TÉCNICO LISBOA

Production and characterization of protein-rich extracts from the red macroalga *Gelidium sesquipedale* and its residues after industrial agar extraction

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I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at the Institute for Bioengineering and Biosciences of Instituto Superior Técnico (Lisbon, Portugal), and developed within the scope of the Smart Valorization of Macroalgae project (FA_05_2017_033) financed by Fundo Azul - Direcção Geral de Política do Mar, during the period March-July 2021, under the supervision of Doctor Maria Teresa Ferreira Cesário Smolders and Professor Marília Clemente Velez Mateus.

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Resumo

A macroalga vermelha *Gelidium sesquipedale* é uma espécie comercialmente explorada para a extração de agar. No âmbito de uma biorrefinaria e economia circular, a implementação de um processo em cascata, que permita valorizar as frações proteica e de agar, contribuindo para a criação de um processo "zero desperdício" seria de enorme importância.

O objetivo deste trabalho consiste em produzir extratos ricos em proteína - a partir da alga inteira bem como dos resíduos de alga que resultam da extração de agar – e caracterizar os extratos proteicos obtidos.

A implementação sequencial de duas extrações aquosas seguida de duas extrações alcalinas resultou numa recuperação de 12,0 ± 1,2% e 15,4 ± 0,9% da proteína total, da alga inteira e dos resíduos industriais, respetivamente. Duas extrações sequenciais assistidas pelas enzimas Celluclast® e Alcalase® seguidas por duas extrações alcalinas resultaram na recuperação de 52,1 ± 1,7% e 36,5 ± 1,3% para a alga inteira e os resíduos industriais, respetivamente. Ensaios *in vitro* de bioacessibilidade dos extratos proteicos, mostraram valores muito superiores aos da biomassa original. Os produtos proteicos obtidos revelaram um elevado conteúdo de hidratos de carbono (~30%). Diferentes métodos de precipitação de proteína foram testados e o rendimento de precipitação calculado – precipitação com sulfato de amónio, por alteração de pH e por adição de etanol. A precipitação por alteração de pH mostrou ser um método simples, rápido e com baixo consumo de reagentes. A técnica de cromatografia de troca aniónica, foi utilizada como primeira tentativa de purificação da fração proteica, demostrando resultados promissores.

Palavras-chave: *Gelidium sesquipedale,* extração de agar, extração proteica, caracterização de proteína, precipitação de proteína, fracionamento cromatográfico.

Abstract

The red macroalga *Gelidium sesquipedale* is a commercially exploited species for agar extraction. Implementing a cascade process, which enables the valorisation of protein and agar fractions and contributes to a “zero waste” process, would be of enormous importance in the context of a biorefinery and circular economy.

This work aims to produce protein-rich extracts from the crude alga and alga residues from the industrial agar extraction process and characterize the protein extracts obtained.

The sequential implementation of two aqueous extractions followed by two alkaline extractions resulted in the recovery of 12.0 ± 1.2% and 15.4 ± 0.9% of the total protein content from the crude alga and the industrial residues, respectively. Two sequential extractions assisted by the enzymes Celluclast® and Alcalase® followed by two alkaline extractions recovered 52.1 ± 1.7% and 36.5 ± 1.3% of the protein from the crude alga and the industrial residues, respectively. *In vitro* bioaccessibility assays showed significantly higher values in the protein extracts compared to the original biomass. The protein products revealed a high content of carbohydrates (~30%). Different protein precipitation methods were assessed – ammonium sulfate precipitation, pH-shift method, and ethanol precipitation. The pH-shift method is shown to be a simple, fast and low reagent consumption method. The anion exchange chromatography technique was used to fractionate proteins for further isolation and purification, showing promising results.

Keywords: *Gelidium sesquipedale,* agar extraction, protein extraction, protein characterization, chromatographic fractionation, protein precipitation.

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List of abbreviations

AA Amino acids

AAA: Amino Acid Analysis

ACE Angiotensin-converting enzymes

AEX Anion Exchange Chromatography

ANOVA a one-way analysis of variance

APC Allophycocyanin

APS Ammonium Persulfate

ASE Accelerated Solvent Extraction

BAPs Bioactive Peptides

CE Capillary Electrophoresis

DEAE Di-Ethyl-Amino-Ethyl

DTT Dithiothreitol

dw Dry Weight

EAA Essential Amino Acids

ESI Electrospray Ionization

FAO Food and Agriculture Organization

FDA Food and Drug Administration

FMOC Fluorenylmethoxy Chloroformate

FTIR Fourier Transform Infrared

GC Gas Chromatography

GP-HPLC Gel Permeation Chromatography in High-Performance mode

GPs Glycoproteins

HPAEC-PAD High-Performance Anion-Exchange chromatography with Pulsed Amperometric Detection

IEF Isoelectric Focusing

IEX Ion Exchange Chromatography

IMTA Integrated Multi-trophic Aquaculture

LAP Laboratory Analytical Procedure

LC Liquid Chromatography

MAAs Mycosporine-like amino acids

MAE Microwave-Assisted Extraction

MALDI-TOF Matrix Assisted Laser Desorption Ionizations/Time-Of-Flight

MS Mass spectrometry

MW Molecular Weight

NAC N-acetyl.L-cysteine

NEAA Non-Essential Amino acids

NF Nanofiltration

NMWCO Normal Molecular Weight cut-off

NREL National Renewable Energy Laboratory's

ODW Oven-dry weight

OPA Ortho Phtalaldehyde

PAR Photosynthetically Active Radiation

PBs Phycobiliproteins

PC Phycocyanin

PE Phycoerythrin

PEC Phycoerythrocyanins

PEFE Pulsed Electric Field Extraction

PLE Pressurized Liquid Extraction

PUFAs Polyunsaturated fatty acids

RO Reverse Osmosis

RP-HPLC Reversed-phase High-Performance Liquid Chromatography

RP-UPLC Reversed-phase Ultra Performance Liquid Chromatography

RT Room Temperature

RuBisCO Ribulose-1,5-bisphosphate carboxylase-oxygenase

SDS-PAGE Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SEC Size Exclusion Chromatography

TEMED Tetramethylethylenediamine

TMP Transmembrane Pressure

UAE Ultrasound-Assisted Extraction

UF Ultrafiltration

v/v volume/volume

w/w weigh/weigh

1. Project motivation and aim

1.1. Motivation

The world population is expected to reach 11.2 billion by 2100 [1], with food requirements estimated to be 70% higher than those in the present [2]. This rapid growth will be manifested in increased food and energy demand, leading to resource-depleting agriculture and fossil fuel exhaustion, with the catastrophic result of irreversible environmental damage [3]. With the awareness level higher than ever, there has been a growing interest in finding alternative protein and energy sources, aiming for a production system where no waste is created, contributing to a sustainable bioeconomy [4].

A biorefinery is described as the sustainable processing of biomass into a spectrum of marketable products and energy [5]. The primary goal of the biorefinery concept is to maximize the use of raw biomass, resulting in a longer lifespan of resources and a lower environmental impact. A cascading approach (Figure 1.1) has been developed to ensure the optimal use of biomass, which promotes the production of products following a decreasing order of value, with bioenergy and biofuels being produced with the leftovers from other production processes. When all the bioresources within the biorefinery structure are used efficiently, including biological leftovers, nearly zero waste is produced [4].

Due to their unique composition, macroalgae, commonly known as seaweed, are an excellent sustainable resource for biorefinery purposes [6], presenting various advantages compared to terrestrial biomass. These include rapid growth and photosynthetic efficiency, low nutrient demands, no competition for agricultural land, cultivation in salted water not requiring fertilization, and conversion to biofuels facilitated by the high carbohydrate and low lignin content exhibited [4].

Regarding protein content, macroalgae are considered a novel and promising source, with some species presenting similar or even higher contents than those in conventional protein sources, such as eggs, soybean, fish, and cereals [7]. In addition, they can exhibit higher protein yield per unit area (2.5-7.5 $t \cdot ha^{-1} \cdot year^{-1}$) when compared to terrestrial crops, such as soybean (0.6-1.2 $t \cdot ha^{-1} \cdot year^{-1}$), legumes grains (1-2 $t \cdot ha^{-1} \cdot year^{-1}$), and wheat (1.1 $t \cdot ha^{-1} \cdot year^{-1}$) [8]. Besides this, marine macroalgae-derived proteins and peptides have demonstrated additional value due to their nutraceutical, pharmaceutical, and cosmeceutical properties such as antioxidant, antihypertensive, immune-modulatory, and anticoagulant substances [9].

To this day, widespread use of seaweed is still hampered by several factors, including harvesting access and rights, the seasonality and geographical location of algae, and the lack of scalable production methods for components isolation from algae [10]. From a biorefinery point of view, seaweed biorefineries are less advanced than those based on terrestrial biomass. Due to the carbohydrate difference, terrestrial biomass-based technology cannot be directly applied to macroalgal biomass, requiring further study for the process design.

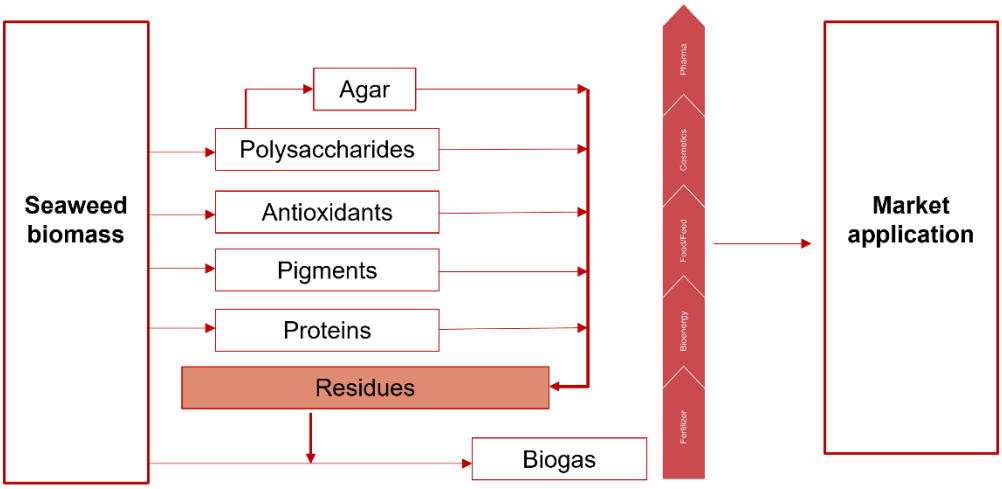

Figure 1.1: Schematic representation of a seaweed biorefinery concept (adapted from [4]*).*

1.2. Aim

Based on the work previously carried out by this research group regarding different strategies for protein extraction from the macroalga *Gelidium sesquipedale* [11], the two most promising approaches were chosen. One likely originates intact proteins, and the other, assisted by enzymes, also originates peptides due to the protease activity. In the scope of a biorefinery concept, the same protein extraction procedures were performed with the residues of *Gelidium* after industrial agar extraction, aiming for a zero-waste process. The resulting extracts were enriched and characterized in terms of composition and molecular weight profile. Bioaccessibility assays were performed by the partner *Instituto Português do Mar e da Atmosfera* (IPMA) to evaluate their possible use in commercial aquafeed. In addition, alternative methods for protein precipitation and purification were also designed to obtain an improved recovery and isolation of the protein fraction.

2. Literature Review

2.1. Macroalgae

2.1.1. Taxonomy and characterization

Macroalgae are a diverse group of multicellular photosynthetic organisms of leaf-like thallus structure and are classified as green, red, and brown based on the colour of their thallus, derived from natural pigments and chlorophylls [10]. In contrast to land plants, which all shared a common ancestor, algal diversity comprises several distantly related groups of photoautotrophic organisms predominantly inhabiting aquatic environments [12].

Green macroalgae are a diverse group of approximately 4500 species included in the *Chlorophyta* phylum and have the identical chlorophyll a/b ratio as terrestrial plants. In fact, it is believed that higher green terrestrial plants (*Embryophyta*) have evolved from this algae group [13].

Red algae belong to a single class – *Rhodophyta* – divided into two subclasses: Florideophycidae and Bangiophycidae. The pigments chlorophyll a, phycoerythrin, and phycocyanin are responsible for the red colour exhibited. There are around 4000–6000 red algae species in over 600 genera, most of which live in tropical marine environments [14].

Brown algae are classified as *Phaeophyceae* under phylum Chrysophyta, and their primary pigments are chlorophyll a and c, *β*-carotene and fucoxanthin. There are approximately 1500–2000 species of brown algae, and their colours, depending upon the proportion of brown pigment (fucoxanthin) to green pigment (chlorophyll), vary from dark brown to olive green [15].

Numerous conditions, including light, temperature, salinity, nutrient availability, pollution, and water turbulence, significantly impact macroalgae's pigment, growth, and chemical composition. Among these conditions, light is a decisive factor since it also conditions the localization of the alga. As macroalgae have their respective pigments that selectively absorb light with specific wavelengths, the classes of macroalgae are vertically distributed from the upper zone (sea surface) to the lower sublittoral zone depending on the pigments exhibited. For instance, with most macroalgae living near the coast, some red algae such as *Gelidium* sp. can inhabit the deep sea area with limited sunlight. This species efficiently absorbs light with wavelengths of photosynthetically active radiation (PAR) that can penetrate seawater to the deep zone due to the presence of phycoerythrin and phycocyanin pigments [16]. In addition, the biological status of algae, including life cycle and developmental stages and macroalgal thallus structure, influences the biochemical composition and, ultimately, the value of the algal source material [12].

As photoautotrophic organisms, macroalgae produce and store organic carbons through CO_2 or HCO_3^- uptake. Because the CO_2 diffusion rate in seawater is extremely low, most macroalgae directly uptake HCO_3^-. The photosynthetic rate greatly depends on the group and species. Green and red macroalgae generally present higher rates compared to brow algae (1-2 order of magnitude higher), and *Enteromorpha* sp (green) and *Porphyra* sp. (red) have the highest photosynthetic rates

known - 1786 and 1808.7 µmol CO_2 consumed/h, respectively [12]. Due to this potential, it is estimated that macroalgae cultivation along the coastline could entrap about 1 billion tons of carbon per year [17].

2.1.2. Sources of algal biomass – wild-harvested *vs* cultivated

The Food and Agriculture Organization of United Nations (FAO) [18] estimates that 1.1 million tons of wild algal stocks are harvested each year, with most biomass used in the phycocolloid and food industries. Due to growing global awareness and a larger pool of scientific evidence of the potential benefits and numerous applications in various sectors, there has been an increased demand for seaweed biomass. Between 1999 and 2009, harvested kelp biomass in Europe increased from about 5 000 tonnes to 30,500 tonnes, with more than 97% of seaweed biomass being harvested from the wild in Norway, France, and Ireland [19]. However, sustainable natural resource exploitation must consider the impact of harvesting from wild populations, which is only known and monitored in a few cases until this date. Also, the wild harvest of macroalgae faces a few other challenges, including the risk of contaminants in the water that may be adsorbed by macroalgae reducing their value or limiting their application, as well as the levels of variability in protein content due to seasons, environmental factors and harvesting location [12].

Therefore, in order to meet the demands, the amount of macroalgae mass-cultivated in the world has continuously increased over the last 10 years, at an average of 10%, rounding 20 million tons produced each year globally. Despite the large macroalgae diversity, with more than 20 000 species reported worldwide, cultivation is limited to around 30 species. Brown and red algae are far more commonly cultivated than green algae, and it has been observed that red algae production has significantly increased while brown algae production has stagnated. Seaweed production is mainly concentrated in Asia, with China being the primary producer, with an estimated annual yield of 13.5 million tons [18].

There are two main approaches for macroalgal cultivation: 1 – seaweeds can be grown on land in tanks or ponds, or 2 – in open sea coastal or offshore environments. Aside from careful site selection, offshore production, by definition, provides only limited opportunities for optimizing the algal growth and composition and other factors such as water quality, access, and conflict of interests of coastal usage [12].

Incorporating macroalgal cultivation in existing aquaculture systems (e.g., fish or mussel farms) has recently gained attention. However, due to the complexity of the various components, only a few fully commercial farms of such integrated multi-trophic aquaculture (IMTA) systems were reported until 2015, and most were in the pilot stage [12]. Under an IMTA approach, thalli from *Rhodymenia pesudopalmata* showed an increase in the protein content (18.7% dry weight (dw)) after 3 days of cultivation in ammonium-rich fishponds effluents than thalli from wild populations (9.4% dw), as well as a fivefold increase in mycosporine-like amino acids (MAAs) total content [20]. The cultivation of *Palmaria palmata* under various cultivation systems and nutrient treatments has also been assessed, suggesting to be a good candidate for IMTA due to its high affinity for NH_4^+, which resulted in a 20% increase in protein in the thalli [19].

It should also be noted that because seaweeds are frequently experiencing stressful conditions and highly fluctuating environments, most algae have mechanisms that allow them to adapt to stressors (e.g., UV radiation, temperature, and salinity) and defend themselves against biological pressures (e.g., competitors, grazers and parasites). This broad range tolerance, combined with their unique cellular structure, predisposes them to growth and development in laboratory and industrial settings [12].

2.1.3. Seaweed chemical composition

2.1.3.1. Carbohydrates

Carbohydrate is the most considerable fraction in macroalgae composition and can vary from 25-50%, 30-60%, and 30-50% dry weight (dw) for green, red and brown algae, respectively [21]. The exact composition varies between species.

Green algae usually contain polysaccharides in the form of starch (1,4-α-glucan). Some species have water-soluble ulvan and insoluble cellulose as a component of their cell walls [22]. Ulvan is characteristic of green algae and is mainly composed of D-glucuronic acid, D-xylose, L-rhamnose, and sulfate [23].

Regarding brown algae, alginic acid (i.e., alginate) is the major polysaccharide found in the cell wall, counting for 40% dw. Alginates are linear unbranched polysaccharides containing β-D-mannuronic acid (M) and α-L-guluronic acid (G) units linked by 1–4 glycosidic bonds. These monomers are mainly arranged in sequences of homopolymeric blocks (MM and GG blocks) and heteropolymeric blocks (MG or GM blocks). Alginates are currently industrially produced from brown seaweed of the genus *Laminaria, Saccharina, Lessonia, Macrocystis, Durvillaea, Ecklonia, and Ascophyllum*. Laminarin (*β*-1,3-glucans) is a unique polysaccharide, characteristic of brown algae, accounting for up to 35% dw, and its structure depends on the degree of branching and polymerization. This polysaccharide is present in high yields in *Laminaria* sp. (up to 32%, depending on the season) and *Saccharina* sp. These molecules are also described in *Ascophyllum*, *Fucus*, and *Undaria* sp. In addition, brown algae also contain fucoidan, glucose, and glyoxylic acid, albeit in smaller amounts [24].

Red algae's main polysaccharide constituents are galactans, such as carrageenan (up to 75% dw) and agar (up to 52% dw) (described in 2.6.1). Distinctly from green and brown algae, they contain floridean starch and floridoside, similar to general starch. Floridean starch is α-1,4-glucosidic linked glucose homopolymer that makes up 80% of the cell volume [25].

2.1.3.2. Lipids

In general, macroalgae present a low lipid content (1–5% dw). Nevertheless, the percentage of ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) in the lipid fraction is higher than that in terrestrial vegetables. PUFAs are known for their anti-hypercholesterolemic, antioxidant, anticancer, antidiabetic, antihypertensive, and anti-inflammatory properties [26].

2.1.3.3. Minerals

The mineral content in seaweed is generally high (8-40% dw), including essential and trace minerals, with high proportions of sulfate (1.3-5.9%), which correspond to elements required for human nutrition. Therefore, the ash content exhibited is also high (21-39% dw) compared to terrestrial plants (5-10% dw). Brown and red algae show the higher ash content (30-1-39.3% and 20.6-21.1% dw, respectively), suggesting their use as fertilizers in agriculture [27].

2.1.3.4. Proteins

In general, red algae present a higher protein content (20-27% dw) compared to green (9-26% dw) and brown (3-15% dw) [28]. These levels are comparable to those found in high-protein vegetables such as leafy greens and legumes (spinach – 26.5% dw, cauliflower – 29.9% dw, soybean – 35 to 40% dw, chickpea – 20 to 25% dw), and cereals (wheat – 8 to 15% dw, barley – 8 to 15% dw, rice – 7 to 9% dw, corn – 9 to 12% dw) [29]. The protein content of marine algae is also affected by the season. For instance, *Palmaria palmata* collected on the French Atlantic coast revealed that the protein content could range between 9 and 25% dw [7]. Protein levels were higher at the end of the winter and in the spring. Season variation in algal protein content has also been reported for other species, including *Laminaria digitata* and *Ulva lactuca* [30].

Algal protein digestibility

The fraction of ingested food components available at the target site of action is referred to as bioavailability. This concept entails the entire process that follows the consumption of a food element, including digestibility and solubility in the gastrointestinal tract, absorption/assimilation of the food element across intestinal epithelial cells and into the circulatory system, and finally incorporation into the target site of utilisation. *In vivo* experiments (animal and human studies) are thus required in studies examining the bioavailability of food elements [31] (Figure 2.1).

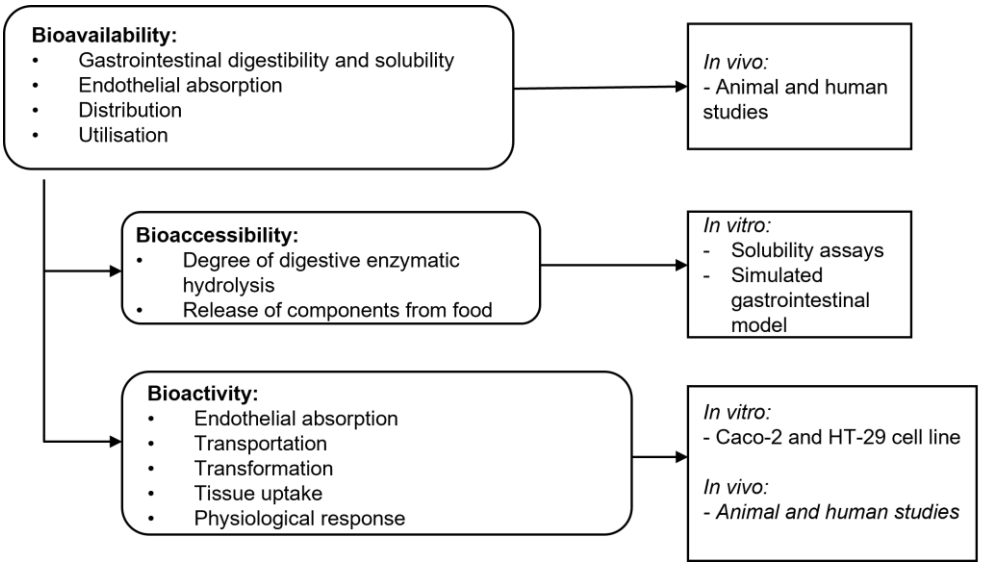

Figure 2.1: Schematic representation of bioavailability description and methods used for bioavailability, bioaccessibility and bioactivity assessment (adapted from [10]).

As shown in Figure 2.1, bioavailability can be subdivided into two stages: bioaccessibility and bioactivity. Most studies on algal protein digestibility have been conducted *in vitro* using proteins extracted under highly alkaline conditions, exposing them to enzyme or enzymatic mixture action such as pepsin, pancreatin, and pronase, and comparing it to casein digestibility (100%). The *in vitro* bioaccessibility of some seaweed species protein extracts has been reported. For example*, Porphyra tenera, Ulva pinnatifida*, and *Ulva pertusa* extracts presented bioaccessibility values of 78%, 87%, and 95%, respectively [7]. Greater *in vitro* digestibility of protein extracts from red seaweeds has also been stated (83%-87%) when compared to brown seaweeds (78.7%-82%) [32]. These results show that seaweed proteins have comparable digestibilities to commonly consumed plants, such as grains (69%–84%), legumes (72%–92%), fruits (72%–92%), and vegetables (68%–80%) [32].

Nevertheless, *in vitro* bioaccessibility studies indicate that unprocessed seaweed proteins are less digestible than other protein sources [33]. This digestibility inhibition is mainly due to high values of soluble fibres (17±33%) [34] - which can block the access of digestive enzymes and decrease the activity of proteolytic enzymes [36] - high polysaccharides content, and polyphenols or trypsin inhibitory compounds [46]. Therefore, treatments that disrupt the cell wall are mandatory to increase protein accessibility or digestibility [28].

Cell wall structure

Because most proteins are found intracellularly, their extraction is critical to their accessibility. Thus, the complex nature and rigidity of the macroalgal cell wall constitutes the main challenge for protein extraction. Polysaccharides are inducers of strong electrostatic interactions, and polyphenols can form reversible hydrogen bonds with proteins or oxidize to quinones, which can bind to proteins irreversibly [35]. Thus, the presence of polysaccharide-bound cell wall mucilage, such as anionic or neutral polysaccharides and polyphenols, reduces protein extractability and requires additional adapted

fractionation and purification steps [36]. When phenolic compounds are oxidized, they can also react to amino acids and form insoluble complexes [37].

Red algae's cell wall structure (Figure 2.2) is particularly complex and can be divided into the fibrillar wall, amorphous matrix, and glycoprotein domain [38]. The fibrillar polysaccharides and glycoprotein domains combine to form a reticulated cell wall embedded in the amorphous matrix [38]. The fibrillar wall is the most inert and resistant component of the cell wall and is mainly constituted by cellulose, with xylan, mannose, and hemicellulose being minor elements [39]. The glycoprotein domain is still little understood, but it is made of glycoproteins with "cellulose-binding domains" that promote polysaccharide fibre crosslinking [38]. The amorphous matrix comprises sulfated galactans, such as carrageenans and agarans, and typically extends to the intercellular spaces between adjacent cells [40] [38]. These polysaccharides are named phycocolloids due to their ability to form aqueous gels [41].

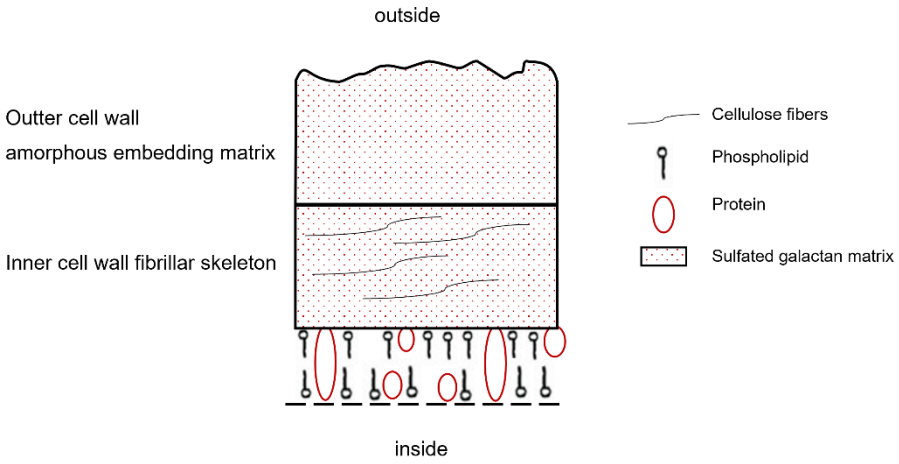

Figure 2.2: Cell wall structure of red algae (adapted from [42]).

Sulfated galactans are polymers composed of two different subunits: A - β-galactose residues with D-conformation and B - α-galactose residues with D and L conformation in carrageenan and agaran, respectively and both form a linear backbone of alternating 3-linked β-D-galactopyranose and 4-linked α-galactopyranose residues [43]. The latter can be present partially or entirely as 3,6-anhydro derivatives. These polysaccharides are frequently bound to sulfate hemiesters, methyl ethers, and pyruvic acid [41].

The number and position of sulfate groups, the presence of 3,6-anhydro-D-galactose, and the pyranosidic ring conformation defines carrageenans structure [25] (Figure 2.3 A). It is worth noting that red seaweed carrageenans can be chemically modified with one or more substitutions in the same molecule in different ratios, resulting in new carrageenans known as "hybrid carrageenans" [38].

Based on the number and position of anionic sulfate groups, approximately 15 types of carrageenans have been recognized, the most commercially important of which are (κ)-,(ι)-, and (λ) - carrageenan, due to their properties as gelling, stabilizing, and thickening agents in the food,

pharmaceutical and cosmetics industries [41]. Carrageenans are all water-soluble and insoluble in organic solvents, oils, and fats. Water solubility is greatly influenced by the number of sulfate groups and their associated cations present in the molecule, which increase hydrophilicity. Sodium, potassium, calcium, and magnesium are the most common cations in carrageenans, but other ions can occur at a lower frequency. The viscosity of solutions and gel strength are determined by the proportion of sulfate fractions and the equilibrium of cations in the water solution [44].

