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Evaluation of the Use of Marine Macroalgae in the Industrial Production of Biorefinery-Derived Biofunctional Products

DOCTORAL THESIS

Nuno Miguel Velosa Nunes
DOCTORATE IN BIOLOGICAL SCIENCES

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Thesis presented to obtain the PhD degree in Biological Sciences, Faculty of Life Sciences, University of Madeira.

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Resumo

A tese de doutoramento aqui apresentada integra a avaliação da composição bioquímica de várias espécies de macroalgas, prospetadas no arquipélago da Madeira e arrojamentos de macroalgas provenientes de Gran Canária com o objetivo principal de determinar a potencialidade destes recursos em integrar estratégias de biorefinaria. Esta metodologia é extremamente importante para a gestão sustentável de recursos e mitigação dos impactos, promovidos pela indústria tradicional e pela exploração intensiva dos recursos naturais, desenvolvendo modelos dinâmicos, lucrativos e confiáveis. Este trabalho está dividido em três capítulos, integrando uma introdução geral, um capítulo composto por artigos científicos e um terceiro capítulo que incorpora uma discussão, conclusão e perspetivas futuras. O capítulo 2 começa por abordar uma análise morfológica em 3 macroalgas, por forma a compreender o seu ciclo biológico. Integra 2 artigos que descrevem a variabilidade bioquímica, nutricional e antioxidante e adicionalmente outros 2 artigos que abordam a avaliação da quantidade de iodo e fucoxantina com precisão, biocompostos de extrema importância económica e nutracêutica, no qual foram desenvolvidas e validadas novas metodologias analíticas para uma rápida análise destes compostos nesta biomassa. A implementação de estratégias de biorefinaria foram conduzidas em 2 macroalgas, *A. taxiformis* e *Z. tournefortii*. Na primeira foram utilizados 4 solventes e 2 metodologias para a extração primária de um composto bioativo, efetuando posteriormente a extração sequencial de lípidos, carragenanas e celulose do resíduo remanescente. Para a segunda, foi utilizado 1 solvente, utilizando o extrator Timatic em conjunto com um modelo estatístico por forma a modelar e otimizar a extração de um composto bioativo, extraindo subsequente do resíduo fucoidano e celulose. Adicionalmente, foi efetuada a avaliação da composição dos ácidos gordos em 3 macroalgas coletadas no arquipélago da Madeira, efetuado uma avaliação por TLC, ATR-FTIR, atividade anticolinesterásica e citotoxicidade *in vitro* na linhagem de células tumorais A549.

Palavras-chave: Macroalgas, biorrefinaria, extração de biocompostos, extratos bioativos, iodo, fucoxantina.

Abstract

This doctoral thesis integrates the evaluation of the biochemical composition of several species of macroalgae, prospected in Madeira archipelago and macroalgae beach casts from Gran Canaria, with the main objective of determining the potential of these resources to integrate in biorefinery strategies. This methodology is extremely important for the sustainable management of resources and mitigation of impact, promoted by the traditional industry and by the intensive exploitation of natural resources, developing dynamic, profitable and reliable models. This work is divided into three chapters, integrating a general introduction, a chapter composed of scientific articles and a third chapter that incorporates a discussion, conclusion and future perspectives. Chapter 2 includes a morphological analysis of 3 macroalgae, in order to understand its biological cycle. Also, 2 articles describe the biochemical, nutritional and antioxidant variability in these resources. Afterwards, 2 other articles address the assessment of the amount of iodine and fucoxanthin with precision. These are compounds of extreme economic and nutraceutical importance, in which new analytical methodologies were developed and validated in this work. The implementation of biorefinery strategies were carried out in 2 macroalgae, *A. taxiformis* and *Z. tournefortii*. In the first, 4 solvents and 2 methodologies were used for the primary extraction of a bioactive compound, subsequently performing the sequential extraction of lipids, carrageenan and cellulose from the remaining residue. For the second, 1 solvent was used, using the Timatic extractor in conjunction with a statistical model in order to optimize the extraction of a bioactive extract, subsequently extracting crude fucoidan and cellulose from the residue. In addition, fatty acid composition was evaluated in 3 macroalgae collected in Madeira archipelago, an evaluation by TLC, ATR-FTIR, anticholinesterase activity and *in vitro* cytotoxicity to the A549 tumor cell line was also performed.

Keywords: Macroalgae, biorefinery, biocompound extraction, bioactive extracts, iodine, fucoxanthin.

Abbreviations

AD – Air-Dried
ANOVA – Analysis of variance
APC – Allophycocyanin
ATR – Attenuated total reflectance
BBD – Box-Behnken design
BHT – Butylated hydroxytoluene
CCD – Central composite designs
CE – Catechol equivalents
DPPH – 2,2-diphenyl-1-picrylhydrazyl
DW – Dry weight
FAME – Fatty acid methyl esters
FAO – Food and agriculture organization
FTIR – Fourier-transform infrared spectroscopy
FW – Fresh weight
GAE – Gallic acid equivalents
GLM – General linear model
HCA – Hierarchical cluster analysis
HPLC – High performance liquid chromatography
IA – Index of atherogenicity
IC_{50} – Inhibitory concentration at 50%
ICP – Inductively coupled plasma
INSA – Instituto Nacional de Saúde Dr. Ricardo Jorge
IT – Index of thrombogenicity
ITC – Instituto Tecnologico de Canarias
L – Lyophilized
MS – Mass spectrometry
MUFAs – Monounsaturated fatty acids
PC – Phycocyanin
PCA – Principal component analysis
PDA – Photo diode array detector
PE – Phycoerythrin
PEAR – Postextraction algal residue
PGE - Phloroglucinol equivalents
PUFAs – Polyunsaturated fatty acids
RDI - Recommended dose intake
RP – Reversed phase
RSM - Response surface methodology
TCC – Total carotenoid content
TChlC – Total chlorophyll content
TFC – Total flavonoid content
TFxC – Total fucoxanthin content
TLC – Thin layer chromatography
TPC – Total phenolic content
SFAs – Saturated fatty acids
UV-Vis - Ultraviolet – visible
VASLME – Vortex assisted solid liquid micro-extraction

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2.1 Sampling, structure identification, cultivation and accessions maintenance of three seaweeds from Madeira Archipelago

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Thesis publications

Nunes, N., Ferraz, S., Valente, S., Barreto, M.C., Pinheiro de Carvalho, M.A.A., 2017. **Biochemical composition, nutritional value, and antioxidant properties of seven seaweed species from the Madeira Archipelago**, in: Journal of Applied Phycology. https://doi.org/10.1007/s10811-017-1074-x

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Nunes, N., Valente, S., Ferraz, S., Barreto, M.C., Pinheiro de Carvalho, M.A.A., 2019. **Validation of a spectrophotometric methodology for a rapid iodine analysis in algae and seaweed casts.** Algal Res. 42, 101613. https://doi.org/10.1016/j.algal.2019.101613

Nunes, N., Rosa, G.P., Ferraz, S., Barreto, M.C., Pinheiro de Carvalho, M.A.A., 2019. **Fatty acid composition, TLC screening, ATR-FTIR analysis, anti-cholinesterase activity and in vitro cytotoxicity to A549 tumor cell line of extracts of 3 macroalgae collected in Madeira.** J. Appl. Phycol. https://doi.org/10.1007/s10811-019-01884-9

Nunes, N., Valente, S., Ferraz, S., Barreto, M.C., Pinheiro de Carvalho, M.A.A., 2019. **Constructing ethanol-derived bioactive extracts using the brown seaweed *Zonaria tournefortii* (J.V.Lamouroux) Montagne performed with Timatic extractor by means of response surface methodology (RSM).** J. Appl. Phycol. https://doi.org/10.1007/s10811-019-01973-9

Nunes, N., Valente, S., Ferraz, S., Barreto, M.C., Pinheiro De Carvalho, M.A.A., 2020. **Biochemical study of attached macroalgae from the Madeira Archipelago and beach-cast macroalgae from the Canary Islands: multivariate analysis to determine bioresource potential**. Botanica marina, 63(3): 283–298. https://doi.org/10.1515/bot-2019-0022

Table of contents

Thesis outline

This thesis is organized in 3 main chapters, namely 1. Introduction, 2. Scientific Articles, and 3. Discussion, Conclusion and Future Perspectives, according to the specific regulation for the 3rd cycle in Biological Sciences from the University of Madeira.

Chapter 1, Introduction, provides the framework for the thesis, providing its main goals and giving coherence to the Scientific Articles, each of which answers specific questions relevant to the global objective of the Thesis stated in its title, i.e., the relevance and feasibility of using marine macroalgae in the industrial production of biorefinery-derived biofunctional products. In this context, the Introduction summarizes the worldwide production of macroalgae, evidences the published biorefinery effort performed to the algae biomass and compares the content of macroalgae biocompounds published by the scientific community.

In Chapter 2, the Scientific Articles section, eigth individual works were integrated. The first work describes the "Sampling, structure identification, cultivation and accessions maintenance of three seaweeds from Madeira Archipelago". This work is being prepared to be submitted and enables the understanding of where are these macroalgae in Madeira Archipelago, identifies its microscopic structures, presents its culture conditions and laboratory maintenance. The second and third works present a biochemical screening of several macroalgae found in the Archipelago and in beach-cast macroalgae. The first paper was published in *Journal of Applied Phycology* 2017, entitled "Biochemical composition, nutritional value and antioxidant properties of seven seaweed species from Madeira Archipelago". The second one, entitled "Biochemical study of attached macroalgae from the Madeira Archipelago and beach-cast macroalgae from the Canary Islands - multivariate analysis to determine bioresource potential" was published in *Botanica Marina*. Two validated methodologies aiming to determine the analytical concentrations of nutritionally relevant algal components were developed. The determination of iodine content, published in 2019 in *Algal Research*, under the title "Validation of a spectrophotometric methodology for a rapid iodine analysis in algae and seaweed casts". The method for fucoxanthin content determination, also published in *Algal Research* in a work entitled "Evaluation of fucoxanthin contents in seaweed biomass by vortex-assisted solid-liquid microextraction using high-performance liquid chromatography with photodiode array detection". Furthermore, separate assays for a biorefinery assessement of two different macroalgae were acomplished. The scientific article entitled "Nutraceutical potential of *Asparagopsis taxiformis* (Delile) Trevisan extracts and assessment of a downstream purification strategy" was published, in *Heliyon* journal (2018). This work reports the production of several biofunctional extracts, using two different methodologies and four distinct solvents. Also, the residue is subjected to a cascade extraction to obtain lipids, carrageenan and cellulose from this biomass. The biorefinery assessement of the brown macroalgae *Zonaria tournefortii* was published in *Journal of Applied Phycology* (2019) under the title "Constructing ethanol-derived bioactive extracts using the brown seaweed *Zonaria tournefortii* (J.V.Lamouroux) Montagne performed with Timatic extractor by means of response surface methodology (RSM)". In this work it was also studied the bioactive properties of this macroalgae, using a statistical analysis to model the production of extracts and applying a cascade approach to the residue, for crude fucoidan and cellulose extraction. The last work included in this thesis was published in 2019, in *Journal of Applied Phycology*, with the title "Fatty acid composition, TLC screening, ATR-FTIR analysis, anti-cholinesterase activity and *in vitro* cytotoxicity to A549 tumor

cell line of extracts of 3 macroalgae collected in Madeira". This work aimed to study the biochemical composition of *U. lactuca*, *A. taxiformis* and *Z. tournefortii* and its potential as a nutraceutical biomass.

Finally, in Chapter 3, Discussion, Conclusion and Future Perspectives, the global discussion of the results with the outlining of conclusions and future research perspectives are presented.

Chapter 1

Chapter 1

1. General introduction

1.1 Introduction

In earlier centuries, coastal populations harvested a wide variety of seaweeds that were used in multiple purposes such as food, animal feed, treatment of diseases (goiter, intestinal afflictions and dilation of the cervix in difficult childbirths) and more recently to fertilize soil and produce gels (Buschmann et al., 2017). Nowadays, harvesting seaweeds is performed exclusively using manpower or recurring to equipment to facilitate this work.

According to the latest data available, in 2015, 730,575 tons of seaweed were harvested worldwide, 52,208 tons of which were in fresh weight and the remaining in dry weight (FAO, 2017). From 2005 till 2015, Chile was the biggest harvester in the world, reaching a gather peak 428,674 tons, in 2013, when the total world harvest was 858,842 tons. Chile contribute for almost 50% of seaweed worldwide harvest in this year (fig. 1).

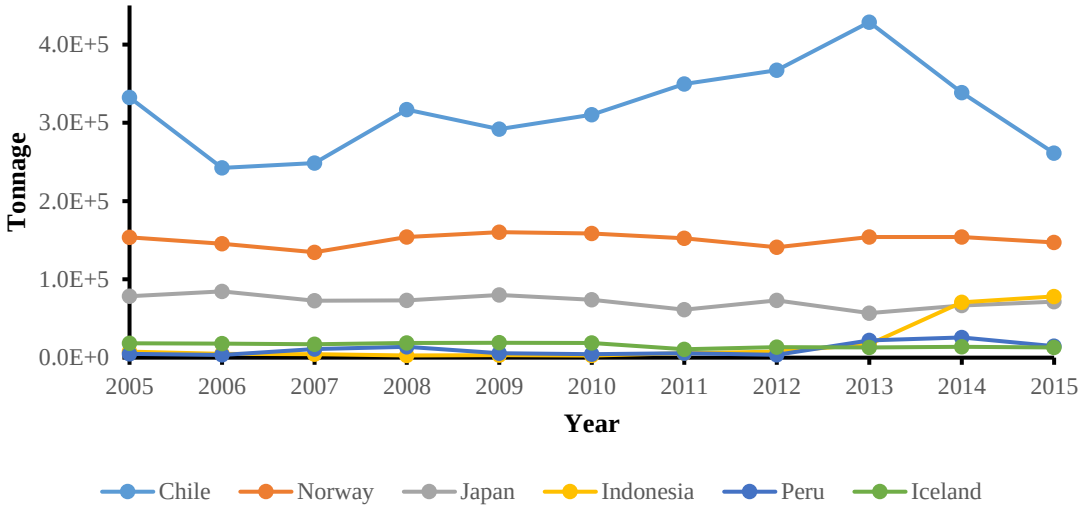

Figure 1 – Plot of FAO (2017) data, yearly tonnage of macroalgae for the six biggest seaweed harvesters worldwide, Chile, Norway Japan, Indonesia, Peru and Iceland, from 2005 till 2015 (harvesters still active in 2015).

In 2015, Chile harvesting capacity decreased to 261,246 tons per year of seaweed, still representing 36% of the worldwide harvesting capacity. In the same period, Norway, the second biggest harvester of seaweed worldwide, maintained its capacity, slightly variating and in 2015 harvested 147,391 tons of seaweed, more than 20% of seaweed world harvest. Japan, Indonesia, Peru and Canada are the following countries in seaweed harvesting, after Chile and Norway. Chile's major harvest was brown seaweeds (Chilean kelp and *Lessonia trabeculata*), followed by red seaweeds (*Gracilaria*) in the southeast Pacific Ocean (fig. 2).

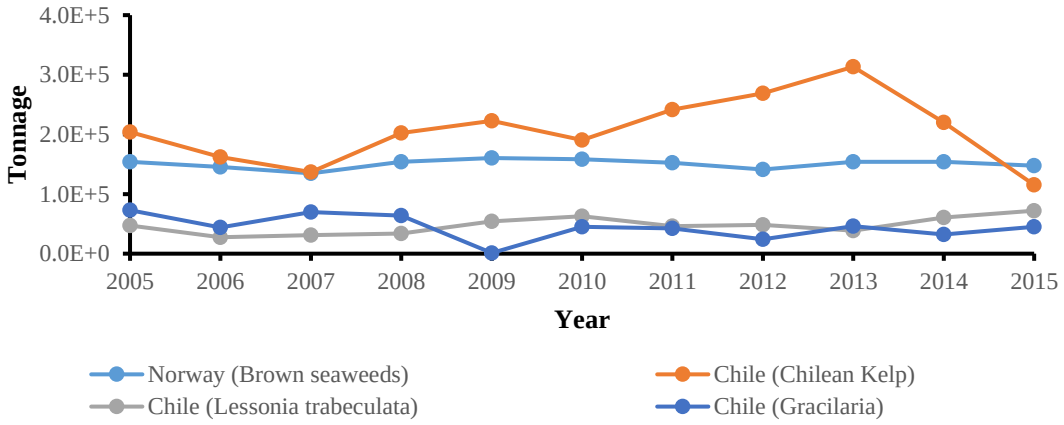

Figure 2 – Plot of FAO (2017) data, tonnage per year, for the four biggest seaweed that were harvested in Norway (brown seaweed) and Chile (Chilean kelp, *Lessonia trabeculata* and *Gracilaria*) from 2005 to 2015 (still active in 2015).

Norway also majorly harvests brown seaweeds which includes *Laminaria hyperborea* and *Ascophyllum nodosum* from northeast Atlantic Ocean. Estimated biomass of *L. hyperborea* situates between 50 and 100 million tons, with an average density of 10 to 15 kg per m^2. *A. nodosum* is estimated in almost 2 million tons along the Norwegian coast, with densities of 4 to 7 kg per m^2 (Meland and Rebours, 2012). According to the FAO (2017), in 2015, the European Union (EU) harvested a total of 45,394 tons, including fresh and dried seaweed, corresponding to 6.21% of worldwide harvesting. Ireland, in 2015, was the largest harvester in the EU, maintaining its harvesting scores of 29,500 tons fresh seaweeds since 2005, (fig. 3), representing 65% of the total EU share. France occupies the 2nd position, with 10,331 tons of fresh seaweed, 22.76% of total EU share, reaching its maximum yield in 2014, with 34,165 tons. Other countries harvesting seaweeds are Spain (2,376 tons - 5.23%), Portugal (1,574 tons - 3.47%), Italy (1,200 tons - 2.64%) and Estonia (413 tons - 0.91%). France presents a variable harvesting tendency, but between 2005 and 2015 the harvesting contribution of the remaining European countries were maintained.

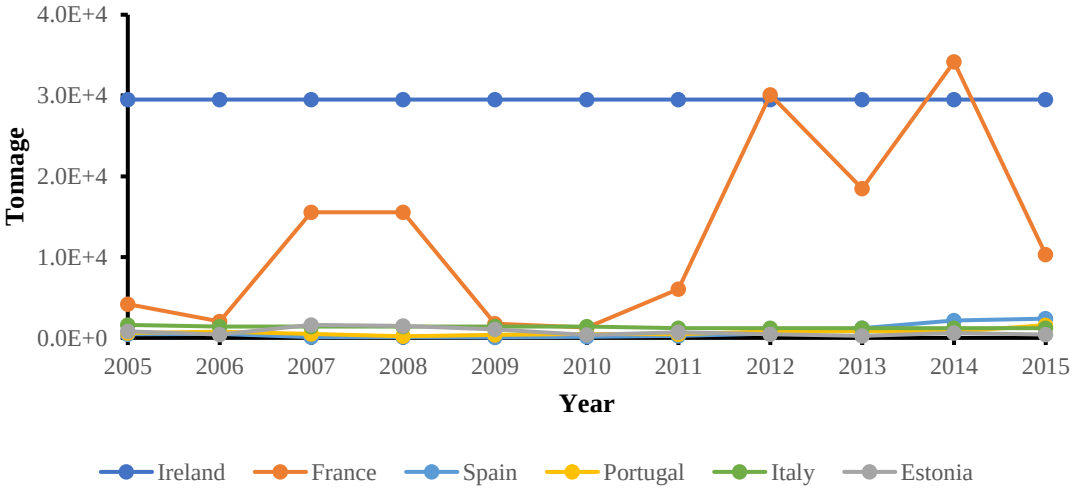

Figure 3 – Plot of FAO (2017) data, yearly tonnage of macroalgae for the only six seaweed harvesters still active in 2015 in European Union, Ireland, France, Spain, Portugal, Italy and Estonia, from 2005 to 2015.

In 2015, China produced almost 50% of the worldwide production of seaweed in aquaculture, which corresponds to 2,152,239.06 tons (fig. 4), majorly constituted by Wakame, *Gracilaria* and indistinct seaweeds (fig. 5). In the same year, Indonesia was the second world aquaculture producer, with 842,852.09 tons of seaweed (fig. 4), corresponding to 19.35% of worldwide production, which mainly corresponds to the production of *Eucheuma* seaweed (fig. 5) for the extraction of carrageenan. Since 2005, China increased seaweed aquaculture production only by 1.12 fold, while in the same period Indonesia increased its production by 6.61 fold, showing a distinct evolution in seaweed aquaculture production in recent years (FAO, 2017). In 2005 Japan was the 2nd largest aquaculture producer, essentially manufacturing Laver, which is square sheet of dried seaweed, in this case Nori, very commonly used in sushi. Over this period we assisted to a decreased production capacity, which has become slightly above Indonesia production in 2015. Korea, Chile and Philippines are the countries showing a significant seaweed aquaculture production after China, Indonesia and Japan (fig. 4) in a worldwide scenario.

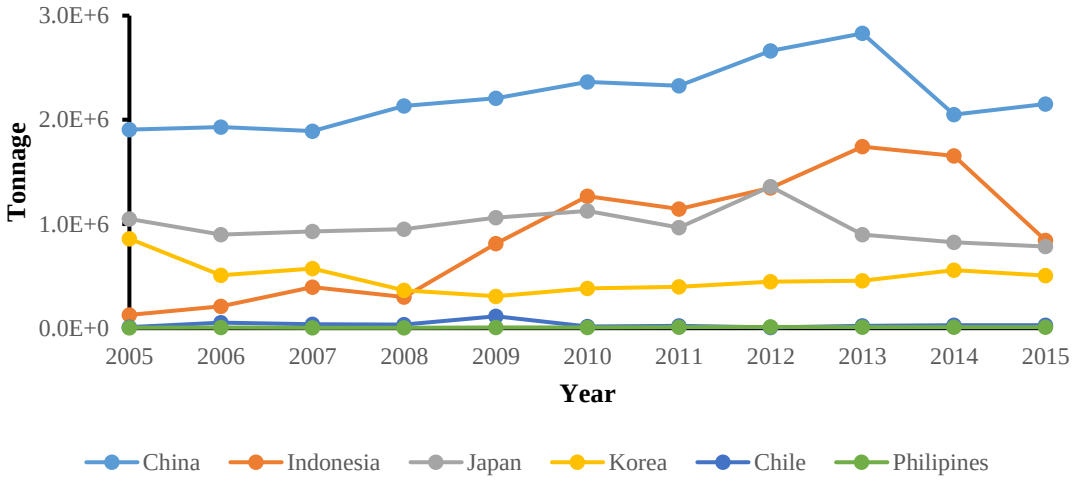

Figure 4 – Plot of FAO (2017) data, yearly tonnage of macroalgae for the six largest seaweed aquaculture producers, China, Indonesia Japan, Korea, Chile and Philippines from 2005 till 2015 (that were still active in 2015).

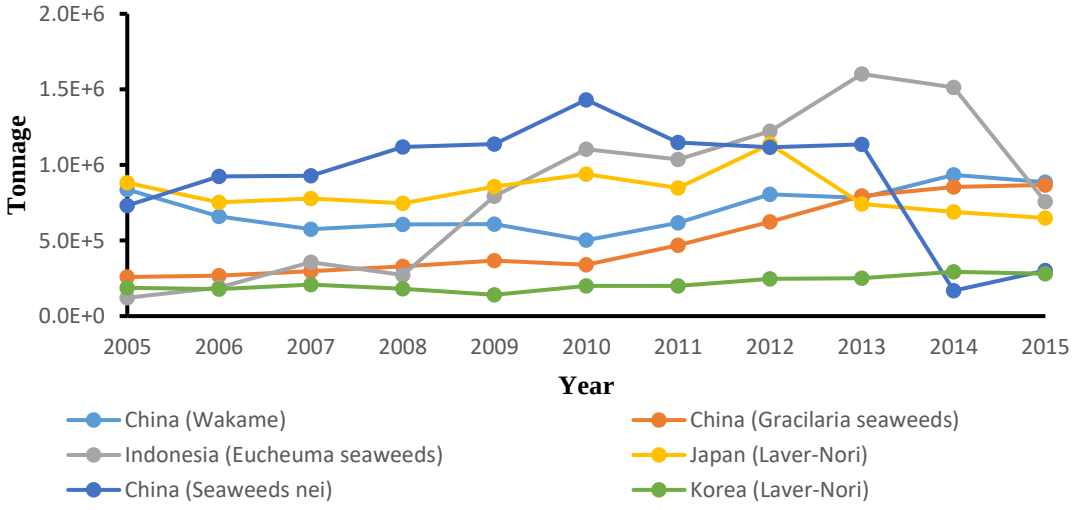

Figure 5 – Plot of FAO (2017) data, tonnage per year, for the six largest aquaculture seaweed produced, China (Wakame, *Gracilaria* and indistinct seaweeds), Indonesia (*Eucheuma* seaweeds), Japan and Korea (Laver-Nori), from 2005 to 2015 (that were still active in 2015).

EU is a small player in seaweed aquaculture production, with a total of 1,951.49 tons in 2015, representing only 0.04% of worldwide production (FAO, 2017). Nevertheless, in 2015, Spain was the largest seaweed producer in EU, with a total share of almost 81%, representing 1,579.92 tons, followed by France that produced 149.8 tons, approximately 7.68%, Portugal 90.56 tons, 4.64%, Ireland 77.67 tons, 3.98%, and Denmark 53.54 tons, 2.74% of total EU seaweed aquaculture production (fig. 6).

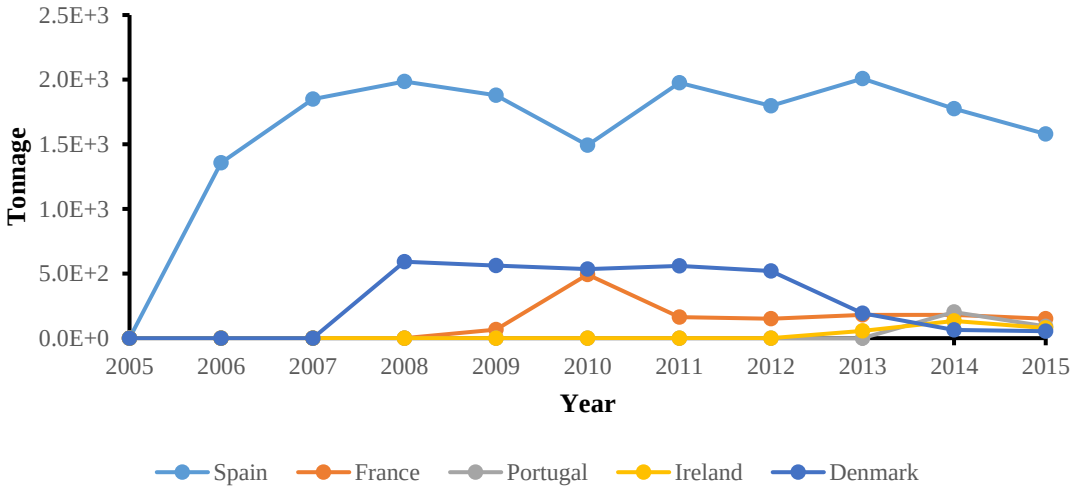

Figure 6 – Plot of FAO (2017) data, tonnage per year, for the only five seaweed aquaculture producers in the European Union, that were still active in 2015, Spain, France, Portugal, Ireland and Denmark from 2005 till 2015.

All of these countries maintain their seaweed aquaculture productions in the northeast of Atlantic Ocean. The aquaculture of seaweed is often considered as an alternative to the harvest of wild seaweeds, because cultivation can provide the intensification of yield and allows to control the quality of the seaweed, crucial for some industries. At the same time, it allows sufficient quantities of seaweed to be produced without inducing pressure on marine resources. Aquaculture is mainly carried out by seaweed farmers, with the particularity of entrancing seaweed propagules in ropes, attached to poles that are firmly secured to the shallow sea bottom. Alternatively, at major depths, reaching up to 30 to 40 meters, seaweed propagules are attached to ropes and suspended, with the assistance of heavy weights, positioned in the sea bottom and maintained in fluctuation together with buoys. In either case, fluctuating structures can be implemented to keep the seaweed at surface or submerged, which can vary in depth with the high and low tide.

The global concept of biorefinary defines it as a network of facilities, equipment and strategies, which integrates biomass for extraction and/or conversion processes to produce biofuels, energy and commodity biochemicals (Moncada et al., 2016). In summary, lignocellulosic feedstock, green and whole crop biorefinaries represent three independent systems that are in general studied and developed to determine their performance and sustainability (Gnansounou et al., 2017). Biorefinary potential is enormous, since there is a great variety of unique feedstock that can be decomposed or converted using multiple step strategies and technologies, available nowadays. Table 1 resumes several biorefinary approaches to extract multiple products, using singular strategies. Furthermore, numerous methods are reported for distinct lignocellulosic feedstock. Silalertruksa et al. (2017) evaluated the potential of sugarcane bagasse in Thailand, which after primary juice extraction for sugar production, was assessed for

steam and electricity, molasses to produce ethanol and vinasse to serve as fertilizer and soil conditioner. Other examples of lignocellulosic based biorefineries are for corn stover (Wang et al., 2017) and wheat-bran (Alonso, 2017).

Table 1 – Biomass used for the extraction of several commodities.

Biomass	Commodities	Reference
Arugula seeds *(Eruca Sativa)*	Methanol Ethanol Biodiesel Biomethane Glycerol	(Rahimi et al., 2017)
Lignocellulose	Lignin Syngas Bio-oil	(Özdenkçi et al., 2017)
Oleaginous yeast (*Rhodosporidium toruloides*)	Refined oil Biogas	(Marques et al., 2017)
Soybean	Refined oil Biodiesel Deffated meal Glycerol Biofuel Dimethyl Furan (DMF) Dimethyl Ether (DME)	(De Pretto et al., 2017) (Abdulkhani et al., 2017)
Sugarcane	Ethanol Sugar Ethanol Steam (electricity)	(Mendes et al., 2017)
Wheat bran	Oil β-glucans Dextrins Glucose Soluble lignin Proteins Xylose Arabinose Cello-oligosaccharides Insoluble lignin Alkylresorcinols (AR's)	(Alonso, 2017)

Also, several strategies using the whole plant are already being established for other crops, such as soybean (*Glycine max*), producing refined oil, biodiesel, deffated meal and energy (Abdulkhani et al., 2017; De Pretto et al., 2017) and more recently arugula (*Eruca sativa*), for the production of energy, biodiesel, ethanol, biomethane and glycerol (Rahimi et al., 2017).

Unicellular photosynthetic microalgae are also studied to be integrated into the biorefinary effort. These organisms have a complex cell-wall, are capable to accelerate their reproduction rate and are considered an ideal material to extract multiple products such as proteins, lipids, carbohydrates, flavonoids, terpenoids and alkaloids (Chua and Schenk, 2017).

Table 2 – Microalgae used to develope biorefinaries strategies to extract several products from this biomass. Primary and secondary applications were determined.

Biomass	**Application**				**Reference**
	Primary	Methodology	Secondary	Methodology	
Chlorella pyrenoidosa	Carbon dioxide bio-capture	------	Oil	Liquefaction	(Wiesberg et al., 2017)
			Bio-syngas	Gasification	
Haematococcus pluvialis	Astaxanthin	Solid-liquid extraction	Biodiesel	Transesterification	(García Prieto et al., 2017)
			Glycerol	Byproduct	
			Poly(hydroxybutyrate) (PHB)	Fermentation by *Cupriavidus necator*	
			Fertilizer	Byproduct	
			Energy	Byproduct	
Isochrysis galbana	Carotenoids Non-polar lipids	Supercritical fluid extraction	Chlorophylls Mid and highly-polar lipids Proteins Sugars	Supercritical fluid extraction	(Gilbert-López et al., 2015)
Nannochloropsis sp.	Eicosapentaenoic acid (EPA)	Solid-liquid extraction	Biodiesel	Transesterification	(Mitra et al., 2015)
Nannochloropsis sp.	Fatty acids (for biodiesel)	Supercritical fluid extraction	Carotenoids Chlorophylls	Supercritical fluid extraction	(Nobre et al., 2013)
			Biogas (hydrogen)	Dark fermentation with *Enterobacter aerogenes*	
Spirogyra sp.	Carotenoids	Solid-liquid extraction	Biogas (hydrogen)	Fermentation using *Clostridium butyricum*	(Pacheco et al., 2015)
			Organic acids	Byproduct	
			Methanol	Chemical reaction between CO_2 and hydrogen	

Table 2 summarizes the bibliography refering microalgae biorefinary approaches to extract various byproducts. Wiesberg and co-workers (2017) considered green microalgae *Chlorella pyrenoidosa* as the starting biomass to integrate in a biorefinary process (table 2).

The primary objective was to assess the economic feasibility of CO_2 bio-capture and storage by these microrganisms, transforming it into valuable products for mankind. Oil was the first product obtained through liquefation processes and secondly the production of bio-syngas applying gasification procedures. They concluded that the major expenditure in this approach, 1/5 of the total cost, was the purchase of photobioreactors. García Prieto et al. (2017) developed an integrated microalgae-based biorefinery through the formulation of a mixed integer nonlinear programming model. They cultivated green microalgae *Haematococcus pluvialis* in open ponds and used a surfactant-chelate to reduce production costs. Primarily, astaxanthin was extracted using a solid-liquid extraction with hexane. Posteriorly, the residual biomass was transformed into other valued products such as biodiesel, throught transesterification of fatty acids and production of polyhydroxybutyrate (PHB) by the fermentation with *Cupriavidus necator*. Three other valuable byproducts were obtained in the final process, glycerol, fertilizer and energy. Additionaly, they designed a detailed capital cost model for process equipments acquisition. They concluded that the extraction of astaxanthin and production of PHB from this microalgae, followed by the production of biodiesel, becomes economically feasible. Mitra et al. (2015) assessed the marine eustigmatophyte *Nannochloropsis* sp. potential to integrate in a biorefinary strategy platform in a two stage cultivation, under photoautotrophic conditions. The cultivation of these microalgae was maintained in a photobioreactor up to the late log phase. Afterwards, they first extracted teicosapentaenoic acid (EPA), using conventional methods of solid-liquid extraction. As a second step of biorefinery, the transesterification of remaining saturated fatty acids (SFAs) and monounsaturatted (MUFAs) for the production of biodiesel was performed. In two phase cultivation, the authors concluded that the temperature and ligth reduction in the second phase can increase EPA production by 3.4 fold, but presents the disadvantage of reducing the biomass, lipid content and productivity. Fractionation of lipids in neutral lipids (NLs), glycolipids (GLs) and phospholips (PLs) demonstrated that the major portion of EPA is presented in PLs class. Nobre and co-workers (2013) also evaluated the potential of *Nannochloropsis* sp. as feedstock in a biorefinary strategy, primarily extracting fatty acids to produce biodiesel, secondly carotenoids and chlorophyll *a* as a high added-value product and thirdly producing biohydrogen, through a dark fermentation with *Enterobacter aerogenes*. The extraction procedures were performed using supercritical fluid extraction, variating temperature, pressure and solvent flow rate in order to determine their effect on extraction yield. They concluded that using only CO_2, at 40 ºC, 300 bar with a flow-rate of 0.62 g/min, they could efficiently extract 33 g of lipids per 100 g of dry biomass. The use of 20% ethanol as co-solvent increased the lipid extraction to 45 g of lipids per 100 g of dry biomass. Gilbert-López and co-workers (2015) assessed the potential of *Isochrysis galbana*, also using supercritical fluid extraction within the same parameters described for *Nannochloropsis* sp. They performed multiple extracts, applying a downstream processing platform, with assistance of various pressurized green solvents (CO_2, ethanol and water) to achieve the isolation of bioactive compounds and compare its yield efficiency with conventional methods. Several extracts were produced, including first carotenoids and non-polar lipids, second carotenoids, chlorophylls, mid and highly-polar lipids, and third proteins and sugars. They also performed a factorial design to optimize the extraction of fucoxanthin, a primary carotenoid found in *Isochrysis galbana*, using 3 different percentages of ethanol, 15, 45 and 75%. Their main target was to determine the similarity between conventional and pressurized extraction during the extraction of bioactive compounds, complying with the principles of green chemistry. This work presents a paradigm shift when adressing the biorefinary of microalgae, targeting only high valuable

products and not bio-fuel. Furthermore, Pacheco and co-workers (2015) evaluated the overall energy consumption and greenhouse gas emissions of pigments extraction and bio-hydrogen production from green microalgae *Spyrogyra* sp.

Seaweed biomass is a potential feedstock for biofuel production, an abundant source of renewable sugars as its growth does not require arable land, fresh water or intense labor (Konda et al., 2015). Table 3 summarizes the information from bibliography refering biorefinary methods to extract several products, using a range of strategies.

Table 3 – Macroalgae used to determine biorefinary strategies with primary and secondary applications.

Biomass	**Application**				**Reference**
	Primary	Methodology	Secondary	Methodology	
Gracilariopsis longissima	Agar	Alkali treatment	Ethanol	Fermentation (*Saccharomyces cerevisiae*)	(Kumar et al., 2013)
			Fertilizer	Byproduct	
Gelidiella acerosa	Bioethanol	Enzymatic hydrolysis (*Saccharomyces cerevisiae*)	Lipids	Solid-liquid extraction	(Baghel et al., 2015)
			Agar	Freezing and thawing	
Gracilaria dura			R-phycoerytrin (R-PE)	Solid-liquid extraction	
			R-phycocyanin (R-PC)	Solid-liquid extraction	
Gelidium pusillum			Liquid fertilizer	Byproduct	
Gracilaria gracilis	Phycobiliproteins	Solid-liquid extraction	Bio-oil	Pyrolysis	(Francavilla et al., 2014)
			Biochar	Byproduct	
Several seaweeds	Bio-oil	Hydrothermal liquefaction	Biogas (CO_2)	Byproduct	(Raikova et al., 2017)
			Ashes	Byproduct	
			Aqueous phase products	Byproduct	
Chaetomorpha linum	Bioethanol	Fermentation (*Saccharomyces cerevisiae*)	Biogas (Biomethane)	Byproduct	(Ben Yahmed et al., 2016)

Ben Yahmed and co-workers (2016) introduced the biorefinery of green seaweed *Chaetomorpha linum*. They investigated an eco-friendly biorefining process to co-produce bioethanol and fermenting the biomass with *Saccharomyces cerevisiae*, obtain biogas, mainly methane as a valuable byproduct. Several pre-treatments of thallus were applyed to determine its desintegration potential. These included acid, neutral and alkali treatments, with 3% NaOH giving the best results, assessed by scanning electron microscope and saccharification tests. After pre-treatment, the biomass would be recovered and subjected to hydrolytic enzymes extracted from *Aspergillus awamari*. Bioethanol yield reached the maximum of 0.41 g per g of reducing sugars. Francavilla and co-workers (2014) performed a cascade approach using red macroalga *Gracilaria gracilis*, to extract and purify phycobiliproteins, using a solid-liquid methodology. R-phycoerytrin, allophycocyanin and phycocyanin were the main phycobiliproteins extracted, yielding 7 mg/g

dry weight (dw), 3.5 mg/g dw and 2 mg/g dw, respectively. This extraction was followed by the production of bio-oil and the residual biomass submitted to a pyrolisis convertion, with optimum temperature between 400 and 600 ºC, obtaining biochar as a byproduct. Bio-oil yield was high, 65 weight (wt) % at 500 ºC, but unfortunely with a high content of nitrogenous compounds, preventing its use as a bio-fuel. Biochar yield was also high, reaching 33 wt% at 400ºC. Baghel and co-workers (2015) selected three seaweeds, *Gelidiella acerosa*, *Gelidium pusillum* and *Gracilaria dura*, to produce bioethanol from the enzymatic hydrolysate using cellulase, followed by a *Saccharomyces cerevisiae* fermentation. Lipids, R-phycoerytrin (R-PE) and R-phycocyanin (R-PC) were extracted from residual pulp using a solid-liquid extraction. Agar was extracted following the freezing and thawing methodology and a liquid fertilizer obtained as a final byproduct. This work envisions a scalable production of valuable products with manageable integrated processes, facilitating the sequencial extraction of major biocomponents of red seaweeds. This strategy shows some advantages over direct extraction, such as the improvement of the agar quality, which increased gel strengh by 1.5 to 3 fold, without alkali or acid pre-treatment. The residue resulting from the sequential extractions was eliminated and chemicals used in cellulose extraction were reduced up to 85 %. Kumar and co-workers (2013) used a different strategy in the biorefinary of red seaweed *Gracilariopsis longissima*, formely knowned has *Gracilaria verrucosa*. Seaweed was harvested at various growth times and agar was primarily extracted by an alkali treatment, yielding 27 to 33 %. The leftover pulp, containing from 62 to 68 % hollocellulose, was subjected to an enzymatic hydrolysis, to obtain 0.87 g of sugars per g of cellulose. This hydrolysate was subjected to fermentation with *Saccharomyces cerevisiae* for the production of bio-ethanol yielding 0.43 g of ethanol per g of sugar. A single final byproduct was obtained, which can be used as a fertilizer or soil conditioner. Raikova and co-workers (2017) applied a hydrothermal liquefaction (HTL) process to produce a bio-crude oil, using thirteen different seaweeds. This work intended to produce a cohesive assessment of the effects which the biochemical composition imprints, when converting seaweed biomass to bio-crude oil. Additionallly, three byproducts were obtained using this methodology, biogas (mainly CO_2), aqueous phase products and ashes. *Ulva lactuca* showed the highest yield for bio-crude oil, 29.9 %. Aqueous phase phosphate concentrations of 236 $mg.L^{-1}$ were obtained using *Solieria chordalis* biomass. A positive correlation between increase of lipids in the biomass and the increase of bio-crude oil yield was obtained. Likewise, the increase of biomass nitrogen will generally contribute to bio-crude oil nitrogen content. This HTL is seen to be a promising biomass convertion method, capable of simultaneously producing fuels and aqueous fertilizers or as auxiliar in the remediation, when producing and using biomass from contaminated marine or fresh water effluents.

1.2 Biocompounds in macroalgae

1.2.1 Phenolic compounds

These compounds are the main secondary metabolites, present in plants, lichens and algae. Structurally, they are formed from one to multiple aromatic rings with one or more hydroxyl groups, totalizing 8,000 different known compounds (Dai and Mumper, 2010). Their main functions include the protection against epiphytes, marine grazers, pathogens, photoprotection and in brown algae they appear cross-linked with alginates to reinforce the cell wall (Audibert et al., 2010). The composition and concentration of the phenolic compounds (PC) tend to fluctuate according to the seaweed location. Seaweed exposed to higher levels of UV radiation and desiccation periods will produce higher content of PC than submerged seaweed (O'Sullivan et al., 2011). Flavonoids are the most consumed polyphenols within human food, containing 15 carbon atoms arranged in three rings, C6-C3-C6. They are divided into six subgroups, which includes anthocyanins, flavanones, flavonols, flavones, flavanols and isoflavones, according to the oxidation status of the central C ring (Dai and Mumper, 2010).

In green and red seaweeds, PC present are mostly bromophenols, but in lower quantity than PC in brown seaweeds, which are mainly phlorotannins (Connan, 2015). Phlorotannins are oligomers or polymers of monomeric units of 1,3,5-trihydroxybenzene (phloroglucinol), interconnected with various linkages, such as dibenzodioxin for eckols and carmalols, aryl–aryl bonds for fucols, ether bonds for phlorethols, hydroxyphlorethols and fuhalols or a combination of both aryl–aryl and ether bonds for fucophlorethols (Rajauria, 2018). These compounds have a wide range of molecular sizes (0.4 to 400 kDa) and can be present in brown seaweeds at various concentrations (Balboa et al., 2013). Special vesicles, named physodes, store these compounds that can be halogenated or sulfated (Stengel and Walker, 2015) and are known to absorb in the UVB range (280–320 nm) (Yuan and Walsh, 2006). Purified phlorotannins are known to present a strong antioxidant activity, due to the presence of phenol rings and to their unique molecular structure, acting as an electron trap, scavenging peroxides, superoxide anions and hydroxyl radicals (Wang et al., 2009). Extracts of phenolic compounds properly purified, fractioned and characterized can represent an important source of bioactive molecules for food, cosmetic and medicine (Audibert et al., 2010). Several epidemiologic data, as well as *in vitro* and *in vivo* studies have reported health benefits when consuming polyphenol rich foods, preventing cardiovascular diseases related to ageing, due to their antioxidant properties, antimutagenic activities and cholesterol-lowering effects (Keyrouz et al., 2011). Additionally, phenolic compounds are found to modulate the activity of a wide range of enzymes and cell receptors (Dai and Mumper, 2010). *In vitro* tests showed that polymeric phlorotannins from brown algae *Eisenia bicyclis* and *Ecklonia kurome* inhibit enzyme activity, such as hyaluronidase (Shibata et al., 2002a), phospholipase A, lipoxygenase and cyclooxygenase-1. This last inhibitory activity was detected in phlorotannins extracted from *Eisenia bicyclis* of the Japan coast (Shibata et al., 2002b). In the food industry, hypopigmentation of products from natural sources is progressively required. The inhibition of tyrosinase or blockade of upstream regulation of melanogenesis for active whitening, can be achieved with phlorotannins without cytotoxic effects (Balboa et al., 2013). Additionally, these compounds have the potential to be used as natural preservatives, due to their antibacterial activity (Audibert et al., 2010). Various solvents and extraction methods are continuously developed due to the diversity of phenolic

compounds and matrix features needed. The most common solvents used for PC extraction include methanol, acetone and ethanol in various concentrations. Several assays for phenolic compounds measurement are available such as Folin-Denis (FD), Folin-Ciocalteu (FC) and 2,4-dimethoxybenzaldehyde (DMBA) assays (Connan, 2015).

Table 4 summarizes the total phenolic content (TPC) in macroalgae from several worldwide places, using different standards to validate TPC content, namely phloroglucinol equivalents (PGE), gallic acid equivalents (GAE) and catechol equivalents (CE).

According to table 4, TPC varies from 0.1 to 24.2 g/100g dw, using PGE as a standard. Also, the highest values for TPC were obtained when extraction from the brown seaweed *Fucus vesiculosus* was performed with 70% acetone, at room temperature, during 24h (Wang et al., 2009). TPC ranges from 20.8 g in *Grateloupia turuturu* and 92.0 g/100g dw in *Codium tomentosum*, a green seaweed, when using CE as a standard and extraction performed with ethyl acetate (Rodrigues et al., 2015). The TPC determined using GAE as a standard, originated values between 0.2 and 0.4 g/100g dw, when extracted with MeOH/H_20 (60/40, v/v) at 40 ºC, during a period of 3h (O'Sullivan et al., 2011). Kumar et al. (2008) determined 2.1 g/100g of dw of TPC in *Kappaphycus alvarezii*, a red seaweed, when extraction was performed using $ChCl_3$/MeOH (2:1, v/v) in a Soxhlet apparatus. Although some variation in TPC can be observed, depending on the seaweed and sampling location, the biggest source of its variation seems to be the solvent system used to obtain the primary extracts and the selected phenolic standard (PGE, GAE or CE). The TPC in *Fucus vesiculosus* from Britanny (France) had 5.8 g PGE /100g dw, using the MeOH/H_2O solvent system, at 40ºC, during 3h to the extraction (Connan et al., 2004). Wang et al. (2009) obtained significantly higher TPC, 24.2 g PGE /100g dw in the same species of seaweed collected in Hafnarfjord (Iceland), but performing the extraction using a 70% acetone solvent system, during 24 h, at room temperature. Also, comparing the TPC in *Fucus vesiculosus*, using the same solvent system, but varying the standards, it was achieved 5.8 g PGE / 100g dw (Connan et al., 2004) or only 0.3 g CE / 100g dw (O'Sullivan et al., 2011).

Table 4 – Total phenolic content described for macroalgae collected in several places worldwide. TPC was extracted differently, using different solvents, temperatures and time. Distinct standards were used to assess TPC content namely Phloroglucinol equivalents (PGE), Gallic acid equivalents (GAE) and catechol equivalents. After individual reference, letters in superscript indicate the state of the biomass for the extraction protocol used by different authors, Fresh (F), Lyophilized (L) or Air-Dried (AD).

Species	Collection site	Extraction methodology	Total phenolic content (TPC) g/100g equivalents DW	References
Ascophyllum nodosum	Portsall, Brittany (France)	MeOH/H_2O 40ºC 3h	5.8 (PGE)	(Lee et al., 2016)^{F}
Ascophyllum nodosum	Hvassahraun, Hafnarfjordur (Iceland)	70% acetone room temp 24h	15.9 (PGE)	(Wang et al., 2009)^{L}
Ascophyllum nodosum	Galway (Ireland)	MeOH/H_2O (60/40, v/v) 40ºC 3h	0.5 (GAE)	(O'Sullivan et al., 2011)^{F}
Bifurcaria bifurcata	Portsall, Brittany (France)	MeOH/H_2O 40ºC 3h	3.7 (PGE)	(Connan et al., 2004)^{F}
Codium tomentosum	Buarcos bay, Figueira da Foz (Portugal)	Ethyl acetate	92.0 (CE)	(Rodrigues et al., 2015)^{AD}
Fucus serratus	Portsall, Brittany (France)	MeOH/H_2O 40ºC 3h	4.3 (PGE)	(Connan et al., 2004)^{F}
Fucus serratus	Hvassahraun, Hafnarfjordur (Iceland)	70% acetone room temp 24h	24.0 (PGE)	(Wang et al., 2009)^{L}
Fucus serratus	Galway (Ireland)	MeOH/H_2O (60/40, v/v) 40ºC 3h	0.4 (GAE)	(O'Sullivan et al., 2011)^{F}
Fucus spiralis	Portsall, Brittany (France)	MeOH/H_2O 40ºC 3h	3.9 (PGE)	(Connan et al., 2004)^{F}
Fucus vesiculosus	Portsall, Brittany (France)	MeOH/H_2O 40ºC 3h	5.8 (PGE)	(Connan et al., 2004)^{F}
Fucus vesiculosus	Hvassahraun, Hafnarfjordur (Iceland)	70% acetone Room Temp 24h	24.2 (PGE)	(Wang et al., 2009)^{L}
Fucus vesiculosus	Galway (Ireland)	MeOH/H_2O (60/40, v/v) 40ºC 3h	0.3 (GAE)	(O'Sullivan et al., 2011)^{F}
Gracilaria gracilis	Buarcos bay, Figueira da Foz (Portugal)	Ethyl acetate	22.8 (CE)	(Rodrigues et al., 2015)^{AD}
Grateloupia turuturu	Buarcos bay, Figueira da Foz (Portugal)	Ethyl acetate	20.8 (CE)	(Rodrigues et al., 2015)^{AD}
Himanthalia elongata	Portsall, Brittany (France)	MeOH/H_2O 40ºC 3h	2.2 (PGE)	(Connan et al., 2004)^{F}
Kappaphycus alvarezii	Port Okha (India)	Chloroform:MeOH (2:1, v/v) Soxhlet	2.1 (GAE)	(Kumar et al., 2008)^{AD}
Laminaria digitata	Portsall, Brittany (France)	MeOH/H_2O 40ºC 3h	0.1 (PGE)	(Connan et al., 2004)^{F}
Laminaria hyperborea	Galway (Ireland)	MeOH/H_2O (60/40, v/v) 40ºC 3h	0.2 (GAE)	(O'Sullivan et al., 2011)^{F}
Osmundea pinnatifida	Buarcos bay, Figueira da Foz (Portugal)	Ethyl acetate	33.7 (CE)	(Rodrigues et al., 2015)^{AD}
Pelvetia canaliculata	Portsall, Brittany (France)	MeOH/H_2O 40ºC 3h	3.4 (PGE)	(Connan et al., 2004)^{F}
Pelvetia canaliculata	Galway (Ireland)	MeOH/H_2O (60/40, v/v) 40ºC 3h	0.4 (GAE)	(O'Sullivan et al., 2011)^{F}
Sargassum muticum	Buarcos bay, Figueira da Foz (Portugal)	Ethyl acetate	49.9 (CE)	(Rodrigues et al., 2015)^{AD}
Saccorhiza polyschides	Buarcos bay, Figueira da Foz (Portugal)	Ethyl acetate	22.4 (CE)	(Rodrigues et al., 2015)^{AD}
Ulva rigida	Sidi Mansour Sfax (Tunisia)	MeOH 70ºC 24h Soxhlet	0.9 (PGE)	(Trigui et al., 2013)^{AD}

1.2.2 Proteins

Macro and microalgae are often consumed due to their high protein content, with a similar or higher content than some plant sources (Fleurence, 1999; Phong et al., 2016). Protein quality depends mainly on its amino acid composition, content variation, proportion, availability and digestibility. There are 20 proteinogenic amino acids and eleven of them are essential for human body functioning and should be obtained through food ingestion, due to our metabolic inability to biosynthesize. These essential amino acids (EAA) exist in many plant sources but often in imbalanced and / or insufficient amounts, with a commonly absence of one or more EAA which are histidine, isoleucine, lysine, leucine, phenylalanine, methionine, tryptophan, threonine and valine (Young and Pellett, 1994). Although the EAA composition in algae fluctuates throughout the year due to seasonality (Fleurence, 1999), they are worthwhile classified as a viable and complete protein source (FAO/WHO, 1991). However, phlorotannins and high polysaccharide content in algae can act as anti-nutrients significantly reducing protein digestibility. Also, the application and widespread food use of algae is limited by harvesting capabilities, seasonality, geographical location, production and methods for protein extraction and purification (Bleakley and Hayes, 2017). Cell disruption methods for protein extraction are commonly achieved through aqueous, acid and alkaline extraction or enzymatic digestion (Galland-Irmouli et al., 1999). Enzymatic assisted protein extraction frequently implies the use of glucanases, amylases, cellulase, endoproteases or proteases. The chemical or subcritical water hydrolysis and more recently, supercritical fluid extraction (SFE), ultrahigh pressure extraction (UPE), pressurized fluid extraction (PFE) and microwave assisted extraction (MAE) were also used for seaweed protein extraction (Kadam et al., 2017).

Table 5 summarizes total protein content in macroalgae collected from several worldwide places, using Kjeldahl's methodology and multiplying the percentage of nitrogen content by a factor of 6.25. Protein content ranges between 5.0 g/100g dw with *Lobophora variegata*, a brown seaweed from Tamil Nadu, India (Verma et al., 2017) and 24.8 g/100g dw with *Porphyra* sp., a red seaweed collected in Azores, Portugal (Paiva et al., 2014).

The bibliographical analysis shows that green seaweed contains between 6.9 and 18.8 g/100g dw of protein, red seaweed from 9.1 to 24.8 g/100g dw and brown seaweed from 5.0 to 16.9 g/100g dw. Interestingly, the highest protein content was observed in red seaweed with *Porphyra* sp., *Gracilaria* sp., *Grateloupia* sp. and *Osmundea* sp., the genus with the highest protein content.

Two different kinds of bioactive algae proteins have been extracted and purified for industrial applications, i.e., phycobiliproteins and lectins, which are commonly extracted from Rhodophyta seaweeds (Bleakley and Hayes, 2017).

Table 5 – Protein content described for macroalgae collected in several places worldwide. The methodology for protein content was applying Kjeldahl method and multiplying by a factor of 6.25. After individual reference, letters in superscript indicate the state of the biomass for the extraction protocol used by different authors, Lyophilized (L) or Air-Dried (AD).

Species	Collection site	Protein content (g/100g) DW (Convertion factor 6.25) Kjeldahl method	References
Caulerpa racemosa	Veraval Coast, Gujarat (India)	12.9	(Kumar et al., 2011)[L]
Caulerpa scalpelliformis	Veraval Coast, Gujarat (India)	10.5	(Kumar et al., 2011)[L]
Caulerpa veravalensis	Veraval Coast, Gujarat (India)	7.8	(Kumar et al., 2011)[L]
Codium tomentosum	Buarcos bay, Figueira da Foz (Portugal)	18.8	(Rodrigues et al., 2015)[AD]
Codium spp.	Barbate estuary, Gulf of Cádiz (Spain)	15.6	(JS and V, 2016)[L]
Dictyota dichotoma	Port Okha, Gujarat (India)	11.9	(Verma et al., 2017)[AD]
Fucus spiralis	São Miguel Island, Azores (Portugal)	9.7	(Paiva et al., 2014)[AD]
Gelidiella acerosa	Port Okha, Gujarat (India)	14.6	(Verma et al., 2017)[AD]
Gelidium micropterum	Mandapam, Tamil Nadu (India)	9.1	(Verma et al., 2017)[AD]
Gracilaria cervicornis	Búzios beach (Brazil)	19.7	(Marinho-Soriano et al., 2006)[AD]
Gracilaria changii	Santubong, Sarawak (Malaysia)	12.6	(Chan and Matanjun, 2017)[L]
Gracilaria fisheri	Pattani bay (Thailand)	11.6	(Benjama and Masniyom, 2012)[AD]
Gracilaria gracilis	Buarcos bay, Figueira da Foz (Portugal)	20.2	(Rodrigues et al., 2015)[AD]
Agarophyton tenuistipitatum	Pattani bay (Thailand)	21.6	(Benjama and Masniyom, 2012)[AD]
Grateloupia indica	Port Okha, Gujarat (India)	10.1	(Verma et al., 2017)[AD]
Grateloupia turuturu	Piriac-sur-Mer (France)	22.9	(Denis et al., 2010)[L]
Grateloupia turuturu	Buarcos bay, Figueira da Foz (Portugal)	22.5	(Rodrigues et al., 2015)[AD]
Halymenia floresii	Barbate estuary, Gulf of Cádiz (Spain)	9.4	(JS and V, 2016)[L]
Lobophora variegata	Mandapam, Tamil Nadu (India)	5.0	(Verma et al., 2017)[AD]
Osmundea pinnatifida	São Miguel Island, Azores (Portugal)	20.8	(Paiva et al., 2014)[AD]
Osmundea pinnatifida	Buarcos bay, Figueira da Foz (Portugal)	23.8	(Rodrigues et al., 2015)[AD]
Padina gymnospora	Port Okha, Gujarat (India)	11.6	(Verma et al., 2017)[AD]
Padina tetrastromatica	Mandapam, Tamil Nadu (India)	12.4	(Verma et al., 2017)[AD]
Porphyra sp.	São Miguel Island, Azores (Portugal)	24.8	(Paiva et al., 2014)[AD]
Saccorhiza polyschides	Buarcos bay, Figueira da Foz (Portugal)	14.4	(Rodrigues et al., 2015)[AD]
Saccorhiza polyschides	Barbate estuary, Gulf of Cádiz (Spain)	7.3	(JS and V, 2016)[L]
Sargassum linearifolium	Port Okha, Gujarat (India)	8.9	(Verma et al., 2017)[AD]
Sargassum muticum	Buarcos bay, Figueira da Foz (Portugal)	16.9	(Rodrigues et al., 2015)[AD]
Sargassum naozhouense	Techeng Island, Guangdong province (China)	11.2	(Peng et al., 2013)[AD]
Sargassum vulgare	Búzios beach (Brazil)	13.6	(Marinho-Soriano et al., 2006)[AD]
Ulva lactuca	Port Okha, Gujarat (India)	12.2	(Verma et al., 2017)[AD]
Ulva reticulata	Mandapam, Tamil Nadu (India)	6.9	(Verma et al., 2017)[AD]
Ulva spp.	Barbate estuary, Gulf of Cádiz (Spain)	7.2	(JS and V, 2016)[L]

1.2.2.1 Phycobiliproteins

These water-soluble macromolecules are a light-harvesting family, existing in the photosynthetic apparatus of cyanobacteria and some groups of eukaryotic algae such as red algae, Glaucophytes and

Cryptomonads (Apt et al., 1995). They are constituents of the phycobilisome multi-molecular complex, linked to the thylakoid membrane (Tandeau De Marsac et al., 2003). These structures present inside the chloroplast structure have the ability to capture the photon energy toward the photosystem (Sonani et al., 2014). Phycobiliproteins (PBPs) are divided in three classes, according to their absorption properties, comprising phycoerytrins (PEs) absorbing light at 495 nm and 540-570 nm, phycocyanins (PCs) at 610–620 nm and allophycocyanins (APCs) at 650–655 nm (Sudhakar et al., 2015). All PBPs have two subunits (α and β) while a third subunit (γ) is only found in PEs as a linker peptide. R-phycoerythrin (R-PE) and B-phycoerythrin (BPE), two PEs, are structurally composed and arranged in (αβ)6γ complex. Meanwhile, PCs and APCs have a structure that can be described as $(\alpha\beta)_3$ complex (Isailovic et al., 2004). PBPs absorption and fluorescence characteristics, in the visible spectrum, result from the presence of photosynthetic pigments named phycobilins and their interactions within polypeptide chains. Individual subunits (α, β and γ) can possess one or more phycobilin chromophores, linked with specific cysteines, present in the polypeptide chains, with a thioester bond (Apt et al., 1995). Nowadays there is a widespread of biotechnological applications for phycobiliproteins, including several fluorescent applications such as labelling, microscopy, immunohistochemistry and flow cytometry analysis (Aneiros and Garateix, 2004). Nonetheless, the majority of industrial applications use low purity compounds (low purity index) as they are used as natural dyes in food and cosmetics (Yamaguchi, 1997). *In vitro* and *in vivo* tests demonstrate the nutraceutical potential of these bioactive compounds, with several patents filed for anti-inflammatory, anti-tumor, anti-oxidative, anti-viral, hepatoprotective and neuroprotective activities (Sekar and Chandramohan, 2008). Numerous extraction and purification techniques have been developed over the years, mainly using phosphate buffer, but also testing distilled water and seawater for phycobiliproteins extraction, applying or not enzyme digestion to facilitate the breakage of the cell wall (Sudhakar et al., 2015).

Table 6 summarizes the bibliographic and methodology information about phycobiliproteins extraction, namely for PEs, PCs and APCs, from macroalgae collected in several places of world. Some variability in phycobiliproteins were extracted from red seaweeds can be observed, ranging from 81.9 to 457.5 mg of phycoerythrins/100g DW of *Champia* spp. and *Scinaia fascicularis*, respectively. Phycocyanins were found to variate between 15.4 mg in *Grateloupia indica* and 282.60 mg/100g DW in *Hypnea musciformis*, both collected in India. The lowest content of allophycocyanins was obtained in *Portieria hornemanni*, 15.2 mg and the highest content of 50 mg/100g DW in *Porphyra* spp., both collected in India. These results were published by Verma et al. (2017). The extraction was performed suspending air-dried seaweed flour in 1M acetic acid-sodium acetate buffer, at pH 5.5, with 0.01% of sodium azide, during 30 minutes in dark condition. Since several researchers published different methodologies for phycobiliproteins extraction, this process could be modulate and optimized. Sudhakar et al. (2015) extracted it from *Gracilaria crassa* collected in India, through suspension of fresh seaweed in water (pH 7), at room temperature. Le Guillard et al. (2015) optimized an ultrasound-assisted enzymatic hydrolysis extraction, during 2h, at 22 ºC, for compounds of *Grateloupia turuturu* from Batz-sur-Mer (France), yielding 360 mg of phycoerythrin/100g DW. Denis et al. (2010) assessed these compounds in the same seaweed from Piriac-sur-Mer, France, suspending lyophilized seaweed flour in phosphate buffer (20mM), pH 7.1, at 4ºC, and obtained a similar phycoerythrin yield, 300 mg/100g DW.

Table 6 – Phycobiliproteins namely phycoerythrins(PEs), phycocyanins (PCs) and allophycocyanins (APCs) content, described for macroalgae collected in several places worldwide. These were assessed differently by different authors and methods and described individually in each reference. All measures are described as dry weight (DW, except when fresh weight (FW) is written after value for individual measurements. After individual reference, letters in superscript indicate the state of the biomass for the extraction protocol used by different authors, Fresh (F), Lyophilized (L) or Air-Dried (AD).

Species	Collection site	Extraction methodology	Phycoerythrins (PEs) mg/100g DW	Phycocyanins (PCs) mg/100g DW	Allophycocyanins (APCs) mg/100g DW	Reference
Botryocladia leptopoda	Port Okha, Gujarat (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	351.10	117.40	28.60	(Verma et al., 2017)^{AD}
Champia spp.	Mandapam, Tamil Nadu (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	81.90	101.90	26.40	(Verma et al., 2017)^{AD}
Gelidiella acerosa	Port Okha, Gujarat (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	335.10	199.80	29.10	(Verma et al., 2017)^{AD}
Gelidium micropterum	Mandapam, Tamil Nadu (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	274.80	85.70	30.10	(Verma et al., 2017)^{AD}
Gracilaria crassa	Rameswaram, Tamilnadu (India)	Suspended in H_2O (pH 7.0) at room temperature	50.00 (FW)	28.00 (FW)	34.00 (FW)	(Sudhakar et al., 2015)^{F}
Grateloupia indica	Port Okha, Gujarat (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	385.50	15.40	28.00	(Verma et al., 2017)^{AD}
Grateloupia turuturu	Batz-sur-Mer (France)	Ultrasound-assisted enzymatic hydrolysis (UAEH) for 2 h at 22 °C	360.00	-----	-----	(Le Guillard et al., 2015)^{F}
Grateloupia turuturu	Piriac-sur-Mer (France)	Suspended in phosphate buffer 20 mM (pH 7.1) at 4ºC	300.00	33.00	-----	(Denis et al., 2010)^{L}
Haloplegma duperreyi	Port Okha, Gujarat (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	89.10	203.20	33.70	(Verma et al., 2017)^{AD}
Halymenia venusta	Port Okha, Gujarat (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	377.10	104.80	31.40	(Verma et al., 2017)^{AD}
Hypnea musciformis	Mandapam, Tamil Nadu (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	271.30	282.60	45.20	(Verma et al., 2017)^{AD}
Hypnea valentiae	Mandapam, Tamil Nadu (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	283.20	236.80	42.70	(Verma et al., 2017)^{AD}
Portieria hornemannii	Mandapam, Tamil Nadu (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	290.40	148.80	15.20	(Verma et al., 2017)^{AD}
Porphyra spp.	Mandapam, Tamil Nadu (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	242.30	67.20	50.00	(Verma et al., 2017)^{AD}
Rhodymenia dissecta	Port Okha, Gujarat (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	141.70	51.10	35.60	(Verma et al., 2017)^{AD}
Scinaia fascicularis	Port Okha, Gujarat (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	457.50	39.50	31.50	(Verma et al., 2017)^{AD}

Scinaia hatei	Port Okha, Gujarat (India)	Suspended in 1 M acetic acid–sodium acetate buffer (pH 5.5) with 0.01% of sodium azide for 30 min in the dark	335.50	98.30	31.70	(Verma et al., 2017)[AD]

1.2.2.2 Lectins

These are important bioactive glycoproteins, responsible for sugar cellular surface recognition in bacteria, plants and animals (Weis and Drickamer, 1996). Lectins are also known as haemagglutinins or agglutinins due to their ability to agglutinate red blood cells (Smit, 2004). Lectins have a non-immune origin and reversibly bind to carbohydrates in a highly specific, non-covalent way (Goldstein and Poretz, 1986). Algae lectins are also known as phycolectins. Their physico-chemical features are distinct from other plant lectins, due to its monomeric structure, with low molecular weight, showing specificity to glycoproteins more than to monosaccharides (Hori et al., 1990). Phycolectins do not require metal ions for its biological activity and possess a high content of acidic amino acids (Singh et al., 2017). Numerous biological processes were undertaken, with lectins participation such as cell-cell communication, host-pathogen interactions, antiviral activities, cancer metastasis and apoptosis induction (Holdt and Kraan, 2011). Over the years, these glycoproteins were successfully assayed in many biotechnological applications such as cytotoxic, mitogenic, antinociceptive, antibiotic, antiadhesion and anti-inflammatory activities (Freitas et al., 2015). In addition, lectin-glycan interactions have been extensively studied has a new therapeutic approach for anti-HIV vaccines, assessing the adherence stage of this virus to lectins from algae sources (Huskens and Schols, 2012).

