing – Electricity; 3B: Purification by freezing - Freezing and thawing – Electricity; 4: Freeze-drying – Electricity; 5: Transport to factory. (B) Effect of location on the global warming potential. ES: Spain; NO: Norway; IE: Ireland.

4. Conclusions

Different extraction protocols, based on a hot water treatment at 90 ºC and alternative ultrasound- and microwave-assisted extractions, were applied for the production of agar-

based extracts from *Gelidium sesquipedale*. Higher purity extracts (refined) were produced by applying an alkaline pre-treatment, while less purified extracts (semi-refined) were obtained by omitting this step. All the extracts were mainly composed of polysaccharides (ca. 50-70%), with agar representing ca. 92-95% and 86-91% of the total carbohydrate in the refined and semi-refined extracts, respectively. Interestingly, the presence of proteins (8-18%) and other minor components such as polyphenols, conferred the semi-refined extracts a relatively high antioxidant capacity (19-24 μmol TE/g extract), highlighting their potential as bioactive ingredients. Despite the greater purity of the refined extracts, the semi-refined methods led to greater agar extraction yields. In particular, the ultrasound-assisted extraction showed promising results, providing higher yields than the standard extraction protocol (up to 19%), while reducing up to 4-fold the extraction time. Due to its greater purity and relatively high molecular weight, the refined extract produced by the standard extraction protocol was able to form much stronger hydrogels than the rest of the extracts. Finally, LCA assessment demonstrated the lower global warming potential of the semi-refined extracts, particularly the ultrasound-assisted extraction of 1h. Overall, the results from this work show the potential of the ultrasound-assisted method for the production of less purified agar-based extracts, with reduced environmental impact and presenting additional functionalities, such as antioxidant capacity, which may be of interest for prospective applications within the food industry.

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Statement of Informed Consent, Human/Animal Rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

CRediT Author Contributions Statement

Marta Martínez-Sanz: Conception and design, Analysis and interpretation of the data, Drafting of the article, Critical revision of the article for important intellectual content, Final approval of the article, Collection and assembly of data.

Laura Pilar Gomez-Barrio: Analysis and interpretation of the data, Drafting of the article, Critical revision of the article for important intellectual content, Final approval of the article, Collection and assembly of data.

Ming Zhao: Analysis and interpretation of the data, Final approval of the article, Collection and assembly of data.

Brijesh Tiwari: Conception and design, Critical revision of the article for important intellectual content, Final approval of the article, Obtaining of funding.

Svein Halvor Knutsen: Conception and design, Analysis and interpretation of the data, Critical revision of the article for important intellectual content, Final approval of the article, Obtaining of funding, Collection and assembly of data.

Simon Ballance: Conception and design, Analysis and interpretation of the data, Critical revision of the article for important intellectual content, Final approval of the article, Obtaining of funding, Collection and assembly of data.

Hanne Kristine Zobel: Analysis and interpretation of the data, Final approval of the article, Collection and assembly of data.

Anna Ekman Nilsson: Analysis and interpretation of the data, Critical revision of the article for important intellectual content, Final approval of the article, Obtaining of funding, Collection and assembly of data.

Christoffer Krewer: Analysis and interpretation of the data, Final approval of the article, Collection and assembly of data.

Karin Östergren: Analysis and interpretation of the data, Final approval of the article, Collection and assembly of data.

Amparo López-Rubio: Conception and design, Analysis and interpretation of the data, Critical revision of the article for important intellectual content, Final approval of the article, Obtaining of funding.

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Supplementary Material

Figure S1. Lab-designed microwave system used for the microwave-assisted extractions**.**

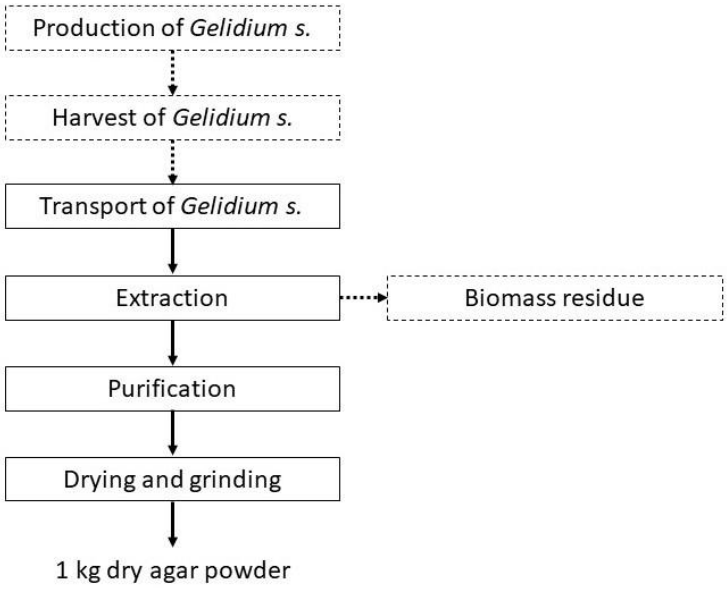

Figure S2. System boundaries for the environmental assessment. Processes in dashed frames are excluded from the assessment.