Agarans are the main components of marine red algae and make up the hot water-soluble portion of the cell wall. These polysaccharides are primarily synthesized by red seaweeds of the genera *Pyropia, Gelidium, Gracilaria*, and *Pterocladia* [45]. The presence of substituent groups such as sulfate, methoxy, and pyruvic provides structural variability (Figure 2.3 B) [46]. According to the initial substitution pattern, the conversion of the 4-linked α-galactopyranose to 3,6-anhydro forms by enzymatic elimination of sulfate from the 6-position may result in the neutral polysaccharide agarose or agarose derivates [47]. Agarose is the primary component of the industrially obtained products “agar-agar” (see section 2.6.1).

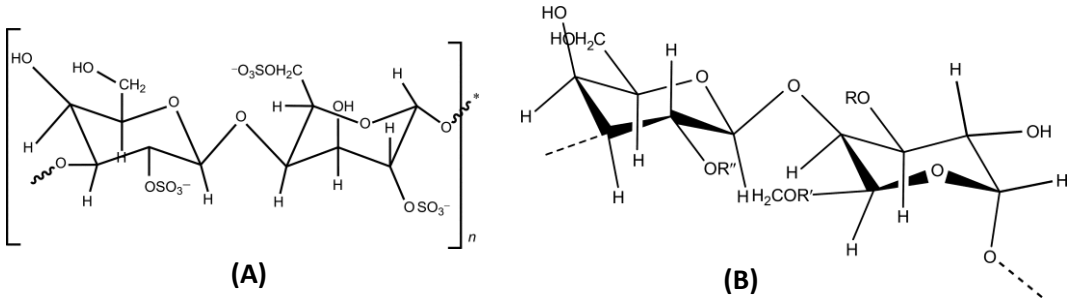

Figure 2.3: Schematic chemical structure of carageen (A) and agaran (B) (adapted from [48]*).*

Amino acid composition

Total amino acids content in seaweed species was reported to be as high as 40 mg/100 mg dw (Table 2.1) [36]. Essential Amino Acids (EAA) - histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine - cannot be synthesized by the human body and must be obtained through diet. In seaweeds, EAA may account for up 50% of total amino acids in some species, such as *Fucus spiralis* (63.5%), *Porphyra sp*. (56.7%), and *Osmundea pinnatifida* (41.6%) [49]. Seaweed proteins contain all amino acids, although tryptophan and lysine are frequently limited in most species. Leucine and isoleucine are generally low in red species, whereas methionine and lysine are often limiting in brown algae. Cysteine is found in low concentrations in many species, being frequently undetectable [50]. Aspartic and glutamic acids account for a sizable portion of total amino acids in numerous seaweed species, contributing significantly to the distinctive bitter taste “umami” flavour associated with seaweed, although it seems lower in red seaweed species [51]. The latter two amino

acids, for example, have been reported to account for 22-44% of total amino acids in *Fucus sp.* and 26-32% in *Ulva* sp. [7]. The seaweed amino acid analysis has revealed profiles similar to ovalbumin (39.5% EAA) and leguminous plants (35.2% EAA), which are comparable to the FAO requirement pattern [29][33]. Overall, the concentration of each amino acid depends on phylum and even on the species within the same genus [38]. The complete analysis of amino acid composition for some seaweed species is represented in Table 2.2.

Due to this attractive nutritional value, seaweed is gaining more attention in the food research and technology field. However, the quality of proteins for human and animal nutrition strongly depends on the digestibility and availability of EAA.

Table 2.1: Amino acid (AA) content and Essential Amino acids/Non-Essential Amino acids (EAA/NEAA) ratio of selected seaweed species.

Species	***Total AA (mg/100 mg of dw)***	***Ratio EAA/NEAA***	***References***
Red algae			
Chondrus crispus	31.2 ± 1.7	1.58 ± 0.05	[52]
Gracilaria sp.	35.5 ± 1.2	1.74 ± 0.05	[52]
Porphyra sp.	38.8 ± 0.9	1.32 ± 0.04	[52]
Gracilaria birdiae	9.1 ± 0.0	0.90	[53]
Green algae			
Ulva sp.	41.3 ± 1.5	1.32 ± 0.05	[52]
Enteromorpha sp.	15.8	0.55	[54]
Brown algae			
Ascophylum nodosum	36.6 ± 1.2	1.06 ± 0.01	[52]
Fucus spiralis	40.2 ± 1.2	1.08 ± 0.01	[52]
Undaria pinnatifida	44.2 ± 1.2	0.87 ± 0.01	[52]
Laminaria filiformis	11.3	0.90	[53]
Laminaria intricata	6.7	0.80	[53]

Table 2.2: Amino acid composition of some seaweed species and two food proteins (in g amino acid/100 g protein)

	Brown algae		***Green algae***		***Red algae***				
Amino acids*	***Laminaria digitata***	***Undaria pinnatifida***	***Ulva armoricana***	***Ulva pertusa***	***Palmaria palmata***	***Porphyra tenera***	***Chondrus crispus***	***Leguminous plants***	***Ovalbumin***
Histidine	1.3	2.7	1.2–2.1	4.0	0.5–1.2	1.4	0.9	3.8–4.0	4.1
Isoleucine	2.7	2.9	2.3-3.6	3.5	3.5-3.7	4.0	1.8	3.6	4.8
Leucine	5.4	5.1	4.6-6.7	6.9	5.9-7.1	8.7	2.9	7.3	6.2
Lysine	3.7	4.3	3.5-4.4	4.5	2.7-5.0	4.5	4.9	6.4-6.5	7.7
Methionine	1.6	2.0	1.4-2.6	1.6	2.7-4.5	1.1	0.5	1.2-1.4	3.1
Phenylalanine	3.2	3.7	5.0-7.1	3.9	4.4-5.3	3.9	1.5	2.4	4.1
Threonine	4.4	2.4	4.5-6.8	3.1	3.6-4.1	4.0	2.2	4.0	3.0
Tryptophan	0.8	0.8	-	0.3	3.0	1.3	-	1.6-1.9	1.0
Valine	4.2	4.1	4.0-5.2	4.9	5.1-6.9	6.4	-	4.5	5.4
Cysteine	1.7	0.5	-	1.2	-	0.3	-	1.1-1.3	1.3
Arginine	0.3	7.5	4.3-8.7	14.9	4.6-5.1	16.4	33.6	13.0-14.0	11.7
Aspartic acid	8.7	5.6	6.0-11.8	6.5	8.5-18.5	7.0	3.8	4.7-5.4	6.2
Glutamic acid	9.4	5.1	11.7-23.4	6.9	6.7-9.9	7.2	4.1	6.4-6.7	9.9
Alanine	14.4	4.8	5.5-7.7	6.1	6.3-6.7	7.4	3.8	-	6.7
Glycine	4.3	4.4	6.3-7.5	5.2	4.9-13.3	7.2	3.5	-	3.4
Proline	3.7	2.8	5.0-10.5	4.0	1.8-4.4	6.4	1.9	-	2.8
Serine	4.0	2.8	5.6-6.1	3.0	4.0-6.2	2.9	2.2	-	6.8
Tyrosine	1.5	1.6	4.4-4.7	1.4	1.3-3.4	2.4	1.0	2.3-2.6	1.8
Alanine	14.4	4.8	5.5-7.7	6.1	6.3-6.7	7.4	3.8	-	6.7
References	[55]	[55]	[7]	[55]	[56]	[55]	[55]	[57]	[57]

**Amino acids written in bold are those recognized as EAA for human nutrition.*

Types of proteins

Peptides

Peptides are protein fragments ranging from 3 to 40 amino acids. These fragments are generated from the parent protein through the gastrointestinal process but can also be produced artificially during fermentation or other processes like enzymatic hydrolysis [58]. As a result, it is expected that seaweed treatment via enzymatic hydrolysis yields enriched peptide extracts.

Enzymes

Enzymes are widely distributed in seaweeds. Alkaline phosphatase (a Zn-containing metalloproteinase that catalyzes the non-specific hydrolysis of phosphate monoesters) is abundant in seaweeds, specifically in *Ulva pertusa* [59]. Alternative oxidases (proteins involved in electron flow through the electron transport chain and regulating the mitochondrial retrograde signalling pathway) have also been identified in *Caulerpa cylindracea* [60]. The fibrinolytic enzyme (trypsin-like serine protease) was reported in green algae, such as *Codium fragile* and *Codium latum* [61]. Rubisco, the bifunctional enzyme known for catalyzing carbon dioxide fixation and oxygenation, was reported in *S. chordalis* [62] and *K. alvarezii* [63].

Glycoproteins and lectins

Glycoproteins (GP) contain glycans (oligosaccharide chains) conjugated to peptide chains through N-glycosyl and/or O-glycosyl linkages. The glycosylation process can happen co- or post-translational modification [64]. GP are located on the cell wall, on the cell surfaces, or are secreted. Their functions include recognition, intercellular interactions, and adhesion [65]. To the moment, a few GP have been isolated through water extraction, from *Ulva* sp. [66], *Ulva lactula* [67], *Codium decorticatum* [68], *Fucus serratus,* and *Fucus vesiculosus* [69], but their structures and functionalities are yet to be known.

Lectins are a type of proteins, described as a structurally diverse group of non-immune origin protein, binding reversibly to specific mono- or oligosaccharides, without modifying the structure of the carbohydrates, found in a variety of organisms [70]. Lectins interact with specific glycan structures present in soluble and membrane-bound glycoconjugates. These protein-carbohydrate interactions result in the lectins participation in various biological processes such as host-pathogen interactions, cell-cell communication, apoptosis induction, cancer metastasis, and cell differentiation [71][72]. In comparison to lectins derived from animals and terrestrial plants, little is known about the biochemical and structural properties of phycolectins [38].

Seaweeds are considered a good source of novel lectins, such as griffithsin, a mannose-specific lectin isolated from the red alga *Griffithsia* sp. [73], SfL-1 and SfL2 from *Solieria filiformis* [74], HRL40

from *Halimeda renschii* [75]. Although some macroalgae lectins have been identified and characterized, little is known about their structural and biochemical properties.

Arabinogalactan proteins are hydroxyproline-rich glycoproteins, which are highly glycosylated proteins found in the cell walls of a few seaweed species, namely in the green seaweeds *C. vermilara* and *C. fragile* [76]. AGPs are involved in the development, signalling and plant-microbe interactions in terrestrial plants [77], but their roles in seaweed remain largely unexplored in lower plants, especially in seaweeds. The first experimental confirmation of AGPs in *Ulva* species showed a unique composition and structure when compared to terrestrial plants, implying a specialized adaptation to the marine environment [78].

Phycobiliproteins

Phycobiliproteins (PBs) are the primary light-harvesting and fluorescent proteins in red seaweeds and the only water-soluble algal pigments (Figure 2.4 A and B), accounting for 20% of dw and representing up to 50% of the total protein content. They are organized in supramolecular structures, called phycobilisomes, located at the stroma, on the external structure of the thylakoid membrane, unlike carotenoids and chlorophylls, located in the lipid bilayer [79]. These proteins enable the transfer of light energy to the living organisms while also surviving in low light conditions [80]. PBs are classified into four types: phycoerythrin (PE), phycocyanin (PC), phycoerythrocyanins (PEC), and allophycocyanin (APC). Red-phycoerythrin is the most common phycobiliprotein found in many red seaweeds and is characterized as an oligomeric chromoprotein with three absorption peaks at 499, 545, and 565 nm [81]. It consists of three protein subunits: a, ß, and γ, with apparent molecular weights of 18, 20, and 30–33 kDa, respectively [82]. These subunits tend to aggregate to form a basic unit with various arrangements. Red-phycoerythrin has an apparent molecular weight of about 240 kDa [38]. PE isolation has been reported from a variety of red algae species, including *Gelidium pusillum* [79], *Furcellaria lumbricalis* [83], and *Gracilaria gracilis* [84].

Mycosporine-like amino acids

Mycosporine-like amino acids (MAAs) are small (<400 Da) secondary metabolites with strong Ultraviolet radiation (UVR) absorption, colourless and stable under changing light, temperature, and pH conditions. UVR protection and antioxidant activity are their primary functions. More than 20 MAAs have been identified in seaweeds, and their ability to synthesize a wide range of MAAs aids in their adaptation to stressful environments [20]. It is noteworthy that red seaweeds biosynthesize MAAs, whereas other marine organisms obtain MAAs through diet or symbiotic or bacterial associations [85]. Some MAAs chemical structures are represented in Figure 2.4 (C-E).

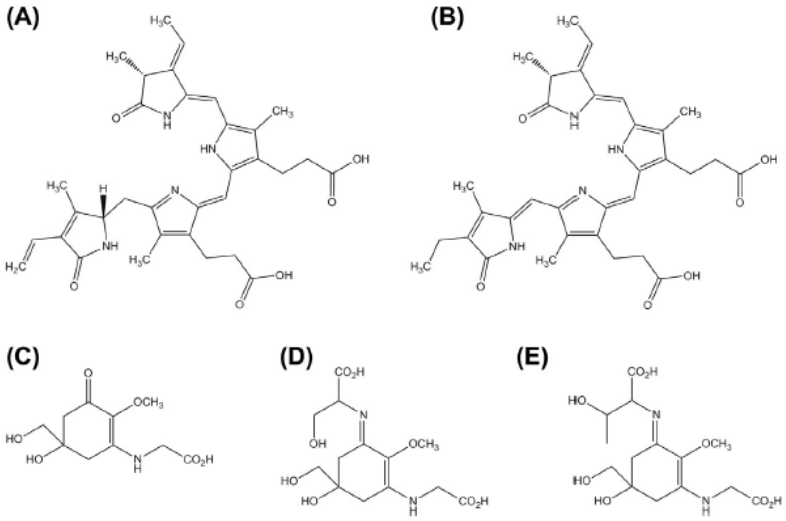

Figure 2.4: Chemical structure of (A) phycoerythrobilin, (B) phycocyanobilin, and the mycosporine-like amino acids: (C) mycosporineglycine, (D) shinorine, and (E) porphyra-3 (adapted from [36]*).*

2.2. Protein extraction methods

2.2.1. Pre-treatment

In most cases, marine algae are harvested from coastal areas. Afterwards, they go through a wash process to remove any salt residues, impurities, or epiphytes. To avoid protein degradation, raw biomass from seaweed must be preserved by drying or freezing or used fresh as soon as possible after harvesting [36] and milled before extraction to ensure uniform mass distribution and a higher surface-to-volume.

2.2.2. Conventional extraction methods

Proteins are traditionally extracted through liquid methods. Various reagents have been employed, such as distilled water, buffers, acid or base solutions, and lysis/surfactant-containing solutions [36]. Protein extraction involving distilled water to enable osmotic shock is recognized as one of the most straightforward extraction conditions. However, the yield is relatively low compared to other liquid-extraction systems, and the large portion of water-soluble proteins somewhat conditions its use, making it inappropriate when a specific type of protein is being targeted [86].

Combining physical methods, such as freeze/thawing and mechanical grinding, can enhance the extraction in some seaweeds. Frozen/thawing, followed by extraction using alkaline solubilization and isoelectric precipitation, showed an increase in protein recovery in *Porphyra umbilicalis* (71%), *S. latissima* (51%), and *U. lactuca* (40%) compared to water extraction alone [87]. Chemical extraction methods, such as two-phase acid and alkali treatments, have been reported as efficient strategies for protein extraction from *A. nodosum* [88]*, Ulva* spp [89]*, L.digitata* [90]. In addition, alkaline solutions combined with reducing agents have been employed to dissociate proteins from polysaccharides. N-acetyl-l-cysteine (NAC) was reportedly used to aid protein extraction from macroalga, resulting in a

protein recovery of approximately 49% from *P. palmata* [91]. Some examples of conventional extraction methods are represented in Table 2.3.

2.2.3. Assisted extraction methods

2.2.3.1. Enzyme-assisted extraction

Enzymes are often used to assist in the protein extraction/hydrolysis from seaweeds [36]. Polysaccharidases such as κ-carrageenase, β-agarase, xylase and cellulase have been used as a cell wall disruption treatment in protein extractions from the red seaweed species *C. crispus, Gracilaria verrucosa*, and *P. palmata* [92]. Different digestion enzymes can be used to release bioactive peptides from parent proteins, with chymotrypsin, trypsin and pepsin being the most commonly used [36]. Combining multiple extraction methods may also aid in extracting algal proteins (Table 2.1). The combination of enzymatic hydrolysis with alkaline extraction increased protein yield in *P. palmata* 1.63 - fold over alkaline extraction alone [91]. Enzyme assisted extraction (EAE) is an eco-friendly and non-toxic approach with a high bioactive yield [93]. However, on a larger/industrial scale, the high amount of enzyme required, and thus the overall cost, may not be economically feasible [86].

2.2.3.2. Ultrasound-assisted extraction

By compressing and decompression through sound waves at frequencies higher than 20 kHz, ultrasound-assisted extraction (UAE) can be used in order to modify plant micronutrients, improve bioavailability, simultaneous extraction, encapsulation, quenching radical sonochemistry to avoid degradation of bioactive, and increasing bioactivity of phenolics and carotenoids by targeted hydroxylation [94]. This technique allows the cell wall breakdown through acoustic cavitation phenomena. Cavitation causes air bubbles to implode, resulting in microturbulence, which converts the sound wave to mechanical energy, disrupting the cell wall. After cell disruption, the solvent can easily penetrate the cells, releasing the intracellular compounds to the bulk solvent [86].

UAE was reported to increase protein extraction of *Ascophyllum nodosum* with acid and alkaline treatment alone by 540% and 27% and reduce the processing time from 60 min to 10 min [88]. Also, UAE implementation followed by ion exchange purification yielded a protein content of 70% in *Ulva* sp. and 86% in *Gracilaria* sp. [95]. In *G. pusillum*, UAE followed by maceration and buffer extraction, allowed the recovery of 77% and 93% for Red-phycoerythrin and Red-phycocyanin, respectively [96]. More examples are presented in Table 2.3.

Table 2.3: Conventional and assisted protein extraction methods for different seaweeds.

Species	***Cell disruption method***	***Extraction method***	***Reagents***	***Conditions***	***Protein recovery yield*** [a]	***Protein quantification method***	**Ref.**
Ulva rigida	Enzymatic hydrolysis	Aqueous polysaccharidase degradation and buffer extraction (sequential)	Phosphate buffer, a mixture of polysaccharidases containing cellulase, hemicellulose and β-glucanase, Tris HCl	***Enzymatic pre-treatment:*** 10 g freeze-dried algal powder; 200 mL of enzymatic medium, pH 6 (6g of polysaccharidase powder in 200 mL of phosphate buffer 0.1 M, pH 6); 30 ºC; 2h. ***Buffer treatment:*** The residue was mixed in 100 mL Tris HCl (0.1 M, pH 7.5); 4 °C.	18.5 ± 2.1%	Kjeldahl method (N × 6.25)	[95]
Ulva rotundata					22.0 ± 1.5%		
Palmaria palmata		Aqueous polysaccharidase degradation and alkaline extraction (sequential)	Deionized water, Cellulase and xylanase, NaOH and NAC	***Enzymatic pre-treatment:*** 1:30 (w/v) of dried, milled seaweed to liquid suspension at pH 5 was pre-incubated (30 min, 40ºC). Enzyme:substrate (E:S) of 48.0 ·10^3 units/100g; reaction incubated at 40 ºC; 24h. ***Alkaline treatment:*** 1:15 (w/v); 0.12 M NaOH; 0.1% (w/v) NAC; stirred for 1h; room temperature.	49% ± 0.5%	AAA [b]	[91]
Ascophyylum nodosum	Osmotic shock	Aqueous and Acid-alkaline Extraction (sequential)	Deionized water HCl and NaOH	***Aqueous treatment:*** Dried, milled seaweed suspended in deionized water (1:20 (w/v); stirred for 16h; 4 ºC. ***Acid and alkaline treatment*** : ***:*** 1:15 (w/v); 0.1M; stirred for 1h; 4 ºC.	59.75 ± 2.44%	Dumas method	[88]
Ulva rigida		Aqueous and alkaline extraction (sequential)	Deionized water and NaOH	***Aqueous treatment:*** 10 g of algal powder; 200 mL deionized water; 4ºC; stirred overnight. ***Alkaline treatment:*** the pellet was treated with NaOH (0.1M) and mercaptoethanol (0.5% v/v); stirred for 1h; room temperature. Supernatant collected after centrifugation (10 000 × g, 20 min, room temperature).	26.8 ± 1.3%	Kjeldahl method (N × 6.25)	[87]
Ulva rotundata					36.1 ± 1.4%		

[a] *Total protein yield expressed as % of total protein (Protein extracted/Total protein x 100);* [b] *AAA: A*mino Acid Analysis

Table 2.3: Conventional and assisted protein extraction methods for different seaweeds (cont.).

Species	Cell disruption method	Extraction method	Reagents	Conditions	Protein recovery yield [a]	Protein quantification method	Ref.
Palmaria palmata	High shear force	Aqueous and alkaline extraction (sequential) with Ultra-turrax	Deionized water, NaOH	***Aqueous high shear force treatment*** Ultra-turrax; 24,000 rpm; 1:20 of weight:volume of deionized water. Following shearing, stirred for 1h; 4ºC. Pellet resuspended in 0.12 M NaOH; 0.1% (w/v); weight:volume of 1:15; stirred for 1h; room temperature.	24 ± 0.1%	Lowry method	[97]
Ulva lactuca		Sonication in aqueous conditions and ammonium sulfate-induced precipitation (sequential)	Ultra-pure water and ammonium sulfate	***Sonication in aqueous conditions*** 10 g of freeze-dried and milled seaweed was suspended in 1 L of ultra-pure water; ultra-sonication for 1h; left to stir overnight; 4°C. Pellet suspended in 200 mL of ultra-pure water and subjected to a second extraction. ***Precipitation:*** Supernatants were combined and brought to 80% (w/v) ammonium sulfate saturation; stirred for 1h; 4°C; centrifuged (20,000 × g, 1h) to precipitate the protein fraction. The precipitates were dialyzed using 3.5- kDa MWCO dialysis tubing; overnight; 4 ºC.	19.6 ± 0.6%	Lowry method	[87]
Saccharina latissima		Aqueous treatment, alkaline solubilization and isoelectric precipitation (sequential)	Deionized water, NaOH and HCl	***Aqueous high shear force treatment*** Dry-milled seaweed in distilled water in a 1:6 (w/v) ratio. Homogenization using Ultra-Turrax; 2 min; 18 000 rpm. Milling with beads; 2 min; 1/30 s. The homogenized sample was stirred for 1h at 8 ºC.	25.1 ± 0.9%	Lowry method	[87]
Porphyra umbilicalis				***Alkaline solubilization and isoelectric precipitation:*** pH adjusted to 12; sample kept in ice. Supernatant collected after centrifugation (at 8,000 × g, 10 min). pH adjusted to 2 and frozen overnight; -20 °C. After thawing and second centrifugation, the pellet was collected	22.6 ± 7.3%		

[a] *Total protein yield expressed as % of total protein (Protein extracted/Total protein x 100);*

2.2.4. Novel methods

Conventional extraction techniques are time-consuming and often not eco-friendly due to the use of organic solvents [93]. The yields obtained are also considered limited. Therefore, some novel methods have been pinpointed as an alternative. These include pulsed electric field extraction (PEFE), microwave-assisted extraction (MAE), sub- and supercritical fluid extraction, and pressurized liquid extraction (PLE). High efficiency, eco-friendly and non-toxic, and short-time requirements have been suggested as some of the advantages. However, there are still some challenges of employing them on an industrial scale as they carry high equipment costs and high energy inputs [86] and are not yet adapted to protein extraction in macroalga, as they have been more widely used to extract carbohydrates, lipids and phenolic compounds [36] [98] [99].

2.3. Protein enrichment and purification methods

Isolation and concentration of proteins from seaweed are relatively unexplored as most literature focus on the extraction approaches only. Purification of extracted proteins, especially for novel proteins, still represents a challenge because of their unknown physicochemical properties [36].
Single or combined methods can be employed, depending on the product's final application and production scale. Precipitation, membrane technologies and chromatography are examples of possible methods [36].

2.3.1. Membrane processes

Membrane technology consists of using a semi-permeable membrane to separate a liquid into two streams (permeate and retentate) by selectively allowing some compounds to pass through while impeding others, usually based on molecular weight criteria [10]. It is inexpensive, simple and scalable. However it faces some limitations, namely the saturation or fouling of membranes [86].

Using the same principles applied in the dairy industry, a combination of membrane technologies, used after a cell disruption technique, could isolate seaweed proteins. Microfiltration (MF) could be used to remove cell wall components, ultrafiltration (UF) to isolate proteins with molecular weights ranging from 1 to 200 kDa, nanofiltration (NF) to remove monovalent salts and reverse osmosis (RO) to reduce volume [100] [58]. Membrane technologies hold great promise for enriching algal proteins as well as developing novel techno-functional and bioactive ingredients and have already been reported used after supercritical CO_2 and ultrasonic-assisted extraction to isolate polysaccharides from *Sargassum pallidum* [101], after hot water extraction in *Ulva fasciata* [102] and in the isolation of phycoerythrin protein from *Grateloupia turuturu* [103].

2.3.2. Chromatography

Chromatography is often a described method in the literature for the purification and isolation of specific proteins, with most studies focusing on the isolation of R-PE and refer to sequential

chromatographic steps. Coupling membrane technology (microfiltration and ultrafiltration) with Size Exclusion Chromatography (SEC) reportedly recovered 60% of R-PE in a protein extract from *Furcellaria lumbricalis.* In *Portieria hornemannii,* 65% of recovery was reported using isoelectric precipitation and Anion Exchange Chromatography (AEX) [105]-[107]. Kazir et al. (2019) showed the possibility of protein purification using IEX to separate the protein from the carbohydrate fraction in *Ulva sp.* and *Gracilara sp.* after alkaline and ultrasound-assisted extractions [95].

2.4. Protein characterization

Direct comparison with standard molecules or literature data allows for the identification and characterization of isolated proteins. However, comprehensive and systematic techniques are required for unknown compounds [36].

2.4.1. Electrophoresis

1D SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is performed in order to identify the molecular weight of dominant protein subunits bands [36]. Protein band resolution is affected by extraction process parameters as well as by the macroalgal species used. For example, in the case of aqueous and alkaline protein extracts from *P. palmata* and *Porphyra dioica*, the aqueous extract demonstrated good resolution of protein bands, whereas the alkaline soluble protein extracts demonstrated low resolution [106] [107]. SDS-PAGE has been reported to be used in the monitorization of R-PE purification from *G. turuturu* by ion-exchange chromatographic [105], to compare the extraction efficiency of different buffers during the protein extraction from *Eucheuma cottoni* [108] and to track differences in protein profiles as a function of the growing season, harvest location, and growth mode (wild vs aquaculture) from *P. palmata* [106], among others. However, it faces some limitations, mainly when applied to seaweed hydrolysates, as due to the low molecular weight of the peptides, they can run off gel or diffuse before staining [86].

2D electrophoresis allows the separation of proteins according to their molecular mass and their isoelectric point. Isoelectric focusing (IEF) is a technique for separating proteins based on their isoelectric point that involves applying a pH gradient and an electrical current across a gel strip. The use of 2D electrophoresis in the separation/characterization of macroalgal proteins is poorly described, probably because non-protein compounds in macroalgal protein extracts interfere with 2D electrophoresis [86]. One study described the use of 2D electrophoresis to optimize protein extraction and characterize the proteome of the edible alga *Gracilaria changii*, resulting in the identification of 15 different protein spots [109]. 2D electrophoretic protocol with laser ablation inductively coupled plasma mass spectrometry (ICP-MS) was designed for the detection of iodine-containing proteins from the red seaweed *P. umbilicalis*, resulting in the detection of iodine-containing protein spots with molecular masses in the region of 10, 20, 28, 40, and 110 kDa and isoelectric point values ranging from pH 8 and 9 [110].

In Capillary electrophoresis (CE), proteins are subjected to an electric field and migrate through electrolyte solutions in a charged capillary tube according to their ionic mobility, where separation is influenced by charge, shape, and size. To this date, the application of CE is limited to the separation and quantification of macroalgal amino acids and MAAs [86].

2.4.2. Chromatography

As already mentioned, different chromatography methods are used to separate and purify various components from seaweeds. The technique chosen is determined by the type of compound being separated and the feasibility of the experiment [111]. Reversed-phase high- and ultra-performance liquid chromatography (RP-HPLC and RP-UPLC), size exclusion chromatography (SEC), ion-exchange chromatography (IEX) are the most commonly used chromatographic methods for the characterization of macroalgal-derived proteins [86]. These methods are often coupled to each other and used sequentially. The amino acid analysis is often done by gas chromatography (GC) or liquid chromatography (LC), requiring a derivatization step with Ortho Phtalaldehyde (OPA) or Fluorenylmethoxy chloroformate (FMOC) treatment [52]. Recently, the possibility of analyzing the amino acid profile without derivatization using anion exchange (HPAEC-PAD) has also been reported [95].