1.2.2.3 Bioactive peptides

Amino acid content and physiological utility after digestion and absorption will determine the nutritional quality of proteins. Bioactive peptides are inactive when integrated in the polypeptide chain of the protein and normally have less than 3 kDa and 2 to 30 amino acids in length. These peptides are released by proteolytic enzymes throughout gastrointestinal digestion or food processing (Vercruysse et al., 2005). The peptides with hormone-like properties exhibit a wide diversity of beneficial physiological activities, including antimicrobial, immunomodulatory, angiotensin-converting enzyme inhibition (ACE), opioid, renin inhibitory, anticoagulant, platelet-activating factor acetylhydrolase inhibitory (PAF-AH), prolyl endopeptidase inhibitory (PEP), satiety-inducing and α-amylase inhibitory activities (Hayes, 2013). Nowadays, milk proteins are the most common source of bioactive peptides. Nonetheless, various bioactive peptides have been identified in animal food products such as meat, fish, eggs, blood, and plant sources including soybean, broccoli, rice, pea, garlic and algae (Bleakley and Hayes, 2017). Algal proteins show high variability throughout the year due to season, temperature and location, becoming a challenge the attempts to obtain bioactive peptides (Joubert and Fleurence, 2008).

1.2.3 Polysaccharides

Polysaccharides are polymers of monosaccharides linked by glyosidic bonds. They are the main structural compounds of macroalgal cell walls and could be involved in the recognition patterns between macroalgae and pathogens (Cardozo et al., 2007). Macroalgae represent the majority of marine organisms producing a large quantity of carbohydrates, due to their unique metabolism and physiological capability to prosper in extreme habitats conditions (Sudha et al., 2014). The term phycocolloid is used to identify the high molecular weight polysaccharides extracted from macroalgae that possess the ability of holding significant amounts of water and the capacity to form gel or viscous substances. They are the principal component of commercial seaweed extracts, with an annual production of 100,000 tons. These extracts have been used for a variety of purposes such as food technology, pharmaceutic, cosmetics and biotechnology (Pangestuti and Kim, 2015). The biological properties of macroalgae polysaccharides have been tested *in vitro* and *in vivo*, showing variable evidences for immunostimulatory, anticoagulant, anti-inflammatory, antiviral, antitumour, antiapoptosis, antioxidant and antiproliferative activities (Garcia-vaquero et al., 2017). Table 7 summarizes the bibliographic information about the total carbohydrates content in macroalgae, collected in several places worldwide. The methodology used and sample condition varies among references, since samples are used lyophilized or air-dried. Methodologies often used for carbohydrates analysis are colorimetric assays, developed by Morse (1947), Morris (1948) and DuBois et al. (1956).

Total carbohydrate content vary between 6.30g/100g DW in *Saccorhiza polyschides*, a brown seaweed (J.S. and V., 2016) and 63.13g/100g dw in *Gracilaria cervicornis*, a red seaweed (Marinho-Soriano et al., 2006). The different colour in seaweed shown specific maximum and minimum carbohydrates yields. Green seaweed carbohydrate varies between 8.80g/100g of DW in *Codium* spp. and 48.95 g/100g of DW in *Caulerpa racemosa* from Gulf of Cádiz (Spain) and Gujarat (India), respectively. Red seaweed shows between 15.50 g/100g dw in *Halymenia floresii* and 63.13g/100g dw in *Gracilaria cervicornis*. For brown seaweed, it varies from 6.30g/100g dw in *Saccorhiza polyschides* and 61.61 g/100g dw found in *Sargassum vulgare*.

Table 7 – Total carbohydrate content described for macroalgae collected in several places worldwide. Carbohydrates were assessed differently, using different solvents, temperatures and time. After individual reference, letters in superscript indicate the state of the biomass for the extraction protocol used by different authors, Lyophilized (L) or Air-Dried (AD).

Species	Collection site	Carbohydrate content (g/100g) DW	Method	References
Caulerpa racemosa	Veraval Coast, Gujarat (India)	48.95	Dubois et al. (1956)	(Kumar et al., 2011)[L]
Caulerpa scalpelliformis	Veraval Coast, Gujarat (India)	38.84	Dubois et al. (1956)	(Kumar et al., 2011)[L]
Caulerpa veravalensis	Veraval Coast, Gujarat (India)	37.23	Dubois et al. (1956)	(Kumar et al., 2011)[L]
Codium tomentosum	Buarcos bay, Figueira da Foz (Portugal)	32.80	Diference method	(Rodrigues et al., 2015)[AD]
Codium spp.	Barbate estuary, Gulf of Cádiz (Spain)	8.80	Anthrone method	(JS and V, 2016)[L]
Dictyota dicotoma	Port Okha, Gujarat (India)	15.00	Dubois et al. (1956)	(Verma et al., 2017)[AD]
Fucus spiralis	São Miguel Island, Azores (Portugal)	17.59	Dubois et al. (1956)	(Paiva et al., 2014)[AD]
Gelidiella acerosa	Port Okha, Gujarat (India)	32.09	Dubois et al. (1956)	(Verma et al., 2017)[AD]
Gelidium micropterum	Mandapam, Tamil Nadu (India)	34.81	Dubois et al. (1956)	(Verma et al., 2017)[AD]
Gracilaria cervicornis	Búzios beach (Brazil)	63.13	Difference method	(Marinho-Soriano et al., 2006)[AD]
Gracilaria changii	Santubong, Sarawak (Malaysia)	41.52	Difference method	(Chan and Matanjun, 2017)[L]
Gracilaria gracilis	Buarcos bay, Figueira da Foz (Portugal)	46.60	Difference method	(Rodrigues et al., 2015)[AD]
Grateloupia indica	Port Okha, Gujarat (India)	22.48	Dubois et al. (1956)	(Verma et al., 2017)[AD]
Grateloupia turuturu	Buarcos bay, Figueira da Foz (Portugal)	43.20	Difference method	(Rodrigues et al., 2015)[AD]
Halymenia floresii	Barbate estuary, Gulf of Cádiz (Spain)	15.50	Anthrone method	(JS and V, 2016)[L]
Lobophora variegata	Mandapam, Tamil Nadu (India)	32.31	Dubois et al. (1956)	(Verma et al., 2017)[AD]
Osmundea pinnatifida	São Miguel Island, Azores (Portugal)	17.61	Dubois et al. (1956)	(Paiva et al., 2014)[AD]
Osmundea pinnatifida	Buarcos bay, Figueira da Foz (Portugal)	32.40	Difference method	(Rodrigues et al., 2015)[AD]
Padina gymnospora	Port Okha, Gujarat (India)	34.45	Dubois et al. (1956)	(Verma et al., 2017)[AD]
Padina tetrastromatica	Mandapam, Tamil Nadu (India)	32.51	Dubois et al. (1956)	(Verma et al., 2017)[AD]
Porphyra sp.	São Miguel Island, Azores (Portugal)	25.37	Dubois et al. (1956)	(Paiva et al., 2014)[AD]
Saccorhiza polyschides	Barbate estuary, Gulf of Cádiz (Spain)	6.30	Anthrone method	(JS and V, 2016)[L]
Saccorhiza polyschides	Buarcos bay, Figueira da Foz (Portugal)	45.60	Difference method	(Rodrigues et al., 2015)[AD]
Sargassum linearifolium	Port Okha, Gujarat (India)	29.82	Dubois et al. (1956)	(Verma et al., 2017)[AD]
Sargassum muticum	Buarcos bay, Figueira da Foz (Portugal)	49.30	Difference method	(Rodrigues et al., 2015)[AD]
Sargassum naozhouense	Techeng Island, Guangdong province (China)	47.73	Difference method	(Peng et al., 2013)[AD]
Sargassum vulgare	Búzios beach (Brazil)	61.61	Difference method	(Marinho-Soriano et al., 2006)[AD]
Ulva lactuca	Port Okha, Gujarat (India)	32.61	Dubois et al. (1956)	(Verma et al., 2017)[AD]
Ulva reticulata	Mandapam, Tamil Nadu (India)	33.35	Dubois et al. (1956)	(Verma et al., 2017)[AD]
Ulva spp.	Barbate estuary, Gulf of Cádiz (Spain)	11.70	Anthrone method	(JS and V, 2016)[L]

1.2.3.1 Alginates

The function of alginate in brown macroalgae (Ochrophyta) is predominantly structural, being located in the cell wall and intercellular matrix, attributing strength and flexibility to withstand sea currents. The molecular arrangement is a linear, anionic block copolymer heteropolysaccharide, comprised of β-D-mannuronic acid (M) and α-L-glucuronic acid (G). The structure of alginates varies according to the specie, season and geographical localization. It is composed by sequential distributed homogeneous M-M (M-blocks) and G-G segments (G-blocks) alternating with M-G segments (MG-blocks) , which vary in relative amounts (D'Ayala et al., 2008). Furthermore, M/G ratio and block structure are crucial for the physicochemical properties of alginates since this parameter is widely used to predict the nature and behavior of gels when calcium ions are added. Moreover, two main factors influence the quality and applicability of alginate gel, since high amount of G-blocks favourably increases gel strength and industrial application will depend on its uronic acid composition (Fertah, 2017). These gels can be synthesized in a wide range of molecular weights, ranging from 50 to 100,000 residues, to better fit different applications. The overall fraction of uronic acid residues is determined in a F_G value within the polymer and the average number of uronic acid residues in the G-blocks are represented as NG (D'Ayala et al., 2008). This polysaccharide is mainly applied in the food industry as a thickener, stabilizer or as an emulsifying agent (Sudha et al., 2014). Alginates extraction from brown seaweed begins with a pre-extraction using hydrochloric acid (HCl), followed by washing, filtration and neutralization with an alkaline product. It forms sodium alginate that is precipitated with ethanol or isopropanol, frequently being re-precipitated in the same way (Laurienzo, 2010).

Sodium alginate shows high ability to remove heavy metals such as cadmium and copper through adsorption (Papageorgiou et al., 2008). This capability is derived from their natural condition, when they are bound with salts present in seawater, particularly Ca^{2+}, Na^{+}, Mg^{2+}, Sr^{2+}, and Ba^{2+} ions (Fertah, 2017). Pharmaceutical and medical interest for alginate applications has increased since it was demonstrated to be efficient in esophageal reflux and it produces multiquality calcium fibers for dermatology and wound healing. It is an active natural disintegrative material, a tablet binder, and an alternative for a sustained release system due to various properties such as the formation of hydrogels under relatively mild pH and temperature. It is considered nontoxic, biodegradable, biocompatible and abundantly available in nature (D'Ayala et al., 2008).

1.2.3.2 Agar

This hydrocolloid is also known as agar-agar, this name being a collective term used to describe gelling polysaccharides. Currently, it is mostly extracted from red species of macroalgae such as *Pterocladiella*, *Gelidium*, *Ahnfeltia*, *Gelidiella*, *Gracilariopsis* and *Gracilaria* (Laurienzo, 2010). Galactans from red species of seaweed were named as agaroids, agarose, agarans and agaroptectin, depending of the conformation and side chains that different agar components can present. The term agarose is nowadays widely used due to later research, that showed a regular polymer of alternating 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose residues. The proportion of 3,6-anhydro-α-L-galactopyranose in the polysaccharides influences the strength and brittleness of the gel. It is widely

used for special preparations in gel electrophoresis, gel permeation chromatography and other applications in biochemical, biomedical and biotechnological laboratories (Knutsen et al., 1994). It is estimated that 70% of agar is comprised of agarose, which presents a linear structure having sulfate ester, methoxyl group and pyruvate ketal as side chain substituents, which can be found in the backbone of agar, influencing its gelling properties. Purified agarose is also commercialized but considerably more expensive, evidencing a significantly lower sulfate content, better optical clarity and increased gel strength comparing with agar (Laurienzo, 2010). Agar is the most abundant cell wall polysaccharide present in red seaweed and its biological function is to provide structural stability with high flexibility to withstand ocean waves and currents. It also provides resistance to pathogens, protect algae against extreme salinity, pH, temperatures and desiccation (Lee et al., 2017). Agar extract is added to boiling water and jellifies at room temperature, forming thermally reversible gels without losing their mechanical and thermic properties, being suitable for multiple applications. The gel structure results from the hydrogen bonds, established in a continuous way, following a coil-double helix transition structure, interacting between themselves and linking bundles of associated right-handed double helices (Norziah et al., 2006). The subsequent three-dimensional network is capable of restraining water molecules in its interstices (Arnott et al., 1974). Agar can be subjected to sodium hydroxide and enzymatic treatments using sulfohydrolase to improve its strength (Freile-Pelegrín and Murano, 2005; Shukla et al., 2011). Additionally, genetic manipulation in the development stages of seaweed has been performed to minimize seasonal variations in plant growth and agar quality (Lee et al., 2016). Recently, the use of agar and agarose beads for sustained release of water soluble drugs was investigated, consisting in dissolving the drug in a hot agar aqueous solution, with a temperature around 70 ºC. The beads are jellified when drops of agar with the dissolved drug are dropped in a cold bath containing a non-solvent for agar, normally acetone or ethyl acetate (Laurienzo, 2010).

1.2.3.3 Carrageenans

This hydrocolloid is widely extracted from Rhodophyta species such as *Kappaphycus*, *Betaphycus*, *Eucheuma*, *Hypnea*, *Gigartina* and *Chondrus*. Carrageenans are mainly defined by alternating 3-linked-β-D-galactopyranose and 4-linked-α-D-galactopyranose units. Carrageenan and agar show a fundamental stereochemical difference between the diastereoisomeric polysaccharide molecules (Anderson et al., 1965). They are classified according to the degree of substitutions occurring on their free hydroxyl groups, which generally could be the addition of ester sulphate or the presence of the 3,6-anhydride on the 4-linking residues (Nanaki et al., 2010). Additionally, carrageenan can present six different forms, Kappa (κ), Iota (τ), Lambda (λ), Beta (β), Mu (μ), Nu (ν), Xi (ξι) and Theta (θ), depending on the seaweed source used for carrageenan extraction (Campo et al., 2009). The first three are the most commercially important, since κ produces strong and rigid gels, τ develops soft gels and λ is used as a thickener as it does not form a gel (Sankalia et al., 2006). Carrageenan properties are primarily influenced by the number and position of ester sulphate groups as well as the content of 3,6-anhydro-galactose. The commercial carrageenan presents a molecular weight that can fluctuate between 100 and 1,000 kDa (Li et al., 2014). κ and τ gel consist of an ordered three-dimensional network of double helices cross-linked with sulphate groups, oriented externally while in λ, the sulphate groups in the 2º position are directed inward, forming the cross-link of an ordered network. Biological properties of carrageenan show potential in pharmaceutics as an anticoagulant,

anticancer, antihyperlipidemic and with immunomodulatory activity (Campo et al., 2009). Although no nutritional value could be attributed to carrageenan, it is widely used as a food additive, functioning as aemulsifier, stabilizer or thickener and has been applied to increase the quality of dairy sweets, puddings, cheese, etc. (Wijesekara et al., 2011; Zia et al., 2017). Also, carrageenan could be used in non-food products such as firefighting foams, air freshener gels and shoe polish (Zia et al., 2017). The economic feasibility is extremely important to choose the proper methodology to extract carrageenan from seaweed. Primarily, seaweed is washed following a hot alkali extraction process, freeing the carrageenan from the cell. Secondly, one of three methods currently used in the industry could be used for carrageenan separation, namely the "freeze–thaw" technique, the "alcohol precipitation" method or the "KCl precipitation" process (Li et al., 2014).

1.2.3.4 Cellulose

This natural biopolymer is one of the most abundant polysaccharides on earth, providing physical structure and strength which can be found in cell walls of almost all plants and seaweed (D'Ayala et al., 2008). There are four types of cellulose available, which include native cellulose (type I), native cellulose with major structural changes caused by treatment with an alkaline solution (type II) and cellulose treated with multiple reagents (type III and IV). These last cellulose derivatives can be amongst others cellulose acetate (CA), ethylcellulose (EC), methylcellulose (MC), hydroxyethylcellulose (HEC), carboxymethylcellulose (CMC) and hydroxypropylcellulose (HPC) (Yang and Li, 2018). It is a versatile material, mainly used to produce paper, pharmaceutical, textile, veterinary, food and cosmetic goods (Lakshmi et al., 2017). Its unique properties such as high strength and modulus, high tensile strength, high surface area, light weight, high aspect ratio, stiffness, cost effectiveness and good biodegradability, allows several industrial applications (Singh et al., 2017). Cellulose type I comprises the general formula $(C_6H_{10}O_5)_n$ composed by β-(1–4)-linked anhydroglucose repeating units, where n can variate from 10,000 to 15,000 depending on the source of raw material (Yang and Li, 2018). It is a homopolysaccharide whose rigid structure depends on the intermolecular and intramolecular hydrogen bonds and hydrophobic interactions (van der Waals) (Lindman et al., 2010). Cellulose type I is considered amphiphilic, because all three hydroxyl groups of the anhydroglucose units possess an equatorial alignment, making it hydrophilic and the hydrogen atoms of C–H bonds have an axial orientation making it hydrophobic. Also, it has highly ordered crystalline regions and disordered amorphous regions along its chain (Nagarajan et al., 2017).

1.2.3.5 Fucoidan

Xylofucoglycuronans, glycuronogalactofucans and fucoidan are the main constituents of biological macromolecules classified as fucans (Phull and Kim, 2017). Fucoidan is a group of fucose-enriched sulphated polysaccharides with a high degree of complexity. These compounds originate in the fibrillary cell wall, where they act as reinforcement molecules and in the intercellular spaces of brown seaweeds, involved in the seaweed protection against the effects of desiccation, when exposed to low tide (Sanjeewa et al., 2017; Senthilkumar et al., 2013). Its chemical structure mainly consists of L-fucose sulphate groups and in smaller extend one or more groups of D-mannose, D-xylose, arabinose, D-galactose,

D-glucuronic acid, L-rhamnose, glucose and acetyl (Wu et al., 2016). Fucoidan molecular structure will vary depending on the species, harvesting time and habitat conditions of seaweed. Fucoidan homofucose backbone consists primarily of 2 types, type I with α(1 → 3)-L-fucopyranose residues or type II with alternating α(1 → 3) and α(1 → 4)-linked L-fucopyranosyls (Cumashi et al., 2007; Sanjeewa et al., 2017). Initially isolated by Kylin in 1913, it was named "fucoidin" and later, according to IUPAC guidelines, the name was changed to "fucoidan". However, other names are used, such as fucan, fucosan or sulphated fucan (Senthilkumar et al., 2013). According to recent reviews by Senthilkumar et al. (2013) and Vo and Kim (2013), several seaweeds have been targeted for the extraction and isolation of fucoidan to multiple biological applications, such as *Analipus japonicus*, *Ascophyllum nodosum*, *Cladosiphon okamuranus*, *Dictyota menstrualis*, *Fucus distichus*, *Fucus evanescens*, *Fucus serratus*, *Fucus vesiculosus*, *Hizikia fusiformis*, *Kjellmaniella crassifolia*, *Pelvetia canaliculata*, *Sargassum kjellmanianum*, *Sargassum stenophyllum* and *Sargassum thunbergii*. Fucoidan extraction and purification often begins with dehydration and milling of seaweeds to obtain a homogeneous biomass with higher surface-to-volume-ratio. A pre-treatment is normally applied for removal of lipids, terpenes and phenols, using a ternary solvent system, composed by methanol/chloroform/water 4:2:1 (v/v), and further applying formaldehyde in an ethanolic solution or washing the seaweed with acetone (Hahn et al., 2012). The extracted fucoidan fraction can be further cleaned with activated charcoal. Typically, the extraction continues with hot aqueous or acidic solution at temperatures ranging from 70 to 100 ºC for several hours (Ale et al., 2011). Further fucoidan purification is achieved either using precipitation with ethanol or dialysis for desalting and removal of low molecular weight compounds. Recently, low pressure anionic or molecular exclusion liquid chromatography has been used to achieve high purified fucoidan fraction (Foley et al., 2011; Hemmingson et al., 2006). Fucoidan biological activity has been extensively studied, including anti-inflammatory, antioxidant, antivirus, antithrombotic and anticoagulant effects, as well preventing various renal, hepatic and uropathic disorders (Senthilkumar et al., 2013). In addition, the antitumor potential was compared with synthetic drugs, where this natural product demonstrated lower side effects (Wu et al., 2016). These results turned fucoidan into a promising substance with multiple applications in the pharmaceutical, cosmeceutical and nutraceutical industries (Phull and Kim, 2017).

1.2.3.6 Ulvans

These are a group of major matrix soluble polysaccharides which appear equally scattered throughout the frond, predominantly within the intercellular space and in the fibrillar wall. This polysaccharide is arranged in a bead-like structure, stabilized by cell wall proteins or strong physical interactions (Alves et al., 2013). Originally, these substances where named as ulvin and ulvacin to designate different fractions of *Ulva lactuca* water-soluble sulphated polysaccharides. Later their designations were changed to ulvan (Lahaye and Robic, 2007). These anionic sulphated polysaccharides are structurally constituted by various proportions of repeating disaccharide units, which include 3-sulphated rhamnose (Rha3S), glucuronic acid (GlcA), iduronic acid (IduA) and some xylose (Xyl) units (Hehemann et al., 2014). Additionally, in their composition, two major aldobiuronic acids disaccharides, which are differentiated in type A and B are present. Type A includes ulvanobiuronic acid 3-sulphate (A3s), a 1,4-linked glucuronic acid with O-3-sulphated rhamnose, and type B, ulvanobiuronic acid 3-sulphate (B3s), a

1,4-linked iduronic acid with O-3- sulphated rhamnose (Glasson et al., 2017). Various biological activities are hypothesized for ulvan, such as osmotic functions, regulating the ionic balance and preventing tissue desiccation, due to its highly hygroscopic capability. Other possible fucntions are protecting seaweed from marine bacterial attacks and mechanically regulating the spore release and adhesivity (Alves et al., 2013). Ulvan molecular structure, macromolecular characteristics and rheological properties are shown to be variable among species and seasons (Robic et al., 2009, a). Classical extraction of ulvan polysaccharides is performed using sodium oxalate or ammonium oxalate aqueous solutions, with temperatures between 80 and 90 ºC, to chelate the Ca^{2+} which crosslinks ulvan strands in the cell wall (Glasson et al., 2017). Althouth, this extraction methodology will dictate the physical and chemical properties of ulvan (Robic et al., 2009, b). Several potential applications has been described for these biologicall active polymers such as anti-hyperlipidemic, anti-oxidant, anti-peroxidative and anti-viral activity (Alves et al., 2013). Additionally, it can function as a heparinoid agent, replacing heparin, treating gastric ulcer or having anti-thrombotic activity (Lahaye and Robic, 2007).

1.2.4 Lipids

Seaweeds are low-energy food, where environmental conditions influence its lipid composition and quantity, as different mechanisms are triggered to allow adaptation (Mišurcová et al., 2011). Although seaweeds have low lipid content, they are considered a potential source of functional lipids due to their large stock in coastal waters. Total lipids (TL) in seaweeds are composed by lipid classes, whose quantity varies according to species, geographical location and environmental factors (Miyashita et al., 2013). Tables 8a and 8b summarize bibliographic and methodology information available about lipids, from macroalgae sampled in different places of the world. The low and high lipid values were found in different red seaweeds, 0.30g/100g dw in *Gracilaria changii*, Sarawak, Malasia (Chan and Matanjun, 2017) and 12.30g/100g dw, found in *Halymenia floresii*, Gulf of Cádiz, Spain, reported by J.S. and V. (2016). The total lipids in green seaweed varies between 1.45g/100g in dw of *Ulva lactuca*, Gujarat, India and 7.10g/100g dw in *Codium* spp., Gulf of Cádiz, Spain. In the brown seaweeds, total lipids oscilate between 0.49g/100g dw in *Sargassum vulgare*, Búzios beach, Brazil and 8.20g/100g dw in *Saccorhiza polyschides*, Gulf of Cádiz, Spain.

Table 8a – Total lipid content described for macroalgae collected in several places worldwide. Lipids were extracted differently, using different solvents, temperatures and time. After individual reference, letters in superscript indicate the state of the biomass for the extraction protocol used by different authors, Lyophilized (L) or Air-Dried (AD).

Species	Collection site	Lipid content (g/100g) DW	Method	References
Caulerpa racemosa	Veraval Coast, Gujarat (India)	2.64	Bligh and Dyer (1959)	(Kumar et al., 2011)[L]
Caulerpa scalpelliformis	Veraval Coast, Gujarat (India)	3.06	Bligh and Dyer (1959)	(Kumar et al., 2011)[L]
Caulerpa veravalensis	Veraval Coast, Gujarat (India)	2.80	Bligh and Dyer (1959)	(Kumar et al., 2011)[L]
Codium spp.	Barbate estuary, Gulf of Cádiz (Spain)	7.10	Soxhlet (n-hexane)	(J.S. and V., 2016)[L]
Codium tomentosum	Buarcos bay, Figueira da Foz (Portugal)	3.60	Soxhlet	(Rodrigues et al., 2015)[AD]
Dictyota dicotoma	Port Okha, Gujarat (India)	2.95	Bligh and Dyer (1959)	(Verma et al., 2017)[AD]
Fucus spiralis	São Miguel Island, Azores (Portugal)	5.23	Folch, Lees & Stanley (1957)	(Paiva et al., 2014)[AD]
Gelidiella acerosa	Port Okha, Gujarat (India)	2.75	Bligh and Dyer (1959)	(Verma et al., 2017)[AD]
Gelidium micropterum	Mandapam, Tamil Nadu (India)	2.20	Bligh and Dyer (1959)	(Verma et al., 2017)[AD]
Gracilaria cervicornis	Búzios beach (Brazil)	0.43	Soxhlet (n-hexane)	(Marinho-Soriano et al., 2006)[AD]
Gracilaria changii	Santubong, Sarawak (Malaysia)	0.30	Soxtec system (petroleum ether) (AOAC 991.36)	(Chan and Matanjun, 2017)[L]
Gracilaria fisheri	Pattani bay (Thailand)	2.20	Bligh and Dyer (1959)	(Benjama and Masniyom, 2012)[AD]
Gracilaria gracilis	Buarcos bay, Figueira da Foz (Portugal)	0.60	Soxhlet extraction	(Rodrigues et al., 2015)[AD]
Agarophyton tenuistipitatum	Pattani bay (Thailand)	2.80	Bligh and Dyer (1959)	(Benjama and Masniyom, 2012)[AD]
Grateloupia indica	Port Okha, Gujarat (India)	1.80	Bligh and Dyer (1959)	(Verma et al., 2017)[AD]
Grateloupia turuturu	Piriac-sur-Mer (France)	2.60	Folch, Lees & Stanley (1957)	(Denis et al., 2010)[L]
Grateloupia turuturu	Buarcos bay, Figueira da Foz (Portugal)	2.20	Soxhlet extraction	(Rodrigues et al., 2015)[AD]

Table 8b – Total lipid content described for macroalgae collected in several places worldwide. Lipids were extracted differently, using different solvents, temperatures and time. After individual reference, letters in superscript indicate the state of the biomass for the extraction protocol used by different authors, Lyophilized (L) or Air-Dried (AD).

Species	Collection site	Lipid content (g/100g) DW	Method	References
Halymenia floresii	Barbate estuary, Gulf of Cádiz (Spain)	12.30	Soxhlet (n-hexane)	(J.S. and V., 2016)^{L}
Lobophora variegata	Mandapam, Tamil Nadu (India)	1.25	Bligh and Dyer (1959)	(Verma et al., 2017)^{AD}
Osmundea pinnatifida	São Miguel Island, Azores (Portugal)	7.53	Folch, Lees & Stanley (1957)	(Paiva et al., 2014)^{AD}
Osmundea pinnatifida	Buarcos bay, Figueira da Foz (Portugal)	0.90	Soxhlet extraction	(Rodrigues et al., 2015)^{AD}
Padina gymnospora	Port Okha, Gujarat (India)	2.28	Bligh and Dyer method (1959).	(Verma et al., 2017)^{AD}
Padina tetrastromatica	Mandapam, Tamil Nadu (India)	1.25	Bligh and Dyer method (1959).	(Verma et al., 2017)^{AD}
Porphyra sp.	São Miguel Island, Azores (Portugal)	8.88	Folch, Lees & Stanley (1957)	(Paiva et al., 2014)^{AD}
Saccorhiza polyschides	Buarcos bay, Figueira da Foz (Portugal)	1.10	Soxhlet	(Rodrigues et al., 2015)^{AD}
Saccorhiza polyschides	Barbate estuary, Gulf of Cádiz (Spain)	8.20	Soxhlet (n-hexane)	(J.S. and V., 2016)^{L}
Sargassum linearifolium	Port Okha, Gujarat (India)	1.93	Bligh and Dyer method (1959).	(Verma et al., 2017)^{AD}
Sargassum muticum	Buarcos bay, Figueira da Foz (Portugal)	1.45	Soxhlet	(Rodrigues et al., 2015)^{AD}
Sargassum naozhouense	Techeng Island, Guangdong province (China)	1.06	Soxhlet	(Peng et al., 2013)^{AD}
Sargassum vulgare	Búzios beach (Brazil)	0.49	Soxhlet (n-hexane)	(Marinho-Soriano et al., 2006)^{AD}
Ulva lactuca	Port Okha, Gujarat (India)	1.45	Bligh and Dyer method (1959).	(Verma et al., 2017)^{AD}
Ulva reticulata	Mandapam, Tamil Nadu (India)	2.50	Bligh and Dyer method (1959).	(Verma et al., 2017)^{AD}
Ulva spp.	Barbate estuary, Gulf of Cádiz (Spain)	3.40	Soxhlet (n-hexane)	(J.S. and V., 2016)^{L}

Total lipids can be divided in classes, which include glycolipids (GL), triacylglycerols (TAG), phospholipids (PL), sterols and pigments. In these classes, fatty acids comprise the largest component, which can bebe present in three different forms such as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). The PUFAs are essential to human body functions and should be obtained through food intake, since they can not be sintesized by our own metabolic processes. Nowadays they are considered an important component of functional food and nutraceutics, promoting health benefits since they are able to reduce the risk of cardiovascular diseases (CVD), diabetes, ostheoporosis and cancer (Mišurcová et al., 2011). GL is the most common lipid class and is comprised of monogalactosyl-diacylglycerols (MGDG), digalactosyl-diacylglycerol (DGDG) and

sulphoquinovosyldiacylglycerol (SQDG) (Holdt and Kraan, 2011). GL is extremely important in photosynthetic membranes of plants, algae and bacteria. In brown seaweeds, GL is the foremost membrane lipid, with high concentration of specific long chain fatty acids such as stearidonic acid (18:4n-3; SDA), eicosapentaenoic acid (20:5n-3; EPA) and arachidonic acid (20:4n-6; ARA) (Miyashita et al., 2013). SDA are produced by the desaturation of α-linolenic acid (ALA), and when consumed it increases eicosapentaenoic acid (EPA) in red blood cell membranes (Lemke et al., 2013). Epidemiological tests demonstrated that the consumption of long chain ω3 fatty acids reduces the risk of cardiovascular disease, particularly sudden heart attack (Albert et al., 1998). The ω6 fatty acids are extremely important in biological systems. ARA participates in the immune response, prevents thrombosis and helps brain function and together with docosahexaenoic acid (DHA), improves cognitive functions and prevention of age-related brain disorders (Hoffman et al., 2009; Kiso, 2011). These unsatturated acids are main components of cell membranes and participate in neuron structure of the central nervous system (Miyashita et al., 2013). Table 9 summarizes bibliographic information about saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) content in total fatty acid methyl esters (FAME) of macroalgae, collected in several places of the world. In table 9 can be observed that SFAs varies between 7.53% in *Gracilaria changii* colected in Sarawak (Malaysia) and 67.4% of the total FAME, in *Grateloupia turuturu*, from Piriac-sur-Mer (France). MUFAs show a variation between 4.97% in *Caulerpa veravalensis*, Gujarat (India) and 48.7% of the total FAME in *Codium* spp., from Gulf of Cádiz (Spain). For PUFAs, these values oscillate between 19.1% of *Grateloupia turuturu* and 51.2% of the total FAME, in *Gracilaria changii*.

Table 9 – Total saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) content in total fatty acid methyl esters (FAME) are described for macroalgae collected in several places worldwide. After individual reference, letters in superscript indicate the state of the biomass for the extraction protocol used by different authors, Fresh (F), Lyophilized (L) or Air-Dried (AD).

Species	Collection site	SFAs (% of total FAME)	MUFAs (% of total FAME)	PUFAs (% of total FAME)	References
Caulerpa racemosa	Veraval Coast, Gujarat (India)	62.15	10.68	27.17	(Kumar et al., 2011)[L]
Caulerpa scalpelliformis	Veraval Coast, Gujarat (India)	50.25	7.00	42.75	(Kumar et al., 2011)[L]
Caulerpa veravalensis	Veraval Coast, Gujarat (India)	55.03	4.97	40.00	(Kumar et al., 2011)[L]
Codium spp.	Barbate estuary, Gulf of Cádiz (Spain)	17.0	48.7	27.2	(J.S. and V., 2016)[L]
Codium tomentosum	Buarcos bay, Figueira da Foz (Portugal)	38.88	18.51	42.60	(Rodrigues et al., 2015)[AD]
Fucus spiralis	São Miguel Island, Azores (Portugal)	33.59	27.10	38.97	(Paiva et al., 2014)[AD]
Gracilaria changii	Santubong, Sarawak (Malaysia)	7.53	38.30	51.20	(Chan and Matanjun, 2017)[L]
Gracilaria gracilis	Buarcos bay, Figueira da Foz (Portugal)	63.54	15.24	21.22	(Rodrigues et al., 2015)[AD]
Grateloupia turuturu	Buarcos bay, Figueira da Foz (Portugal)	42.74	11.54	45.72	(Rodrigues et al., 2015)[AD]
Grateloupia turuturu	Piriac-sur-Mer (France)	67.4	11.6	19.1	(Denis et al., 2010)[L]
Halymenia floresii	Barbate estuary, Gulf of Cádiz (Spain)	18.8	37.0	32.6	(J.S. and V., 2016)[L]
Osmundea pinnatifida	São Miguel Island, Azores (Portugal)	56.78	20.64	22.23	(Paiva et al., 2014)[AD]
Osmundea pinnatifida	Buarcos bay, Figueira da Foz (Portugal)	58.07	18.92	23.01	(Rodrigues et al., 2015)[AD]
Porphyra sp.	São Miguel Island, Azores (Portugal)	54.32	17.31	24.96	(Paiva et al., 2014)[AD]
Saccorhiza polyschides	Buarcos bay, Figueira da Foz (Portugal)	36.42	29.09	34.49	(Rodrigues et al., 2015)[AD]
Saccorhiza polyschides	Barbate estuary, Gulf of Cádiz (Spain)	20.5	37.6	27.7	(J.S. and V., 2016)[L]
Sargassum binderi	Straits of Malacca near Port Dickson (Malaysia)	31.17	13.65	36.91	(Jaswir et al., 2012)[F]
Sargassum duplicatum	Straits of Malacca near Port Dickson (Malaysia)	29.49	15.39	33.10	(Jaswir et al., 2012)[F]
Sargassum muticum	Buarcos bay, Figueira da Foz (Portugal)	42.17	21.13	36.70	(Rodrigues et al., 2015)[AD]
Sargassum naozhouense	Techeng Island, Guangdong province (China)	33.63	10.42	18.84	(Peng et al., 2013)[AD]
Ulva spp.	Barbate estuary, Gulf of Cádiz (Spain)	24.0	45.5	24.2	(J.S. and V., 2016)[L]

1.2.4.1 Pigments

Among the most abundant pigments found in nature, carotenoids account for approximately 600 compounds. They are lipid soluble methylated polyene derivatives or unsaturated terpenoids with multiple colours, due to the delocalization of the excited state π-electron energy, stated by the variation of conjugated carbon double bonds (Ruban, 2010). The molecular structure comprises a backbone of 40 carbon polyene chain, attributing their distinctive structure, chemical properties, and light-absorbing features. Cyclic groups could exist in its extremities (one or both) including functional groups that contain oxygen (Del Campo et al., 2007). Carotenoids are essential for photosynthesis, contributing to light harvesting, quench chlorophyll triplet states, scavenge reactive oxygen species, dissipate excess energy, maintain the structure and function of photosynthetic complexes (Demmig-Adams and Adams, 2002). The contribution of carotenoids to light harvesting is often greater in algae than in higher plants due to the smaller amount of light that reaches their photosynthetic tissue (Britton et al., 2008). The total carotenoid content (TCC) in several seaweed is described in table 10**.** TCC ranges between 7.42 mg, in *Gracilaria changii*, Sarawak (Malaysia) and 265.80 mg/100g DW in *Sargassum linearifolium* from Gujarat (India). Green seaweed was found to contain between 35.70 mg, in *Ulva reticulata*, Tamil Nadu (India) and 141.70 mg/100g dw in *Caulerpa vervelansis* also from the same location. Red seaweed contains between 7.42 mg, in *Gracilaria changii* and 192.80 mg/100g dw of *Botryocladia leptopoda*, from Gujarat (India). Finally, brown seaweed shows TCC between 32.70 mg in *Stoechospermum marginatum*, Gujarat (India) and 265.80 mg/100g DW in *Sargassum linearifolium*.

1.2.4.1.1 Chlorophylls

These are greenish pigments directly linked to photosynthesis, synthesized by phototrophic organisms in structures called chloroplasts, within flattened vesicles (thylakoids), to absorb sunlight. They are the most abundant pigments on earth and generally carry magnesium as the central metal ion and a phytol (C_{20}) esterified with the propionic acid moiety at C_{17} (Roca et al., 2016). These pigments belong to the tetra pyrroles organic molecules group together with hems, bellins and corrins. Tetra pyrroles are constituted by four pyrrole rings with five atoms each, one of which is nitrogen. Alone, a pyrrole molecule does not absorb light, but when four pyrroles ring merge, linked by unsaturated methane groups, it becomes a molecule rich in conjugated double bonds which can strongly absorb light. Additionally, the four nitrogen atoms are oriented towards the centre of the structure and became capable of accepting coordinating metal ions above or below the plane of the macromolecule. The stabilized oxidation states, solubility and geometry of these metals (which can be manganese, magnesium, cobalt, iron, zinc, vanadium and nickel), together with the ring structure of tetra pyrroles, mediates interactions with proteins in living organisms (Smith and Witty, 2002). Three main classes of chlorophylls are nowadays known, which are distinguished by their unsaturation degree. These are phytoporphyrins, with complete unsaturated macrocycle (chlorophyll *c*), bacteriochlorins with a double saturation of the C7-C8 and C17-C18 bonds (bacteriochlorophylls *a*, *b*, and *g*) and chlorins with a saturated bond between C17 and C18 (chlorophyll *a*, *b*, *d* and bacteriochlorophylls *c*, *d*, *e*, *f*) (Roca et al., 2016). The biochemical pathway of chlorophyll synthesis is tightly controlled since it can become a potential cellular phototoxin. In certain circumstances, when excess of light occurs, the photosynthetic apparatus can be overexcited, resulting in additional

absorbed energy transferred to oxygen, with the formation of reactive oxygen species (ROS). These ROS could also accumulate as a result of the inhibition of chlorophyll biosynthesis or of its degradation during cellular death (Hörtensteiner and Kräutler, 2011). Additionally, it is essential that a correct stoichiometry is achieved between chlorophylls and apoproteins that naturally link with this pigment (Stenbaek and Jensen, 2010). Green seaweed produce chlorophyll a and b, red seaweeds only chlorophyll a and brown seaweeds are known to produce chlorophyll *a* and *c* (Takaichi, 2013). Each person is estimated to consume up to 86 mg of chlorophyll per day. This consumption can provide some health benefits such as antioxidant activity (Lanfer-Marquez et al., 2005) and good ability to chelate some chemical carcinogens or mutagens, reducing cancer risk (Chen and Roca, 2018). *Porphyra* (red seaweed) and *Enteromorpha* (green seaweed) species have been successfully assessed as a strong suppressor against genotoxin-induced umuC gene expression in *Salmonella typhimurium*, which is possibly correlated with carcinogenesis (Okai et al., 1996). In processing food, chlorophylls can be converted into pheophytin, pyropheophytin and pheophorbide, known to prevent cancer (Holdt and Kraan 2011). Pheophorbide *a*, which results from chlorophyll breakdown, was isolated from *Grateloupia elliptica* and successfully inhibited the viability of U87MG, SK-OV-3 and HeLa cancer line cells (Cho et al., 2014). Pheophytin, a chlorophyll without the central magnesium ion (Mg^{2+}), was isolated from *Sargassum fulvellum*, a brown seaweed that demonstrated potential to treat neurodegenerative diseases such as Alzheimer's disease, due to its capability to promote the differentiation of phaeochromocytoma (PC12) cells (Ina et al., 2007). For these reasons, chlorophylls and its derivatives, isolated from seaweed, have become an attractable biomass source for food, cosmetic and pharmacology applications (Chen et al., 2017). Table 10 summarizes bibliographic and methodology information about total chlorophyll *a* (TChl a) content in macroalgae collected in several places of world. TChl *a* varies between 50.40 mg in *Halymenia venusta*, Gujarat (India) and 2,357.90 mg/100g DW in *Caulerpa vervelansis*, collected in Tamil Nadu (India). Green seaweed varies its TChl *a* content between 203.50 mg of *Acrosiphonia orientalis* from Gujarat (India) and 2,357.90 mg/100g DW in *Caulerpa vervelansis*. Red seaweed TChl *a* content fluctuates between 50.40 mg in *Halymenia venusta* and 426.20 mg/100g dw of *Porphyra* spp., collected in Maharashtra (India). Brown seaweed TChl *a* content varies between 113.00 mg in *Iyengaria stellata*, Gujarat (India) and 502.60 mg /100g dw of *Stoechospermum marginatum*, collected in Gujarat (India).

Table 10 – Total chlorophyll *a* and carotenoid content described for macroalgae collected in several places worldwide. Chlorophyll a and carotenoid were assessed differently by different authors and methods are described individually in each reference. After individual reference, letters in superscript indicate the state of the biomass for the extraction protocol used by different authors, Lyophilized (L) or Air-Dried (AD).

Specie	Collection site	Carotenoid content (mg/100g) DW	Chlorophyll *a* content (mg/100g) DW	Method	References
Acrosiphonia orientalis	Port Okha, Gujarat (India)	140.50	203.50	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Boodlea composita	Port Okha, Gujarat (India)	39.80	1151.30	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Botryocladia leptopoda	Port Okha, Gujarat (India)	192.80	374.70	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Caulerpa vervelansis	Mandapam, Tamil Nadu (India)	141.70	2357.90	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Champia spp.	Mandapam, Tamil Nadu (India)	73.90	177.50	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Dictyopteris australis	Port Okha, Gujarat (India)	159.50	355.30	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Dictyota dicotoma	Port Okha, Gujarat (India)	140.60	263.10	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Gelidium micropterum	Mandapam, Tamil Nadu (India)	74.60	392.90	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Gelidiella acerosa	Port Okha, Gujarat (India)	75.10	71.2	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Gracilaria changii	Santubong, Sarawak (Malaysia)	7.42	57.79	Kumar, Ramakritinan, and Kumaraguru (2010)	(Chan and Matanjun, 2017)^{L}
Grateloupia indica	Port Okha, Gujarat (India)	31.70	89.60	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Haloplegma duperreyi	Port Okha, Gujarat (India)	178.10	344.70	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Halymenia venusta	Port Okha, Gujarat (India)	27.10	50.40	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Hypnea musciformis	Mandapam, Tamil Nadu (India)	123.80	268.00	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Hypnea valentiae	Mandapam, Tamil Nadu (India)	119.20	296.40	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Iyengaria stellata	Port Okha, Gujarat (India)	82.00	113.00	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Lobophora variegata	Mandapam, Tamil Nadu (India)	115.00	222.50	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Padina gymnospora	Port Okha, Gujarat (India)	207.10	343.90	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Padina tetrastromatica	Mandapam, Tamil Nadu (India)	249.60	340.40	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Porphyra spp.	Shrivardhan, Maharashtra (India)	58.00	426.20	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Portieria hornemannii	Mandapam, Tamil Nadu (India)	41.50	89.50	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Rhodymenia dissecta	Port Okha, Gujarat (India)	118.00	347.90	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Sargassum linearifolium	Port Okha, Gujarat (India)	265.80	239.80	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Scinaia fascicularis	Port Okha, Gujarat (India)	31.80	53.10	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Scinaia hatei	Port Okha, Gujarat (India)	71.10	66.60	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Spatoglossum asperum	Port Okha, Gujarat (India)	37.90	409.70	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Stoechospermum marginatum	Port Okha, Gujarat (India)	32.70	502.60	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Turbinaria spp.	Mandapam, Tamil Nadu (India)	153.90	260.00	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Ulva lactuca	Port Okha, Gujarat (India)	36.90	909.20	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Ulva reticulata	Mandapam, Tamil Nadu (India)	35.70	904.50	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}
Valonia utricularis	Port Okha, Gujarat (India)	51.50	687.40	Duxbury and Yentach (1956)	(Verma et al., 2017)^{AD}

1.2.4.1.2 Fucoxanthin

This is a photosynthetically active molecule that in brown algae integrates in a 'chlorophyll-carotenoid-protein complex' in the thylakoids structure (Haugan and Liaaen-Jensen, 1992). This xanthophyll is a major marine carotenoid, contributing to more than 10% of the estimated total production of carotenoids in nature (Kim et al., 2012). It contains six oxygen atoms in its structure, which are organized in epoxy, hydroxyl, carbonyl and carboxyl moieties in its molecule, resulting in a highly polar carotenoid (Peng et al., 2011). At each end of the molecule, a cyclic polar group is located, increasing the coordination of the molecule in the membrane, and regulating the interaction patterns with protein membrane-spanning helixes. Together with violaxanthin, lutein, neoxanthin and zeaxanthin, they are the most common xanthophylls (Ruban, 2010). It possesses an unusual highly reactive allenic bound, found rarely in carotenoids, contributing to its unique features (Peng et al., 2011). This pigment has an orange colour and was first isolated from brown seaweed *Fucus*, *Dictyota* and *Laminaria* (Willstätter and Page, 1914). Based on its unique molecular structure, fucoxanthin is considered an important nutraceutical biocompound with various therapeutic activities (Fariman et al., 2016). Its major applications are as an antioxidant (Rajauria et al., 2016), anti-obesity (Miyashita and Hosokawa, 2017) and anti-cancer agent (Gutiérrez-Rodríguez et al., 2017; Ma et al., 2017), but costs associated with production and purification are a major drawback for its industrialization and commercialization (Guedes et al., 2011). Table 11 summarizes bibliographic and methodology information about fucoxanthin extraction and quantification, determined in brown seaweed, collected in several places of the world. Fucoxanthin extracted and determined in fresh weight (FW), shows values ranging between 2.20 mg, in *Hizikia fusiformis*, collected in Tokushima (Japan) and 26.00 mg/100g FW of *Eisenia bicyclis*, collected in South Korea. Dry weight (DW) fucoxanthin values varied from 17.90 mg in *Iyengaria stellate*, from Gujarat (India) and 101.00 mg/100g DW of *Sargassum duplicatum*, from Straits of Malacca (Malaysia).

Table 11 – Fucoxanthin content (mg/100g dry weight (DW) or fresh weight (FW) when stated after individual value for macroalgae collected in several places worldwide. Fucoxanthin content was assessed differently by different authors and methods are described individually in each reference. After individual reference, letters in superscript indicate the state of the biomass for the extraction protocol used by different authors, Fresh (F) or Air-Dried (AD).

Specie	Collection site	Extraction methodology	Fucoxanthin mg/100g DW	References
Eisenia bicyclis	South Korea	LC-MS and HPLC (ethanol)	26.00 (FW)	(Kim et al., 2012)[F]
Eisenia bicyclis	Naruto, Tokushima (Japan)	HPLC (methanol)	7.70 (FW)	(Kanazawa et al., 2008)[F]
Hizikia fusiformis	Naruto, Tokushima (Japan)	HPLC (methanol)	2.20 (FW)	(Kanazawa et al., 2008)[F]
Laminaria japonica	Hakodate, Hokkaido (Japan)	HPLC (methanol)	18.70 (FW)	(Kanazawa et al., 2008)[F]
Sargassum binderi	Straits of Malacca near Port Dickson (Malaysia)	HPLC (methanol)	73.00	(Jaswir et al., 2012)[F]
Sargassum duplicatum	Straits of Malacca near Port Dickson (Malaysia)	HPLC (methanol)	101.00	(Jaswir et al., 2012)[F]
Sargassum fulvellum	Kasumi, Hyogo (Japan)	HPLC (methanol)	6.50 (FW)	(Kanazawa et al., 2008)[F]
Sargassum plagiophyllum	Straits of Malacca near Port Dickson (Malaysia)	RP-HPLC (Cold acetone-methanol, 7:3 v/v)	71.00	(Jaswir et al., 2013)[F]
Turbinaria turbinata	Straits of Malacca near Port Dickson (Malaysia)	RP-HPLC (Cold acetone-methanol, 7:3 v/v)	59.00	(Jaswir et al., 2013)[F]
Undaria pinnatifida	Naruto, Tokushima (Japan)	HPLC (metanol)	11.10 (FW)	(Kanazawa et al., 2008)[F]
Dictyopteris australis	Port Okha, Gujarat (India)	Spectrophotometer (Acetone 90%)	22.70	(Verma et al., 2017)[AD]
Dictyota dicotoma	Port Okha, Gujarat (India)	Spectrophotometer (Acetone 90%)	18.40	(Verma et al., 2017)[AD]
Iyengaria stellata	Port Okha, Gujarat (India)	Spectrophotometer (Acetone 90%)	17.90	(Verma et al., 2017)[AD]
Lobophora variegata	Mandapam, Tamil Nadu (India)	Spectrophotometer (Acetone 90%)	22.90	(Verma et al., 2017)[AD]
Padina gymnospora	Port Okha, Gujarat (India)	Spectrophotometer (Acetone 90%)	43.50	(Verma et al., 2017)[AD]
Padina tetrastromatica	Mandapam, Tamil Nadu (India)	Spectrophotometer (Acetone 90%)	41.10	(Verma et al., 2017)[AD]
Sargassum linearifolium	Port Okha, Gujarat (India)	Spectrophotometer (Acetone 90%)	37.50	(Verma et al., 2017)[AD]
Spatoglossum asperum	Port Okha, Gujarat (India)	Spectrophotometer (Acetone 90%)	57.80	(Verma et al., 2017)[AD]
Stoechospermum marginatum	Port Okha, Gujarat (India)	Spectrophotometer (Acetone 90%)	36.90	(Verma et al., 2017)[AD]
Turbinaria spp.	Mandapam, Tamil Nadu (India)	Spectrophotometer (Acetone 90%)	43.10	(Verma et al., 2017)[AD]

1.3 Conclusions

Seaweed presents itself as a new feedstock with great potential for multi-products extraction. Over the past decade, it has become more sustainable, with the development of new technologies, producing valuable marketable products and energy. The biorefinary approach increases the sustainability, efficiently extracting more compounds and reducing waste, which allows the creation of a bioeconomy, based in multi-product production from a single source. This enables an increase of the biomass conversion efficiency, reducing the amount of raw material discarded. Additionally, the implementation of a biorefinary centered in a cascade extraction, could provide a better profitability and improve residue management of the feedstock, mitigating the environmental impacts. Several seaweed species can be integrated in the biorefinary approach, providing enough material to a designed cascade extraction. This improves sustainability, economic growth and market development. Numerous economic appealing products can be extracted, due to the singular properties of seaweed. Among these products are included proteins, lipids with distinct fatty acids, sugars and polysaccharides with bioactive properties, antioxidants and other high value compounds, which can be used by the biotechnology industry. The remaining residue, after biomass exhaustion, could be successfully processed to produce bioenergy, in the form of biochar and bio-oil or even integrate a bio-fertilizer for crop production. Moreover, seaweed was not domesticated, having natural defences against epiphytes and marine grazers, reducing the inputs during aquaculture and minimizing the environmental impact. Their production does not occupy land area, reducing the deforestation pressure. Furthermore, it is of extreme importance, when studying different seaweed species to integrate in a biorefinary, that sufficient research is performed to design a specific strategy that copes with the feedstock availability and integrates technological processes and pathways available for product extraction, adapting this industry to the local conditions. Moreover, a biorefinary concept enables the interaction between different bioeconomy sectors, forming partnerships between different stakeholders, enabling higher social, environmental and economic benefits. Still, some apprehensions exist, concerning the sustainability principle, since seaweed availability oscillates with climate, season, local conditions and legal requirements, posing a challenging factor. However, with the increasing development of new cost-effective bioprocessing routes and employing eco-friendly downstream processes in the biorefinary complex, it could be the key factor for global successful implementation.

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Chapter 2

Chapter 2

2 Scientific articles

2.1 Sampling, structure identification, cultivation and accessions maintenance of three seaweeds from Madeira Archipelago

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Manuscript in preparation for submission

2.1.1 Abstract

In this work, three macroalgae, *Asparagopsis taxiformis* (Delile) Trevisan 1845: 45, *Zonaria tournefortii* (J.V.Lamouroux) Montagne 1846: 32 and *Ulva lactuca* Linnaeus 1753: 1163 from Madeira archipelago were selected to assess their *ex situ* cultivation, characterize and identify microscopic structures and life cycle, identify prominent sites for sampling and assess the viability to maintain these accessions in the germplasm bank. During seaweed prospection, it was observed that the red macroalgae *A. taxiformis* are usually completely submerged, but exposed to sunlight, in small communities, attached to sand. Individuals found were dioecious and the algae tetrasporophyte stage known as "*Falkenbergia hillebrandii*", was also found in the wild. Several microscopic details were observed in this species, namely: the three cell row in the apical part of the gametophyte; the cystocarps filled with carpospores in the female gametophyte; and spermatangia structures in the male gametophyte. The brown macroalgae *Z. tournefortii* is mainly present in shady places, also completely submerged, and attached to basalt substrates, in small communities or isolate. Small selected fragments of this macroalgae were fundamental to identify if it was a sporophyte or gametophyte, since the individuals are morphologically identical at the naked eye. It was also determined that the blade margin has a smooth appearance, the presence of paraphyses in the sporophyte and antheridia in the male gametophyte. Green macroalgae *U. lactuca* were found in intertidal areas, subjected to intermittent waves and periods of desiccation, preferring the basaltic substrate, with some individuals exposed to sunlight and some covered from it. For these three macroalgae, higher number of individuals are found in early spring, decreasing in number when seawater temperature arises. The microscope observations have shown individual non-differentiated cells, being considered complex cellular colonies due to the lack of plasmodesmata. Cultivation tests were performed using variable strategies and media. For *U. lactuca*, two forms of cultivation success were achieved, through vegetative propagation and spore production. Protoplast production was also tested for this species but with low hematocrit values, preventing a successful production of *U. lactuca* propagules. For *A. taxiformis*, propagules were obtained when cystocarps released the carpospores or the tetrasporophyte its spores, but the *in vitro* cultivation was often contaminated by microalgae, preventing the propagules development. For *Z. tournefortii*, cultivation was not successful. It was determined that axenic cultures were difficult to obtain at this stage due to some difficulties in separating only the spores and eliminating sources of contamination. For the germplasm maintenance of these species in bank, the accession establishment was successful, since cultures were maintained for several months, with temperature maintained below 20 ºC, with nutrients provided weekly.

2.1.2 Introduction

The increasing need for new sources of biomass that can be used for multiple industrial applications enable the study on macroalgae singular features, allowing their cultivation and intrinsic capabilities in biomass production, during its life cycle. The species selected for this work include *Ulva lactuca* Linnaeus 1753:1163, *Asparagopsis taxiformis* (Delile) Trevisan 1845: 45 and *Zonaria tournefortii* (J.V.Lamouroux) Montagne 1846: 32. These are multicellular, photosynthetic organisms which can vary in their pigmentation, enabling their separation in three phyla: Chlorophyta that includes green macroalgae; Rhodophyta comprising red macroalgae; and Ochrophyta, brown macroalgae (Ellen et al., 2015). They occur in the intertidal and subtidal ocean areas around the world, comprising the aquatic euphotic zones. Mankind use of macroalgae has evolved since the beginning of civilization and aquaculture represents a recent development, since the need to minimize human impact in the terrestrial environment and the search for new food sources, to cope with an increasing population. According to the Portuguese Navy (2015), Madeira archipelago has 10,823 km^2 of sea area and only 810 km^2 of land, enabling an enormous potential for exploring sea resources and development of related industries, whereas macroalgae aquiculture could play an important role in biomass production. This could represent an emerging economical effort for this insular region. Macroalgae cultivation can be achieved through vegetative propagation and/or by sexual reproduction, depending on their reproductive features. *Ulva* species life cycle alternate the haploid gametophyte with the diploid sporophyte, isomorphic structures, which are not distinguishable to the naked eye, due to its morphological similarity (Potter et al., 2016). *Ulva lactuca* presents distromatic green thalli, which can vary from lanceolate to oval shape, tangled along the edge (Sherrington, 2013). The inexistence of plasmodesma, i.e. microscopic channels which allow the communication between plant cells, signifies that *Ulva* spp. are complex cellular colonies (Hoek et al., 1995). Cells among the blade edges, with the ability to form gametangia or sporangia are upper regulated by two sporulation inhibitors, comprising a glycoprotein and a factor with high and low molecular weight, respectively (Vesty et al., 2015). *Ulva lactuca* life cycle is represented in figure 1, adapted from Hoek et al. (1995).

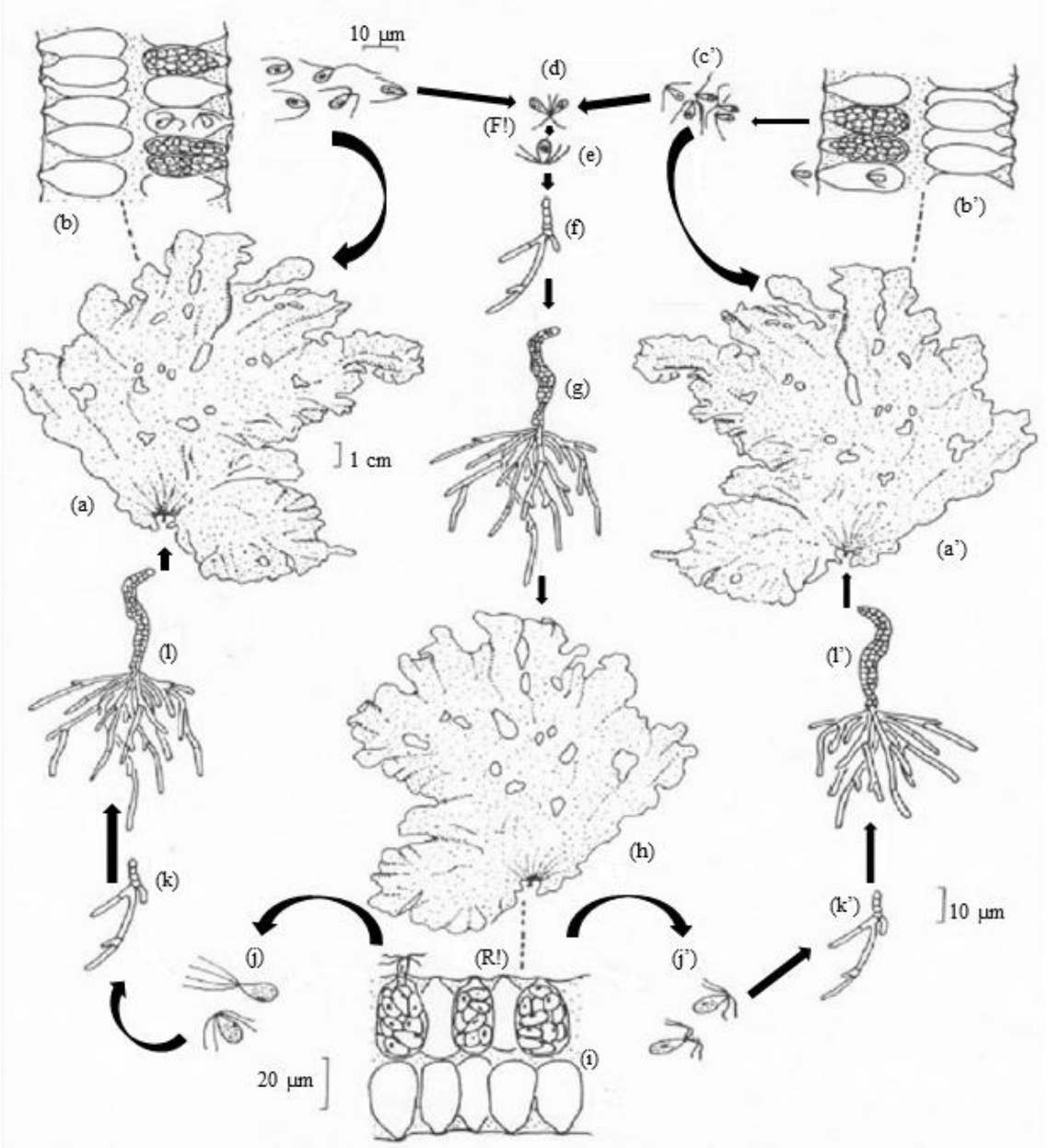

Figure 1 – Schematic illustration of *Ulva lactuca* Linnaeus 1753 life cycle, adapted from Hoek et al. (1995). The letters a and a' represent the haploid gametophyte thalli which enable the production through mitosis (b and b') of anisogamous biflagellate gametes labelled by c and c'. From the fusion (d) between the male and female biflagellated gametes results the zygote egg (e), which germinates into a filament (f) and further in the formation of pluriseriate filament represented by the letter g. Finally a distromatic thallus of the sporophyte (h) is formed and through meiosis in the sporangia (i) the quadriflagellated haploid zoospores (j and j') are produced, which develop into new haploid gametophyte generations.

The red macroalgae *A. taxiformis* has a diplohaplontic heteromorphic life cycle with a major gametophytic life stage. The diploid phase includes the formation of cystocarps, through fecundation of the female gamete and subsequent development of an epiphytic stage known as *Falkenbergia hillebrandii* (Bornet) Falkenberg (figure 2).

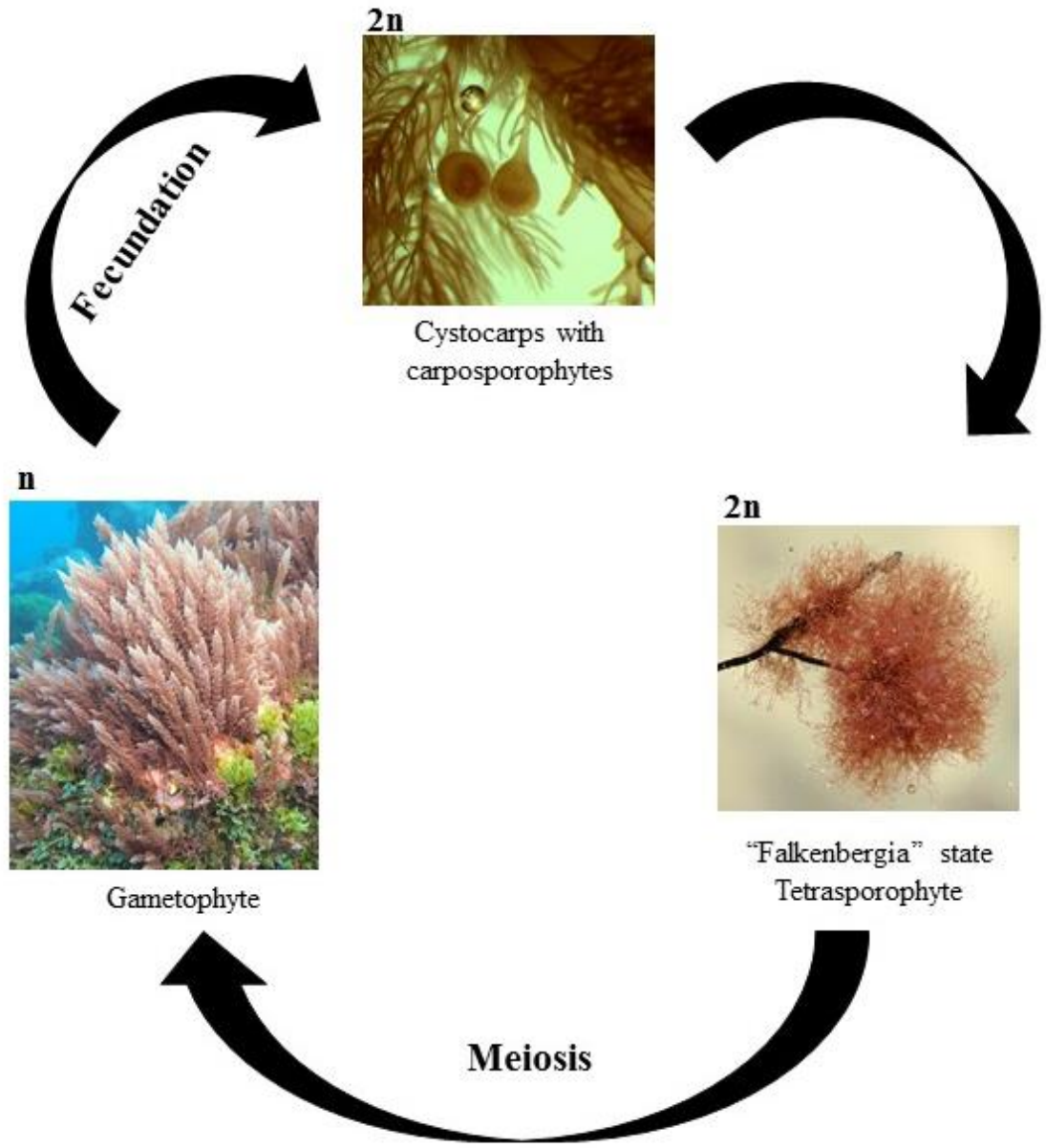

Figure 2 – Schematic illustration of *Asparagopsis taxiformis* (Delile) Trevisan de Saint-Léon 1845 life cycle, adapted from Zanolla and Andreakis (2016). The **n** and **2n** represents the haploid and diploid phases.

This stage represents a tetrasporophyte composed by tightly ramified filaments, each with 2 cell rows (Andreakis et al., 2004; Zanolla and Andreakis, 2016). This species gametophytes possess unspecific branches ascending from stoloniferous branches and the preferred substrate for anchorage is typically sand, due to its well-developed rhizomatous structure (Bonin and Hawkes, 1988). Species distribution ranges from tropical to warm temperate waters around the globe (Andreakis et al., 2004; Barone et al., 2013). The brown macroalgae *Z. tournefortii* is characterized by 2 isomorphic life stages which includes dioecious haploid gametophyte and diploid sporophyte, which can be differentiated only through the observation of their mature reproductive structures (Montañés et al., 2006), represented in figure 3. This macroalgae is attached to the substrate by means of a basal disc with flamboyant branches and upright stems (Ángeles Montañés et al., 2002). The thallus comprises a darker tissue, forming zones, common to the Dictyotales, primordially originating the generic designation of "*Zonaria*" (Dahl, 1971). The main objective of this work

was to sample, characterize and identify the morphological structures of these macroalgae, to establish and maintain *in vivo* accessions in germplasm bank, with the ultimate goal to perform their *ex situ* cultivation.

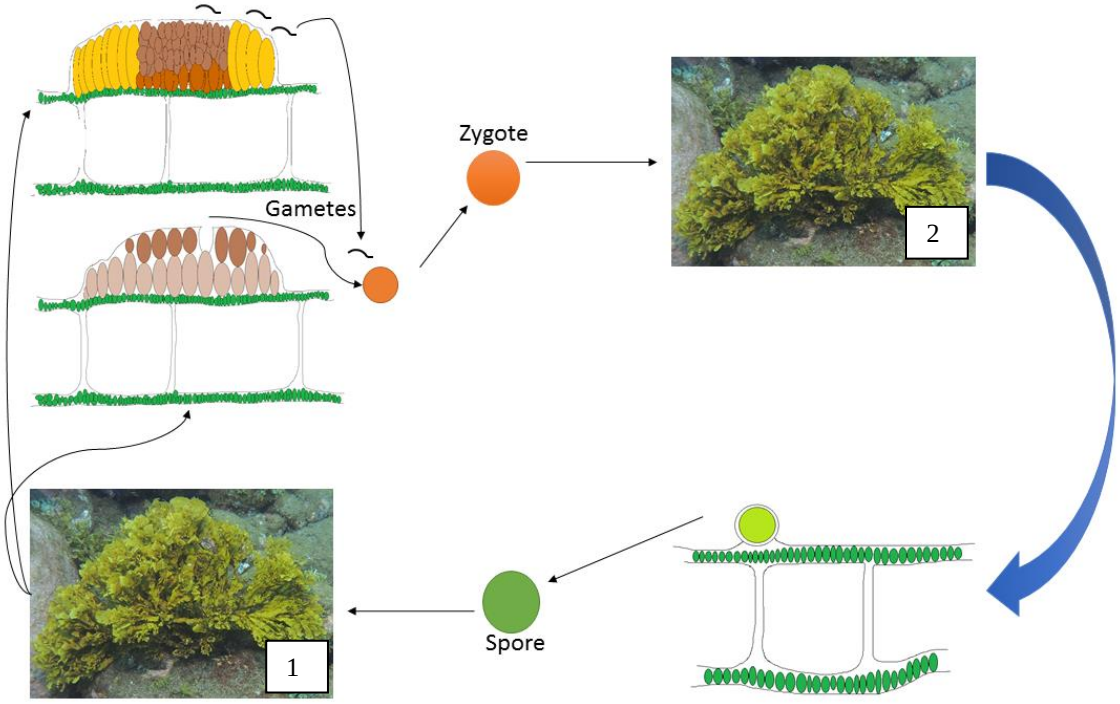

Figure 3 – Schematic illustration of *Zonaria tournefortii* (J.V.Lamouroux) Montagne 1846 life cycle. Includes the representation of the gametophyte (1) and the sporophyte (2).

2.1.3 Materials and Methods

Macroalgae used in this study were collected in two places in the Madeiran south coastline from intertidal zone to a maximum of a 10-meter depth dive (figure 4). These species *Ulva lactuca*, collected at Santa Cruz (figure 5), *Asparagopsis taxiformis* and *Zonaria tournefortii* collected in Reis Magos beach (figure 6) were the most common macroalgae in these places.

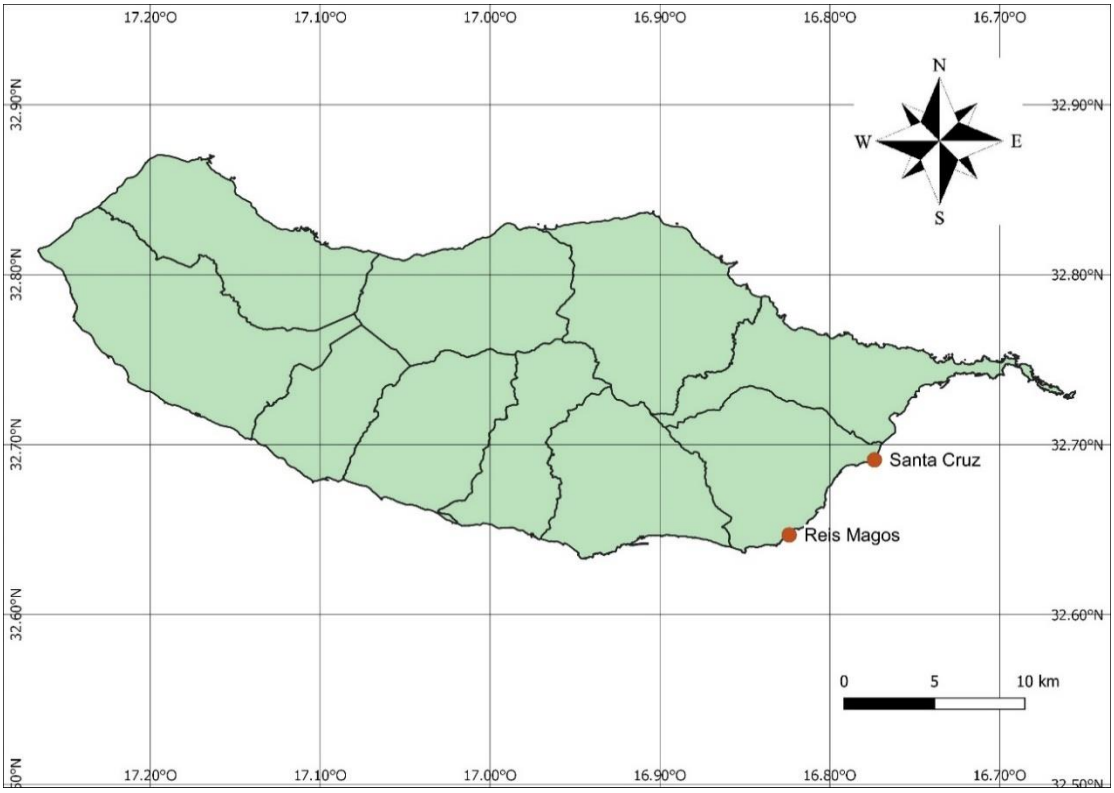

Figure 4 – Localization of the prospection and sampling sites of 3 macroalgae in Madeira Island. The macroalgae *Asparagopsis taxiformis* and *Zonaria tournefortii* were collected in Reis Magos and *Ulva lactuca* in Santa Cruz.

Figure 5 – Macroalgae prospection at Santa Cruz. The flask at the left and right contain *Ulva lactuca* and middle flask *Chondrus crispus*.

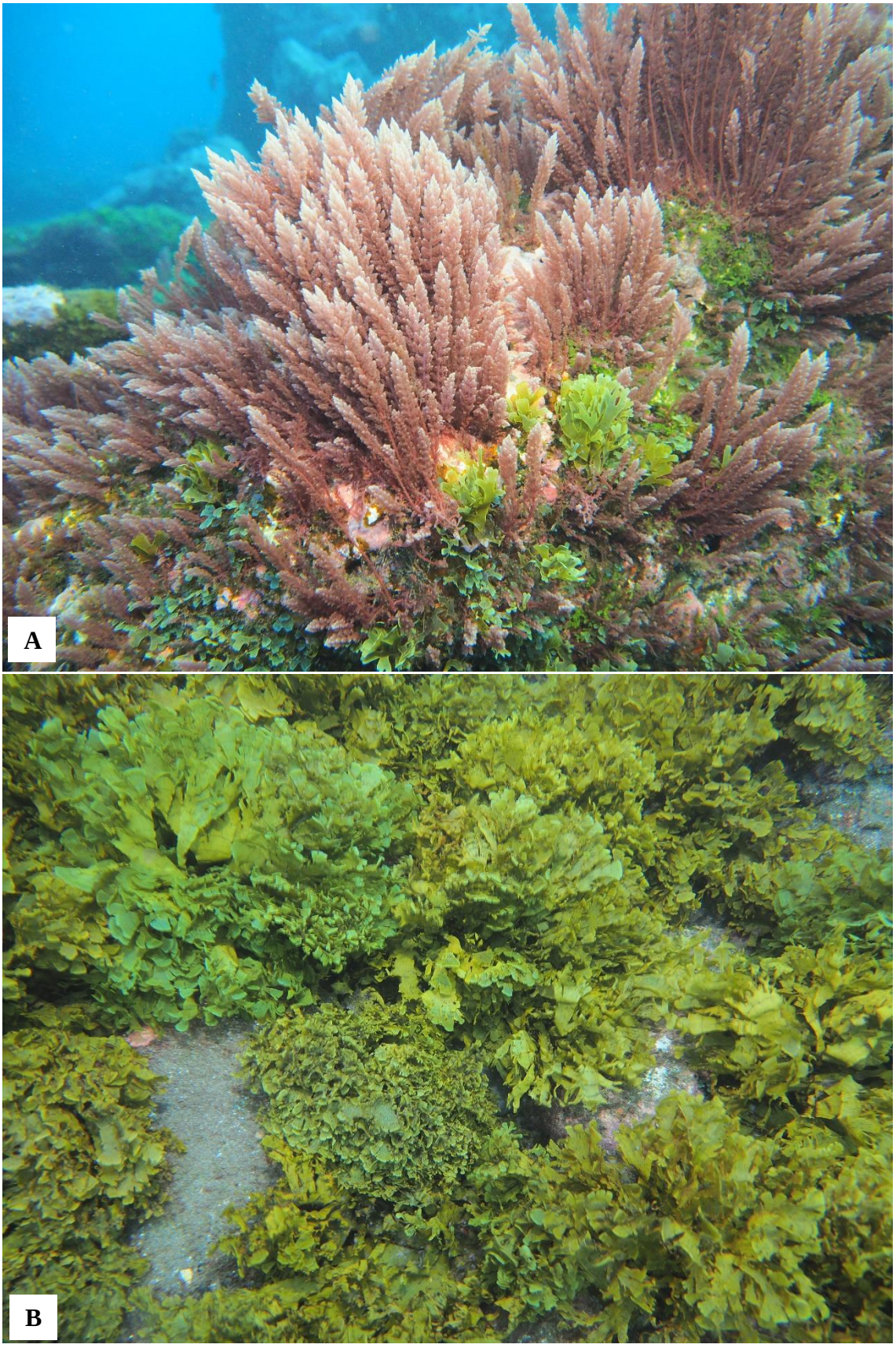

Figure 6 – Macroalgae prospection at Reis Magos. A - *Asparagopsis taxiformis* (Delile) Trevisan 1845: 45; B - *Zonaria tournefortii* J.V.Lamouroux) Montagne 1846: 32.

The samples were separated and transported in seawater to the laboratory. Seawater (further designated as natural seawater) was collected and filtered. Additionally, artificial seawater was produced through the addition of sodium chloride to deionized water (dH_2O) and the salinity controlled until 30 ‰ was achieved. Macroalgae were cleaned, using a fine brush, to remove any strange material or epiphytes that could be attached. Furthermore, macroalgae structure and life stage were analyzed in the laboratory, by microscope observations, using an optical and stereo microscope, which were subsequently photographed. Macroalgae cultivation was assayed, using different techniques and growth conditions, namely liquid and solid media, temperature ranges and light intensity regimes. Several media for macroalgae cultivation were tested, namely an F/2 medium (Guillard, 1975; Guillard and Ryther, 1962) (see annex 1), MPI medium (Suto, 1959) (see annex 2) and PES medium (Provasoli, 1968) (see annex 3). Solid medium was prepared adding 1.5% agar. When diatomaceous algae were detected, GeO_2 (germanium dioxide) was added to mitigate contamination (see annex 4). Different algae material was also used, from adult blade fragments to reproductive structures (gametophytes and sporophytes). *U. lactuca* cultivation was performed by cutting small pieces of the thallus and inserting these fragments into solid (agar plates) or liquid (F/2) media, with natural seawater. Both cultivation variants were transferred to a growth chamber at 16 ± 2 ºC with the photoperiod adjusted to 12h:12h (l/d). Additional protoplast production from this biomass was assayed, according to Reddy and Seth (2018) (see annex 5).

Asparagopsis taxiformis cultivation under different conditions was tested. In a first set of experiments, entire and apical part of the macroalgae were cultivated in natural and artificial seawater. *A. taxiformis* cultures were maintained, at room temperature, 23 ± 2 ºC, using artificial light (light intensity: 200 µE m^{-2} s^{-1}). Additionally, entire *A. taxiformis* was left, in natural seawater, for 24h, to develop a stress situation that promotes the macroalgae gametes release. This seawater was transferred, after 24 h, and placed at room temperature, about 24 ± 2 ºC, under natural light, which intensity varied during the day between 112 and 680 µE m^{-2} s^{-1}. The gametes were isolated and transferred to flasks with natural seawater, for cultivation in a growth chamber at 16 ± 2 ºC, photoperiod 12h:12h (l/d). The sporophyte ("*Falkenbergia hillebrandii*" state) was cultivated, with small fragments placed in individual agar plates to allow spore release. Additionally, the sporophyte was immersed in seawater for 1 hour, and then the spores were inoculated in petri plates and test tubes, with liquid medium (PES medium). These cultivation experiments were transferred to a growth chamber at 16 ± 2 ºC, with a photoperiod of 12h:12h (l/d).

The *Z. tournefortii* cultivation consisted in the use of small apical fragments obtained using a scalpel, placed into a petri dish filled with natural seawater. These fragments were cut into smaller pieces and transferred to petri dishes or test tubes with F/2 liquid medium, with assistance of stereo microscope. Other cultivation experiments, using the entire or apical part of the macroalgae in artificial seawater were also performed. These cultures were maintained under room temperature, about 23 ± 2 ºC, using artificial light (light intensity: 200 µE m^{-2} s^{-1}). Additionally, it was also tested the cultivation of the entire macroalgae in natural seawater in a growth chamber at 16 ± 2 ºC and photoperiod of 12h:12h (l/d). Nutrients and vitamins were added to each culture weekly to achieve accessions maintenance in the genetic bank.

2.1.4 Results and Discussion

2.1.4.1 Prospection sites and macroalgae sampling

Ulva lactuca was harvested in Santa Cruz (figure 5), in intertidal zone, attached to the basaltic substrate, together with *Chondrus crispus*, a red seaweed. In the spring, this site holds a high number of fully matured *U. lactuca* individuals (± 40 individuals/m^2) in a total area of 20 m^2. This number decreases during summer and autumn and little propagules begin to appear in the winter. In this area, *U. lactuca* is only submerged for brief periods of time, in high tide, being most of the time subjected to intermittent waves and desiccation periods. Specimens can reach 15 cm in length and are strongly attached to the substrate, frequently exposed to direct sunlight. *A. taxiformis* and *Z. tournefortii* (figure 6) are found in the subtidal zones of Reis Magos beach, being always fully submerged and can be found from 1 to 10 meters depth, frequently exposed to strong waves. These populations of macroalgae occurs close to each other. Specimens have a similar size, about 30 cm in length, but appear exposed to different conditions. The red macroalgae *A. taxiformis* develops in small communities, exposed to sunlight, mainly attached to sand, which was also reported by Bonin and Hawkes (1988) in which ± 60 individuals could be found in each m^2 in about 100 m^2 for this populated spot. The gametophytes of this species are dioecious, with the development of cystocarps and spermatangia in different individuals as reported by Barone et al. (2013). Tetrasporophyte state of *A. taxiformis*, known as the "*Falkenbergia hillebrandii*" was found in Reis Magos beach, in the south coast of Madeira island. These are diploid organisms, pink coloured with about 1.5 cm in diameter, with a spherical clump shape, attached by means of loosely branched filaments. These tufts comprise of tangled, branched trisiphonous filaments (Bonin and Hawkes, 1988). The brown macroalgae *Z. tournefortii* prefers covered locations, rarely reached by direct sunlight. In the prospection site, ± 15 individuals were found in each m^2, in a populated spot, covering about 50 m^2. Sporophyte and gametophyte in this species cannot be distinguished in the field, due to their isomorphism. This can only be achieved using a microscope. These are normally found attached to rocky basalt substrate, present in small communities or alone. They have a green colour when submerged, but out of the water, exhibit a brown colour. A high number of individuals is also detected at the beginning of spring, it begins to decrease at the end of spring and disappear in summer, when water temperatures arises.

2.1.4.2 Microscopic assessment

The microscope observations were realized to evaluate the sampled macroalgae, to analyze their cell structure, determine the phase or stage of life cycle, detect and determine the presence of reproductive structures and assess its maturation. Similar individual cells were visualized in *U. lactuca* blade (figure 7), showing no differentiation and comparatively bigger than vegetative cells of other macroalgae species. Their structure is frequently quadrate with several levels of anticlinal elongation. Individuals have a fibrillary organization, capable to realize photosynthesis and sexual reproduction, mainly constituted by carbohydrates, including cellulose (Sherrington, 2013). According to Hoek et al. (1995), cells with nucleus, cup-shaped parietal chloroplast, with one or more pyrenoids inside these last with sub-cellular micro-compartments for CO_2 fixation, which were also observed in this work. They also state that *Ulv*a genus individuals are a complex single cellular colony, due to the absence of plasmodesmata, microscopic conduits which enable communication and metabolite transportation between cells.

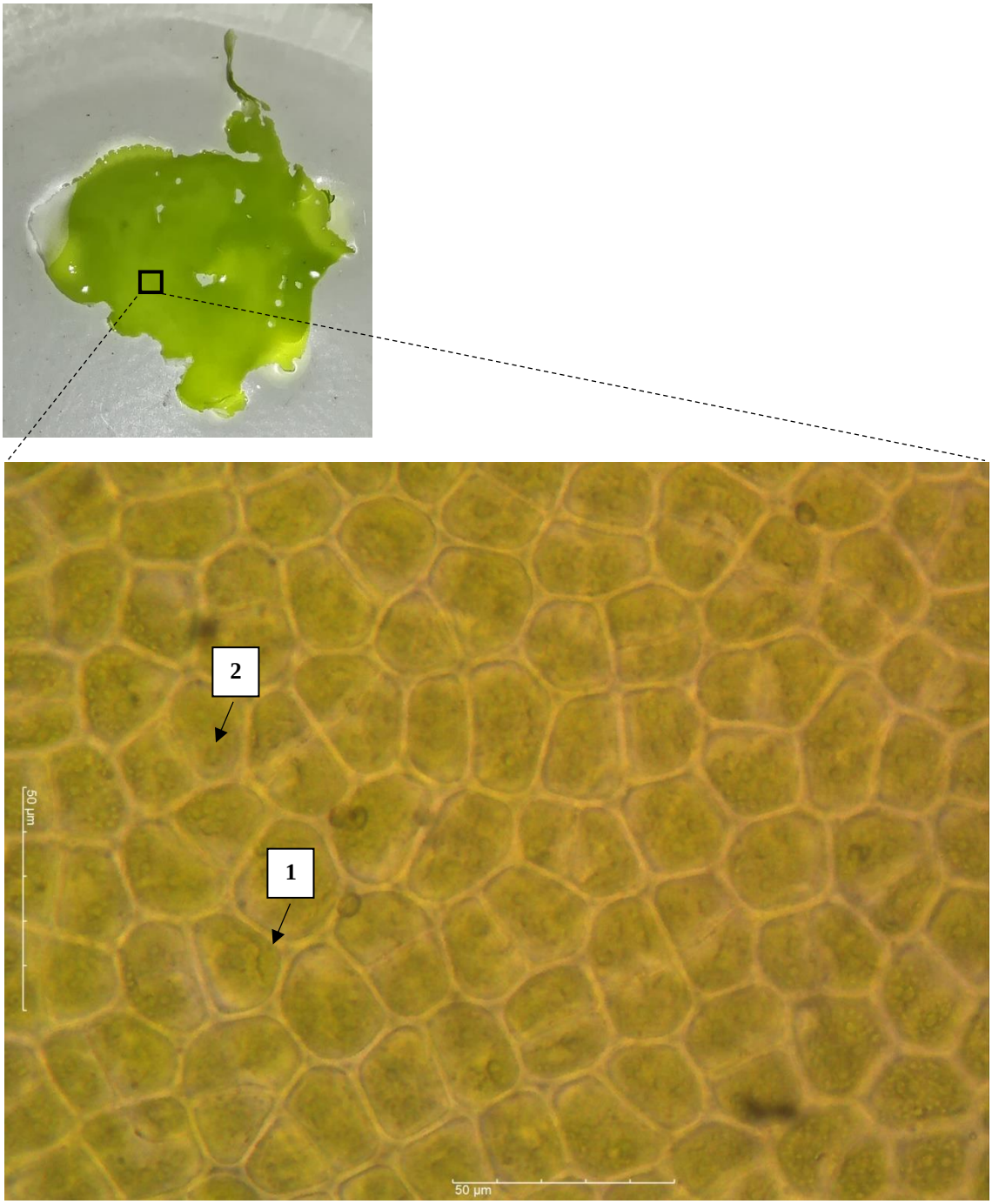

Figure 7 – Microscopic photograph of *Ulva lactuca* thallus evidencing the cellular structure. The number 1 points to the cup-shaped parietal chloroplast and number 2 to a pyrenoid. Photograph has a 50 µm scale.

The microscopic details of *A. taxiformis* structure was achieved and presented in figure 8. The apical filamentous branches were composed by three cell rows and can be observed in figure 8 (A). Cystocarps were also detected. Two cystocarps are developed in the lower part of major algae branch as described by Bonin and Hawkes (1988). In figure 8 (B) the carpospores, within the cystocarp, can also be observed. Additionally, when the cystocarp is fully matured, numerous gonimoblast branches arise from a large fusion of cells, similar to a tree trunk. At the end of these gonimoblast filaments, an ovoid-shaped pigmented carposporangium is present, which are released through an apical or subapical pore (Bonin and Hawkes, 1988). The formation of tetrasporagium in the sporophyte (figure 8, C), begins with a single periclinal division of a mature periaxial cell. This undergoes an anticlinal division to produce 4 tetrasporangia which are subsequently released, due to a rupture in the cell wall (Bonin and Hawkes, 1988). This tetrasporophytic life stage is completely epiphytic (Andreakis et al., 2004). *A. taxiformis* is a dioecious species, meaning that male and female sexual organs are present in different individuals. These individuals have a gametophytic period in a diplohaplontic heteromorphic life cycle (Andreakis et al., 2004). In figure 8 (D) a male spermatangium can be observed, within a specialized male branch. According to Bonin and Hawkes (1988), male branches are normally narrow, long and covered completely with spermatangia, excluding close to the base, which seems as a stalk.

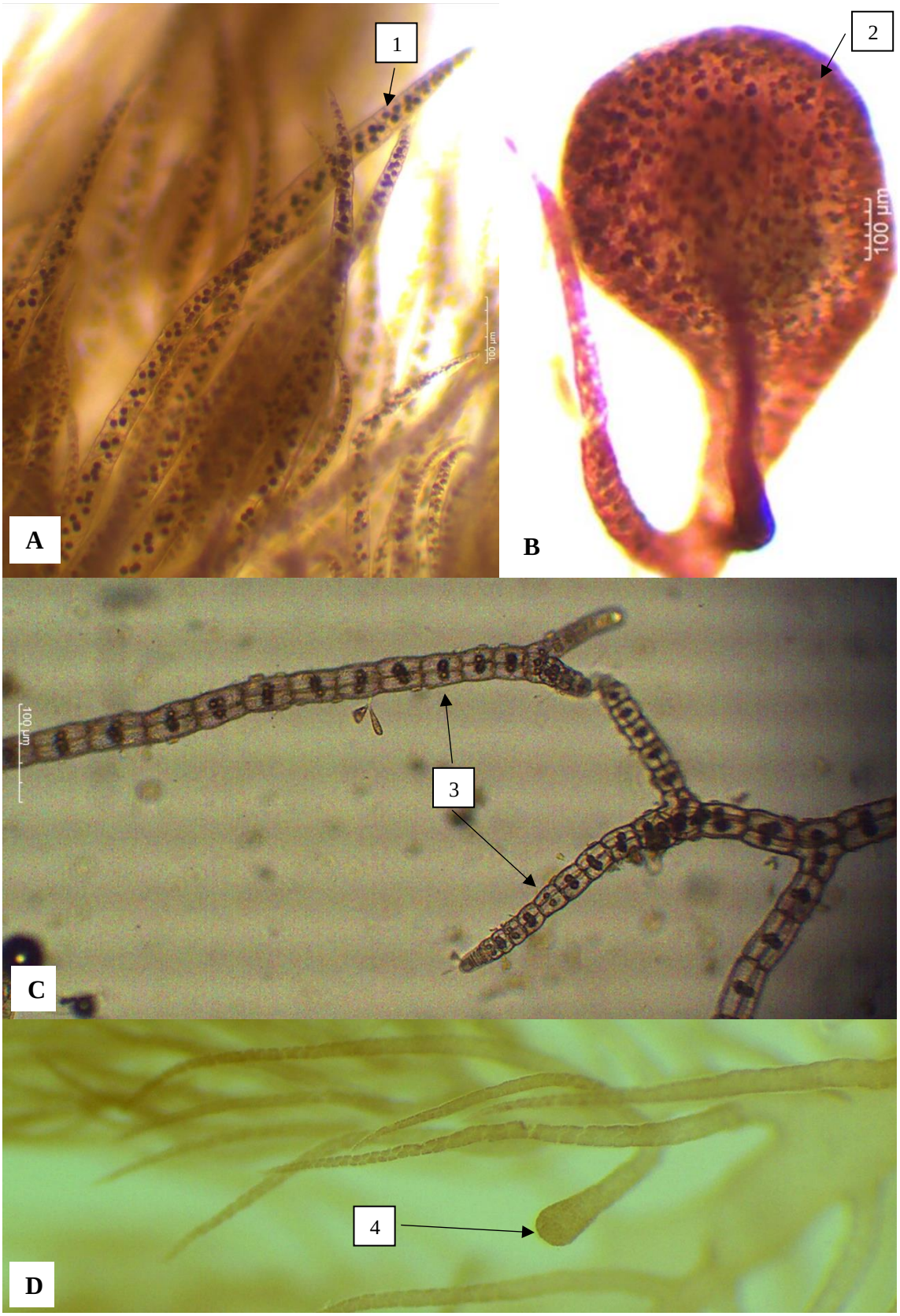

Figure 8 – Photographs with the structural details of *Asparagopsis taxiformis*. A – Apical cells (1) located in branches, 100 µm scale; B – Cystocarp with visible carpospores (2), 100 µm scale; C – Microscopic details of "Falkenbergia" sporophyte state with 2 cells row (3), 100 µm scale; D – Detailed structure of spermatangium, male organ (4).

Figure 9 shows the microscopic structures in the brown macroalgae *Z. tournefortii*. The macroalgae blade margin appears with a smooth surface (figure 9, A), that can have a wavy or serrated form. Also, in figure 9 (B) can be observed the presence of paraphyses, a dark transversal line in the macroalgae blade, originated from cortical cells. This organism has a life cycle composed by alternate isomorphic gametophyte and sporophyte generations, being these identical and could be ascertained only with a microscope observation, identifying the sexual structures (Ángeles Montañés et al., 2002). The sporangia is dispersed within the *indusiate sori* own numerous cortical cells, which could have a cylindrical or ovoid shape, deprived of a basal stalk cell, enclosed by paraphyses (Phillips, 1997). The mature sporangium is coated with a dense mucilage, which accumulates beneath and the protoplast splits into 8 spores (Phillips and Nelson, 1998). Male gametophytes form numerous antheridia, gathered in irregular shaped *indusium sorum*, with a mucilaginous matrix, arranged in transverse whitish lines (figure 9, C). Each antheridia is originated from a cortical cells transformed into antheridia. The mature antheridia are cylindrical and in the periphery, some could be slightly elongated and hyalines (Ángeles Montañés et al., 2002). In figure 9 (D), rectangular vegetative cells with similar size can be observed, displayed in radial lines of the macroalgae blade. They have a nucleus with 5µm in diameter with a distinctive nucleolus, bordered by several organelles with physodes, which are vesicles containing tannins (Dahl, 1971). These cells are formed from the meristematic cells, which are bigger and more densely pigmented when located in the border of the meristematic layer (Ángeles Montañés et al., 2002).

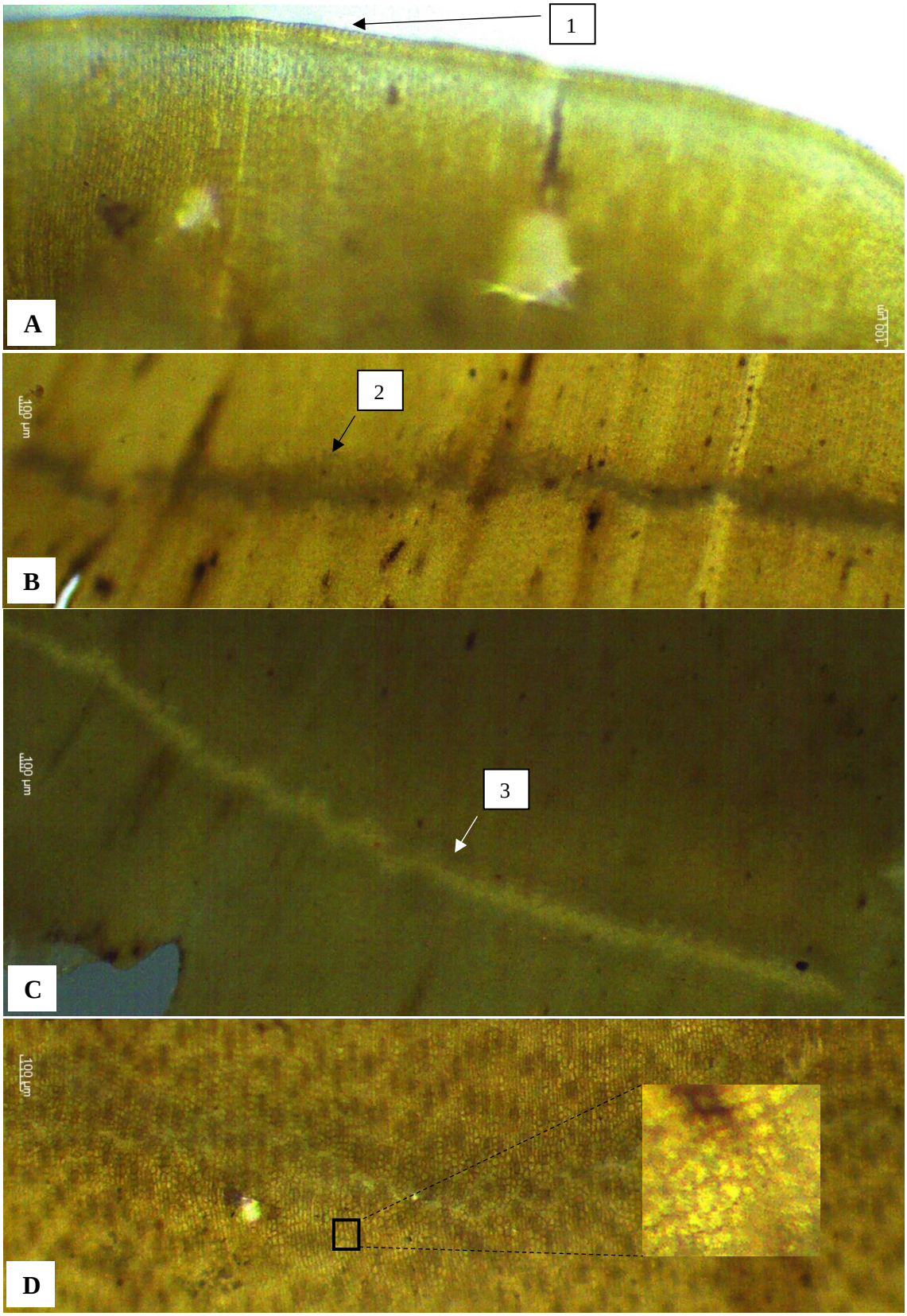

Figure 9 – Figure shows the structure detail of *Zonaria tournefortii* using a 100 µm scale. A – Blade apical extremity, with growth cellular layer (1); B – Dark line of paraphyses (2) evidenced in the blade of the sporophyte; C – Male gametophyte with antheridia, represented by the whitish line (3); D – Rectangular cells, with similar size, present in radial lines in the macroalgae blade.

2.1.4.3 Macroalgae cultivation and maintenance of accessions in the germplasm collection of the bank

Cultivation trials were performed to determine and optimize conditions to produce viable propagules which would grow into juvenile organisms and transferred from the laboratorial facilities to scale up tanks or transferred to the sea offshore facilities. These propagules are derived from spores or zygotes, produced, during the life cycle of each macroalgae species. To achieve this goal, a deeper knowledge of each macroalgae life cycle is imperative. Over the years, several strategies were developed, which use numerous abiotic factors to induce the reproductive maturity, achieving sporulation outside the normal life cycle (Gupta et al., 2018). Several cultivation media were developed to support macroalgae vegetative propagules or reproductive structures, providing nutrition for optimal physiological development. These are the F/2 medium (annex 1) developed by Guillard and Ryther (1962), the MPI medium (annex 2) developed by Suto (1959) and PES medium (annex 3), developed by Provasoli (1968). They have different recipes and compositions, which provides macronutrients, trace metals (micronutrients) and vitamins. These media were prepared in fresh filtered and autoclaved seawater. Furthermore, to prevent or eliminate fouling by diatoms, a GeO_2 solution (annex 4) was added, since Goldschmidt (1937) hypothesized that its presence results in the exchange of silica by germanium, inhibiting the silica frustule mineralization and preventing the formation of diatoms wall. Different techniques were applied to produce the propagules of these 3 macroalgae, namely using solid and/or liquid media. In the first case 1.5 % agar was introduced as substrate and a liquid phase to provide nutrients to the macroalgae. Or, only a liquid medium, in which the entire organism or pieces of it would be free, unattached, with a more dynamic contact with the involving medium.

For the red macroalgae *A. taxiformis* (figure 10, A), it was tested the use of reproductive structures produced by the apical part of this macroalgae or using the entire organism, which were maintained in liquid medium with artificial or natural seawater. A total of 16 trials were performed and the development of microscopic structures was observed, but their maintenance and development was difficult, due to the microalgae appearance and contamination, which become a problem after some time. The use of stress conditions was also assessed. This stress consisted on leaving the macroalgae in dark conditions for a period of 24h to oblige *A. taxiformis* to release gametes and induce the fecundation. Although, after 5 attempts, the method did not give the expected results. Recently, we have tried to release the spores from the tetrasporophyte "*Falkenbergia hillebrandii*" (figure 10, B), inducing its sporulation, which we think is the most promising way to produce an *ex situ* culture of individuals. The whole organism was introduced in liquid medium or little fragments were cut, to induce sporulation. The development of some spores was observed, but after some time, the culture became contaminated, creating the problem for its long-term maintenance and development. Axenic cultures were difficult to obtain at this stage due to some difficulty in separating the spores from sources of contamination, such as microalgae.

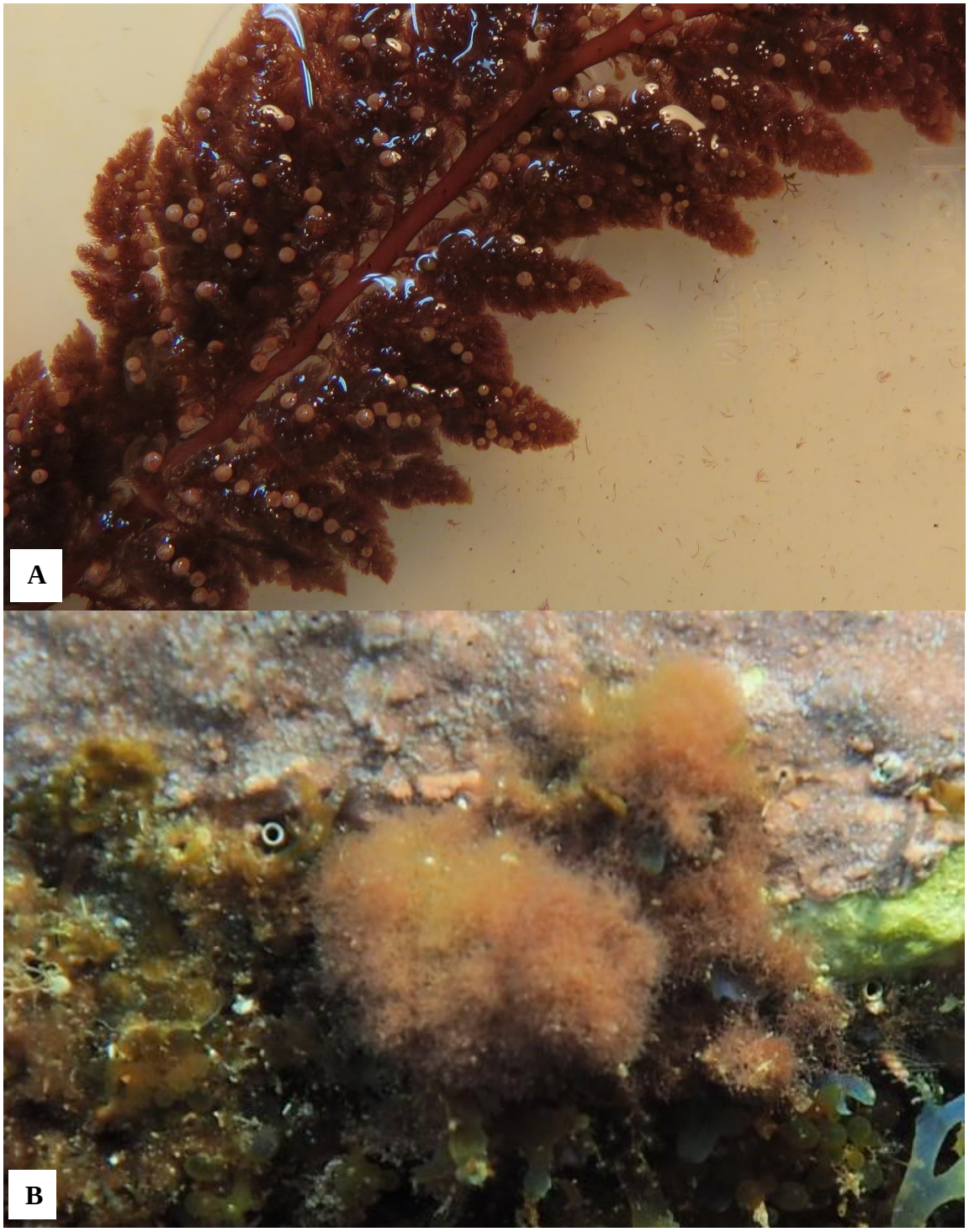

Figure 10 – *Asparagopsis taxiformis ex situ* cultivation. A – Testing the ability to use the cystocarps to release carposporophyte and form the tetrasporophyte; B – Used to test the ability to use "Falkenbergia" state for spores release to form gametophytes.

Apical fragments (figure 11, A) of the brown macroalgae *Z. tournefortii* were tested for meristematic activity and growth in media, using natural and artificial seawater, light intensity of 200 µE m^{-2} s^{-1}, photoperiod of 12 hours and temperature of 23 ± 2 ºC (figure 11, B). The entire macroalgae was also cultivated (figure 11, C), using natural seawater at 16 ± 2 ºC with the same light intensity and photoperiod. Furthermore, a different experiment, leaving the whole macroalgae for a period of 24 hours, in the dark, followed by its transference into light was performed to force the macroalgae to release the reproductive cells into the water. A total of 7 trials were performed and no release or germination of the reproductive structures was observed. This could be attributed to specific timings that this macroalgae has to produce spores or specific lunar cycles that enable its release, as Phillips and Clayton (1997) observed for this genus.

Figure 11 – *Zonaria tournefortii ex situ* cultivation. A - Cut and identification of the reproductive structures and spores from the apical part of the macroalgae blade; B – Small fragments of apical part of macroalgae in the growth medium for spore release; C – Cultivation of whole macroalgae for spore production and release.

The green macroalgae *U. lactuca* was cut into small pieces (figure 12, A) and these were introduced in natural seawater, enriched with F/2 medium and maintained at a temperature of 16 ± 2 ºC, 12 hour photoperiod. Cultivation, as a result of vegetative propagation or spores release, was succeeded. A total of 11 tests were performed on this species, with a time frame of 2 months each, with 10 subsequent subcultures. In the first case small cuts of *U. lactuca* grown until the macroalgae biomass filled the entire container. The second method of cultivation was attempted, through the production of spores, released from algae fragments. These spores, after some time, developed into new small propagules, which grew into algae juveniles (figure 12, B). The biomass production was assessed every 2 months during the trials. For a period of 6 months, initiating the cultivation with 5 grams (FW) of biomass, about 35 grams could be produced every month. However, this procedure requires further research to improve the conditions, which enable specimen's maturity and sporulation, being these processes highly species specific. In certain cases, new biotechnological approaches enable the production of viable protoplasts. It uses individualized cells, obtained from the macroalgae thallus, after its digestion by a mix of lytic enzymes, used to remove the polysaccharide cell wall (Reddy et al., 2006). All obtained protoplasts are identical cells, capable to develop into adult organisms, produced from a small part of biomass (Gupta et al., 2018). This approach applied to certain macroalgae species, allows to substitute the production of spores or zygotes as the main source of propagules (Reddy et al., 2006). This methodology was implemented to obtain protoplasts from *Ulva lactuca*, firstly cutting very small pieces of macroalgae, with less than 1 mm^2, to improve lysis efficiency (figure 12, C). After, the lysis the biomass was collected into test tubes (figure 12, D) to quantify the number of protoplasts, using a microscope and a hemocytometer. A total of 3 trials were performed and 1500 protoplasts were accounted in each µL. Our difficulties in this procedure were in the optimization of the lysis procedure, since our protoplasts number were very low and in the cultivation tests, propagation was not observed.

Figure 12 – Aspect of *Ulva lactuca* cultivation. A – Freshly cut pieces of macroalgae, during the cleaning procedure to remove epiphytes; B – Development of *Ulva lactuca* propagules; C – Preparation of macroalgae for protoplast production; D – Test tube with resulting protoplasts.

For the three macroalgae integrated in this work, it was possible to maintain, for some time (2 to 3 months) the whole seaweed as viable germplasm in the bank (figure 13). This result is particularly important, because it allows to perform germination testing for long time periods, as well as to maintain an *ex situ* collection of macroalgae for research and cultivation purposes. *A. taxiformis* and *Z. tournefortii*, even have been hold as viable accessions for longer periods if the natural substrate was collected at same time than the macroalgae samples, as demonstrated in figure 13 (E) for *Z. tournefortii*. *U. lactuca*, was maintained as viable accessions for at least for 1 year, providing macroalgae material for new cultures and

experiments, with parts of specimens cut and disposed in a new growth media, at least once each two months.

Figure 13 – Aspect of macroalgae germplasm collection maintained in the bank. A – General view of germplasm maintenance; B – Fresh *Ulva lactuca* cultures founded by macroalgae pieces cuts and transference for new containers; C – 50 L aquarium with entire specimens of *Ulva lactuca* maintained in the bank; D – *Asparagopsis taxiformis* entire specimens maintained in the bank; E – *Zonaria tournefortii* entire specimens maintained in the bank.

2.1.5 Conclusion

We can conclude that for *U. lactuca* and *A. taxiformis* the best periods for prospection and sampling of material is early spring. However, for *Z. tournefortti*, this period is probably not appropriate, since material collected, during this period shows some propagation difficulties, which allow us to hypothesize that late winter would be probably preferable for this macroalgae sampling. Microscopic observation enabled to visualize the cellular and reproductive structures, life phases and cycle of these macroalgae. For instance, it was possible to observe individual cells in *U. lactuca*, these cells participate in the blade formation, but lack of plasmodesmata. The *A. taxiformis* structure was observed, with identification of apical cells, cystocarps, with carpospores, spermatangia, with the male gametophyte and the cellular structure of the tetrasporophyte filaments. For *Z. tournefortii*, the microscopic details of the blade margin, where apical meristematic activity occurs was observed, as well as the paraphyses in the sporophyte, the antheridia in the male gametophyte and the identical rectangular cells within radial disposition in the macroalgae blade.

The experiments for macroalgae cultivation showed that the use of agar as solid substrate is not essential, since it was not observed any improvement using this compound as an artificial substrate. We have also determined that artificial seawater was not suitable for cultivation assays, since we could not discard the possibility that observed difficulties in macroalgae cultivation do not arise from its use. Furthermore, the F/2 medium was considered the most adequate for macroalgae cultivation since this could be kept workable for long time periods of time, refrigerated at 4 ºC. Moreover, *A. taxiformis* propagules were obtained in laboratorial conditions, although with major difficulties, when testing the gametophytes and using the tetrasporophyte "*Falkenbergia hillebrandii*" state, to produce viable spores. These, after some time, would become contaminated, from which axenic cultures were impossible to obtain. It was also determined that the "*Falkenbergia hillebrandii*" state is the most suitable biomass to produce spores in a controlled environment, but further optimization is required to obtain pure cultures that can be maintained for long time periods. Furthermore, whole macroalgae were successfully maintained in cultivation under controlled conditions, in the presence of its natural substrate. For *Z. tournefortii*, spore production was not achieved and this fact could result from the lack of information about its life cycle in the wild, requested to adequately program its sampling periods or to obtain the spore release. Moreover, it was possible to maintain accessions of this macroalgae in the germplasm bank for long time periods, again, when it was brought attached to the natural substrate and maintained in natural seawater, additivated with F/2 medium. For *U. lactuca* species, vegetative growth and propagules were successfully achieved after some optimization. Germplasm maintenance was also effectively maintained for long time periods, free floating in a 50 L aquarium using natural seawater, fortified with F/2 medium. Protoplast production was limited, and further optimization is required to obtain higher amount of protoplasts, after blade lysis procedure.

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2.1.5 Annexs

Annex 1

F/2 Medium (Guillard and Ryther 1962, Guillard 1975)

The following reagents were added to 950 mL of filtered natural seawater, and final volume performed to 1L with filtered natural seawater. Sterilization by autoclave.

Reagents	Stock Solution ($g.L^{-1}$ dH_2O)	Quantities, mL	Concentration in Final Medium (M)
$NaNO_3$	75	1	8.82×10^{-4}
NaH_2PO_4 H_2O	5	1	3.62×10^{-5}
Na_2SiO_3 $9H_2O$*	30	1	1.06×10^{-4}
Trace metals Solution	(see recipe below)	1	-
Vitamins Solution	(see recipe below)	0.5	-

* Added only in diatom cultivation.

F/2 Trace Metals Solution

The EDTA and other reagents were dissolved in 950 mL of dH2O, and the final volume adjusted to 1L with dH2O. Storage at 4 ºC.

Reagents	1º Stock Solution ($g.L^{-1}$ dH_2O)	Quantities, g or mL	Concentration in Final Medium (M)
$FeCl_3$ $6H_2O$	-	3.15	1.17×10^{-5}
Na_2EDTA $2H_2O$	-	4.36	1.17×10^{-5}
$MnCl_2$ $4H_2O$	180.0	1.0	9.10×10^{-7}
$ZnSO_4$ $7H_2O$	22.0	1.0	7.65×10^{-8}
$CoCl_2$ $6H_2O$	10.0	1.0	4.20×10^{-8}
$CuSO_4$ $5H_2O$	9.8	1.0	3.93×10^{-8}
Na_2MoO_4 $2H_2O$	6.3	1.0	2.60×10^{-8}

F/2 Vitamins Solution

The thiamine HCl, and 1mL of the stocks solutions were dissolved in 950 mL of dH2O, and the final volume adjusted to 1 L with dH2O. Filter-sterilization and frozen storage.

Reagents	1º Stock Solution ($g.L^{-1}$ dH_2O)	Quantities, mg or mL	Concentration in Final Medium (M)
Thiamine HCl (vitamina B_1)	-	200.0	2.96×10^{-7}
Biotin (vitamina H)	1.0	1.0	2.05×10^{-9}
Cyanocobalamin (vitamina B_{12})	1.0	1.0	3.69×10^{-10}

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Annex 2

MPI Medium (Suto. S. 1959)

Stock solution, volume 100 mL (store in amber bottles)

$NaNO_3$ - 10 g
Na_2HPO_4 $12H_2O$ - 2g

PI Metals stock solution, volume 1 L (Autoclave and store at cool condition in dark)

EDTA-Na_2 - 3 g
$FeCl_3$ $6H_2O$ - 387 mg
$MnCl_2$ $4H_2O$ - 432 mg
$ZnCl_2$ - 31mg
$CoCl_2$ $6H_2O$ - 12mg
$CuSO_4$ $5H_2O$ - 5mg
Na_2MoO_4 $2H_2O$ - 126mg
H_3BO_3 - 3.53g

Medium Preparation from stock solution

$NaNO_3$: 1ml
Na_2HPO_4 $12H_2O$: 1ml
PI Metals stock solution : 1ml
Seawater (filtered seawater) : 950ml
Distilled water : 50ml

Boiled at 80ºC for 5 minutes.

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Annex 3

PES Medium (Provasoli, L. 1968)

PES stock solution

$NaNO_3$	: 350 mg
Glycerine Phosphate.Na	: 50 mg
FeEDTA (2Na)	: 18.8 mg
*Vitamin mixture	: 1 mL
**PII metals	: 25 mL
Tris	: 500 mg
pH	: 7.8
Distilled water (Milli Q)	: 100 mL

*** Stock solutions of vitamins, volume 100 mL**

Biotin	: 50 mg
Cyanocobalamin	: 10 mg
Thiamin HCl	: 500 mg

***Preparation of vitamin mixture**

Add 1 mL of stock solution of Biotin, 10 mL of Cyanocobalamin and 10 mL of Thiamin HCl and adjust the mixture volume up to 100 mL.

****PII metal stock solution, volume 1 L**

EDTA-Na_2	: 1 g
$FeCl_3$ $6H_2O$	: 48.5 mg
$MnCl_2$ $4H_2O$	: 143 mg
$ZnCl_2$	: 10 mg
$CoCl_2$ $6H_2O$	: 4 mg
H_3BO_3	: 1.176 g
Distilled water	: 1000 mL

Medium Preparation: Add 20 mL of the above stock to 980 mL of filtered seawater or autoclaved seawater.

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Annex 4

A saturated stock solution can be prepared dissolving:
250 mg of GeO_2 in 1 L of deionized water (dH2O).

This stock solution is then added to culture media at a concentration of 2 mL/L of seawater.
Stock solution should be stored in a refrigerator and properly labeled.

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Annex 5

Protoplast Preparation Protocol (Reddy, CRK and Seth, A. 2018)

Chemicals including enzymes:

- Sodium chloride (NaCl)
- 2-[N-Morpholino] ethane sulfonic acid (MES)
- Dextran sulphate sodium salt
- Mannitol
- Cellulase Onozuka R-10
- Macerozyme R-10
- Deionised water (Millipore water)

Equipment's/other materials

- Weighing balance (analytical type)
- Weighing boats
- Magnetic stirrer with stirring rod and bar
- pH meter
- Refrigerator centrifuge
- Centrifuge tubes
- Surgical blades
- Orbital platform shaker
- Glass funnel
- Glass beakers
- Petri dish
- Glass pipettes
- Microscope
- Refrigerator

Preparation of enzyme mix:

- Cellulase Onozuka R-10 2%
- Dextran sulfate 0.5%
- NaCl 1%
- Mannitol 0.8 M
- MES 25 mM
- pH 6.0

Dissolved in deionized water.
Note: When the protoplast yield is unsatisfactory, the addition of 1% Macerozyme R-10 is recommended.

The enzyme mixture is prepared on the same day as the algae harvested:

- In Pre-cooled (4 ºC) deionized water;
- Dissolve 2% of Cellulase Onozuka R-10;
- 1% Macerozyme R-10;
- 1% of NaCl;
- 25 mM of MES;
- 0.5% of dextran sulfate;
- Adjust pH to 6.0
- Centrifuge at 10,000 x g for 20 min at 4 ºC
- Remove the clear supernatant
- Add 0.8 Mannitol at supernatant just before commencing the protoplast isolation.

Caution: Ensure that the enzyme mix is maintained at 4 ºC.

Isolation and Purification of Protoplasts:

1. In autoclaved seawater (ASW) clean the thallus with a brush under a microscope;
2. With a sterilized blade cut the clean thallus into small pieces ($1mm^2$);

3. Transferred the cut pieces to test tubes containing ASW;
4. Leave the material to stand at room temperature;
5. The tissues at the bottom of the tubes are again rinsed with ASW;
6. The tissues are transferred to petri dish (60 x 15 mm) containing 5mL of enzyme mixture;
7. Place the petri dish in rotary shaker (40 - 50 rpm) in the dark at 20 ± 1 ºC for 2h;
8. Softly passed the contents of petri dish through a nylon mesh (25 - 30µm pore size);
9. Centrifuged the suspension (120 x g) for 5 minutes;
10. Replaced half of the supernatant with the same volume of ASW;
11. Repeat the points 9 and 10 twice;
12. The pelleted protoplasts are resuspended in 1mL of ASW.

Protoplasts Culture:

1. Using a hemocytometer estimated the protoplast yield;
2. Prepare an aliquot of protoplast suspension (~ 1.7 x 10^3 protoplasts cm^{-2});
3. Dispense the aliquot in 10 ml sterilized PES medium;
4. Maintained at 21 ± 1 ºC at 15µmol photons $m^{-2}s^{-1}$ with a 12:12h light: dark photoperiod;
5. Replenished the culture medium weekly;
6. On day 5 count the dividing cells to calculate the protoplasts regeneration rates.

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2.2 Biochemical composition, nutritional value and antioxidant properties of seven seaweed species from Madeira Archipelago

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2.2.1 Abstract

Biochemical composition and antioxidant activity was assessed in seven seaweeds from Madeira Archipelago, namely Green (*Ulva lactuca*), red (*Asparagopsis taxiformis*, *Chondrus crispus*, *Galaxaura rugosa*, *Grateloupia lanceola* and *Nemalion elminthoides*) and brown seaweed (*Zonaria tournefortii*). Seaweeds mineral content varied from 16.60 g to 84.16 g/100g dw. Organic matter, composed by fiber and matricial polysaccharides (8.33 g to 54.04 g/100g dw), starch (1.95 g to 25.41 g/100g dw), protein (2.80 g to 17.55 g/100g dw) and fat (1.46 g to 12.04 g/100g dw) was also determined. *A. taxiformis* was found to have substantial quantities of protein, fat, fiber and matricial polysaccharides, compared to the analysed seaweeds. Analysis of antioxidant components included the measurement of chlorophyll a (28.81 mg to 244.3 mg/100g dw), total carotenoids (0 to 297.8 mg/100g dw), total phenolic compounds (0 to 2154 mg GAE/100g dw) and total flavonols (7.27 mg to 604.8 mg QE/100g dw). *Z. tournefortii*, was found to possess the highest values of chlorophyll a, total carotenoids, total phenolic content (TPC) and antioxidant activity, determined through ferric reduction antioxidant potential (FRAP), ferrous ion chelating (FIC), free radical scavenging assay (FRSA) and β-carotene bleaching (β-CB). Statistical analysis developed thirty-eight significant correlations between various biochemical and antioxidant parameters or activity and determined that fat content developed the highest quantity of correlations. Overall this study gave us better understanding of Madeira autochthonous seaweeds in their potential of being introduced as a raw material for nutrient supplementation in various food products or to produce functional foods using seaweeds natural properties.

Keywords: Total carotenoids. Antioxidant activity. Nutritional and biochemical evaluation. TPC. Flavonols. Chlorophyll *a*.

2.2.2 Introduction

Portugal has an exclusive economic zone of 1,720,560 km^2 of sea and only 91,763 km^2 of land area, representing an enormous potential for sea economy development. Madeira archipelago where this study was carried out, has 10,823 km^2 of sea and only 810 km^2 of land, showing a similar potential (Portuguese Navy 2015). The increase of world population and constrains determined by global climate change and limitation of terrestrial resources for food and energy supply, raises serious concerns about global food security (Rosegrant and Cline 2003) and lead us to explore new food sources. A worldwide quest to explore and utilize non-conventional food sources, of both terrestrial and marine origin, to improve the nutritional quality of food is underway (Kumar et al. 2011). To achieve this goal, a deeper understanding of seaweed resources, their biochemical and antioxidant composition is required.

Seaweed as an alternative food source depends of its biochemical composition, nutrients and calories supplied for man's diet. They represent an excellent raw material of nutrients and bioactive compounds such as minerals, dietary fiber, protein, essential fatty acids, vitamins and carotenoids (Kılınç et al. 2013). Seaweed protein content varies greatly depending on the species and factors such as season and environmental conditions (Dawczynski et al. 2007). Seaweed is a source of dietary fiber, largely soluble and known to prevent obstipation, colon cancer, cardiovascular disease and obesity (Dreher 1987). Protein from seaweed contains all essential amino acids, although some seasonal variations in their concentrations can be observed (Galland-Irmouli et al. 1999). Fatty acids of certain seaweeds are predominantly unsaturated and show antiviral activity (Kendel et al. 2015). Seaweeds are considered to be natural sources of hydrosoluble and liposoluble vitamins, such as thiamine and riboflavin, β-carotene and tocopherols, which may reduce the risk of heart disease, thrombosis and atherosclerosis (Mishra et al. 1993).

Carotenoids and phenolics are plants and seaweeds antioxidants that can rapidly neutralize the free radicals and retard or decrease the extent of oxidative deterioration (Miyashita 2014). The reactive oxygen species (ROS) formed in human tissues can promote an extensive oxidative damage that leads to age related degenerative processes, cancer and a wide range of other human diseases (Aruoma 1999). For this work, we have selected *Ulva lactuca*, *Asparagopsis taxiformis*, *Chondrus crispus*, *Galaxaura rugosa*, *Grateloupia lanceola*, *Nemalion elminthoides* and *Zonaria tournefortii*. Various bioactive compounds were determined in these seaweeds, showing antifouling, antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory properties, as well as antitumor and anticoagulant activity (Pereira 2015). Several of these seaweeds are known to be consumed by humans such as *A. taxiformis*, *N. elminthoides*, *C. crispus* and *Ulva* spp. were no special regulation exists (Mahadevan 2015), but only *C. crispus* and *Ulva* spp. are permitted by French legislation and recognized as food (Holdt and Kraan 2011). Due to their commercial value, *U. lactuca* and *C. crispus* have integrated in the seaweed cultivation efforts, implementing different strategies and technics for bioremediation or yield increase (Zertuche-González et al. 2001; Ale et al. 2011; Nielsen et al. 2012; Castelar et al. 2014).

In this work, seaweeds were subjected to several assays including the biochemical composition, to assess moisture content, total mineral, protein, starch, fat, fiber and matricial polysaccharides. Nutritional value was also determined in some seaweeds, with a history of human consumption or related to these, calculating the % of contribution to the recommended dose intake (RDI) regarding protein, carbohydrates and fat. For antioxidant compounds analysis, chlorophyll a, total carotenoids, total phenolic and flavonols were evaluated. To assess the antioxidant activity, two single electron transfer tests (FRAP

and FRSA), one hydrogen atom transfer (β-CB) and one chelating assay (FIC) were included. This is the first study carried out in Madeira to evaluate autochthonous seaweeds in their potential of being introduce as alternative or functional foods in the agri-food industry.

2.2.3 Materials and methods

Seaweeds used in this study were collected in Madeiran south coastline from intertidal zone to a maximum of a 10-meter depth dive. Seven of the most common species were included in this study, namely: *Ulva lactuca* Linnaeus 1753, *Asparagopsis taxiformis* (Delile) Trevisan de Saint-Léon 1845, *Chondrus crispus* Stackhouse 1797, *Galaxaura rugosa* (J.Ellis & Solander) J.V.Lamouroux 1816, *Grateloupia lanceola* (J.Agardh) J.Agardh 1851, *Nemalion elminthoides* (Velley) Batters 1902 and *Zonaria tournefortii* (J.V.Lamouroux) Montagne 1846. Samples were transported in seawater and gently rinsed with filtered fresh water. Afterwards, a primary drying was applied in which seaweeds were frozen at -35ºC and lyophilized, under reduced pressure (4 x 10^{-4} mbar) with a cooling trap set at -56ºC for 5 days. Samples were milled to 200 mesh particle size, vacuum packed and stored in the dark until use. All samples were analyzed in triplicates in all tests carried out.

2.2.3.1 Chemicals

Methanol, trichloroacetic acid, chloroform, boric acid, potassium sulphate and sulfuric acid (95-97%) were purchased from Sigma-Aldrich. Folin Ciocalteu reagent, aluminium chloride, sodium acetate, monopotassium phosphate, dipotassium phosphate, ethylenediamine tetraacetic acid (EDTA), anthrone and sodium carbonate were purchased from Fluka. Gallic acid, quercetin, ferric chloride hexahydrate, butylated hydroxytoluene (BHT), linoleic acid and β-carotene were purchased from Sigma. 2,2-diphenyl-1-picrylhydrazyl (DPPH), hexacyanoferrate (III) potassium, ferroin and selenium were purchased from Aldrich. Ascorbic acid, soluble starch and ferric sulphate were purchased from Merck. Tween 20 was purchased from Pharmacia Biotech and hydrochloric acid (37%) from Riedel de Haen. All chemicals were of analytical grade.

2.2.3.2 Apparattus

Velp Scientifica DK 8S Heating Digester, Kjeldahl (Distillation and Titration Unit Velp Scientifica UDK 152), analytical humidity scale (Kern MRS 120-3), analytical scale (Sartorius MC1, Research RC 210P), Vulcan 3-550 furnace, Heratherm OMS 180 oven (Thermo Scientific), Ultrasonic-h bath (P Selecta), 5430R Eppendorf centrifuge and UV/Vis spectrophotometer (2401 PC, Shimadzu) were used.

2.2.3.3 Samples chemical analysis

Moisture content was determined according with AOAC 925.10 (2000). Ashes were determined through AOAC 930.22 (2005). Lipids were quantified as described by Folch et al. (1957), with the modifications suggested by Bligh and Dyer (1959). Protein content was determined as described by AOAC 978.04 (2005), adjusting the conversion factor of nitrogen into protein to 5.58 for *U. lactuca* (Shuuluka et al. 2013), 5.38 for brown seaweed and 4.59 for red seaweed (Lourenço et al. 2002) except for *A. taxiformis*

that was used 4.51 (Diniz et al. 2011). Starch analysis was conducted according to the Hodge and Hofreiter method (1962). Fiber and matricial polysaccharides were calculated as the difference between 100% of dry matter and the sum of other parameters values.

For the antioxidant quantification and activity experiments, 1 g (gram) of dry seaweed was weighed and mixed with 25 mL of 50% methanol (v/v), under continuously stirring for 1 hour. Extracts were sonicated and centrifuged, 10 min, at 2,935 g. The supernatant was stored and pellet used in a second extraction. The supernatants were combined, brought up to 50 mL with 50% methanol (v/v) and stored at -20° C. Total phenolic content (TPC) using Folin Ciocalteu method was conducted as described by Chew et al. (2008), chlorophyll a and total carotenoids determined according to Wellburn (1994) and Kumar et al. (2010). Flavonol quantification was conducted according with the method developed by Kumaran and Karunakaran (2006). The free radical-scavenging assay was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) described by Yen and Chen (1995) and Duan et al. (2006), ferric reducing antioxidant power (FRAP) assay performed following Oyaizu (1986) and Yuan et al. (2005) publications. Ferrous ion chelating (FIC) assay was based on Decker and Welch (1990) and Chew et al. (2008), β-carotene bleaching (β-CB) assay conducted as described by Velioglu et al. (1998) and Ismail and Tan (2002).

2.2.3.4 Statistical analysis

To analyse data, the statistical program SPSS 23.0 for Windows was used. All values are expressed as mean of three replicate determinations ± standard deviation. Data was analysed using one-way analysis of variance (ANOVA), were it was determined its homoscedasticity followed by Pearson's test ($p \leq 0.01$) to assess correlations between means and a Tukey's test ($p \leq 0.01$) to determine statistical variance between seaweeds in each parameter.

2.2.4 Results

The biochemical evaluation of the seven seaweeds analysed are presented in table 1. Values for all biochemical parameters analysed were expressed on a dry weight basis. Moisture content varied between 1.47 g in *G. rugosa* and 4.50 g/100g in *A. taxiformis* in red seaweeds, *U. lactuca* had a moisture content of 3.41 g/100g and *Z. tournefortii* 4.13 g/100g. Total mineral (ashes) content showed great variation depending on the seaweed. Red seaweeds varied their content between 16.60 g in *G. lanceola* and 84.16 g/100g dw for *G. rugosa*. The protein content in red seaweed ranged from 2.80 g in *G. rugosa* and 17.55 g/100g dw in *A. taxiformis*, *U. lactuca* had 7.16 g and *Z. tournefortii* 9.44 g/100g dw. Starch content alternated in red seaweeds from a minimum of 1.95 g in *G. rugosa* to 25.41 g/100g dw in *G. lanceola*, *U. lactuca* had 9.34 g and *Z. tournefortii* 4.07 g/100g dw. Fiber and matricial polysaccharides content alternated in red seaweed from 8.33 g in *G. rugosa* to 47.96 g/100g dw in *G. lanceola*, *U. lactuca* contained 54.04 g and *Z. tournefortii* 44.50 g/100g dw. Lipid content showed a variation between 1.46 g dw in *G. rugosa* and 6.62 g/100g dw in *A. taxiformis* in red seaweed, *U. lactuca* had 2.36 g and *Z. tournefortii* contained 12.04 g/100g dw.