RP-HPLC method combined with UV detectors was used for the identification and isolation of folates [112], phenolic compounds [113] and the pigments chlorophyll and carotenoids from brown algae [114]. SEC was reportedly used to recover C-phycocyanin (CPC) from *Oscillatoria tenuis* species [115]. IEX is mainly reported as a tool for R-PE isolation from *Polysiphonia urceolata* [116], *Mastocarpus stellatus* [117], *Gelidium pusillum* [79], among others.

2.4.3. Spectrometry

Fourier Transform Infrared (FTIR) spectroscopy provides information about the structural composition of proteins, particularly the secondary structural composition [36]. In the obtained FTIR spectrums of protein extracts from *M. pyrifera* and *C. chamissoi*, the bands at 3281 cm^{-1} and 3274 cm^{-1} may correspond to an amide of the protein polypeptide skeleton due to the N-H vibrations; bands at 1637 cm^{-1} and cm^{-1} could be identified as C=O vibrations of the peptide bond of proteins. While bands at 1220 cm^{-1} and 1243 cm^{-1} possibly correspond to S=O vibrations from sulfated polysaccharide (fucoidan and carrageenan) and could indicate the presence of polysaccharides co-precipitated with proteins [118].

Mass spectrometry (MS) can be used to accurately identify and characterize proteins due to its ability of mass and purity determination, sequence analysis, post-translational modification analysis, and protein-protein interactions. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) have emerged as useful tools [36]. MALDI-TOF/MS was used to confirm the presence of protein part in fractions rich in glycoproteins obtained from *Ulva* sp. [119]. The bioactive peptides in *P. palmata* and *S. chordalis* were identified by ESI-MS/MS [62]. The fibrinolytic enzyme codiase was determined by MS and SDS-PAGE in *C. fragile* [61].

2.5. Applications of seaweed proteins and derivatives

As macroalgae are rich sources of protein, minerals, vitamins, polyunsaturated fatty acids, and phycocolloids, they can be used as functional health-promoting ingredients in food, especially in animal feed (aquaculture, farm animals and pets). Macroalgae and macroalgal extracts incorporated in the animal feed have been shown to improve the specific growth rate of fish [120] and the immune system and intestinal health of several farm animal species, including pigs [121], cows [122] and broiler chickens [123]. Although human consumption of seaweed is currently underdeveloped, particularly in Western countries, the relatively high protein content of dome species and favourable essential amino acid profile make seaweed a promising protein source prepared for future growth [10]. At the laboratory scale, seaweed has been successfully incorporated as a functional ingredient into some foods [124] [125].

2.5.1. Bioactive proteins

Lectins and phycobiliproteins are two bioactive algal protein families that have been extensively used in various industrial applications. The main biological activities of lectins are blood grouping, anti-viral, cancer biomarkers, and targets for drug delivery [126]. Phycobiliproteins are a powerful and highly sensitive fluorescent reagent used in fluorescent labelling, flow cytometry, fluorescent microscopy, and fluorescent immunohistochemistry [127]. Also, a growing number of studies have demonstrated PBs health-promoting properties in a wide range of pharmaceutical applications, such as anti-antioxidant, anti-inflammatory, neuroprotective, hypocholesterolemic, hepatoprotective, anti-viral and anti-tumour [128]. Their main application is in the food and cosmetic industry as colourants.

In addition, several studies reported that MAAs are the most common sunscreen compound in seaweeds [129] and play a role as antioxidants, and some may protect the skin from UVR [130].

2.5.2. Bioactive peptides

Bioactive peptides (BAPs) are inactive when encoded in the parent protein sequence and can be released through various mechanisms. Enzymatic hydrolysis is one of the most commonly used approaches for BAP release due to its mild operating conditions and the availability of a wide range of proteolytic enzyme preparations [86]. BAPs have been shown to exhibit properties such as antihypertensive, antioxidant, anticoagulant, antiproliferative, immunomodulatory, and anti-microbial [44][133][134]. Among hypertension-related processes, angiotensin-converting enzymes (ACE) play a critical role in blood pressure regulation by promoting the conversion of angiotensin-I to the vasoconstrictor angiotensin-II and inactivating the vasodilator bradykinin, which has a depressor effect in the renin-angiotensin system. As a result, ACE inhibition is thought to be a practical therapeutic approach in treating hypertension [131]. Some examples of seaweed-derived peptides and their properties are presented in Table 2.4.

Table 2.4: Sequence and respective bioactivity of several peptides derived from seaweeds.

Species	***Peptide Sequence [a]/Name***	***Activity***	***References***
Palmaria palmata	IRLIIVLMPILMA	Renin inhibitory, antihypertensive	[133]
	SDITRPGGNM	Antioxidant	[134]
	VYRT; LDY; LRY; FEQDWAS	ACE-I inhibitory	[135]
Porphyra yezoensis	AKYSY; MKY		[136]
Undaria pinnatifida	AIYK; YKYY; KFYG		[137]
Porphyra haitanensis	VPGTPKNLDSPR; MPAPSCALPRSVVPPR	Antiproliferative	[138]
Bryopsis sp.	Kahalalide F	Antitumoral	[139]

[a] A = alanine, R = arginine, N = asparagine, D = aspartic acid, C = cysteine, E = glutamic acid, Q = glutamine, G = glycine, H = histidine, I = isoleucine, L = leucine, K = lysine, M = methionine, F = phenylalanine, P = proline, S = serine, T = threonine, W = tryptophan, Y = tyrosine, V = valine

2.6. Recovery of proteins and polysaccharides from *Gelidium* species

Gelidium sp is a genus of a thalloid of single-axis red algae, belonging to the Gelidiaceae family, and it is currently the most speciose genus in the family with 134 species [140]. *Gelidium* natural populations are exploited worldwide to extract technical agars (bacteriological agar and agarose) and are an essential raw material source for the phycocolloid industry since *Gelidium* aquaculture is not yet feasible on a large scale [141]. Even though agar obtained from *Gelidium* species only accounts for 1.6% of global agar production [142], its natural high gelling strength and low gelling temperatures make it more attractive than agars extracted from other species. Some examples of *Gelidium* species are represented in Figure 2.5.

In 2009, approximately 90% of all agar produced globally was destined for food applications, and the remaining was used for medical and scientific applications (e.g. medium for cells culture) [143].

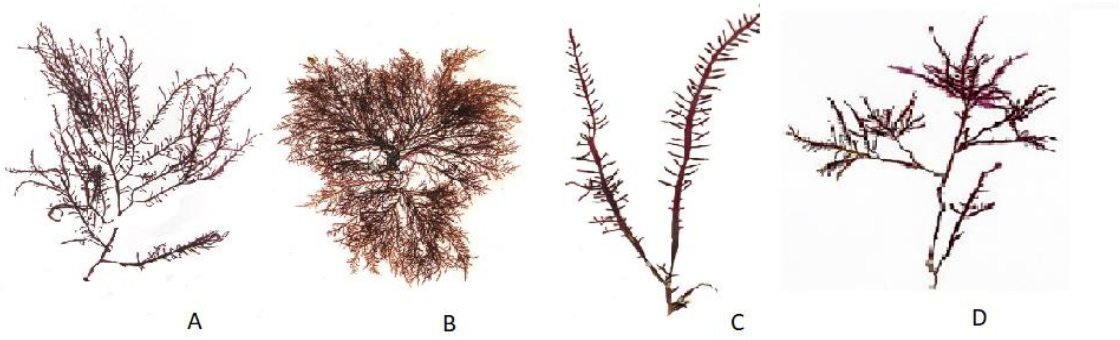

Figure 2.5: A - Gelidium johnstonii; B – Gelidium allanii; C – Gelidium koshikianum; D- Gelidium sesquipedale. (adapted from [144]*).*

The annual demand for bacteriological agar and agarose from *Gelidium* has increased from 250 and 15 tons in 1991 to around 700 and 50 tons in 2018, respectively [145], causing the demand to be greater than supply and resulting in prices to be nearly triple as before scarcity [146]. In the ’90s of the last century, Spain, Portugal, and Morocco accounted for roughly 50% of *Gelidium* harvest worldwide.

Japan and South Korea contributed around 14%, while Mexico and Indonesia contributed 10% and 7%, respectively [147]. However, due to the decreasing wild population, especially in Portugal and Japan, the focus of global production shifted to Morocco, detaining 82% of total production in 2018 [146].

2.6.1. Agar extraction

Agar is a heterogeneous mixture of molecules with a backbone structure of two repeating units, 1,3-linked p-D-galactopyranose and 1-4-linked 3,6-anhydro-oc-L-galactopyranose, with a few variations in the level of substitution of hydrophobic (methoxyl) and polar (sulfate, pyruvate) groups [148].

It is commonly defined as a mixture of two polysaccharide fractions (Figure 2.6). Agarose is the neutral, low sulfate/methoxyl substituted polysaccharide fraction of agar with a high gelling capacity. Agaropectin is the charged, heterogeneous mixture of smaller molecules and highly substituted polysaccharide fractions with a low gelling capacity [148]. The pattern of substitution and the ratio of agarose to agaropectin are affected by several factors, including environmental factors such as hydrodynamic conditions, the availability and quality of light and nutrients, physiological factors such as reproductive stage and nutritional state, and alga processing and isolation conditions during agar extraction [149].

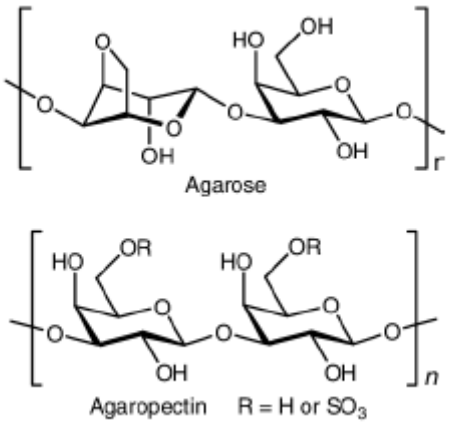

Figure 2.6: Structural features of agar – agarose and agaropectin [143]

The extraordinary gelling power of agar is due to the hydrogen bonds formed among its galactan chains, which provide excellent reversibility, with gelling and melting temperatures typically differing by about 45 ºC [143]. Agar is insoluble in cold water and requires heating at above 85 ºC to dissolve [142].

Agar polysaccharides are mainly exploited from *Gracilaria, Gelidium* and *Pterocladia,* also known as agarophytes. Agar isolated from *Gracilaria* is typically more sulfated than the others, with the pattern of sulfation dominated by the esterification of C-6 of the linked galactose L-unit. Before industrial agar extraction, a chemical desulfation step by alkaline hydrolysis is necessary to increase gel strength and quality, whereas desulfation occurs as a natural internal transformation in *Gelidium* and *Pterocladia* via an enzymatic process [143].

Agar production can be divided into five key steps: pretreatment, extraction, filtration, concentration, and dehydration.

Depending on the genus, seaweeds go through a different pretreatment stage. For *Gelidium,* it consists of mild alkali treatment, usually with sodium carbonate (0.5% (w/v), to remove the phycoerythrin pigment and to macerate the seaweed for better extraction. It usually lasts for 30 minutes at 90 ºC. For *Gracilaria,* as mentioned, to promote desulfation and increase gel quality, the seaweed undergoes an alkali treatment with a sodium hydroxide solution, at concentrations ranging from 0.5% to 7%, heated for 1–2 hours at 85–90 ° C. Then, they are washed with water or a weak acid to neutralize residual alkali [150].

Agar extraction requires cooking the seaweeds in excess of water at the boiling point. Careful addition of acid to adjust the pH to 6.3–6.5 is usually needed. Extraction under pressure reduces processing time while increasing agar yield. However, these conditions can degrade the extracted agar. As a result, optimal extraction conditions for each type of seaweed must be established. The dissolved agar in water must be filtered to remove the residual seaweed, and the hot filtrate must be cooled to form a gel. The gel may be bleached (e.g., sodium hypochlorite) to reduce any colour [150].

After washing out the bleach, the gel contains about 1% agar. The remaining 99% is water and must be removed, either by a freeze-thaw process or by squeezing it out using pressure (syneresis). After thawing and straining, the concentrated extract contains 10-12% of agar – a tenfold increase. Syneresis is an alternative method based on squeezing in a hydraulic press to remove water, improving agar purity by removing a more significant amount of water and soluble impurities with low energy consumption [150].

Although the industrial process has improved in terms of energy requirements, large amounts of water and solvents are still used in the current industrial practice of agar extraction during bleaching, pre-treatment, and alkaline modification. These steps must be carefully monitored to avoid the significant pollution caused by the effluents. As a response, a novel agar extraction process has been reported, named photobleaching, and it exploits sunlight to photochemically degrade the coloured organic matter (CDOM) [151]. In addition, microwave assisted extraction has also been suggested as an option to integrate into the process since it reduces the consumption of solvents (in comparison to solvent consumption of traditional methods), lowers the level of energy required, and reduces the extraction time [152].

Even though the genus *Gelidium* is one of the most exploited for agar extraction, the carbohydrate content and, therefore, the agar content depends on the species. Some examples are presented in Table 2.5.

Table 2.5: Carbohydrate content of some Gelidium species.

Species	***Carbohydrate content (% dw)***	***Agar content (% dw)***	***Quantitative method***	***Ref***
Gelidium amansii	59.9	~30%	Acid hydrolysis; liquid chromatography	[153]

Gelidium pusillum	40.6 ± 2.2	31.6 ± 3.0	Weight difference using crude protein, lipid, fibre, moisture and ash content data	[154][155]
Gelidium pluma	47.4	36.3	Weight difference using crude protein, lipid, fibre, moisture and ash content data	[156]
Gelidium sesquipedale	55.7	~40	Phenol-sulphuric acid colourimetric method	[157]

2.6.2. Protein extraction

Reported protein extraction strategies from *Gelidium sesquipedale* [11] [158] are presented in Table 2.6. Implementing re-extractions steps of both aqueous and alkaline treatments reportedly increased protein recovery compared to single aqueous followed by alkaline extractions (22.4% vs 14.7%). However, for *G. sesquipedale*, the addition of a sonication step before alkaline extraction did not enhance protein release compared to the conventional extraction method. Regarding enzyme assisted extraction within different enzymes (Viscozyme, Celluclast and Alcalase), an aqueous extraction with Alcalase followed by an alkaline extraction resulted in a 32.2 % yield, whereas with Celluclast and Viscozyme values of 20.3 and 22.2% were reported [11].

Faraj *et al.* reported protein recovery yields in crude *Gelidium sesquipedale* and in its residues after agar extraction following two different extraction and precipitation procedures. For both biomasses, higher protein recovery yields were attained when using alkaline extraction followed by acid precipitation than extraction with distilled water and ammonium sulfate precipitation [158]. However, the yield of each step was not detailed.

Table 2.6: Examples of protein extraction strategies employed in Gelidium sesquipedale.

Biomass	Extraction method	Reagents	Conditions	Initial Protein content (% dw)	Protein recovery yield (%)	Protein Quantification method	Ref
Gelidium sesquipedale	Aqueous extraction and alkaline extraction (sequential)	Distilled water; NaOH	***Aqueous treatment:*** 10 g of algal powder suspended in 200 ml of distilled water; stirred for 16 at 4ºC; centrifugation. ***Alkaline treatment:*** pellet resuspended in 100 ml of NaOH 0.1 M and stirred for 1h at RT; centrifugation	14.8	14.7 ± 2.3	Lowry Method	[11]
	Aqueous extraction, aqueous re-extraction, alkaline extraction, and alkaline re-extraction (sequential)		***Aqueous treatment*****:** same conditions as above; re-extraction following the same conditions. ***Alkaline treatment:*** same conditions as above; re-extraction following the same conditions;	14.8	22.4 ± 0.1	Lowry Method	[11]
	Sonication-assisted aqueous extraction and alkaline extraction (sequential)		***Sonication-assisted aqueous treatment***: 10 g of algal powder suspended in 100 ml of distilled water: The algal cells were disrupted using an ultrasonic cell disruptor (TT 13 probe, Bandelin Sonoplus) for 10 min, 50W and a 5s/10s on and off-cycle. ***Alkaline treatment:*** addition of 50 mL of NaOH 0.1 M, stirred for 1h at RT; centrifugation	14.8	16.6 ± 1.0	Lowry Method	[11]
	Enzyme-assisted aqueous extraction using Celluclast and alkaline extraction (sequential)	Distilled water; Cellulase®; NaOH	***Enzyme treatment:*** 10 g of algal powder in 200 ml of distilled water, pH 8. Enzyme substrate 0.2% (g enz/g algae); stirred for 16h at 50 ºC; centrifugation; ***Alkaline treatment:*** equal conditions as above	14.8	20.3 ± 2.2	Lowry Method	[11]
	Enzyme-assisted aqueous extraction using Alcalase® and alkaline extraction (sequential)	Distilled water; Alcalase®; NaOH	***Enzyme treatment:*** Same conditions as enzyme treatment with celluclast, except working pH at 4.5. ***Alkaline treatment:*** pellet resuspended in 100 mL of NaOH 0.1 M stirred for 1h at RT; centrifugation	14.8	32.2 ± 2.2	Lowry Method	[11]

Table 2.6: Examples of protein extraction strategies employed in Gelidium sesquipedale (cont.).

Biomass	Extraction method	Reagents	Conditions	Initial Protein content (% dw)	Protein recovery yield (%)	Protein Quantification method	Ref
***Gelidium sesquipedale* (crude)**	Alkaline solubilization; acid precipitation	Sodium sulfate; NaOH; HCl	***Alkaline solubilization*:** Suspension in 0.3% sodium sulfate (ratio of algal powder:solution = 1:10); adjustment to pH 12 using NaOH 1N; agitation for 1 h. Centrifugation. ***Acid precipitation*:** adjustment of the supernatant's pH to 4.5 using HCl 1N. Centrifugation, washing, neutralization (pH 7) and filtration of the precipitate. Lyophilization.	30	75.6	Kjeldahl method (conversion factor of 6.25).	[158]
	Solubilization in distilled water; ammonium sulfate precipitation.	Distilled water; ammonium sulfate	***Aqueous treatment:*** Suspension in distilled water; agitation for 1h; pH 7-8. Two sequential centrifugations. ***Ammonium sulfate precipitation***: 1 volume of ammonium sulfate 50% to 1 volume of the combined supernatants; centrifugation. Washing the pellet with distilled water; dissolution of the pellet in distilled water at pH 7; dialysis against distilled water at pH 7.		51		
***Gelidium sesquipedale* (agar extraction residues)**	Alkaline solubilization; acid precipitation	Sodium sulfate; NaOH; HCl	***Alkaline solubilization:*** Equal conditions as described above. ***Acid precipitation:*** Equal conditions as described above.	25	63	Kjeldahl method (conversion factor of 6.25).	[158]
	Solubilization in distilled water; ammonium sulfate precipitation.	Distilled water; ammonium sulfate	***Aqueous treatment:*** Equal conditions as described above. ***Ammonium sulfate precipitation:*** Equal conditions as described above.		40		

2.6.3. Additional applications of agarophytes

Besides the classical uses as agar producers, there has also been evidence of *Gelidium* species beneficial use in other fields. Even though agar is widely used in the food industry (primarily as a texture modifier and thickening agent) and in microbiology, alternative applications, such as the development of biodegradable films, hybrid biopolymeric nanofibers, encapsulation structures, or components with bioactive functionalities are currently being investigated [159]. These soluble polysaccharides and algae extracts have demonstrated antioxidant, antiviral, antiproliferative, anti-inflammatory, phyto-stimulant, and bioremediation properties (Table 2.7).

Table 2.7: Examples of main bioactivities of Gelidium species (adapted from [160]*).*

Species	Extracts	Bioactivity	Ref
Gelidium sesquipedale	Agar-based biofilm	Antimicrobial	[161]
	Methanolic extracts Dichloromethane-methanol extracts	Antifungal	[162]
	Agar-based aerogel	Antioxidant	[163]
	Ethanol/aqueous extracts		[164]
	Chloroform extracts		[165]
	Ethanolic extracts	Anti-inflammatory	[166]
	Dichloromethane-methanol extracts	Antiproliferative	[167]
	Oligosaccharides	Phyto stimulant	[166]
	Biomass	Bioremediation	[168]
Gelidium foliaceum	Polysaccharides	Antioxidant	[169]
Gelidium amansii	Ethyl acetate, acetone, methanol extracts	Antioxidant	
	Phosphate-buffered saline, methanol extracts	Antiproliferative	[170]
Gelidium pulchellum ***Gelidium spinulosum***	Aqueous extracts	Antiviral	[171]

Besides the use in the typical food and biotechnological applications, the agar fraction could be used in the pharmaceutical industry, where has been found to be effective to treat obesity, hypercholesterolemia, diabetes, and intestine cancer [172], in the food industry as packaging because of the possibility to produce stable gels with antimicrobial and antioxidant activities [163], as well as in the cosmetic industry as a photo protectant and antioxidant [164]. Furthermore, the phytostimulant effect of the agaro-oligosaccharides, obtained by hydrolysis of agar, opens up the possibility of its use in agriculture as a substitute for chemical fertilizers [166].

With this evidence, it is urgent to start taking advantage of the *Gelidium sesquipedale* products from agar extraction, as they show tremendous potential for application in different industries [160].

3. Materials and Methods

Most experiments described here were performed in the Institute for Bioengineering and Biosciences facilities in Instituto Superior Técnico between February 22nd and July 31st, 2021. Some analyses, adequately identified, were carried out by the partner IPMA.

3.1. Materials

3.1.1. Chemicals and solutions

An AG245 digital analytical laboratory scale (Mettler Toledo) was used for weighing solutes, and a FiveEasy F20 pH/mV meter (Mettler Toledo) was used for pH measurements and adjustments.

The chemicals used were hydrochloric acid 37% solution in water (Honeywell Fluka), sulfuric acid 96% solution in water (ACROS Organics), sodium hydroxide pellets 98% (Thermo Fisher Scientific), calcium carbonate ≥ 99% (Merck, Germany), sodium carbonate ≥ 99.5% (Farma-Quimica Sur Sl, Spain), ammonium sulfate ≥ 99% (Panreac, USA), TRIS base ≥ 99.8% (Thermo Fisher Scientific), Tween 20 (Sigma-Aldrich, Germany), phosphate buffered saline 10x (Panreac, USA), sodium chloride ≥ 99.5 % (Merck, Germany), 40% acrylamide/bis stock solution (37.5:1) (BioRad, USA), TEMED (Sigma-Aldrich, Germany), APS (Sigma-Aldrich, Germany), Laemmli sample buffer (BioRad, USA), DTT (Sigma-Aldrich, Germany), acetic acid glacial 99.5% (Panreac, USA), ethanol absolute 99.8% (Thermo Fisher Scientific), ethanol 96% (Panreac, USA), sodium thiosulfate > 99.99% (Merck, Germany), silver nitrate > 99% (Sigma-Aldrich, Germany), formaldehyde 37% solution in water (Panreac, USA), potassium sodium tartrate tetrahydrate ≥ 99% (Panreac, USA), copper (II) sulfate pentahydrate ≥ 99.5% (Panreac, USA), Folin & Ciocalteu's phenol reagent 2N (Sigma-Aldrich, Germany), bovine serum albumin lyophilized powder ≥ 96% (Sigma-Aldrich, Germany), D(+) glucose anhydrous 99.5% (Thermo Fisher Scientific), and D(+) galactose ≥ 98% (Carl Roth Chemicals, Germany).

3.1.2. Biomass

Gelidium sesquipedale crude was acquired from Iberagar – Sociedade Luso-Espanhola de Colóides Marinhos S.A. This company kindly provided resultant solid residue after industrial agar extraction.

According to Iberagar, the annual collection takes place between July 15th and November 15th. Fresh seaweed is washed with water immediately after harvesting and sun-dried in the summer until a moisture content of 20% (w/w). When these conditions are met, the storage at room temperature can be held for up to 2 years. After the agar extraction process, the solid residue is washed once again with water and dried. Both dried alga and dried residue were ground into a fine powder with an average granulometry of 0.25mm.

3.2. Experimental methods

3.2.1. Protein loss in the industrial agar extraction process

As mentioned in section 2.6.1, a pre-treatment with a mild alkaline sodium carbonate solution is performed during agar extraction from *Gelidium*. Under the same conditions described in an FAO publication in 1987 by R. Armisen and F. Galatas [173], the alkaline washing step of biomass before agar extraction was reproduced to determine how much protein is potentially lost in this step. 300 mL of sodium carbonate 0.5% was added to 10 g of algal powder in a 500 mL Erlenmeyer flask (n=2), heated to 90 ºC and held for 30 min with gentle stirring (7×30mm cylindrical magnetic stir bar; RO 5 Power IKAMAG magnetic stirrer, IKA Werke, Germany, coupled with an SP Bel-Art thermometer). The suspension was centrifuged at 10,000 × g for 30 min at 4 ºC (Sorvall™ RC 6 centrifuge, SLC-3000 rotor, Thermo Fisher Scientific, USA). The supernatant was collected and stored for protein assay

3.2.2. Up-scaled protein extraction

Previously developed work focused on different strategies for extracting the protein fraction from *Gelidium sesquipedale* [11]. In this study, two strategies were selected, which featured a sequence of 4 extraction steps, and the extraction was up-scaled to a final volume of 5 L (Table 3.1).

At the end of the sequential extraction steps, the pooled extracts (i.e., supernatants) were combined, the pH was adjusted to a neutral value (approximately 7) and stored for further processing. A sample was retrieved for protein assay.

Table 3.1: Protein extraction procedures implemented in the up-scaled process.

Procedure	***Extraction Conditions***
A	Aqueous extraction, aqueous re-extraction, alkaline extraction, and alkaline re-extraction (sequential)
B	Enzyme-assisted aqueous extraction using Celluclast® BG, enzyme-assisted aqueous extraction using Alcalase®, alkaline extraction and alkaline re-extraction (sequential)

3.2.2.1. Aqueous extraction

Algal biomass powder (100 g) was suspended in 2 L of deionized water in a 5 L beaker (n=1). The suspension was stirred at 600 rpm (10x70mm cylindrical magnetic bar; RO 5 Power IKAMAG magnetic stirrer, IKA Werke, Germany) for 16h at 4 ºC. Afterwards, the suspension was centrifuged (Sorvall™ RC 6 centrifuge, SLC-3000 rotor, Thermo Fisher Scientific, USA) at 10,000 × g for 30 min at 4 ºC. The supernatant was collected, stored and a sample was withdrawn for protein assay. The

aqueous re-extraction consists of applying the same extraction conditions to the resultant pellet of the previous aqueous extraction.

3.2.2.2. Enzyme assisted aqueous extraction

The enzymes were mixed with 1.5 L of deionized water in a 2 L Erlenmeyer flask (n=1). A sample was withdrawn for protein assay to account for the protein content derived from the addition of the enzyme. Algal biomass powder (75 g) was added to the solution, and the pH was adjusted to the value for optimal activity defined by the manufacturer, either using hydrochloric acid (HCl) or sodium carbonate (Na_2CO_3). Table 3.2 presents the enzyme concentration used and the optimum operating conditions (pH and temperature) of each enzyme. The suspension was stirred at 600 rpm (10x70mm cylindrical magnetic bar; RO 5 Power IKAMAG magnetic stirrer, IKA Werke, Germany) in an incubator (Agitorb 160E, Aralab, Portugal) for 16h at 50 ºC. Afterwards, the suspension was centrifuged at 10,000 × g for 30 min at 4 ºC (Sorvall™ RC 6 centrifuge, SLC-3000 rotor, Thermo Fisher Scientific, USA). The supernatant was collected, stored and a sample was withdrawn for protein assay.

Regarding the extraction step using Alcalase®, as its use results in the hydrolysis of peptide bonds, the resultant supernatant before being stored was subjected to a heat treatment at 100 ºC during 10 min in a water bath (HB4 basic, IKA® WERKE, Germany) to guarantee the total inactivation of the enzyme, in order to avoid further hydrolysis. The enzyme assisted extraction with Alcalase® consists of applying the extraction conditions to the resultant pellet of the previous extraction with Celluclast®.

Table 3.2: Enzyme name, supplier, concentration, and recommended operating conditions (pH and temperature) for each enzyme used in the extractions.

Enzyme	***Supplier***	***Concentration (g_{enz}/100 g_{alga})***	***pH***	***Temperature (ºC)***
Celluclast® BG	Novozymes	0.2	4.5	50
Alcalase®	Merck. Germany		8.0	

3.2.2.3. Alkaline extraction

The pellet resultant from the centrifugation of the previous step was resuspended in 1 L of 0.1 M NaOH and stirred at 600 rpm (10x70mm cylindrical magnetic bar; RO 5 Power IKAMAG magnetic stirrer, IKA® WERKE, Germany) for one hour at room temperature (RT). Then, the suspension was centrifuged at 10,000 × g for 30 min at 4 ºC (Sorvall™ RC 6 centrifuge, SLC-3000 rotor, Thermo Fisher Scientific, USA). The supernatant was collected stored, and a sample was withdrawn for protein assay. The alkaline re-extraction consists of applying the same extraction conditions to the resultant pellet of the previous alkaline extraction.