Table 1 – Biochemical content, color and uses

Seaweeds	**Color**	**Uses** (Pereira, 2015)	**Moisture** g (100 g)$^{-1}$ in dw	**Total minerals** g (100 g)$^{-1}$ in dw	**Protein** g (100 g)$^{-1}$ in dw	**Starch** g (100 g)$^{-1}$ in dw	**Fiber and matricial polysaccharides** g (100 g)$^{-1}$ in dw	**Fat** g (100 g)$^{-1}$ in dw
Asparagopsis taxiformis	Red	Seasoning, source of pharmaceutical and bioactive compounds	4.50 ± 0.20a	23.76 ± 0.48a,e	17.55 ± 0.11a	8.03 ± 0.38a	32.47 ± 1.04a	6.62 ± 0.54a
Chondrus crispus	Red	Extraction of polysaccharide carrageenan	3.26 ± 0.11a,b	23.11 ± 0.13a	6.71 ± 0.07b	18.23 ± 0.46b	46.39 ± 0.79b,d	2.46 ± 0.14b,c
Galaxaura rugosa	Red	Extracts with antiviral, antifungal, anti-inflammatory and antimicrobial activity.	1.47 ± 0.30b	84.16 ± 0.08b	2.80 ± 0.06c	1.95 ± 0.11c	8.33 ± 0.08c	1.46 ± 0.06b
Grateloupia lanceola	Red	---------	3.00 ± 0.91a,b	16.60 ± 0.03c	4.97 ± 0.02d	25.41 ± 0.91d	47.96 ± 1.00d	2.97 ± 0.12c
Nemalion elminthoides	Red	Utilized as food and extracts with antiherpetic activity	4.10 ± 0.50a	60.64 ± 0.02d	3.80 ± 0.05e	5.36 ± 0.21e	26.07 ± 0.12e	2.17 ± 0.13b,c
Ulva lactuca	Green	Used as food worldwide, in cosmetics and extratcs have biological activity	3.41 ± 1.05a	25.18 ± 0.48e	7.16 ± 0.03f	9.34 ± 0.87a	54.04 ± 0.53f	2.36 ± 0.12b,c
Zonaria tournefortii	Brown	Antioxidant and antitumor activity in extracts	4.13 ± 0.05a	25.18 ± 1.10e	9.44 ± 0.06g	4.07 ± 0.41e	44.50 ± 1.77b	12.04 ± 0.81d

Data are mean ± standard deviation in grams per 100 grams of seaweed on a dry weight basis. All determinations were carried out in triplicate. Different letters within the same column indicate significant differences ($p \leq 0.01$).

The antioxidant quantification and potential of the seven seaweeds are given in the table 2. Chlorophyll a content varied between 28.81 mg in *A. taxiformis* and 184.5 mg/100g dw in *G. lanceola* in red seaweed, *U. lactuca* presented a value of 92.72 mg and *Z. tournefortii* 244.3 mg/100g dw. Also, a wide variation in carotenoid content was observed, in red seaweed these values alternated between 8.99 mg in *N. elminthoides* and 131.1 mg/100g dw in *G. lanceola* with no significant amounts detected in *G. rugosa*, *U. lactuca* had 20.41 mg and *Z. tournefortii* had the highest value, 297.8 mg/100g dw. Total phenolic compounds (TPC) showed as well high degree of variability. Red seaweeds diverged from 25.79 mg in *G. lanceola* and 65.52 mg GAE (gallic acid equivalents)/100g dw in *N. elminthoides* with again no significant amounts detected in *G. rugosa*, *U. lactuca* presented 55.61 mg and *Z. tournefortii* showed the highest TPC content of 2,155 mg GAE/100g dw. Flavonol content varied from 7.27 mg in *G. rugosa* to 206.1 mg QE (quercetin equivalents)/100g dw in *G. lanceola* for red seaweeds, the highest result was determined in *U. lactuca* 604.8 mg and *Z. tournefortii* presented a value of 157 mg QE/100g dw.

Table 2 – Antioxidant compounds

Seaweeds	**Chlorophyll a** mg (100 g)$^{-1}$ in dw	**Carotenoids** mg (100 g)$^{-1}$ in dw	**TPC** mg GAE (100 g)$^{-1}$ in dw	**Flavonols** mg QE (100 g)$^{-1}$ in dw
Asparagopsis taxiformis	28.81 ± 3.25a	13.14 ± 2.63a,b	57.63 ± 3.92a	19.26 ± 0.95a
Chondrus crispus	62.51 ± 4.09b	21.25 ± 1.56b	36.28 ± 4.64a	79.17 ± 1.44b
Galaxaura rugosa	32.30 ± 0.52a	ND	ND	7.27 ± 1.30a
Grateloupia lanceola	184.55 ± 5.90c	131.13 ± 5.23c	25.79 ± 2.13a	206.06 ± 9.04c
Nemalion elminthoides	89.09 ± 6.27d	8.99 ± 1.15a,b	65.52 ± 8.20a	85.76 ± 9.63b
Ulva lactuca	92.72 ± 2.88d	20.41 ± 1.68b	55.61 ± 4.13a	604.77 ± 15.73d
Zonaria tournefortii	244.25 ± 8.48e	297.77 ± 10.14d	2154.57 ± 119.27b	156.99 ± 11.37e

Data are mean ± standard deviation in milligrams per 100 grams of seaweed on a dry weight basis. All determinations were carried out in triplicate. Different letters within the same column indicate significant differences ($p \leq 0.01$). Not detected (ND).

Antioxidant activities are given in the table 3. Ferric reduction antioxidant potential (FRAP) assay showed dispersed results that ranged from 71.69 mg in *A. taxiformis* to 1,896 mg AAE (ascorbic acid equivalents)/100g dw in *G. lanceola* for red seaweeds, *U. lactuca* had 238.8 mg and *Z. tournefortii* developed the highest value 6,078 mg AAE/100g dw. In ferrous ion chelating (FIC) assay, red seaweed varied their chelating potential from 4.00% in *G. lanceola* to 45.64% in *G. rugosa*, *U. lactuca* had 4.28% and *Z. tournefortii*, a brown seaweed had the highest chelating activity, 77.93%. Free radical scavenging assay (FRSA) in red seaweeds showed values alternating from 18.69 mg in *G. rugosa* to 63.97 mg AAE/100g dw in *C. crispus*, it was determined in *U. lactuca* 59.97 mg and *Z. tournefortii* presented the highest value of 3,928 mg AAE/100g dw. β-CB assay was also employed and in red seaweeds, values ranged from 3.01% in *A. taxiformis* to 12.12% in *C. crispus*, *U. lactuca* showed 2.88% and *Z. tournefortii* presented again the highest value, 95.03%. β-CB assay was followed spectrophotometrically during 120 minutes and compared with BHT standard at 0.010 mg mL^{-1}.

Table 3 – Antioxidant activity

Seaweeds	FRAP mg AAE (100 g)$^{-1}$ in dw	FIC (% Chelating activity)	FRSA (AEAC) mg AAE (100 g)$^{-1}$ in dw	β-CB (% Antioxidant activity in dw)
Asparagopsis taxiformis	71.69 ± 2.11a	16.95 ± 1.18a	21.29 ± 1.21a	3.01 ± 0.14a
Chondrus crispus	100.96 ± 9.16a	40.38 ± 1.39b	63.97 ± 1.32a	12.12 ± 1.15b
Galaxaura rugosa	149.15 ± 4.99a,b	45.64 ± 0.81b	18.69 ± 1.65a	9.04 ± 0.65b,c
Grateloupia lanceola	1895.51 ± 29.51c	4.00 ± 0.28c	31.64 ± 0.99a	6.24 ± 0.20a,c
Nemalion elminthoides	107.26 ± 3.95a	12.57 ± 1.10a	43.46 ± 1.27a	4.17 ± 0.34a
Ulva lactuca	238.79 ± 9.86b	4.28 ± 0.86c	59.97 ± 2.97a	2.88 ± 0.04a
Zonaria tournefortii	6078.29 ± 89.63d	77.93 ± 4.62d	3927.83 ± 47.59b	95.03 ± 3.50d

Data are mean ± standard deviation in milligrams per 100 grams of seaweed on a dry weight basis or percentage. All determinations were carried out in triplicate. Different letters within the same column indicate significant differences ($p \leq 0.01$).

The results presented in figure 1, show that *G. rugosa*, *A. taxiformis*, *N. elminthoides* and *U. lactuca*, in decreasing order of antioxidant activity and the last seaweed comparable with control, with no antioxidant mechanism to protect linoleic acid. *Z. tournefortii*, *C. crispus* and *G. lanceola* showed the highest antioxidant potential, presenting *Z. tournefortii* higher antioxidant activity than BHT (0.010 mg mL^{-1}) at the end of the 120 minutes of the assay.

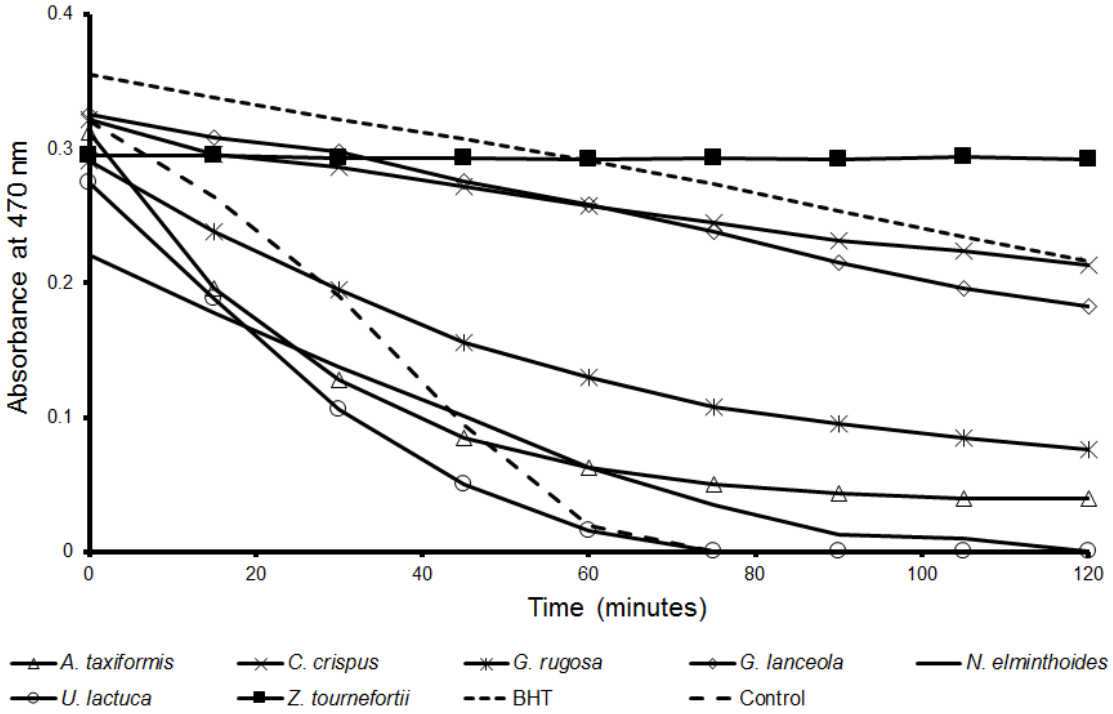

Figure 1 – β-carotene bleaching assay plot of seaweeds methanol extracts, control and BHT (0.010 mg mL^{-1}) to determine the antioxidant activity during 120 minutes.

For statistical analysis, a Tukey's test ($p \leq 0.01$) was applied to determine statistical variance between seaweeds in each given parameter. All the seaweeds expressed below were statistically different from each other in each parameter, also the names of the seaweeds appear from highest to the lowest content. Biochemical composition and nutritional values determined here, developed some interesting statistical results. Three red seaweeds namely *G. rugosa*, *N. elminthoides* and *G. lanceola*, were identified to be statistically different when comparing their total mineral content. Protein quantity in all seaweeds were statistically different, presenting *A. taxiformis* the highest content and *G. rugosa* the lowest. Starch content showed that *G. lanceola*, *C. crispus* and *G. rugosa* are statistically different. *U. lactuca*, *A. taxiformis*, *N. elminthoides* and *G. rugosa* have statistically different fiber content. Also, the higher fat content in *Z. tournefortii* and *A. taxiformis* revealed to be statistically different. When applying this statistical analysis to the antioxidant compounds, *Z. tournefortii*, *G. lanceola* and *C. crispus* displayed statistical differences in their chlorophyll a content. *G. lanceola* and *Z. tournefortii* exhibited a carotenoid content statistically distinct from all the other seaweeds and only *Z. tournefortii* continue to demonstrate this distinction for TPC content. For flavonols, *U. lactuca*, *G. lanceola* and *Z. tournefortii* were statistically different between each other and all the other seaweeds used in this work. For the antioxidant activity, *Z. tournefortii* and *G. lanceola* were found to have statistical different values for FRAP assay and only *Z. tournefortii* continued to demonstrate its dissimilarity for the other parameters, such as FIC, FRSA and β-CB assays. Also, it was performed a Pearson coefficient test ($p \leq 0.01$) to determine the existence of correlations between composition and antioxidant parameters, demonstrated in table 4.

Table 4 – Statistical analysis using Pearson correlation to determine relationships between different parameters

	Moisture	Starch	Minerals	Fiber	Fat	Protein	Chlorophyll *a*	Carotenoids	Flavonols	TPC	FRSA	FRAP	FIC	β-CB
Moisture			**- e**		**+ d**	**+ e**				**+ e**	**+ e**			**+ e**
Starch			**- d**	**+ e**										
Minerals	**- e**	**- d**		**- a**										
Fiber		**+ e**	**- a**						**+ d**					
Fat	**+ d**					**+ e**	**+ d**	**+ b**		**+ b**	**+ b**	**+ b**	**+ d**	**+ b**
Protein	**+ e**				**+ e**									
Chlorophyll a					**+ d**			**+ a**		**+ c**	**+ c**	**+ b**		**+ c**
Carotenoids.					**+ b**		**+ a**			**+ a**	**+ a**	**+ a**	**+ d**	**+ a**
Flavonols				**+ d**										
TPC	**+ e**				**+ b**		**+ c**	**+ a**			**+ a**	**+ a**	**+ c**	**+ a**
FRSA	**+ e**				**+ b**		**+ c**	**+ a**		**+ a**		**+ a**	**+ c**	**+ a**
FRAP					**+ b**		**+ b**	**+ a**		**+ a**	**+ a**		**+ d**	**+ a**
FIC					**+ d**			**+ d**		**+ c**	**+ c**	**+ d**		**+ b**
β-CB	**+ e**				**+ b**		**+ c**	**+ a**		**+ a**	**+ a**	**+ a**	**+ b**	

Statistical significance at 0.01 level; Signalling, + or – reveal the type of relation between parameters in their positive or negative correlation, respectively; Letters indicate the interval of the linearity correlation (R^2) – a (1.000 – 0.900); b (0.899 – 0.800); c (0.799 – 0.700); d (0.699 – 0.600); e (0.599 – 0.500).

Thirty-eight significant correlations between different biochemical and antioxidant parameters or activity were identified. Seven correlations occur between biochemical compositional parameters and three of these correlations were negative, involving total minerals with starch, moisture content and fibers. The correlation between minerals and fiber content presented a coefficient of R^2 = -0.905. The total content of lipids presents itself as the biochemical parameter that shows the highest number of correlations with antioxidant compounds or activity, with nine significant positive correlations which five are R^2 from 0.800 to 0.899 with carotenoids and antioxidant activity assays (TPC, FRSA, FRAP and β-CB). Twenty-four correlations showed values of linearity higher than R^2 = 0.700 and twenty-three of them are between antioxidant compounds and activity parameters. The antioxidant compounds analyzed, TPC, carotenoids and chlorophyll *a*, showed eleven positive correlations with antioxidant capacity assays performed in this work, of which ten of these correlations are higher than R^2 = 0.700. Carotenoids correlates with FRSA, measuring scavenge of free radicals (R^2 = 0.912). Total phenolic compounds appear correlated with total carotenoids (R^2 = 0.908) and with less extend to chlorophyll a (R^2 = 0.759). TPC also is highly correlated with antioxidant activities such as FRSA (R^2 = 0.998), β-CB (R^2 = 0.990) and FRAP (R^2 = 0.951). FRAP assay developed a positive correlation between total carotenoids (R^2 = 0.992) and chlorophyll a (R^2 = 0.934).

2.2.5 Discussion

The biochemical composition results, presented in table 1, demonstrates the variability between seaweeds in each given parameter. Seaweed dehydration and processing into flour have successfully developed moisture content less than 13 g/100g in all seaweeds processed and according to Van Hal (2000), the lower limit for development of microorganisms. Total mineral (ashes) content shows great variability and *G. rugosa* presented the highest value, 84.16 g/100g dw, due to its coralligenous composition. *A. taxiformis* showed almost twice the value demonstrated by El-Baroty et al. (2007), 23.76 g/100g dw. *G. lanceola* developed similar results (16.60 g/100g dw) with the same genera, *Grateloupia turuturu*, used in the work of Denis et al. (2010) and Munier et al. (2013). Green seaweed, *U. lactuca*, developed equivalent results with Mageswaran and Sivasubramaniam (1984) work, 25.18 g/100g dw, but more than twice the work of Ortiz et al. (2006), probably due to different sea conditions or seasonality. Protein quantification showed that *A. taxiformis*, a red seaweed, presented the highest content between all the seaweed used in this study, 17.55 g/100g dw, potentially providing 31.34% of the recommended dose intake (RDI) (table 5). Seaweeds are only considered staple food in few countries and regions, but this raw material has the potential of being introduced as an ingredient in various food related items, increasing its nutritional quality and helping some food products to meet the recommended dose intake (RDI). Also Dawczynski et al. (2007) and Diniz et al. (2011) showed that protein of red seaweed has in its composition all essential aminoacids and high essential amino acid index (EAAI), rich in lysine, but can be poor in some aminoacids such as methionine or tryptophan. For *G. lanceola* used in this work, it did not develop any resemblance in protein content (4.97 g/100g dw) with other studies using the same genera (*G. turuturu*), presenting in some cases more than 4 times less than other reported studies (Denis et al. 2010; Chandraprabha et al. 2012; Munier et al. 2013). Using same genera but different species for these comparisons may probably be the cause for these high degrees of discrepancy, but few published works exist using the same species and

analysis, forcing a genera comparison in most cases. Interestingly, using same species also develops protein content fluctuations between works (Hardouin et al. 2013). *U. lactuca* presented in this work 7.16 g/100g dw and in the works of Mageswaran and Sivasubramaniam (1984), Ortiz et al. (2006), Manivannan et al. (2008) and Yaich et al. (2011), it varies from 3.30 g to 27.20 g/100g dw. *G. lanceola* and *C. crispus* showed the highest starch content, 25.45 g and 18.23 g/100g dw, respectively. Although starch content is lower than some terrestrial crops, they can be used to fortify foodstuff as a source of energy were other food sources are not available all year around. *G. turuturu*, same genera as *G. lanceola*, known to be consumed by humans (Denis et al. 2010), presents in Chandraprabha et al. (2012) report, 5.52 g/100g dw in starch content, differing greatly from the analysed *G. lanceola*, 25.41 g/100g dw. These differences can be attributed to starch fluctuations throughout the season, quality of a reserve substance. Fiber content showed great variability between seaweeds used in this work, ranging from 8.33 g to 54.04 g/100g dw, in *G. rugosa* and *U. lactuca*, respectively. Yaich et al. (2011) and Ortiz et al. (2006) reported similar values for *U. lactuca*. Fiber consists of different polysaccharides fractions, such as hemicelluloses, celluloses, lignins, oligosaccharides or pectins. Some of which are used in the food industry as texture modifiers and known to decrease the risks of some human diseases, such as coronary. Considering human or animal consumption, fiber and matricial polysaccharides are a positive feature in seaweeds. High content in consumed seaweeds can be positively correlated with low incidence of colorectal cancer (Hoshiyama et al. 1993). Indigestible viscous seaweed polysaccharides such as alginates, carrageenans and funorans, have shown positive effects on serum lipid levels in rats (Jiménez-Escrig and Sánchez-Muniz 2000). Fat content showed some variability, ranging from 1.46 g and 12.04 g/100g dw, between *G. rugosa* and *Z. tournefortii*. *A. taxiformis* demonstrated to have the second higher fat content among the seaweed used (6.62 g/100g dw), contributing with 9.46% RDI (table 5) when consumed by humans.

Table 5 – Mean values for some nutritional parameters to seaweeds known to be edible and percentage of Recommended Dose Intake (RDI) that each seaweed can deliver when consumed.

	Protein (g)		**Carbohydrate** (g)		**Fat** (g)		
Recommended Dose Intake (RDI)[1]	56		130		70		
Species	**Protein** (g/100 dw)	**%RDI**	**Carbohydrate** (g/100g dw)	**%RDI**	**Fat** (g/100g dw)	**%RDI**	**Energy** (Kcal)
Asparagopsis taxiformis[2]	17.55	31.34	40.50	31.15	6.62	9.46	181.84
Chondrus crispus[2,3]	6.71	11.98	64.61	49.70	2.46	3.51	116.73
Grateloupia lanceola [4]	4.97	8.88	73.37	56.44	2.97	4.24	122.07
Nemalion elminthoides[2]	3.80	6.79	31.43	24.18	2.17	3.10	68.15
Ulva lactuca[2,3]	7.16	12.79	63.38	48.75	2.36	3.37	115.21

[1] Data from Trumbo et al. (2002). [2] Seaweed knowned to be consumed by humans (Pereira, 2015). [3] Permitted seaweed for human consumption in Europe (Meland and Rebours 2012). [4] Seaweed from the same genus as seaweeds knowned to be consumed by humans (Athukorala et al. 2003; Munier et al. 2013; Seo et al. 2013).

This seaweed is known as "limu kohu" in Hawaii, consumed as a staple food and part of their tradition (Burreson et al. 1976). El-Baroty et al. (2007) analysed the lipid composition concluding that ω3 linolenic acid is the major fatty acid of the lipid fraction in *A. taxiformis*. This fatty acid has the ability to prevent cardiovascular diseases (Mozaffarian 2005). *G. lanceola* fat content (2.97 g/100g dw) has some resemblance with the same genera, *G. turuturu* (2.81 g and 5.44 g/100 dw) published in the work of Munier et al. (2013) in two separated locations. *U. lactuca* showed in this work lower value of fat content (2.36 g/100g dw) than Yaich et al. (2011) work (7.87 g/100g dw), higher than Ortiz et al. (2006) (0.30 g/100g dw) but similar with Kendel et al. (2015) using *U. armoricana* (2.62 g/100g dw). According to Manivannan et al. (2008), seaweeds are known for their low fat content which varies significantly throughout the year. This biochemical parameter, developed the highest number of correlations with antioxidant compounds (TPC, carotenoids, chlorophyll a) and activity (FRSA, β-CB, FRAP), suggesting that the chemical component that provide part of the antioxidant capabilities are of lipophilic nature.

Antioxidant quantification of seven seaweeds is given in the table 2 and includes the determination of chlorophyll a, total carotenoids, total phenolic compounds (TPC) and flavonols. Brown seaweeds are known to produce chlorophyll *a* and *c*, red seaweed only chlorophyll *a* and green seaweed produces chlorophyll *a* and *b* (Takaichi 2013). Chlorophyll a content ranged from 28.81 mg in *A. taxiformis* to 244.3 mg/100g dw in *Z. tournefortii*. *U. lactuca* presented a value of 92.72 mg/100g dw of chlorophyll a, higher than the work of Abd El-Baky et al. (2008) (18.00 and 28.00 mg/100g dw), cultivated in natural or artificial seawater, respectively**.** For the remaining seaweeds, our measurements represent the first results for chlorophyll a parameter. Chlorophylls are greenish pigments, synthesized by phototrophic organisms to promote photosynthesis, that in consumed seaweed have bioactivity as an antioxidant (Lanfer-Marquez et al. 2005) and in processed food can be converted in pigments such as pheophytin, pyropheophytin and pheophorbide, known to prevent cancer (Holdt and Kraan 2011). Carotenoids, terpenoid pigments and their oxigenated derivatives (xanthophylls) act as antioxidants, neutralizing reactive oxygen species (ROS) during metabolic processes (von Elbe and Schwartz 1996). Although seaweeds synthesize β-carotene, brown seaweeds synthetize mainly fucoxanthin and violaxanthin. Red seaweeds produce essentially lutein, α-carotene and zeaxanthin. Green seaweed produce mostly lutein, violaxanthin, neoxanthin and zeaxanthin (Holdt and Kraan 2011; Pereira 2015). In this work seaweeds demonstrated a wide range in carotenoid content, bringing the highest value to 297.8 mg/100g dw (*Z. tournefortii*) and the lowest to below the detection capability of the equipment with *G. rugosa*. A. *taxiformis* had a carotenoid content (13.14 mg/100g dw) more than ten times lower than the work of Ragonese et al. (2014), 137.2 mg/100g dw, showing enormous variability between seaweed of the same species and suggesting that carotenoids content is more likely induced by external factors than from species or genera variability. For *G. lanceola*, carotenoid content reached the value of 131.1 mg/100g dw, that is more than twice the published content of Chandraprabha et al. (2012) in *G. turuturu*, 60.00 mg/100g dw. *U. lactuca* carotenoid content (20.41 mg/100g dw) demonstrated to be similar from the values determined by Abd El-Baky et al. (2008), that ranged between 13.00 mg to 24.00 mg/100g dw. Carotenoid content is very important due to their nutraceutical and antioxidative properties, showing bioactivity in the prevention of pathologies caused by oxidative stress (Okuzumi et al. 1993). In this work, total carotenoids were statistically highly correlated with FRAP assay ($R^2 = 0.992$), possibly due to higher contents of astaxanthin, fucoxanthin, lutein and zeaxanthin (Rodrigues et al. 2012), acting as iron reducing antioxidants. Carotenoids also correlates with

FRSA ($R^2 = 0.912$), a mechanism that applies electron transfer to convert free radicals into more stable compounds (Matanjun et al. 2008). Phenolic compounds are a major molecular group, contributing to the tissue antioxidant activity (Chan et al. 2007). They are important in defending against invading bacteria, wounding or excessive radiation (Cox et al. 2010). *Z. tournefortii* showed the highest TPC content between the seaweeds used in this work, 2,155 mg GAE/100g dw, approximately twice the amount reported by Chkhikvishvili and Ramazanov (2000) (1,060 mg GAE/100g dw). Brown seaweeds TPC content are mainly constituted by polyphenols such as fucol, fucophlorethol, fucodiphloroethol G, ergosterol and phenol compounds namely phlorotannins (Holdt and Kraan 2011). Protocatechnic, gentisic and hydroxybenzoic phenolic acids were determined at higher quantities in red and green seaweeds, in the work of Farvin and Jacobsen (2013), when evaluating 11 different phenolic acids in 16 seaweeds from the Danish coast. In their work, using ethanol for extraction, they have obtained 236.5 mg GAE/100g dw in *U. lactuca*, that represents 4.3 folds more than the TPC exibihted in this work (55.61 mg GAE/100g dw), using 50% methanol. *Grateloupia filicina*, a source of food in Korea and Japan, exhibited in the work of Athukorala et al. (2003) 198.0 mg GAE/100g dw, seven times greater than *G. lanceola* used in this work, 25.79 mg GAE/100g dw, demonstranting high variability within same genera. *N. elminthoides*, colected at Yucatan Peninsula (Mexico), showed a TPC content of 1,870 mg GAE/100g dw in Zubia et al. (2007) work, presenting increadibly higher results than the same seaweed analysed in this work, 65.52 mg GAE/100g dw. This discreapance can be attributed to extrinsic factors that influence greatly in TPC content, namely UV radiation, grazing, bacterial infection and epiphytism (Tanniou et al. 2013). According to Pereira (2005) report, Madeira Archipelago have lower limits of solar irradiance (5 MJ/m^2) than Yucatan Peninsula (10 MJ/m^2) described by Quej et al. (2017), attributing to this abiotic factor the possibility of being responsible for TPC variation within the same species of seaweed. The statistical analysis revealed a high correlation between TPC with total carotenoids ($R^2 = 0.908$), chlorophyll a ($R^2 = 0.759$) and with antioxidant activities such as FRSA ($R^2 = 0.998$), β-CB ($R^2 = 0.990$) and FRAP ($R^2 = 0.951$). The high correlation between TPC and FRSA in seaweeds was also demonstrated in the work of Ragan and Glombitza (1986), evidencing the relationship of the polyphenolic content in seaweed and the activity of free radical scavengers. The positive correlation between TPC and β-CB evaluates the contribution of lipophilic compounds for antioxidant activity, also demonstrated by Chew et al. (2008). These findings are in agreement with the work of Matanjun et al. (2008), that determined the phenolic content and lipophilic antioxidant activity in 8 species of seaweed. Flavonols and flavonol glycosides, a class of compounds of phenolic origin, with chemical and biological activities are known to be scavengers of ROS and inhibitors of lipid peroxidation (Cox et al. 2010). According to the work of Yoshie-Stark et al. (2003), when analysing 27 seaweeds from the sea of Japan, flavonols such as morin were identified in all seaweeds and myricetin mainly present in brown and red seaweeds. Flavonol glycosides such as rutin were in higher content in red seaweeds and quercitrin only present in brown seaweeds. Flavonol content in this work varied from 7.27 mg QE in *G. rugosa* to 604.8 mg QE/100g dw in *U. lactuca*. For this last seaweed contrasting values of flavonol content have been published, 800.0 mg (Sarojini et al. 2012), 135.0 mg (Meenakshi et al. 2009) and 2.36 mg QE/100g dw (Elmegeed et al. 2014). Although flavonol content determined in this work were similar to Sarojini et al. (2012) reported values, they greatly differ from other data published, highlighting the variability between distinct areas, climate conditions and seasonality. Statistical evaluation showed that flavonols did not correlate with TPC or with β-CB, suggesting that flavonol is a minor constituent in

phenolic compounds and does not play a significant role in lipid antioxidant protection in seaweeds used in this work.

Antioxidant activity was also measured and results are given in table 3, which include the determination of primary antioxidants capacity with ferric reduction antioxidant potential (FRAP) assay and free radical scavenging assay (FRSA) using mechanisms based on the single electron transfer (SET) (Prior et al. 2005). β-CB assay, a scavenge peroxyl radical test that uses mechanisms of hydrogen atom transfer (HAT) for proton relocation (Dawidowicz and Olszowy 2010). And ferrous ion chelating (FIC) assay, that measures the ability of secondary antioxidants to inhibit oxidation through an indirect approach, in this case chelating metal (Kristinsson 2014). FRAP activity measures the ability of seaweeds antioxidant compounds to reduce iron ions involved in Fenton and Haber-Weiss reaction (Li et al. 2006). Results obtained for this assay range from 71.69 mg in *A. taxiformis* to 6,078 mg AAE/100g dw in *Z. tournefortii*. For *U. lactuca*, the value obtained was 238.8 mg AAE/100g dw, being relatively similar to the work of Stern et al. (1996), 310.0 mg AAE/100g dw. FIC assay developed also a high variability in the results, ranging from 4.28% in *U. lactuca* to 77.93% in *Z. tournefortii*. Phlorotanins usually present in brown seaweeds, are responsible for the strong chelation of heavy metals, developing strong FIC activity demonstrated by *Z. tournefortii* (Toth and Pavia 2000). However, the chelating activity determined by Wang et al. (2009) reached 40% in *U. lactuca*, presenting almost ten times higher that we have obtained. They also tested *C. crispus* that developed 30% chelating activity, lower than our results, 40.38%. Metal ion-chelating activity of seaweed extract have higher binding ability preventing the generation and free movement of these oxidative radicals in the tissues (Kumar et al. 2010). FRSA measures the ability of seaweed antioxidant compounds, e.a. carotenoids and chlorophyll, to scavenge and neutralize ROS and proton radicals, generated in tissues as result of oxidative stress (Chew et al. 2008). The lowest scavenging activity was developed by *G. rugosa* (18.69 mg AAE/100g dw) and the highest by *Z. tournefortii* (3,928 mg AAE/100g dw), being this brown seaweed distinct due to its immense activity in scavenging free radicals. Seaweeds used for these tests demonstrated high variability, inherent for their individual characteristics, and due to lack of publications found for these seaweeds in this assay, it could not be presented any comparison. Statistical analysis, applying a Pearson correlation test (table 4) determined a high positive relationship between FRSA and β-CB ($R^2 = 0.995$), also documented by Zhang et al. (2007), using 28 seaweed species from Qingdao Cost, China, indicating that free radical scavenging activity is mainly due to lipophilic compounds present in seaweed. For β-CB activity of lipophilic antioxidants, that protect unsaturated fatty acid of peroxidation by ROS, a great variability between seaweeds is also found, ranging from 2.88% for *U. lactuca* and 95.03% for *Z. tournefortii*.

In summary, this work represents the first effort to increase our knowledge about the biochemical composition, nutritional value, antioxidant potential and activity of seven seaweeds from Madeira. *A. taxiformis* showed the highest content of protein and significant quantities of biochemical and nutritional parameters. Together with its history of human consumption, this report could allow us to select this seaweed as a raw material for food supplementation assessments. *G. lanceola*, developed the highest results of starch and fiber content among red seaweeds, we can assume that being from same genera that *G. turuturu* and *G. filicina*, could be suitable to human consumption and be introduced in food-related products. For *Z. tournefortii*, an understudied brown seaweed, important results were obtained, such as its highest content of fat and antioxidant components and activity among the seaweeds used in this report. It can be suitable for antioxidant extraction and purification for food applications, but more studies should be carried to determined specific components that enable its antioxidant activity. Using statistical analysis, we determined thirty-eight significant correlations between different biochemical and antioxidant parameters or activity, giving us better perception how biochemical, antioxidant components or activity are related and the degree of relationship. Interestingly fat content is highly correlated with antioxidant compounds and activity parameters, suggesting that most of the antioxidant capabilities of seaweeds are from a lipophilic origin.

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2.3 Biochemical study of attached macroalgae from the Madeira Archipelago and beach-cast macroalgae from the Canary Islands - multivariate analysis to determine bioresource potential

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2.3.1 Abstract

Fifteen attached macroalgae from the Madeira Archipelago, comprising three green, three red and nine brown algal species, as well as two beach-cast macroalgal samples, collected along the north shore of Gran Canaria, were assessed for their biochemical properties. The analysis included the determination of total minerals, total carbohydrates, protein, lipids, chlorophyll a, total carotenoids, total phenolic content, fucoxanthin and phycobilins (allophycocyanin, phycocyanin and phycoerythrin). The results showed a high variability of biochemical composition, allowing for the targetting of specific bioresources for particular purposes, including functional foods. This work provides the foundation for a biorefinery strategy implementation plan, for which specific macroalgae may be targeted for valuable and beneficial compounds.

Keywords: Carotenoids; Fucoxanthin; Lipids; Phycobilins; Protein.

2.3.2 Introduction

Marine macroalgae have a high potential as an alternative source of biochemical compounds since they possess several specific metabolic pathways. They contain nutrients, such as proteins, minerals, fiber, carotenoids, vitamins and fatty acids, all of which provide beneficial bioactivity (Kılınç et al. 2013). For instance, macroalgal protein is considered an excellent source of essential amino acids necessary for human metabolism (Galland-Irmouli et al. 2000). Similarly, fiber is recognized in preventing the occurrence of colon cancer, obstipation, obesity and cardiovascular diseases (Dreher 1987). Additionally, liposoluble and hydrosoluble vitamins such as tocopherols, β-carotene, thiamine, and riboflavin have been shown to reduce the risk of thrombosis, atherosclerosis and heart disease (Mishra et al. 1993). Furthermore, phenolics and carotenoids, found in these resources, have the ability to neutralize free radicals, delaying oxidative degradation (Miyashita 2014). These compounds could neutralize reactive oxygen species (ROS) produced in human metabolism, related to degenerative processes associated with ageing, cancer and other human diseases (Aruoma 1999).

In-depth studies on the potential of bioresources may yield several, as yet undiscovered, valuable compounds, making a biorefinery industry potentially feasible. Screening the biochemical composition would be the first step in predicting an alga's potential for further investigation. Presently, the macroalgal industry is focused mainly on single-product (e.g. xanthophylls, polysaccharides, hydrocolloids, proteins) extractions and more recently, the production of biofuels, with little to no use for the remaining biomass (Van Hal et al. 2014). A cascade extraction envisions a primary extraction of a valuable compound followed by a set of subsequent extractions of other compounds found in the target bioresource, in order to maximize extraction efficiency and monetize the process and resource. This method coupled with an efficient fragmentation of the resource increases the potential for a cost-effective biocompound industry (Gilbert-López et al. 2015). For example, a coupled effect of an efficient biorefinery and an eco-friendly process could co-produce both bioethanol and biogas, using the green macroalga *Chaetomorpha linum*, (Ben Yahmed et al. 2016). Similarly, a cascade extraction study was conducted using *Gelidiella acerosa*, *Gelidium pusillum* and *Gracilaria dura*, to determine the feasibility of these red macroalgae as a multi-product bioresource to produce purified bioethanol, lipids, agar, R-phycoerythrin (R-PE), R-phycocyanin (R-PC) and liquid fertilizer (Baghel et al. 2015). More recently Nunes et al. (2018) showed the potential of *Asparagopsis taxiformis* for integration into a cascade extraction for the production of a bioactive extract, lipids, carrageenan and cellulose, with minimum waste.

Madeira Archipelago has 810 km^2 of land surface, but an exclusive economic zone of 10,823 km^2 of sea area (Portuguese Navy 2015). There is thus great potential for new sea-related economic industries. Macroalgae are usually collected in the intertidal and subtidal areas, although several nutrient-enriched areas exist that develop large masses of free-floating, beach-cast macroalgae. These beach-casts have a huge economic impact as they tend to cover large expanses of the coastline, which inevitably harms traditional fishery, negatively impacts on tourism and disturbs aquaculture production (Oyesiku and Egunyomi 2014). It is thus imperative to develop mitigation strategies to effectively reduce the negative impact of such beach-casts (Smetacek and Zingone 2013). One strategy could be to use these beach-casts as raw materials (e.g. biocompound extraction) for industrial purposes (Nunes et al. 2019a and 2019b). Still

other applications could be the direct use of the beach-cast macroalgae as soil fertilizers for crop production (Franzén et al. 2019).

In view of ongoing mitigation strategies, this study aimed to: 1) assess and compare the biochemical composition of three green (Chlorophyta), three red (Rhodophyta) and nine brown (Ochrophyta, Phaeophyceae) macroalgae from the intertidal and subtidal zone of the Madeira Archipelago and two samples of beach-cast macroalgae from the Island of Gran Canaria; and 2) identify potential new sources of valuable biocompounds that can be extracted and purified from these resources.

2.3.3 Materials and methods

2.3.3.1 Bioresources from Madeira Archipelago and Canary islands

Macroalgal samples were collected in the spring of 2017 to a maximum depth of 10 m by means of free-diving round the Madeiran Archipelago; collection sites included the Madeira and Porto Santo Islands. Macroalgal samples comprised three green, three red and nine brown species. The following macroalgae were collected: green, *Dasycladus vermicularis* (Scopoli) Krasser, *Ulva intestinalis* Linnaeus and *Ulva* sp.; red, *Asparagopsis taxiformis* (Delile) Trevisan, *Corallina officinalis* Linnaeus and *Halopithys incurva* (Hudson) Batters; brown, *Cystoseira compressa* (Esper) Gerloff & Nizamuddin, *Cystoseira humilis* Schousboe ex Kützing, *Cystoseira usneoides* (Linnaeus) M.Roberts, *Dictyota dichotoma* (Hudson) J.V.Lamouroux, *Halopteris filicina* (Grateloup) Kützing, *Halopteris scoparia* (Linnaeus) Sauvageau, *Lobophora variegata* (J.V.Lamouroux) Womersley ex E.C.Oliveira, *Padina pavonica* (Linnaeus) Thivy and *Sargassum vulgare* C.Agardh.

2.3.3.2 Study sites

In spring, the Madeira Archipelago sea surface temperature varies from 18.5 to 22.5 ºC, salinity from 32 to 39, conductivity from 54.8 to 55.4 $mS.cm^{-1}$, pH from 8.1 to 8.2 and the dissolved oxygen from 8.7 to 9.2 $mg.l^{-1}$ which were determined using a seawater multiparameter device (Multi 3430 Set G, WTW, Weilheim, Germany). The seawater here is within an oligotrophic range, with phosphate (0 – 0.8 µM), silicic acid (0.2 – 9.2 µM), nitrite (0 – 0.6 µM) and nitrate (0 – 34 µM) present in low concentrations throughout the year (Kaufmann and Maranhão 2017). The photosynthetically active radiation (PAR) at a depth of 11 m ranges from 0 to 26 % of the surface PAR (Kaufmann and Maranhão 2017). Collection sites are shown in Figure 1A.

Beach-cast macroalgae were collected in Playa de Las Canteras, along the northern shore of the Island of Gran Canaria, Canary Islands. Beach-cast macroalgae 1 was composed of 95% *Lobophora variegata* and 5% others, while beach-cast macroalgae 2 was composed of 57% *Halophytis incurva*, 32% *Dictyota* sp., 10.7% *Lobophora variegata* and 0.3% others. At this site, the sea surface temperature averages 23 ºC and salinity 36 (Pelegrí et al. 2005). The seawater here is also oligotrophic, with 0.4 µM of nitrate, 0.3 µM of silicon dioxide and 0.01 µM of phosphate, with an average pH of 8.0 (Pérez et al. 2001). The collection site is shown in Figure 1B.

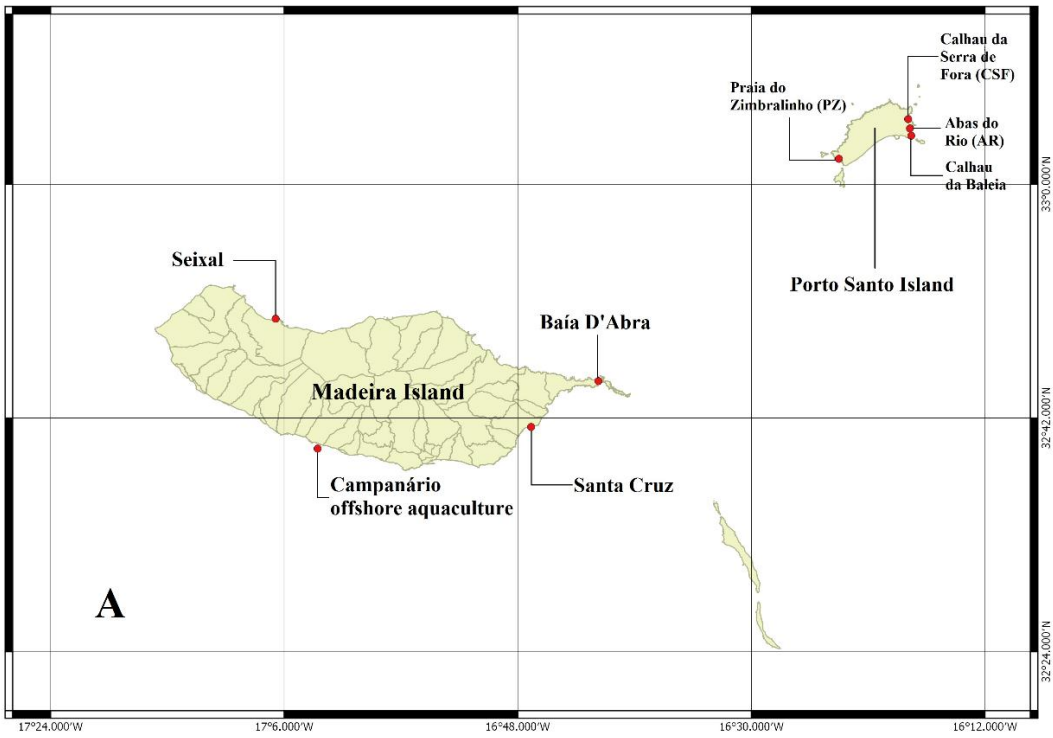

Figure 1 – Maps of both locations showing sampling sites from which macroalgae or beach-cast macroalgae were collected. (A) Madeira Archipelago, including Madeira and Porto Santo Islands; (B) Gran Canaria Island.

2.3.3.3 Treatment samples

After harvesting the attached macroalgae, samples were transported to the laboratory in seawater, where they were gently rinsed with filtered freshwater. Thereafter the macroalgae were frozen at -35 ºC and freeze-dried under reduced pressure (4 x 10^{-4} mbar) with a cooling trap (Scanvac Coolsafe Model 55-4, Labogene, Lynge, Denmark) set at -56 ºC for 5 days. Finally, lyophilized samples were milled to 200 mesh particle size in an electric mill (IKA Werke Model M20, Staufen, Germany), vacuum-packed with a vacuum sealer (AudionVac Model VMS 153, Derby, UK) and stored at -35 ºC until further use. Beach-cast macroalgae were cleaned of sand and epiphytes and rinsed with fresh water. The different species were identified, and subsequently air dried at ambient temperature.

2.3.3.4 Major constituent analysis

Moisture content was determined according to AOAC 925.10 (2000). Samples were oven (Memmert Model UF 260, Schwabach, Germany) dried at 105 ºC until a constant weight was achieved. Total minerals were determined according to AOAC 923.03 (2005). Samples were calcinated in a furnace (Vulcan Model 3-550, NEY, USA) at 550 °C for 5 h and the mineral content determined gravimetrically. Protein content was determined as described by AOAC 978.04 (2005). The process began with the digestion of the samples at 420 °C in a Velp Scientifica digester (DK 8S Digester Heater, Velp Scientifica, Usmate, Italy) using potassium and selenium sulfate. The ammonium sulfate that formed in the reaction was titrated in a distillation and titration unit (Model UDK 152, Velp Scientifica, Usmate, Italy) using sodium hydroxide. Ammonia condensation, using boric acid with bromocresol green and methyl red indicators, allowed for the quantification of total nitrogen. The conversion factors used to convert nitrogen to protein were 5.13 for green, 5.38 for brown and 4.59 for red macroalgae (Lourenço et al. 2002). The protein content (nitrogen-to-protein conversion factor) calculated for the beach-cast macroalgae was determined by considering the % variability of the different macroalgal compositions in each of these beach-cast masses. Lipids were quantified as described by Folch et al. (1957). Initially, freeze-dried milled macroalgae were mixed with $CHCl_3$:MeOH (2:1, v/v), sonicated for 10 min and then centrifuged at room temperature. The extract was filtered, added to a 0.9% NaCl solution, vortexed and centrifuged again for phase separation. The upper phase was removed and the remaining lipophilic phase evaporated in a rotary evaporator (Heidolph, Model Hei-Vap HL, Schwabach, Germany) at 35 ºC and then weighed on a scale (Precisa, Model ES 225SM-DR, Dietikon, Switzerland). Total carbohydrates were calculated as the difference between 100% of dry matter and the sum of the other biocomponents.

2.3.3.5 Minor constituent analysis

Chlorophyll *a* (Chl-*a*) and total carotenoids (TCC) were extracted with methanol and absorbance read on a spectrophotometer (Shimadzu, Model UV-2401 PC, Kyoto, Japan) at 470, 652.4 and 665.2 nm as per Kumar et al. (2010) and their contents calculated using equations 1 and 2, respectively. Total phenolic content (TPC) was measured as per Chew et al. (2008) and expressed in gallic acid equivalents (GAE). Phenol extraction was performed using 50% methanol and quantified by mixing with Folin Ciocalteu reagent and sodium carbonate at 7.5% (w/v), followed by the absorbance reading at 765 nm.

1. $Chlorophyll\ a\left(\frac{\mu g}{mL}\right) = 16{,}72(A665{,}2) - 9{,}16(A652{,}4)$ (Equation 1)

2. $Cx + c\left(\frac{\mu g}{mL}\right) = (1000(A470)) - (1{,}63(Chl\ a)) - (104{,}96(Chl\ b))$ (Equation 2)

Phycobilins from red macroalgae were extracted with 1 M acetic acid-sodium acetate buffer (pH 5.5) and 0.01% of sodium azide as per Francavilla et al. (2014), and absorbance read at 498.5, 614 and 651 nm, to quantify the allophycocyanin (equation 3), phycocyanin (equation 4) and phycoerythrin (equation 5) content, using the equations developed by Kursar et al. (1983).

3. $APC\ \left(\frac{\mu g}{mL}\right) = 181{,}3(A651) - 22{,}3(A614)$ (Equation 3)

4. $PC\ \left(\frac{\mu g}{mL}\right) = 151{,}1(A614) - 99{,}1(A651)$ (Equation 4)

5. $PE\ \left(\frac{\mu g}{mL}\right) = 155{,}8(A498{,}5) - 40(A614) - 10{,}5(A651)$ (Equation 5)

Fucoxanthin from brown macroalgae was extracted using 80% ethanol at 40 °C for 1 h, the absorbance read at 445nm, and the content calculated using equation 6 as per Wu et al. (2014).

6. $Fucoxanthin\left(\frac{mg}{g}\right) = \frac{A445\times n\times V\times 1000}{A\times m\times 100}$ (Equation 6)

2.3.3.6 Statistical analysis

Data are expressed as an average of three replicates ± standard deviation, using the SPSS 24 software. Tests included the post hoc Tukey's b test, with a significance level of $p < 0.05$ applied to assess the statistical variance of individual compounds between macroalgae. A Pearson correlation was also performed to determine the relationship between the biochemical parameters. STATISTICA 10 software was used to perform a principal component analysis (PCA), frequently used for establishing predictive models and exploratory data analysis. PCA improved the perception of the results, since a score plot (Figure 2) was performed, enabling a visual analysis of the influence of each biochemical parameter in the 2D distribution of the samples. A loading plot (Figure 3) was used to explain a probable sample grouping. A dendrogram (Figure 4) was constructed, based on Euclidean distances, to determine the linkage distance between the different samples.

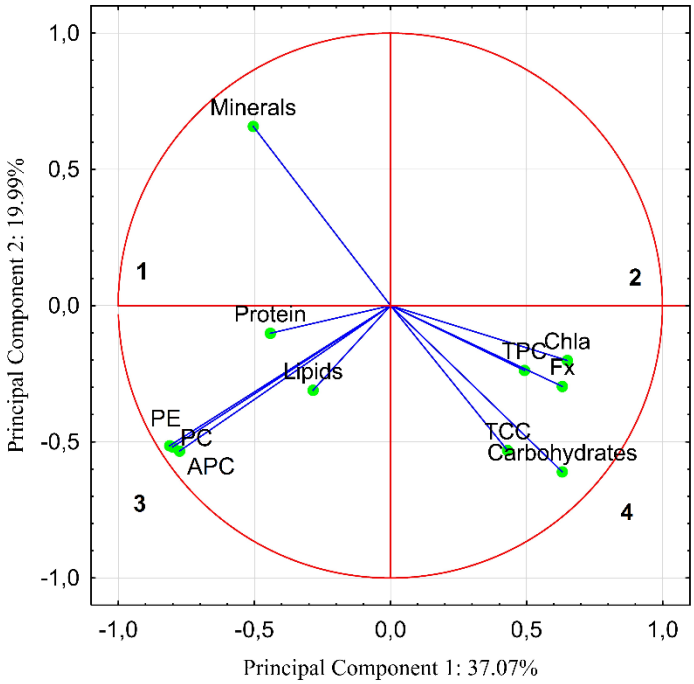

Figure 2 – Principal Component Analysis of the biochemical analysis carried out for the 19 samples of macroalgae from the Madeira Archipelago and beach-cast macroalgae from Gran Canaria, with a projection of the variables on the factor-plane. Quadrant 1 – minerals; Quadrant 3 – protein, lipids and phycobilins, including allophycocyanin (APC), phycocyanin (PC) and phycoerythrin (PE); Quadrant 4 – chlorophyll *a* (Chl*a*), total phenolic content (TPC), fucoxanthin (Fx), carbohydrates and total carotenoid content (TCC).

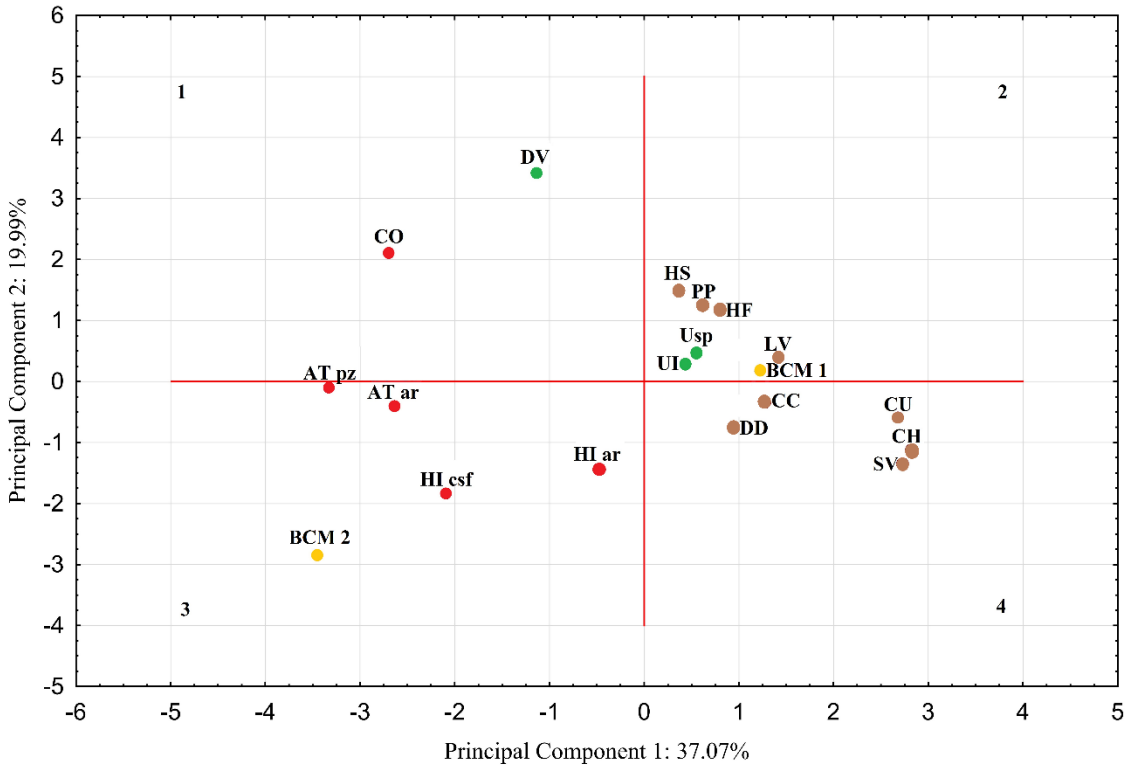

Figure 3 – Principal Component Analysis of the biochemical analysis carried out for the 19 samples of macroalgae from the Madeira Archipelago and beach-cast macroalgae from Gran Canaria, with a projections of the samples on the factor-plane. Quadrant 1 – *Corallina officinalis* (CO) and *Dasycladus vermicularis* (DV); Quadrant 2 – *Halopteris scoparia* (HS), *Padina pavonica* (PP), *Halopteris filicina* (HF), *Ulva* sp. (Usp), *Ulva intestinalis* (UI), *Lobophora variegata* (LV), Beach-cast macroalgae 1 (BCM 1); Quadrant 3 – *Asparagopsis taxiformis* from Praia do Zimbralinho (AT pz) and Abas do Rio (AT ar), *Halopithys incurva* from Calhau da Serra de Fora (HI csf) and Abas do Rio (HI ar), Beach-cast macroalgae 2 (BCM 2); Quadrant 4 – *Cystoseira compressa* (CC), *Dictyota dichotoma* (DD), *Cystoseira usneoides* (CU), *Cystoseira humilis* (CH), *Sargassum vulgare* (SV). The colour of each dot represents the colour of each macroalga, except for beach-cast macroalgae that are shown in yellow, since these could contain all three groups of macroalgae.

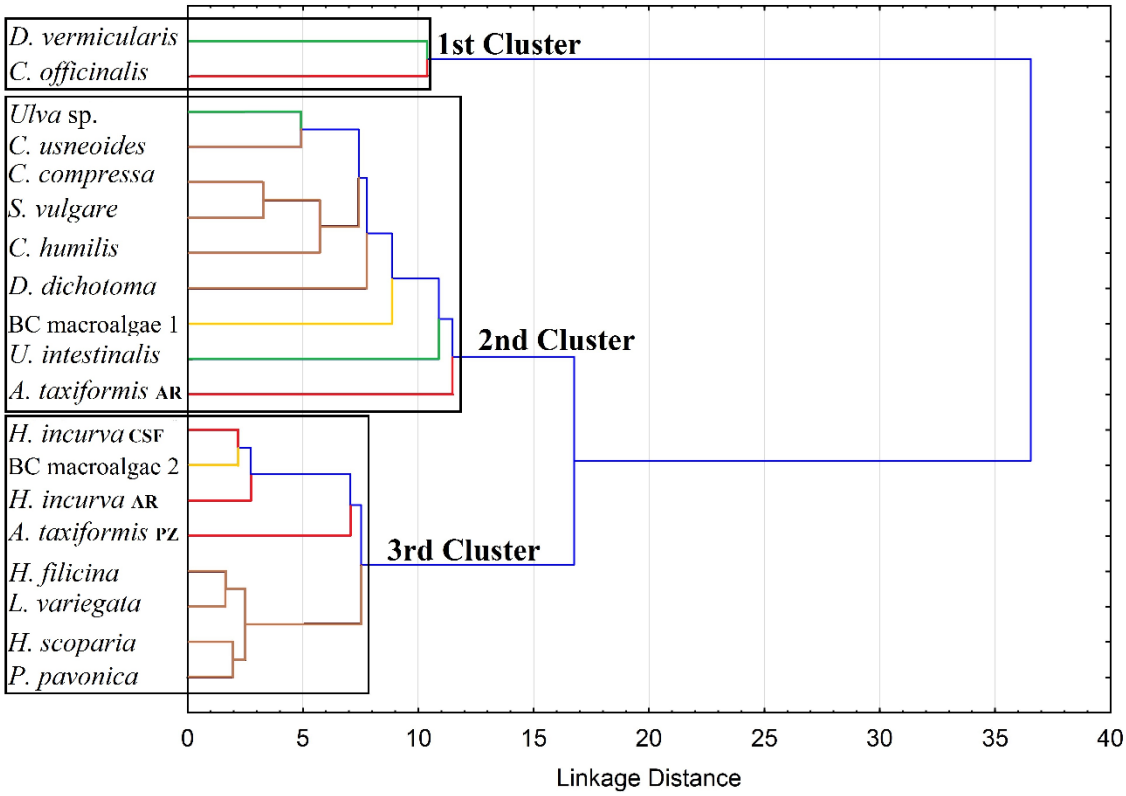

Figure 4 – Hierarchical cluster analysis in a dendrogram format, for a single linkage, using Euclidean distance. The biochemical analysis carried out for the 19 samples of macroalgae from the Madeira Archipelago and beach-cast macroalgae (BC macroalgae) from Gran Canaria were included. The colour of each line represents the colour of each macroalga, except for beach-cast macroalgae which are shown in yellow. Blue lines indicate mixed algal groups. Algal species names as in Figure 3; BC = beach-cast, AR = Abas do Rio, CSF = Calhau da Serra de Fora, PZ = Praia do Zimbralinho.

2.3.4 Results

2.3.4.1 Total minerals

Among the macroalgae analysed, the highest mineral content was observed in *Dasycladus vermicularis* and in *Corallina officinalis*, and the lowest mineral content in *Cystoseira humilis* (Table 1). The red macroalgae *Asparagopsis taxiformis* from Abas do Rio (AR) (32.37 g.100g^{-1} dry weight (dw)) and Praia do Zimbralinho (PZ) (50.12 g.100g^{-1} dw), collected in Porto Santo Island (Table 1), showed significant differences in mineral content. Beach-cast macroalgae 1 and 2 had statistically different mineral contents, 38 and 46 g.100g^{-1} dw (Table 1).

Table 1 – Biochemical constituents of attached macroalgae from the Madeira Archipelago and beach-cast macroalgae from the Canary Islands

	Bioresource	Moisture ($g.100g^{-1}$ dw)	Total minerals ($g.100g^{-1}$ dw)	Protein ($g.100g^{-1}$ dw)	Lipids ($g.100g^{-1}$ dw)	Total carbohydrates ($g.100g^{-1}$ dw)
	Green macroalgae					
1	*Dasycladus vermicularis*(1)	3.64 ± 1.03^{a}	86.96 ± 1.27^{n}	10.14 ± 0.36^{abc}	2.11 ± 0.63^{a}	0.00^{a}
2	*Ulva* sp.(2)	8.15 ± 0.23^{def}	30.25 ± 0.12^{e}	2.76 ± 0.02^{a}	5.85 ± 0.83^{ef}	53.73 ± 1.97^{gh}
3	*Ulva intestinalis*(3)	8.83 ± 0.79^{cdef}	26.10 ± 0.39^{d}	16.85 ± 0.59^{c}	5.29 ± 0.5^{cdef}	41.81 ± 10.68^{fgh}
	Red macroalgae					
4	*Asparagopsis taxiformis* AR(4)	7.77 ± 0.50^{bcdef}	32.37 ± 1.08^{e}	15.83 ± 0.16^{bc}	7.88 ± 0.64^{g}	39.73 ± 10.49^{efg}
5	*Asparagopsis taxiformis* PZ(5)	6.35 ± 0.47^{abcdef}	50.12 ± 0.40^{i}	11.76 ± 0.12^{abc}	6.97 ± 0.79^{fg}	28.68 ± 6.72^{cde}
6	*Corallina officinalis*(5)	3.64 ± 1.60^{a}	85.19 ± 0.17^{n}	2.69 ± 0.06^{a}	5.24 ± 0.91^{cdef}	6.02 ± 1.89^{ab}
7	*Halopithys incurva* AR(4)	5.89 ± 0.89^{abcd}	48.88 ± 1.45^{hi}	9.41 ± 0.19^{abc}	3.83 ± 0.35^{abcd}	34.70 ± 4.62^{ef}
8	*Halophytis incurva* CSF(1)	6.78 ± 0.63^{bcdef}	47.88 ± 0.67^{gh}	7.69 ± 0.04^{abc}	5.05 ± 0.86^{cde}	33.35 ± 4.92^{cdef}
	Brown macroalgae					
9	*Cystoseira compressa*(2)	9.08 ± 0.41^{f}	23.58 ± 0.92^{c}	4.05 ± 0.17^{a}	5.61 ± 1.12^{def}	56.55 ± 1.34^{gh}
10	*Cystoseira humilis*(6)	7.60 ± 0.74^{bcdef}	20.44 ± 0.18^{b}	4.68 ± 0.03^{ab}	2.95 ± 0.10^{ab}	63.51 ± 4.01^{h}
11	*Cystoseira usneoides*(6)	8.20 ± 1.83^{def}	30.70 ± 0.14^{e}	3.78 ± 0.14^{a}	3.50 ± 0.54^{abc}	51.50 ± 4.28^{gh}
12	*Dictyota dichotoma*(5)	5.04 ± 1.47^{ab}	27.51 ± 0.71^{d}	7.22 ± 0.01^{abc}	10.00 ± 0.67^{h}	49.76 ± 1.39^{fgh}
13	*Halopteris filicina*(4)	6.06 ± 1.96^{abcde}	55.34 ± 0.50^{jk}	5.47 ± 0.05^{ab}	3.10 ± 0.65^{ab}	31.43 ± 1.16^{cde}
14	*Halopteris scoparia*(7)	5.20 ± 0.70^{abc}	57.20 ± 1.69^{km}	5.54 ± 0.01^{ab}	3.64 ± 0.44^{abc}	29.86 ± 4.06^{cde}
15	*Lobophora variegata*(8)	5.05 ± 0.90^{ab}	54.67 ± 0.47^{j}	5.92 ± 0.04^{ab}	4.20 ± 0.99^{bcde}	30.88 ± 3.76^{cde}
16	*Padina pavonica*(6)	5.66 ± 0.65^{abcd}	57.85 ± 1.68^{m}	3.74 ± 0.15^{a}	3.50 ± 0.60^{abc}	30.17 ± 2.83^{cde}
17	*Sargassum vulgare*(9)	5.79 ± 0.49^{abcd}	22.74 ± 0.16^{c}	5.49 ± 0.01^{ab}	5.06 ± 0.07^{cde}	59.17 ± 0.74^{h}
	Miscellaneous					
18	Beach-cast macroalgae 1(10)	6.80 ± 0.64^{bcdef}	37.88 ± 0.82^{f}	6.32 ± 0.09^{abc}	3.67 ± 0.37^{abc}	48.27 ± 6.06^{fgh}
19	Beach-cast macroalgae 2(10)	6.26 ± 0.65^{abcdef}	46.43 ± 1.29^{g}	8.23 ± 0^{abc}	4.80 ± 0.26^{bcde}	34.04 ± 1.72^{def}

Data are means ± standard deviation in grams per 100 grams of sample ($g.100g^{-1}$) on a dry weight (dw) basis. All determinations were carried out in triplicate (n=3). Different letters within the same column indicate significant differences ($p < 0.05$) determined using Tukey b test. Collection locations are identified by superscript values after each scientific or sample name as follows: (1) Calhau da Serra de Fora, Porto Santo; (2) Praia do Zimbralinho, Porto Santo; (3) Campanário, Madeira; (4) Abas do Rio, Porto Santo; (5) Praia do Zimbralinho, Porto Santo; (6) Piscinas naturais Seixal, Madeira, (7) Baía D'Abra, Madeira; (8) Calhau da Baleia, Porto Santo; (9) Santa Cruz, Madeira; (10) Playa de Las Canteras, Gran Canaria.

2.3.4.2 Protein

The highest protein content was found in the green alga *Ulva intestinalis* (16.85 g of protein per 100g dw) from Madeira Island, sampled in the offshore fish cages located at Campanário (Table 1). The lowest protein content was found in the red alga *Corallina officinalis* (2.69 $g.100g^{-1}$ dw; Table 1). Protein content in the red alga *Asparagopsis taxiformis* varied between 12 and 16 $g.100g^{-1}$ dw in the samples collected in Abas do Rio and Praia do Zimbralinho, in Porto Santo Island (Table 1). Beach-cast macroalgae showed protein contents that ranged from 6 to 8 $g.100g^{-1}$ dw (Table 1).

2.3.4.3 Lipid content

The highest lipid content was found in the brown alga *Dictyota dichotoma* (10.00 g.100g^{-1} dw) and the lowest was found in the green alga *Dasycladus vermicularis* (2.11 g.100g^{-1} dw), both collected from Porto Santo Island (Table 1). A high lipid content was observed in the red alga *Asparagopsis taxiformis*, collected in Abas do Rio and Praia do Zimbralinho, ranging between 7 and 8 g.100g^{-1} dw (Table 1). Beach-cast macroalgae from Playa de Las Canteras, Gran Canaria, had a moderate lipid content, containing from 4 to 5 g.100g^{-1} dw (Table 1).

2.3.4.4 Carbohydrates

The highest carbohydrate contents were found in the brown macroalgae *Cystoseira humilis* and *Sargassum vulgare* (Table 1). Low carbohydrate contents were found in *Dasycladus vermicularis* and in *Corallina officinalis* (Table 1). Beach-cast macroalgae showed a medium carbohydrate content, between 34 and 48 g.100g^{-1} dw (Table 1).

2.3.4.5 Chlorophyll *a*

The analysis of macroalgal samples shows that higher concentrations of chlorophyll *a* were present in the Chlorophyta *Ulva intestinalis* (15.23 mg.g^{-1} dw) and Phaeophyceae *Cystoseira usneoides* (11.84 mg.g^{-1} dw; Table 2). Lowest chlorophyll *a* contents were detected in *Asparagopsis taxiformis* from both locations, Abas do Rio and Praia do Zimbralinho (Table 2). Chlorophyll *a* content in Canary Islands beach-cast macroalgae varied between 7 and 8 mg of chlorophyll *a* per g dw (Table 2).

2.3.4.6 Total phenolic content

TPC was assessed in macroalgae from Madeira Archipelago and was not detected in *Ulva* species, *Asparagopsis taxiformis*, *Corallina officinalis*, and *Padina pavonica*. The highest content of TPC was determined in *Cystoseira usneoides*, 35.20 mg.g^{-1} dw (Table 2). The two beach-cast macroalgae analysed showed distinct amounts of TPC, varying between 1 and 3 mg per g of dw (Table 2).

2.3.4.7 Total carotenoid content

Among the macroalgae analysed, the highest TCC was determined in *Halopithys incurva* from Abas do Rio and Calhau da Serra de Fora (Porto Santo Island) with values ranging from 5 to 7 mg.g^{-1} dw (Table 2). The brown algae *Sargassum vulgare* and *Lobophora variegata* also had high TCC, with values around 4 mg.g^{-1} dw (Table 2). For beach-cast macroalgae, TCC was between 3 and 4 mg.g^{-1} dw (Table 2).

Table 2 – Chlorophyll, carotenoids and phenolic compounds of attached macroalgae from the Madeira Archipelago and beach-cast macroalgae from the Canary Islands

	Bioresource	Chlorophyll *a* (mg.g^{-1} dw)	TCC (mg.g^{-1} dw)	TPC (mg GAE.g^{-1} dw)
	Green			
1	*Dasycladus vermicularis*	4.97 ± 0.06^{cdef}	0.26 ± 0.01^{a}	17.31 ± 0.36^{f}
2	*Ulva* sp.	5.61 ± 0.66^{defg}	1.69 ± 0.11^{b}	0.00^{a}
3	*Ulva intestinalis*	15.23 ± 0.33^{n}	0.00^{a}	0.00^{a}
	Red			
4	*Asparagopsis taxiformis* AR	0.58 ± 0.04^{a}	0.26 ± 0^{a}	0.00^{a}
5	*Asparagopsis taxiformis* PZ	0.88 ± 0.04^{a}	0.31 ± 0.02^{a}	0.00^{a}
6	*Corallina officinalis*	3.43 ± 0.06^{bcd}	0.30 ± 0.01^{a}	0.00^{a}
7	*Halopithys incurva* AR	11.38 ± 0.43^{m}	6.59 ± 0.42^{g}	16.52 ± 0.76^{f}
8	*Halophytis incurva* CSF	7.07 ± 0.43^{fghi}	4.58 ± 0.25^{f}	14.99 ± 0.19^{e}
	Brown			
9	*Cystoseira compressa*	4.53 ± 0.38^{bcde}	2.88 ± 0.10^{bcde}	9.11 ± 0.33^{d}
10	*Cystoseira humilis*	9.65 ± 0.09^{jkm}	4.05 ± 0.09^{def}	33.14 ± 1.41^{g}
11	*Cystoseira usneoides*	11.84 ± 0.86^{m}	2.90 ± 0.18^{bcde}	35.20 ± 2.22^{g}
12	*Dictyota dichotoma*	7.82 ± 1.41^{ghij}	2.74 ± 0.17^{bcd}	2.38 ± 0.20^{bc}
13	*Halopteris filicina*	8.85 ± 1.09^{hijk}	2.77 ± 0.09^{bcd}	1.98 ± 0.02^{bc}
14	*Halopteris scoparia*	8.15 ± 0.16^{hij}	2.15 ± 0.08^{bc}	1.90 ± 0.17^{bc}
15	*Lobophora variegata*	9.04 ± 1.49^{ijk}	4.27 ± 0.18^{ef}	8.99 ± 0.70^{d}
16	*Padina pavonica*	7.83 ± 0.26^{ghij}	3.60 ± 0.13^{cdef}	0.00^{a}
17	*Sargassum vulgare*	11.07 ± 2.72^{km}	4.37 ± 0.26^{f}	14.60 ± 1.03^{e}
	Miscellaneous			
18	Beach-cast macroalgae 1	7.78 ± 0.25^{ghij}	3.58 ± 0.10^{cdef}	3.22 ± 0.20^{c}
19	Beach-cast macroalgae 2	6.49 ± 1.17^{efgh}	2.88 ± 0.25^{bcde}	1.42 ± 0.10^{ab}

Data are mean ± standard in milligrams per gram (mg.g^{-1}) of sample on a dry weight (dw) basis. All determinations were carried out in triplicate (n=3). Different letters within the same column indicate significant differences ($p < 0.05$) determined using Tukey b test. TCC, total carotenoids; TPC, total phenolic compounds; GAE, gallic acid equivalents. Collection sites: AR, "Abas do Rio"; CSF, "Calhau da Serra de Fora"; PZ, "Praia do Zimbralinho".

2.3.4.8 Phycobilins

In the red macroalgae analysed, the allophycocyanin (APC) content varied between 0.12 mg.g^{-1} dw in *Corallina officinalis* and 0.33 mg.g^{-1} dw in *Halopithys incurva* from Calhau da Serra de Fora (Table 3). On the other hand, phycocyanin (PC) oscillated between 0.08 mg.g^{-1} dw in *Asparagopsis taxiformis* from Abas do Rio and 0.14 mg.g^{-1} dw in *H. incurva* from Calhau da Serra de Fora (Table 3). Finally, phycoerythrin (PE) presented the lowest value in *C. officinalis*, 0.46 mg.g^{-1} dw and the highest in *H. incurva* from Calhau da Serra de Fora, 0.85 mg.g^{-1} dw (Table 3). Among the two samples of beach-cast macroalgae, only beach-cast macroalgae 2 showed phycobilins due to the presence of Rhodophyta macroalgae (57% *H. incurva*). APC, PC and PE values for this resource, were the highest detected in this work (Table 3).

Table 3 – Phycobilins of attached macroalgae from the Madeira Archipelago and beach-cast macroalgae from the Canary Islands

	Bioresource	APC (mg.g^{-1} dw)	PC (mg.g^{-1} dw)	PE (mg.g^{-1} dw)
	Red			
1	*Asparagopsis taxiformis* AR	0.13 ± 0.02^{a}	0.08 ± 0.01^{a}	0.51 ± 0.03^{a}
2	*Asparagopsis taxiformis* PZ	0.21 ± 0.02^{b}	0.12 ± 0.01^{ab}	0.67 ± 0.03^{b}
3	*Corallina officinalis*	0.12 ± 0.03^{a}	0.09 ± 0.01^{a}	0.46 ± 0.03^{a}
4	*Halopithys incurva* AR	0.12 ± 0.06^{a}	0.010 ± 0.01^{a}	0.82 ± 0.05^{c}
5	*Halophytis incurva* CSF	0.33 ± 0.03^{c}	0.14 ± 0.02^{b}	0.85 ± 0.07^{c}
	Miscellaneous			
6	Beach-cast macroalgae 2	0.48 ± 0.06^{d}	0.27 ± 0.03^{c}	1.14 ± 0.06^{d}

Data are mean ± standard deviation in milligrams per gram (mg.g^{-1}) of sample on a dry weight (dw) basis. All determinations were carried out in triplicate (n=3). Different letters within the same column indicate significant differences ($p < 0.05$) determined using Tukey b test. APC, allophycocyanin; PC, phycocyanin; PE, phycoerythrin. Collection sites: AR, "Abas do Rio"; CSF, "Calhau da Serra de Fora"; PZ, "Praia do Zimbralinho".

2.3.4.9 Fucoxanthin

Fucoxanthin content was assessed in the nine brown macroalgae and the two beach-cast macroalgae. Fucoxanthin yield varied from 0.34 mg.g^{-1} dw in *Halopteris scoparia* to 1.19 mg.g^{-1} dw in *Sargassum vulgare* (Table 4). Both beach-cast macroalgae, due to the presence of brown seaweeds in their composition, were also assessed. Fucoxanthin values for beach-cast macroalgae were between 0.52 and 0.65 mg.g^{-1} dw (Table 4).

Table 4 – Fucoxanthin content of attached brown macroalgae from the Madeira Archipelago and beach-cast macroalgae from the Canary Islands

	Bioresource	Fucoxanthin (mg.g^{-1} dw)
	Brown	
1	*Cystoseira compressa*	0.49 ± 0.01^{a}
2	*Cystoseira humilis*	0.59 ± 0.01^{ab}
3	*Cystoseira usneoides*	0.75 ± 0.0^{abc}
4	*Dictyota dichotoma*	0.77 ± 0.03^{abc}
5	*Halopteris filicina*	0.59 ± 0.04^{ab}
6	*Halopteris scoparia*	0.34 ± 0.02^{a}
7	*Lobophora variegata*	1.07 ± 0.07^{bc}
8	*Padina pavonica*	0.39 ± 0.01^{a}
9	*Sargassum vulgare*	1.19 ± 0.07^{c}
	Miscellaneous	
10	Beach-cast macroalgae 1	0.52 ± 0.01^{a}
11	Beach-cast macroalgae 2	0.65 ± 0.03^{ab}

Data are mean ± standard deviation in milligrams per gram (mg.g^{-1}) of sample on a dry weight (dw) basis. All determinations were carried out in triplicate (n=3). Different letters within the same column indicate significant differences ($p < 0.05$) determined using Tukey b test.

2.3.4.10 Statistics

The Pearson correlation coefficients (Table 5) discriminated the macroalgal samples by negative or positive correlations between the compositional parameters. A significant negative correlation was found between total minerals and total carbohydrates (R^2 = -0.948). Furthermore, significant positive correlations were found between chlorophyll *a* and TCC (R^2 = 0.463), and between fucoxanthin and TCC (R^2 = 0.462).

Table 5 - Pearson correlation coefficients showing relationships among different chemical components of attached macroalgae from the Madeira Archipelago and beach-cast macroalgae from the Canary Islands

	Total minerals	Protein	Lipids	Carbohydrates	Chlorophyll *a*	TCC	TPC	Fucoxanthin
Total minerals	1							
Protein	-0.085	1						
Lipids	-0.356**	0.294*	1					
Carbohydrates	-0.948**	-0.105	0.208	1				
Chlorophyll *a*	-0.319*	-0.046	-0.350**	0.372**	1			
TCC	-0.247	-0.398**	-0.267*	0.340**	0.463**	1		
TPC	-0.216	-0.235	-0.427**	0.268*	0.377**	0.429**	1	
Fucoxanthin	-0.349**	-0.419**	-0.061	0.416**	0.371**	0.462**	0.276*	1

Statistical significance at 0.01 level (**) or at 0.05 level (*) bilateral using Pearson correlation test; Signalling (–) reveal the negative relation between parameters, or, in its absence, their positive correlation. Values presented are for R^2. TCC, total carotenoid content; TPC, total phenolic content. Data for allophycocyanins (APC), phycocyanins (PC) and phycoerythrins (PE) were not in compliance with Pearson coefficient assumptions and therefore are not shown in this table.

The PCA (Figures 2 and 3) determined a total of 57.06% of the cumulative variance with two principal components (PC), in which PC 1 had 37.07% and PC 2 19.99%. This projection (Figure 2) showed that protein, lipids and phycobilins were positioned in the 3rd quadrant. The TPC, chlorophyll *a*, fucoxanthin, TCC and carbohydrates were grouped in the 4th quadrant and mineral content was isolated in the 2nd quadrant. Three parameters, namely chlorophyll *a*, TPC and fucoxanthin were located close together, as were TCC and carbohydrates. The phycobilins overlapped in the projection, due to the intrinsic correlation of these compounds. Furthermore, the projection of the cases for individual species (Figure 3) shows that the macroalgae were scattered in the projection plane according to their biochemical composition. *Dasycladus vermicularis* and *Corallina officinalis*, are located in the 1st quadrant, influenced by their high mineral content. Furthermore, the green macroalgae, *Ulva* sp. and *Ulva intestinalis* were located close together in the 2nd quadrant, due to their biochemical resemblance. Two more distinct groups were located in this quadrant: the brown algae *Halopteris scoparia*, *Padina pavonica* and *Halopteris filicina*, and the brown alga *Lobophora variegata* with the beach-cast macroalgae 1. This is due to their intermediate biochemical content, with no distinct parameters. Phycobilins accounted for the isolation of

beach-cast macroalgae 2 in the 3rd quadrant, due to its high content of *Halopithys incurva* (57%). In the 3rd quadrant, protein content also imprinted a strong influence, separating *Asparagopsis taxiformis* from its two locations. Similarly, lipids imprinted a strong influence, separating *H. incurva* from its two locations. In the 4th quadrant, two groups of brown algae were formed, namely *Cystoseira compressa* and *Dictyota dichotoma*, and also *Cystoseira usneoides*, *Cystoseira humilis* and *Sargassum vulgare*. These two groups were separated due to the influence of five biochemical parameters, namely chlorophyll *a*, TPC, fucoxanthin, TCC and carbohydrates.

With regard to the distance between samples, three well-defined clusters were obtained (Figure 4). The 1st cluster (Euclidean linkage distance of 36) included *D. vermicularis* and *C. officinalis* that were well separated from the remaining samples due to their high mineral content. The 2nd and 3rd clusters have a Euclidian linkage distance of 16. The 2nd cluster comprised nine samples, namely two green (*Ulva* sp. and *U. intestinalis*), five brown (*C. usneoides*, *C. compressa*, *S. vulgare*, *C. humilis* and *D. dichotoma*), one red (*A. taxiformis* AR) macroalgae, and the beach-cast macroalgae 1. The 3rd cluster comprised eight samples, namely three red (*H. incurva* CSF, *H. incurva* AR and *A. taxiformis* PZ), four brown (*H. filicina*, *L. variegata*, *H. scoparia* and *P. pavonica*) macroalgae and the beach-cast macroalgae 2.

2.3.5 Discussion

2.3.5.1 Mineral composition

The mineral content of macroalgae is an essential trait to determine their nutritional, industrial, pharmaceutical, or agricultural uses. Macroalgae are recognized as a rich source of minerals, mainly iodine and iron, and have a high potential to become a source for daily nutrition or to be used as a nutraceutical product (Mišurcová et al. 2011a).

The mineral content in macroalgae can fluctuate due to endogenous factors intrinsic to a specific species, or due to several exogenous environmental factors such as the concentration of minerals, the temperature and the pH of seawater (Circuncisão et al. 2018). This may explain why *Asparagopsis taxiformis*, collected along the southern shore of Porto Santo Island, displayed a mineral content that fluctuated between 1.36 and 2.11, values higher than those previously reported for the species collected on Madeira Island (Nunes et al. 2017). The variation in mineral content between these samples of *A. taxiformis* (Nunes et al. 2017, this study) was potentially a result of the differences in the ecological conditions of the intertidal and subtidal zones between Madeira and Porto Santo Islands. Additionally, *A. taxiformis* was rich in bioaccumulated iodine, which could be used to produce nutraceutical iodine-rich supplements (Nunes et al. 2018). *Corallina officinalis* and *Dasycladus vermicularis* collected in Porto Santo Island contained the highest mineral contents, similar to *Galaxaura rugosa* (Rhodophyta) collected from Reis Magos beach in Madeira Island (Nunes et al. 2017). Similarly, Marsham et al. (2007) found a high mineral content (78 $g.100g^{-1}$ dw) in *C. officinalis* collected from North Yorkshire, UK. Moreover, *Dictyota dichotoma* had more than twice the mineral content of the same species collected in India, although *Lobophora variegata* had a similar content compared with Verma et al. (2017). The two beach-casts of macroalgae differed in their mineral yields notably due to the variation in macroalgal composition collected at different periods.

2.3.5.2 Potential as a protein source

Ulva intestinalis collected in offshore cages at the *Sparus aurata* fish farm was rich in protein. Protein contents for the species reported in this study were similar to those reported for the species from Southern Thailand by Benjama and Masniyom (2011). The high protein content in our study is likely the result of unconsumed feed from the fish farm that dissolves into the surrounding waters, providing available nutrients. This is known as eutrophication, which can be substantially reduced with the incorporation of macroalgae that then increase in growth rate and protein content as a consequence (Shpigel et al. 2018). This strategy reduces the environmental impact of fish aquaculture and enables the development of a macroalgal resource that has a market potential (Ellis and Tiller 2019).

Asparagopsis taxiformis collected from Porto Santo Island (this study) has also proven to be a prominent protein source, but with slightly lower values than those collected on the south coast of Madeira Island (Nunes et al. 2017). The differences in the protein contents of the two *A. taxiformis* populations can likely be attributed to the higher human impact (increased eutrophication through increased population density and industrialization) on Madeira Island. Furthermore, the protein content of *Corallina officinalis* in this study was significantly lower than that reported by Marsham et al. (2007) for the species. Verma et al. (2017) assessed a total of 30 macroalgae collected in India and measured a higher protein content (12 $g.100g^{-1}$ dw) in *Dictyota dichotoma*. The beach-cast macroalgae in this study were considered a moderate protein source, being similar to the pelagic macroalgal mixtures of *Sargassum natans* and *S. fluitans* (Oyesiku and Egunyomi 2014). These pelagic masses stay afloat, and previous research by Oyesiku and Egunyomi (2014) demonstrated that these masses contain a moderate protein yield. These could be extensively collected, when located near to the coastline, highlighting its potential use as a protein source for multiple applications.

Macroalgae are regularly consumed whole or in supplement form due to their protein content, which is comparable to other plant sources (Phong et al. 2016). Some selected species of macroalgae are a staple food in some Asian countries. Increased protein content increases the potential of a macroalga to be introduced as a nutraceutical ingredient, providing essential amino acids. The fortification of several food products is a viable strategy to improve the products' nutritional value and thus achieve the recommended daily intake (RDI).

2.3.5.3 Low-fat bioresource

The macroalgae investigated in this study had a low lipid content, not exceeding 10%. Such low lipid contents were previously reported by Lorenzo et al. (2017) who evaluated the edible brown macroalgae *Ascophyllum nodosum* and *Fucus vesiculosus*. Nonetheless, the lipid composition of macroalgae is an important determinant because bioactive lipids, in particular, activate several biochemical mechanisms in the consumers of macroalgae (Mišurcová et al. 2011b). The lipid content of *Asparagopsis taxiformis* from Madeira Island was previously determined by Nunes et al. (2018). This macroalga is known to be a traditional staple food in Hawaii, identified as "limu kohu" (Burreson et al. 1976). Nunes et al. (2019c) recently published the nutraceutical and bioactive potential of lipid extracts obtained from three macroalgae collected from Madeira Island, identifying their fatty acid composition, anti-cholinesterase activity, and *in vitro* cytotoxicity to the A549 tumour cell line. Moreover, in this study, *Corallina officinalis*

had a higher lipid content than the same species examined by Marsham et al. (2007), demonstrating the biochemical dissimilarity of these resources when propagated under different conditions. Similarly, in this study the lipid content of *Dictyota dichotoma* and *Lobophora variegata* were approximately triple those found in these macroalgae by Verma et al. (2017). The beach-cast macroalgae in this study were not a noticeable lipid source. In contrast, Oyesiku and Egunyomi (2014) described a higher lipid content in pelagic macroalgal masses, highlighting the influence of the macroalgal composition on the lipid yield. These comparisons show the importance of assessing different resources due to the biochemical differences that can occur. Total lipid content and composition can vary between species/assemblages and can fluctuate due to differing environmental conditions and geographical positions (Miyashita et al. 2013).

2.3.5.4 Carbohydrate content inference

Vizetto-Duarte et al. (2016) assessed the biochemical composition of five *Cystoseira* species, collected on the mainland of Portugal. They found that *Cystoseira humilis* had 64.09 g of carbohydrates per 100 g dw, similar to our results. The beach-cast macroalgae in this study showed a high polysaccharide content, which was lower than the macroalgal pelagic masses collected offshore of the coast of Nigeria (Oyesiku and Egunyomi 2014). The variation in carbohydrate content is likely related to the different species composition of these algal masses, ranging from almost monospecific to mixed species compositions. Additionally, specific biochemical pathways and habitat features force these organisms to biosynthesize different contents and types of polysaccharides (Sudha et al. 2014).

2.3.5.5 Chlorophyll *a* as a potential nutraceutical

Chlorophyll *a* is an important feature for macroalgae due to the capability of this molecule to chelate several chemical carcinogens and mutagens, thus potentially decreasing the risk of cancer in consumers (Chen and Roca 2018). Chlorophyll *a* content was highly variable in the different species sampled in this study, with *Ulva intestinalis* being the most prominent source. Furthermore, *Dictyota dichotoma* and *Lobophora variegata* contained significantly higher chlorophyll *a* values than those reported for these species from India (Verma et al. 2017). This could indicate, at least for these species, that the production of chlorophyll *a* is higher in Madeira Archipelago, promoting this location for producing or collecting macroalgae when targeting higher chlorophyll *a* biomass.

2.3.5.6 Total phenolic content (TPC) in brown macroalgae

Phenolic compounds are used by algae as protection against marine grazers, epiphytes, pathogens, and in photoprotection mechanisms (Audibert et al. 2010). In this work, TPC analysis demonstrated a high content of these compounds in *Cystoseira humilis* and *Cystoseira usneoides*. This finding corroborates the statement by Deniaud-Bouët et al. (2014) who stated that brown macroalgae will tend to have higher TPC contents due to the possible enzymatic cross-linking of alginates by phenols in regulating the strengthening of the cell wall. Habitat conditions greatly influence the composition and concentration of phenolic compounds in macroalgae. The biosynthesis and accumulation are determined by the action of long

desiccation periods in the intertidal zone, high levels of UV radiation (O'Sullivan et al. 2011), or grazing activity (Alstyne 1988).

2.3.5.7 Macroalgae as a source of carotenoids with antioxidant potential

TCC is an important parameter that can determine the selection of macroalgae as a source of antioxidants and provide functional activity. In this work, elevated amounts of TCC were detected in the red macroalga *Halopithys incurva* and the browns *Sargassum vulgare* and *Lobophora variegata*. They could be selected for further analysis to determine the specific carotenoids that comprise the physiological composition of these macroalgae. In our study, *Dictyota dichotoma* and *L. variegata* had considerably higher TCC values than isolates of the same species investigated by Verma et al. (2017). Beach-cast macroalgae were not a prominent source of TCC, and the variation was strongly imprinted by the algal composition. Macroalgae are known to biosynthesize β-carotene and other carotenoids that are specific to the algal class. These terpenoids and their oxygenated derivatives, xanthophylls are antioxidants, scavenging reactive oxygen species (ROS) throughout the metabolic processes (von Elbe and Schwartz 1996).

2.3.5.8 Beach-cast macroalgae and *Halopithys incurva* as prominent sources of phycobilins

Phycobilins are water-soluble macromolecules present in red algae, which have different bioactivities (Apt et al. 1995). Biological tests performed *in vitro* and *in vivo* have shown their performance for anti-tumour, anti-viral and anti-inflammatory activity, as well as their hepatoprotective or neuroprotective potential (Sekar and Chandramohan 2008). *Halopithys incurva* from Calhau da Serra de Fora possessed the highest content of all the three classes of phycobilins among the red algae analysed in this study. Interestingly, beach-cast macroalgae 2, which comprised 57% of *H. incurva*, had a higher phycobilin content than *H. incurva* from Calhau da Serra de Fora, possibly due to the different ecosystem dynamics (e.g. available nutrients, exposure to sunlight, hydrodynamics, grazing, epiphytes, etc) that these macroalgae were exposed to. Similar values for allophycocyanin were found in this study to those reported by Verma et al. (2017), who assessed 14 red macroalgae collected from India, but with higher values for phycocyanin and phycoerythrin than those presented in this study.

2.3.5.9 Environmental effects on fucoxanthin yield

Fucoxanthins are exclusively present in the photosynthetic complex of brown algae (Haugan and Liaaen-Jensen 1992), contributing to about 10% of the total carotenoids found in nature (Kim et al. 2012). Different environmental conditions can trigger different adaptation mechanisms, enabling strategic metabolic pathways and leading to an increase or decrease in carotenoid production. Any environmental factor that influences carotenoid production will thus influence fucoxanthin production. The brown macroalga *Sargassum vulgare* had the highest fucoxanthin content in this study. Although beach-cast macroalgae 1 had significantly more brown macroalgae (95% *Lobophora variegata*) than beach-cast macroalgae 2 (32% *Dictyota* sp. and 10.7% *L. variegata*), no significant difference in fucoxanthin content was observed between them. These results are in line with the brown macroalgae analysed in this study.

Moreover, *Dictyota dichotoma* and *L. variegata* from this study had approximately four times more fucoxanthin than the same species collected from India (Verma et al. 2017). Due to their biological and therapeutic activities (e.g. anticancer, antidiabetic, antitumour, etc.; Rajauria et al. 2016), macroalgae are screened to find new and abundant sources of these compounds.

2.3.5.10 Statistical analysis of the biochemical parameters

Correlation analysis between the biochemical parameters (Table 5) showed fifteen correlations significant at 1% and five correlations significant at 5%. Between these, three significant correlations at 1%, with the highest Pearson coefficient value were assessed between five parameters (carbohydrates, chlorophyll *a*, fucoxanthin, TCC and total minerals). Carbohydrates were found to be negatively correlated with the mineral content. Marinho-Soriano et al. (2006) also found this correlation in their assessment of two tropical macroalgae collected in the northwest of Brazil. Moreover, in a previous study, Nunes et al. (2017), examining seven macroalgae from the Madeira Archipelago, also found two similar correlations, the previously mentioned negative correlation between carbohydrates and minerals and a significant positive correlation between chlorophyll *a* and TCC. Additionally, fucoxanthin was found to have a positive correlation with TCC. This could be attributed to the fact that fucoxanthin contributes to the total amount of carotenoids in macroalgae resources.

Principal component analysis closely related the phycobilins with protein content. Verma et al. (2017) reported similar results. Phycobilins are linked to proteins, forming phycobiliproteins, which are light-harvesting pigment-protein complexes present in red algae (Sudhakar et al. 2015). In the projection of algal samples (Figure 3), several small groups were formed, due to their biochemical similarity. A total of eight groups and one single sample were displayed in the four quadrants. *Dasycladus vermicularis* and *Corallina officinalis* formed one group, as did the *Asparagopsis taxiformis* and *Halopithys incurva* collected in different locations. Also, the two species of *Ulva* were located close together, but BCM 2 did not form any group with the remaining macroalgae. The dendrogram (Figure 4) elucidates the biochemical relations between this heterogeneous group of macroalgae and beach-cast macroalgae. These samples formed three distinct clusters, based on their biochemical analysis, and environmental conditions were found to influence their linkage distance. *Halopithys incurva* from the two locations, Calhau da Serra de Fora and Abas do Rio, were closely related, but *A. taxiformis* from Abas do Rio and Praia do Zimbralinho were separated between clusters 2 and 3, in contrast to their close positions in Figure 3. This discrepancy can be attributed to the two different statistical analyses. Furthermore, the two samples of beach-cast macroalgae were statistically different from each other in terms of their phycobilin, total mineral and TPC content, and this demonstrates that if this resource is considered for biocompound extraction or any economic utilization, a deeper study is needed to understand the compositional variability of these resources throughout the year, to improve its industrial integration.

2.3.6 Conclusion

The green macroalga *Ulva intestinalis* is a good potential source of protein, lipids, carbohydrates and chlorophyll *a*. *Ulva* as a genus is permitted for food consumption in the European Union, and it can be harvested in offshore seabream aquaculture on Madeira Island. This macroalga has the potential to be upscaled into an integrated *Ulva*-fish aquaculture system, increasing profitability by providing an additional commercial resource and reducing nitrate emissions to the environment, by capturing this nutrient. The brown macroalga *Dictyota dichotoma* has potential as a source of lipids and could be used to produce lipid-related supplements. Similarly, the red macroalga *Halopithys incurva* can be used for phycobilin extraction. This species could be used for extracting these compounds with a lower purity index for the food industry, functioning as a natural dye or as a source of bioactive compounds. The brown macroalga *Sargassum vulgare* has potential as a source of fucoxanthin and can be used to produce rich fucoxanthin extracts using "Green Chemistry" techniques, supplements or additives for food products. Beach-cast macroalgae were found to be a source of several biocompounds, but their value is strongly dependent on their algal composition, which is unpredictable; further study is needed here.

Several significant correlations were found including the negative correlation between carbohydrates and minerals, and positive correlations between TCC, chlorophyll *a* and fucoxanthin. Principal component analysis and dendrogram analysis helped to determine the biochemical parameters that strongly characterize these macroalgae and their biochemical groups. It is of the utmost importance to determine the biochemical composition of these new bioresources, thus determining the potential applications and strategies appropriate to each resource. Moreover, multi-compound extraction could be optimized for these resources, implementing biorefinery strategies to maximize extraction and improve income and sustainability. It is important to link this biochemical knowledge with an efficient and eco-friendly extraction design in order to build a profitable and sustained, long-lasting industry.

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2.4 Validation of a spectrophotometric methodology for a rapid iodine analysis in algae and seaweed casts

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2.3.8 Abstract

Iodine plays an important role in human metabolism and its deficiency is particularly harmful in pregnancy and childhood. It remains a major public health concern in many countries, especially in Portugal. The main purpose of this work was to develop a validated spectrophotometric analysis for a fast and reliable iodine quantification in algal samples. Absorbance was determined at 410 nm demonstrating a good linearity ($R^2 \approx 1.0$) in the range of 0 – 0.06 mg I/ 100g. LOD and LOQ were 1.7×10^{-3} and 5.0×10^{-3} mg I/ 100g, respectively. Accuracy was determined using recovery and varied between 101 and 118 %. For precision analysis, an intra-day test performance (RSD = 8.7 %) and a repeatability assay (RSD = 3.8 %) were performed. Matrix effect assessment demonstrated that this had a negligible effect (3.2 %) in the iodine quantification. The spectrophotometric method was externally validated, for iodine quantification in algal samples, by INSA certified laboratory. The correlation coefficient between external iodine quantification and our work was $R^2 \approx 0.9$, showing a good correlation. Applicability was assessed in 25 macroalgae species (5 green, 9 red and 11 brown), 12 seaweed casts, collected in Canary Islands and 1 microalga (*Isochrysis galbana*) provided by ITC (Instituto Tecnologico de Canarias).

2.4.2 Introduction

In many areas of the world, soil surface is becoming gradually impoverished in iodine content due to leaching processes (EFSA, 2006). This important mineral is essential for human biochemistry and physiology due to its incorporation in thyroid hormones T3 (3,5,3-triiodothyronine) and T4 (thyroxine or 3,5,3,5-tetraiodothyronine), which, in turn, are responsible for the regulation of several processes of cellular metabolism and energetic balance, including mitochondrial metabolism, thermoregulation, and the catabolism of carbohydrates, lipids and protein (Schroeder et al., 2014). Iodine is particularly needed during the early stages of growth and maturation of most organs (EFSA, 2006). Iodine is now recognized as playing a protective role against fibrocystic breast disease and breast cancer (Patrick, 2008). A relationship has also been hypothesized between iodine deficiency and a number of other health issues such as attention deficit hyperactivity disorder, psychiatric disorders (Nhmrc, 2005) and nonspecific disease categories such as chronic fatigue and depressed immunity (El Din and El-Sherif, 2012). Due to the high iodine content in seaweed, its supplementation use increases the thyroid stimulating hormone (TSH) in healthy postmenopausal women (Teas et al., 2009) and slightly decreases serum free thyroxine FT4 (Miyai et al., 2008; Mohamed et al., 2012).

Iodine deficiency remains a major public health concern in many countries, including some European ones (Andersson et al., 2007; Andersson et al., 2012). In 2011, it was estimated that 44 % of the European population, which would be about 393 million people, had insufficient iodine intake, evidenced by the concentration of urinary excretion less than 100 µg/ L (Andersson et al., 2012). Mainland Portugal and Portuguese islands are no exception. According to Limbert et al. (2010), in a study with 3631 pregnant women, 83.2 % in the mainland and 94.6 % in Madeira and Azores islands have a urinary iodine content below 150 µg/ L. The proximity to the sea does not prevent iodine deficiency. The same study was carried out on 311 children from Madeira Island and 676 children from Azores of both sexes, aged between 6 and 12 years, with 67.8 % and 78.4 %, respectively, showing insufficient iodine excretion. The high rainfall, with its leaching effect removing iodine from soils, may explain part of the iodine deficiency found. This gives seaweed great potential as a health promoting ingredient in the functional food industry (Holdt and Kraan, 2011). Also, seaweed extracts could be used to bio-fortify soils and for animal production, allowing an indirect path for iodine supplementation of man food intake.

New analytical methods were developed over the years, with state-of-the-art equipment's that efficiently analyze the iodine content and determine the iodine speciation in several different biological samples. These methodologies include neutron activation analysis, atomic absorption spectrometry, inductively coupled plasma with optical emission spectrometry (ICP-OES), x-ray fluorescence, electrochemical, potentiometric probes and inductively coupled plasma with a mass spectrometer (ICP-MS) (Brix et al., 2017; Jerše et al., 2018). But all of these equipment's are not available for most of the laboratories, due to its prohibitive costs or maintenance. When iodine speciation is not required, the spectrophotometric method for iodine analysis in algae samples, properly validated, still represent a significant advantage due to its low cost and its fairly easy methodology.

The main purpose of this research was to conjugate previous published methodologies based on Sandell and Kolthoff (1937) method for iodine quantification and develop a validated spectrophotometric

method, based on available low-cost equipment present in most laboratories, to enable a rapid and reliable quantitation of the iodine content in algae samples.

2.4.3 Materials and methods

2.4.3.1 Seaweeds from Madeira Archipelago

Samples of 25 seaweeds were collected in a 10-meter maximum depth dive in the Madeira archipelago. The following green seaweeds (Chlorophyta) were collected: *Caulerpa racemosa* (Forsskål) J.Agardh 1873: 35, *Dasycladus vermicularis* (Scopoli) Krasser in Beck & Zahlbruckner 1898: 459, *Ulva intestinalis* Linnaeus 1753: 1163, *Ulva lactuca* Linnaeus 1753: 1163 and *Ulva* sp. Red seaweeds (Rhodophyta) comprise *Asparagopsis armata* Harvey 1855: 544, *Asparagopsis taxiformis* (Delile) Trevisan 1845: 45, *Chondrus crispus* Stackhouse 1797: xxiv, *Corallina officinalis* Linnaeus 1758: 805, *Galaxaura rugosa* (J.Ellis & Solander), *Grateloupia lanceola* (J.Agardh) J.Agardh 1851: 182, *Halopithys incurva* (Hudson) Batters 1902: 78, *Laurencia obtusa* (Hudson) J.V.Lamouroux 1813: 130 and *Nemalion elminthoides* (Velley) Batters 1902: 59. Brown seaweeds (Phaeophyta) include *Cystoseira compressa* (Esper) Gerloff & Nizamuddin 1975: 342, *Cystoseira humilis* Schousboe ex Kützing 1860: 18, *Cystoseira usneoides* (Linnaeus) M.Roberts 1968: 259-261, *Dictyopteris polypodioides* (A.P.De Candolle) J.V.Lamouroux 1809: 332, *Dictyota dichotoma* (Hudson) J.V.Lamouroux 1809: 42, *Halopteris filicina* (Grateloup) Kützing 1843: 293, *Halopteris scoparia* (Linnaeus) Sauvageau 1904: 349, 377, *Lobophora variegata* (J.V.Lamouroux) Womersley ex E.C.Oliveira 1977: 217, *Padina pavonica* (Linnaeus) Thivy in W.R.Taylor 1960: 234, *Sargassum vulgare* C. Agardh 1820: 3 and *Zonaria tournefortii* (J.V.Lamouroux) Montagne 1846: 32.

The samples were collected in summer and transported in seawater to laboratory and gently rinsed with filtered fresh water. Afterwards, the seaweeds were frozen at -35 ºC and freeze-dried under reduced pressure (4 x 10^{-4} mbar), with a cooling trap set at -56 ºC for 5 days. Lyophilized samples were milled to 200 mesh particle size, vacuum packed and stored at -35 ºC until use.

2.4.3.2 Seaweed beach cast and *Isochrysis galbana*

Seaweed beach casts were collected in Canary Islands, in the north shore of the island of Gran Canaria in “Playa de Las Canteras”, from November 2016 till October 2017. These were collected in the same day of appearance, transported, washed with fresh water and sun-dried. Also, two lots of *Isochrysis galbana* Parke 1949:265, a brown microalgae (Haptophyta) cultivated and harvested at the “Instituto Tecnológico de Canarias” (ITC) were included in this study. *I. galbana* was originally cultivated in a chamber with two posterior inoculations to small scale raceways and finally transferred into a 6000 L raceway inside a greenhouse with light intensity varying between 400 and 1700 µmols photons m^2 s^{-1}. Culture medium was a standard f/2 composed in seawater.Iodine quantification

Iodine assessment was initiated with seaweed incineration, adopting the method described by Mahesh et al. (1992) with modifications. About 0.5 g of sample was weighted into porcelain crucibles and 0.5 mL

of potassium hydroxide solution (6 M) mixed with a metal rod and placed in an oven at a temperature of 95 °C, during 1h. Afterwards, 0.5 mL of zinc sulphate solution (0.5 M) was added and the sample returned to the oven for a 1 h at 95 °C. The crucibles were then placed in a muffle furnace to reach the 600 ºC for a period of 1h. After, it was reconstituted in deionized water, filtered to remove particles and the volume adjusted accordingly. The oxidation process and spectrophotometric measurement were performed according to Pino et al. (1996) with modifications. Aliquots of 0.2 mL of sample or standard were added to test tubes, followed by addition of 1.0 mL of ammonium persulfate (1 M). Samples underwent a process of oxidation during 30 min in a water bath at 95 °C. Subsequently, 2 mL of arsenic acid (25.3 x 10^{-3} M), 1 mL of sulfuric acid (1.3 M) and 1 mL of water were sequentially added. The tubes were placed in a water bath at 32 °C for 10 min, then 0.5 mL of ceric ammonium sulphate (15.8 x 10^{-3} M) was added, vortexed and placed again in the water bath for 10 min. A standard solution A performed with KIO_3 to a concentration of 7.9 M (1000 µg/ mL). Standard solution B prepared by diluting the standard solution A to 100 times to an iodine concentration of 78.7 mM (10.0 µg/ mL). Working standards with concentration of 0.8, 1.6, 2.4, 3.2, 3.9 mM were prepared in distilled water. After incubation the transmittance of the samples or standards was read at 410 nm in a spectrophotometer. The calibration curve was prepared daily by placing transmittance (%) against iodine concentration (mg of iodine per 100g). All samples were analysed in triplicates in all tests carried out.

2.4.3.4 Validation methodology

To validate the spectrophotometric iodine quantification method, linearity, sensitivity, accuracy, precision, matrix effect and applicability were determined. Linearity included a linear regression, linear concentration range and correlation coefficient. Sensitivity included limit of detection (LOD) and limit of quantification (LOQ). Accuracy was performed evaluating the recovery percentage of spiked samples, adding to the incinerated biomass standard solutions of KIO_3 with several concentrations (0.01 – 0.05 mg I/ 100g) to assess instrument recovery capability. Precision included the analysis of intra-day variability and repeatability and results are provided in relative standard deviation (RSD, %). Intermediate precision was performed calculating the recovery yield using different spectrophotometers (Shimadzu PC-1601 and Shimadzu PC-2401) and calibration curves in different days. Matrix effect (% ME) was also calculated to determine if the seaweed matrix interfered in the iodine extraction and this was calculated relating the slope of a fortified sample (before incineration) with different concentrations of KIO_3 and the slope of a standard curve of KIO_3 (equation 1). Sample incineration was measured for its variability robustness, using different times. Applicability of the validated methodology was assessed in 26 algae and 12 seaweed beach cast samples. All samples were analyzed in triplicates in all tests carried out.

$$\%\ \mathrm{ME} = \left(\frac{\text{slope fortified sample}}{\text{slope standard curve}} \times 100\right) - 100 \qquad \text{(Equation 1)}$$

Nine samples were assessed in triplicate by an external laboratory (INSA – Instituto Nacional de Saúde Dr. Ricardo Jorge), certified for iodine analysis in biological samples to determine the analytical deviation of the iodine content between an ultraviolet – visible (UV-Vis) spectrophotometric method (this work) and a validated inductively coupled plasma mass spectrometric method (ICP-MS - INSA).

2.4.3.5 Statistical analysis

All values are expressed as mean of three replicates ± standard deviation except for the LOD and LOQ assessment performed to *I. galbana*, which were performed ten replicates. The statistical data analysis was performed, using SPSS 24.0 program for Windows. Data were analysed using one-way analysis of variance (ANOVA) and determined its homoscedasticity. Tukey's b test ($p \leq 0.05$) was performed to determine statistical variance between algae and seaweed beach casts. Also, using Excel from Microsoft Office 2013, we have performed several linear regressions.

2.4.4 Results and discussion

2.4.4.1 Methodology validation

Prior to the iodine content measurement in the selected algae and seaweed beach cast samples, the spectrophotometric method was properly validated (Table 1 and 2). Linearity, sensitivity, accuracy, precision and matrix effect were determined at 410 nm wavelength, plotting transmittance against the concentration of iodine.

Table 1 – Summarized validation results obtained for the iodine quantification through the spectrophotometric methodology.

	Parameter	**Result**
Linearity	Linear regression (y = mx + b)	703.6x + 34
	Linear concentration range	0 – 0.06 mg I/ 100g
	R^2	1.0
Sensitivity	LOD (standard analytical solution 0.01 mg I/100g)	1.7×10^{-3} mg I/ 100g
	LOQ (standard analytical solution 0.01 mg I/100g)	5.0×10^{-3} mg I/ 100g
	LOD (sample with lowest iodine content, *Isochrysis galbana*, 10 readings)	6.0×10^{-2} mg I/ 100g
	LOQ (sample with lowest iodine content, *Isochrysis galbana*, 10 readings)	1.8×10^{-1} mg I/ 100g
Accuracy	Recovery	%
	SW + 0.01 mg I/ 100g	103
	SW + 0.02 mg I/ 100g	118
	SW + 0.03 mg I/ 100g	104
	SW + 0.04 mg I/ 100g	101
	SW + 0.05 mg I/ 100g	102
Precision	Intra-day (% RSD)	8.7
	Repeatability (% RSD)	3.8
Matrix effect	Variation (%)	3.2

All determinations were carried out in triplicate except the LOD and LOQ using *Isochrysis galbana*, which 10 readings were performed.

Table 2 – Macroalgae samples fortified with standard analytical solutions of KIO_3 from 50 to 200 % of its iodine content, before incineration.

Macroalgae	**KIO_3 fortification** (%)	**Result** (%)	**Difference** (%)
Ulva sp.	50	53.6	3.6 (+)
(Green)	100	94.8	5.2 (-)
	200	185.8	14.2 (-)
Grateloupia lanceola	50	51.5	1.5 (+)
(Red)	100	102.5	2.5 (+)
	200	193.6	6.4 (-)
Cystoseira usneoides	50	61	11 (+)
(Brown)	100	99.0	1 (-)
	200	184.8	15.2 (-)

All determinations were carried out in triplicate. Signal + or – indicate if the difference in the result % is positive or negative, respectively.

Linearity was achieved plotting five different concentrations of KIO_3 and including a blank. The equation obtained was $y = 703.6x + 34$ with a correlation coefficient (R^2) close to 1.0 (Fig. 1).

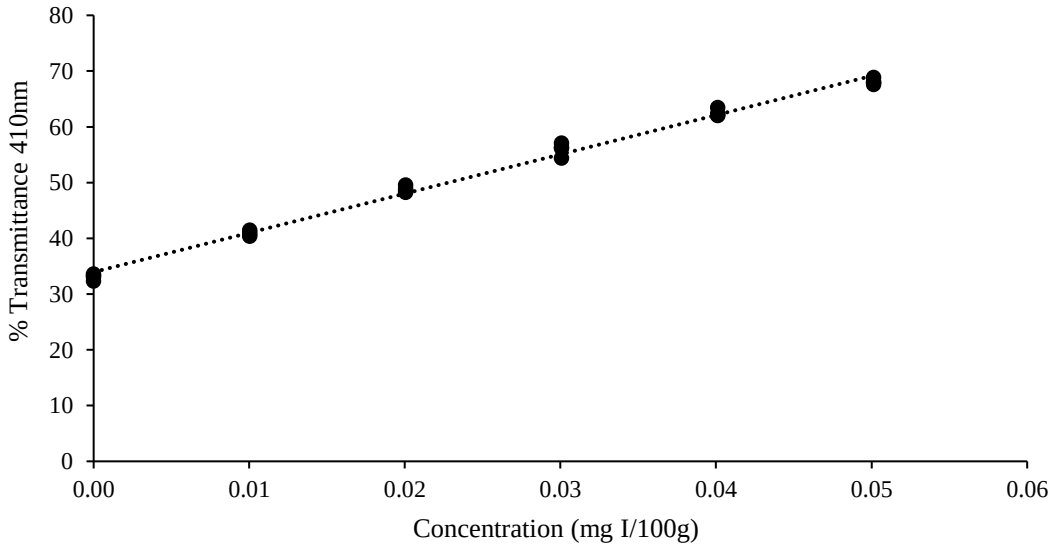

Fig. 1 – Linear regression of potassium iodide determining the relation between concentration of compound (iodine) and transmittance (%) in the spectrophotometer. All determinations were carried out in triplicate.

Linear concentration ranged between 0 and 0.05 mg of I/ 100g. Sensitivity included a limit of detection (LOD) of 1.7 x 10^{-3} mg I/ 100g and a limit of quantification (LOQ) of 5.0 x 10^{-3} mg I/ 100g, when evaluating a macroalgae sample (*U. lactuca*), fortified with the lowest concentration of the standard analytical solution (0.01mg I/ 100g) and subjected to the entire procedure. The macroalgae iodine content is subtracted to the total iodine determined and LOD is calculated by multiplying the standard deviation (σ) by 3.3 and LOQ multiplying the standard deviation (σ) by 10. Also, we have calculated the LOD and LOQ, using the sample with the lowest iodine content (*I. galbana*), analysed it 10 times and applying the same

equations as before, the LOD was 6.0 x 10^{-2} mg I/ 100g and LOQ 1.8 x 10^{-1} mg I/ 100g. All samples were above the LOQ calculated in this work. Accuracy was performed, spiking incinerated samples with standard solutions of KIO_3 with several concentrations (0.01 – 0.05 mg I/ 100g) and recovery values varied between 101 and 118 %. Precision was determined calculating the intra-day variability of spiked samples, determining a value of 8.7 % and repeatability using the same sample measured for five times in the same day resulted in a relative standard deviation (RSD) of 3.8 %. The matrix effect was calculated to determine the interaction that seaweed matrix has on iodine assessment and found to have a 3.2 % variability. Also, for validation purposes, we have performed the fortification of three macroalgae (1 green, 1 red and 1 brown) with 50, 100 and 200 % of its iodine content, with KIO_3 standard solutions, before incineration (table 2). This was performed to assess recovery of the added iodine, measuring the method efficiency. For 50 % fortification, values determined were between 51.5 and 61 %. When 100 % fortification is implemented, 94.8 to 102.5 % could be assessed. Highest fortification (200 %), iodine recovery oscillated between 184.8 and 193.6 %. These recovery values were considered suitable for the proposed methodology since the fortification process is implemented before incinerating the samples. The intermediate precision was evaluated by assessing the recovery yield, using the described method with two spectrophotometers, with different calibration curves in different days. The recovery yield graphic (Fig. 2) plots the theoretical *versus* real iodine concentrations with the measurement of concentration for each point.

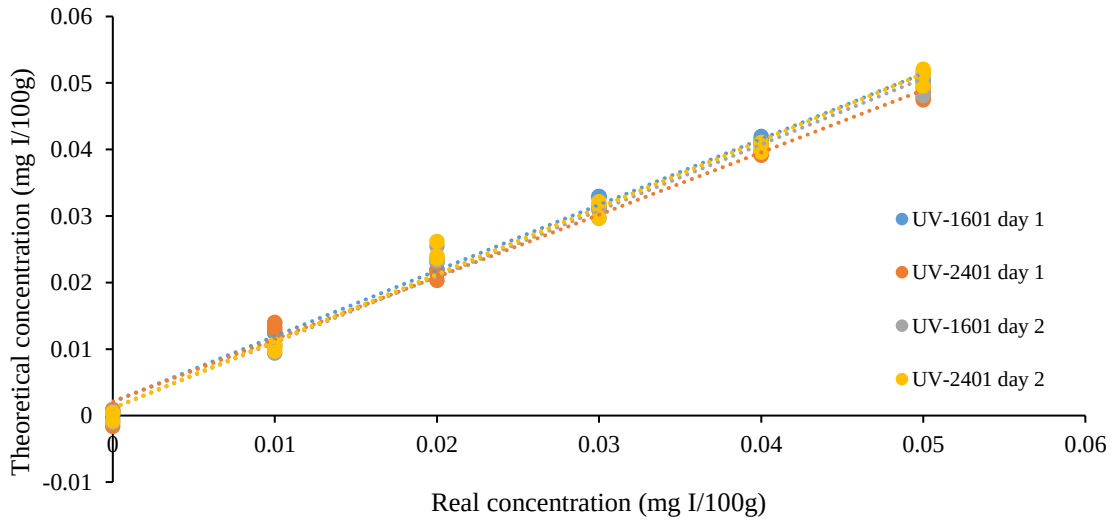

Fig 2 – Plot of theoretical and real (measured) concentration of iodine in the recovery yield testing. Spiked samples were measured using two different spectrophotometers (UV-1601 and UV-2401) in two different days. Values for plotting originate from subtracting the seaweed iodine content, remaining the fortified iodine content. All determinations were carried out in triplicate.

The obtained concentration represents the amount of iodine used in increasing fortification of spiked samples, with the amount of the seaweed iodine calculated and subtracted to the total iodine concentration. The R^2 for the four linear regressions was around 1.0 which resulted in an excellent fitting. The incineration time was tested for its robustness, to determine if incineration time would influence iodine content in seaweed samples (Table 3). The times tested were 1h, 1:30h, 2h and 2:30h. The average iodine content was 10.6 ± 0.3 mg I/ 100 g of dry weight (dw) with an RSD of 3%. These results demonstrated that samples incineration was complete with this methodology and that increasing the incineration time would

not influence the mineralization of the samples. This assessment is of extreme importance, since some iodinated compounds could be volatized by the high temperatures. For biological matrices, dry incineration should be performed in the presence of alkaline agents to transform iodinated compounds into non-volatile species (Kučera and Krausová, 2007).

Table 3 – Assessment of the iodine content, variating the time of incineration in the muffle oven at 600 ºC, to test the applicability of the ultraviolet – visible (UV-Vis) spectrophotometry to seaweed matrices.

Nº	Time of incineration	Mean ± SD (mgI/100g of dw)	RSD (%)
1	1h	10.3 ± 0.7	6.4
2	1h30m	10.4 ± 0.3	2.4
3	2h	11.1 ± 1.2	6.6
4	2h30m	10.6 ± 0.5	4.7
Average		10.6 ± 0.3	3.0

Data are mean ± standard deviation in milligrams per 100 grams of algae on a dry weight basis (dw). All determinations were carried out in triplicate.

To validate the spectrophotometric methodology, the iodine content of nine algal samples was determined using inductively coupled plasma mass spectrometry (ICP-MS). The ICP-MS methodology have higher sensitivity and lower detection limit, and can be used to analyse different types of samples. Normally, the iodine is analysed in alkaline medium, adding ammonia solution, to form a non-volatile NH_4I (Brix et al., 2017; Jerše et al., 2018). The comparison between these two techniques are described in detail in Table 4 and graphically represented in Figure 3.

Table 4 – Comparison of the iodine content of 9 selected macroalgae samples determined using UV-Vis spectrophotometry and ICP-MS. The ICP-MS analysis has performed with the assistance of INSA (Instituto Nacional de Saúde Dr. Ricardo Jorge).

Scientific name	Spectrophotometer (Our study) (mg I ± SD/ 100g dw)	ICP-MS (INSA) (mg I ± SD/ 100g dw)
Asparagopsis taxiformis	1162.7 ± 33.1	1268 ± 124
Cystoseira humillis	6.8 ± 0.1	5.7 ± 0.2
Galaxaura rugosa	41.8 ± 0.9	24.5 ± 0.1
Grateloupia lanceola	13.6 ± 0.2	8.2 ± 0.5
Halopteris scoparia	71.3 ± 1.3	85.8 ± 8.1
Padina pavonica	6.7 ± 0.2	5.7 ± 0.2
Ulva intestinalis	6.3 ± 0.2	5.6 ± 0.3
Zonaria tournefortii	16.9 ± 0.3	15.4 ± 1.2
SC 14.11.2016	24.7 ± 0.4	27.7 ± 2.7

Data are mean ± standard deviation in milligrams per 100 grams of algae on a dry weight basis. All determinations were carried out in triplicate. SC-Seaweed Cast. Instituto Nacional de Saúde Dr. Ricardo Jorge is a certified Portuguese institute for iodine analysis.

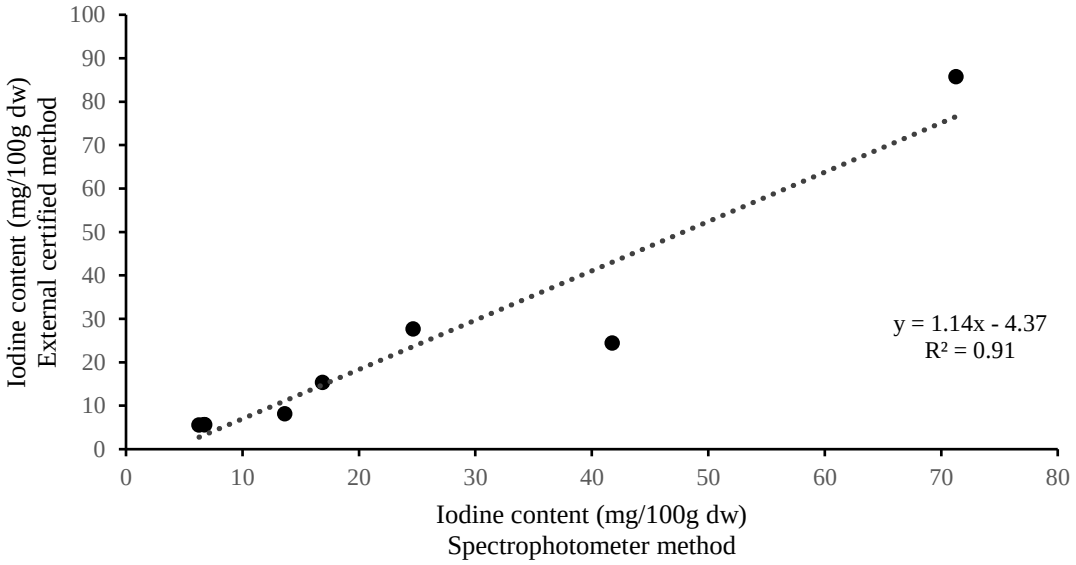

Fig. 3 – Linear regression model determining the relation between the iodine content determined in 8 macroalgae samples analyzed by an external certified method, using an ICP/MS (INSA) and our work (spectrophotometer method). All determinations were carried out in triplicate.

The comparison of the iodine yields obtained by these two methodologies showed that they were very close, although some discrepancies were detected more prominently in *Galaxaura rugosa* and *Grateloupia lanceola*, with around 40% deviation between techniques. Judprasong et al. (2016) also compared the ICP-MS and spectrophotometric technics, to assess the iodine content in some selected Thai foods, using a previous alkali ashing. It is referred in this work that some bias effect is to be expected from the spectrophotometric technic when iron and/or sodium is present, which can produce positive iodine content variance. This false positive effect is also described by Todorov et al. (2018), which also compared these two technics to assess the iodine content in general food products. The measurement of remaining samples showed similar results of iodine content, with deviations between 8 and 17%, representing a positive validation of the spectrophotometric method. The linearity between spectrophotometric and ICP-MS results, using 8 samples, was strong with an R^2 around 0.9 (Fig. 3). The ninth sample that we have not introduced in the linear regression is the red macroalgae *A. taxiformis*. Due to its extreme high iodine content, it was not suitable for this regression. However, the iodine content determined by spectrophotometric and ICP-MS was 1162.7 ± 33.1 and 1268 ± 124 mg I/ 100 g dw, respectively. These results have a difference of 105.3 mg, around 9.1%, meaning that they are very similar to each other. Other methods for iodine quantification and speciation were developed and validated over the years. Nitschke and Stengel (2015) developed an isocratic method using high performance liquid chromatography (HPLC) to quantify iodine in macroalgae and macroalgae products as iodide (I^-) with an ultraviolet (UV) detector set to 223 nm, with a previous dry alkaline incineration. Yeh et al. (2014) also developed a validated methodology using gas chromatography linked to an electron capture detector (GC-ECD) to quantify iodine in macroalgae samples, previously derivatized with 3-pentanone. Sun et al. (2015) developed a pressure-driven capillary electrophoresis methodology using an ion-pairing reagent to improve iodine speciation and discriminate between iodine containing molecules in macroalgae. This technique was externally validated with HPLC-ICP-MS, achieving good results.

2.4.4.2 Method applicability

Marine systems and organisms, such as seaweeds, contain and bio accumulate the majority of the available iodine (Saenko et al., 1978). Chemical iodine species in seaweeds seems to be mainly I^-, organic iodine and in minor amounts IO_3^- (Hou et al., 1997). Twenty five seaweed samples, representative of the algal diversity of Madeira Archipelago, were assessed for their iodine content, comprising 5 green, 9 red and 11 brown seaweeds (Table 5). Green seaweed presented an average of 9.8 mg I/ 100g dw, which varied between 2.6 ± 0.2 mg I/ 100g dw and 22.0 ± 0.3 mg I/ 100 g dw, in *Ulva* sp. and *C. racemosa*, respectively. Hou and Yan (1998), working with neutron activation analysis, have determined several inorganic elements in 35 seaweed species collected in China, in which *U. pertusa* contained between 1.3 and 3.3 mg I/ 100 g dw, *U. lactuca* 5.4 mg I/ 100 g dw and *U. intestinalis* 11.4 mg I/ 100 g dw. These results fit closely into the range described in this work. Red seaweed showed an average of 331.3 mg I/ 100 g dw, varying between 13.6 mg I/ 100 g dw and 1162.7 mg I/ 100 g dw, for *G. lanceola* and *A. taxiformis*, with the last specie presenting the highest iodine content. *A. taxiformis* was collected in three different locations of Madeira archipelago, two in Porto Santo and one in Madeira. Across sites, this alga specie, constantly presented considerably high iodine content when compared with the remaining seaweeds, 936.4 ± 172.4 mg I/ 100 g dw. This specie was previously studied by Kaliaperumal (2003) and identified as a “seaweed rich in iodine”

used to ameliorate goitre disease. McConnell and Fenical (1977) determined the biological purposes of the halogenated metabolites in *A. taxiformis*, which are kept in specialized gland cells (Paul et al., 2006), frequently functioning as protection compounds or as antioxidants. These are connected with environmental adaptations, since *A. taxiformis* usually grows where high number of seaweed herbivores occurs, in subtropical and tropical waters. Research performed in India reported a high iodine accumulation by *A. taxiformis*, 499.3 mg I/ 100 g dw, and *Asparagopsis* genus, 556.7 mg I/ 100 g dw (CMFRI, 1987). Hou and Yan (1998) also determined that *Corallina pilulifera* possess 16.1 mg I/ 100g dw, very near to our values for *Corallina officinalis*, 18.1 ± 0.3 mg I/ 100 g dw, suggesting a close genetic relation in iodine bioaccumulation among seaweeds of this genus. Comparing with red seaweeds already in the food markets, these usually contain lower iodine concentration, between 7.2 to 29.3 mg I/ 100 g dw in *Palmaria* sp. (Roleda et al., 2018) and 2.9 to 4.6 in *Porphyra* sp. seaweeds (Yeh et al., 2014). In this work, brown seaweeds presented an average iodine content of 30.1 mg I/ 100 g dw, ranging between 5.8 ± 0.2 mg I/ 100g dw and 71.3 ± 1.3 mg I/ 100g dw in *Cystoseira usneoides* and *Halopteris scoparia*, respectively. *Cystoseira* species presented the lowest iodine values in contrast to the *Halopteris* species. *Sargassum vulgare* included in this work had an iodine content of 62.3 ± 1.4 mg I/ 100 g dw very close to the lowest content described by Hou and Yan (1998), which included in their work 6 *Sargassum* species varying from 11.1 to 593.9 mg I/ 100 g dw, demonstrating a very broad iodine content for this genera. These results are comparable with extensively commercialized brown seaweed species such as *Alaria* sp., ranging from 17.1 till 107 mg I/ 100 g dw, *Saccharina* genus, from 155.6 to 720.8 mg I/ 100 g dw (Roleda et al., 2018), *Undaria* varying from 9.4 till 18.5 mg I/ 100 g dw and *Laminaria* ranging from 24.1 till 492.1 mg I/ 100 g dw (Yeh et al., 2014).

For microalga *Isochrysis galbana*, the average value for iodine content was determined to be 0.32 ± 0.08 mg I/ 100 g dw, varying from 0.25 ± 0.03 mg I/ 100 g dw and 0.39 ± 0.04 mg I/ 100 g dw (Table 5). Although other works assessing *Isochrysis galbana* iodine content are not known, other microalgae species were already evaluated. For instance *Arthrospira platensis* Gomont, a cyanobacteria, was evaluated by Mosulishvili et al. (2002) and was determined to reach a maximum of 200 mg I/ 100 g dw, but the evaluation performed by Romarís-Hortas et al. (2012) only determined 1.2 mg I/ 100 g dw. Niedobová et al. (2005) evaluated *Chlorella* genus using inductively coupled plasma - optical emission spectrometry (ICP-OES) in vacuum UV methodology and determined that iodine content can vary from 10 to 130 mg I / 100 g dw. It seems that iodine content can fluctuate in microalgae depending in the abiotic and biotic factors inherent to their environment or cultivation conditions.

Table 5 – Iodine content in 26 species of algae, with 25 macroalgae collected in Madeira archipelago and 1 species of microalgae cultivated by ITC (Instituto Tecnologico de Canarias).

Scientific name	Color	Collection site	Island	Iodine (mg ± SD/ 100g dw)
Caulerpa racemosa	Green	Calheta	Porto Santo	22.0 ± 0.3^{ab}
Dasycladus vermicularis	Green	Calhau da Serra de dentro	Porto Santo	5.3 ± 0.8^{a}
Ulva intestinalis	Green	Campanário	Madeira	6.3 ± 0.2^{a}
Ulva lactuca	Green	Santa Cruz	Madeira	12.9 ± 0.5^{ab}
Ulva sp.	Green	Porto das Salemas	Porto Santo	2.6 ± 0.2^{a}
Average				**9.8**
Asparagopsis armata	Red	Reis Magos	Madeira	938.7 ± 5.6^{e}
Asparagopsis taxiformis	Red	Abas do Rio	Porto Santo	833.1 ± 5.8^{d}
Asparagopsis taxiformis	Red	Praia do Zimbralinho	Porto Santo	813.5 ± 47.1^{d}
Asparagopsis taxiformis	Red	Reis Magos	Madeira	1162.7 ± 33.1^{f}
Corallina officinalis	Red	Praia do Zimbralinho	Porto Santo	18.1 ± 0.3^{ab}
Chondrus crispus	Red	Santa Cruz	Madeira	22.1 ± 0.7^{ab}
Galaxaura rugosa	Red	Reis Magos	Madeira	41.8 ± 0.9^{bc}
Grateloupia lanceola	Red	Campanário	Madeira	13.6 ± 0.2^{ab}
Halopithys incurva	Red	Abas do Rio	Porto Santo	42.5 ± 1.2^{bc}
Halopithys incurva	Red	Calhau da Serra de fora	Porto Santo	61.8 ± 0.7^{c}
Laurencia obtusa	Red	Porto das Salemas	Porto Santo	13.6 ± 0.5^{ab}
Nemalion elminthoides	Red	Baía D'Abra	Madeira	13.9 ± 0.6^{ab}
Average				**331.3**
Cystoseira compressa	Brown	Porto das Salemas	Porto Santo	13.2 ± 0.7^{ab}
Cystoseira humillis	Brown	Seixal	Madeira	6.8 ± 0.1^{a}
Cystoseira usneoides	Brown	Porto das Salemas	Porto Santo	5.8 ± 0.2^{a}
Dictyopteris polypodioides	Brown	São Vicente	Madeira	10.0 ± 0.1^{a}
Dictyota dichotoma	Brown	Abas do Rio	Porto Santo	8.2 ± 0.1^{a}
Halopteris filicina	Brown	Abas do Rio	Porto Santo	66.0 ± 0.4^{c}
Halopteris scoparia	Brown	Baía D'Abra	Madeira	71.3 ± 1.3^{c}
Lobophora variegata	Brown	Porto dos Frades	Porto Santo	64.2 ± 2.5^{c}
Padina pavonica	Brown	Seixal	Madeira	6.7 ± 0.2^{a}
Sargassum vulgare	Brown	Santa Cruz	Madeira	62.3 ± 1.4^{c}
Zonaria tournefortii	Brown	Reis Magos	Madeira	16.9 ± 0.3^{ab}
Average				**30.1**
Isochrysis galbana 12.05.2017	Brown	ITC	Gran Canaria	0.3 ± 0.0^{a}
Isochrysis galbana 26.05.2017	Brown	ITC	Gran Canaria	0.4 ± 0.0^{a}
Average				**0.3**

Data are mean ± standard deviation in milligrams per 100 grams of algae on a dry weight basis. All determinations were carried out in triplicate. Different letters indicate significant differences ($p \leq 0.05$) determined in SPSS 24.0 using Tukey b test. SC-Seaweed Cast

Twelve samples of seaweed beach casts, collected in Canary Islands, in the island of Gran Canaria, were evaluated for their iodine content. Iodine in these samples varied from 14.8 ± 0.7 mg I/ 100 g dw and 45.5 ± 1.5 mg I/ 100 g dw, averaging 32.2 ± 8.9 mg I/ 100 g dw (Table 6). Oyesiku and Egunyomi (2014) evaluated pelagic masses of *Sargassum* collected offshore in Ondo State, Nigeria and determined that iodine content was 0.04 mg I/ 100 g dw. These seaweed beach casts, also known as "seaweed tides" or floating offshore masses of seaweed, directly harm traditional fishery (Oyesiku and Egunyomi, 2014), cause financial disorder to tourism and aquaculture productions, with increasing reports over the years, justifying the need to develop mitigation strategies to reduce the negative impact (Smetacek and Zingone, 2013) and create new industrial applications for their economic development. Extracts of these seaweed masses or their direct application for soil fertilization, crop and animal production could be studied, introducing an iodine bio-fortification program to overcome iodine deficiency.