3.2.3. Concentration of the protein extracts

In both sequential extracting procedures, the combination of the resultant supernatant from each extraction step resulted in a high volume, which was not compatible with the conditions required for further analysis and characterization assays. In that way, it was necessary to reduce it, implementing a concentration step. The choice between an ultrafiltration or nanofiltration system was taken regarding the expected molecular weights of the proteins/peptides present in each extract.

The concentration steps were carried out with retentate recycling and permeate removal occurring at the same time. The permeate volumetric flux (J), the volume concentration factor (VCF) and the average rejection coefficient (σ) for each membrane were calculated using Equations 3.1, 3.2, and 3.3, respectively.

$$J = \frac{Q_p}{A} \qquad \textit{Equation 3.1}$$

Where Q_p is the permeate filtration rate (L/s), and A is the effective membrane area (m^2)

$$VCF = \frac{Volume_{retentate}}{Volume_{feed}}.100 \qquad \textit{Equation 3.2}$$

$$\sigma = 1 - \frac{[Protein]_{permeate}}{[Protein]_{feed}} \qquad \textit{Equation 3.3}$$

3.2.3.1. Ultrafiltration

Ultrafiltration was carried out using a lab-scale QuixStand Benchtop tangential-flow filtration system coupled with a hollow fiber cartridge (GE Healthcare, USA) of 10 kDa nominal molecular weight cut-off (NMWCO) with an effective membrane surface area of 110 cm^2. Figure 3.1 shows the schematic representation of the system used.

Inlet and outlet pressures were adjusted by a peristaltic pump (Sci-Q 323, Watson-Marlow, UK) and a backpressure valve to maintain an average transmembrane pressure (TMP) along the length of the membrane. The system was equipped with 2 pressure gauges with a 0-2 barg range (0-30-psig) (Anderson-Negele, USA) to monitor feed and retentate pressures. The pressure was maintained below 1 barg. The filtration was carried out until the desired final volume was obtained (~400 mL), which resulted in a VCF of 10. Samples of both final retentate and permeate were taken and stored for protein analysis.

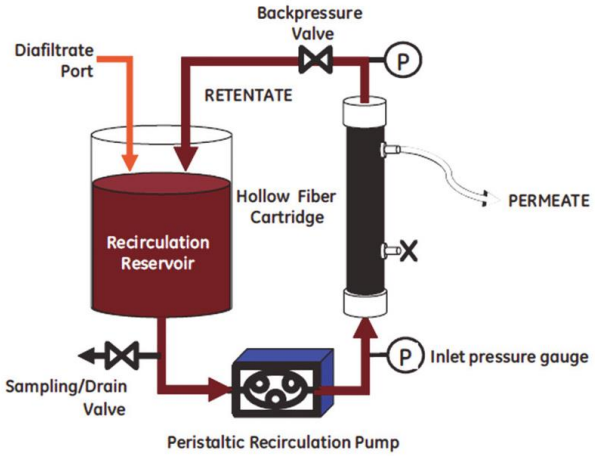

Figure 3.1: Graphic representation of QuixStand benchtop system flow diagram (adapted from [174]).

3.2.3.2. Nanofiltration

Due to the use of Alcalase®, the resultant extract from procedure B is expected to have small peptides with a correspondent low molecular weight, which would result in a significant protein loss if using the same ultrafiltration membrane as above. A nanofiltration membrane composed of polyamide with an NMWCO of 400 Da (FilmTec™ NF270, Dupont) was chosen. One stainless steel module was set up, coupled with one plate of 0.23 m diameter containing two-disc filters, resulting in an effective membrane area of approximately 0.083 m^2.

The transmembrane pressure was regulated by a piston pump (Rannie, Denmark) and a needle valve on the exit of the membrane. The pressure was monitored in the inlet and outlet of the membrane module by 2 pressure gauges with a range of 0-100 barg (Wika, Germany) and regulated between 20-30 bar. The conductivity of the permeate collected at various operation times and in the final retentate was measured by a conductivity meter (Oakton Instruments, USA). The filtration was carried out until the minimum volume possible was achieved, resulting in a VCF of approximately 5. A sample of both final retentate and permeate was taken and stored for protein analysis.

Cleaning of the membranes

Before each filtration, the water permeability was measured. At the end of each run, membranes were rinsed by pumping distilled water, and their water permeability was measured one more time. After rinsing, the membranes in the ultrafiltration system were cleaned by passing 2 L of a NaOH solution 1 M through the membrane, followed by 2 L distilled water. Regarding the nanofiltration system, 2 L of a caustic detergent (99% NaOH) solution (1 g/L) was recirculated for 20 min and rinsed again with distilled water until the solution leaving the system was clear. Both systems were then operated with distilled water to seek for any changes in the water permeability.

3.2.4. Effect of pH during storage of protein extracts in the size of peptides

After the 4 sequential steps for protein extraction, the pH of the extracts was strongly alkaline (~13) due to the implementation of the last two alkaline extraction steps. This high pH might cause hydrolysis of the proteins when the "pool" resulting from the extraction is stored at these conditions. The effect of pH on the size of peptides when the extract was stored at the initial pH (~13) or at a neutral pH was assessed. This study was only conducted for the extract after procedure A (Table 3.1) since the one obtained with enzyme-assisted extraction is expected to have small peptides and free amino acids that would be lost or not detected in the approaches used for this study. Also, the studies were only performed with the protein extract from *Gelidium sesquipedale* crude "powder". To assess peptide size through electrophoresis and chromatography, the extracts needed to be concentrated to improve the amount of protein in the sample.

3.2.4.1. Protein precipitation using ammonium sulfate

The combined supernatants obtained after protein extraction (30 mL) were transferred to 50 mL Falcon conical centrifuge tubes (n=3 for each concentration value), and a sample was withdrawn for protein assay (initial protein concentration). The mass of ammonium sulfate required to achieve the desired concentration in the protein extract (70%, 75%, 80%, 85% of saturation) calculated by Equation 3.4 was gradually added while stirring (4.5×15mm cylindrical magnetic stir bar in a RO 5 Power IKAMAG magnetic stirrer, IKA Werke, Germany). After adding the total mass of ammonium sulfate, the tubes were placed at 4 ºC for 16 h with stirring, followed by centrifugation at 15,000 x g for 30 min at 4 ºC. (Centrifuge 5810 R with a fixed-angle rotor, Eppendorf, Germany). The supernatants were collected for protein assay, and the pellet was resuspended in 15 mL of Tris HCL 10 mM pH 7 (buffer) for further diafiltration, and a sample was taken for protein quantification.

To understand if the presence of ammonium sulfate interfered with the Lowry-protein quantification method, solutions with the same salt concentration were prepared with distilled water.

$$Mass\ of\ (NH_4)_2SO_4\ (g) = \frac{G_{sat}.(S_2-S_1)}{1-P.S_2}.V_{initial} \qquad \textit{Equation 3.4}$$

Where G_{sat} (g/L) are the grams of $(NH_4)_2SO_4$ in 1 litre of saturated solution, S_1 and S_2 are the initial and final ammonium sulfate concentration, as % of complete saturation, respectively, P= $(specific\ volume\ (mL/g)\ \cdot Gsat)$/1000, and $V_{initial}$ is the initial sample volume in litres. The parameters used in Equation 3.4 are described in Table 3.3. G_{sat} was determined considering the molarity of a saturated solution at 4 ºC (3.93 M [175]) and the MW of ammonium sulfate (132.14 g/mol). At 4 ºC, the specific volume was estimated to be 0.53 mL/g [175].

Table 3.3: Parameters used in equation 3.4, the mass of ammonium sulfate added, and the correspondent expected final volume upon addition.

G_{sat} (g/L)	S_1 (%)	S_2 (%)	P	$V_{initial}$ (mL)	Mass of $(NH_4)_2SO_4$ (g)	Vfinal (mL)
519.1	0	70	0.275	30	13.51	37.2
		75			14.73	37.8
		80			15.98	38.5
		85			17.29	39.2

The protein precipitation yield was determined using Equation 3.5

$$Precipitation\ Yield\ (\%) = \frac{(Protein\ Mass)_{pellet}}{[Protein]_{initial} \cdot V_{initial}} \cdot 100 \qquad \textit{Equation 3.5}$$

The protein mass in the pellet was calculated through a mass balance in the liquid phase (Equation 3.6).

$$Protein\ Mass_{pellet}(g) = [\text{Protein}]_{initial} \cdot V_{initial} - [\text{Protein}]_{supernatant} \cdot V_{supernatant} \qquad \textit{Equation 3.6}$$

3.2.4.2. Diafiltration and concentration

Diafiltration of the redissolved pellet after precipitation with 85% saturated ammonium sulfate solution was carried out using an Amicon Ultra-15 Centrifugal Unit (MWCO of 3 kDa, regenerated cellulose, 15 mL, 7.6 cm^2, Merck, Germany). All centrifugations were performed at 3220 x g and 4 ºC (Centrifuge 5810 R with an A-4-62 swing-bucket rotor, Eppendorf, Germany).

The membrane was first washed with 5% Tween 20 (10 mL, 10 min), then with MilliQ water (2x, 10 mL, 10 min), and finally with Tris-HCl 10 mM pH 7 buffer (10 mL, 10 min). Afterwards, 10 mL of the redissolved pellet were loaded with the addition of 5 mL of Tris-HCl 10 mM pH 7 buffer to avoid protein aggregation and deposition onto the membrane surface. The time required for each centrifugation was dependent on the volume of permeate collected. When approximately 5 mL of permeate was collected, the same amount of buffer was added to restore the retentate volume to 15 mL. This procedure was repeated until about 50 mL of cumulative permeate volume was collected. Samples of the finals retentate and permeate were withdrawn for protein quantification. The membrane was washed with MilliQ water (10 mL, 20 min) and stored in 20% (v/v) ethanol until further use.

After diafiltration, the protein samples were concentrated until a final protein concentration around 0.6 g/L, suitable for gel electrophoresis and small injection volumes in the chromatography assays.

In such concentration step, the same centrifugal filtration unit was used. The retentate volume was reduced by centrifugation (Centrifuge 5810 R with an A-4-62 swing-bucket rotor, Eppendorf, Germany) at 3220 x g at 4 ºC for 5 min. The procedure was repeated until the desired volume was obtained (~3 mL). Samples of the final retentate and permeate were withdrawn for protein quantification.

The mass balance equation, the diafiltration yield, the number of diavolumes (n_D), the rejection coefficient (σ) and the volume concentration factor (VCF) were calculated using Equation 3.7, Equation 3.8, Equation 3.9, Equation 3.3 and Equation 3.2, respectively.

$$(Protein\ Mass)_{load} = (Protein\ Mass)_{retentate} + (Cumulative\ Protein\ Mass)_{permeate} \qquad \textit{Equation 3.7}$$

$$Yield\ (\%) = \frac{(Protein\ Mass)_{retentate}}{(Protein\ Mass)_{load}} . 100$$ *Equation 3.8*

$$n_D = \frac{(Cumulative\ Volume)_{permeate}}{Volume_{load}}$$ *Equation 3.9*

3.2.4.3. Size exclusion chromatography

Size exclusion chromatography (SEC) experiments were performed using a *Biosep-SEC-s3000* column from Phenomenex (silica resin, 300 x 7.80 mm, 5.0 µm, 290 Å) with an exclusion range from 5-700 kDa on the ÄKTA 100 Purifier system (GE Healthcare, USA), equipped with a Pump P-900 (assembled with 4 pump heads in 2 pump modules), a UV-900 detector and an autosampler (P-960 Sample Pump) with a 1 mL loop. The loop was first washed with the mobile phase phosphate buffered saline (PBS) 1x composed of 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 pH 7.5. 1 mL of sample was injected into the system. Measurements were performed at a flow rate of 1 mL/min, and the absorbance at 280 nm was recorded using the *Unicorn 5.31 software.* After analysing the obtained chromatograms, the fractions corresponding to each peak were combined and collected to be characterized by electrophoresis.

After each run, the column was washed with Milli-Q water, and it was stored in 20% (v/v) ethanol solution at RT until further use.

3.2.5. Alternative protein precipitation methods

3.2.5.1. Protein precipitation using a pH-shift method

The development of a pH-shift protein precipitation method for *Gelidium sesquipadale* was adapted from a previous study relative to protein isolation in the brown seaweed *Saccharina latissima* [176].

For both extraction procedures, A and B from crude *Gelidium*, 30 mL of the combined supernatants were transferred to 50 mL Falcon conical centrifuge tubes (n=3 for each pH value), and a sample was withdrawn for protein assay (initial protein concentration). HCl 6 M was slowly added while stirring, at RT, to stepwise adjust the pH to values in the range 1 and 6, whereas another was left at pH 7. All the samples were left to incubate for 30 min with stirring at 4 ºC (4.5×15mm cylindrical magnetic stir bar in a RO 5 Power IKAMAG magnetic stirrer, IKA Werke, Germany). At each pH examined, samples were centrifuged at 10,000 x g for 10 min at 4 ºC (Centrifuge 5810 R with a fixed-angle rotor, Eppendorf, Germany). The supernatants were collected for protein quantification, and the pellets were first redissolved in 10 mL of Tris-HCl 10 mM pH 7 and then collected for protein assay.

Optimization of pH-shift protein precipitation method

An additional approach was implemented to optimize protein precipitation yield by recovering the precipitated proteins from the successively collected supernatant between pH adjustments. After analysis from the previous results, the interval of pH values registering the highest precipitation yield

was identified. For both extraction procedures, A and B, 30 mL of the protein extract was transferred to 50 mL Falcon conical centrifuge tubes (n=3 for each pH value). The pH of the extract was directly adjusted to the highest value of the interval with the addition of HCl 1 M, incubated and centrifuged as described in section 3.2.5.1. The supernatant was recovered before each new pH shift, and a sub-sample was collected for protein assay. Then, the pH adjusted to the next decided value, and the process was repeated until the last value of the interval was reached. To maximize the protein recovery, the pH values were adjusted in 0.5 pH steps.

3.2.5.2. Organic solvent-driven precipitation

To test this alternative precipitation method, 10 mL of extraction procedure A supernatants were transferred to 500 mL Thermo Scientific™ Nalgene™ PPCO centrifuge bottles (n=3). A sample was withdrawn for protein assay (initial protein concentration). Protein precipitation was assessed for different final ethanol concentrations. Precipitation in 90% (v/v) ethanol was carried out after adding 9 volumes of cold ethanol (cooled until -20 ºC for 2 hours) to 1 volume of aqueous protein solution. For 80% (v/v) and 70% (v/v), 8 and 7 volumes of cold ethanol were added to 1 volume of aqueous protein extract, respectively [177].

The mixture was homogenized by vigorous vortexing (laboratory shaker 444-1378, VWR) and left to incubate at –20 ºC for 2 hours. The samples were then centrifuged at 10,000 × g for 30 min at 4 ºC (Sorvall™ RC 6 centrifuge, SLC-3000 rotor, Thermo Fisher Scientific, USA). The supernatants were collected for protein quantification, and the pellets were left in the recipients without the lid at RT for 3h to allow residual ethanol to evaporate. Afterwards, the ethanol-free pellets were dissolved in 10 mL of Tris-HCl 10 mM pH 7 and collected for protein assay. Solutions with the same ethanol concentrations were prepared with distilled water to evaluate whether the presence of the organic solvent interfered with the protein quantification method.

3.2.6. Quantification of total carbohydrates co-eluted during protein extraction

This study was only performed for the protein extraction procedures A and B (Table 3.1) applied to whole *Gelidium sesquipedale.* It was not carried when using industrial *Gelidium* residues since co-extraction of significant amounts of residual carbohydrate fraction is not expected.

After each extraction step of the above described sequential procedures, a sample of the algae residue was taken and placed in a convection oven at 40 ºC (Function Line, Heraeus) for 7 days to guarantee its complete drying. Total carbohydrate content in the dried residue was determined as described in section 3.3.1.2.

3.2.7. Anion exchange chromatography

Purification of the proteins in the products obtained after diafiltration and concentration was done by anion exchange chromatography (AEX). It was carried out with the protein products (α and β) resulting from protein extracts stored at different pH values (13 and 7, respectively). This process allows the separation of the co-extracted neutral carbohydrates in the extracts.

The protein isolation by AEX was evaluated using two prepacked columns: *1 mL HiTrap DEAE FF* and *1 mL HiTrap Q Sepharose FF* columns (*HiTrap™ IEX Selection Kit,* from GE Healthcare) in the ÄKTA 100 Purifier system (GE Healthcare, USA). The absorbance at 280 nm and conductivity of buffer solutions exiting the column were measured during the runs, and the chromatograms were recorded using *Unicorn 5.31 software.*

During each run, the following steps were performed: equilibration of the column with the binding buffer; sample loading; wash of the column with the same buffer; and elution and regeneration of the column with the elution buffer. The binding/washing buffer consisted of 10 mM Tris-HCl (pH 7.1) and the elution/regeneration buffer 10 mM Tris-HCl (pH 7.1) with 1 M NaCl. Two types of operational modes of elution were tested – linear gradient and step wise elutions.

Linear elution

The followed method was the same for both types of columns and protein products. The column was first equilibrated with 5 column volumes (CV). Then,1 mL of the sample was injected into the column (with a 1 mL loop). In order to remove the unbounded material, the column was washed with 2 CV of binding buffer. To perform the linear gradient elution, 0 to 100%, 20 CV of elution buffer were used.

Step wise elution

After analysing the chromatograms obtained from each column using the linear elution, the AEX method was altered, as indicated below. All the eluted fractions were collected, and the ones corresponding to peaks were pooled, concentrated, quantified, and characterized by SDS-PAGE.

1 mL HiTrap Q Sepharose FF column

For the protein product α (extract stored at pH 13), 5 CV of binding buffer were used to equilibrate the column, and 1 mL of extract was injected. With 5 CV of elution buffer, one step was induced at 140 mM NaCl and the second at 1 M of NaCl. After elution, the column was regenerated with 15 CV of elution buffer with 1 M NaCl to remove the remaining protein attached. For the protein product extract β, the method was the same with the difference that 3 steps were induced, the first at 60 mM NaCl, the second at 220 mM NaCl and the last at 1 M of NaCl.

1 mL HiTrap DEAE FF

For the protein product α, the method performed only differed from the *HiTrap Q Sepharose FF* column in the way that the first step was programmed to occur at 60 mM NaCl. The method followed for protein product β was the same as for the *HiTrap Q Sepharose FF* column.

3.2.8. Protein characterization

3.2.8.1. Concentration of the protein fractions

Aiming at protein characterization by SDS-PAGE, the collected fractions after SEC and AEX separations needed to undergo a concentration step. Due to the small volumes necessary for the electrophoresis, it was decided to perform the concentration step by evaporating the solvent under vacuum in a DNA SpeedVac System (Thermo Fischer Scientific, USA) until the desired volume was obtained (~50 µL). A 2 µL drop was placed in the NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fischer Scientific, USA) to assess the final concentration by reading the absorbance at 280 nm.

3.2.8.2. SDS-PAGE

Samples were characterized by one-dimensional SDS-PAGE, which was carried out using a Mini Protean Cell Electrophoresis unit (BioRad, USA) in a 4% (w/v) stacking gel and a 12% (w/v) acrylamide resolving gel, prepared from a 40% acrylamide/bis stock solution (37.5:1). The resolving gel was composed by 11.68% acrylamide/bis-acrylamide 40% solution, 1x resolving buffer (0.375 M Tris-HCl (pH 8.8), 0.1% SDS), 0.05% TEMED and 0.25% APS. The stacking gel was composed by 3.89% acrylamide/bis-acrylamide 40% solution, 1x stacking buffer (0.125 M Tris-HCl (pH 6.9), 0.1% SDS), 0.01% TEMED and 0.05% APS.

The samples preparation consisted of mixing 32 µL of protein sample with 12 µL of Laemmli sample buffer and 5 µL of DTT. This mixture was heated until 100°C for 10 min to ensure total denaturation and then cooled at RT.

For the electrophoresis run, each gel lane was loaded with 20 µL of the concentrated sample and run at 120 mV using a running buffer composed of glycine 192 mM, Tris 25 mM pH 8.3 and 0.1% SDS. The molecular weights of bands of the protein fractions were compared to Precision Plus Protein Standards Dual Color marker, 250-10 kDa (BioRad, USA). The gels were silver stained, as described in 3.2.8.3.

3.2.8.3. Silver staining

First, the bands were fixated using a 30% (v/v) ethanol and a 10% (v/v) acetic acid solution for 2 h. After that, the gel was washed by 3 washing steps. The 1st with 30% (v/v) ethanol for 10 min followed by 2 washing steps with Milli-Q water of 10 min each. The sensibilization step was completed in 1 minute

with 0.02% (w/v) sodium thiosulfate, and after being washed 3 times with Milli-Q water for 30s each, the gels were incubated in a silver nitrate solution of 0.15% (w/v) for 30 min, followed by the last washing step with Milli-Q water for 1 min. The development step was carried out with a 3% (w/v) sodium carbonate and 0.05% (v/v)) formaldehyde solution. This solution was added until the bands started to appear. The reaction was stopped using a 5% (v/v) acetic acid solution for 15 min. The gels were scanned and analysed using Quantity One® 1-D Analysis Software (BioRad, USA).

3.3. Analytical methods

3.3.1 Biomass characterization

3.3.1.1. Total solids, moisture and ash content

The content of total solids, moisture and ash present in the biomass (alga and alga residues after agar extraction) were determined using the National Renewable Energy Laboratory's (NREL) "Determination of Total Solids and Ash in Algal Biomass" Laboratory Analytical Procedure (LAP) [178], with some minor modifications. Crucibles were pre-conditioned overnight in a 575 ºC muffle furnace (L 24/11 Nabertherm, Germany) and allowed to cool to room temperature in a desiccator. Each crucible was weighted (Mettler Toledo AG245) to the nearest 0.1 mg, and 100 mg of biomass was added (n=3). The crucibles were then placed in a convection drying oven at 60 ºC and atmospheric pressure for 18 hours (Function Line, Heraeus®). After withdrawing the samples, they were allowed to cool to room temperature in a desiccator. The crucibles with the oven-dried samples were weighed. The percentages of total solids, moisture, and oven-dry weight (ODW) were determined using equations 3.10, 3.11 and 3.12, respectively.

$$Total\ Solids\ (\%) = \frac{weight_{crucible+dried\ sample} - weight_{crucible}}{weight_{biomass}} . 100 \qquad \textit{Equation 3.10}$$

$$Moisture\ (\%) = 100 - Total\ Solids\ (\%) \qquad \textit{Equation 3.11}$$

$$ODW_{sample} = \frac{weight_{dried\ sample} \cdot Total\ Solids(\%)}{100} \qquad \textit{Equation 3.12}$$

After oven drying, the samples were reduced to ashes in the same muffle furnace programmed with a ramping program: from RT to 105 ºC holding for 12 min, to 250 ºC at 10 ºC/min holding for 30 min and finally to 600 ºC at 20 ºC/min holding for 16 hours. The crucibles were allowed to cool to room temperature in a desiccator and were weighed. Equation 3.13 was used to determine the ash content.

$$Ash\ (\%) = \frac{weight_{crucibles+ash} - weight_{crucibles}}{ODW_{sample}} . 100 \qquad \textit{Equation 3.13}$$

3.3.1.2. Total carbohydrate content

Total carbohydrates were determined according to the NREL's "Determination of Total Carbohydrates in Algal Biomass" LAP [179], with some minor modifications.

Algal biomass (0.5 g) was weighted (AG245 digital analytical laboratory scale, Mettler Toledo) into a 250 mL Schott Duran® laboratory glass bottle (n=3). Each bottle received 5 mL of 72% (w/w) sulfuric acid and was incubated in an orbital agitator (Agitorb 160E, Aralab, Portugal) for 1h at 30 ºC and 250 rpm. Afterwards, by adding 138.5 mL of Milli-Q water, the sulfuric acid concentration was reduced to 4% (w/w). The bottles were autoclaved for 1h at 121 ºC. After that, they were allowed to cool to RT for approximately 1h. After vortexing (laboratory shaker 444-1378, VWR), aliquots of 10 mL were transferred to 50 mL Falcon conical centrifuge tubes and neutralized until a pH of 6-8 with the slow addition of calcium carbonate (powder). The samples were centrifuged for 10 min, 4000 x g (Centrifuge 5810 R, A-4-62 swing-bucket rotor, Eppendorf, Germany) and a supernatant sample of 2 mL was taken and stored at -20 ºC until further analysis.

High-Performance Liquid Chromatography (HPLC; Hitachi LaChrom Elite) was used to quantify monosaccharides after the two-step sulfuric acid hydrolysis (section 3.3.2). Samples were analysed in an HPLC system equipped with a Rezex ROA Organic acid H + 8% (30mm × 7.8mm) column, a Hitachi LaChrom Elite L-2200 autosampler, a Hitachi LaChrom Elite L-2130 pump, and a Hitachi L-2490 refraction index detector. The sample injection volume was 20 μL, a 5mM solution of H_2SO_4 was used for the elution, and the flow rate of the pump was set to 0.5 mL/min. The column was kept at 65 ºC (heater for large columns connected externally to the HPLC system, Croco-CIL 100-040-220 P, 40cm × 8cm × 8 cm, 30–99ºC). The samples were first centrifuged for 5 min at 10,000 x g (115 P microcentrifuge, Sigma-Aldrich, Germany), then 200 µL of the resultant supernatant were diluted with 200 µL of 50 mM H_2SO_4. The mixture was vortexed and centrifuged again. To prepare the HPLC vials, the supernatant was diluted 10x with 50 mM H_2SO_4 for a final volume of 1 mL. The same method was previously used to prepare standards of glucose and galactose in order to obtain the calibration curves, presented in *Appendix A.2*.

The cellulose and the agar content were determined using Equations 3.14 and 3.15, respectively, where 162 is the MW of glucose and galactose monomeric units in polymeric glucan and galactan, 180 is the MW of glucose and galactose, and 1.27 is the weight ratio between L-3,6-anhydro galactose (AHG) and D-galactose in agar [179].

$$Cellulose = \frac{c_{glucose} \cdot \frac{162}{180}}{c_{biomass} \cdot ODW} \cdot 100 \qquad \textit{Equation 3.14}$$

$$Agar\ (\%) = \frac{(c_{galactose} + 1.27 \cdot c_{galactose}) \cdot \frac{162}{180}}{c_{biomass} \cdot ODW} \cdot 100 \qquad \textit{Equation 3.15}$$

3.3.1.3. Total protein content

Total protein content in samples of 0.1 g (n=3) was determined at IPMA (partner in the project Smart Seaweed), using a nitrogen analyser FP-528 DSP LECO (LECO, St. Joseph, USA) calibrated with EDTA ($y = 8.4 \cdot 10^{-1}x - 2.3 \cdot 10^{-3}$) using the Dumas method [180] with a nitrogen-to-protein conversion factor of 4.59 [181].

3.3.1.4. Lipid content

Lipid content in samples of 0.2 g (n=3) was determined at IPMA, using the Soxhlet method. The content is determined by gravimetry after extraction with the solvent ether and its evaporation.

3.3.2. Protein products characterization

3.3.2.1. Chemical composition

The concentrated extracts resultant from procedures 3.2.2.1 and 3.2.3.2 were lyophilized for 48h (Alpha 1-2 LD plus, Martin Christ, Germany) at an initial and final temperature of -41ºC and -39ºC, respectively. Then, the powders were characterized regarding total solids, moisture, ash, protein and lipid content as described in section 3.3.1, except for carbohydrate content that was determined using the Dubois method [182], also known as the phenol-sulfuric method, with some minor modifications.

Briefly, to 200 µL of sample or standard, 200 µL of 5% phenol solution and 1 mL of concentrated sulfuric acid solution 96% were rapidly added. The mixture was vortexed and incubated for at least 60 min at RT. Using 104-QS 10mm Hellma Analytics cuvettes, the absorbance was read at 485 nm in a DR3900 spectrophotometer (Hach Lange, USA). A calibration curve ranging from 0 to 0.1 g/L was previously obtained using a stock solution of standard glucose of 1 mg/mL, presented in *Appendix A.3.*

3.3.2.2. Protein bioaccessibility

Protein bioaccessibility was determined by *In vitro* digestion procedure and was performed by IPMA. The method followed was based on the work developed by C. Versantvoort *et al*. [183]. *In vitro* digestion models recreate the digestion process in the human gastrointestinal tract by using physiologically based conditions, such as the chemical composition of digestive fluids, pH, and residence times typical for each digestion fraction. The digestion starts by adding 6 mL of saliva to 0.3 g of sample and is incubated for 5 min. Afterwards, 12 mL of gastric juice was added, and the mixture was incubated for 2 h under agitation. Finally, 12 mL of duodenal juice, 6 mL of bile and 2 mL of bicarbonate solution are added, and the mixture is incubated for another 2 h under agitation. At the end of the *in vitro* digestion process, the digestion tubes are centrifuged for 5 min at 2750 x g, enabling the recovery of the chyme (supernatant), where the nitrogen content was measured and the digested matrix (pellet). Bioaccessibility is determined as the fraction of external dose released from its matrix. Table 3.4 show

the composition of the solutions used to mimic the human gastrointestinal process. The protein content was determined as described in 3.3.1.3.