Table 6 – Iodine content in Seaweed beach casts, collected in the island of Gran Canaria.

Sample	Iodine (mg/100g ± SD dw)
SC 14.11.2016	24.7 ± 0.4^{a}
SC 29.05.2017	45.5 ± 1.5^{b}
SC 23.06.2017	31.0 ± 0.5^{c}
SC 26.06.2017	26.8 ± 0.8^{d}
SC 05.07.2017	35.8 ± 0.4^{e}
SC 12.07.2017	30.5 ± 0.6^{c}
SC 20.07.2017	39.0 ± 1.2^{f}
SC 08.08.2017	22.9 ± 0.4^{i}
SC 21.08.2017	14.8 ± 0.7^{j}
SC 18.09.2017	34.0 ± 0.6^{k}
SC 06.10.2017	40.5 ± 0.5^{fm}
SC 10.10.2017	41.2 ± 0.2^{m}
Average ± SD	**32.2 ± 8.9**

Data are mean ± standard deviation in milligrams per 100 grams of seaweed beach cast on a dry weight basis. All determinations were carried out in triplicate. Different letters indicate significant differences ($p \leq 0.05$) determined in SPSS 24.0 using Tukey b test. SC- Seaweed Cast.

2.4.5 Conclusion

The iodine yield in algae is an important nutraceutical factor for humans, since this mineral is becoming scarce in terrestrial food due to lixiviation processes and intensive cropping. Consequently, algae could become a natural supplementation of this essential mineral, preventing iodine deficiency related disorders. The validated method performed in this study demonstrated a good working range (0 – 0.06 mg I/ 100g), with an excellent correlation coefficient ($R^2 \approx 1.0$). Sensitivity was determined, achieving a low LOD (1.7×10^{-3} mg I/ 100g) and LOQ (5.0×10^{-3} mg I/ 100g) values, using the lowest concentration of the analytical standard solution. Also Accuracy was assessed calculating recovery and acceptable values were determined (101 and 118 %). Precision was considered optimal, due to intra-day (RSD = 8.7 %) and repeatability (RSD = 3.8 %) assessment. Matrix effect was considered neglectable (3.2 %) and considered not to imprint significant variation for iodine quantification. Also, fortification recovery assessment (50 – 200 %), was performed to the 3 different macroalgae colours, adding KIO_3 standard solutions before sample mineralization. Results were considered suitable, since recovery % was not distant from the initial fortified value. This indicate that iodine volatilization in the incineration procedure, oxidation process and iodine dilution do not impose a significant variation. Iodine content in algae varies greatly between different species. This is demonstrated by the range of values obtained for macroalgae, between *Ulva* sp., 2.6 ± 0.2 and *A. taxiformis*, 1162.7 ± 33.1 mg I/ 100 g dw, with the other seaweeds being distributed between these values. This is a positive remark since it allows targeting seaweeds with a specific iodine content for precise market applications. These results suggest that *A. taxiformis*, a red macroalgae, is an iodine rich source, reaching values similar to a brown macroalgae known as Kelp, from the Laminariales. *Isochrysis galbana*, on the contrary, demonstrated a very low iodine content when compared with macroalgae and could be used for people with iodine sensibility. Seaweed beach casts varied their iodine content from 14.8 ± 0.7 to 45.5 ± 1.5 mg I/ 100 g dw, which is probably due to the seaweed species variations throughout the year. The external validation, performed with the INSA assistance was extremely helpful to determine the accuracy between very different methodologies for iodine quantification. These results showed that spectrophotometric method is still a reliable technique for rapid determination of iodine content in algae samples. With this work, it evidences the potential of using seaweed or beach cast, to produce bioactive extracts that could function directly or indirectly for iodine supplementation. These could be consumed directly by humans or applied in irrigated crops or even in animal production. It is therefore important to determine the iodine level present in each sample, using a robust and reproducible method, and in the present work we have proved that the method used herein has the qualities required for this purpose. Due to the prevalent awareness of this important micronutrient and nowadays potential of functional foods, these assessments are of great importance to prevent life threatening diseases, using local products or unused biomass.

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2.5 Evaluation of fucoxanthin contents in seaweed biomass by vortex-assisted solid-liquid microextraction using high-performance liquid chromatography with photodiode array detection

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2.5.1 Abstract

Fucoxanthin is considered an important marine bioactive compound with biological properties with promising effects, namely on health. A simple and efficient analytical methodology is proposed for its quantification in seaweed biomass by using vortex-assisted solid-liquid microextraction (VASLME) followed by reversed phase high-performance liquid chromatography (RP-HPLC) photodiode array detection (PDA) analysis. This microextraction uses reduced quantities of sample (25 mg) and solvent (300 µL of ethanol) to efficiently extract this high-valued xanthophyll, in a vortex time of 15 min. These extraction parameters were optimized performing a Central Composite Design (CCD) analysis, running 32 individual experiments. In turn, the method validation was assessed. The linearity of the method was confirmed ($R^2 = 0.99998$) in a concentration range from 12 to 3600 µg.g^{-1} dw. Also, good sensitivity and accuracy results were observed through the LOD (3.33 µg.g^{-1}), LOQ (10.09 µg.g^{-1}) and recovery (varied from 95 to 97%) assessments. Good precision was also verified, with intra-day variation within 2.0 - 3.3%, and inter-day within 1.0 - 3.8%. Matrix effect was also evaluated and an acceptable variation of 3.4% was found. The method applicability was confirmed by the analysis of 22 seaweed biomass samples and fucoxanthin content was found to vary from about 10 to 853 µg.g^{-1} dw. This method demonstrated a good performance and can be successfully implemented for a rapid, reliable and accurate screening of fucoxanthin in seaweed biomass.

2.5.2 Introduction

Fucoxanthin is a high-value commercial xanthophyll (about 11 €/mg) firstly extracted by Willstätter and Page in 1914 from *Dictyota*, *Fucus* and *Laminaria* brown seaweeds, subsequently also found in other brown seaweeds and diatoms (microalgae). It is one of the most abundant carotenoids, estimated to comprise 10% of the total found in nature (Miyashita and Hosokawa, 2017). The fucoxanthin content oscillates according to season and life cycle (Balboa et al., 2013). This carotenoid is linked to chlorophyll *a* and specific proteins of these marine plants, playing an important role on their light harvesting and photoprotection (Kim et al., 2012). Its molecular structure includes an unusual allenic bond and oxygenic functional groups, that together constitute a unique arrangement (Peng et al., 2011) well developed to capture blue and green photons, predominantly present in deeper ocean waters (Caron et al., 1996). Fucoxanthin's distinct molecular structure is also responsible for its exceptional biological activity, particularly for its antioxidant properties, that are mainly related with the free radical scavenging and singlet oxygen species quenching (Miyashita and Hosokawa, 2017). These properties are quite promising on the prevention and treatment of oxidative stress-related diseases (Fariman et al., 2016). Additionally, fucoxanthin also demonstrates anti-inflammatory, neuroprotective, antiangiogenic, skin protective, anti-obesity, anti-diabetic, anti-cancer, hepatoprotective, cardiovascular and cerebrovascular protective effects (Kraan, 2013; Miyashita and Hosokawa, 2017; Gutiérrez-Rodríguez et al., 2017; Ma et al., 2017; Zhang et al., 2015). Recently, other pharmacological activities have been attributed to fucoxanthin, particularly promising for the therapy of the pulmonary fibrosis (Ma et al., 2017), cerebral ischemic/reperfusion injury (Hu et al., 2018), hyperglycemia, hyperlipidemia and insulin resistance (Zhang et al., 2018), glycaemic control (Zaharudin et al., 2019) and liver cancer (Foo et al., 2019). These biological properties suggest its high potential for application in human and animal food, health and cosmetics. Thus, there is high interest not also in fucoxanthin high purity extracts but also in fucoxanthin rich supplements to be used as a natural antioxidant for food or beverages preservation.

Due to its high potential, research developments have been improving the industrial potential to purify fucoxanthin from algae biomass. Macroalgae *Saccharina japonica* (formerly *Laminaria japonica*) waste parts have been researched to determine their potential as a commercial-scale fucoxanthin resource using conventional extraction methods (Kanazawa et al., 2008). Later, new technological approaches such as pressurized liquid methodology (Shang et al., 2011) and supercritical CO_2/ethanol extraction (Gilbert-López et al., 2015) have been suggested to obtain larger quantities of extracts rich in fucoxanthin from macro and microalgae and to overcome high production costs, namely reducing solvent-usage and time. Other authors proposed microwave (Pasquet et al., 2011) and ultrasound (Raguraman et al., 2018) assisted techniques as alternatives. Different extraction conditions have also been investigated, varying the solvent type, solvent-to-solid ratio, extraction time, temperature and the extraction technologies, including maceration, ultrasound-assisted extraction, Soxhlet extraction and pressurized liquid extraction (Kim et al., 2012; Wen et al., 2018). Most of these extraction procedures, besides being costly, time-consuming and labour-intensive, often use large amounts of solvent, generating waste and contaminating samples. Also, few studies are devoted to optimize a valid green analytical procedure for the fucoxanthin analysis in algae biomass.

Thus, the main purpose of this work was to develop a simple, fast, cost-effective and environmentally friendly analytical method to rapidly and efficiently assess the fucoxanthin content in

seaweed biomass, to determine its potential as a fucoxanthin resource. Therefore, a vortex-assisted solid-liquid microextraction (VASLME) for sample preparation before HPLC-PDA quantification is proposed.

2.5.3 Materials and methods

2.5.3.1 Chemicals

All chemicals and standards had a purity grade higher than 95%. Methanol UPLC grade and formic acid were supplied by Panreac (Barcelona, Spain), ethanol by Aga (Portugal) and fucoxanthin standard from Sigma (China). Type 1 ultrapure water was obtained with a Simplicity® UV apparatus from Millipore (Milford, MA, USA).

2.5.3.2 Sample collection and preparation

Two seaweed biomass sample sets were collected. The first comprises brown macroalgae samples from Madeira archipelago (Portugal) and Galway (Ireland), collected between the intertidal and the subtidal zone up to a 10-meter maximum depth dive. *Dictyopteris polypodioides* (A.P.De Candolle) J.V. Lamouroux 1809: 332, *Dictyota dichotoma* (Hudson) J.V.Lamouroux 1809: 42, *Halopteris filicina* (Grateloup) Kützing 1843: 293, *Halopteris scoparia* (Linnaeus) Sauvageau 1904: 349, *Lobophora variegata* (J.V.Lamouroux) Womersley ex E.C.Oliveira 1977: 217, *Padina pavonica* (Linnaeus) Thivy in W.R.Taylor 1960: 234, *Sargassum vulgare* C. Agardh 1820: 3 and *Zonaria tournefortii* (J.V.Lamouroux) Montagne, 1846 were collected in Madeira archipelago (Porto Santo and Madeira Islands). *Ascophyllum nodosum* (Linnaeus) Le Jolis 1863: 96 and *Fucus vesiculosus* Linnaeus 1753: 1158 were collected in Galway. These samples were transported in seawater and gently rinsed with filtered fresh water except for Galway seaweed, which were air-dried. Afterwards, a primary drying was applied in which seaweed was frozen at -35 ºC and freeze-dried under reduced pressure (4×10^{-4} mbar), with a cooling trap set at -56ºC for 5 days. Samples were milled to 200 mesh particle size, vacuum packed and stored at -35 ºC until use. These samples were visually identified using the book publications performed by Cabioc'h et al. (1992), Braune and Guiry (2011), Rodríguez Prieto (2013) and Pereira (2016). The second set is composed by 10 samples of beach-cast seaweed collected in the north shore of the island of Gran Canaria (Canary Islands), in "Playa de Las Canteras" from May 29 till October 10, 2017. These were air-dried, milled, packed and sent to our laboratory. The compositional details of these biomasses are presented in Table 1. Seaweed casts are masses of several seaweeds that stay stranded in beaches, affecting tourism, residents, local ecosystems and artisanal fishery. These macroalgae were identified using the book publications of Carrillo and Sansón (1999), Haroun et al. (2003) and Espino et al. (2006).

Table 1 – Composition of seaweed beach cast samples collected between May 29 and October 10, 2017, in "Playa de Las Canteras", Gran Canaria.

Seaweed code	Prospection date	Seaweed composition
1	29-May-2017	*Dictyota* sp. (32%), *H. incurva* (57%), *L. variegata* (10.7%) and others (0.3%)
2	23-Jun-2017	*A. taxiformis* (16.6%), *C. barbata* (8.4%), *Dictyota* sp. (30.6%), *Jania* sp. (30.5%) and *L. variegata* (13.9%)
3	26-Jun-2017	*A. taxiformis* (50%), *Dictyota* sp. (41.6%) and *H. scoparia* (8.4%)
4	12-July-2017	*A. taxiformis* (45%), *Dictyota* sp. (21%), *L. variegata* (25%) and *H. scoparia* (9%)
5	20-July-2017	*A. taxiformis* (34.8%), *Dictyota* sp. (39.1%), *Jania* sp. (4.3%) and *L. variegata* (21.8%)
6	8-Aug-2017	*A. taxiformis* (30%), *Dictyota* sp. (36%), *L. variegata* (24%) and *H. scoparia* (10%)
7	21-Aug-2017	*A. taxiformis* (28%), *Dictyota* sp. (42%), *L. variegata* (22%) and *H. scoparia* (8%)
8	18-Sep-2017	*A. taxiformis* (33.8%), *C. barbata* (14.5%), *Dictyota* sp. (22.6%), *Laurencia* sp. (0.5%) and *L. variegata* (28.6%)
9	6-Oct-2017	*A. taxiformis* (23.8%), *C. barbata* (22.2%), *Dictyota* sp. (22.2%) and *L. variegata* (31.8%)
10	10-Oct-2017	*C. barbata* (10%), *Dictyota* sp. (20%), *Jania* sp. (25%) and *L. variegata* (45%)

2.5.3.3 Vortex Assisted Solid-Liquid Micro-Extraction optimization

A Central Composite Design (CCD) was implemented to determine the optimum conditions of three parameters of the analytical extraction procedure, namely: sample amount, solvent volume and vortex time. The selected solvent was ethanol at 96% and extraction performed with a Vortex Genie 2, from Scientific Industries. The brown macroalgae *Z. tournefortii* was used for the optimization and validation assessments. For more information, please consult the supplementary material.

2.5.3.4 Chromatographic conditions

A Nexera X2 UHPLC system composed by two binary LC-30AD pumps, a DGU-20 A5 degassing unit, a CTO-20A column oven, a SIL-30AC autosampler and a PDA detector (200-800 nm) SPD-M20A was used for chromatographic analysis. The UV/Vis spectrum of fucoxanthin was used for identification and the 454 nm detection wave-length was used for quantification purposes. A gradient elution with methanol (solution A) and ultra-pure water acidified with 0.1% of formic acid (solution B) was used at 0.3 mL/min flow rate. The gradient started with 6 min of 20% solution A, then, it was gradually set up to 90% in 11 min changed to 100% in 1 min and maintained for 6 min. Finally, solution A was reduced to 5% in 1 min and held for 5 min to prepare the next injection, with a total injection time of 30 min. The mobile phases were previously filtered through a hydrophilic polypropylene 0.2 µm pore size membrane filter (Pall Corporation, Ann Arbor). Sample extracts were separated in a reversed phase Sunshell C18 column (150 x 2.1 mm, 2.6 µm) from ChromaNik Technologies Inc (Osaka, Japan), thermostated at 30 ºC, with an injection volume of 1 µL. All samples were extracted in triplicate and injected twice.

2.5.3.5 Method validation

The optimized methodology was validated, assessing linearity, sensitivity, matrix effects, selectivity, precision (repeatability and reproducibility) and accuracy. Linearity was calculated based on linear regression analysis, through correlation coefficient (R^2). Sensitivity was evaluated by the limit of detection (LOD) and limit of quantification (LOQ) according to equations 1 and 2, respectively, where σ is the standard deviation of the y-intercept and *b* the curve slope.

$$\mathrm{LOD} = 3.3\frac{\sigma}{\mathrm{b}} 3\sigma/\mathrm{b} \qquad (1)$$

$$\mathrm{LOQ} = 10\frac{\sigma}{\mathrm{b}} \qquad (2)$$

For the calibration curve, eight working standard solutions were prepared within 12 - 3600 μg.g^{-1} dw concentration range, by spiking ethanol with the fucoxanthin standard solution.

Matrix effects were also evaluated, based on the ratio between two slope curves: one with the response of the direct injection of fucoxanthin standard working solutions (curve 1) and the other obtained from the extracts of the seaweed biomass (*Z. tournefortii*) spiked with the fucoxanthin standard working solutions (curve 2), as described by Matuszewski (2006) (eq. 3).

$$\%\mathrm{ME} = \left[\frac{(\text{slope of calibration curve 1} - \text{slope of calibration curve 2})}{\text{slope of calibration curve 1}}\right] \qquad (3)$$

These analyses intended to determine if the seaweed matrix (ME - matrix effect) had influence on the fucoxanthin extraction.

The method selectivity was verified by checking the absence of interferences at the fucoxanthin retention time of the chromatograms of all samples and standard solutions.

Repeatability and reproducibility were assessed by intra and inter-day analysis, respectively, of *Z. tournefortii* sample and two ethanol standard solutions of fucoxanthin (480 and 2400 μg.g^{-1} dw). The assessment of repeatability was obtained through the variation coefficient of ten successive extractions of these samples. The reproducibility was evaluated by the analysis of five extractions of the same samples in three different days, in a time span of 10 days. These results were expressed in percentage to the relative standard deviation (%RSD).

Accuracy was determined through the evaluation of a recovery study, spiking a macroalgae sample (*Lobophora variegata*) at three different fucoxanthin concentrations (24, 480 and 2400 μg.g^{-1} dw). Recovery was calculated according to equation 4 where SWS is the measured fucoxanthin concentration in a spiked sample, SW is the measured concentration in the sample and S is the concentration of fucoxanthin added to the sample.

$$\text{Recovery (\%)} = \frac{\mathrm{SWS} - \mathrm{SW}}{\mathrm{S}} \times 100\ (\%) \qquad (4)$$

Finally, the method was applied to 10 different seaweeds (12 samples) and 10 beach-cast seaweeds (10 samples) containing different species of brown seaweed in its composition, in order to confirm the applicability of the proposed methodology for the determination of fucoxanthin in seaweed biomass.

2.5.3.6 Extract stability

The extract stability was evaluated at 0, 5 and 10 days after extraction to determine if these extracts could be considered stable. *Z. tournefortii* extracts at two fucoxanthin concentrations, 480 and 2400 µg.g^{-1} dw, were used for this assay. These were kept at 10 ºC in amber vials. Additionally, two ethanol fucoxanthin standard solutions at same concentration were also tested.

2.5.3.7 Statistical analysis

Samples were evaluated using two replicas and three injections, being expressed as mean of six measurements ± standard deviation. Definitive Screening Design (DSD) for design matrix and subsequent data analysis (model estimation and optimization) was achieved using the JMP® ver. 11.1.0 (32-bit) (SAS Institute Inc.).

2.5.4 Results and discussion

2.5.4.1 Vortex Assisted Solid-Liquid Micro-Extraction optimization

Methanol, acetone and ethanol are the most common solvents used for the extraction of marine pigments (Ragumaran et al., 2018). In this study, methanol was not considered due to its inherent toxicity (class 2 solvent). Kim et al. (2012) tested different solvents (water, ethyl acetate, acetone and *n*-hexane) for the extraction of fucoxanthin from the diatom *Phaeodactylum tricornutum* and reported that the best extraction yield was obtained when high purity grade ethanol was used. Although ethanol (class 3 solvent – low toxicity) is not as widely used as acetone (also class 3 solvent) for the extraction of microalgae pigments, it has revealed greater yield for fucoxanthin extraction (Kim et al., 2012). For all these reasons ethanol was chosen as the extraction solvent to develop the experimental layout. With the purpose of developing a green extraction procedure, solvent microvolumes were considered. In order to assist the microextraction, ultrasound and vortex were investigated. Tests with ultrasound bath revealed lack of repeatability, therefore, it was decided to proceed with vortex assistance. The use of small quantities of sample were also intended. The design matrix to determine the best extraction conditions for fucoxanthin was established by a Central Composite Design (CCD), with three factors, volume of ethanol (µL), vortex time (min) and sample amount (mg) at three levels, with center points. For more information about the design, please consult the supplementary material.

2.5.4.2 Method validation

The VASLME followed by HPLC-PDA method was validated for the rapid determination of fucoxanthin content in seaweed biomass. The results are expressed in Table 2. The validation parameters assessed were linearity, sensitivity, matrix effects, selectivity, precision and accuracy, after determining the optimal extraction conditions of fucoxanthin.

No matrix effect was observed, %ME was 3.4%. Therefore, the calibration curve adopted was the one performed by spiking fucoxanthin (Fx) stock solution in ethanol, according to equation 5.

$$\text{Fx area} = 2306667 \times \text{Fx concentration } \mu\text{g.g}^{-1} + 6595 \quad (5)$$

A good correlation coefficient of R2=0.99998 was found, supporting the method linearity. Also, excellent sensitivity was obtained, LOD = 3.33 µg.g-1 and LOQ = 10.09 µg.g-1. These values are quite lower than the values typically found in macroalgae (about 100 – 1000 mg.g-1 dw) (Wang et al., 2018). The method also revealed good precision: 2 to 3.3% of variation in intra-day analyses and 1 to 3.8% for the inter-day analysis. Additionally, the results of the recovery study ranged between 95 and 97%, as summarized in Table 2, demonstrating the accuracy of the method.

Table 2 – Validation results for VASLME methodology to quantify fucoxanthin in seaweed.

	Parameter	**Result**
Linearity	Linear regression (y = mx + b)	2306667x + 6595
	Linear concentration range	12 - 3,600 µg.g^{-1}
	R^2	0.99998
Sensitivity	LOD (µg.g^{-1})	3.33
	LOQ (µg.g^{-1})	10.09
Accuracy	Recovery	%
	SW + 24 µg.g^{-1}	96
	SW + 480 µg.g^{-1}	95
	SW + 2,400 µg.g^{-1}	97
Precision	Intra-day (% RSD)	2.0 - 3.3
	Inter-day (% RSD)	1.0 - 3.8

All determinations were the result of two replicas each injected three times. LOD – Limit of Detection; LOQ – Limit of Quantification; SW – Seaweed; RSD – Relative Standard Deviation.

After assessing the figures which resulted from the Response Surface Methodology (RSM) assessed in this work and are presented as supplementary material, fucoxanthin was quantified in 22 samples of algae biomass. The fucoxanthin contents (Tables 3 and 4) were found to vary between 10.1 ± 0.3 and 852 ± 12 µg.g^{-1} dw, which are within the calibration range. Figure 1 shows the typical chromatograms (Fx retention time at 19.9 min) of a standard solution (240 µg.g^{-1} dw) and a seaweed biomass sample (brown algae), confirming the method selectivity. It also revealed that the variation between sample replicates never exceeded the 7%, even at levels close to LOQ.

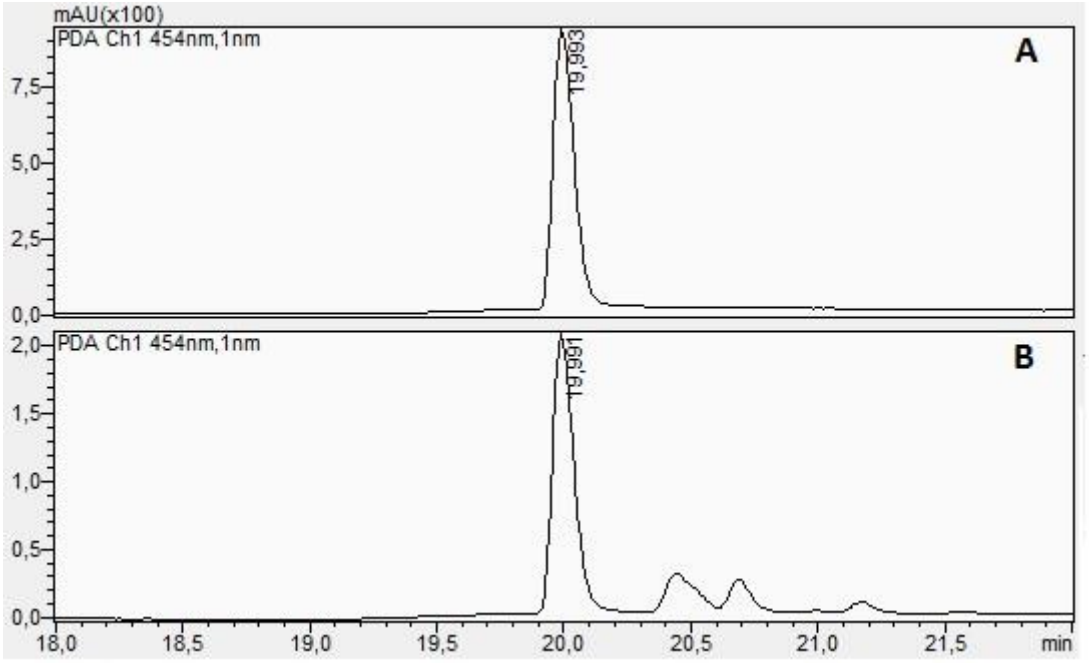

Figure 1 - Chromatograms of a fucoxanthin standard solution of 240 µg.g^{-1} dw (A) and a brown seaweed sample (B).

Table 3 – Seaweed samples for fucoxanthin yield testing.

Seaweed	**Colection site**	**Prospection date**	**Fucoxanthin content** (µg.g^{-1} dw ± SD)
A. nodosum	Galway (Ireland)	Jun/2018	21.6 ± 0.9
D. dichotoma	Porto Santo	Mar/2017	12.2 ± 0.4
D. dichotoma	Madeira	Aug/2018	514 ± 5
D. polypodioides	Madeira	Aug/2018	597 ± 30
F. vesiculosus	Galway (Ireland)	Jun/2018	22 ± 1
H. filicina	Porto Santo	Mar/2017	17.3 ± 0.3
H.scoparia	Madeira	Jan/2016	10.1 ± 0.3
L. variegata	Porto Santo	Mar/2017	40.9 ± 0.8
P. pavonica	Madeira	July/2016	10.2 ± 0.3
S. vulgare	Madeira	Jun/2017	400 ± 14
Z. tournefortii	Madeira	Aug/2016	852 ± 12
Z. tournefortii	Madeira	July/2017	381 ± 3

Data are mean ± standard deviation in micrograms of fucoxanthin per 1 gram of algae on a dry weight basis (dw). All determinations were the result of two replicas each injected three times. dw – Dry Weight; SD – Standard deviation.
dw – Dry Weight; SD – Standard deviation.

Table 4 – Seaweed beach casts tested for fucoxanthin concentration.

Seaweed Beach Cast code	**Prospection date**	**Fucoxanthin concentration** (µg.g^{-1} dw ± SD)
1	29-May-2017	20.3 ± 1.3
2	23-Jun-2017	14.2 ± 0.6
3	26-Jun-2017	13,2 ± 0,51
4	12-July-2017	33.3 ± 0.8
5	20-July-2017	27.6 ± 1.7
6	8-Aug-2017	32.6 ± 1.2
7	21-Aug-2017	19.6 ± 1.0
8	18-Sep-2017	49.4 ± 2.0
9	6-Oct-2017	22.9 ± 0.6
10	10-Oct-2017	28.4 ± 1.1

Data are mean ± standard deviation in micrograms of fucoxanthin per 1 gram of algae on a dry weight basis (dw). All determinations were the result of two replicas each injected three times. dw – Dry Weight; SD – Standard deviation.
dw – Dry Weight; SD – Standard deviation.

2.5.4.3 Extract Stability

Sample extracts kept at 10 ºC, enclosed in amber vials, were tested for stability and considered suitable due to the low variation detected between initial (0 days), middle (5 days) and the end (10 days) of the assay. For *Z. tournefortii*, the variation of fucoxanthin content was 0.8% and 0.3% for 5 and 10 days, respectively. The fucoxanthin ethanolic solution at 480 µg.g^{-1} dw was found to vary 1.3% and 4.3% in 5 and 10 days respectively. For the fucoxanthin solution with 2400 µg.g^{-1} concentration, the variation was found to be 0.7% in both dates.

These results indicate that ethanolic extracts of fucoxanthin are stable in the 10 days after sample extraction, regardless of its concentration. Thus, an accurate evaluation of the fucoxanthin concentration in seaweed biomass can be obtained at least for 10-day period at 10 ºC and light protected. This result is very important not only for performing the simultaneous extraction of multiple samples and stock before HPLC analysis, but also for eventual industrial applications.

This methodology enables to accurately assess the fucoxanthin content in macroalgae biomass, using small quantities of biomass, allowing sample shipping to dedicated laboratories and perform several extractions at once, reducing extraction time. Stability of the extract is favourable since sample sets could be extracted and analysed with precision within 10 days when kept at 10 ºC in amber vials. Table 5 summarizes some fucoxanthin analysis methods. These are compared for the quantity of algal resource needed, solvent and volume used for extraction, health and environmental concerns relating the use of these solvents, extraction methodology and analysis apparatus. The resulting ratio (solvent/algal quantity) presented by other works are usually greater than this work, using higher solvent quantities to extract fucoxanthin from selected samples, producing more waste. Some of these solvents are health concerns or

environmentally aggressive, being necessary safer and greener options. Also, simplicity is achieved when smaller quantities of algae, solvent and fewer steps are needed to perform fucoxanthin analysis, resulting in larger number of samples, which can be handled at the same time. Performing the methodology described in this work, it is possible to analyse 50 individual vials in a 3 day period.

Table 5 – Summarized methods for fucoxanthin analysis

Reference	Quantity of algal resource	Solvent and volume	Health and environmental concerns*	Extraction methodology	Analysis apparatus (duration)
Present work	25 mg (dw)	Ethanol (96%), 0.3 mL	9/10 - 7/10	Vortex assisted extraction (RT, 15 min)	HPLC-PDA (21 min)
Pasquet et al. (2011)	50 mg (dw)	Acetone, 30 mL	6/10 - 7/10	1. Soaking (RT, 120 min); 2. Hot soaking (56 ºC, 120 min); 3. UAE (12,2 W, 10 min); 4. VMAE and MAE (50 W, 5 min)	HPLC-PDA (20 min)
Petrushkina et al. (2017)	10 mg (dw)	Acetonitrile, 1 mL	2/10 - 4/10	Shaken (1800 rpm, 20 min)	HPLC-PDA (15 min)
Sun et al. (2019)	4 g (dw)	Methanol, 120 mL	4/10 - 8/10	Vortex assisted extraction (20 min)	UPLC-PDA-TWIMS-QTOF-MS (16 min)

* Retrieved from Curzons et al. (2002), higher numbers are preferable. RT – Room Temperature; UAE – Ultrasound Assisted Extraction; VMAE – Vacuum Microwave Assisted Extraction; MAE – Microwave Assisted Extraction; UPLC-PDA-TWIMS-QTOF-MS – Ultra Performance Liquid Chromatography coupled to Photodiode Array detector/Travelling Wave Ion Mobility mass Spectrometry/Quadrupole Time-Of-Flight Mass Spectrometry.

2.5.4.4 Fucoxanthin content in seaweed biomass

Table 3 reports the evaluation results of 12 samples of 10 different marine brown macroalgae species. The fucoxanthin contents varied significantly between samples, from 10.1 ± 0.3 μg.g^{-1} dw to 852 ± 12 μg.g^{-1} dw. Samples with high fucoxanthin concentrations, varying from 400 ± 14 to 852 ± 12 μg.g^{-1} dw (*Z. tournefortii, D. polypodioides, D. dichotoma, S. vulgare*), were all collected in Madeira Island seas. These species are comparable to those found by Jaswir et al. (2012) in *Sargassum aquifolium* (as *Sargassum binderi*) and *Sargassum ilicifolium* (as *Sargassum duplicatum*), collected in the Straits of Malacca, near Port Dickson (Malaysia), between 730 and 1010 μg.g^{-1} dw of fucoxanthin and higher contents than those reported by Kim et al. (2012) in *Ecklonia bicyclis* (as *Eisenia bicyclis*) collected in South Korea (260 μg.g^{-1} dw). On the other hand, the majority of the brown macroalgae samples evaluated exhibited low fucoxanthin contents (lower than 40.9 ± 0.8 μg.g^{-1} dw). It is interesting to notice that this result cannot be attributed to the species, since the same species revealed different concentration levels at different locations (*D. dichotoma*) and collection dates (*Z. tournefortii*). This result might also be related to seasonality, as it has been recently published (Fariman et al., 2016). Table 4 presents the screening results of 10 beach-cast seaweeds, composed by different brown seaweeds and others (Table 1), sampled in "Playa de Las Canteras", Gran Canaria within the studied collecting period (May 29 and October 10, 2017). The fucoxanthin content was found to vary between 13.2 ± 0.5 μg.g^{-1} dw in sample 3 (June 26, 2017) and 49 ± 2 μg.g^{-1} dw in sample 8 (September 18, 2017). The fucoxanthin levels of this biomass resource varies along the year and are even comparable to some brown macroalgae samples collected in Madeira Islands and Galway. These algae wastes can be valorised as a relevant bioresource of high-valued bio-compounds for eventual industrial applications, namely through the implementation of biorefinery strategies.

2.5.5 Conclusion

A simple and reliable analytical method was successfully optimized and validated to quickly quantify fucoxanthin in seaweed biomass, using reduced amounts of sample and extraction solvent. Simultaneous seaweed biomass VASLME can be performed prior to RP-HPLC-PDA analysis, which takes 30 min. CCD was crucial to optimize the fucoxanthin extraction yield, pointing out that 25 mg of sample with 300 μL of ethanol and vortexed for 15 min are the best experimental combination for the proposed sample preparation procedure. Good results were obtained for all the validation parameters, particularly in terms of sensitivity (LOQ = 10.09 μg.g^{-1}) and precision (maximum variation of 3.8%). Ethanolic extracts are stable at least for a 10-day period at 10 ºC and light protected, allowing the simultaneous extraction of multiple samples and stock them before HPLC analysis. The method proved to be an accurate tool for the evaluation of the fucoxanthin concentration in seaweed biomass, as it was demonstrated by the analysis of 22 samples. Fucoxanthin concentration was found to vary from about 10 to 852 μg.g^{-1} dw.

Samples collected in Madeira presented the highest contents (400 - 852 μg.g-1 dw), namely those from *Z. tournefortii, D. polypodioides, D. dichotoma* and *S. vulgare* brown macroalgae. In beach-cast seaweeds from Gran Canaria the fucoxanthin levels (<49 μg.g^{-1} dw) are comparable to some brown macroalgae samples collected in Madeira Islands and Galway. This information can contribute to the development of sustainable strategies to valorise these algae wastes as a relevant bioresource of high-valued bio-compounds for eventual industrial applications.

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2.5.7 Supplementary material

Experimental conditions established from a Central Composite Design (CCD) and the respective fucoxanthin RP-HPLC-PDA chromatographic areas.

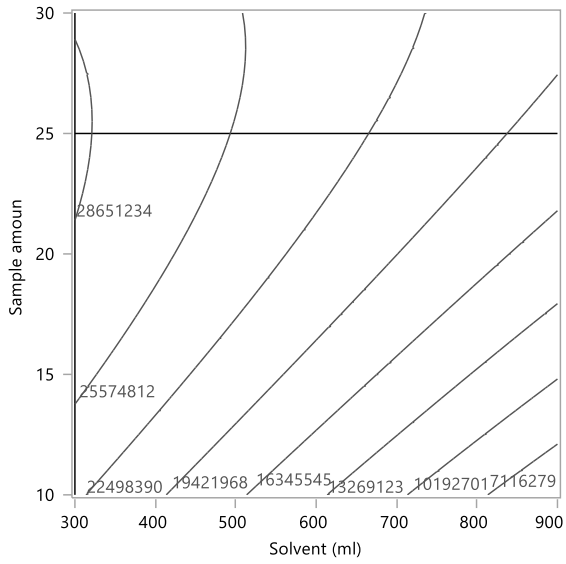
	Solvent quantity (µL)	Vortex time (min)	Sample amount (mg)	HPLC/PDA area
1	600	9	20	22233772
2	900	15	30	19114407
3	300	15	10	20223121
4	900	15	10	7116279
5	300	3	30	19158317
6	300	15	30	31261583
7	600	3	20	20213123
8	600	9	20	21692425
9	900	9	20	15766652
10	600	9	20	17390568
11	600	9	20	23359660
12	600	9	30	22655579
13	600	3	20	22777463
14	300	3	10	22021212
15	900	3	30	21430062
16	900	15	30	20289597
17	600	15	20	24992966
18	900	3	30	20469040
19	600	9	10	16860054
20	300	15	30	31727656
21	300	9	20	26541141
22	300	3	30	17881422
23	900	15	10	8583696
24	900	3	10	7520164
25	900	3	10	8480648
26	900	9	20	13985455
27	300	9	20	26521514
28	600	9	10	13877981
29	600	15	20	24545455
30	300	3	10	25813955
31	300	15	10	23666416
32	600	9	30	22021312

Data for each point is the average result of two measurements in the HPLC-PDA.

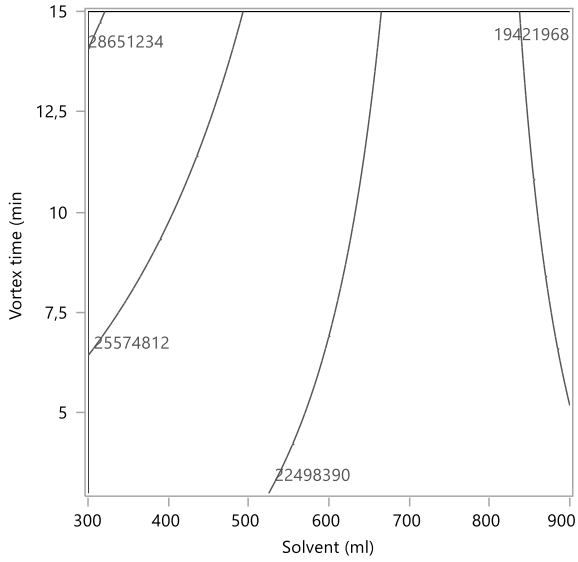
Results from General Linear Model (GLM) with all effects ANOVA and Pure Error analysis.

Factor	SS	DF	MS	F value	ρ value
Solvent volume (µL) 1L	5.208156 E14	1	5.208156 E14	189.0235	0
Vortex time (min) 2L	3.316798 E13	1	3.316798 E13	12.0379	0.002931
Sample amount (mg) 3L	2.580884 E14	1	2.580884 E14	93.6700	0
1L by 2L	3.843939 E13	1	3.843939 E13	13.9511	0.001647
1L by 3L	1.065955 E14	1	1.065955 E14	38.6875	0.000009
2L by 3L	4.795863 E13	1	4.795863 E13	17.4060	0.000639
Pure Error	4.684002 E13	17	2.755295 E12		
Total SS	1.186024 E15	31			

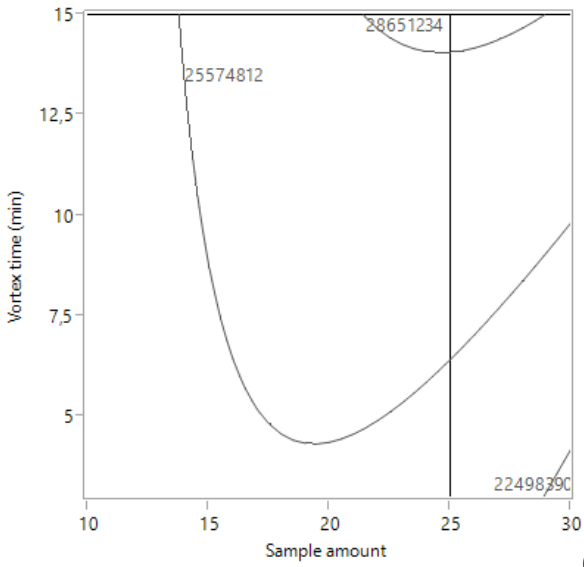
Statistical information resulted from the design matrix analysis, which 32 single points were injected twice in HPLC-PDA chromatography apparatus. SS – Sum of Squares; DF – Degrees of Freedom; MS – Mean Squares.



(A)

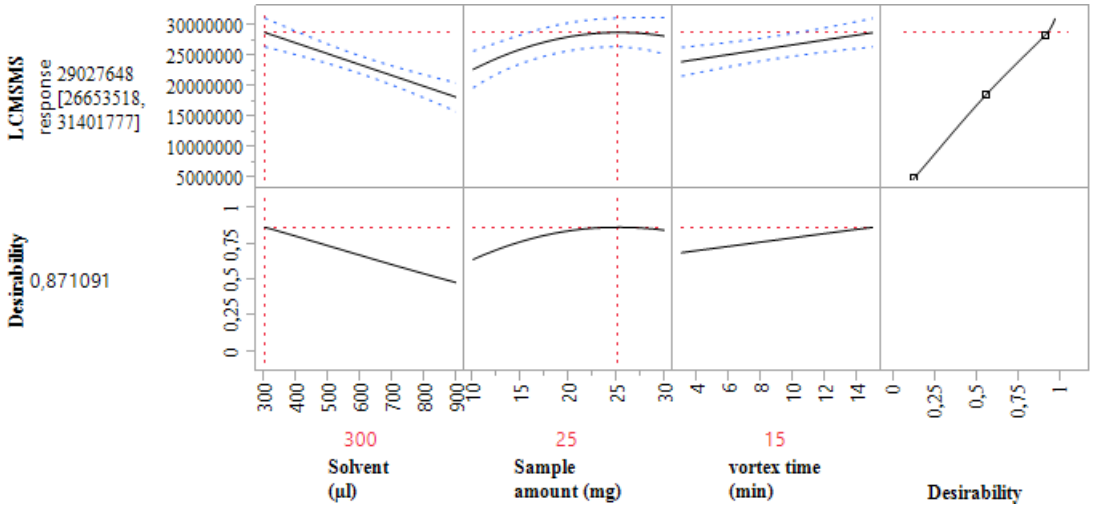


(B)



(C)

Contour plot set from response surface analysis.



Prediction profiler with maximum desirability set from the response surface Analysis.

2.6 Nutraceutical potential of *Asparagopsis taxiformis* (Delile) Trevisan extracts and assessment of a downstream purification strategy

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2.6.1 Abstract

The main goal of the present work was to determine the nutraceutical potential *of Asparagopsis taxiformis* D. extracts from Madeira Archipelago south coast. Extraction methodologies consisted either/or in 72 hours stirring, at room temperature (M1), or 6 cycles of Soxhlet extraction (M2), both with re-extraction. Solvents used were distilled water, ethanol, methanol and ethyl acetate. M1 allowed to obtain the highest values for extraction yield (31.65 $g.100g^{-1}$ dw) using water, whereas iodine content (3,372 $mg.100g^{-1}$ dw), TPC (1,707 mg GAE.$100g^{-1}$ dw) and chlorophyll a (45.96 $mg.100g^{-1}$ dw) were obtained using ethanol, and TCC (36.23 $mg.100g^{-1}$ dw) with methanol. Extracts that showed higher reduction activity in M1 were derived from ethanol extraction (1,908 mg AAE.$100g^{-1}$ dw). Water and ethanol were the best solvents for higher DPPH scavenging activity in M2, both with same result (IC50 1.37 $mg.mL^{-1}$). The lowest value of IC50 for chelating activity (1.57 $mg.mL^{-1}$) was determined in M1, using ethyl acetate. The remaining residue was used to obtain other products, i.e. lipid extraction (M1, 2.05 $g.100g^{-1}$ dw), carrageenans (M2, 21.18 $g.100g^{-1}$ dw) and cellulose (M1, 23.81 $g.100g^{-1}$ dw) with subsequent FTIR ATR analysis. Our results show that *A. taxiformis* is a valuable source of bioactive compounds. The M1 extraction methodology using ethanol is the most effective solvent to produce an iodine rich bioactive extract with potential of being used as a nutraceutical supplement. Also, we have demonstrated a possible downstream strategy that could be implemented for multiple compound extraction from *A. taxiformis* residue. This has a vital importance for future feasibility, when using this biomass as an industrial feedstock for multiple products production. Statistical analysis, using SPSS 24.0, was also performed and important correlations were found between assays and methods.

Keywords: Iodine; Downstream; Antioxidant capacity; Antioxidant compounds; Asparagopsis taxiformis; Biorefinery; Seaweed; Macroalgae.

2.6.2 Introduction

Asparagopsis taxiformis, has been earlier researched by Kaliaperumal (2003) [1] and referred to as a "seaweed rich in iodine" which can be used to ameliorate goitre disease. McConnell and Fenical (1977) [2] identified the biological functions of the halogenated metabolites in *A. taxiformis*, which include bromine (Br) and iodine (I) in their molecular structures. These metabolites could be stored in specialized gland cells [3] and often function as defense compounds, correlated with environmental adaptations, due to *A. taxiformis* abundance in subtropical and tropical waters, where high seaweed herbivory occurs. In India, this species was found to accumulate 499.30 mg I/100g dw (dry weight) and its genus 556.70 mg I/100g dw [4]. In many areas of the world, soil surface is progressively becoming poorer in iodine content due to leaching processes [5]. The majority of the available iodine is supported by marine systems and marine organisms such as seaweeds, which can accumulate substantial quantities of iodine [6]. Chemical iodine species in seaweeds seems to be mainly I^-, organic iodine and in minor quantity IO_3^- [7]. There is a widespread interest for functional foods that might promote health benefits, such as reducing the risk of chronic diseases and enhancing the ability to promote health, thus improving the quality of life. Seaweeds potential as a functional food can be used to produce supplements for the food industry [8]. To determine the nutraceutical quality of the extracts produced, nine parameters were assessed, namely extract yield, iodine, total phenolic compounds (TPC), flavonoids, chlorophyll a, total carotenoid content (TCC), reducing activity (RA), free radical scavenging assay (FRSA) and ferrous ion chelation (FIC). TPC are present in seaweeds as a major molecular group, with an important action in defending against bacteria, wounding or excessive radiation and contributing to the antioxidant activity of the tissue [9,10]. These compounds are attractive due to their nutraceutical and antioxidative properties, showing bioactivity that is indicative of preventing pathologies caused by oxidative stress [11].

Numerous approaches are being developed for industrial crops or biomass applications, considering that the chemical composition of seaweeds and their fast growth enables a large set of strategies for biorefinary implementation. Nowadays, chemical production or extraction using seaweed as feedstock is mainly focused on single products, such as the extraction and purification of hydrocolloids, polysaccharides, xanthophylls, proteins and production of biofuels, discarding the remaining biomass [12]. Integrating sustainable strategies to cascade processing with efficient disintegration of biomass to obtain valuable biocompounds could be the answer for a profitable industry[13]. Taking this into consideration, a downstream process was also applied on the *A. taxiformis* residue after primary extraction. This remaining biomass is also known as PEAR (post-extracted algal residue). Three distinct compounds of commercial interest were extracted and quantified from PEAR, namely lipids, carrageenans and cellulose, as a first step towards a biorefinary strategy.

In this work, several solvents were tested, namely distilled water, ethanol, methanol and ethyl acetate and two distinct extraction processes for the development of seaweed nutraceutic production, using biomass from the south cost of Madeira. The directive 2009/32/CE for food applications allows the use of these solvents, under good working practices and quality control. Although methanol has some restrictions, it can be used if its maximum presence does not reach 10 mg/kg in the final product. FTIR-ATR was considered for spectra comparison between direct extraction and PEAR extraction of lipids, carrageenan and cellulose. This is a powerful technic, widely used for detection and characterization of various types of molecules,

delivering a unique signature to each compound analyzed. The variability of transmittance along the correspondent wavenumber is caused due to the relative mass and geometry of the atoms. The conformation of the molecules causes the resonance between vibrations that further modulates the spectra [14].

2.6.3 Materials and methods

2.6.3.1 Seaweed biomass

Asparagopsis taxiformis (Delile) Trevisan de Saint-Léon 1845 samples were collected in a 10-meter maximum depth dive of the Madeiran south coastline, coordinates 32,646951 - 16,823967. Samples were collected in august 2016 and were transported in seawater and gently rinsed with filtered fresh water. Afterwards, a primary drying was applied in which seaweed was frozen at -35 ºC and freeze-dried under reduced pressure (4 x 10^{-4} mbar), with a cooling trap set at -56 ºC for 5 days. Samples were milled to 200 mesh particle size, vacuum packed and stored at -35 ºC until use.

2.6.3.2 Preparation of extracts

Two methods of extract production were used. Ten grams of lyophilized *A. taxiformis* were added to 300 mL of one of the following solvents: distilled water, ethanol, methanol or ethyl acetate (1:30, w/v). The first extraction method (M1) included stirring the mixture in a borosilicate flask, under sonication, during 90 min. Afterwards, continuously stirring at 1,100 rpm was carried out at approximately 20 ºC for 72 hours. Final suspension was centrifuged for 10 min at 7200xg. The supernatant was kept for analysis, as extract. In the second extraction method (M2) a soxhlet extractor was used to perform six complete extraction cycles. A re-extraction was performed in both methodologies and extracts were pooled together, according to the respective solvent and method. Extracts were partly evaporated in a rotary evaporator and dehydrated completely in an oven, at 40 ºC. The post-extracted algal residue (PEAR) was recovered and dried in an oven, at 40 ºC. Both extracts and PEAR were stored under vacuum at -35 ºC until use.

2.6.3.3 Extract analysis

A. taxiformis extracts were subjected to eleven analysis parameters. Total phenolic content (TPC) was determined using Folin Ciocalteu method described by Chew et al. (2008) [15] and results were expressed as grams of gallic acid equivalents (GAE) per 100 grams of dw of extract. Chlorophyll *a* and total carotenoids content (TCC) were determined according to Wellburn (1994) and Kumar et al. (2010) [16,17]. Flavonoids were quantified according to Chan et al. (2015) [18], using aluminium chloride colorimetry and results were expressed as grams of quercetin equivalents (QE) per 100 grams dw of extract. The free radical-scavenging activity (FRSA) was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Yen and Chen (1995) and Duan et al. (2006) [19,20], also using butylated hydroxytoluene (BHT) as a positive control and results were expressed in milligrams IC_{50} per millilitre. Reducing activity was performed following Yuan et al. (2005) [21] work and results were expressed as grams of ascorbic acid equivalents (AAE) per 100 grams dw of extract. Ferrous ion chelating activity (FIC assay) was measured based on Chew et al. (2008) and Decker and Welch (1990) [15,22], using EDTA as a positive control,

results were expressed as milligrams IC_{50} per millilitre. All samples were analyzed in triplicates in all tests carried out.

2.6.3.4 Iodine quantification

Iodine quantification was initiated with seaweed incineration, adopting the method described by Mahesh et al. (1992) [23] with some modifications. About 0.5 g of sample is weighted into porcelain crucibles and 0.5 ml of potassium hydroxide solution (6M) mixed with a metal rod and placed in an oven at a temperature of 95 °C for 1h. After, it is added 0.5 ml of zinc sulphate solution (0.52M) and returned to the oven for a further 1 h at 95 °C. The crucibles were then placed in a muffle furnace to reach the 600 °C for a period of 2h. After, it is reconstituted in deionized water, filtered to remove particles and the volume adjusted accordingly. The oxidation process and spectrophotometric determination were performed according to Pino et al. (1996) [24] with some modifications. Aliquots of 0.2 ml of sample or standard was added to test tubes, followed by addition of 1.0 ml of ammonium persulfate (1M) to all tubes. The samples undergo a process of oxidation during 30 min in a water bath at a temperature of 95 °C. It was sequentially added 2 ml of arsenic acid (0.0253 M), 1 ml of sulfuric acid (1.25M) and 1 ml of water. The tubes are placed in a water bath at 32 °C for 10 min, then 0.5 ml of ceric ammonium sulfate (0.0158M) is added, vortexed and incubated exactly for 10 minutes in the water bath at the same temperature as before. A standard solution A is performed with KIO_3 to a concentration of 7.87 M (1000 µg/ml). Standard solution B is prepared by diluting the standard solution A to 100 times, to an iodine concentration of 78.74 mM (10.0 µg/ml). The working standards are prepared by diluting with distilled water in a volumetric flask of 100 ml amount of 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml and 5.0 ml of standard solution B resulting in the concentration of 0.78, 1.57, 2.36, 3.18, 3.94 mM (0.02, 0.04, 0.06, 0.08 and 0.10 µg/0.2 ml). After incubation the reactions are read at 410 nm in a spectrophotometer cuvettes of 10 mm. The calibration curve is prepared daily by placing % transmittance vs. iodine content in µg of iodine on the axis X.

2.6.3.5 Downstream product analysis

Lipids were quantified as described by Folch et al. (1957) [25]. Carrageenan was determined according to Tasende et al. (2012) [26] and cellulose was quantified following Baghel et al. (2015) [27]. In order to facilitate the comprehension of the multiple analysis and extractions performed in this work, a flowchart was created (figure 1). For extractions, residues and by-products a simple code was attributed, presenting results and discussion in a more perceptible way.

FTIR-ATR spectra of the samples were obtained with a Perkin Elmer Spectrum Two coupled with a Diamond ATR accessory (DurasamplIR II, Smiths Detection, UK). Thirty-two scans were acquired in transmittance mode in the range of 4000-650 cm^{-1}, with a wavenumber resolution of 1 cm^{-1}. All samples were analyzed in triplicates in all tests carried out.

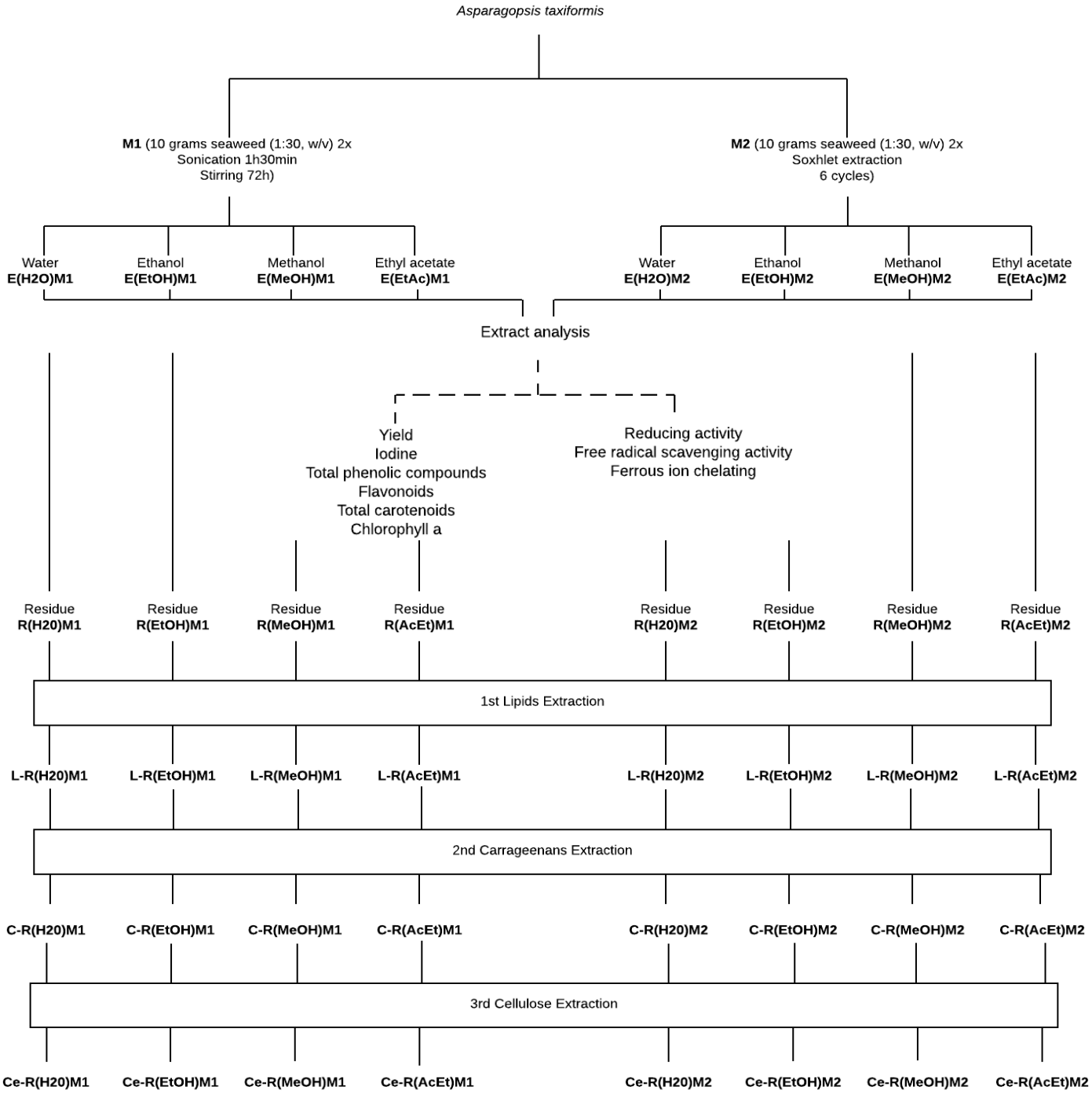

Figure 1 – Schematic representation of extract production, using 4 solvents permitted by the food industry and two methodologies, with posterior extraction of lipids, carrageenan and cellulose from PEAR biomass. Codes were attributed to each product for simplicity purposes when discussing results.

2.6.3.6 Statistical analysis

All values are expressed as mean of three replicates ± standard deviation. The statistical data analysis was performed, using SPSS 24.0 program for Windows. Data were analysed using one-way analysis of variance (ANOVA), and determined its homoscedasticity followed by Pearson's test ($p \leq 0.01$) to assess correlations between means. Tukey's test ($p \leq 0.01$) was also performed to determine statistical variance between seaweeds in each parameter.

2.6.4 Results and Discussion

In total, eight *A. taxiformis* extracts were assessed. These were produced using four distinct solvents (ethanol, methanol, water and ethyl acetate) considered safe for extracts production and two methods. Results for extract yield, TPC, flavonoids, chlorophyll a and TCC are presented in table 1. For simplicity reasons, figure 1 was created, coding extractions, residues and by-products in an intuitive way, so it would became clear all of the steps involved in this research.

Table 1 – Yield of extract, iodine content and antioxidant composition from *Asparagopsis taxiformis* (Delile) Trevisan

Solvent	Extract Yield		Iodine		TPC		Flavonoids		Chlorophyll a		TCC	
	g (100 g)$^{-1}$ in dw		g (100 g)$^{-1}$ in dw		g GAE (100 g)$^{-1}$ in dw		g QE (100 g)$^{-1}$ in dw		mg (100 g)$^{-1}$ in dw		mg (100 g)$^{-1}$ in dw	
	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
Water	31.65 ± 0.79a	18.93 ± 0.69a	1.71 ± 0.03a	2.92 ± 0.05a	0.62 ± 0.01a	0.47 ± 0.04a	0.04 ± 0a	0.04 ± 0a	ND	ND	ND	1.66 ± 0.26a
Ethanol	10.92 ± 1.77b	9.69 ± 0.21bc	3.37 ± 0.01b	3.13 ± 0.01b	1.71 ± 0.13b	1.43 ± 0.08b	2.51 ± 0.03b	3.51 ± 0.06b	45.95 ± 0a	14.34 ± 2.69a	22.72 ± 0.69a	23.23 ± 1.04b
Methanol	11.98 ± 0.66b	14.49 ± 4.09ac	2.38 ± 0.07c	1.82 ± 0.02c	0.57 ± 0.02a	0.52 ± 0.03a	4.02 ± 0.11c	2.85 ± 0.02c	8.65 ± 1.98b	34.80 ± 1.98b	36.23 ± 1.05b	26.62 ± 0.11c
Ethyl acetate	5.32 ± 0.67c	3.19 ± 0.06b	0.69 ± 0.01d	0.59 ± 0d	0.07 ± 0.01c	ND	24.25 ± 0.11d	7.75 ± 0.04d	8.10 ± 1.35b	13.90 ± 1.96a	36.13 ± 1.72b	23.74 ± 0.67b

Data are mean ± standard deviation in grams or milligrams per 100 grams of seaweed extract on a dry weight basis. All determinations were carried out in triplicate. Different letters within the same column indicate significant differences ($p \leq 0.01$) determined in SPSS 24.0 using Tukey b test. Not detected (ND).

2.6.4.1 Extract yield

Extract yield was higher using distilled water (31.65 ± 0.79 g/100g dw) E(H2O)M1 and (18.93 ± 0.69 g/100g dw) for E(H2O)M2 (table 1). Methanol and ethanol solvents also provided high extraction yields in E(MeOH)M2 (14.49 ± 4.09 g/100g dw) and E(EtOH)M1 (10.92 ± 1.77 g/100g dw), presented in table 1. Mellouk et al. (2017) [28] also provided some insight of the antioxidant properties of *A. taxiformis* from the Algerian coast and water extraction resulted in the highest yield (24.0 g/100g dw), followed by methanol extract (21.3 g/100g dw) and ethanol extract (18.1g/100g dw). Although comparing *A. taxiformis* from different locations and time of harvest, the extract yield were in similar order with higher extraction yield using water, followed by methanol and ethanol solvents. In order to determine the *in vitro* anti-methanogenic activity of *A. taxiformis* extracts, Machado et al. (2016) [29] performed initial extractions with four different solvents, water, methanol, DCM and hexane. They have obtained higher yield using water and methanol, 24.9 g/100g dw and 10.2 g/100g dw, demonstrating some similarity with our results. Chan et al. (2015) [18] used similar solvents to produce extracts from *Gracilaria changii*, collected in Sarawak, Malaysia, varying from 13.06 ± 1.14 g/100g dw using ethanol (comparing with our data 10.92 ± 1.77 g/100g dw) and 3.15 ± 0.45 g/100g dw using ethyl acetate (comparing with our data 5.32 ± 0.67 g/100g dw).

2.6.4.2 Iodine quantification

For whole *A. taxiformis*, iodine content reached 1.16 ± 0.03 g/100g dw. The highest iodine content was detected using ethanol as solvent in M1, E(EtOH)M1 and M2 E(EtOH)M2 extractions, representing 3.37 ± 0.01 g/100g dw and 3.13 ± 0.01 g/100g dw, respectively (see table 1). McConnell and Fenical (1977) [2] also used ethanol as a primary step to extract halogenated compounds from fresh *A. taxiformis* and *Asparagopsis armata*, obtaining high yield. Iodine is extensively integrated in these compounds, due to its biogenic functions. Statistical analysis showed that all values of iodine content in the extracts are independent, suggesting that the iodine has variable affinity, depending on the solvent used. The positive correlation (R^2=0.77) (table 2) between M1 and M2 extraction methods means that iodine extraction is strongly influenced by the solvents used in this work. Determining iodine content in seaweed extracts are of extreme importance, when considering these extracts to human supplementation. Iodine is essential for human physiology due to its integration in the composition of thyroid hormones T3 (3,5,3-triiodothyronine) and T4 (thyroxine or 3,5,3,5-tetraiodothyronine). These hormones are responsible for regulating key metabolic processes, such as catabolism of carbohydrates, lipids and protein, cellular respiration, thermoregulation, intermediary metabolism, and nitrogen retention [30]. Iodine plays an important role in human physiology, and it is known that its low intake is particularly harmful in childhood and pregnancy, contributing for developmental deficiencies and diseases. Iodine deficiency remains a major public health concern in many countries of the world [31]. Portugal is not an exception and the proximity to the sea does not avoid iodine deficiency. A study involving 3,631 pregnant women has shown that 83.2% in the mainland and 94.6% in Madeira and Azores Archipelagos had urinary iodine levels below 150 µg.L^{-1} [32], which is considered to be an indicator of insufficient iodine intake [33].