Table 3.4: Composition of the synthetic juices of the in vitro digestion model.

	Saliva (pH 6.8)	*Gastric juice (pH 2)*	*Duodenal juice (pH 8)*	*Bile juice (pH 8)*
Inorganic solution	10ml KCl 89.6 g/l 10ml KSCN 20 g/l 10ml NaH_2PO_4 88.8 g/l 10ml $NaSO_4$ 57 g/l 1.7ml NaCl 175.3 g/l 20ml $NaHCO_3$ 84.7 g/l	15.7ml NaCl 175.3 g/l 3.0ml NaH_2PO_4 88.8 g/l 9.2ml KCl 89.6 g/l 18ml $CaCl_2$ 22.2 g/l 10ml NH_4Cl 30.6 g/l 6.5ml HCl 37% g/g	40ml NaCl 175.3 g/l 40ml $NaHCO_3$ 84.7 g/l 10ml KH_2PO4 8 g/l 6.3ml KCl 89.6 g/l 10ml $MgCl_2$ 5 g/l 180 µl HCl 37% g/g	30ml NaCl 175.3 g/l 68.3ml $NaHCO_3$ 84.7 g/l 4.2ml KCl 89.6 g/l 150 µl HCl 37% g/g
Organic solution	8ml urea 25 g/l	10ml glucose 65 g/l 10ml glucuronic acid 2 g/l 3.4ml urea 25 g/l 10ml glucoseamine hydrochloride 33 g/l	4ml urea 25 g/l	10ml urea 25 g/l
Add to mixture organic + inorganic solution	290 mg α-amylase 15mg uric acid 25mg mucin	1 g BSA 2.5 g pepsin 3 g mucin	9ml $CaCl_2$ 22.2 g/l 1 g BSA 9g pancreatin 1.5 g lipase	10ml $CaCl_2$ 22.2 g/l 1.8 g BSA 30 g bile

3.3.3. Protein quantification

The protein concentration in the extracts was determined using the Lowry method [184]. To 0.1 mL of sample or standard, 0.1 mL of 2N NaOH was added. Hydrolysis took place for 10 min at 100ºC in a digital heating block (Biotrace International). The hydrolysate was allowed to cool to room temperature before adding 1 mL of freshly prepared complex-forming reagent [2% (w/v) Na_2CO_3 in distilled water + 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water + 2% (w/v) sodium potassium tartrate in distilled water] in the proportion 100:1:1 (by volume). After waiting 10 min at RT, 0.1 mL of 1 N Folin reagent was added. The mixture was vortexed and incubated at RT for 30 min. The absorbance was read at 750 nm in a DR3900 spectrophotometer (Hach Lange, USA) using 104-QS 10mm Hellma Analytics cuvettes. In order to obtain a calibration curve, with standards ranging from 0 to 0.5 mg/L of protein, a bovine serum albumin stock solution containing 2 mg/mL of protein in distilled water was used. The calibration curve is shown in *Appendix A.1.*

The protein extracted and the protein extraction yield were calculated using Equations 3.16 and 3.17, respectively.

$$[\text{Protein}](g/100\ biomass\ \text{dw}) = \frac{[\text{Protein}]_{\text{supernatant}}(g/L).\text{V}_{\text{supernatant}}(L).\text{dilution factor}}{ODW\ (g)}.100 \qquad \textit{Equation 3.16}$$

$$\text{Protein Extraction Yield}\ (\%) = \frac{[\text{Protein}]_{\text{supernatant}}(g/L).\text{V}_{\text{supernatant}}(L).\text{dilution factor}}{ODW\ (g).\frac{Total\ Protein\ (\%)}{100}}.100 \qquad \textit{Equation 3.17}$$

3.4. Statistical analysis

MS Excel was used for statistical analysis of the results. Data are presented as mean values ± standard deviation (SD), with most experiments performed in triplicate (n=3). A one-way analysis of variance (ANOVA) was used when comparing more than two sets of experimental data, with a significance level of p=0.05.

4. Results and Discussion

4.1. Biomass characterization

The chemical composition of the starting biomass is presented in Table 4.1.

Table 4.1: Chemical composition of Gelidium sesquipedale (crude) and industrial residues after agar extraction (dry weight basis). Values are expressed as mean ± standard deviation, n=3.

Component	***Gelidium sesquipedale***	***Industrial Residues***
Total Solids	92.8 ± 0.3%	89.4 ± 0.2%
Moisture	7.2 ± 0.2%	10.6 ± 0.2%
Total Carbohydrates (% dw)	62.1 ± 2.5	34.9 ± 3.4
Cellulose	10.5 ± 1.4	22.4 ± 1.3
Agar	51.6 ± 1.2	12.5 ± 3.0
Protein (% dw)	14.8 ± 0.2	20.6 ± 0.7
Lipids (% dw)	0.7 ± 0.1	0.7 ± 0.1
Ash (% dw)	19.8 ± 0.9	18.5 ± 0.7

The high total solids content and, as a result, the low moisture content in both biomasses are consistent with the drying treatment performed on the alga and on the residues, which guarantees a moisture content of less than 20%. Even though sun-drying is not the best drying method to avoid the deterioration of sensitive bioactive compounds, it is still used for seaweed drying prior to polysaccharides extraction.

The total carbohydrate content for *Gelidium sesquipedale* (62.1 ± 1.7% dw) matches the statement that carbohydrates comprise 30% to 60% of the dw of red algae [48], and it is in agreement with the value previously reported for this species, 62.4 ± 3.5% dw [11]. For *Gelidium amansii*, a value of 59.9% dw was obtained using a similar quantification method (HPLC after saccharification) [153]. Nevertheless, it is higher than some values reported in the literature for other *Gelidium sp.* species. Most of the values reported in Table 2.5 were obtained by performing different quantification methods. Phenol-sulfuric acid often fails to match HPLC quantification of even a simple mixture of sugars [179]. The determined agar content is also higher than the values reported in Table 2.5. Because the L-3,6-anhydro galactose (AHG) content was calculated indirectly, a galactose assay kit could also be used to confirm this result. The cellulosic content obtained is comparable to those reported for *Gelidium* species, such as *Gelidiella acerosa* and *Gelidium pusillum*, which have cellulosic contents of 13.7% and 9.3%, respectively [153]. Regarding the industrial residues, as expected, the total carbohydrate content is lower than the value obtained for the crude due to the agar extraction process. A higher value of 44.2% dw basis carbohydrate content has been reported for *Gelidium sesquipedale* waste biomass obtained after agar extraction [185], determined using the same quantification method.

The protein content obtained (14.8 ± 0.2% dw) is below the average reported for red macroalga, generally located between 20-30% dw [28]. Nevertheless, it is comparable with the values of protein content in *Gelidium* species. Values of 10.5 ± 0.1% dw, 11.3 ± 1.0% dw, 23.4 ± 0.7% dw and 13.4%

have been reported for *Gelidium amansii* [173], *Gelidium pusillum*, *Gelidium microdon* e *Gelidium corneum*, respectively [153]. Although these values were also determined using total nitrogen quantification - Kjeldahl method - a nitrogen-to-protein factor of 6.25 was used instead. The use of this general factor is frequently unsuitable because it overestimates the protein content. A study considering 9 different strains of red algae under various growth conditions reported a factor of 4.59 as a better estimation [181], hence the chose of a conversion factor of 4.59 to be used in this work. In addition, it is also known that the protein content varies along the time of the year, and for most seaweeds, it is higher during winter and early spring [16]. Although this correlation has not been assessed for *Gelidium sesquipedale,* the harvest of the biomass took place between July and November, which may also influence the low obtained value. Even though these values are not comparable to those in high-protein vegetables, as mentioned in section 2.1.3.4, they are still comparable to protein content in cereals (7-15% dw). Concerning the industrial residues, as the carbohydrate fraction was partially withdrawn, the protein content increased (20.6 ± 0.7% dw) on a dry weight basis. A higher value of 25% dw of protein was reported in *Gelidium sesquipedale* residues after agar extraction, using a conversion factor of 6.25 in the Kjeldahl method [158].

The lipid content obtained was the same for both biomasses (0.7 ± 0.1% dw), suggesting that the agar extraction process resulted in some lipid loss. Otherwise, the lipid content in the industrial residues should be higher than in the crude alga. These values are in agreement with the values found in the literature that reported a lipid fraction accounting between 0.7% to 2.2% dw in *Gelidium* species [186].

The ash fraction in red algae species varies according to geographical location and season and it is related to mineral content. It is much higher than that found in terrestrial vegetables, partially due to the high sodium content resulting from the contact with seawater. The ash contents obtained for both algal biomasses are comparable to the values reported in the literature. Content of 21.2% dw was reported for *Gelidium pusillum* [154], and 34 red algae strains showed an average of 22.9 ± 11% dw [187]. The lower content in the industrial residues is a result of mineral loss during the agar extraction process. If there were no removal of minerals during the extraction process, then a higher ash content in the residues would be expected due to the decrease in dry weight.

It should be noted that the sum of all components content from the industrial residues biomass does not comprise the value of the total solids.

4.2. Pre-treatment of *Gelidium sesquipedale* in agar extraction

As mentioned in section 2.6.1, the macroalgae are subjected to a pre-treatment to optimize phycocolloid extraction in the agar extraction industry. In the case of *Gelidium* species, it consists of a mild alkali treatment to eliminate phycoerythrin pigment and macerate the seaweed aiming for a better extraction. However, these conditions can simultaneously induce protein solubilization, diminishing the protein content of the residue. The possibility of protein recovery from alga residue is of great interest in the scope of a cascade approach of a biorefinery, contributing towards a “no waste” agar extraction industrial process. In order to quantify the amount of protein potentially lost in this step, a procedure under similar conditions, i.e., algal suspension in a mild solution of sodium carbonate heated to 90 ºC

for 2 h, was carried out. After centrifugation of the algal suspension, a protein concentration of 1.29 ± 0.04 g/L was determined in the resultant supernatant, corresponding to a loss of 3.26 g protein/100 g of alga. Protein loss results are presented in Table 4.2.

Table 4.2: Supernatant volume (L) and protein concentration (g/L) determined after centrifugation of the algal suspension subjected to an alkaline treatment, using the Lowry method (n=3) and average protein loss on this step, considering the initial protein content of Gelidium sesquipedale and initial protein mass. Values are expressed as mean ± standard deviation, n=2.

$(Protein\ content)_{initial}$ (% dw)	$(Protein\ mass)_{initial\ (g)}$	$V_{inicial}$ (L)	$V_{collected}$ (L)	$(Protein\ concentration)_{supernatant}$ (g/L)	Average Protein Loss (%)
14.8 ± 0.2	1.48 ± 0.2	0.30	0.26	1.29 ± 0.04	22.8 ± 0.2

This result could not be compared with values in the literature since other processes for the pre-treatment step in agar extraction are reported using different conditions, e.g., solvents, temperature, and process time, resulting in different protein solubilization and consequent loss. For example, alkaline pre-treatments were also reported using a 5-10% (w/v) sodium hydroxide solution [159] [188] during a range of time from 1h to 12h [188], and a range of working temperatures from 80 ºC to 100 ºC [143]. The use of an 80% ethanol solution was also reported for *Gelidium latifolium* [189]*,* Here the loss of protein might even be higher due to the possible presence of alcohol-soluble proteins*.* Using a stronger alkaline solution (1M NaOH) in the pre-treatment of seaweed has resulted in higher gel strength for *Gelidium, floridanum* [190]. However, it could result in the hydrolysis of the protein, compromising, even more, the protein content in the residues.

The possible protein loss in the cooking in boiling water step during agar extraction was not assessed, but a protein extraction procedure in the same conditions should also be performed, as the hot aqueous medium can also induce protein solubilization.

4.3. Up-scaled protein extraction

The two most promising procedures previously designed for the protein extraction and recovery from *Gelidium sesquipedale* [11] were repeated with higher processing capacity; on the one hand, for the assessment of the feasibility of the process, and in the other to obtain sufficient extracted protein to perform further characterization studies. Since procedure A likely originates intact proteins and procedure B, due to the use of a protease, results in a rich peptide extract, the extracts are expected to have different properties. The chosen extraction strategies were also assessed with the industrial residues of *Gelidium* after agar extraction to characterize the protein fraction still present and evaluate the possibility of further upgrading this biomass.

Starting with 100 g of algal biomass, procedure A consisted of the sequential aqueous and alkaline extractions, with respective re-extractions (same extraction conditions applied to the pellet obtained after previous extraction step). Aqueous extractions were executed at 4 ºC for 16h, while alkaline extractions were performed at RT for 1h. At the end of the process, the pH of the algal suspension was 12.8 ± 0.2.

Procedure B was carried out starting with 75 g of algal biomass and included the sequential steps of 1) enzymatic-assisted aqueous extraction with Celluclast®, 2) enzymatic-assisted aqueous extraction with Alcalase®, 3) alkaline extraction and 4) alkaline re-extraction. The enzyme assisted extractions also consisted of a hot aqueous extraction once they were carried out at 50 ºC for 16h. The initial pH of the aqueous extraction with Celluclast® was set to 4.5 ± 0.02, and with Alcalase® to 8 ± 0.01. At the end of the procedure, the pH of alkaline re-extraction reached values of 13.2 ± 0.3. Due to the protease action of Alcalase®, the resultant extract after the enzyme assisted extraction was subjected to a thermal shock treatment to assure the enzyme inactivation and avoid further hydrolysis of the peptides during extract storage. Additional extraction parameters, including volume, extraction time, protein mass extracted, protein concentration and recovery yield, are presented in Table 4.3. Figures 4.1 and 4.2 present the total protein extracted in grams per 100 grams of algal biomass (dry weight) obtained with procedures A and B, respectively.

Table 4.3: Protein content, solvent volume used, volume collected, total protein mass extracted, protein concentration in the obtained extract and protein recovery yield of procedures A and B for both crude and industrial residues from Gelidium sesquipedale. Protein recovery yield is expressed in % of total protein (Equation 3.14). Protein concentration in the pooled extracts (combined supernatants) was determined using the Lowry method (n=3). Values are expressed as mean ± standard deviation, n = 2

Algal Biomass	*Procedure*	*(Protein mass)$_{available}$ (g)*	*V_{used} (L)*	*$V_{collected}$ (L)*	*Concentration (g/L)*	*Total Mass (g)*	*Protein Recovery Yield (%)*
Crude	A	14.8 ± 0.2	6	5.2	$0.35 \pm 1 \cdot 10^{-3}$	1.78	12.0 ± 1.2
	B	20.6 ± 0.7	4.5	4.2	$1.39 \pm 2 \cdot 10^{-2}$	5.77	52.1 ± 1.7
Industrial Residues	A	11.1 ± 0.2	6	5.4	$0.58 \pm 1 \cdot 10^{-3}$	3.16	15.4 ± 0.9
	B	15.5 ± 0.7	4.5	4.1	$1.38 \pm 8 \cdot 10^{-3}$	5.64	36.5 ± 1.3

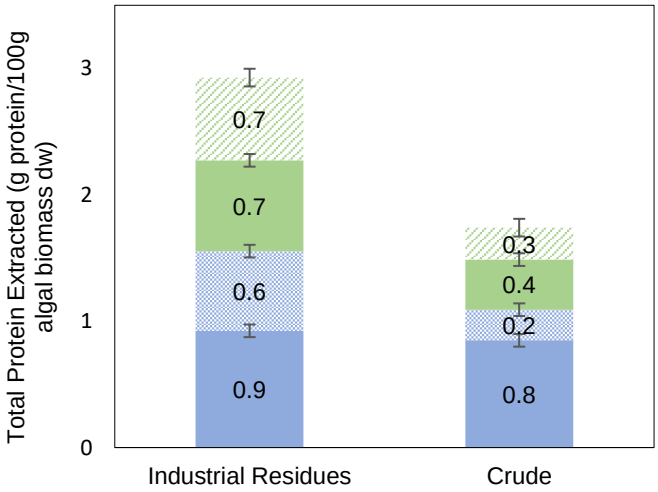

Figure 4.1: Total protein extracted in procedure A (one aqueous extraction, one aqueous re-extraction, one alkaline extraction and one alkaline re-extraction), in grams of protein per 100 grams of algal biomass (dryweight), for Gelidium sesquipedale crude and industrial residues using the Lowry method (n=3). Values are expressed as mean ± standard deviation, n = 2. (■ - aqueous extraction (16h, 4ºC); ■ - aqueous re-extraction (16h, 4ºC); ■ - alkaline extraction (1h, RT); ■ - alkaline re-extraction (1h, RT).

Sequential aqueous and alkaline extractions yielded 1.78 ± 0.03 g protein/100 g algal biomass dw (12.0% of protein recovery) for the crude alga and 3.16 ± 0.01 g protein/100 g algal biomass (15.4% of protein recovery) for the industrial residues. These values are in agreement with those determined for *Gelidium sesquipedale,* 14.7 ± 2.3% of protein recovery yield [11] and reportedly for *Porphyra umbilicalis,* 15 ± 4.8% [191] but are lower than some found in the literature for this type of extraction protocol. For *Ulva rigida, a* protein recovery yield of 26.8 ± 1.3% was reported [87]. However, protein content and yields differ significantly between groups of algae, so it may be improper to compare results directly. A similar extraction method employed in the agar industrial residues from *Gelidium sesquipedale* obtained a considerably higher protein recovery yield of 40%. However, the authors used the Kjeldahl method with a conversion factor of 6.25 as the protein quantification method [87]. As already stated, this value often results in protein overestimation.

Comparing the extraction procedure for both algal biomasses, no significant differences were observed ($p>0.05$), even though it was possible to obtain a higher amount of protein from the industrial residues (3.16 g *vs* 1.58 g obtained with the crude alga). In reality, it is not reflected on significantly higher recovery yield value (15.4 ± 0.9 obtained with the industrial residues *vs* 12.0 ± 1.2 with the crude alga) as the residue exhibits a higher protein content, in terms of % of dw, than the crude (20.6% vs 14.8% in the crude alga)

When comparing the water-soluble and the alkaline soluble proteins, the protein extracted during the aqueous process was significantly higher ($p<0.05$), suggesting that most proteins are soluble in water, specifically the protein pigment complexes, like the phycobiliproteins, as the obtained extract exhibited a reddish colour. Nevertheless, alkaline solutions (NaOH) have been shown to effectively solubilize and aid in the extraction of highly water-insoluble proteins from macroalgae [97]. Increasing the pH of alkaline extractions usually reflects an increased extraction yield due to the neutralization of side amine groups of basic amino acid acids such as lysine and arginine at a higher alkaline pH, which increases protein total negative charge, resulting in increased protein-solvent interaction and protein solubility [192]. However, in this range of pH values, protein denaturation is expected and inevitable, so this extraction method can only be applied when the proteins do not need to be in their active form. Incorporation of reducing agents, such as β-mercaptoethanol [29], L-cysteine-hydrochloride-monohydrate and NAC [97], which dissociate proteins from polysaccharides, have been reported to increase the yield of alkaline soluble proteins extraction. However, care should be taken when dealing with products for feed purposes, as β-mercaptoethanol is not considered food grade, and the others oblige to extra purification steps.

In procedure B, sequential hot aqueous assisted by enzymes and alkaline extractions led to a protein extraction of 7.2 ± 0.01 g/100 g algal biomass (36.5 ± 0.01% of protein extraction yield) and 6.7 ± 0.02 g/100 g algal biomass (52.1 ± 0.02% of protein extraction yield), for industrial residues and crude alga, respectively. Following this method, protein extraction significantly reached higher values than procedure A ($p<0.01$).

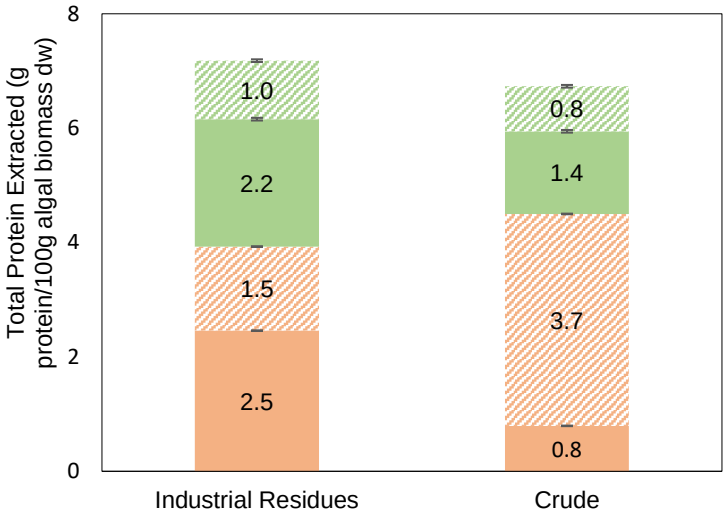

Figure 4.2: Total protein extracted in procedure B in grams of protein per 100 grams of algal biomass (dry weight), for Gelidium sesquipedale crude and industrial residues using the Lowry method (n=3). Values are expressed as mean ± standard deviation, n = 2. (■ - enzyme assisted extraction with Celluclast® (16h, 50ºC); ■ - enzyme assisted re-extraction with Alcalase® (16h, 50ºC) ; ■ - alkaline extraction (1h, RT); ■ - alkaline re-extraction (1h, RT)

Even though the overall procedure was not significantly different between the two algal biomasses ($p>0.05$), it is observable that the different enzymes resulted in different outcomes for each biomass. Celluclast® BG consists of a mixture of endocellulase responsible for catalyzing the breakdown of the algal cell wall cellulose into simple sugars. By disrupting the cell wall, allows proteins to be more accessible. The aqueous extraction assisted by this enzyme resulted in a total protein extraction of 2.5 ± 0.01 g/100 g algal biomass and 0.8 ± 0.03 g/100 g algal biomass for the industrial residues and the crude alga, respectively. This result suggests that the agar extraction process from this alga greatly influences the algal cell wall structure and its partial disruption, resulting in improved use of Celluclast®. However, when comparing the results from Alcalase® use, the inverse phenomenon is observed. Alcalase belongs to the endopeptidase enzymes class, and its use leads to the hydrolysis of peptide bonds that link amino acids in the polypeptide chain, which has been shown to improve protein extraction significantly. The disappointing results of this extraction step on industrial residues may be due to the probable previous protein hydrolysis in the agar extraction process. So, other enzymes from different classes should be investigated to improve protein extraction from the residues.

The obtained results with this extraction procedure agree with those previously obtained for *G. sesquipedale* following the same conditions (38.5 ± 3.5% of protein recovery yield). However, they were inferior to those reported. In *Palmaria palmata,* the use of Alcalase® alone significantly increased the protein extraction (54.3-55.2%), and the combination of Celluclast® and Alcalase® resulted in extraction yield values as high as 90% [91].

The results attained in this work are not entirely comparable to those described in the literature because of differences in algae genus, the extracting parameters or even the protein quantification method used. It is also worth mentioning that due to the hygroscopic nature of seaweed, some water is absorbed during the extraction process, resulting in lower collected volumes than the initial solvent

volume. In this way, the obtained protein recovery yield may not correspond to the maximum possible to attain with this algal biomass.

4.3.1. Protein extracts concentration

The up-scaled extraction procedures resulted in high-volume diluted extracts, challenging in terms of downstream processing. For the characterization analysis intended to be implemented, a concentration step was necessary. The strategy followed depended on the expected molecular weights of the proteins/peptides present in each extract.

Regarding procedure A, as the protein extract is expected to contain intact proteins, an ultrafiltration system equipped with a 10 kDa NMWCO membrane was chosen. The process was carried on until the desired retentate volume was obtained, corresponding to a VCF of approximately 10. Imposing a maximum working pressure of 1 bar, the average permeate volumetric flux registered was 11.4 ± 3 $L.h^{-1}.m^{-2}$. For procedure B, as the extract is expected to have small peptides, a nanofiltration process resorting to a 400 Da NMWCO membrane was selected to ensure the minimum protein loss possible. Due to the significant dead volume of the system, the maximum VCF possible to attain was approximately 5. At an approximate working pressure of 25 bar, the average permeate volumetric flux was 3.2 ± 1.2 $L.h^{-1}.m^{-2}$. Additional process parameters are represented in Table 4.4.

Table 4.4: Concentration step using an ultrafiltration membrane with an NMWCO of 10 kDa for extraction procedure A and a nanofiltration membrane with an NMWCO of 400 Da for extraction procedure B (n=2 for each concentration strategy) - initial and final protein mass, initial and final volume, protein mass in the retentate, protein retention yield, and average rejection coefficient. The initial and final protein mass in the feed and retentate was determined using the Lowry method (n=3).

Concentration strategy	*Algal biomass*	*(Protein mass) initial (g)*	*$V_{initial}$ (L)*	*V_{final} (L)*	*(Protein mass) final (g)*	*Protein Retention Yield (%)*	*Average Rejection coefficient (σ)*
Ultrafiltration	Crude	1.78 ± 0.03	5.20	0.32	1.26 ± 0.01	70.7 ± 1.2	0.63 ± 0.08
	Industrial Residues	3.16 ± 0.01	5.40	0.45	2.23 ± 0.04	70.3 ± 1.5	0.73 ± 0.07
Nanofiltration	Crude	5.78 ± 0.02	4.20	0.95	5.19 ± 0.05	90.0 ± 1.9	0.90 ± 0.05
	Industrial Residues	5.64 ± 0.08	4.10	0.82	5.02 ± 0.05	89.2 ± 2.8	0.91 ± 0.03

The concentration of the protein extracts obtained from procedure A showed a protein loss of 0.52 g (29.3% of the initial protein mass) and 0.93 g (29.7% of the initial protein mass) from de crude alga and industrial residues extract, respectively. Even though both extracts are expected to have intact protein, a membrane with an NMWCO of 10 kDa may be too wide since some proteins possibly have a lower molecular weight and can pass through the membrane pores. The similar protein retention yield suggests that the extracts contain proteins with an identical molecular weight profile. The processing was highly time-consuming, taking around five days to be completed, so a system that would provide a higher membrane-active surface area and/or bear a higher working pressure should be investigated.

When comparing the nanofiltration results, a protein loss of 0.58 g (9.9% of the initial protein mass) and 0.52 g (10.8% of the initial protein mass) was registered from the crude, and industrial residues extract, respectively. The protein retention yield and the average rejection coefficient determined for both show that this membrane is suitable for the process. Even though the processing was not as time-consuming as the ultrafiltration, due to the higher pressure possible to apply, it still would be possible to improve by adding more plates and membrane discs to the system.

At the end of each filtration process, the system was rinsed with water and cleaned with an alkaline solution or caustic detergent for ultrafiltration and nanofiltration systems, respectively. Their water permeability was measured again to verify an eventual reversible membrane clogging or irreversible fouling effect. It took more than one cleaning cycle to restore the initial permeability, but none of the membranes showed irreversible fouling. Different process conditions (e.g., pH) should be assessed to minimize the protein and/or polysaccharides interactions with the membrane surface.

4.3.2. Overall process

At the end of the concentration process, considering the protein recovery yield obtained (Table 4.4), the combined extracts from procedure A resulted in a 1.26 ± 0.01 g of protein (2.81 g/L) from crude alga and 2.23 ± 0.04 g of protein (4.95 g/L) from their agar extraction residues, corresponding to an overall yield of 8.62% and 10.8%, respectively (Table 4.5). Concerning procedure B, a total of 5.19 ± 0.05 g (4.95 g/L) of protein and 5.02 ± 0.05 g of protein (5.15 g/L) were achieved for the crude and industrial residues alga, respectively, which corresponds to an overall yield of 46.8% and 33.5% respectively (Table 4.5).

Table 4.5: Total protein recovery yield (Protein recovered/Total protein·100) after each process step (extraction and concentration) and resultant overall yield for extraction procedures A (one aqueous extraction, one aqueous re-extraction, one alkaline extraction and one alkaline re-extraction) and B (one enzymatic-assisted aqueous extraction with Celluclast®, one enzymatic-assisted aqueous extraction with Alcalase®, one alkaline extraction and one alkaline re-extraction) for both crude and industrial residues biomass.