Table 2 – Statistical analysis using Pearson correlation to determine relationships between different parameters

		Extraction yield		Iodine		TPC		Reduction activity		FRSA		FIC		Chlorophyll a		TCC		Flavonoids		PEAR Yield		Lipids		Carrageenans		Cellulose	
		M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
Extraction yield	M1	1	,849**	0,028	,615*	0,034	0,024	-0,362	-,738**	,637*	-0,321	-,831**	-,986**	-0,400	-,587*	-,922**	-,936**	-,661*	-,877**	-,950**	-,850**	0,227	0,153	-,722*	-,876**	,739**	,624*
	M2	,849**	1	0,369	,661*	0,226	0,267	-0,129	-0,289	0,257	0,102	-0,489	-,800**	-0,202	-0,127	-,705*	-,636*	-,843**	-,952**	-,924**	-,869**	-0,315	-0,391	-,915*	-,988**	,755*	,751*
Iodine	M1	0,028	0,369	1	,770**	,927**	,951**	,817**	,584*	-,745**	0,002	0,518	0,010	,765**	0,254	-0,110	0,194	-,740**	-0,445	-0,111	-0,282	-,813**	-,840**	-0,666	-0,354	0,330	-0,101
	M2	,615*	,661*	,770**	1	,805**	,801**	0,497	-0,062	-0,193	-0,399	-0,117	-,607*	0,446	-0,332	-,717**	-0,471	-,911**	-,812**	-,602*	-0,574	-0,359	-0,427	-,894**	-,711*	,738**	0,166
TPC	M1	0,034	0,226	,927**	,805**	1	,993**	,910**	0,434	-,700*	-0,331	0,450	-0,045	,879**	-0,049	-0,248	0,074	-,626*	-0,346	-0,034	-0,137	-0,558	-,593*	-0,586	-0,191	0,408	-0,313
	M2	0,024	0,267	,951**	,801**	,993**	1	,908**	0,477	-,724**	-0,272	0,477	-0,026	,872**	0,011	-0,215	0,107	-,648*	-0,359	-0,036	-0,165	-,618*	-,648*	-0,579	-0,208	0,369	-0,287
Reduction activity	M1	-0,362	-0,129	,817**	0,497	,910**	,908**	1	,658*	-,883**	-0,252	,732**	0,342	,994**	0,117	0,111	0,415	-0,277	0,063	0,375	0,215	-0,540	-0,541	-0,203	0,254	0,044	-,610*
	M2	-,738**	-0,289	,584*	-0,062	0,434	0,477	,658*	1	-,921**	0,511	,959**	,791**	,638*	,798**	,730**	,897**	0,005	0,342	,592*	0,343	-,805**	-,768**	0,146	0,399	-0,416	-0,389
FRSA	M1	,637*	0,257	-,745**	-0,193	-,700*	-,724**	-,883**	-,921**	1	-0,171	-,942**	-,651*	-,872**	-0,547	-0,505	-,753**	0,121	-0,248	-0,553	-0,329	,750**	,718**	0,009	-0,382	0,251	0,529
	M2	-0,321	0,102	0,002	-0,399	-0,331	-0,272	-0,252	0,511	-0,171	1	0,329	0,435	-0,292	,872**	,620*	0,542	0,048	0,098	0,097	-0,096	-0,547	-0,524	0,234	-0,042	-0,370	0,304
FIC	M1	-,831**	-0,489	0,518	-0,117	0,450	0,477	,732**	,959**	-,942**	0,329	1	,855**	,737**	,677*	,744**	,919**	0,158	0,501	,748**	0,557	-,656*	-,606*	0,237	0,595	-0,496	-,587*
	M2	-,986**	-,800**	0,010	-,607*	-0,045	-0,026	0,342	,791**	-,651*	0,435	,855**	1	0,374	,678*	,954**	,969**	,611*	,834**	,924**	,805**	-0,312	-0,243	0,678	,825**	-,753**	-,581*
Chlorophyll a	M1	-0,400	-0,202	,765**	0,446	,879**	,872**	,994**	,638*	-,872**	-0,292	,737**	0,374	1	0,091	0,135	0,430	-0,203	0,131	0,435	0,271	-0,479	-0,475	-0,140	0,341	-0,002	-,673*
	M2	-,587*	-0,127	0,254	-0,332	-0,049	0,011	0,117	,798**	-0,547	,872**	,677*	,678*	0,091	1	,801**	,811**	0,057	0,246	0,372	0,108	-,750**	-,697*	0,094	0,185	-0,501	0,069
TCC	M1	-,922**	-,705*	-0,110	-,717**	-0,248	-0,215	0,111	,730**	-0,505	,620*	,744**	,954**	0,135	,801**	1	,945**	,608*	,774**	,812**	,665*	-0,331	-0,254	0,630	,719*	-,800**	-0,347
	M2	-,936**	-,636*	0,194	-0,471	0,074	0,107	0,415	,897**	-,753**	0,542	,919**	,969**	0,430	,811**	,945**	1	0,417	,681*	,827**	,655*	-0,527	-0,459	0,479	,696*	-,688*	-0,471
Flavonoids	M1	-,661*	-,843**	-,740**	-,911**	-,626*	-,648*	-0,277	0,005	0,121	0,048	0,158	,611*	-0,203	0,057	,608*	0,417	1	,931**	,741**	,770**	0,540	,604*	,979**	,867**	-,710**	-0,477
	M2	-,877**	-,952**	-0,445	-,812**	-0,346	-0,359	0,063	0,342	-0,248	0,098	0,501	,834**	0,131	0,246	,774**	,681*	,931**	1	,929**	,906**	0,257	0,328	,942**	,961**	-,778**	-,667*
PEAR Yield	M1	-,950**	-,924**	-0,111	-,602*	-0,034	-0,036	0,375	,592*	-0,553	0,097	,748**	,924**	0,435	0,372	,812**	,827**	,741**	,929**	1	,914**	-0,025	0,044	,750*	,953**	-,720**	-,785**
	M2	-,850**	-,869**	-0,282	-0,574	-0,137	-0,165	0,215	0,343	-0,329	-0,096	0,557	,805**	0,271	0,108	,665*	,655*	,770**	,906**	,914**	1	0,248	0,322	0,783	,964**	-,663*	-,721*
Lipids	M1	0,227	-0,315	-,813**	-0,359	-0,558	-,618*	-0,540	-,805**	,750**	-0,547	-,656*	-0,312	-0,479	-,750**	-0,331	-0,527	0,540	0,257	-0,025	0,248	1	,992**	0,457	0,247	0,015	-0,104
	M2	0,153	-0,391	-,840**	-0,427	-,593*	-,648*	-0,541	-,768**	,718**	-0,524	-,606*	-0,243	-0,475	-,697*	-0,254	-0,459	,604*	0,328	0,044	0,322	,992**	1	0,505	0,315	-0,085	-0,128
Carrageenans	M1	-,722*	-,915*	-0,666	-,894**	-0,586	-0,579	-0,203	0,146	0,009	0,234	0,237	0,678	-0,140	0,094	0,630	0,479	,979**	,942**	,750*	0,783	0,457	0,505	1	,908**	-,757*	-0,602
	M2	-,876**	-,988**	-0,354	-,711*	-0,191	-0,208	0,254	0,399	-0,382	-0,042	0,595	,825**	0,341	0,185	,719*	,696*	,867**	,961**	,953**	,964**	0,247	0,315	,908**	1	-,641*	-,876**
Cellulose	M1	,739**	,755*	0,330	,738**	0,408	0,369	0,044	-0,416	0,251	-0,370	-0,496	-,753**	-0,002	-0,501	-,800**	-,688*	-,710**	-,778**	-,720**	-,663*	0,015	-0,085	-,757*	-,641*	1	0,402
	M2	,624*	,751*	-0,101	0,166	-0,313	-0,287	-,610*	-0,389	0,529	0,304	-,587*	-,581*	-,673*	0,069	-0,347	-0,471	-0,477	-,667*	-,785**	-,721*	-0,104	-0,128	-0,602	-,876**	0,402	1

Statistical significance at 0.01 level (**) or at 0.05 level (*) bilateral, using Pearson correlation test in SPSS 24.0; Signalling (–) reveal the negative relation between parameters or in its absence, their positive correlation. Values presented are for R^2.

2.6.4.3 Antioxidant quantification

2.6.4.3.1 Total phenolic compounds (TPC)

The highest value for total phenolic content (TPC) (table 1) was determined in ethanol extracts, E(EtOH)M1 (1.71 ± 0.13 g GAE/100g dw) and E(EtOH)M2 (1.43 ± 0.08 g GAE/100g dw). The TPC presence in ethyl acetate extracts varied from non-detectable quantity in E(EtAc)M2 to a slightly higher content, 0.07 ± 0.01 g GAE/100g for E(EtAc)M1, suggesting a weak affinity. Farvin and Jacobsen (2013) [34] evaluated eleven different seaweeds from the coast of Denmark and found that protocatechnic, gentisic and hydroxybenzoic phenolic acids were the major compounds in TPC for red and green seaweeds. A positive Pearson correlation (R^2=0.80 to R^2=0.95), presented in table 2, between iodine and TPC was detected in the extracts. According to Hou et al. (2000) [35], iodine could be predominantly found in seaweed tissues bound with proteins, polyphenols and pigments.

2.6.4.3.2Total flavonoid content (TFC)

Flavonoids, a subdivision of TPC, are secondary metabolites constituted by anthocyanidins, chalcones, flavones, flavanols, flavanones, isoflavones and flavonols, differentiated according to their biosynthetic origin and presenting a general structure of two aromatic rings linked together by a 3-carbon bridge [36]. Total flavonoid content (TFC) determined in the extracts was inversely related to the polarity of the solvents used in the extraction. Highest TFC content was determined in ethyl acetate extract (table 1), E(EtAc)M1 24.25 ± 0.11 g QE/100g dw and lowest with water extraction, 0.04 ± 0.0 g QE/100g dw, both in E(H2O)M1 and E(H2O)M2. Solvent efficiency for TFC extraction in M1 apparently decreases from E(EtAc)M1 > E(MeOH)M1 > E(EtOH)M1 > E(H2O)M1, or in M2 from E(EtAc)M2 > E(EtOH)M2 > E(H2O)M2 > E(MeOH)M2. This suggests that flavonoids extraction, using M1 method is inversely correlated with the solvent polarity, ethyl acetate having strong affinity to flavonoids. According to Stankovic et al. (2011) [37], when using solvents like chloroform, dichloromethane, diethyl ether or ethyl acetate, polar flavonoids such as flavonols, methylated flavones, isoflavones and flavanones are extracted. Still, the use of alcohols or alcohol-water mixtures, allows the extraction of flavonoid glycosides and more polar aglycones. Tukey b test showed that all values of flavonoids were independent from each other in both extraction methods. The positive correlation in table 2 (R^2=0.931) between M1 and M2 methods suggests, for *A. taxiformis*, that solvents are more likely to influence flavonoid extraction than the extraction methods used.

2.6.4.3.3 Chlorophyll *a*

Chlorophylls are greenish pigments that have antioxidant bioactivity in consumed seaweed [38]. These pigments in processed food can be converted into compounds such as pheophytin, pyropheophytin and pheophorbide, known to have preventive action against cancer [8]. The only chlorophyll type found in red seaweeds is chlorophyll *a* [39] and maximum yield of this pigment was obtained using ethanol (table 1), E(EtOH)M1 (45.95 ± 0 mg/100g dw) and methanol in E(MeOH)M2 (34.80 ± 1.98 mg/100g dw). When using water, no chlorophyll *a* was detected. Using Pearson's correlation analysis, no correlation could be found when comparing the two different methodologies, M1 and M2, in their chlorophyll content,

indicating that chlorophyll a content is strongly dependent of the method applied to obtain the resulting extract. This effect can be easily observed when comparing chlorophyll a content in ethanol extract, E(EtOH)M1, 45.95 ± 0 mg/100g dw and in E(EtOH)M2, 14.34 ± 2.69 mg/100g dw, a decrease of 3.2 times. Methanol demonstrated the inverse behavior, extracting 8.65 ± 1.98 mg/100g dw in E(MeOH)M1 and increasing to 34.80 ± 1.98 mg/100g dw in E(MeOH)M2, a four time increase. This fact can be due to solvent efficiency influenced when heated in the Soxhlet extractor. Ethanol decreases its efficiency and methanol increases. Also, comparing the amount of chlorophyll a content in whole seaweed *A. taxiformis*, 28.81 ± 3.25 mg/100g dw, described in our previous work [40], only ethanol extract (45.95 ± 0 mg/100g dw) using E(EtOH)M1 and methanol extract (34.80 ± 1.98 mg/100g dw) using E(EtOH)M2, allowed a higher concentration of chlorophyll *a*, demonstrating the higher affinity of chlorophyll *a* for these two solvents, using different methods.

2.6.4.3.4 Total carotenoids content (TCC)

Carotenoids are terpenoid pigments and their oxygenated derivatives are called xanthophylls. These compounds have the ability to function as antioxidants, neutralizing reactive oxygen species (ROS) during metabolic processes [41]. Red seaweeds as *A. taxiformis* essentially produce lutein, α-carotene and zeaxanthin [8,42]. Total carotenoids content (TCC) varied according to the solvents used to produce the extracts. The highest value was obtained with methanol extract (table 1) in E(MeOH)M1, 36.23 ± 1.05 mg/100g dw and the lowest with water extract, with no amount detected. Comparing with the work of Chan et al. (2015) [18], the highest amount achieved in their work was using ethyl acetate extract, 73.44 ± 14.87 mg/100g dw and in our work, ethyl acetate was also very close to the highest value, 36.13 ± 1.72 mg/100g dw in E(EtAc)M1. This demonstrates that carotenoids in *A. taxiformis* have similar affinity to methanol and ethyl acetate when method 1 is applied and that ethyl acetate presents a high content of carotenoids, not only demonstrated in this work but also in the work of Chan et al. (2015) [18]. Therefore, it can be observed that the sequence of total carotenoids contents according to the solvents used is exactly the same when comparing the two different methods. M1 and M2 have the same order, E(MeOH) > E(EtAc) > E(EtOH) > E(H2O), but M1 has shown highest efficiency when extracting total carotenoids. In a previous work [40], we have demonstrated that *A. taxiformis* whole seaweed had 13.14 ± 2.63 mg/100g dw, and comparing this value with the ones obtained in the extracts, all of the extracts have higher concentration of TCC, except in water derived extracts, that presented from 1.66 ± 0.26 mg/100g in E(H2O)M2 to no amount detected in E(H2O)M1. Pearson's correlation evidenced a high degree of correlation between the two methods (R^2=0.945) in table 2, permitting to conjecture that TCC extraction is strongly defined by the solvent used and not from the methods applied in this work.

2.6.4.4 Antioxidant activity

The antioxidant activity, which includes reducing activity (RA), free radical scavenging activity (FRSA) and ferrous ion chelation (FIC), was also determined and results are presented in table 3.

Table 3 – Antioxidant activity of extracts from *Asparagopsis taxiformis* (Delile) Trevisan

Solvent	Reducing Activity (RA) mg AAE(100 g)$^{-1}$ in dw		FRSA (DPPH) mg IC 50 (mL)$^{-1}$		FIC mg IC 50 (mL)$^{-1}$	
	M1	M2	M1	M2	M1	M2
Water	233.15 ± 5.15a	174.38 ± 11.65a	4.65 ± 0.29a	1.37 ± 0.03a	113.01 ± 10.62a	74.00 ± 1.81a
Ethanol	1908.44 ± 59.15b	1156.86 ± 13.87b	1.54 ± 0.07b	1.37 ± 0.04a	5.26 ± 0.27b	10.49 ± 0.44b
Methanol	584.46 ± 15.36c	1161.47 ± 14.43b	2.69 ± 0.03c	1.64 ± 0.01b	8.36 ± 0.29c	10.07 ± 0.18b
Ethyl acetate	409.60 ± 10.84d	707.42 ± 98.78c	3.62 ± 0.04d	1.44 ± 0.08a	1.57 ± 0.03d	5.88 ± 0.26c

Data are mean ± standard deviation in milligrams per 100 grams of seaweed on a dry weight basis, mg IC 50 (mL)$^{-1}$ or percentage. All determinations were carried out in triplicate. Different letters within the same column indicate significant differences ($p \leq 0.01$) determined in SPSS 24.0 using Tukey b test.

2.6.4.4.1 Reducing activity (RA)

This antioxidant assay incorporates L-ascorbic acid as a standard and evaluates the potential of the compounds that comprise the extract to function as single electron transfer (SET) through primary antioxidation. The results are presented in L-ascorbic acid equivalents. The RA reaches the highest values of 1,908.44 ± 59.15 mg AAE/100g dw in E(EtOH)M1 and of 1,161.47 ± 14.43 mg AAE/100g dw in E(MeOH)M2 extracts. The lowest RA was obtained with distilled water E(H20)M1 and E(H2O)M2, 233.15 ± 5.15 and 174.38 ± 11.65 mg AAE/100g dw, respectively. RA in M1 shows a decrease in activity from E(EtOH)M1 > E(MeOH)M1 > E(EtAc)M1 > E(H2O)M1. In M2, there was a change in the most efficient solvent, presenting a different order, E(MeOH)M2 > E(EtOH)M2 > E(EtAc)M2 > E(H2O)M2. This discrepancy between M1 and M2 demonstrates the influence of the methods applied when comparing the same solvent extraction. Comparing ethanol extraction in the two methods employed, the antioxidant capacity assessed by RA obtained in E(EtOH)M1 was 1.7 times higher than E(EtOH)M2, distinguishing the simple stirring method for 72 hours with ultra-sounds as a better choice. In this assay we used BHT as a common antioxidant standard. The comparative RA of the extracts was between 573 and 52 times weaker than BHT, using E(H2O)M2 and E(EtOH)M1, respectively. Statistical analysis using Tukey b test showed that RA values in M1 were independent, each solvent having a different degree of reducing activity, but in M2 ethanol and methanol are closely related. Using Pearson's correlation (table 2), it was determined that M1 has some degree of correlation with M2 (R^2=0.658), suggesting that the solvents characterize the RA in the extract but methodology also imprints some variation.

2.6.4.4.2Free radical scavenging activity (FRSA)

FRSA uses DPPH as a stable radical to measure the ability of seaweed antioxidant compounds, e.g. carotenoids and chlorophyll, to scavenge and neutralize ROS and proton radicals, generated in tissues as result of oxidative stress [15]. It uses mechanisms based on the single electron transfer (SET) [43]. The

IC_{50} values demonstrated that E(H2O)M2 and E(EtOH)M2 were the most efficient extracts (table 3), with values of 1.37 ± 0.03 mg/ml and 1.37 ± 0.04 mg/ml, respectively. E(H20)M1 was the least efficient extract, having the highest value for IC_{50}, 4.65 ± 0.29 mg/ml. It can be observed how two different methodologies greatly influence the performance of the final extract, although using the same solvent. In M1, a clear differentiation was detected, resulting in E(EtOH)M1 < E(MeOH)M1 < E(EtAc)M1 < E(H2O)M1, with E(EtOH)M1 needing 3 times less extract to develop the same activity as E(H2O)M1. For M2, although some variation occurred in the sequence, the values are very similar, demonstrating high efficiency. The crescent order for M2 is E(H2O)M2 < E(EtOH)M2 < E(EtAc)M2 < E(MeOH)M2, with the three first solvents presenting better results than E(EtOH)M1. This in an evidence that M2, independently of the solvent used, has a higher ability of producing extracts with better IC_{50} results. In M1, the results will vary greatly, depending of the solvent used to produce the extract. Mellouk et al. (2017) [28] also evaluated *A. taxiformis* of which water, methanol and ethanol extracts also produced good DPPH scavenging activity, using an extract concentration lower than 0.2 mg/ml. Statistical analysis was performed, and Tukey's test determined that in M1 there are four distinct groups, highlighting the intrinsic capability of each solvent to produce a different extract with singular scavenging activity. In M2, only 2 distinct groups were formed, of which E(H2O)M2, E(EtOH)M2 and E(EtAc)M2 are one and E(MeOH)M2 the second group. No correlation between methods was determined using Pearson's test (table 2), showing a clear independence and with no correlation between equal solvents.

2.6.4.4.3Ferrous ion chelation (FIC)

The ferrous ion chelation (FIC) assay measures the ability of secondary antioxidants to inhibit oxidation through an indirect approach, in this case metal chelating [44]. It was demonstrated, for method 1 (table 3), that E(EtAc)M1 developed 50% of chelating activity with the lowest concentration of extract, 1.57 mg/ml and E(H20)M1 the highest, 112.32 mg/ml. For method 2, E(EtAc)M2 presented the lowest concentration needed to achieve 50% of chelating activity, 5.88 mg/ml, and E(H20)M2 the highest concentration, 73.97 mg/ml. In M1, in increasing order of concentration to achieve 50 % of chelating activity, E(EtAc)M1 < E(EtOH)M1 < E(MeOH)M1 < E(H2O)M1. In M2, the order was slightly different, E(EtAc)M2 < E(MeOH)M2 < E(EtOH)M2 < E(H2O)M2. Except for water and ethyl acetate, the other extracts varied inversely, due to the change in the methodology. Statistical analysis using Tukey's b test resulted in four distinct groups in M1, showing independence in the results obtained in each extract and in M2 only three groups were formed, of which extracts produced with ethanol and methanol are in the same. Pearson's correlation (table 2) between M1 and M2 is $R^2 = 0.855$, showing strong relation between methodologies and attributing similar activity between different extracts, using the same solvent, in both methods. Also, when evaluating Pearson's correlation between parameters, FIC was determined to have a strong linear correlation with TCC, highlighting that carotenoid content is linked to the antioxidant capacity, when evaluating the ability of carotenoids to function as chelators, with a secondary antioxidant mechanism.

2.6.4.5 Downstream extractions

In order to test a downstream extraction, the remaining seaweed residue was kept and dried in an oven at 40 ºC and weighed. Residue derived from seaweed extracts are denominated as PEAR (post-extracted algal residue) or simply as algal cake. This strategy was implemented to overcome some problems that often occur, which are the discharge of residue, low profitability and high input of biomass. This approach intends to understand how much can be extracted from PEAR, using multiple steps, to extract and purify high value seaweed derived products. Three extraction procedures in a sequential way were assessed. Each time PEAR was subjected to an extraction protocol, the residue was dried, grounded and weighed to determine the real yield that can be obtained from the subsequent residue. Lipids were primarily extracted from PEAR, followed by carrageenan and cellulose. Results are shown in table 4.

2.6.4.5.1 Residue quantification

R(EtAc)M2 presented the highest amount of PEAR as a starting material, 92.75 ± 1.00 g/100g dw and lowest from R(H2O)M1, 58.73 ± 5.54 g/100g dw. Using M1, in decreasing order of PEAR yield, R(EtAc)M1 > R(EtOH)M1 > R(MeOH)M1 > R(H2O)M1. In M2, the order was exactly the same only varying the quantity of PEAR yield. Statistical analysis, using Tukey's b analysis, showed that in M1, all PEAR yield formed statistically different groups and in M2 no individual groups were formed. We can assume that M1 makes a clear distinction between different PEAR yields, however this cannot be observed in M2, due to the variation of extraction using Soxhlet apparatus, defining its efficiency. This is intrinsically linked to the method rather than the solvent previously used. Pearson's correlation test demonstrated that M1 and M2 are close related (table 2), which originated a correlation of $R^2 = 0.914$. Also, it was determined that PEAR yield is positively correlated with cellulose, with flavonoids and TCC measured in the primary extract.

2.6.4.5.2 Lipids extraction

Lipids were the first product to be extracted from PEAR, using the solvent extraction procedure described by Folch et al. (1957) [25]. Highest lipid content was determined in L-R(EtAc)M1, 2.05 ± 0.03 g/100g dw and the lowest quantity in L-R(MeOH)M1, 0.26 ± 0.03 g/100g dw. For M1, resulted in the following sequence, in decreasing order of lipids quantity, L-R(EtAc)M1 > L-R(H2O)M1 > L-R(EtOH)M1 > L-R(MeOH)M1. The order was exactly the same in M2, with quantities varying slightly. We have also performed direct extraction of lipids, using same procedure, using whole seaweed *A. taxiformis*, and found that 5.42 ± 0.23 g/100g dw of lipids could be extracted. Comparing the results of downstream processing with direct extraction of whole seaweed *A. taxiformis*, we found that from whole seaweed, two to fourteen times higher lipids yield could be extracted. However, lipids extracted from PEAR could be a solution to increase profitability from the same biomass. According to Mellouk et al. (2017) [28], 2.85 g/100g dw of lipids could be extracted from Algerian *A. taxiformis* of which 91% are FAME, comprised of 23% saturated and 68% unsaturated fatty acids. The different yield obtained in our work could be due to different extraction technics or due to the natural variations in biocompounds within the *A. taxiformis* seaweed, that occur throughout the year or being located in different sites, in this case in the Atlantic Ocean and

Mediterranean Sea, respectively. El-Baroty et al. (2007) [45], analysed the lipid composition concluding that ω3 linolenic acid is the major fatty acid of the lipid fraction in *A. taxiformis* and Mozaffarian (2005) [46] demonstrating that this fatty acid has the ability to prevent cardiovascular diseases. According to these previous studies, lipids extracted from *A. taxiformis* could became an important product when assessing its nutraceutical applicability.

All lipid extractions were subjected to FTIR-ATR scan for comparative analysis (figure 2). A direct extraction of lipids from *A. taxiformis* was performed permitting a visual comparison between this extraction and lipids extracted from PEAR. Eighteen major peaks were identified from 650 to 4000 cm^{-1} wavenumber. This technique was used to evaluate the variability that occurs when extracting lipids from PEAR and perform a primary characterization of lipids extracted from *A. taxiformis* producing a fingerprint spectra. Peaks represent the percentage of transmittance in corresponding wavenumber measured in cm^{-1}. The IR spectra is majorly divided in two groups. From wavenumber 650 to 1800 cm^{-1}, fifteen major peaks can be observed and correlated with lipids polar head groups and from wavenumber 2800 to 4000 cm^{-1}, a second group is observed, with 3 major peaks. Bands from 1 to 9 can also be correlated with high concentration of halogens in *A. taxiformis* [2]. According to Burreson et al. (1975) [47], which initially evaluated the haloforms in the essential oil of *A.taxiformis*, collected in Waikiki (Hawai), due to their interest in the odoriferous constituents, they have determined that the main component of this essential oil is the bromoform CHBr3, followed by smaller amounts of numerous chlorine and iodine haloforms. Recently, Machado et al. (2016) [29], evaluated whole and several extracts of *A. taxiformis* potential to inhibit methanogenesis. They have extracted a high yield of the bromoform CHBr3 using DCM in dried *A. taxiformis* (172.32 mg/100g dw) which successfully reduced the *in vitro* total gas production, using concentrations ≥ 5μM. Additionally, new highly brominated cyclopentenones were recently discovered in *A. taxiformis* extracts. These new molecules are called “Mahorones” and were discovered while Greff et al. (2014) [48] were initiating a chemical ecological study to determine the function of secondary metabolites in their interaction with native species.

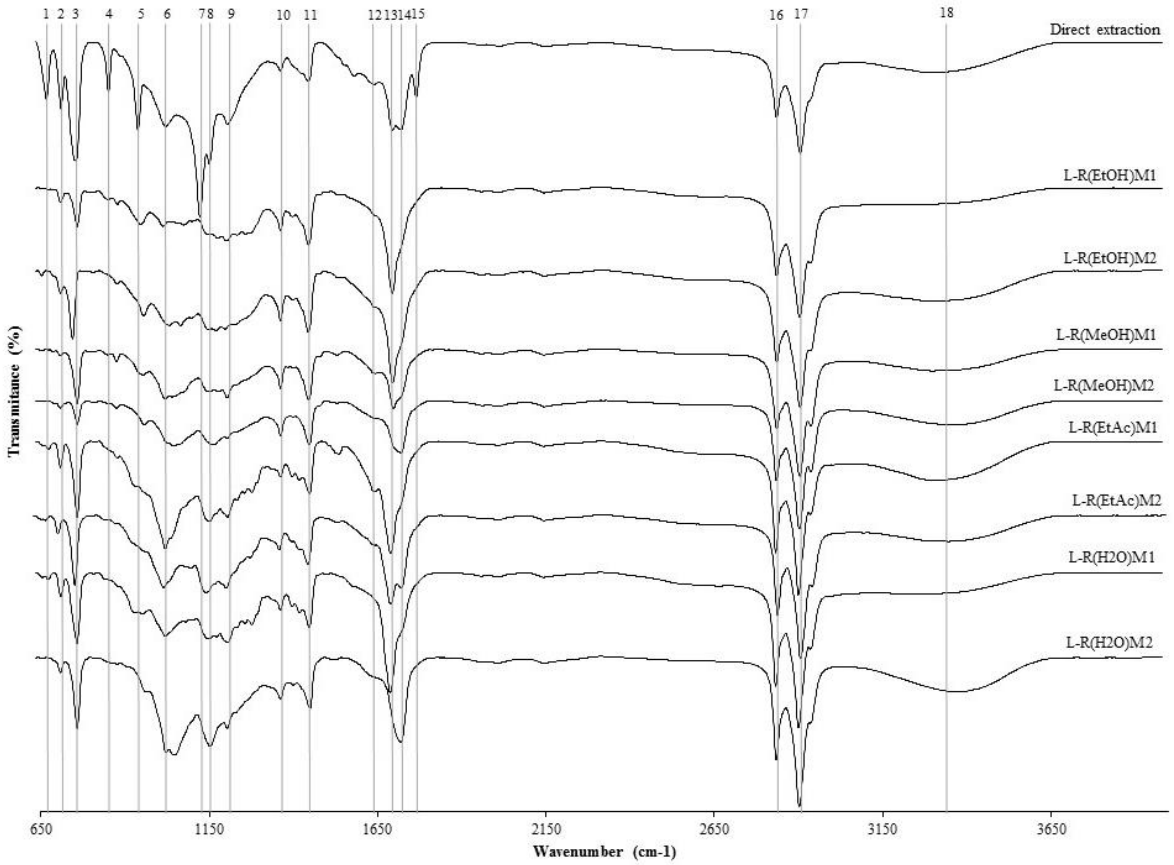

Figure 2 – FTIR ATR spectra, plotting wavenumber (cm^{-1}) by transmittance (%), of lipids extracted using Folch et al. (1957) [40] methodology of whole seaweed (direct extraction) or from PEAR.

Band 2 at 720 cm^{-1} can be attributed to CH2 rocking and band 11 could also be attributed to CH2 bending. Band 16 and 17 are due to C-H stretching in CH3 and CH2. Peaks within 1070 and 1250 cm^{-1} (band 7 and 8) are corresponding to C-O-C stretching and band 14 to C=O stretching in esters [14]. The wider peak (band 18) is due to some water content in lipid extract. Comparing the FTIR-ATR spectra, it becomes possible to visualize the fluctuation of major peaks intensity between direct extraction and lipid extracted from the downstream residue. This technic should be suitable for rapid quality control with further studies in lipid composition of *A. taxiformis*.

Statistical analysis, using Tukey's b test, demonstrated that all of the values in M1 and M2 are independent, presenting each PEAR as unique biomass in terms of lipid extractability. Pearson's correlation test showed that M1 and M2 have an R^2 = 0.992 (table 2), also showing that the lipid extraction is independent of this two methodologies and strongly dependent of the solvent primarily used.

2.6.4.5.3Carrageenan extraction

Carrageenan was the second product to be extracted from PEAR and highest yield (table 4) was obtained in C-R(EtAc)M2, 21.18 ± 0.81 g/100g dw and the lowest quantity obtained in C-R(H2O)M2, 2.28 ± 0.47 g/100g dw. The resulting sequence, in decreasing order in M1, was C-R(EtAc)M1 > C-R(MeOH)M1 > C-R(EtOH)M1 > C-R(H2O)M1. In this sequence, where the solvent has direct contact for 72 hours with the biomass, it can be observed that carrageenan yield increase is inversely correlated with the polarity

index of the solvent used for extraction, having ethyl acetate 4.4, methanol 5.1, ethanol 5.2 and water 9.0. For M2, the polarity index was not as linear, and changed the order of the sequence resulting in C-R(EtAc)M2 > C-R(EtOH)M2 > C-R(MeOH)M2 > C-R(H2O)M2. We have performed the extraction of carrageenan from whole *A. taxiformis* and found that 20.11 ± 1.09 g/100g dw (data not shown) could be extracted. Comparing this result with PEAR carrageenan extraction, a quantity of one to nine times higher could be extracted from the whole seaweed that the amount which is extracted from PEAR. Although, PEAR could present a slightly higher result of carrageenan yield when comparing to whole seaweed due to the concentration process. When extracting several compounds from the initial biomass, this residue will inevitably become more concentrated in certain compounds that were not previously extracted. This will make carrageenan more concentrated in PEAR than in whole seaweed. C-R(EtAc)M1 and C-R(EtAc)M2 have carrageenan yield similar to whole seaweed and C-R(H2O)M2 about 8.8 times less than whole seaweed. Comparing with the work of Hung et al. (2009) [49], 49% of the dw of carrageenan-rich *Kappaphycus alvarezii* cultivated in Camranh Bay, Vietnam, are carrageenans. Although *A. taxiformis* and its extracts present far less carrageenan, bioactivity could be an important factor to assess special features that would increase its applicability.

Table 4 – Yield of PEAR and quantification of subsequent extraction of lipids, carrageenan's and cellulose from *Asparagopsis taxiformis* (Delile) Trevisan residue

Solvent	PEAR Yield		Lipids		Carrageenans		Cellulose	
	g (100 g)$^{-1}$ in dw		g (100 g)$^{-1}$ in dw		g (100 g)$^{-1}$ in dw		g (100 g)$^{-1}$ in dw	
	M1	M2	M1	M2	M1	M2	M1	M2
Water	58.73 ± 5.54a	76.08 ± 1.87a	1.80 ± 0.04a	1.30 ± 0.03a	3.75 ± 1.56a	2.28 ± 0.47a	23.81 ± 0.89a	20.74 ± 0.68a
Ethanol	75.43 ± 3.65b	87.30 ± 1.18bc	0.65 ± 0.06b	0.58 ± 0.03b	6.98 ± 1.56a	14.30 ± 1.46b	21.65 ± 2.69a	18.13 ± 0.52b
Methanol	72.47 ± 0.39c	81.20 ± 5.59ab	0.26 ± 0.03c	0.35 ± 0.03c	7.77 ± 1.83a	6.85 ± 3.07a	20.30 ± 0.89a	20.59 ± 0.37a
Ethyl acetate	82.71 ± 2.86d	92.75 ± 1.00c	2.05 ± 0.03d	1.57 ± 0.08d	20.78 ± 1.88b	21.18 ± 0.81c	18.59 ± 0.49a	18.47 ± 0.54b

Data are mean ± standard deviation in grams per 100 grams of seaweed residue on a dry weight basis. All determinations were carried out in triplicate. Different letters within the same column indicate significant differences ($p \leq 0.01$) determined in SPSS 24.0 using Tukey b test.

Carrageenan extracted from PEAR and obtained with a direct extraction were subjected to FTIR-ATR scan for comparative analysis between 650 to 1500 cm^{-1} (figure 3). Band 1 (694 cm^{-1}), between band 2 (741 cm^{-1}) and 3 (770 cm^{-1}), peaks are usually assigned to skeleton bending of pyranose ring. This is well observed in direct extraction and C-R(EtOH)M1 spectra. According to Knutsen et al. (1994) [50], that implemented a nomenclature for red seaweed polysaccharides, absorption at 805 cm^{-1} (band 4) are manifestations of 3,6-anhydro-D-galactose-2-sulphate (DA2S) and can be assigned to iota hybridization of carrageenan. Band 4 appeared prominently as a shoulder in direct extraction and C-R(EtOH)M1 readings. In the same extractions, a strong absorption at 820 cm^{-1} (band 5) and 867 cm^{-1} (band 8), indicates the presence of D-galactose-6-sulphate (D6S), attributed to mu carrageenan, a precursor of kappa. At 825 cm^{-1} (band 6) is assigned to D-galactose-2,6-disulphate (D2S, 6S), the nu carrageenan, a precursor of iota. At 845 cm^{-1} (band 7), assigned to the presence of D-galactose-4-sulphate (G4S), is representative of kappa carrageenan. This appeared as a peak in direct extraction and C-R(EtOH)M1 and in a lesser extend in the

other carrageenan extractions. The degree of hybridization between iota and kappa are usually performed, dividing the value obtained at 805 cm^{-1} by the value obtained at 845 cm^{-1}, originating a ratio of carrageenan hybridization [51]. This is particularly important due to the variation that occurs with carrageenan that is constituted mostly by kappa hybridization, resulting in a strong but brittle gel and iota carrageenan in soft and elastic gel. Differences in these ratios, defines the application of carrageenan extractions, majorly in the food industry, for example, in the production of chocolate milk [52]. Ratios obtained were between 1.02 in C-R(EtOH)M2 and 1.19 in direct extraction, with C-R(EtOH)M1 obtaining the highest ratio, 1.11 within carrageenan extracted from PEAR. This indicates the majority of iota hybridization in carrageenan extracted from *A. taxiformis*. Absorption of IR between 928 cm^{-1} (band 9) and 933 cm^{-1} (band 10) with a shoulder at 1070 cm^{-1} (band 13) represents the presence of 3,6anhydro-D-galactose (DA). Absorption between 1010 cm^{-1} (band 11) and 1030 cm^{-1} (band 12) with an additional band at 1150 cm^{-1} (band 14) may be attributed to C-C and C-O stretching vibrations of pyranoid ring, common to all polysaccharides [53]. Considering absorptions of IR between 1210 cm^{-1} (band 15) and 1260 cm^{-1} (band 16), it is attributed to sulphate ester ($O\text{-}SO^{-}_{3}$) (S). This is particularly important due to the rheological properties that are influenced by the degree of sulfation within the polymer. This characterization can be well observed in direct extraction and C-R(EtOH)M1. Carrageenan extracted from C-R(EtOH)M1 presents a pattern very similar to carrageenan extracted from *A. taxiformis* direct extraction.

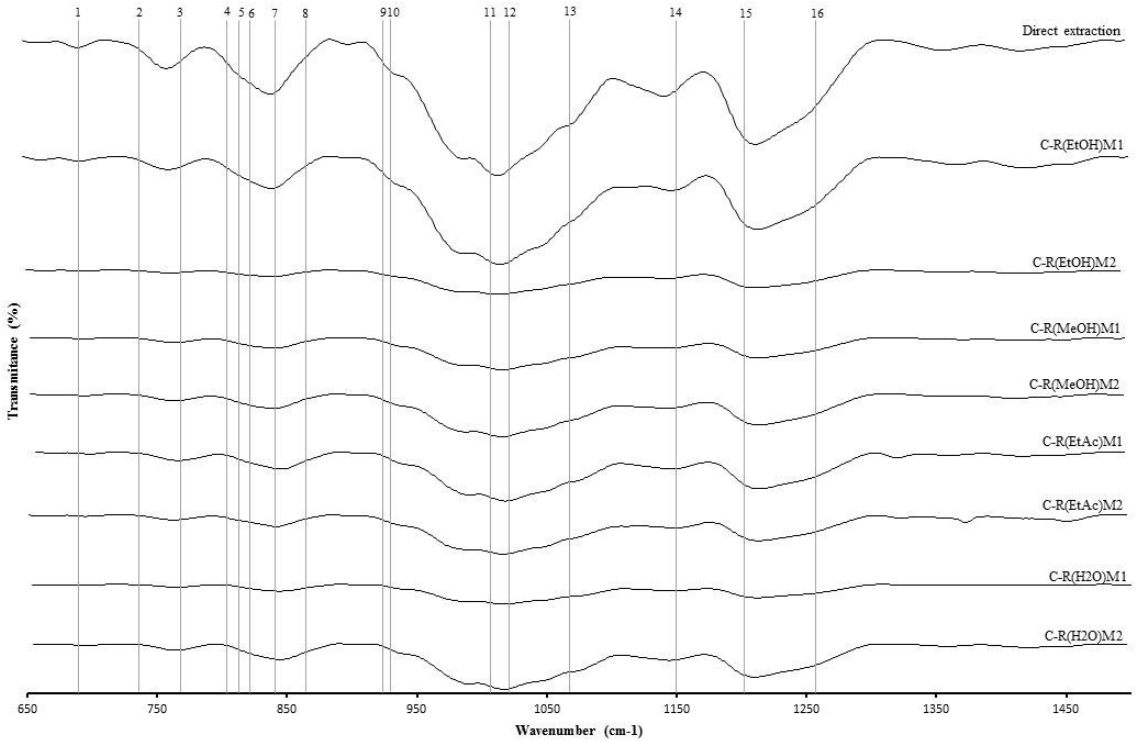

Figure 3 – FTIR ATR spectra, plotting wavenumber (cm^{-1}) by transmittance (%), of carrageenan extracted using Tasende et al. (2012) [41] methodology of whole seaweed (direct extraction) or from PEAR.

Statistical analysis using Pearson correlation (table 2), demonstrated an inverse proportionally between extraction yield and carrageenan extraction yield but positively related with flavonoids content in the primary extract. Tukey's b test showed that in M1, 2 groups are formed, C-R(H20)M1, C-R(EtOH)M1 and C-R(MeOH)M1 and second group only C-R(EtAc)M1. For M2, 3 groups were formed, of which one was formed by C-R(H20)M2 and C-R(MeOH)M2, C-R(EtOH)M2 and C-R(EtAc)M2.

2.6.4.5.4Cellulose extraction

Thirdly, we have successfully extracted cellulose from the remaining residue and the highest cellulose value is presented in Ce-R(H20)M1, 23.81 ± 0.89 g/100g dw and the lowest in Ce-R(EtOH)M2, 18.13 ± 0.52 g/100g dw (table 4). The sequence, from the highest content to the lowest was Ce-R(H2O)M1 > Ce-R(EtOH)M1 > Ce-R(MeOH)M1 > Ce-R(EtAc)M1. Here, we can observe that the extraction yield of cellulose is proportional to the polarity index of the solvents used. For M2, this was not observed, and the sequence resulted in Ce-R(H2O)M2 > Ce-R(MeOH)M2 > Ce-R(EtAc)M2 > Ce-R(EtOH)M2. We also determined the cellulose content from whole seaweed *A. taxiformis*, and determined that 9.76 ± 0.15 g/100g dw could be obtained from direct extraction. Comparing with PEAR residue, cellulose could be extracted from PEAR 1.9 to 2.4 times higher than from direct extraction. The motive of the increase ratio, comparing PEAR with whole seaweed, is due to an increase of cellulose concentration in final PEAR, due to selectively extracting other compounds. In each time that we extracted a different compound from initial PEAR, we would dry, grind and weight, and each time we did this, cellulose would get concentrated in final PEAR. According to Siddhanta et al. (2009) [54], 2.5 to 12.5% of cellulose could be determined in 12 different seaweeds from India, evidencing the potential that PEAR has in extracting and purifying cellulose. Long polymeric chains of cellulose can be extracted from seaweed and hydrolyzed forming nanocellulose [55]. Nanocristaline cellulose has an enormous potential, with multiple applications such as the production of films with barrier properties, biocomposites, and systems for controlled drug release [56], which justifies the interest of extracting cellulose from PEAR.

Using FTIR ATR analysis we developed a fingerprint spectra for cellulose extracted from red seaweed *A. taxiformis* (figure 4). Cellulose extracted and purified from PEAR was compared with cellulose obtained from direct extraction from whole seaweed *A. taxiformis*. Fifteen major bands were identified from 650 to 4000 cm^{-1} in FTIR ATR spectra which can be used for comparability. Decrease of transmittance % at 900 cm^{-1} (band 3) are due to the absorption of IR from glycosidic linkages between the anhydroglucose rings in the cellulose. This peak can be observed in every cellulose extracted from PEAR but better distinguish in Ce-R(EtOH)M2. Between 1000 cm^{-1} (band 4) and 1030 (band 5) decreases in transmittance could be due to C-O-C bending in which Ce-R(EtOH)M2 demonstrates prominently. At 1054 cm^{-1} (band 6) a strong decrease of transmittance could be observed and can be assigned to the skeletal vibration of C-O-C pyranose ring skeleton in cellulose fibers. This peak was visible in all of the cellulose extracted from PEAR and directly extracted from whole seaweed but with different intensities. At 1160 cm^{-1} (band 8), a decrease in transmittance can be assigned to C-O-C asymmetric stretching of cellulose. This can be well observed in cellulose obtained directly from whole seaweed but more distinguish in Ce-R(EtOH)M2. A decrease in transmittance at 1432 cm^{-1} (band 12) could be due to C-H bending and at 1642 cm^{-1} (band 13) IR absorbed could be assigned to O-H bending in water molecules. An sp3 C-H stretching vibration was detected at 2903 cm^{-1} (band 14) and a broad peak appeared at 3316 cm^{-1} (band 15) which could be assigned

as a specific absorbance of O-H stretching, demonstrating an hydrophilic tendency of the cellulose fibers [57]. This peak is visible in all cellulose extracted from PEAR and whole seaweed *A. taxiformis* but prominently visible in Ce-R(EtOH)M2.

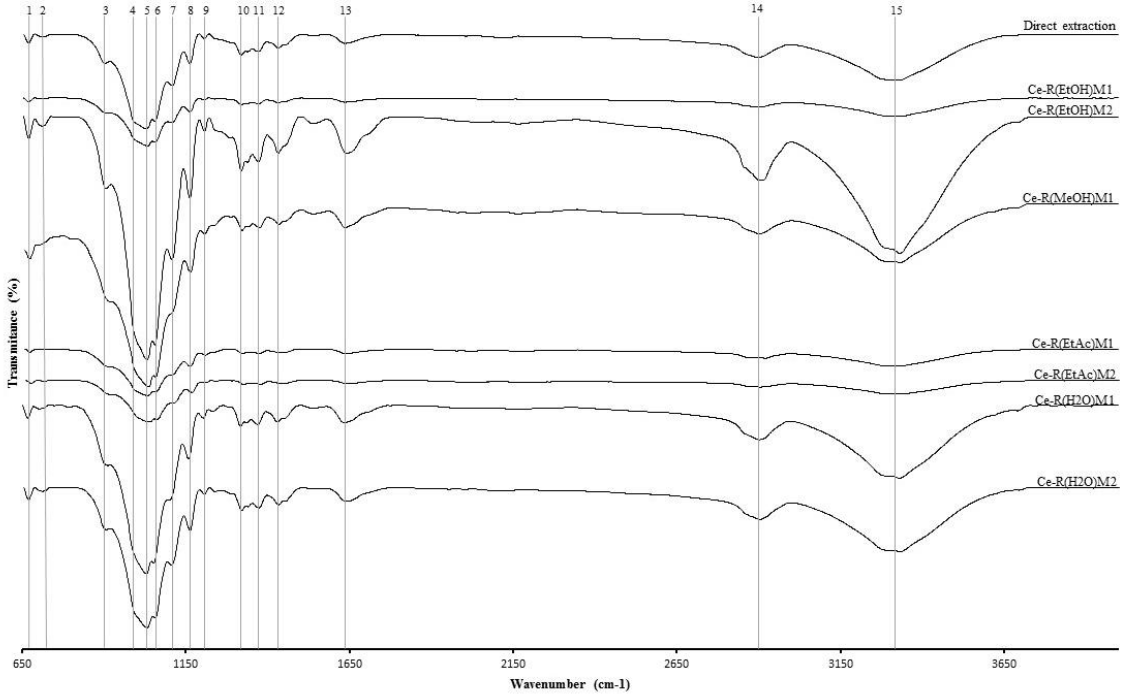

Figure 4 – FTIR ATR spectra, plotting wavenumber (cm^{-1}) by transmittance (%), of cellulose extracted using Baghel et al. (2015) [42] methodology of whole seaweed (direct extraction) or from PEAR

Statistical analysis showed that applying Tukey's b test, M1 only formed one group, suggesting that these values are not statistically different and in M2 two groups were formed. One group includes Ce-R(H2O)M2 and Ce-R(MeOH)M2, the second group the remaining Ce-R(EtOH)M2 and Ce-R(EtAc)M2. The Pearson's correlation test (table 2) demonstrated that there isn't correlation between methodologies, and cellulose is negatively correlated with residue yield proving the statement previously described that cellulose will be increasingly concentrated after supplement, lipids and carrageenan extraction.

2.6.5 Conclusion

The perspective of this research work was to increase the knowledge of the nutraceutical potential of *A. taxiformis* from Madeira archipelago, as a whole or, its extracts. Also, the development of a downstream strategy, to develop procedures to purify valuable compounds from *A. taxiformis* PEAR, was performed, attributing applicability and yield of important products to remaining residue. This seaweed demonstrated to be an excellent source of natural iodine, utilizing four solvents permitted in the food industry with two distinct methodologies. Method 1 demonstrated to be the most predictable, since certain parameters could be related with the polarity index of the solvents used. Since our main effort was to produce an iodine rich extract, ethanol demonstrated to be the most efficient solvent in having the most concentrated iodine content. Evaluation of the antioxidant compounds in E(EtOH)M1 resulted in the highest values for TPC and chlorophyll a content. Also, in three parameters analysed for antioxidant capacity, E(EtOH)M1 developed the best results for RA and FRSA antioxidant capacity tests. For the downstream strategy adopted in this work, lipids could be further studied to determine their fatty acids composition, carrageenan studied in their gelling properties, purity and bioactivity. Cellulose could be the precursor from low to high end products, since this could be the starting material to produce paper, bioethanol or micro and nano-crystalline cellulose, a multifunctional ingredient in the food and pharmaceutical industry. When targeting iodine rich extracts, we have determined, using statistical analysis, that iodine content is positively correlated with TPC and negatively correlated with flavonoids, allowing us to extrapolate in the future, which extracts would have higher concentration of iodine based on TPC or flavonoids analysis. FTIR ATR analysis demonstrated high potential for spectra comparison and quality control due to its simplicity and time saving. It produced a fingerprint signature for lipids, carrageenan and cellulose extracted directly from whole seaweed *A. taxiformis*, used for comparison between same products extracted from PEAR.

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2.7 Constructing ethanol-derived bioactive extracts using the brown seaweed *Zonaria tournefortii* (J.V.Lamouroux) Montagne performed with Timatic extractor by means of response surface methodology (RSM)

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2.7.1 Abstract

Understanding the biochemical and antioxidant composition and capacity of a target biomass is the first step to its selectivity as functional food, which can enhance the ability to promote health by reducing the risk of chronic diseases. The main purpose of this work was to employ Response Surface Methodology (RSM) to determine the effect of the independent variables, % of ethanol (50 – 96 %), time of sonication (0 – 20 min) and number of extraction cycles (6 – 18 cycles) in the primary extract of brown seaweed *Zonaria tournefortii*, studying the yield variation of some bioactive compounds, assessing the potential of these bioextracts to integrate as a natural additives or supplements in the functional food industry. The extractions were performed employing "Green Chemistry" technics, executed with the Timatic extractor, which applied pressurized ethanol solution at a maximum pressure of 8.5 bar, through milled dehydrated biomass in the extraction vessel. Several parameters were assessed in the primary bioactive extract, which included extract yield (11.56 – 28.49 g (100 g)$^{-1}$ dw), total chlorophyll content (0.14 – 1.42 g (100 g)$^{-1}$ dw), total carotenoid content (0.35 – 0.80 g (100 g)$^{-1}$ dw), total fucoxanthin content (0.04 – 0.13 g (100 g)$^{-1}$ dw), total phenolic content (3.58 – 5.84 g (100 g)$^{-1}$ dw), total flavonoid content (0.22 – 4.70 g (100 g)$^{-1}$ dw), DPPH (56.05 – 76.45 %) and reducing activity (3.83 – 6.04 g (100 g)$^{-1}$ dw). A second objective was to determine residue suitability for subsequent extraction of valuable compounds such as fucoidan (4.87 to 6.59 g (100 g)$^{-1}$ dw) and cellulose (18.88 to 20.27 g (100 g)$^{-1}$ dw), implementing the first step to a biorefinery strategy, using a cascade approach.

Keywords: Functional food; Bioactive extract; Biorefinery; Madeira Archipelago; Brown macroalgae; Fucoidan.

2.7.2 Introduction

Nowadays, intensive research is conducted worldwide to increase knowledge concerning the integration and use of non-conventional food sources, of both terrestrial and marine origin, to improve the nutritional quality of food (Kumar et al. 2011). The understanding of the biochemical composition and antioxidant capacity of the target biomass is the first step to its selectivity as a functional food, promoting health benefits and reducing the risk of chronic diseases. Seaweeds represent an excellent raw material for nutrients, food ingredients and bioactive compounds which are increasingly exploited (Kılınç et al. 2013). Intensive research concerning biorefinary strategies implementation to seaweed biomass has been published, which this resource is introduced in a cascade extraction for multiple product production, increasing the biomass usability and reducing waste. Sustainable strategies for cascade extraction, linked to an efficient disintegration of biomass to obtain valuable biocompounds, can lead to a profitable industry (Gilbert-López et al. 2015). Kumar et al. (2013), used *Gracilariopsis longissima*, a red seaweed, to extract primarily agar and subsequently produce ethanol and a fertilizer, using the remaining biomass residue. Baghel et al. (2015) studied the implementation of a cascade extraction in 3 selected seaweeds, *Gelidiella acerosa*, *Gelidium pusillum* and *Gracilaria dura*. Their research demonstrated that bioethanol could be efficiently produced from this biomass as a primary product which afterwards lipids, agar, phycobilins and a liquid fertilizer could be extracted from the residual pulp. In a previous work (Nunes et al., 2018), we have studied the potential of a red seaweed, *Asparagopsis taxiformis* to integrated in a cascade extraction to primarily extract a bioactive extract followed by the extraction of lipids, carrageenan and cellulose from the residue. The green seaweed, *Ulva lactuca*, was also studied by Bikker et al. (2016) which envisioned the production of a rich protein extract to be used as an animal feed. Also, applying an anaerobic fermentation to the hydrolysate, acetone, butanol, ethanol and 1,2-propanediol could be produced. Brown seaweed was also introduced in the research effort which Kostas et al. (2017) demonstrated that *Laminaria digitata* as the potential to be a valuable source of alginates and fucoidan. Furthermore, a waste liquor was obtained as a byproduct, which demonstrated antioxidant and antimicrobial activity and finally ethanol could be produced through fermentation. In this work, *Zonaria tournefortii*, a common brown seaweed present in the south shore of Madeira island was the target seaweed. In a previous work, we have characterized seven macroalgae collected in Madeira Archipelago, which *Z. tournefortii* was included, which demonstrated the highest antioxidant activity and lipid content (Nunes et al., 2017). The main objective of this work was to determine the variation of some bioactive compounds, in a primary extract of the brown seaweed *Zonaria tournefortii*, when varying three independent variables (% of ethanol, time of sonication and number of extraction cycles) according to a Box-Behnken design. A second objective was to determine the suitability of the residue for subsequent extraction of two valuable biocompounds, fucoidan and cellulose, implementing a biorefinery strategy, using a cascade approach.

2.7.3 Materials and methods

2.7.3.1 Seaweed biomass

Zonaria tournefortii (J.V.Lamouroux) Montagne 1846 samples were collected in a 10-meter maximum depth dive of the Madeiran south coastline, coordinates 32,646951 - 16,823967. Samples were collected in august 2016 and were transported in seawater and gently rinsed with filtered fresh water to remove salt and debris. Afterwards, a primary drying was applied in which seaweed was frozen at -35 ºC and freeze-dried under reduced pressure (4 x 10^{-4} mbar), with a cooling trap set at -56 ºC for 5 days. Samples were milled to 200 mesh particle size, vacuum packed and stored at -35 ºC until use.

2.7.3.2 Experimental design and statistical analysis

Optimization of extraction conditions was performed using STATISTICA 10.0 software for Windows, adopting a Box-Behnken design, with three-factor and three-level of second-order regression. The number of experiments were calculated according with the equation 1:

$$N = k^2 + k + cp \qquad (1)$$

where k is the factor number and cp is the replicate number of the central points. This resulted in 15 experimental runs with three central points. The parameters and levels employed were 50, 73 and 96 % ethanol extractions, time of sonication (0, 10 and 20 min) and Timatic cycle extractions (6, 12 and 18 cycles). These independent variables were assessed in order to determine the maximum yield of several parameters used to evaluate the primary extract. A response surface methodology (RSM) was employed and fitted to a second-order polynomial model, according with equation 2:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i + \sum \sum_{i<j=1}^{3} \beta_{ij} X_i X_j \qquad (2)$$

where, Y is the predicted response, β_0 is the constant coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the cross product coefficients, X_i and X_j are independent variables. The statistical method of RSM is used for optimizing several industrial processes.

In order to visualize the correlation between the response and the independent variables, the fitted polynomial regression equations were expressed graphically. Analysis of variance (ANOVA) was performed to distinguish significant differences between dependent variables ($p < 0.05$). Two dimensional contour plots were developed, while holding one variable constant in the second-order polynomial model. The validity of the model was determined by comparing the experimental and predicted values.

2.7.3.3 Preparation of ethanol “Green Chemistry” extracts

Extractions were performed with 50, 73 or 96% ethanol, using a Timatic semiautomatic extractor (Technolab, Spello, Italy, represented in figure 1, set at a constant pressure and static phase. Program was set as follows: solvent capacity 1L, solvent to biomass ratio 50:1, compression time 3 min, decompression

time 6 min, minimum pressure 6 bar, compression pressure 8.5 bar at room temperature (± 23 ºC). Total process time varied with the nº of cycles, 6 cycles ran for 54 min, 12 cycles for 108 min and 18 cycles for 162 min. Twenty (20) gram samples were placed in a 100µm pore bag and placed inside a 0.5 L extracting vessel. At the end of each extraction, extracts were filtered through 11µm Whatman filter, partly evaporated in a rotary evaporator and dehydrated completely in an oven, at 40 ºC. *Z. tournefortii* extracts were subjected to analysis of eight parameters at a concentration of 1 mg.ml^{-1}. The post-extracted algal residue (PEAR) was recovered and dried in an oven at 40 ºC, for further downstream assessment. Both extracts and PEAR were stored under vacuum at -35 ºC until use.

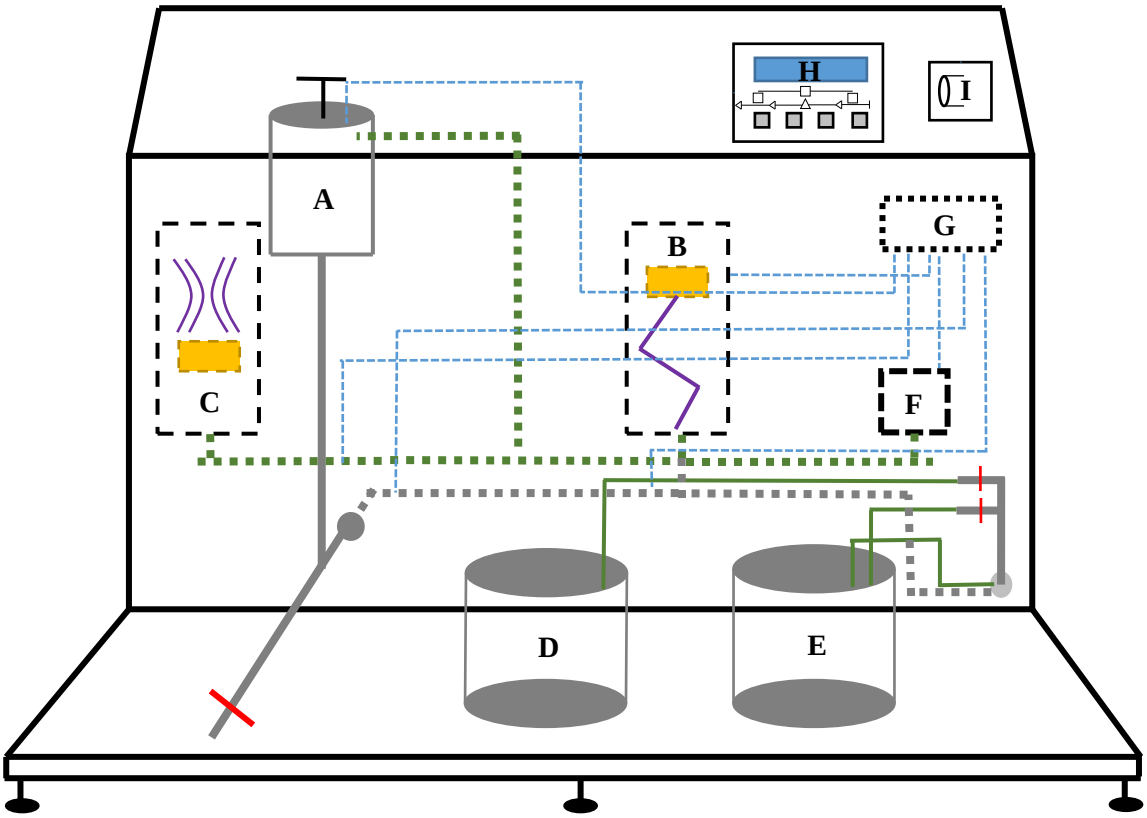

Figure 1 – Timatic Micro extractor schematic diagram, A: Sample vessel; B: Active pressure piston; C: Secondary pressure piston; D: Discharge; E: Discharge/Circulation; F: Pump; G: Pneumatic valve; H: Electronic display and access buttons; I: Printer.

2.7.3.4 Extract analysis

2.7.3.4.1 Total phenolic content (TPC)

TPC was determined using Folin Ciocalteu method described by Chew et al. (2008), adding 1.5 ml of Folin Ciocalteu's phenol reagent to 1.2 ml of Na_2CO_3 (7.5% w/v) and then adding 0.3 ml of sample extract (1 mg.ml^{-1}). The reaction mixture was incubated in the dark for 30 min and the absorbance was measured at 765 nm. Results were expressed as grams of gallic acid equivalents (GAE) per 100 grams in dry weight (dw) of extract. The calibration equation for gallic acid was $Y = 0.0076853x + 0.069$ $R^2 = 0.9969$.

2.7.3.4.2Pigments measurements

Total chlorophyll, carotenoids and fucoxanthin content in *Z. tournefortii* extracts were determined through the equations described in Verma et al. (2017) using a spectrophotometer, which the wavelength is described in each equation. These were assessed in triplicate and equations were as follows:

$$Total\ Chlorophyll\ \left(\frac{\mu g}{mL}\right) = 20.2(A645) + 8.02(A663)$$

$$Carotenoids\left(\frac{mg}{g}\right) = 4.2(OD452.5) - (0.0264(Chl\ a)) + (0.426(Chl\ b))$$

$$Fucoxanthin\left(\frac{mg}{g}\right) = A470 - 1.239\big(A631 + A581 - 0.3(A664)\big) - 0.0275(A664)/141$$

2.7.3.4.3Total Flavonoid Content (TFC)

The TFC of *Z. tournefortii* extracts was determined by the aluminium chloride colorimetry test according to Chan et al. (2015). Initially, 0.5 ml of sample (1 $mg.ml^{-1}$) or standard was stirred with 1.5 ml of methanol, 0.1 ml of aluminium chloride (10 %), 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The mixture was then incubated for 30 min in the dark at room temperature. Readings were carried out in a Shimadzu PC-2401 spectrophotometer at 415 nm and quercetin was used as a standard, ranging from 0.05 to 0.15 $mg.ml^{-1}$. The results were expressed as grams of quercetin equivalent (QE) per 100 grams dw of extract (g QE $(100g)^{-1}$ dw). The calibration equation for quercetin was $Y = 0.0059x - 0.0225$, R^2 = 0.9955. All measurements were performed in triplicate.

2.7.3.4.4Free radical-scavenging assay (DPPH)

For the DPPH assay, the 2,2-diphenylpicrylhydrazyl stable radical was used following the methods described by Yen and Chen (1995) and Duan et al. (2006) with modifications. An aliquot of 2 ml of freshly prepared 0.16 mM methanolic solution of DPPH was added to 2 ml of seaweed extract (1 $mg.ml^{-1}$) solution. The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark. Readings were accomplished in a Shimadzu PC-2401 spectrophotometer at 517 nm. An equal amount of methanol and DPPH served as control and ascorbic acid was used as positive control. All measurements were performed in triplicate. The result was calculated and expressed as % of radical scavenging.

$$\%\ Radical\ Scavenging = \left[1 - \left(\frac{A\ sample - A\ sample\ blank}{A\ control}\right)\right] \times 100$$

Where the *A* control is the absorbance of the control (DPPH solution without sample), the *A* sample is the absorbance of the test sample (DPPH solution plus test sample) and the *A* sample blank is the absorbance of the sample (sample without DPPH solution).

2.7.3.4.5Reducing activity (RA)

The RA assay was determined following the method of Oyaizu (1986) and Yuan et al. (2005) with modifications. A volume of 1.25 ml of potassium phosphate buffer (0.2 M, pH 6.6) was added to an equal

volume of potassium hexacyanoferrate at 1% (w/v). This solution was mixed with 0.5 ml of extract at 1 $mg.ml^{-1}$. The reaction mixture was incubated in a water bath at 50 ºC, during 20 min. Afterwards, 1.25 ml of trichloroacetic acid (10%, w/v), 1.25 ml of water and 0.25 ml of ferric chloride hexahydrate (0.1%, w/v) were added to 1.25 ml of the reaction mixture. The solution was incubated at room temperature, during 10 min for color development, in the dark. The absorbance was measured at 700 nm in a Shimadzu PC-2401 spectrophotometer. The RA value was expressed as grams of ascorbic acid equivalents (AAE) per 100 g of dw. The calibration equation for ascorbic acid was $Y = 0.0121x + 0.0047$, $R^2 = 0.9996$.

2.7.3.5 Downstream product analysis

To assess the potential of a downstream extraction, the seaweed PEAR (Post Extracted Algal Residue) was dried in an oven at 40 ºC and weighed. PEAR was subjected to two extraction procedures, using a cascade approach, firstly extracting fucoidan and secondly cellulose.

2.7.3.5.1 Fucoidan extraction

After the primary extraction, PEAR was used for fucoidan extraction. This method was followed according to the Chale-Dzul et al. (2015) with modifications. Two grams of dried residue were initially extracted with 50 mL of ethanol (1:25, w/v) at 80 % (v/v) at 70 °C, during 12 h. The residue was separated from the supernatant and used in the following extractions. Three aqueous extractions, using distilled water with a biomass:water ratio of 2:25 (w/v) were performed, with primary extraction conducted at room temperature, during 7 h, followed by a second extraction for 7 h extraction at 70 ºC and finally a third extraction for 4 h at 70 ºC. The aqueous extracts were pooled together and the residue kept and dried at 40 ºC for subsequent cellulose extraction. The liquid was treated with calcium chloride (2M) at room temperature in order to precipitate alginates. After alginate removal by centrifugation at 10,000 rpm for 30 min, the supernatant was dialyzed for 48 h to decrease salinity, changing distilled water every 12 hours. Resulting extracts were freeze-dried, analyzed in ATR-FTIR spectra analyzer and compared with fucoidan obtained by direct extraction from the biomass and with standard fucoidan extracted from *Fucus vesiculosus* (≥ 95% purity) purchased from Sigma-Aldrich.

2.7.3.5.2 Cellulose extraction

After fucoidan extraction, the remaining residue was integrated in the final biorefinery step to perform the cellulose extraction. The methodology was adapted from Baghel et al. (2015) with modifications. Initially this residue was soaked in acetate buffer (1:15, w/v) containing 36% of sodium chlorite. The mixture was bleached at 60 °C, during 8 h. Afterwards, samples were washed with water until neutrality and treated with sodium hydroxide (0.5M) solution (1:6, w/v) at 60 °C for 12 h. Afterwards, the mixture was washed with water until neutrality, re-suspended in hydrochloric acid at 5% (1:4, w/v) and heated up to boiling (100 ºC), maintaining it for 5 minutes. This mixture was left to rest overnight at room temperature, washed with water to remove acid excess and dried. The obtained cellulose was then characterized with ATR-FTIR and compared with cellulose directly extracted from the starting biomass.