Procedure	*Algal Biomass*	*Protein Extraction Yield (%)*	*Protein Retention Yield (%)*	*Overall Yield (%)*
A	Crude	12.0 ± 1.2	70.7 ± 1.7	8.62 ± 2.9
	Industrial Residues	15.4 ± 1.7	70.3 ± 2.1	10.8 ± 2.8
B	Crude	52.1 ± 0.9	90.0 ± 1.5	46.8 ± 1.4
	Industrial Residues	36.5 ± 1.3	89.2 ± 1.4	32.5 ± 2.7

It should be noted that ideally, during the downstream processing, a further purification step should have been performed, at the very least protein precipitation. However, due to time limitations and the urgency to deliver the protein products to the project partners, it was impossible to do it. In that sense, the obtained concentrated extracts are not considered purified protein products.

4.3.3. Protein products characterization

4.3.3.1. Chemical composition

After concentration, the protein extracts were lyophilized for 48h and afterwards characterized regarding the chemical composition. Figure 4.3 shows the obtained results.

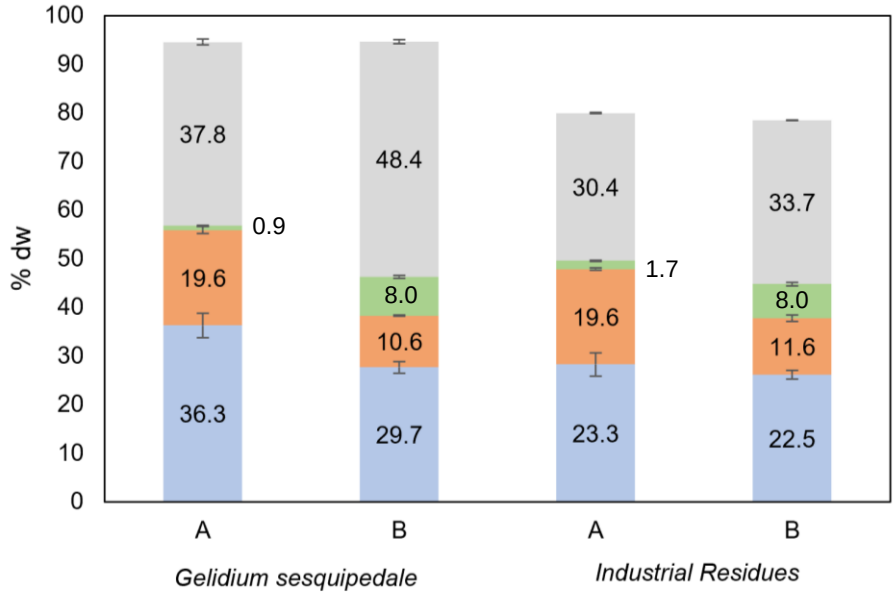

Figure 4.3: Chemical composition of the protein products (dry weight basis) obtained after extraction procedures A and B, using Gelidium sesquipedale (crude) and industrial residues after agar extraction as biomass. Values are expressed as mean ± sd, n = 3 (- Total Carbohydrates; - Protein; - Lipids; - Ash)

The protein content found in the lyophilized extracts was lower than expected, showing that the performed extractions also extracted partially the carbohydrate fraction. The protein and carbohydrates content in the extracts obtained with extraction procedure A are comparable to those found in a study conducted by M. Kazir *et al.*, which reported protein content of 20% dw and carbohydrate content of 37% dw in an extract obtained after aqueous and alkaline sequential extractions from *Ulva sp* and protein precipitation using ammonium sulfate [95]. An isolation/purification step, other than protein precipitation with ammonium sulfate that, as reported, failed to increase protein concentration must be performed in order to remove other components and increase the protein content. No values could be found reporting of protein content in the resultant extracts after enzyme assisted extractions.

The carbohydrate fraction was determined using the Dubois method, also known as the phenol-sulfuric acid method. Even though the saccharification followed by HPLC is considered a more reliable method, it is not advised when the samples exhibit an ash content > 10% [179]. The phenol-sulfuric acid is rapid and straightforward, however, and it is highly dependent on the sugar used for calibration. Not all carbohydrates exhibit a similar colourimetric response, and some do not even show any, so an over- or underestimation of the carbohydrate content can happen [193]. In the present case, a probable underestimation of the carbohydrate content in the products with origin in the industrial residues as biomass happened, as the sum of all components does not comprise the total solids content. An alternative carbohydrate quantification should be assessed.

The lipid content was significantly higher ($p<0.01$) in the extracts obtained with procedure B than in the initial biomass (8.0% vs 0.7% in both initial biomasses). Perhaps, the extractions performed at 50 ºC enhanced the lipid solubilization. In *Ulva lactuca,* the rise of temperature has been found to enhance oil extraction, with the highest yield obtained at 55 ºC [194]. Regarding the extracts obtained with procedure B, no significant differences lipid content differences were observed compared to the initial biomass.

The lowest ash content was found in the extracts generated using the industrial residues biomass. Nevertheless, the ash content was significantly higher ($p<0.01$) than that in the respective biomass, indicating that the performed extraction steps were found to concentrate minerals in the resultant extracts. These high values could also be due to the use of two alkaline sequential steps with sodium hydroxide. When assessing the conductivity of the pooled extracts after procedure B for both crude and residues biomass, values of 5410 and 4800 µS/cm were registered, respectively. In a rough estimation, if considering that is all due to sodium hydroxide, it gives an approximate concentration of salts of 1 g/L and 880 mg/L, respectively. Since the membrane used in nanofiltration cannot remove the divalent ions in the solution, a desalting step should be performed to lower the ash fraction. Harrysson et al. (2018) found that when performing protein extraction by sonication in water, subsequent ammonium sulfate precipitation and dialysis against water at 4 ºC, the ash content in the obtained extract was significantly lower than in the initial biomass [87].

To obtain protein products with a higher protein content, a protein isolation step should be carried out.

4.3.3.2. Protein Bioaccessibility

Protein products were digested *in vitro,* simulating the human digestion process to assess protein bioaccessibility. The method was also applied in the crude alga and in the industrial residues to verify the effectiveness of the protein extraction procedures. Figure 4.4 shows the obtained results.

All the protein products showed an improved protein bioaccessibility when compared to their initial biomass. The high fibre content (17-33%) of the algal cell wall is considered one of the primary reasons for reduced protein digestibility in unprocessed seaweed since it can block the access of digestive enzymes and decrease the activity of proteolytic enzymes. Similarly, phenolic compounds can form insoluble compounds when reacting to amino acids, also decreasing bioaccessibility. The alga industrial residues show accessibility significantly lower than the crude alga, suggesting that the agar extraction process causes structural differences in the alga components, jeopardizing access to the protein fraction.

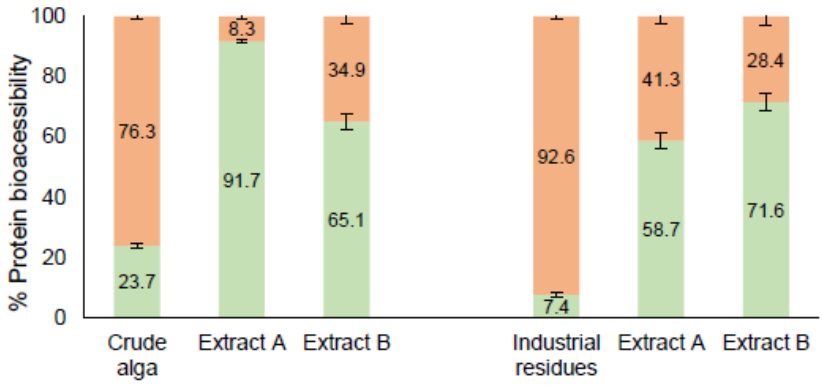

Figure 4.4: Protein bioaccessibility (%) obtained after In vitro digestion process in the initial biomass (crude and industrial residues) and in the protein products obtained after extraction procedure A ((one aqueous extraction, one aqueous re- extraction, one alkaline extraction and one alkaline re-extraction) and B (two enzymes assisted extractions with Celluclast® and Alcalase®, one alkaline extraction and one alkaline re-extraction) from both biomasses. Values are expressed as mean ± sd (n=3) (■ - not bioaccessible; ■ - bioaccessible)

The result of procedure A in the crude *Gelidium sesquipedale* is comparable to those reported in the literature for this type of extract. Assays using *Ulva pinnatifida* and Ulva pertusa protein extracts found 87% and 95% bioaccessibility [28]. Even though the obtained values of the remaining products were lower, they are still comparable to those found in commonly consumed plants, such as grains (69%–84%), legumes (72%–92%), fruits (72%–92%), and vegetables (68%–80%) [32]. Nevertheless, other extraction conditions should be investigated to improve these results, namely enzymes and solvents concentration.

4.4. Influence of the pH of the stored extracts on the size of the peptides

Due to the implementation of one alkaline extraction and one alkaline re-extraction, the pH of the pooled extracts was strongly alkaline. The effect of storage in these conditions or at neutral pH (result from neutralization of the final extract) in the size of peptides was assessed. The proteins present in the extract were precipitated, diafiltrated and concentrated, and finally characterized by size exclusion chromatography and gel electrophoresis.

4.4.1. Protein precipitation using ammonium sulfate

One of the most common methods for protein concentration and partial purification from a solution is ammonium sulfate precipitation. In solution, proteins form hydrogen bonds with water molecules via their exposed polar and ionic groups. The solubility of globular proteins increases when salt (<0.15 M) is added – salting in [175]. However, when large amounts of small, highly charged ions are added, such as ammonium sulfate, these groups compete with proteins to bind to the water molecules, removing the water molecules from proteins and decreasing their solubility, resulting in precipitation – salting-out [195]. The number and position of polar groups, the molecular weight of the protein, the pH of the solution, and the temperature are all critical factors in determining the concentration at which a specific protein will precipitate.

In order to find the suitable ammonium sulfate saturation for the proteins precipitation, 30 mL of the pooled extracts were subjected to increased ammonium sulfate concentration in solution – 70% to 85% saturation induced precipitation - for 16h at 4 ºC, with stirring. Table 4.6 and Figure 4.5 present the precipitation results.

Table 4.6: Ammonium sulfate precipitation parameters from the extract of procedure A (one aqueous extraction, one aqueous re-extraction, one alkaline extraction and one alkaline re-extraction). Initial protein mass in the pooled extract, protein mass re-dissolved in Tris-HCl 10 mM, and in the supernatant was quantified using the Lowry method (n=3). Protein mass in the pellet was calculated indirectly using equation 3.6. Values are expressed mean ± standard deviation, and n=3 for each saturation.

Ammonium sulfate saturation (%)	*V_{pool} (mL)*	*(Protein mass)$_{initial}$ (mg)*	*$V_{supernatant}$ (mL)*	*(Protein mass)$_{pellet}$ (mg)*	*(Protein mass) $_{re\text{-}dissolved}$ (mg)*	*Precipitation Yield) $_{supernatant}$ (%)*
70	30	12.84 ± 0.2	36	3.22 ± 0.05	4.01	25.1 ± 0.5
75			36.5	3.64 ± 0.03	4.31	28.4 ± 1.1
80			37.8	5.36 ± 0.1	5.47	41.7 ± 0.4
85			38.5	5.84 ± 0.07	6.47	45.5 ± 0.4

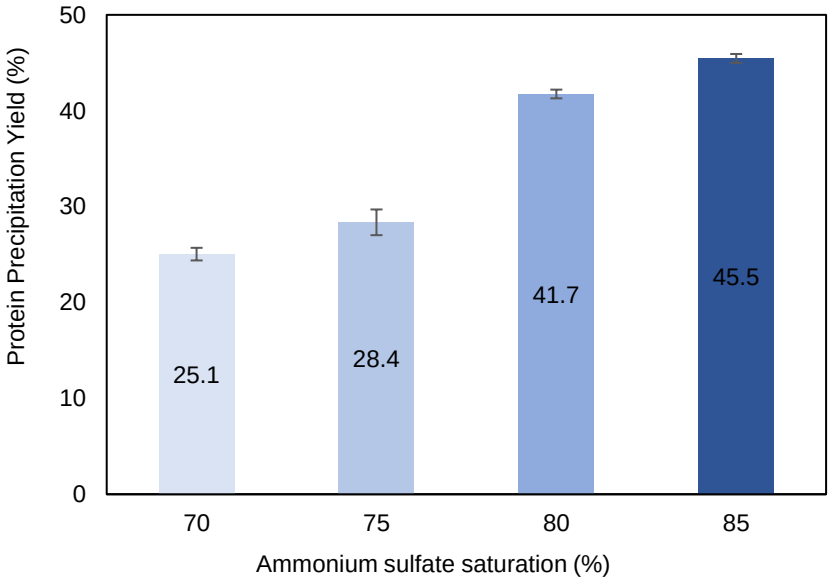

Figure 4.5: Graphic representation of the precipitation yield obtained for each ammonium sulfate saturation (70 to 85%) after incubation for 16h at 4ºC. Protein was quantified in the respective supernatants after centrifugation using the Lowry method (n=3). Values are expressed as mean ± standard deviation and n=3 for each saturation.

The pellets that resulted from precipitation of each saturation were re-dissolved in 15 mL of Tris HCl 10 mM pH 7, and a sample was withdrawn for protein quantification. The theoretical protein mass precipitated was also calculated through Equation 3.6. The determined protein mass re-dissolved in the buffer was higher and led to significantly different ($p<0.01$) precipitation yields, probably due to analytical errors and/or interferences. However, as Tris can be considered an interference in the Lowry method

[196] and despite Tris distortion being corrected with blank correction, it was established for precipitation yield purposes to consider the protein mass in the pellet determined indirectly through Equation 3.6 to avoid protein overestimation. A saturation of 85% led to a higher protein precipitation yield, and significant differences were observed between the yields obtained with 70% and 85% ($p<0.01$), 75% and 85% ($p<0.01$) and 80% and 85% ($p<0.05$).

The obtained results concerning the procedure A extract are also significantly higher than those obtained *for Gelidium sesquipedale* protein extract after enzyme assisted and alkaline extraction (identical to procedure B), which registered a yield of 24.6 ± 4.9 at 85% of saturation [11]. This result was already expected, given that extract B has smaller peptides due to protease activity, which might hamper protein-protein interactions during salting-out. Also, for *Gelidium sesquipedale*, protein precipitation with ammonium sulfate after suspension in distilled water rendered 11.5%, however, it was performed with a 50% saturation solution [158].

Precipitation with 85% ammonium sulfate was repeated to assess differences in the protein precipitation yield between the extract stored at alkaline pH (after extraction procedure A without any adjustments) or at neutral pH (after neutralization to consider eventual conditioning for further processing and storage purposes). Table 4.7 shows the obtained results.

Table 4.7: Parameters of the ammonium sulfate (85% of saturation) precipitation of the extract A (one aqueous extraction, one aqueous re-extraction, one alkaline extraction and one alkaline re-extraction) at neutral (~7) and alkaline (~13) pH. Initial protein mass in the pooled extract was determined using the Lowry method (n=3). Protein mass in the pellet was calculated indirectly using Equation 3.6. Values are expressed as mean ± standard deviation, n=3.

pH	*V_{pool} (mL)*	*$(Protein\ mass)_{initial}$ (mg)*	*$V_{supernatant}$ (mL)*	*$(Protein\ mass)_{pellet}$ (mg)*	*Protein Precipitation Yield (%)*
7	30	12.8 ± 0.5	38.5	5.8 ± 0.7	45.8 ± 2.5
13		12.6 ± 0.3		6.1 ± 0.8	48.7 ± 1.4

Comparing the yield values obtained for each pH value tested, no significant differences were observed ($p>0.05$), suggesting that the extracts pH and the consequent differences in protein-protein interactions apparently did not influence the precipitation yield.

In section 4.5, alternative precipitation methods were studied to try to improve protein recovery.

4.4.2. Extract diafiltration and concentration

The precipitated pellets were re-dissolved in 15 mL of Tris HCl 10 mM pH 7 in order to be desalted. Diafiltration was carried by centrifugal ultrafiltration, with an NMWCO of 3 kDa, using the same buffer.

To avoid protein aggregation and consequent sedimentation on the membrane surface, from the 15 mL available, only 10 mL of the re-dissolved pellet were loaded into the centrifugal filter units, which has resulted in an initial concentration of 0.39 ± 0.01 g/L and 0.38 ± 0.01 g/L for the sample at neutral pH and alkaline pH, respectively. After diafiltration final concentrations of 0.31 ± 0.03 g/L and

0.32 ± 0.02 g/L, respectively were determined. Diafiltration was carried out until 5.51 and 5.53 diafiltration volumes (n_D) for the samples at pH 7 and pH 13, respectively. Additional results are presented in Table 4.8.

Table 4.8: Diafiltration using an Amicon Ultra-15 centrifugal filter device (3 kDa NMWCO) - initial and final protein mass, initial and final retentate volume, cumulative permeate volume, cumulative protein mass in the permeate, protein retention yield and average rejection coefficient, for samples at neutral pH and alkaline pH. Protein mass in the permeate was determined using the Lowry method (n=3), and the protein mass in the retentate was calculated using Equation 3.7. Values are expressed as mean ± standard deviation, n=3.

pH	*$V_{initial}$ (mL)*	*(Protein mass)$_{initial}$ (mg)*	*V_{final} (mL)*	*$V_{cumulative\ permeate}$ (mL)*	*(Protein mass) $_{final}$ (mg)*	*(Protein mass) $_{cumulative\ permeate}$ (mg)*	*Retention Yield (%)*	*Average Rejection Coefficient (σ)*
7	10	3.76 ± 0.1	9.3	55.1	2.86 ± 0.02	0.91 ± 0.01	68.5 ± 1.2	0.83 ± 0.07
13		3.75 ± 0.2	9.6	55.3	3.01 ± 0.3	0.71 ± 0.02	76.4 ± 1.4	0.85 ± 0.09

No significant differences ($p<0.05$) are observed concerning the average rejection coefficient results between the two pH values. The differences in the rejection yield could be due to the different pH values of the solutions, as proteins in different conformations interact differently with the membrane surface. Another likely explanation is that modules with very small filtration areas, as in the present case, cannot guarantee that the membranes have the same pore size distribution. It is worth mentioning that most desalting steps described in the literature are accomplished through dialysis against deionized water, generally at low temperatures. The few found reporting the use of diafiltration concerned different alga species, extraction parameters and diafiltration conditions, making it unreasonable to compare the obtained results directly.

After diafiltration, the resultant extract was too diluted for further characterization assays. So, a concentration step with VCFs of 38.7 and 40.6 for the sample at neutral pH and alkaline pH, respectively, using the same centrifugal unit was performed (Table 4.9).

Table 4.9: Concentration of DF retentate using an Amicon Ultra-15 centrifugal filter device (3 kDa NMWCO) – initial and final protein concentration, initial and final retentate volume protein mass in the retentate and cumulative permeate for samples at neutral pH and alkaline pH. Protein mass in the permeate was determined using the Lowry method (n=3), and the protein mass in the retentate was calculated using Equation 3.7. Values are expressed as mean ± standard deviation, n=3.

pH	*[Protein]$_{initial}$ (g/L)*	*Vi_{nitial} (mL)*	*[Protein]$_{final}$ (g/L)*	*V_{final} (mL)*	*(Protein mass)$_{retentate}$ (mg)*	*(Protein mass)$_{permeate}$ (mg)*
7	0.31 ± 0.03	9.3	0.59 ± 0.05	3.6	2.11 ± 0.02	0.75 ± 0.09
13	0.32 ± 0.02	9.6	0.61 ± 0.03	3.9	2.34 ± 0.3	0.71 ± 0.03

4.4.3. Size exclusion chromatography

In order to determine the molecular weight profile of the proteins in the extracts, a size exclusion chromatography (SEC) experiment was performed. SEC is a non-destructive technique that separates molecules based on their physical size (i.e., molecular weight) in decreasing order. Protein SEC separations are performed under native conditions, preserving the macromolecule's biological activity [197]. Proteins are susceptible to interact with surface charged sites of stationary chromatographic phases, which can cause protein adsorption, shifts in retention time, and peak tailing asymmetry. These non-ideal interactions in SEC can be minimized with the correct choice of stationary and mobile phases. Due to its superior mechanical strength, non-swelling nature, and inertness under a wide range of conditions, derivatized porous silica is often the preferred stationary phase in SEC. Increasing the ionic strength or salt concentration of the mobile phase is also a standard method for reducing electrostatic interactions, and in this way, the phosphate buffered saline (PBS) was chosen as the mobile phase.

After column equilibration, each run started by injecting 1 mL of sample and was carried out at a flow rate of 1 mL/min. Absorbance at 280 nm was measured (Figure 4.6 (A)), and the fractions corresponding to the identified peaks were collected and characterized by SDS-Page (Figure 4.6 (B)).

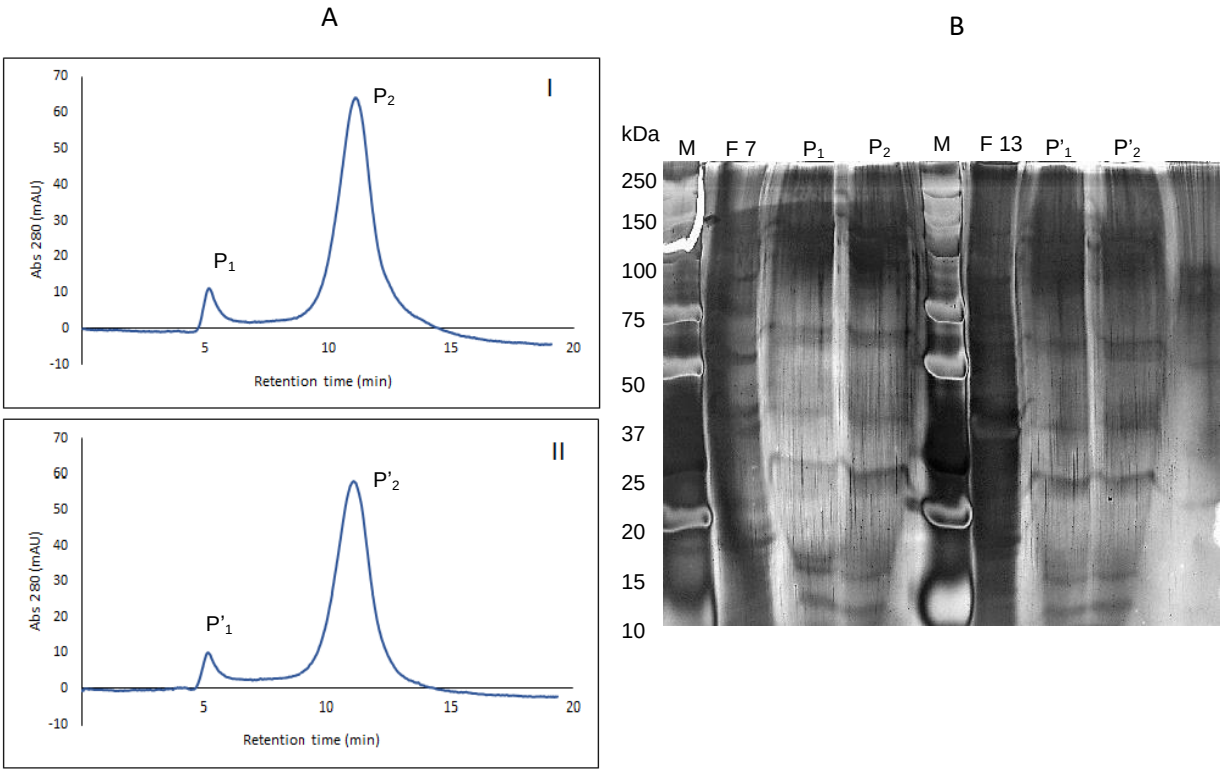

Figure 4.6: (A) Chromatograms obtained from the SEC experiments for the extract at pH 7 (I) and pH 13 (II), using a Biosep-SEC-s3000 column 300x7,8 mm, 5 µm, 290 Å, with a 1 mL loop, isocratic run at 1mL/min. PBS 1x was used as the mobile phase (B) Silver stained SDS-PAGE 12% acrylamide gel of the peak fractions collected from the SEC column. M – molecular weight marker; F 7 – Feed at pH 7 injected in the column (16 µg); P_1 (3 µg) – first peak; P_2 (7 µg) – second peak; F 13 – Feed at pH 13 injected in the column (17 µg); P'_1 (4 µg) –first peak; P'_2 (7 µg) – second peak.

Comparing the results with the elution times of standard proteins provided by the column manufacturer (*Appendix B)*, the first detected peak of both chromatograms appears to correspond to a protein of approximately 600 kDa, which might be Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) enzyme. As already mentioned, this enzyme is responsible for carbon dioxide fixation and as photosynthetic organisms, algae are expected to exhibit it. In its native form, RuBisCo has a molecular weight of 550 kDa and comprises 16 subunits – eight of them large and the remaining eight small [198]. The molecular weight of each polypeptide chain has been widely reported. In RuBisCo isolated from the green alga *Chlamydomonas reinhardtii*, the molecular weight determined by SDS-PAGE (denaturants conditions) of each subunit was 55 kDa and 16 kDa, respectively [199].

When looking at P_1 and P'_1 lanes in the gel, it is possible to see bands in the approximately 60 and 14 kDa region, which probably correspond to the enzyme subunits. However, as other bands are present in the lanes, corresponding to other proteins probably bound to the column, it is challenging to analyse. To correctly identify the presence of the enzyme, the extract should be subjected to prior RuBisCo isolation techniques, and SEC should be used as a polishing step in the purification.

Regarding the second peaks of both chromatograms, no significant differences in the molecular weight of the proteins are noticed between the extracts stored at different pH, as the peaks are registered in the same range of retention times. This result might be because the column that was used fractionates the samples over a vast molecular weight range, with the peaks spanning retention times between 9 and 13 minutes – so the peak will present molecules between 250 kDa to molecules as small as 244 Da. In this sense, a column with smaller pores should be more suitable for small proteins.

It is also worth noting that a desalting step should have been implemented before concentration. The collected fractions were diluted in the mobile phase that contained salt, and when concentrated in the Speed Vac, the salt content increased, which caused interferences when performing the SDS-PAGE, resulting in the “crooked” lines. Also, due to the volume of the combined fractions, the concentration step was very time-consuming when working in the SpeedVac equipment, suitable for minor sample volumes. In addition, as the concentrated sample volumes were extremely small, it was not possible to quantify the protein concentration with the Lowry method. As an alternative, the spectrophotometer Nanodrop® was used. It allows for rapid measurement, but the protein concentrations given are rather a rough estimation. Other protein quantification methods suitable for small sample volumes should be investigated.

4.5. Alternative protein precipitation methods

Protein precipitation with ammonium sulfate concentrates the protein fraction but does not purify it, requiring additional processing for the salt removal. Also, scaling up the process requires a large amount of salt. Therefore, other relatively simple alternative protocols were explored to assess the feasibility of protein fraction recovery and its implementation in an industrial process.

4.5.1. Protein precipitation using a pH-shift method

The pH of a solution at which the net charge of a protein is zero is known as the isoelectric point (pI). When the pH of the solution is higher than the pI, the protein's surface is mostly negatively charged, exhibiting repulsive forces. Similarly, the protein surface becomes predominantly positively charged when the pH falls below the pI, resulting in protein repulsion. At the pI, the proteins have a zero net charge, as the negative and positive charges are balanced, reducing repulsive electrostatic forces and enhancing aggregation and consequent precipitation [200].

To map the protein precipitation yield at different pH values, the pH of 30 mL of the pooled extracts from both procedures A and B was step adjusted to values between 1 and 6 by slowly adding 6 M of HCl, whereas one was left with the initial pH value (pH 7). The solutions were left to incubate for 30 min at 4 ºC, with stirring. To understand if the acid pH values interfered with the protein quantification method, aqueous hydrochloric acid solutions were prepared with distilled water with each pH value examined and diluted using the same dilution factor as the protein assay supernatants. When the Lowry method was used, these solutions had absorbance values similar to distilled water, so it was assumed that no significant interference occurred for a dilution factor of 2 and 5. The obtained precipitation results for each pH value for both protein extracts are represented in Figure 4.7.

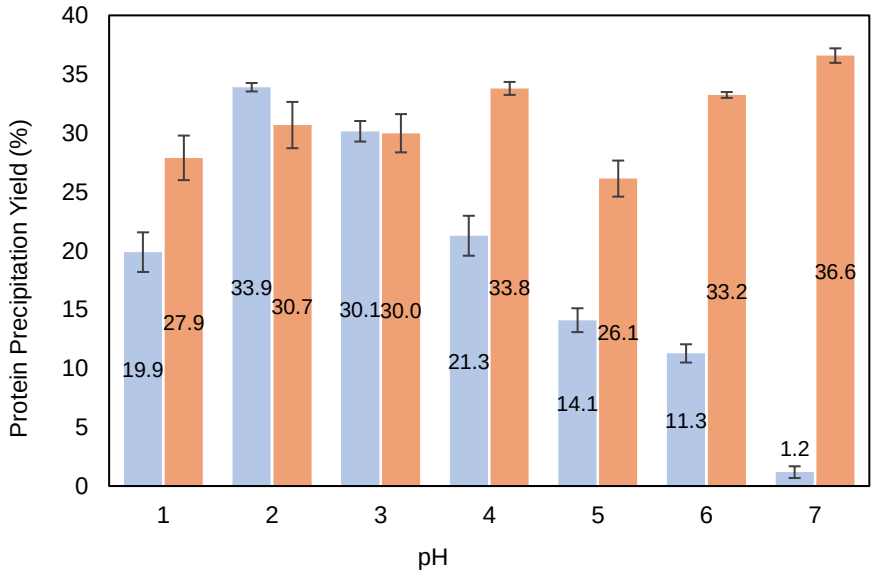

Figure 4.7: Graphic representation of the precipitation yield obtained for different pH values after incubation for 30 min at 4 ºC. Protein was quantified in the respective supernatants after centrifugation using the Lowry method (n=3). The original extract with a pH value of 7 was used as a control. Values are expressed as mean ± standard deviation and n=3 for each pH value. (- extract from procedure A (one aqueous extraction, one aqueous re-extraction, one alkaline extraction and one alkaline re-extraction); - extract from procedure B (Enzyme-assisted aqueous extraction using Celluclast®, enzyme-assisted aqueous extraction using Alcalase®, alkaline extraction and alkaline re-extraction)).

Regarding procedure A extract results, the highest yields were obtained when the pH was adjusted to 2 and 3, resulting in 33.9 ± 0.4% and 30.2 ± 0.9% of precipitated proteins, respectively, which are not significantly different ($p>0.05$). The other values were significantly different from these ($p<0.05$), and the protein precipitation decreased with the increase of the pH value. This tendency can

be sustained because algae proteins pI appears to be lower than other biomasses. For example, values of 3.5 and 4 have been reported for *Scenedesmus acutus* [201] and *Tetraselmis sp*. [202], respectively.

The pH-shift process was mainly used to extract protein from fish muscle [196] but more recently, to design a protein recovery process economically feasible and straightforward to implement in the industry has been adapted to algal proteins. It was first applied to *S.latissima* by Veide Vilg and Undeland (2017), where it was observed that 34.5% of the solubilized proteins at pH 12 were precipitated at pH 2 [176]. Later, and based on the previously mentioned study, maximum protein precipitation of 22.7 ± 8.01% and 33.3 ± 0.9% was reported for *Ulva lactuca* and *Porphyra umbilicalis* protein extracts, respectively, by adjusting the pH directly from 12 to 2 [87].

Although ammonium sulfate precipitation 85% saturated yielded 45.5 ± 0.4% of protein precipitation (Figure 4.5), it still requires the desalting process, where more protein mass is lost, as confirmed in section 4.4.1 (protein retention yield = 68.5 ± 1.2), which results in an overall protein recovery of 31.2 ± 0.5%. This result is not significantly different ($p>0.05$) from the ones obtained with pH value adjustment to 2 and 3, and it is more time and reagent consuming.

On the contrary to what was observed previously, in procedure B extract results, it is impossible to identify a linear trend between the pH value and the protein precipitation yield, probably because of the use of the Alcalase®. Its protease activity results in various peptides and amino acids with different pI, making it a challenge to precipitate the majority of the protein fraction using a single step adjusted pH value. Nevertheless, pH 7 yielded the best result, with 36.6 ± 0.6% of the protein fraction precipitated, although it was not statistically different ($p>0.05$) from the results regarding pH 4 and 6, with 33.8 ± 0.6% and 33.3 ± 2.2% of precipitation yield, respectively.

A.Naseri et al. (2020) used the pH-shift method to recover the protein fraction from *Palmaria palmata* after an enzyme-assisted extraction with Celluclast® and Alcalase®, among other combinations, followed by alkaline treatment like performed in the present work. By adjusting the pH value to 2, it was only possible to recover approximately 20% of the protein fraction, which was lower than the value obtained in this work (30.7 ± 2.0%) [91]. Even using the same enzyme concentration, many other factors influence this method, so it is expected that the results are not precisely concordant. The yields for other pH values were not assessed. Moreover, they concluded that it is easier to recover the protein when a lower concentration of enzyme combinations with Alcalase® is used, possibly because it is less hydrolysed. Comparing the pH-shift process with ammonium sulfate precipitation, reported by M. Gordalina (2020), performed after the same extraction procedure B, more promising results were obtained with this method since 85% ammonium sulfate saturation only resulted in 24.6 ± 4.9% of proteins recovery [11].

The addition of flocculating/precipitating agents has been pointed as possible precipitation yield improving agents [176], as long as they are considered food grade. To improve the overall yield, the g-forces of centrifugation could be increased, with the awareness that would raise the energy costs when implemented in an industrial process or extension of the precipitation incubation time. The irreversible denaturation caused by the mineral acids used to adjust the pH is the most significant disadvantage of isoelectric point precipitation [203].

Optimization of pH-shift protein precipitation method

This method was designed to optimize the recovery of proteins with slightly different pI values. During the pH adjustment performed in steps of 1 unit, if the pI values of some proteins were surpassed, the candidate proteins to precipitate would be solubilized again. Looking at Figure 4.8, the interval of pH values corresponding to the highest precipitation yield were identified for both extracts. The pH ranges went from 4 to 1.5 and 7 to 3.5, respectively, to procedures A and B.

In that way, 30 mL of each extract was directly adjusted to the highest value of the identified interval (i.e., extract from procedure A was adjusted to 4 and from procedure B was left at pH 7), incubated and centrifuged as described in the previous method. The resultant supernatant was recovered, and a sub-sample was withdrawn for protein quantification. The pH was adjusted with a 0.5 step to the following value, and the process was repeated until the last value of the interval was reached, successively recovering the supernatant. Figure 4.8 shows the obtained results, and the support calculations for the figure construction are presented in *Appendix C.*

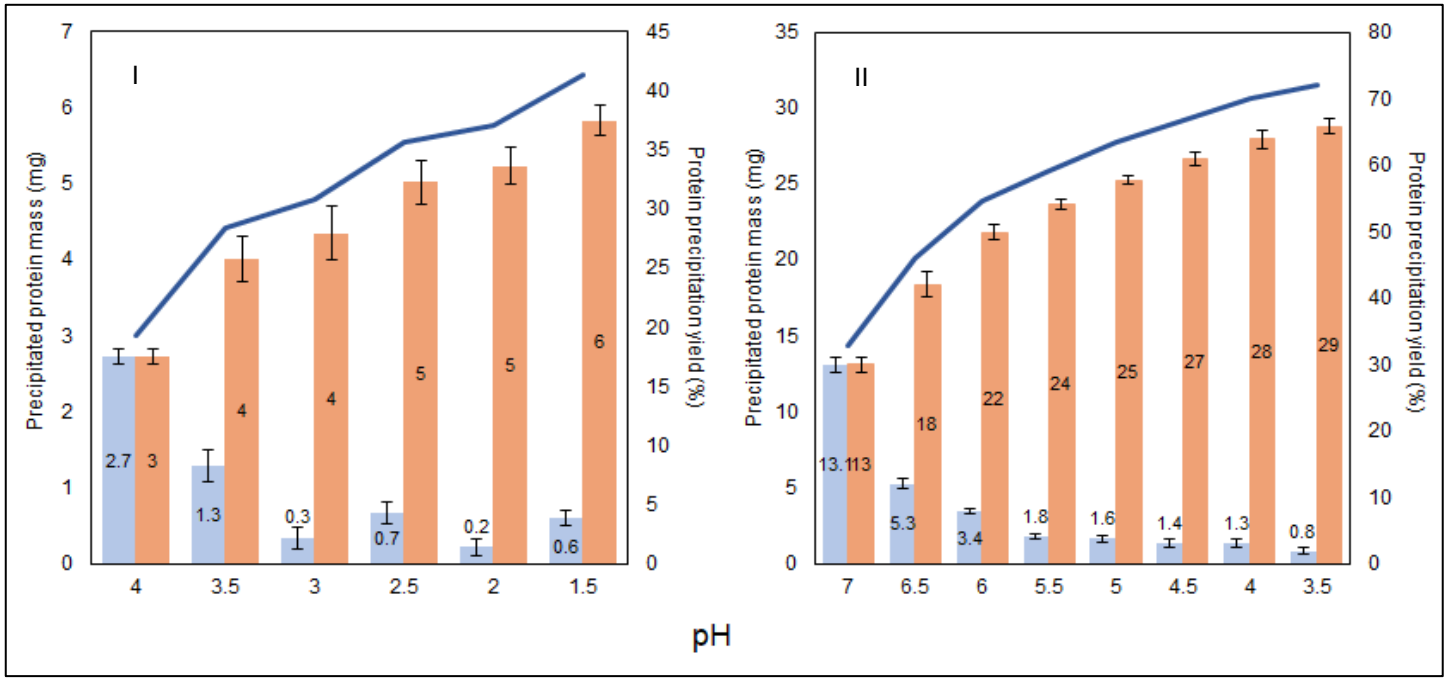

Figure 4.8: Graphic representation of the precipitated protein mass (mg), cumulative protein mass (mg) and correspondent protein precipitation yield (%) obtained in each examined pH value for the resultant extracts from extraction procedure A (one aqueous extraction, one aqueous re-extraction, one alkaline extraction and one alkaline re-extraction) (I) and B (Enzyme-assisted aqueous extraction using Celluclast®, enzyme-assisted aqueous extraction using Alcalase®, alkaline extraction and alkaline re-extraction) (II), after incubation for 30 min at 4ºC. The adjustment of pH was successively performed in the recovered supernatant of the previous adjustment. Protein was quantified in the respective supernatants after centrifugation using the Lowry method (n=3). Values are expressed as mean ± standard deviation and n=3 for each pH value. (- precipitated protein mass (mg); - cumulative protein mass (mg); (——) – protein precipitation yield (%).

The overall recovered protein mass and correspondent precipitation yield increased significantly for both extracts when performing this method. In the pooled extract from procedure A, it was possible to recover 2 ± 0.2 mg of protein more, resulting in a precipitation yield approximately 11 percentual points higher than the previous method. Regarding the pooled extract from procedure B, 29 ± 0.8 mg of protein was recovered, corresponding to the double of previously obtained and resulting in a precipitation yield 40 percentual points higher. This method notably resulted in the extract obtained using the protease due to the vast diversity of pI values. One can conclude that in this extract, the present proteins had similar pI values, and pH steps of 1 did not allow their precipitation.

Nevertheless, it should be noted that this process was significantly more time-consuming, making it difficult to adapt to an industrial environment. In addition, working with low protein mass quantities could lead to the propagation of errors. Therefore, as a suggestion, this assay could be repeated for the pooled extract of procedure A with a higher initial volume, as it presents a lower protein initial concentration.

4.5.2. Organic solvent-driven precipitation

The addition of miscible organic solvents, such as ethanol, to a solution, may result in protein precipitation. As the organic solvent gradually displaces water from the protein surface, the solvation layer around proteins will decrease. With thinner layers, proteins can aggregate by attractive electrostatic and hydrophobic forces. The temperature should be kept below 0 degrees during the process, although lengthy exposure to this temperature range can lead to protein denaturation [200].

Protein precipitation using ethanol was assessed for three different final organic solvent concentrations (90%, 80% and 70% (v/v)). To meet these conditions, 10 mL of the pooled extracts from procedure A were subjected to adding nine, eight and seven volumes of cold ethanol to one volume of protein extract, respectively. Table 4.10 presents additional parameters, and Figure 4.9 shows the obtained results.

Table 4.10: Parameters of the protein precipitation by ethanol-driven precipitation of procedure A pooled extracts (one aqueous extraction, one aqueous re-extraction, one alkaline extraction, and one alkaline re-extraction) Protein mass in the pellet was calculated indirectly using Equation 3.10. Values are expressed as mean ± standard deviation (n=3).

Procedure	*V_{pool} (mL)*	*(Protein mass) $_{initial}$ (mg)*	*Ethanol final concentration [%(v/v)]*	*$V_{supernatant}$ (mL)*	*(Protein mass) $_{pellet}$ (mg)*	*Precipitation yield (%)*
A	10	5.8 ± 0.1	90	100 ± 0.1	4.4 ± 0.3	77.5 ± 1.9
			80	90 ± 0.2	3.5 ± 0.2	61.2 ± 0.7
			70	80 ± 0.1	1.6 ± 0.1	27.8 ± 2.7

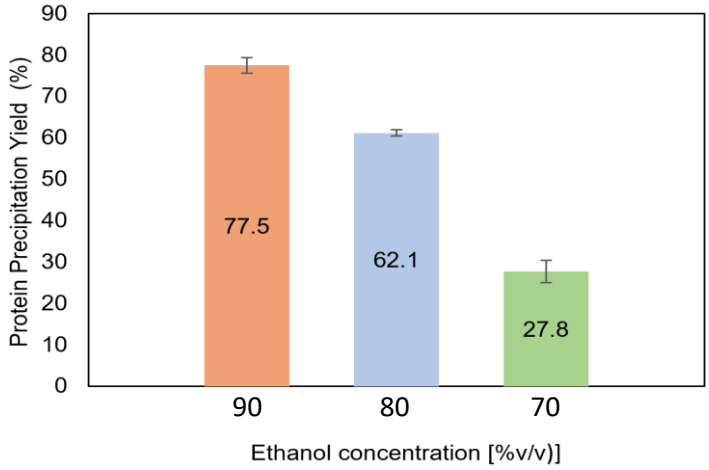

Figure 4.9: Graphic representation of the precipitation yield obtained for each ethanol concentration (90%, 80% and 70% (v/v)) after incubation for 2h at -20 ºC. Protein was quantified in the respective supernatants after centrifugation using the Lowry method (n=3). Values are expressed as mean ± standard deviation and n=3 for each concentration.

This method led to outstanding results when subjecting the extract to a concentration of 90% (v/v) of ethanol. The obtained protein precipitation yield was significantly different from the one obtained with 80% (v/v) ($p<0.01$), although this last one was still significantly higher than the results obtained with ammonium sulfate and pH-shift precipitation techniques applied to the same extract. Notwithstanding the obtained results, due to the high consumption of reagents required, this method would not be applied in an industrial approach of protein recovery from this type of extract. Knowing this, the method was not assessed for the extract from procedure B.

In fact, this precipitation method is mainly applied in proteomic experiments with a low working volume of samples, generally regarding body fluids like serum or plasma [204]. These contain a high concentration of the so-called “ballast proteins” (accounting for up to 90% proteins), such as serum albumin, immunoglobulins, transferrin, etc. and the scientific interest is now focused on the remaining 10% of proteins that may contain potential markers. To avoid immunodepletion of these highly abundant proteins, which represents a time consuming and costly process, ethanol precipitation has been pinpointed as an alternative to fractionation. Studies report that 60-80% (v/v) ethanol concentration widely precipitates the ballast proteins, leaving the considered “interesting” protein fraction in solution [203].

Acetone is another organic solvent broadly used for protein precipitation purposes. It is generally used in lower concentrations than ethanol, with 40% (v/v) reported [205]. However, as it is only considered safe for use as an indirect food additive in adhesives and food-contact coatings by FDA [206], it was not considered in the scope of animal feed production from *Gelidium sesquipedale*.

4.6. Carbohydrate co-elution in protein extraction

Although protein extractability is enhanced with the extraction steps performed, carbohydrate co-elution also occurs during both the procedures, as evidenced in section 4.3.3. Even if the resulting extracts exhibit an attractive nutritional value and biological activity, when it comes to *Gelidium sesquipedale* processing in the scope of a biorefinery, the conservation of the agar fraction is imperative when designing a “no waste” agar extraction industrial process.

The solid residues obtained after each protein extraction step for both A and B procedures were oven-dried for seven days, and a carbohydrate quantification method by sulfuric hydrolysis and HPLC analysis was performed. Figure 4.10 shows the obtained results. Note that the values are expressed in grams of carbohydrate per 100 grams of residual algal biomass (dw).

Table 4.11: Cellulose and agar content, in grams of carbohydrate per 100 grams of residual biomass (dw) in the resultant solid residues after each protein extraction step from procedures A and B, after being oven-dried for seven days. Carbohydrate quantification was determined by a two-step sulfuric hydrolysis followed by HPLC. Values are expressed as mean ± standard deviation (n=3).

Extraction Procedure	*Extraction Step*	*Cellulose Content (g cellulose/g residual biomass dw)*	*Agar content (g agar/g residual biomass dw)*
A	Aqueous extraction	9.6 ± 1.4	48.6 ± 1.2
	Aqueous re-extraction	9.3 ± 1.3	45.9 ± 0.9
	Alkaline extraction	8.8 ± 1.5	43.6 ± 2.7
	Alkaline re-extraction	8.1 ± 1.8	39.8 ± 2.5
B	Enzyme assisted extraction with Celluclast®	8.9 ± 1.2	46.2 ± 1.6
	Enzyme assisted extraction with Alcalase®	8.4 ± 1.5	42.1 ± 3.0
	Alkaline extraction	7.4 ± 1.4	40.0 ± 2.2
	Alkaline re-extraction	6.4 ± 0.9	36.1 ± 1.2

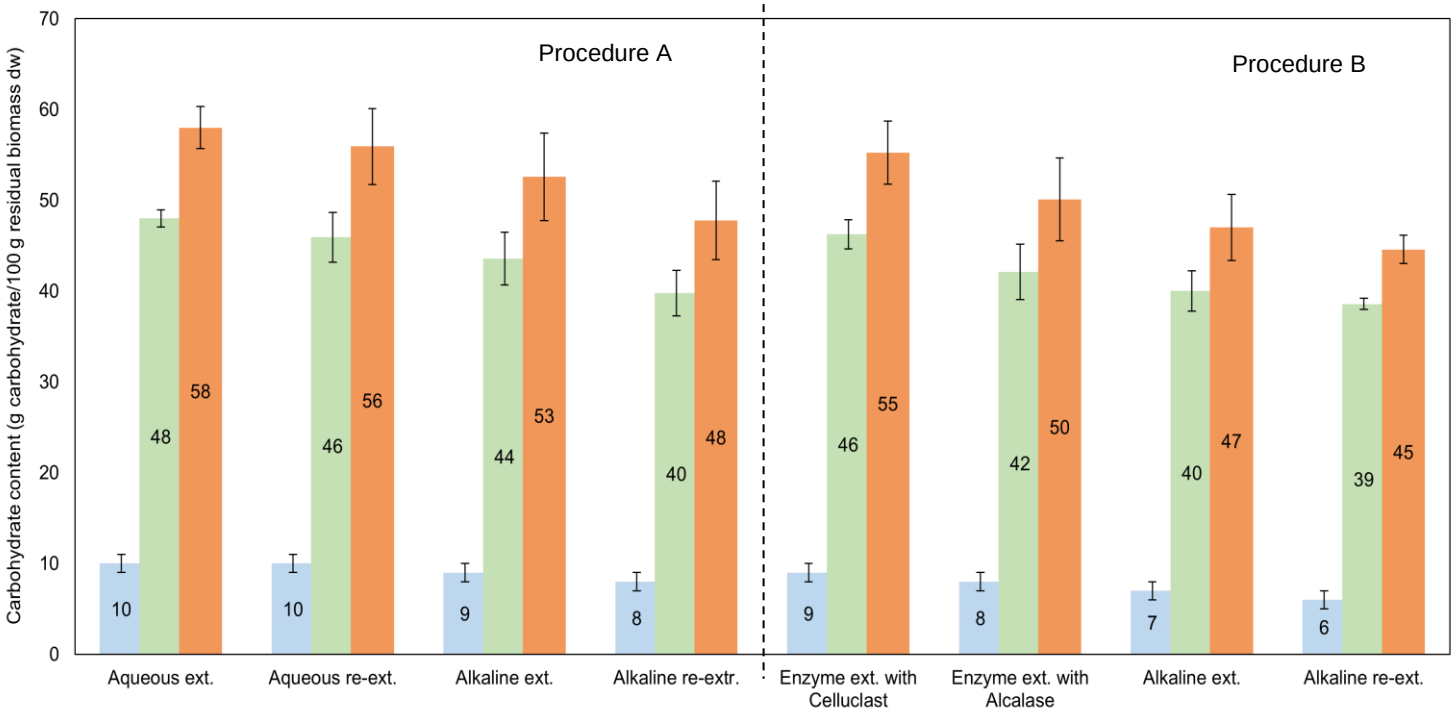

Figure 4.10: Cellulose, agar and total carbohydrate content, in grams of carbohydrate per 100 grams of residual biomass (dw) in the resultant solid residues after each protein extraction step from procedures A (one aqueous extraction, one aqueous re-extraction, one alkaline extraction and alkaline re-extraction) and B (one enzymatic-assisted aqueous extraction with Celluclast, one enzymatic-assisted aqueous extraction with Alcalase®, one alkaline extraction and one alkaline re-extraction). Carbohydrate quantification was determined by a two-step sulfuric hydrolysis followed by HPLC. Values are expressed as mean ± standard deviation, n=3. (- Cellulose; - Agar; - Total)

The most substantial losses in the cellulose content occurred during the alkaline extractions and re-extractions for the two procedures and in the enzyme assisted extraction with Celluclast® concerning procedure B. A Significant difference was observed between cellulose content in the residue after the aqueous extraction from procedure A and the enzyme assisted extraction with Celluclast® from procedure B. As mentioned, this enzyme is responsible for the catalytic hydrolysis of cellulose and hemicellulose by breaking down β-glucosidases bonds, so it is only expected to observe a more considerable loss when compared with the loss by osmotic shock caused by the suspension in distilled water. Higher concentrations of Celluclast® (60 µL/3 g of algal biomass) have been used for the saccharification of carbohydrates in microalgal biomass previous to bioethanol production [207]. Thus, care should be taken when using this enzyme if the carbohydrate content is to be preserved. In addition, a significant difference ($p<0.01$) was reported in the alkaline re-extraction step between the two procedures, meaning that the cellulose content is significantly different between the two final residues.

All the steps account for some agar loss (Table 4.11). Looking at the obtained results after aqueous extraction from procedure A and after enzyme assisted extractions from procedure B, no significant differences were observed ($p>0.05$), suggesting that the hot protein extraction performed at 50 ºC did not enhance the carbohydrate co-extraction, presumably because the perfect conditions for agar extraction from *Gelidium sesquipedale* were not met, as the optimal temperature is close to water

boiling point and the pH is typically close to 6 [150]. No significant difference is observed in the agar content of the algae after both protein extraction procedures A and B. ($p>0.05$).

Regarding the total carbohydrate content, no difference was reported between the sequential extraction steps in each procedure (i.e., aqueous extraction compared with enzyme assisted extraction with Celluclast, aqueous re-extraction compared to enzyme assisted extraction with Alcalase®, etc.).

It was not possible to establish the weight ratio between the residual and the initial biomass for each protein extraction step, only for the final residues after procedures A and B. The weight ratios found were 0.71 (g residue dw/g of initial biomass dw) and 0.58 (g residue dw/g of initial biomass dw) for extraction procedures A and B, respectively, which results in total carbohydrate content of 34.1 g carbohydrate/100 g of initial algal biomass and 26.1 g carbohydrate/100 g of initial algal biomass dw, after procedures A and B, respectively. Converting the agar content, concentrations of 28.4 g/100 g initial algal biomass dw and 22.6 g/100 g initial algal biomass dw were obtained. Despite this reduction in the agar content, the values, even though inferior, can still be compared to those found in other *Gelidium* species exploited in industrial extraction, with reported agar content of ~30% [155]. However, because some biomass was probably lost during transfers, the ratios found may not correspond to the actual weight ratio, so the carbohydrates concentration determined may be underestimated.

To the best of my knowledge, no similar studies are available in the literature. Thus, an additional method for determining total carbohydrates should be performed to confirm the obtained results.

4.7. Fractionation of the protein extracts by anion exchange chromatography

As stated above, a considerable fraction of the carbohydrates is co-eluted during the protein extraction procedures, which might not be compatible with some biological applications requiring purified protein products. As a result, another step was implemented to assess the feasibility of protein fractionation by anion exchange chromatography. This assay was performed in the extracts obtained after ammonium sulfate precipitation, diafiltration and concentration, described in sections 4.4.1. and 4.4.2. Even though ammonium sulfate precipitation is already considered an isolation step, the co-eluted carbohydrates are likely bound to the proteins during the extraction procedure. Therefore, they are expected to precipitate with the protein fraction and to be retained by the membrane during the diafiltration and concentration steps.

Two different cation resins were tested. One is considered a strong anion exchanger – Quaternary Ammonium (Q) Sepharose, and the other a weak anion exchanger - Di-Ethyl-Amino-Ethyl (DEAE). When a cation resin is used, negatively charged molecules bind to the resin and are later eluted with increasing salt concentrations in the aqueous phase. The pH must be carefully chosen to ensure that both proteins and polysaccharides are negatively charged, allowing them to attach to the resin and, hopefully, with different strengths of interaction with the charged resin. The characteristic polysaccharides from red macroalga are more negatively charged than proteins and are expected to bind more strongly to the resin [95]. Thus, proteins are likely to be eluted first.

4.7.1 Linear elution

The first assays were performed under a linear elution mode from 0 to 1 M of NaCl (i.e., 0 to 100% of NaCl) using the two types of cationic resins and assessed for the different pH extracts. The registered chromatograms are shown in Figure 4.11.

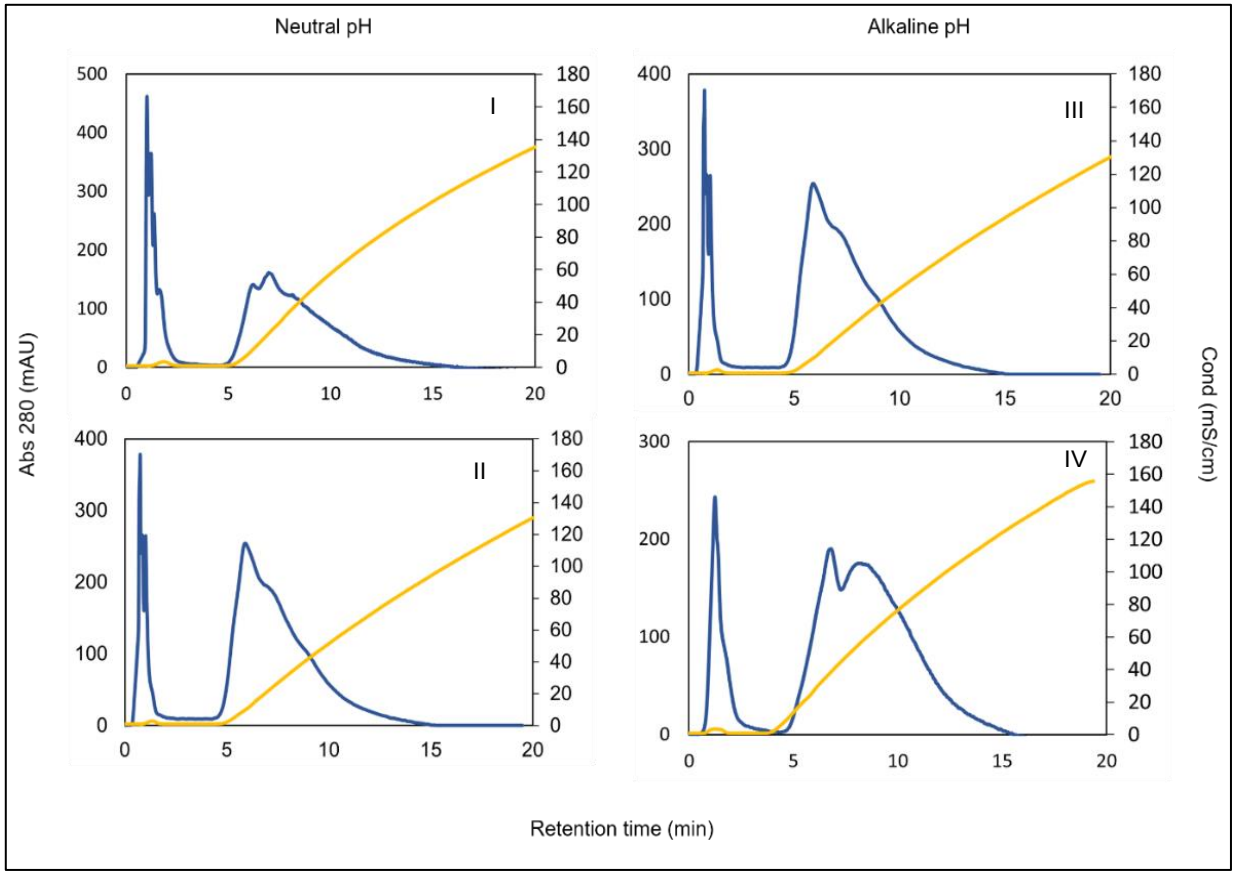

Figure 4.11: Chromatograms obtained for the extracts stored at two pH values using two different cationic resins in a linear elution mode (0 to 1 M NaCl), 1 mL loop and a flow rate of 1 mL/min. (——) represents the absorbance at 280 nm, and (——) represents the conductivity of the eluent in mS/cm. (I and II) - Q Sepharose and DEAE prepacked columns, respectively, for the extract stored at neutral pH. (III and IV) – Q Sepharose and DEAE prepacked columns, respectively, for the extract stored at alkaline pH.

The pH of the solution strongly influences proteins interactions, not only between each other but also with the polysaccharides and, especially, with the resin. Even though after diafiltration with Tris-HCl 10 mM buffer, the extract samples that initially were stored at alkaline pH at this point also presented a neutral pH value, if the proteins suffered alkaline hydrolysis, differences can be noticed in these assays, as the alkaline hydrolysis is considered irreversible. Therefore, considering the same column, it is unsurprising that the registered chromatograms will differ depending on the pH. It is also observable that the signal of unbound protein peak (i.e., the first registered peak immediately after sample injection) is always stronger for the sample at neutral pH than at alkaline pH, regardless of the column tested,

which might suggest that in neutral conditions, there are more positively charged proteins that did not bind to the column.

4.7.2 Step wise elution

Considering the results obtained with the linear elution mode, a step elution method was designed to optimize the resolution of the elution peaks. For this method, the elution steps were programmed based on the maximum conductivity registered for each elution peak from the chromatograms represented in Figure 4.11. Figures 4.12 and 4.14 show the chromatograms from the fraction of the protein extracts stored at neutral and alkaline pH, respectively. The fractions corresponding to the identified peaks were collected, combined, and characterized by SDS-PAGE (Figures 4.13 and 4.15)

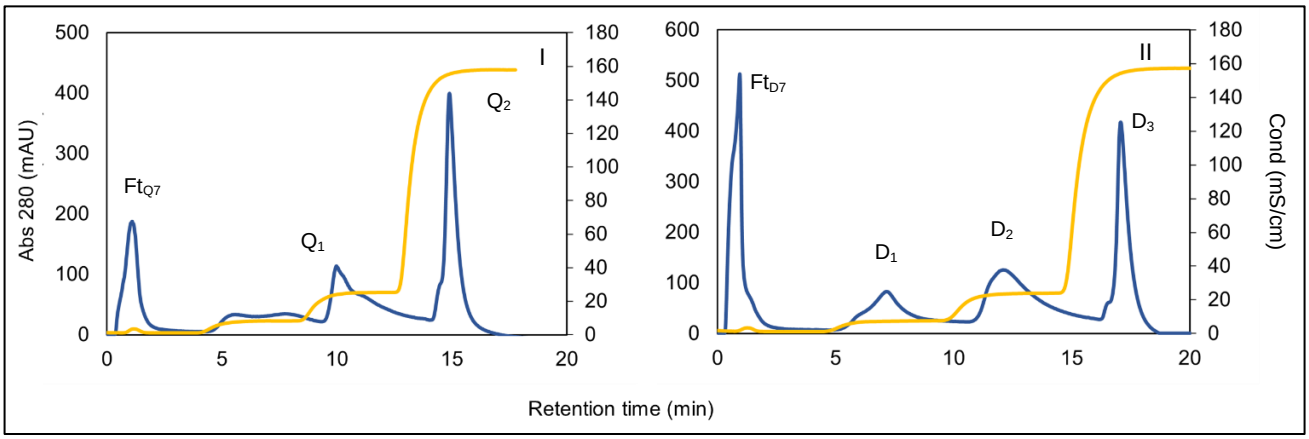

Figure 4.12: Chromatograms obtained for the extracts stored at neutral pH using two different cationic resins – Q sepharose prepacked column (I) and DEAE prepacked column (II) in a step elution mode (from 0 to 1 M of NaCl), 1 mL loop and a flow rate of 1 mL/min. (——) represents the absorbance at 280 nm, and (——) represents the conductivity of the eluent in mS/cm.

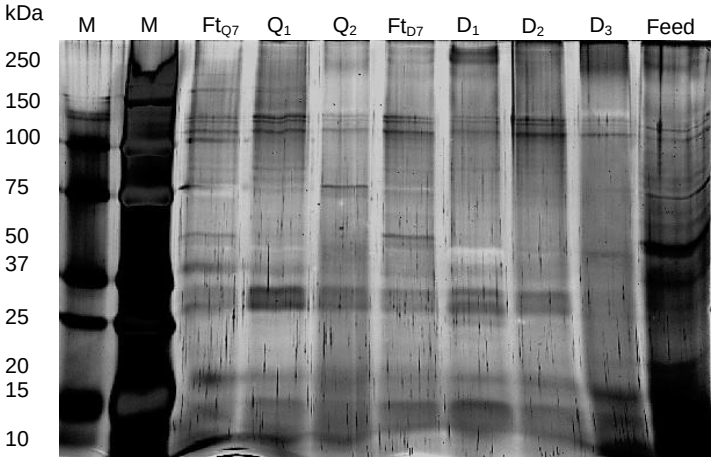

Figure 4.13: Silver stained SDS-PAGE 12% acrylamide gel of the peak fractions collected from the Q Sepharose and DEAE prepacked columns for the extract stored at neutral pH. M – molecular weight marker; Feed – Feed at pH 7 injected in the columns (18 µg); Ft_{Q7} (7 µg) – Flowtrough pH 7; Q_2 (5 µg) – first elution peak; Q_2 (3 µg) – second elution peak - from the Q Sepharose prepacked column; Ft_{D7} (4 µg) – Flowthrough pH 13; D_1 (5 µg) – first elution peak; D_2 (5 µg) – second elution peak; D_3 (1.5 µg) – third elution peak – from the DEAE prepacked column.

As observed in the chromatograms I and II from Figure 4.11, the elution peaks from each type of column happened approximately simultaneously, i.e., at the same percentage of elution buffer for the samples stored at neutral pH. Therefore, the step elution method chosen for these assays was the same, and three elution steps were programmed. The first happened when approximately 60 mM of NaCl was attained (6.5 mS/cm) and the second at 220 mM of NaCl (18.30 mS/cm). Comparing the chromatograms of the two different columns (Figure 4.9), it is observable that following this method, for the DEAE ligand column (II), it was possible to reach a better peak resolution between the first and second elution peaks than with the Q Sepharose column. For this reason, when collecting the fractions corresponding to the peaks from assay I, it was decided to combine all the fractions corresponding to the eluted volume between 5 and 12 mL and consider them as one only peak. Gradient steepness has a significant impact on the retention time and selectivity of the column. Thus, in this case, the step choice should be reviewed and optimized.

Similar to what was already discussed in section 4.4.3, the protein fraction present in the recovered samples was too diluted in the mobile phase, and so the same concentration under vacuum strategy was carried out. The protein mass in the concentrated sample was also quantified using the Nanodrop®. In this way, the values indicated in Figure 4.13 consist of an approximation of the mass loaded in each gel lane. Nevertheless, it is possible to see well-defined protein bands in the gel, and roughly all the protein content was recovered through the different fractions for both columns.

A step elution method with two steps was performed for the assays with the samples stored at alkaline pH. For the Q Sepharose column, the first step was programmed to happen at 140 mM of NaCl, and regarding the DEAE column, the step was set at 60 mM of the salt.

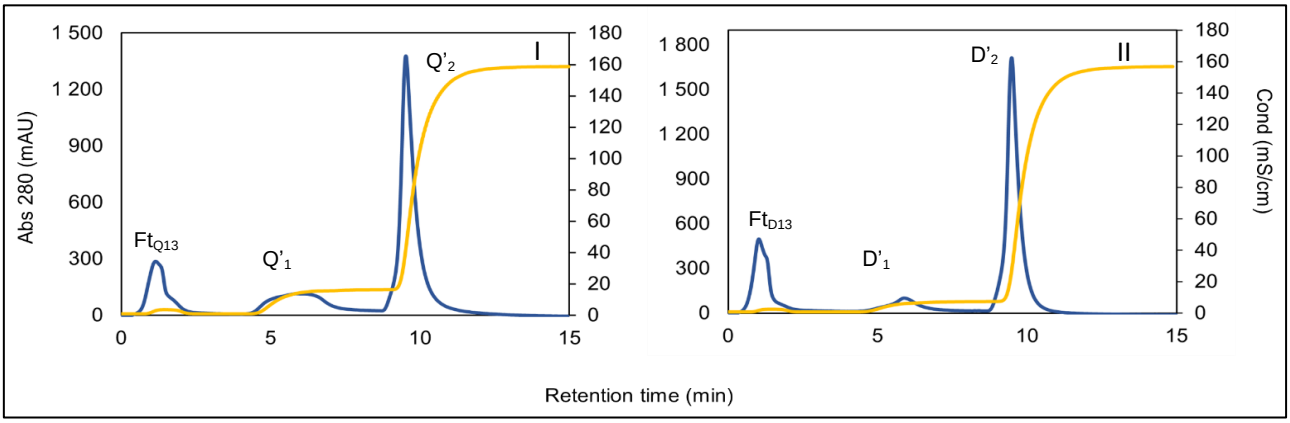

Figure 4.14: Chromatograms obtained for the extracts stored at alkaline pH using two different cationic resins – Q sepharose prepacked column (I) and DEAE prepacked column (II) in a step elution mode (from 0 to 1 M of NaCl), 1 mL loop and a flow rate of 1 mL/min. (——) represents the absorbance at 280 nm, and (——) represents the conductivity of the flowthrough in mS/cm.

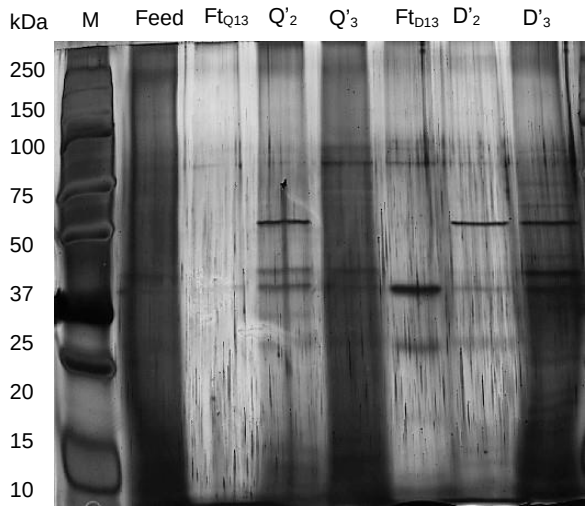

Figure 4.15: Silver stained SDS-PAGE 12% acrylamide gel of the peak fractions collected from the Q Sepharose and DEAE prepacked columns for the extract stored at alkaline pH. M – molecular weight marker; Feed – Feed at pH 13 injected in the columns (20 µg); Ft_{Q13} *- (0.5 µg) – Flowthrough pH 7; Q'$_1$ (2 µg) – first elution peak; Q'$_2$ (5 µg) – second elution peak - from the Q Sepharose prepacked column* Ft_{D13} *- (5 µg) – Flowthrough pH 13; D'$_1$ (1 µg) – first elution peak; D'$_2$ (8 µg) – second elution peak – from the DEAE prepacked column.*

Similar to what was observed in the assays for the samples stored at neutral pH, it was possible to achieve a better peak resolution using the DEAE ligand column (II). Based on the estimated protein concentration in each concentrated sample, a minor recovery of the protein fraction was registered for the Q Sepharose column, confirmed by the less intense bands in the gel presented in Figure 4.15.

This fractionation strategy is mainly used when one pretends to isolate one specific protein rather than the total of the protein fraction. Red-Phycoerythrin separation and purification from red macroalga through anion exchange chromatography have been extensively reported in the literature. For instance, from *Gracilaria gracilis,* using a DEAE Sepharose fast flow chromatography, R-PE was recovered at 200 mM of NaCl [104]. Also, as an alternative to common gradient elution of ionic strength, R-PE purification from *Polysiphonia urceolata* was reported using the same column but with a gradient elution of pH [116]. Only one similar study was found in the literature for a protein extract from *Ulva sp* with an initial carbohydrate content of approximately 25% [95]. Those authors report that after using a TOYOPEARL® DEAE-650S resin it was possible to remove most carbohydrates to a final value of 1%.

These assays consisted of a first approach to assess the feasibility of separating the protein and polysaccharides fractions by anion exchange chromatography. Even though not strictly required for the purposes of animal feed production from *Gelidium sesquipedale*, it might be necessary to implement when highly purified protein products are required for a specific application. The results were promising, especially using the DEAE ligand column. Nonetheless, the percentage of polysaccharides that could be separated and accurately quantify the protein recovery yield remains to be determined. For this, the assays should be repeated with higher pre-purified extracts volume.

5. Conclusions and future perspectives

Macroalgae are considered a novel and promising protein source and exhibit other components attractive to various industries. Due to their unique composition, they are an excellent sustainable resource for biorefinery purposes. In the scope of a biorefinery, the main goal of the present work was to implement different protein extraction procedures from crude *Gelidium sesquipedale* and from industrial residues that result from the agar extraction process to assess the feasibility of a “no waste” industry. The protein extracts were characterized, and, additionally, different methods were tested to improve protein recovery and isolation.

Regarding protein extraction, two different approaches were performed on a 5 L scale. Procedure A consisted of the conventional method with four sequential steps: two aqueous extractions (deionized water, 16h, 4ºC) and two alkaline extractions (0.1M NaOH, 1h, RT). This procedure led to a protein recovery of 12.0 ± 1.2% of the crude alga's total protein content and 15.4 ± 0.9% from the industrial residues, respectively. Procedure B consisting of two sequential extractions assisted by the enzymes Celluclast® and Alcalase® followed by two alkaline extractions recovered 52.1 ± 1.7% and 36.45 ± 1.3% from the crude alga and the industrial residues, respectively. These results show that even with the partial loss of protein during the pre-treatment of the alga for the agar extraction process (roughly 22.8%), it is still possible to obtain similar yield values between the different starting biomasses.

Nevertheless, it should be noted that protein extraction is also expected to occur during the agar extraction process, so this value does not represent the total protein loss in the process. When protein extraction is to be performed with industrial residues, a different enzyme combination should be assessed to improve protein extraction, as the use of Alcalase® showed disappointing results compared to the values obtained with the crude alga. Future work also includes optimizing extraction parameters for *Gelidium sesquipedale* crude and residues, such as extraction time, reagent and/or enzyme concentration, agitation speed and the dimensioning of the necessary equipment to scale up the process.

In both extraction procedures A and B using crude *Gelidium,* a fraction of the alga carbohydrates was lost in each protein extraction step, with a total loss of approximately 30% of the total carbohydrate content. Despite a reduction of 20%-30% in the agar content, using procedures A and B, respectively, the values, even though inferior, can still be compared to those found in other *Gelidium* species exploited in industrial extraction. The agar quality should be assessed to understand if the protein extraction procedures jeopardize the remaining agar fraction.

The protein extracts were concentrated by crossflow filtration and lyophilized for further characterization. Regarding chemical composition, all the protein products showed a higher content of carbohydrates than desirable, confirming that the procedures followed also enhanced the co-elution of this component. For that reason, to obtain protein-rich extracts, efforts should be made to develop novel protein extraction techniques that limit the co-extraction of other compounds and implement protein recovery and isolation methods suitable for the process. The ash content was also considerably high. The bioaccessibility of the protein fraction in the products was assessed by the partner IPMA and

showed a greater improvement when compared to the bioaccessibility in the initial biomass (23.7 ± 1.0% and 7.4 ± 0.6% in the crude alga and industrial residues, respectively). The greatest protein bioaccessibility (91.7 ± 0.7%) was found in the protein product obtained after extraction procedure A using crude *Gelidium.*

Other characterization assays included the molecular weight profile of the proteins in extracts stored at different pH values – at the original value obtained after the multi-step extractions (strongly alkaline) or at a neutral pH value obtained after neutralization of the extracts – to assess if the alkaline conditions had a hydrolysis effect on the peptides. The proteins present in the extract were precipitated with ammonium sulfate, diafiltrated to remove the salt and concentrated, and finally characterized by size exclusion chromatography and gel electrophoresis. In the chromatograms from the SEC experiments, no significant differences in the molecular weight of the proteins were noticed between the extracts at different pH, as the peaks are registered in the same range of retention times. Nevertheless, the column used fractionates the samples over a vast molecular weight range (5-700 kDa). A different column with smaller pores should be used. The electrophoresis gel did not show any differences as well.

Alternative protein precipitation methods other than using the salting-out process were also assessed. The pH-shift method showed promising results. Regarding procedure A extract results, the traditional pH-shift method yielded 33.9 ± 0.4% of protein recovery when the pH was adjusted to 2. Even though the yield obtained with ammonium sulfate precipitation 85% saturated (45.5 ± 0.4%) was higher, it still requires the desalting process, where also some protein is lost (protein retention yield = 68.5 ± 1.2%), the overall protein recovery yield is approximately the same. In procedure B extracts, adjusting the pH to 7 resulted in 36.6 ± 0.6% of the protein fraction precipitated, higher than the one previously reported for this extract (24.6 ± 4.9%). This method is simple, straightforward to implement in industrial processes and does not require high consumption of reagents. Ethanol 90% (v/v) precipitation of the procedure A extracts led to the highest precipitation yield (77.5 ± 1.9%). However, due to the high consumption of reagents required, this method would not be applied in an industrial approach.

Anion exchange chromatography was used as a first approach to fractionate the protein product obtained after precipitation with ammonium sulfate and salt-washing from the carbohydrate fraction still present and obtain a purified protein product. The obtained results using Q Sepharose and DEAE ligands columns were promising, as it was possible to recover roughly all the protein content through the different fractions. Future work includes the quantification of the separated polysaccharides.

The present work proves the potential of using *Gelidium sesquipedale* in a “no waste” extraction process, providing insights into this macroalga's valorisation that goes beyond agar extraction. Marine algae are abundant, renewable and can be obtained from the oceans on a large scale at a low cost. It is of great urgency to start taking advantage of these sustainable resources.

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Appendix A – Calibration Curves

A.1. Protein Quantification

The protein samples were diluted with distilled water, whenever necessary, to be within the curve validity range.

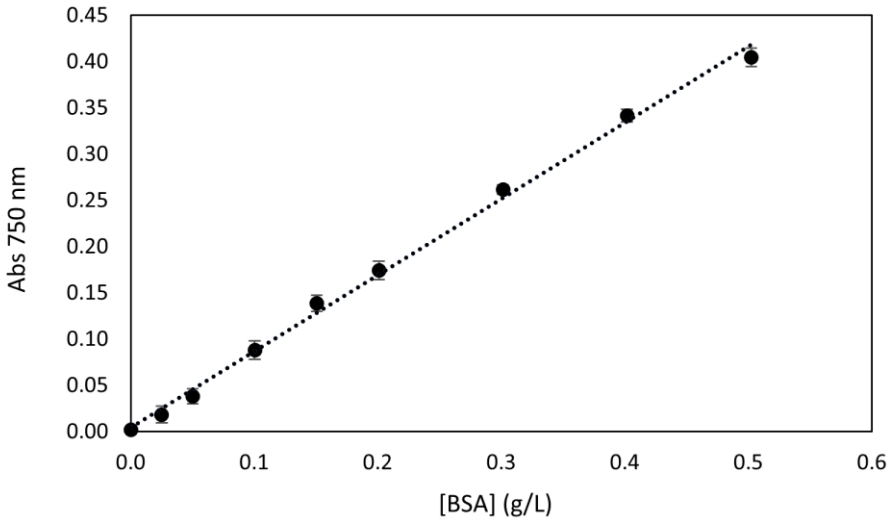

Figure A.1: Calibration curve for protein quantification using Lowry's method prepared with BSA for a working range of 0-0.5 g/L. Values are presented as mean± sd (n=3).

$Abs_{750\,nm} = 8.25 \cdot 10^{-1} \cdot [Protein]\left(\frac{g}{L}\right) + 4.20 \cdot 10^{-3}$ *with a correlation factor of* 0.997

A.2 Monosaccharides Quantification

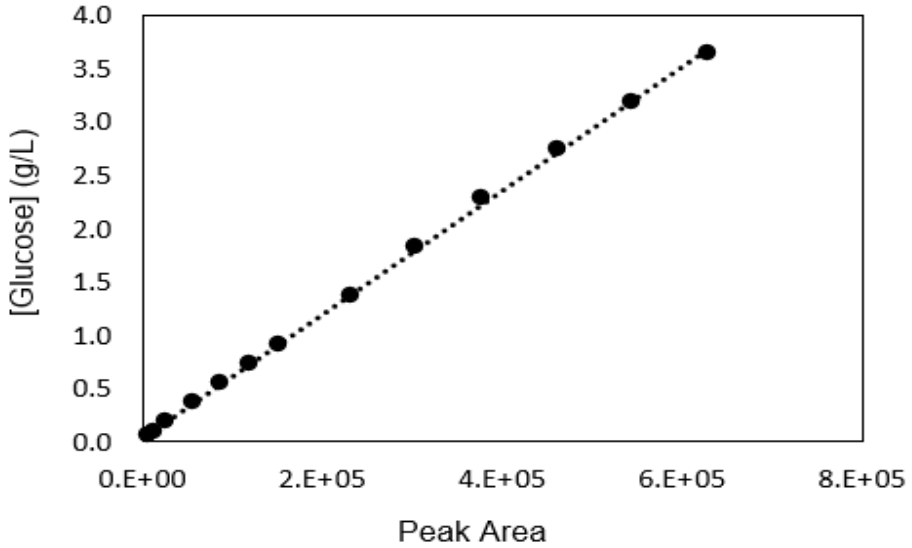

Figure A.2: Calibration curve for glucose quantification using High-Performance Liquid Chromatography prepared for a working range of 0-3.64 g/L.

$[Glucose]\left(\frac{g}{L}\right) = 5.80 \cdot 10^{-6} \cdot Peak\ Area + 2.88 \cdot 10^{-2}$ *with a correlation factor of* 0.999

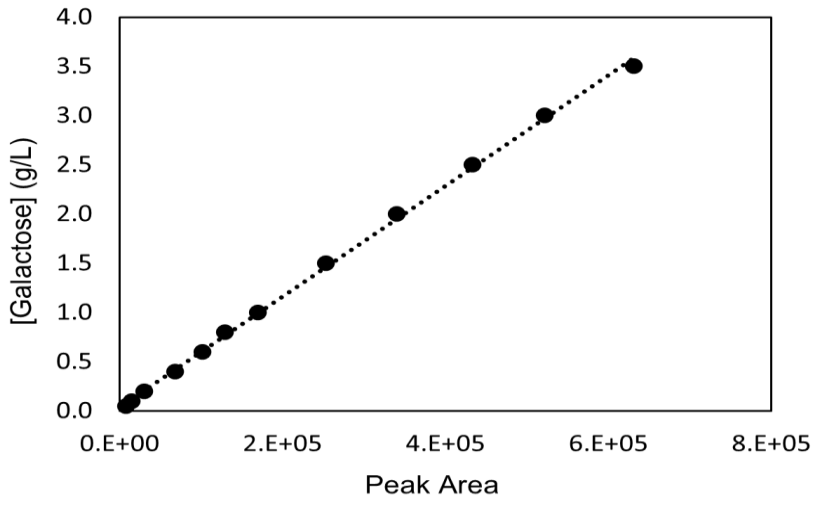

Figure A.3: Calibration curve for galactose quantification using High-Performance Liquid Chromatography prepared for a working range of 0-3.5 g/L.
$[Galactose](g/L) = 5.62 \cdot 10^{-6} \cdot Peak\ Area + 3.87 \cdot 10^{-2}$ *with a correlation factor of* 0.999

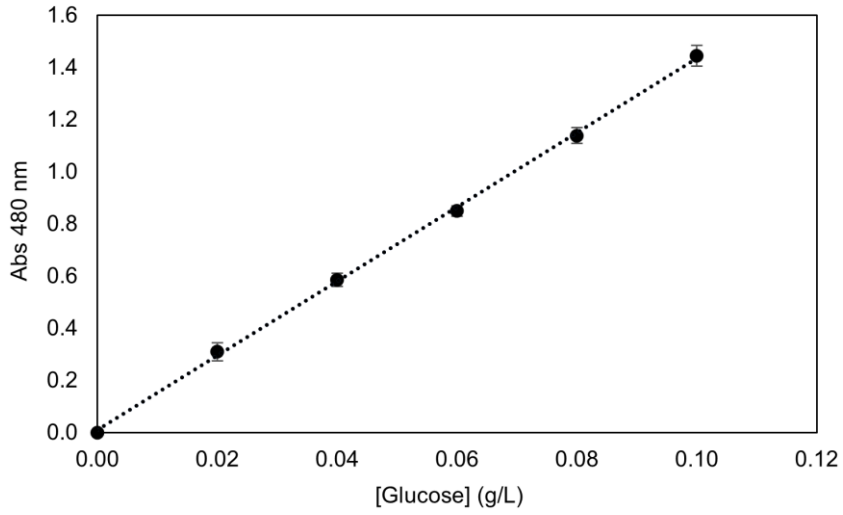

Figure A.4: Calibration curve for glucose quantification using Dubois method prepared with glucose standard for a working range of 0-1.0 g/L.
$Abs_{485\,nm} = 14.2 \cdot [Glucose](g/L) + 9.3 \cdot 10^{-3}$ *with a correlation factor of* 0.999

Appendix B – SEC of standards proteins

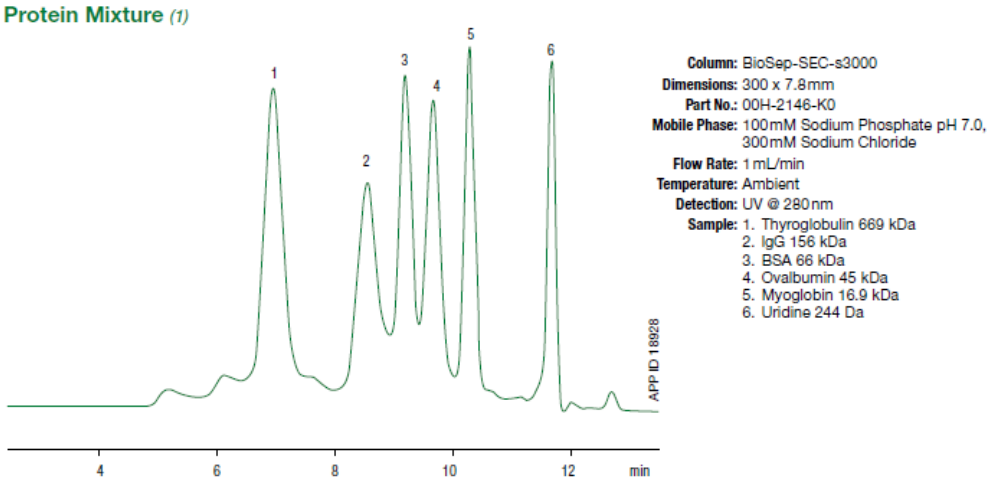

Figure B.1: Elution times of standard protein mixture provided by the column manufacturer

Appendix C – pH-shift precipitation method

Table C.1: Parameters of the protein precipitation by pH-shift by subsequent recovery of the supernatant applied to the pooled extracts of procedures A (one aqueous extraction, one aqueous re-extraction, one alkaline extraction and one alkaline re-extraction) and B (one enzymatic-assisted aqueous extraction with Celluclast®, one enzymatic-assisted aqueous extraction with Alcalase®, one alkaline extraction and one alkaline re-extraction). Protein mass in the pellet was calculated indirectly using Equation 3.10. Values are expressed as mean ± standard deviation (n=3).

Procedure	*V_{pool} (mL)*	*(Protein mass)$_{initial}$ (mg)*	*pH*	*(Protein mass)$_{pellet}$ (mg)*	*(Protein mass) cumulative (mg)*	*Precipitation Yield (%)*
A	30	14.1 ± 1.2	**4**	2.7 ± 0.1	3 ± 0.1	19.3 ± 2.1
			3.5	1.3 ± 0.2	4 ± 0.3	28.5 ± 2.7
			3	0.3 ± 0.2	4 ± 0.4	30.9 ± 1.8
			2.5	0.7 ± 0.1	5 ± 0.3	35.6 ± 2.5
			2	0.2 ± 0.3	5 ± 0.4	37.1 ± 2.7
			1.5	0.6 ± 0.2	6 ± 0.5	41.3 ± 2.1
B	30	39.9 ± 1.0	**7**	13.1 ± 0.5	13 ± 0.5	32.9 ± 1.2
			6.5	5.3 ± 0.3	18 ± 0.8	46.1 ± 1.1
			6	3.4 ± 0.3	22 ± 0.5	54.7 ± 1.5
			5.5	1.8 ± 0.3	24 ± 0.6	59.2 ± 2.0
			5	1.6 ± 0.4	25 ± 0.7	63.3 ± 2.2
			4.5	1.4 ± 0.3	27 ± 0.7	66.8 ± 1.3
			4	1.3 ± 0.3	28 ± 0.6	70.1 ± 1.5
			3.5	0.8 ± 0.5	29 ± 0.8	72.1 ± 1.1