2.7.3.5.3Spectroscopic analysis

The samples ATR-FTIR spectra were obtained with a Perkin Elmer Spectrum Two, coupled to a Diamond ATR (DurasamplIR II, Smiths Detection, UK). Thirty-two scans were acquired in transmittance mode, spectra range 4000-650 cm^{-1}, with a wave resolution of 1 cm^{-1}. All samples were analyzed in triplicates for all tests carried out.

2.7.4 Results and discussion

2.7.4.1 Box-Behnken design (BBD)

The experimental design matrix (table 1) was constructed using Box-Behnken design (BBD), with three factors (ethanol %, sonication time and number of cycles) and three levels (-1, 0, 1) in a second-order regression (equation 1), resulting in 15 runs with 3 center points (0,0,0). This set of experiments allowed us to determine the extraction conditions that produces a significant effect in the extracts bioactivity and to understand how the biocompounds are influenced. The percentage of ethanol varied from 50 to 96%, mixed with distilled water, previous sonication of sample was performed from 0 to 20 minutes and the Timatic extractor functioned between 6 to 18 cycles. The BBD is a spherical model, with all points describing a sphere, not containing any point at the vertices of the cubic region, created by the maximum and minimum limits of each variable. This design is advantageous when the points on the corners of the cube determine factor level combinations impossible to test. The BBD employed in this work enabled the prediction of the biocompounds in the bioextract (supplement table A), varying the independent variables (factor levels). The prediction of the bioactive compounds includes extract yield (Yield), total chlorophyll content (TChlC), total carotenoids content (TCC), total fucoxanthin content (TFxC), total phenolic content (TPC) and total flavonoids content (TFC). Also, it was integrated two dependent variables for antioxidant assessment, free radical-scavenging assay (DPPH) and reducing activity (RA).

Table 1 – Box-Behnken design (BBD) for 3 factors (independent variables, A, B and C) with 3 levels (-1, 0,+1). Design resulted in 15 runs with 3 center points (0,0,0). Ethanol used was 50, 73 or 96%. The selected number of cycles in the Timatic was 6, 12 or 18. The extraction was subjected to 0, 10 or 20 minutes of sonication.

Run	(A) Ethanol (%)	(B) Cycles (nº)	(C) Sonication (min)
1 (+1,-1,0)	96 (+1)	6 (-1)	10 (0)
2 (-1,-1,0)	50 (-1)	6 (-1)	10 (0)
3 (-1,0,-1)	50 (-1)	12 (0)	0 (-1)
4 (+1,0,+1)	96 (+1)	12 (0)	20 (+1)
5 (0,0,0)	73 (0)	12 (0)	10 (0)
6 (0,0,0)	73 (0)	12 (0)	10 (0)
7 (-1,+1,0)	50 (-1)	18 (+1)	10 (0)
8 (-1,0,+1)	50 (-1)	12 (0)	20 (+1)
9 (+1,+1,0)	96 (+1)	18 (+1)	10 (0)
10 (+1,0-1)	96 (+1)	12 (0)	0 (-1)
11 (0,-1,+1)	73 (0)	6 (-1)	20 (+1)
12 (0,-1,-1)	73 (0)	6 (-1)	0 (-1)
13 (0,+1,+1)	73 (0)	18 (+1)	20 (+1)
14 (0,0,0)	73 (0)	12 (0)	10 (0)
15 (0,+1,-1)	73 (0)	18 (+1)	0 (-1)

2.7.4.2 Influence of the independent variables in the biocompounds

Yield values (supplement table A) were higher with 50% ethanol extractions, 28.49 g (100 g)$^{-1}$ dw and decrease in an inverse correlation with the increase of ethanol percentage, reaching its minimum with 96% ethanol, 11.60 g (100 g)$^{-1}$ dw. For TChlC, TCC, TFxC, TPC and TFC, the highest values were obtained when performing the extraction with 96% ethanol, reaching 1.41, 0.79, 0.13, 5.81 and 4.34 g (100 g)$^{-1}$ dw, respectively. A direct correlation was determined between the percentage of ethanol used to perform the extraction and the yield of these biocompounds in the bioactive extract, reaching its lowest content when performing the extraction with 50% ethanol, which TChlC TCC, TFxC, TPC and TFC were 0.14, 0.35, 0.04, 3.63 and 0.45 g (100 g)$^{-1}$ dw. The DPPH did not show this linear behavior since the highest antioxidant capacity was demonstrated in the bioactive extract performed with 96% ethanol, 76.01 % and the lowest when performing the extract using 73% ethanol, 56.85%, when performing the free radical scavenging assay. The highest values for RA were obtained when the extractions were performed with 50% ethanol (5.88 g (100 g)$^{-1}$ dw), decreasing to minimal values when 73% ethanol (3.87 g (100 g)$^{-1}$ dw) was used.

2.7.4.3 Statistical model analysis

An ANOVA analysis was done to determine the relationship between the response and significant variables with 95% confidence with 0.05 significance level. High F-values associated with low ρ-values, generally less than 0.05, indicate that this model could be used to predict the biocompounds responses. The ρ-values greater than 0.05 indicate that the specific variable, integrated in the model, cannot be predicted accurately. In the statistical analysis of model (supplement table B), all F-values and respectively ρ-values were considered acceptable except for TFxC (ρ-value=0.072) and TFC (ρ-value=0.064). The Lack-of-fit performance analysis indicates the goodness of fit and ρ-values greater than 0.05 (non-significant) suggest that there is no lack of fit. This statistical test indicated that all of the dependent variables were adequate for this model and could predict the responses accurately, except for TFxC ($\rho = 0.016$).

2.7.4.4 Correlation matrix

A correlation matrix was obtained (supplement table C), which analysis demonstrated the relation between individual parameters. Significant negative statistical correlations were found between yield and TPC (-0.96), TChlC (-0.92), TCC (-0.82), TFxC (-0.78) and TFC (-0.74). Moreover, TChlC had also significant positive correlations between TPC (0.91), TFxC (0.88), TCC (0.86) and TFC (0.83). TCC demonstrated the same behavior with TFxC (0.96), TFC (0.82) and TPC (0.80). TFxC had additional statistical significant correlations with TFC (0.80) and TPC (0.79). Additionally, TPC demonstrated some correlation with TFC (0.72). These high correlation coefficients evidence that selected model has good relationship between the response and independent variables.

2.7.4.5 Model Optimization

Graphical analysis of the response surface was determined (data not shown), plotting firstly the ethanol % vs nº of cycles (constant 10 min of sonication), secondly the ethanol % vs time of sonication (constant of 12 cycles) and thirdly the nº of cycles vs time of sonication (constant 73% ethanol), to predict optimal extracting conditions for all parameters used. These constants (10 min sonication, 12 cycles in the Timatic extractor and 73% ethanol for extraction) correspond to the central level of each factor. For extraction yield, the RSM clearly predicts that the percentage of ethanol is inversely correlated with extract yield. Lower percentage of ethanol used to produce the extract will correspond to higher yields due to the usage of water as a co-solvent. Water has higher dielectric constant than ethanol, 80 and 25 at 20 ºC, respectively, thus representing higher polarity (Mohsen-Nia et al. 2010). Water can increase the density of the fluid mixture and cause swelling of the solid particles, improving the diffusion and thereby the solubilization of polysaccharides (Saravana et al. 2017). Higher extract yield could be achieved, performing the extraction exclusively under higher sonication time or alternatively increasing the number of cycles. The optimization of the independent variables enables maximum values for the studied parameters (supplement table D). The reducing activity (RA) is also higher when the ethanol percentage is 50%. This means that when targeting an extract with a higher antioxidant capacity with this specific activity, a lower percentage of ethanol would be preferable, reducing ethanol costs. For the extraction of the remaining biocompounds, the highest ethanol percentage (96%) is preferable. The extraction cycles could be significantly reduced for almost all of the biocompounds studied, except for DPPH and TChlC, reducing the time of extraction. Furthermore, the previous sonication performed to the biomass with the correspondent extract solvent could be neglected when targeting TCC and TPC or reduced to 8 or 13 min when higher DPPH and RA activity are preferable, respectively. The optimization of the conditions to perform the extract, enable to target specific characteristics, reducing costs, time of extraction and equipment usage. Plotting all independent variables, in order to determine the best conditions (total desirability) of the overall extraction (figure 2), these were found to be 96% ethanol, 14.4 cycles and 20 min sonication, which desirability value reached 0.72 which maximum value is 1.

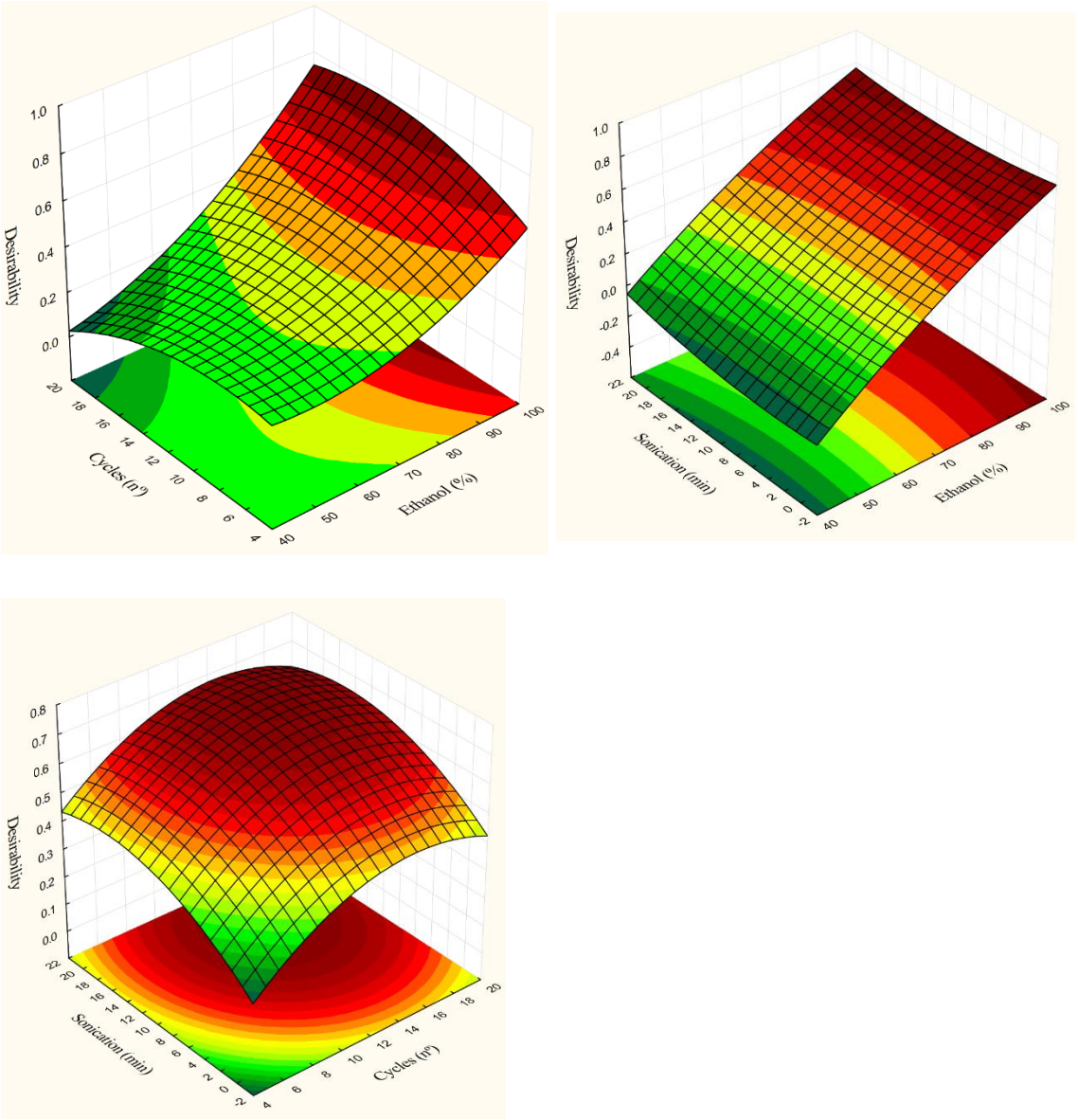

Figure 2 – Desirability surface plots, using Quadratic fit methodology, for the statistical assessment of the biochemical and antioxidant assays. Overall desirability was 0.72 for the designed model.

2.7.4.6 Model Validation

The mean, minimum, maximum and standard deviation for each individual extraction parameter was determined (supplement table E). The goodness of the model and RSM was verified using the multiple R, R^2 and adjusted R^2 correlation coefficients. The correlation coefficients for yield, TChlC, TPC and RA varied from 1 to a minimum of 0.9. These were found to possess the highest correlation between predicted and experimental values (supplement table A). For TCC, TFxC and DPPH, the correlation coefficients oscillated from 0.95 and a minimum of 0.84. The lowest correlation found was in TFC which varied from 0.91 to a minimum of 0.77. In all parameters, the predicted correlation was in close agreement with the adjusted coefficient of correlation.

2.7.4.7 Model Predictability

Surface plots were performed to predict the behavior and identify important relationships between the dependent variables. Five relevant relationships were found, which enabled a comprehensive understanding of how these parameters are linked.

In the figure 3 (A), it can be visualized a linear increase of TFxC when TChlC and TCC increase simultaneously. TFxC reaches the highest value, 0.17 g $(100\ g)^{-1}$ dw when TChlC and TCC is 1.6 and 0.9 g $(100\ g)^{-1}$ dw, respectively. Also, higher values of TFxC (0.14 g $(100\ g)^{-1}$ dw) is achieved when TChlC and TPC are at its maximum values, 1.6 and 6.0 g $(100\ g)^{-1}$ dw, respectively, presented in figure 3 (B). Again, higher TFxC value is achieved also when TPC and TFC are at its maximum value, 6.0 and 5.0 g $(100\ g)^{-1}$ dw, respectively, presented in figure 3 (C). These results demonstrate that higher TChlC, TCC, TPC and TFC values are preferable when constructing extracts with higher fucoxanthin contents. Furthermore, higher TPC values in the extract were found when higher TChlC and lower TCC values are present, figure 3 (D). TPC could reach 7.0 g $(100\ g)^{-1}$ dw when TChlC is at 1.6 g $(100\ g)^{-1}$ dw and TCC 0.3 g $(100\ g)^{-1}$ dw. Plotting TPC against TFC, figure 3 (E), it can be determined that DPPH percentage achieves maximum (100% of scavenging activity) when TPC it's at maximum value of 6.0 g $(100\ g)^{-1}$ dw and TFC at minimum value. This result means that the scavenging activity in the extract is not influenced by flavonoids but possibly by other phenolic compounds present in the extract.

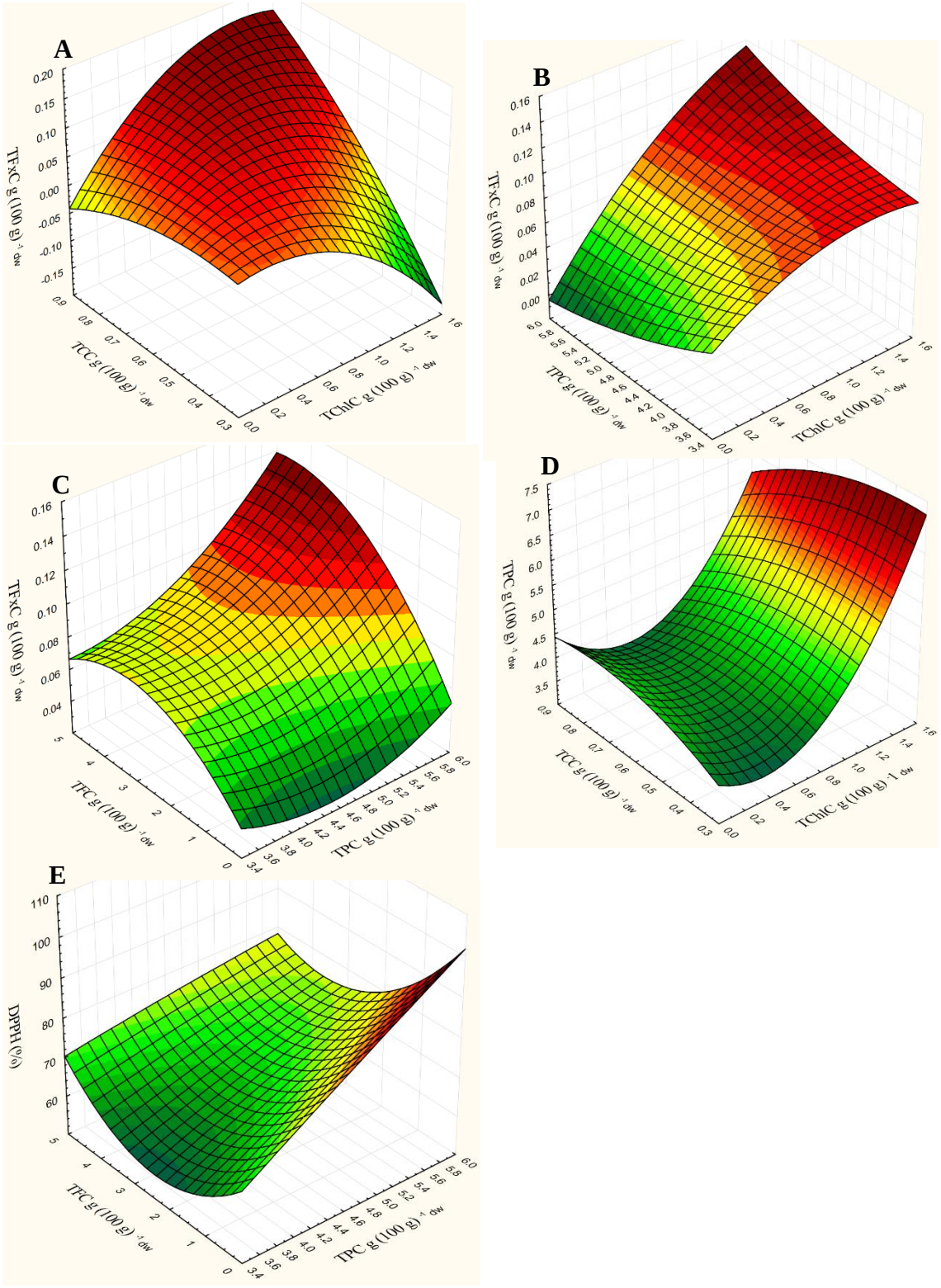

Figure 3 – Surface plots with predicted equation at the top and color scale for quantity. Values are described in g (100 g)$^{-1}$ dw except for DPPH which is presented in % of antioxidant capacity. (**A**) TFxC (Z) against TChlC (X) and TCC (Y), equation TFxC = -0,0164-0,0786*x+0,2592*y-0,1152*x*x+0,4313*x*y-0,319*y*y; (**B**) TFxC (Z) against TChlC (X) and TPC (Y) equation TFxC = 0,2045-0,0056*x-0,0598*y-0,0301*x*x+0,0235*x*y+0,0042*y*y; (**C**) TFxC (Z) against TPC (X) and TFC (Y) equation TFxC = 0,232-0,0922*x+0,0113*y+0,0103*x*x+0,0046*x*y-0,0042*y*y; (**D**) TPC (Z) against TChlC (X) and TCC (Y) equation TPC = 2,4294-1,1033*x+5,6895*y+2,387*x*x-1,8531*x*y-3,8494*y*y; (**E**) DPPH (Z)

against TPC (X) and TFC (Y) equation DPPH = 19,2021+15,2059*x-3,2193*y-0,2388*x*x-1,982*x*y+2,0789*y*y.

2.7.4.8 Cascade approach

The PEAR residue was reserved for the downstream extractions. This biorefinery effort was applied to create a possibility to overcome an industrial problem which is the high input of biomass, large discharge of residue and low profitability. Several scientific studies concerning this effort were recently published. Bikker et al. (2016), assessed a biorefinary strategy for the green macroalgae *Ulva lactuca*. They performed an aqueous treatment and an enzymatic hydrolysis at 50 ºC, primarily extracting a protein rich fraction, which could be used for animal feed. Applying an anaerobic fermentation to the liquid extract, they determined that biofuels, acetone, butanol, ethanol and 1,2-propanediol could be obtained. Also, Magnusson et al. (2016), studied *Ulva ohnoi* and *Ulva tepida* to integrate in a biorefinary effort, primarily extracting seaweed salts, dehydrating the water used to wash these macroalgae and hypothesized the use of the the remaining biomass to produce compost, used for crop production, feed mill utilized for animal production or to produce biocrude for renewable fuels.

The methodology presented in this work intends to assess the quantity of crude fucoidan and cellulose that can be extracted from PEAR. Each time PEAR was exposed to an extraction protocol, the residue was dehydrated, ground and weighed to assess the real yield that can be obtained from the subsequent residue. Also, to assess the lost biomass using this biorefinary strategy, table 2 was performed. The initial biomass, used to perform the extraction, was 20 g from which the highest bioactive extract content (5.7 g) originated from performing the extract with the lowest ethanol percentage in the extraction solvent (50%). Moreover, higher yield of crude fucoidan was extracted from the 73% and 96 % ethanol derived residue (1.3 g) and cellulose from the 50 % ethanol remaining residue (4.0 g). The lost or unused biomass varied from 8.4 to 10.9 g, from which higher losses resulted from the primary usage of the highest ethanol percentage in the extraction solvent (96%).

Table 2 – Biorefinery efficiency.

% of ethanol in the extraction solution	**Initial biomass weight** (g)	**Moisture** (g)	**Biomass extract weight** (g)	**Crude fucoidan weight** (g)	**Crude cellulose weight** (g)	**Unused biomass** (g)
50%	20	0.8	5.7	1.0	4.0	8.5
73%	20	0.8	5.4	1.3	4.1	8.4
96%	20	0.8	3.2	1.3	3.8	10.9

Data is a mean value in grams. Assessment of the biorefinary efficiency when performing the proposed cascade extraction, firstly removing a bioactive extract, followed by crude fucoidan and cellulose extraction.

2.7.4.8.1 Fucoidan

The fucoidan crude extract from PEAR of *Z. tournefortii* was quantified between 4.87 ± 0.33 and 6.59 ± 0.43 g (100 g)$^{-1}$ dw, presented in table 3, for the 50% and 73% ethanol previous extractions, respectively. Fucoidan extracted from whole seaweed *Z. tournefortii* was 5.66 ± 0.45 g (100 g)$^{-1}$ dw. PEAR from 73% and 96% ethanol extracts gave 6.59 ± 0.43 and 6.38 ± 0.21 g (100 g)$^{-1}$ dw, respectively, presenting more fucoidan than the seaweed biomass. This fact is due to the fucoidan enrichment in the residue, when performing a previous selective extraction to the seaweed biomass, resulting in a more concentrated source of fucoidan. It comprises fucose-enriched sulfated polysaccharides with a high degree of complexity. They are present in the fibrillar cell wall as structural molecules and in the intercellular spaces of brown seaweeds reinforcing tissue cohesion. Several biological activities can be attributed to fucoidan, including antioxidant, anti-inflammatory antithrombotic, anticoagulant, antivirus and also possess effect against various renal, hepatic and uropathic disorders (Senthilkumar et al. 2013). Nowadays it is used as an additive in a few products such as healthy food, drinks and cosmetics (Pádua et al. 2015). Fletcher et al. (2017) assessed 3 seaweeds from the coast of Aberystwyth (UK) and found that the percentage of fucoidan was 7.5, 8.9 and 12.2 for *F. serratus*, *A. nodosum* and *F. vesiculosus*, respectively. Skriptsova et al. (2010) determined that the yield of fucoidan extracted from *U. pinnatifida*, collected from Peter the Great Bay (Russia) from April till July, oscillated between 3.21 and 16.00 g (100 g)$^{-1}$ dw, depending on the month which was collected. Yuan and Macquarrie (2015) assessed a biorefinery strategy, using *A. nodosum*, collected in the coast of Shetland as a starting biomass, which determined that fucoidan yield could reach up to 14.09 g (100 g)$^{-1}$ dw. *Laminaria digitata*, collected in Cornwall, England, was also integrated in a biorefinary strategy application (Kostas et al. 2017), in which crude fucoidan was primarily extracted in which yield reached 6.55 g (100 g)$^{-1}$ dw, similar to this work.

Table 3 – Downstream extracted products from PEAR. Extracted and purified yield of fucoidan and cellulose, represented in g (100 g)$^{-1}$ in dw for the direct extraction from biomass and from PEAR (50, 73 and 96 % ethanol remaining residue).

	Fucoidan yield (g (100 g)$^{-1}$ dw ± SD)	**Cellulose yield** (g (100 g)$^{-1}$ dw ± SD)
Direct extraction	5.66 ± 0.45	8.75 ± 0.13
50% ethanol	4.87 ± 0.33	20.24 ± 0.10
73% ethanol	6.59 ± 0.43	20.27 ± 0.15
96% ethanol	6.38 ± 0.21	18.88 ± 0.26

The ATR-FTIR analysis of standard fucoidan purified from *F. vesiculosus* (≥95%), purified fucoidans resulting from the direct seaweed extraction and from PEAR extracts was performed (figure 4). Fifteen important bands were detected from 650 to 4000 cm^{-1} and used for fucoidan evaluation. IR absorption at 694 cm^{-1} (band 1) possibly indicates a twisting movement of C-O linkage. At 836 cm^{-1} (band 2) it was observed an intense peak due to a symmetric C-O-S bending vibration of sulfate substitutes at axial C-4 position. Absorption of IR occurred at 920 cm^{-1} (band 3) could indicate a rocking vibration of CH_3, peak at 974 cm^{-1} (band 4) an antisymmetric vibration of C-O-S and a rocking vibration of CH_3. An intense peak at 1,009 cm^{-1} (band 5), shoulder at 1,048 cm^{-1} (band 6), small peak at 1,137 cm^{-1} (band 7) and a more intense peak at 1,158 cm^{-1} (band 8) could be due to the stretching in a pyranoid ring and C-O-C stretching of glycosidic bonds. An intense peak of IR absorption at 1,222 cm^{-1} (band 9) is a characteristic peak for fucoidan, due to the vibration of S=O linkage. A shoulder present in band 9 with 1,256 cm^{-1} (band 10) could indicate an asymmetric O=S=O stretching vibration of sulfate esters. Small intensity peaks at 1,380 cm^{-1} (band 11) might be due to symmetric bending vibrations of CH_3 and at 1,455 cm^{-1} (band 12), scissoring vibrations of CH_2 and asymmetric vibrations of CH_3. At wavelength 1,635 cm^{-1} (band 13) a medium intensity peak was detected and could be due to the asymmetric stretching of carboxylate vibration. At 2,948 cm^{-1} (band 14) wavenumber, a low intensity peak aroused and could be attributed to C-H stretching. At 3,417 cm^{-1} (band 15), an intense band was detected and is characteristic of water presence in the fucoidan extract and standard.

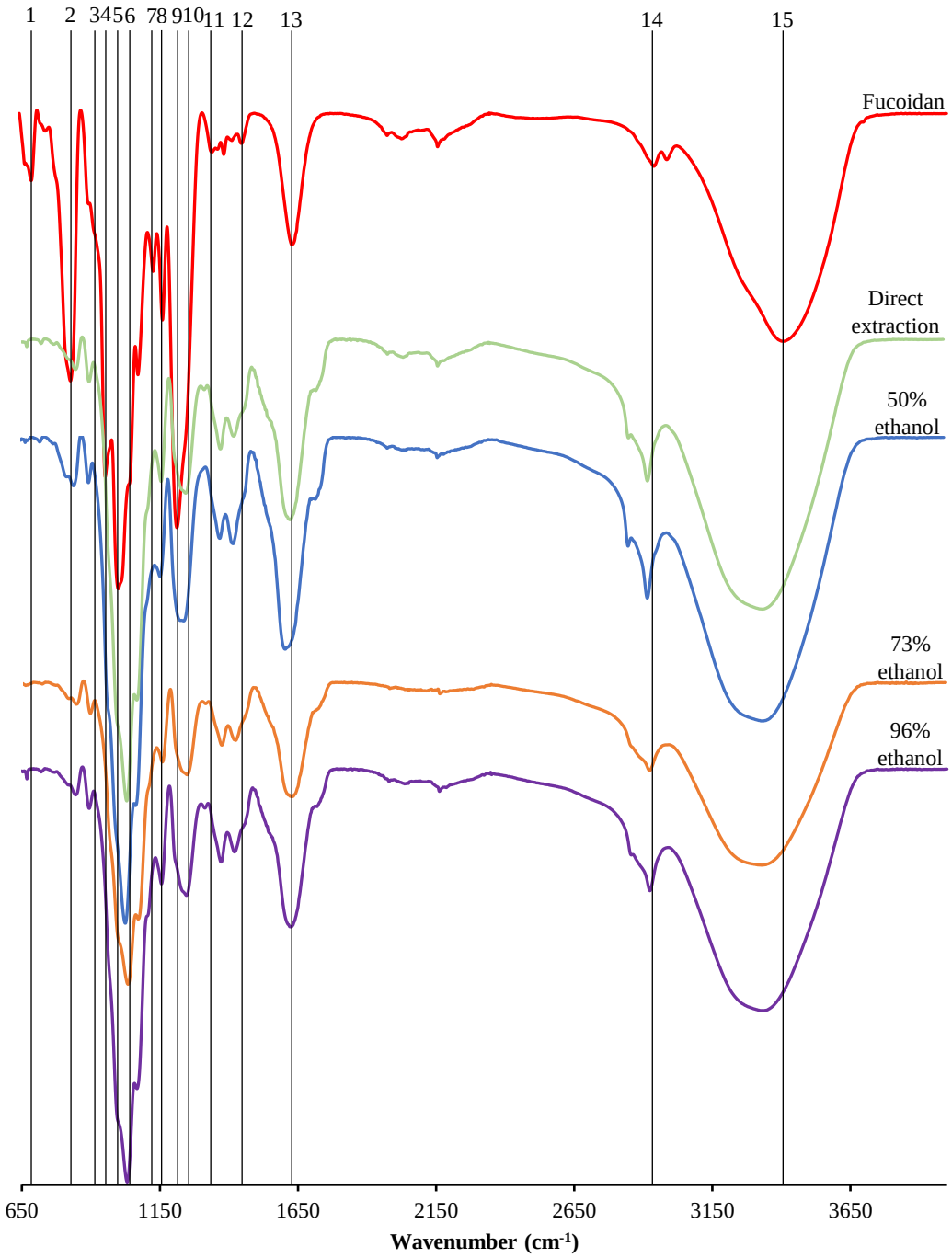

Figure 4 – ATR-FTIR spectra, plotting wavenumber (cm^{-1}) by transmittance (%), of fucoidan extracted using Chale-Dzul et al. (2015) methodology of whole seaweed (direct extraction) or from PEAR (50, 73 and 96% ethanol remaining residue) and comparing with fucoidan standard.

2.7.4.8.2 Cellulose extraction

The cellulose extracted from PEAR varied from 18.88 ± 0.26 to 20.27 ± 0.15 g (100 g)$^{-1}$ dw, presented in table 3, for the 96% and 73% ethanol remaining residues, respectively. The cellulose yield from whole seaweed *Z. tournefortii* was determined to be 8.75 ± 0.13 g (100 g)$^{-1}$ dw. The cellulose extracted from PEAR was about 2 times higher than from the direct extraction. The reason of the higher ratio is due to an increase in cellulose concentration in PEAR, which resulted from selective extractions of other compounds, as referred above for fucoidan. Each time another compound was extracted from the initial PEAR, it was dried, ground and weighed, and each time, cellulose was concentrated in the final PEAR. Cellulose extracted from seaweeds have long polymeric chains that could be hydrolyzed to produce nanocellulose (Chen et al. 2016). It has potential to be introduced in several applications being the production of films with barrier properties, biocomposites and systems for controlled drug deliver and release are some examples (Ditzel et al. 2017). Siddhanta et al. (2009) determined in 12 seaweed species from India, which cellulose varied from 2.5 to 12.5%, demonstrating the potential that PEAR has for selectively extract and purify cellulose. In a previous work (Nunes et al. 2018), we have demonstrated the applicability of a biorefinary strategy to the red macroalgae *Asparagopsis taxiformis*, in which 21.65 g (100 g)$^{-1}$ dw of cellulose could be extracted from the residue, when performing the initial extraction using ethanol.

The ATR-FTIR analysis technic developed a fingerprint spectra for the cellulose purified from *Z. tournefortii* (figure 5). Eleven important bands were identified from 650 to 4,000 cm^{-1} and used for comparison. The decrease of transmittance % at 902 cm^{-1} (band 1) was a consequence of glycosidic linkages between the anhydroglucose rings in the cellulose. IR absorption from 1,000 cm^{-1} (band 2) to 1,031 cm^{-1} (band 3) could be attributed to C-O-C bending. At 1,055 cm^{-1} (band 4), a reduction of transmittance could be detected and assigned to the vibration of C-O-C pyranose ring skeleton and at 1,160 cm^{-1} (band 5) due to C-O-C asymmetric stretching of cellulose. Transmittance reduction at 1,428 cm^{-1} (band 6) could be attributed to C-H bending and at 1,641 cm^{-1} (band 7) IR absorbed could be due to O-H bending in water molecules. Peak at 1,735 cm^{-1} (band 8) could be attributed to C=O and peak at 2,162 cm^{-1} (band 9) could be due to stretch in triple bonds between carbons. A sp3 C-H stretching vibration was identified at 2,899 cm^{-1} (band 10) and a stretching of O-H was responsible for IR absorption and consequent decrease of transmittance % at 3,343 cm^{-1} (band 11) due to some water present in the purified cellulose.

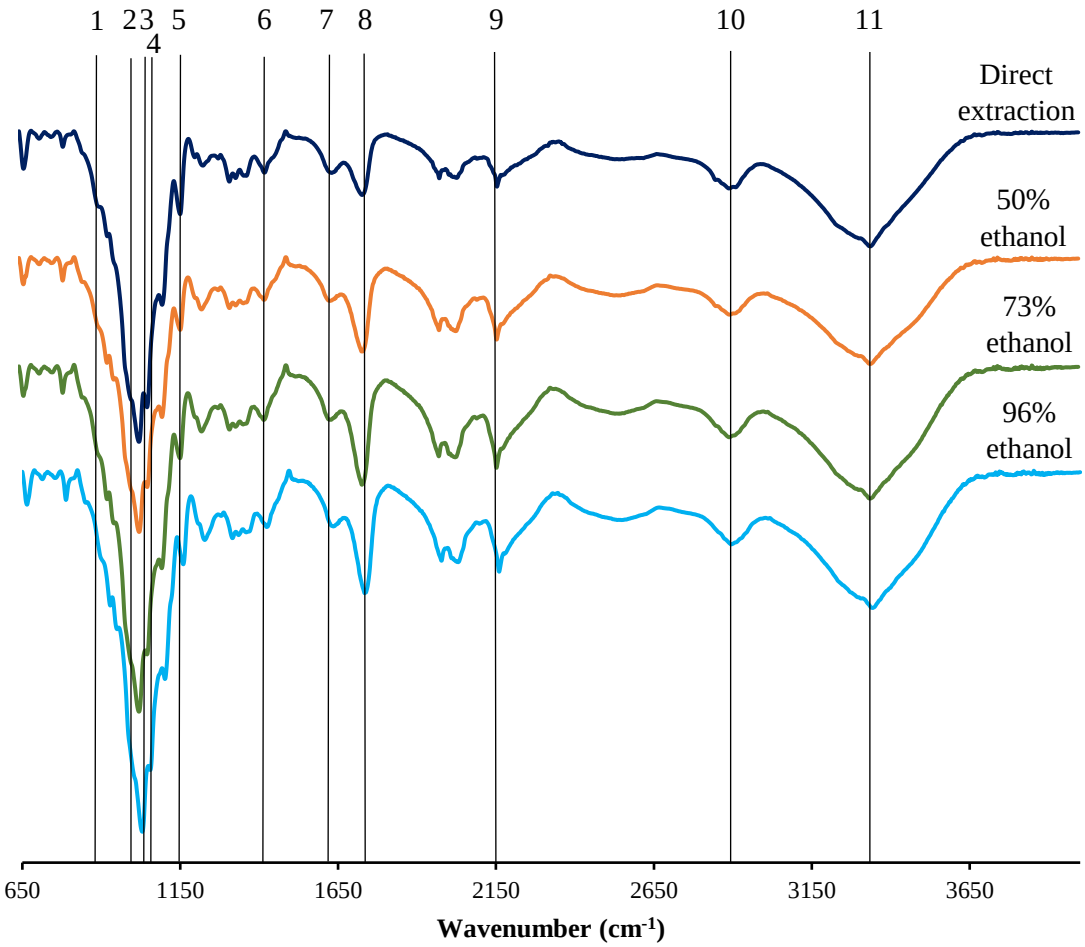

Figure 5 – ATR-FTIR spectra, plotting wavenumber (cm^{-1}) by transmittance (%), of cellulose extracted using Baghel et al. (2015) methodology of whole seaweed (direct extraction) or from PEAR (50, 73 and 96% ethanol remaining residue).

2.7.5 Conclusions

The outcome of this work enabled us to understand the usefulness of the Timatic equipment to produce a bioactive extract, using the macroalgae *Z. tournefortii*, applying a BBD to increase efficiency. An overall prediction suggests that higher yield of biocompounds and antioxidant activity are obtained in the primary extract, when performing the extraction with higher ethanol percentages, although extract yield presented an opposite behaviour. These extracts could be further purified to obtain specific compounds. For example, purified fucoxanthin and phenolic compounds could be further purified from this primary extract, since these are value-added products, known for their bioactive capabilities, which potentiates their integration in food products as natural antioxidants. Furthermore, these extracts could be considered as a whole for nutraceutical applications, optimizing the extraction to increase or decrease specific compounds or activities. Further studies would be necessary to firstly test the potential of these extracts in specific bioactivities *in vitro*, followed by *in vivo* assessments. The cascade approach allowed an evaluation of the extractability and purification potential of fucoidan and cellulose in the remaining biomass residue. Fucoidan yield of *Z. tournefortii* was found to be lower than some reported works, but this lower value could be attributed to the collection period, since it has been reported that these values tend to be superior

in macroalgae collected in autumn and winter. The reason could be attributed to the presence of fucoidan in the fibrillar cell wall as a reinforcement molecule, possibly to support stronger currents and movement in these periods. This biocompound could be efficiently extracted from PEAR, increasing income and reducing waste, since this product could be commercialized as a nutraceutical or food supplement due to several positive bioactivities already reported in previous works. Also, cellulose yield extracted from PEAR presented higher values than the direct extraction from the biomass and other reported works, evidencing the PEAR potential for cellulose extraction and purification. Cellulose could be further purified to integrate in value-added products such as an excipient in food supplements or as nanocellulose, used to produce cosmetics and aerogels, between other numerous applications. ATR-FTIR analysis allowed the comparison of fucoidan and cellulose with direct extractions from the biomass and standard. The obtained spectra were essential to determine the key IR absorptions that distinguish these compounds and revealed to be very close to the direct extractions from the biomass and in the case of fucoidan, similar to its standard.

2.7.6 Acknowledgements

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2.7.8 Supplementary material

Supplement table A – Experimental and predicted values performed with Box-Behnken design (BBD) using Response Surface Methodology (RSM) in STATISTICA 10.0 software.

	Yield g (100 g)$^{-1}$ dw		**Total Chlorophyll** (TChlC) g (100 g)$^{-1}$ dw		**Total Carotenoid Content (**TCC) g (100 g)$^{-1}$ dw		**Fucoxanthin** (TFxC) g (100 g)$^{-1}$ dw		**Total Phenolic Content** (TPC) GAE g (100 g)$^{-1}$ dw		**Total Flavonoid Content** (TFC) QE g (100 g)$^{-1}$ dw		**Free radical-scavenging assay** (DPPH) % scavenging activity		**Reducing activity** (RA) AAE g (100 g)$^{-1}$ dw	
Run	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	15.67	15.34	1.14	1.24	0.58	0.67	0.07	0.10	5.16	5.39	2.99	3.41	69.98	72.92	4.64	4.95
2	28.32	28.77	0.14#	0.10	0.38	0.34	0.05	0.03	4.03	3.80	0.94	0.56	72.69	67.74	5.88*	5.70
3	28.49*	26.92	0.14#	0.20	0.36	0.41	0.04#	0.05	3.69	3.93	0.45#	0.94	66.90	68.10	5.41	5.54
4	15.80	15.08	1.41*	1.37	0.77	0.75	0.13*	0.12	5.46	5.47	4.34*	3.58	70.66	71.51	4.97	4.96
5	25.85	26.08	0.41	0.52	0.62	0.59	0.08	0.08	3.98	4.10	2.25	2.71	64.84	62.08	4.68	4.53
6	25.88	26.08	0.44	0.52	0.64	0.59	0.08	0.08	3.89	4.10	2.88	2.71	56.85#	62.08	4.66	4.53
7	27.88	28.59	0.15	0.09	0.35#	0.32	0.04#	0.03	3.63#	3.60	0.99	1.07	63.32	68.09	5.39	5.39
8	28.12	28.52	0.19	0.23	0.40	0.42	0.05	0.06	3.85	3.88	0.92	0.73	67.36	66.34	5.66	5.70
9	15.99	15.16	1.23	1.23	0.67	0.65	0.10	0.09	5.15	5.19	4.05	3.92	76.01*	73.26	4.78	4.64
10	11.60#	13.48	1.39	1.33	0.79*	0.74	0.13*	0.12	5.81*	5.52	3.32	3.79	74.31	73.27	4.96	4.79
11	26.75	26.48	0.75	0.74	0.55	0.54	0.08	0.08	4.02	4.06	1.29	1.89	60.66	59.88	4.53	4.52
12	24.73	24.88	0.74	0.70	0.57	0.53	0.09	0.08	4.14	4.11	2.75	2.10	58.85	61.64	4.49	4.35
13	25.72	26.30	0.71	0.72	0.52	0.52	0.08	0.08	3.94	3.86	2.05	2.40	59.28	60.22	4.24	4.21
14	26.51	26.08	0.70	0.52	0.51	0.59	0.08	0.08	4.42	4.10	3.01	2.71	64.54	62.08	4.25	4.53
15	25.16	24.70	0.64	0.69	0.47	0.51	0.07	0.08	3.84	3.91	2.92	2.61	64.93	61.98	3.87#	4.04

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Supplement table B – Results from General Linear Model (GLM) with all effects ANOVA and Lack of fit analysis, using STATISTICA 10.0 software.

	Sum of squares	DF	Mean square	F-value	p-Value
Yield					
Model	444.174	9	49.353	76.082	0.000
Residual	3.243	5	0.649		
Lack of fit	2.964	3	0.988	7.071	0.126
Pure Error	0.279	2	0.140		
Total SS	447.418	14			
TChlC					
Model	2.711	9	0.301	19.202	0.002
Residual	0.078	5	0.016		
Lack of fit	0.027	3	0.009	0.347	0.800
Pure Error	0.052	2	0.026		
Total SS	2.789	14			
TCC					
Model	0.249	9	0.028	5.836	0.033
Residual	0.024	5	0.005		
Lack of fit	0.014	3	0.005	0.969	0.544
Pure Error	0.010	2	0.005		
Total SS	0.272	14			
TFxC					
Model	0.009	9	0.001	3.944	0.072
Residual	0.001	5	0.000		
Lack of fit	0.001	3	0.000	63.572	0.016
Pure Error	0.000	2	0.000		
Total SS	0.010	14			
TPC					
Model	6.602	9	0.734	11.961	0.007
Residual	0.307	5	0.061		
Lack of fit	0.148	3	0.049	0.618	0.666
Pure Error	0.159	2	0.080		
Total SS	6.909	14			
TFC					
Model	17.779	9	1.975	4.219	0.064
Residual	2.341	5	0.468		
Lack of fit	2.005	3	0.668	3.976	0.207
Pure Error	0.336	2	0.168		

Total SS	20.120	14			
DPPH					
Model	425.244	9	47.249	4.804	0.049
Residual	49.178	5	9.836		
Lack of fit	8.194	3	2.731	0.133	0.932
Pure Error	40.984	2	20.492		
Total SS	474.422	14			
RA					
Model	4.131	9	0.459	10.214	0.010
Residual	0.225	5	0.045		
Lack of fit	0.106	3	0.035	0.599	0.675
Pure Error	0.118	2	0.059		
Total SS	4.356	14			

Supplement table C – Correlation matrix for the Box-Behnken design (BBD) dependent variables using STATISTICA 10.0 software.

	Yield	**TChlC**	**TCC**	**TFxC**	**TPC**	**TFC**	**DPPH %**	**RA**
Yield	1.00	-0.92	-0.82	-0.78	-0.96	-0.74	-0.61	0.15
TChlC	-0.92	1.00	0.86	0.88	0.91	0.83	0.40	-0.41
TCC	-0.82	0.86	1,00	0.96	0.80	0.82	0.24	-0.35
TFxC	-0.78	0.88	0.96	1.00	0.79	0.80	0.23	-0.38
TPC	-0.96	0.91	0.80	0.79	1.00	0.72	0.66	-0.07
TFC	-0.74	0.83	0.82	0.80	0.72	1.00	0.25	-0.50
DPPH	-0.61	0.40	0.24	0.23	0.66	0.25	1.00	0.42
RA	0.15	-0.41	-0.35	-0.38	-0.07	-0.50	0.42	1.00

Supplement table D – Profiles determined for each variable and overall desirability, predicted by the Response Surface methodology (RSM) to achieve maximum values (desirability), using STATISTICA 10.0 software.

	Ethanol (%)	Cycles (nº)	Sonication (min)
Yield	56.9	11.4	19
TChlC	96	17.4	20
TCC	96	12.6	0
TFxC	96	14.4	20
TPC	96	11.4	0
TFC	96	15.6	20
DPPH	96	18	8
RA	50	6	13
Overall desirability	96	14.4	20

Supplement table E – Statistical values and correlation coefficients, determined using Response Surface Methodology (RSM) with STATISTICA 10.0 software determined for each variable.

	Mean	Minimum	Maximum	Std.Dev.	Multiple R	Multiple R^2	Adjusted R^2
Yield	23.50	11.60	28.49	5.52	1.00	0.99	0.99
TChlC	0.68	0.14	1.42	0.44	0.99	0.97	0.96
TCC	0.55	0.35	0.80	0.14	0.95	0.91	0.89
TFxC	0.08	0.04	0.13	0.03	0.94	0.88	0.84
TPC	4.33	3.58	5.84	0.69	0.98	0.95	0.94
TFC	2.34	0.22	4.70	1.22	0.91	0.82	0.77
DPPH	66.08	56.05	76.45	5.72	0.94	0.89	0.86
RA	4.83	3.83	6.04	0.55	0.96	0.92	0.90

2.8 Fatty acid composition, TLC screening, ATR-FTIR analysis, anti-cholinesterase activity and *in vitro* cytotoxicity to A549 tumor cell line of extracts of 3 macroalgae collected in Madeira

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2.8.1 Abstract

Three macroalgae collected in Madeira Island were included in this study to determine their potential for drug, nutraceutical, food or supplement application. Fatty acid content was higher in *Z. tournefortii* (12.32 $mg.g^{-1}$ dw) with 16.58% of PUFAs, eicosapentaenoic acid (C20:5ω3) and arachidonic acid (20:4ω6) having concentrations of 2.59% and 1.17%, respectively. The anti-thrombogenic and anti-atherogenicity potential was higher for *Z. tournefortii* due to relevant fatty acids in the biochemical composition this macroalgae. Lipid classes were assessed in the lipid extract and neutral lipids (NL) were in higher yield in *A. taxiformis* (51.16%) and lower in *Z. tournefortii* (26.96%). The glycolipids (GL) were between 36.03% and 16.11% in *Z. tournefortii* and *U. lactuca*. Phospholipids (PL) fraction varied from 35.91% and 31.60% in *A. taxiformis* and *Z. tournefortii*. TLC screening identified that *U. lactuca* contains phytol and cholesterol in its NL, digalactosyldiacylglycerol in its GL, cardiolipin and L-α-phosphatidylcholine in its PL. *Z. tournefortii* contains, phytol and cholesterol in its NL classes, and the PL classes contain L-α-phosphatidylethanolamine and 1-(3-sn-phosphatidyl)-rac-glycerol. The macroalgae *A. taxiformis* revealed cholesterol in its NL fraction and the same phospholipids as *Z. tournefortii* in its PL fraction. ATR-FTIR analysis enabled a "fingerprint" spectra and important sulfation absorption bands were identified, revealing the functional polysaccharides within these macroalgae. Anticholinesterasic activity was assessed in *A. taxiformis*, with a low IC_{50} for AChE (8.92 ± 0.43 $\mu g.mL^{-1}$) and BuChE (13.96 ± 0.32 $\mu g.mL^{-1}$), demonstrating dual inhibitory activity, justifying the interest to identify the active principle which may be the scaffold of a novel drug.

Keywords: Seaweeds; *Asparagopsis taxiformis*; *Ulva lactuca*; *Zonaria tournefortii*; Lipids; PUFAs; MUFAs; SFAs.

2.8.2 Introduction

Nowadays, intensive research is conducted worldwide to explore and determine the nutritive, nutraceutical, pharmaceutical and cosmeceutical potential of innumerous extracts from organic materials. Seaweeds have been included in this effort due to their intrinsic biochemical composition, derived from evolutionary adaptation to the harsh conditions of high salt concentration, desiccation and herbivory, extreme variations in sunlight and temperature fluctuation. The sea currents and wave energy also cause motional pressure to these organisms, since they must remain attached to the substrate, subjected to additional environmental stress, adjusting the metabolic network and developing new metabolites to permit adaptation to this variable habitat (Mišurcová et al. 2011).

Seaweeds contain a low lipid content but are considered a potential source of functional lipids due to their enormous stock in coastal waters. Among total lipids (TL), lipid composition and quantity varies according to species, geographical origin and environmental conditions (Miyashita et al. 2013). TL are further divided in lipid classes such as glycolipids (GL), triacylglycerols (TAG) and phospholipids (PL). In these classes, fatty acids exist as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). These last (PUFAs) are essential to human nutrition, since algae produce PUFAs in *de novo* synthesis and cannot be biosynthesized by almost all heterotrophic organisms, having to be ingested in the diet. Nowadays they are also considered as functional food and nutraceuticals with health benefits, since they are able to reduce the risk of cardiovascular diseases (CVD), diabetes, ostheoporosis and cancer (Mišurcová et al. 2011). GL is the most common lipid group and includes monogalactosyl-diacylglycerols (MGDG), digalactosyl-diacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) (Holdt and Kraan 2011). GL are important in photosynthetic membranes of higher plants, in algae and bacteria. In brown seaweeds, GL are the primary membrane lipids, with high concentration of specific long chain fatty acids, which include stearidonic acid (18:4n-3) (SDA), eicosapentaenoic acid (20:5n-3) (EPA) and arachidonic acid (20:4n-6) (ARA) (Miyashita et al. 2013). SDA is formed by the desaturation of α-linolenic acid (ALA), and when consumed increases the level of eicosapentaenoic acid (EPA) in erythrocyte membranes (Lemke et al. 2013). Epidemiological experiments demonstrated that ingesting long chain ω3 fatty acids decreases cardiovascular diseases, mainly unexpected heart attacks (Albert et al. 1998). The ω6 fatty acids are particularly important in biological systems. ARA participates in the immune response, prevents thrombosis, helps brain function, and together with docosahexaenoic acid (DHA), is correlated with a reduction in age-related disorders of the brain and cognitive functions (Hoffman et al. 2009; Kiso 2011). These are main components of cell membranes and participate in neuron structure in the central nervous system (Miyashita et al. 2013).

Macroalgae are also an interesting source of novel molecules which may be the scaffolds of more efficient drugs, namely against age-related diseases (Barbosa et al., 2014). Alzheimer's disease (AD) is a progressive neurodegenerative disorder, affecting roughly 2% of the population in industrialized countries, which is characterized by synapse degeneration and neuronal death in regions of the brain responsible for learning and memory processes (Mattson 2004). Two main strategies exist to improve the cholinergic function in AD, which are stimulating cholinergic receptors or increasing the availability of acetylcholine into the neuronal synaptic cleft, inhibiting the enzyme acetylcholinesterase (AChE, E.C. 3.1.1.7) (Howes et al. 2003). Several pharmaceuticals are available for this second strategy and include tacrine, rivastigmine,

galantamine and donepezil, however the use of these drugs only slows down the progress of the illness and causes negative side effects, highlighting the need for new sources of anti-acetylcholinesterasic compounds (Arruda et al. 2012). The inhibition of butyrylcholinesterase or pseudocholinesterase (BuChE, EC 3.1.1.8) may also contribute to the treatment of AD. BuChE is produced mainly in the liver and catalyzes butyrylcholine more rapidly, although it is also able to hydrolyze acetylcholine (Colovic et al. 2013).

Seaweed extracts and individualized compounds have been researched for their cytotoxic effect in human A549 lung cancer cellular lines, due to their unique chemical structures that could be used as novel drugs (Alwarsamy et al. 2016). Lung cancer is the most prominent cause of cancer deaths, accounting more than one million deaths worldwide per annum (Lee et al. 2011). This cancer development is characterized by proliferation of anaplastic cells without restrain, invading and metastasizing to the surrounding tissues and organs (Marudhupandi et al. 2015). Several factors are responsible for this illness and include unhealthy diet, inherited genetic mutations, drug use, hormones, environmental toxins, infectious organisms and immune conditions, the treatment being the obliteration of the tumour cells (Gutiérrez-Rodríguez et al. 2017). Standardized treatment of this malignancy is the application of synthetic compounds in chemotherapy, which attacks the cancer cells but at the same time is cytotoxic to normal cells (Alwarsamy et al. 2016). It is therefore of the outmost importance to discover new sources of bioactive compounds that can reduce this malignancy without the nefarious side effects.

This work intended to determine the potential of these 3 seaweeds, due to their significant abundance in Madeira Island. This is an effort to search new sources of bioactive compounds that would help develop purification strategies to research for new pharmaceutical compounds, namely displaying anti-cholinesterasic and / or cytotoxic activity against A549 tumour cell lines. This study will also determine if these macroalgae are good sources of essential fatty acids. These assessments could permit to develop new drugs, new nutraceuticals or to envision new food sources with multiple health applications.

2.8.3 Materials and methods

2.8.3.1 Collection of samples

The chlorophyta macroalgae *Ulva lactuca* Linnaeus 1753:1163 was collected at 15 of May 2017, in "Santa Cruz" at coordinates 32º41'27"N and 16º46'25"W. Rhodophyta *Asparagopsis taxiformis* (Delile) Trevisan 1845: 45 was collected at 8 of September 2017 and phaeophyta *Zonaria tournefortii* (J.V.Lamouroux) Montagne 1846: 32 collected at 7 of July 2017 in "Reis Magos" beach, coordinates 32º38'48"N and 16º49'26"W in a 10-meter maximum depth dive of the Madeiran archipelago. Samples were transported in seawater and gently rinsed with filtered fresh water. Afterwards, a primary drying was applied in which seaweed was frozen at -35 ºC and freeze-dried for 5 days under reduced pressure ($4x10^{-4}$ mbar), with a cooling trap set at -56 ºC. Samples were milled to 200 mesh particle size, vacuum packed and stored at -35 ºC until use.

2.8.3.2 Fatty acid determination

The fatty acids (FAs) composition was determined according to Lepage and Roy (1986) with the modifications suggested by Cohen et al. (1988) and assessed as FA methyl esters (FAME). Initially, for the conversion of FAs to FAMEs, a mixture of ethyl acetate-methanol (1:19 v/v) was added to the dried samples. These were incubated at 80 °C for 1 h and followed by an extraction using heptane. The analytical apparatus consisted in gas chromatography (Agilent HP 6890), fitted with a mass selective detector (Agilent 5973) and a capillary column DB-225J&W (30 m × 0.25 mm inner diameter, 0.15 μm film thickness), also from Agilent. Chromatographic settings were followed as described, helium was used as the carrier gas set to a flow rate of 2.6 mL.min^{-1}, initial temperature of the oven was set to 35 °C for a period of 0.5 min, subsequently incremented by 25 °C min^{-1} to 195 °C, followed by 3 °C min^{-1} to 205 °C and 8 °C min^{-1} to achieve a maximum temperature of 230 °C for a period of 3 min. Injector temperature was set to 250 °C and the transfer line set to 280 °C with the split ratio at 1:100. FAMEs were identified, comparing the retention times and mass spectra fragmentation to standards (bacterial acid methyl esters CP mix and Supelco 37 component FAME mix). The internal standard was heneicosanoic acid (C21:0). Two measures of each sample were performed, presented as mean values ± standard deviation (SD) of FAME and results expressed in mg.g^{-1} of dry weight (DW).

The index of thrombogenicity (IT) and the index of atherogenicity (IA), in which lower results indicate a lower risk to develop those pathologies, was calculated as proposed by Ulbricht and Southgate (1991) using the following equations:

$$\mathrm{IT} = (14{:}0 + 16{:}0 + 18{:}0)/[(0.5 \times \Sigma\mathrm{MUFA} + 0.5 \times \mathrm{PUFA}\omega6 + 3 \times \mathrm{PUFA}\omega3) + (\mathrm{PUFA}\omega3/\mathrm{PUFA}\omega6))]$$

$$\mathrm{IA} = [(12{:}0 + (4 \times 14{:}0) + 16{:}0)]/[\Sigma\mathrm{MUFA} + \mathrm{PUFA}\omega6 + \mathrm{PUFA}\omega3]$$

2.8.3.3 Extraction and fractionation

2.8.3.3.1Lipid extraction (LE)

The methodology of Folch et al. (1957), with some modifications, was used to perform the lipid extractions (LE) from the macroalgae. Initially, 2 grams of freeze-dried milled macroalgae were mixed with 40 mL of $CHCl_3$:MeOH (2:1). Subsequently, it was sonicated for 10 min at 37 kHz at 100% and centrifuged at 7197 g at room temperature. The liquid extract was filtered with 8 μm pore filter paper, added to 8 mL of 0.9% NaCl solution, vortexed and centrifuged at 470 g for phase separation. The upper phase was removed and discarded, remaining the lipophilic phase. The majority of the solvent was evaporated in a rotary evaporator and the residual solvent evaporated in an oven at 35 ºC overnight and weighted. These extracts will from now on be abbreviated as LE.

2.8.3.3.2 Ethanol extraction (EE)

The ethanol extracts (EE) were performed with 96% ethanol, using a Timatic semiautomatic extractor (Technolab, Spello, Italy) set at a constant pressure and static phase. The program was set as follows: solvent capacity 1L, solvent to biomass ratio 50:1, compression time 3 min, decompression time 6 min, minimum pressure 6 bar, compression pressure 8.5 bar at room temperature (± 23 ºC). The number of cycles was set to 12 cycles, which corresponded to 108 min for each extraction. Each sample of 20 grams was placed in a 100 µm mesh bag and placed inside a 0.5 L extracting vessel. In the end of each extraction, the liquid extracts were filtered through 8 µm Whatman filter and were partly evaporated in a rotary evaporator and dehydrated completely in an oven, at 35 ºC until complete dryness. After, these were stored under vacuum at -35 ºC until use.

2.8.3.4 Lipid classes

LE were fractioned into neutral (N), glycolipid (GL) and phospholipid (PL) classes. Initially, 50 mg of extract were solubilized in 2 mL of chloroform and applied to a glass column with 10 g of 200 to 400 mesh silica particles. Silica was previously packed with chloroform, with quartz sand beneath and underneath for protection. Fractionation was performed using 100 mL of a specific solvent for each class. Neutral lipids and sterols were extracted with chloroform, glycolipids with acetone and phospholipids with methanol. For each extraction, the corresponding solvent containing the lipid class was collected and dried, initially in a rotary evaporator, afterwards in an oven at 35 ºC. The residue was weighted and stored at -35 ºC until use.

2.8.3.5 Thin layer chromatography (TLC)

The separated lipid classes were assessed using TLC with MACHEREY-NAGEL Pre-coated TLC sheets POLYGRAM® SIL G/UV_{254} (0.20 mm silica gel 60). The TLC mobile phase for neutral lipids was hexane: diethyl ether: acetic acid (85:15:0.1, v/v), and TLC plate was revealed with vanillin solubilized in sulfuric acid (1:100, w/v), with posterior heating at 120 ºC in an oven until complete dryness of the TLC plate. The standards were cholesterol acetate, cholesterol, β-carotene and phytol. The mobile phase for glycolipids was dichloromethane: methanol (85:15, v/v) and the TLC plate was revealed with α-naphthol: sulfuric acid: water: ethanol (2.4:10:10:80, w/v), and the TLC plate heated at 120 ºC for 3 to 5 min, until pink and purple spots were detected. The standard used was digalactosyldiacylglycerol (DGDG). Phospholipids were separated by polarity and charge. The mobile phase was chloroform: methanol: ammonium hydroxide (65:25:4, v/v), revealed with sprayed 10% copper and 8% phosphoric acid solubilized in water, and placed in an oven at 120 ºC until spots appeared. The standards used were L-α-Phosphatidylcoline, L-α-Phosphatidylethanolamine, 1-(3-sn-Phosphatidyl)-rac-glycerol sodium salt, 1,2-Diacyl-sn-glycerol-phospho-L-serine, Cardiolipin sodium salt and Lysophosphatidylcholine. The retention factor (equation 1) was calculated in millimetres for the separated compounds within each lipid class, as this is calculated by dividing the distance travelled by the compound and the distance travelled by the solvent front.

2.8.3.6 Bioactivities

2.8.3.6.1 Anti-cholinesterase activity

The assays to assess anti-acetylcholinesterase (AChE) and anti-butyrylcholinesterase activities (BuChE) were adapted from Ellman et al. (1961) modified by Arruda et al. (2012). In each microplate, serial dilutions of the extracts were prepared in 100 mM phosphate buffer pH 8, in order to obtain concentrations between 0-150 $\mu g.L^{-1}$. Afterwards, 0.25 $U.mL^{-1}$ acetylcholinesterase or butyrylcholinesterase were added to each microwell, and incubated for 5 min. The reaction was started by the addition of a substrate mixture, composed by equal parts of 3 mM 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB) and 75 mM acetylthiocholine iodide (ATChI) for AChE or butyrylthiocholine iodide (BuTChI) for BuChE. The absorbance at 415 nm was read at 0, 150, 300 and 450 sec in a Bio Rad Model 680 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Enzyme inhibition was determined as the percentage activity of reaction media containing samples and the activity of control without inhibitor. All samples were tested in quadruplicate, the mean ± SD determined and results expressed as IC_{50} ($\mu g.mL^{-1}$) and also as % inhibition at the highest concentration tested (150 µg/mL), since it allows comparison between extracts that did not achieve 50% inhibition but nonetheless present different activities.

2.8.3.6.2 *In vitro* cytotoxicity

The cytotoxicity against A549 human adenocarcinoma tumour cell line was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 96 wells plates were seeded with 20,000 A549 cells /well in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2% fetal bovine serum (FBS) and 1% penicillin–streptomycin mixture (10,000 UI/mL), in the presence of known concentrations of the extracts or standard previously dissolved in Dimethyl sulfoxide (DMSO). The microplates were incubated for 48 h at 37 ºC, 5% CO_2 and 98% humidity. Afterwards, 10 µL of 5 $mg.mL^{-1}$ MTT were added and the microplate was again incubated at 37 ºC for 3 to 4h. The culture medium was removed and DMSO was added to the wells to dissolve formazan crystals. The absorbance was read in a Bio Rad Model 680 microplate reader at 550 nm. All samples were tested in quadruplicates, the mean ± SD determined and results were expressed as IC_{50} ($\mu g.mL^{-1}$) and also as % inhibition at the highest concentration tested (200 µg/mL), as referred above.

2.8.3.7 Spectroscopic analysis

ATR-FTIR spectra of the samples were obtained with a Perkin Elmer Spectrum Two, coupled with a Diamond ATR accessory (DurasamplIR II, Smiths Detection, UK). Thirty-two scans were acquired in transmittance mode in the range of 2,000-650 cm^{-1}, with a wavenumber resolution of 1 cm^{-1}. All samples were analyzed in triplicates.

2.8.4 Results

2.8.4.1 Lipid screening

The amounts of total lipids from each algal species and lipid classes separated from the total extracts are presented in table 1. Total lipids were higher in *Z. tournefortii* (9.66 ± 0.30 g/100g dw), followed by *A. taxiformis* (6.08 ± 0.45 g/100g dw) and *U. lactuca* (1.33 ± 0.03 g/100g dw). Concerning lipid classes, neutral lipids and sterols were presented in a similar proportion in the *U. lactuca* and *A. taxiformis* extracts (50.63 ± 6.10 and 51.16 ± 4.85 %, respectively) and in a lower proportion in *Z. tournefortii* (26.96 ± 8.22 %). The glycolipids fraction was between 36.03 ± 8.08 % and 16.11 ± 1.36 % in *Z. tournefortii* and *U. lactuca*, respectively. The phospholipids fraction did not differ much between species, varying from 35.91 ± 2.36 % to 31.60 ± 1.40 % in *A. taxiformis* and *Z. tournefortii*, respectively.

Table 1 – Total lipid content and class fractionation of *Ulva lactuca*, *Zonaria tournefortii* and *Asparagopsis taxiformis*

Sample		Average ± standard deviation
Ulva lactuca (Chlorophyta)	Total lipids	1.33 ± 0.03 g.100g^{-1} dw
	Neutral and Sterols	50.63 ± 6.10 %
	Glycolipids	16.11 ± 1.36 %
	Phospholipids	35.84 ± 3.40 %
Asparagopsis taxiformis (Rhodophyta)	Total lipids	6.08 ± 0.45 g.100g^{-1} dw
	Neutral and Sterols	51.16 ± 4.85 %
	Glycolipids	23.68 ± 1.92 %
	Phospholipids	35.91 ± 2.36 %
Zonaria tournefortii (Phaeophyta)	Total lipids	9.66 ± 0.30 g.100g^{-1} dw
	Neutral and Sterols	26.96 ± 8.22 %
	Glycolipids	36.03 ± 8.08 %
	Phospholipids	31.60 ± 1.40 %

Data for total lipids are mean values ± standard deviation (SD) in g.100g^{-1} of dry weight (dw). Lipid class fractionation (neutral and sterols, glycolipids and phospholipids) are described as percentage (%) of total lipid content.

The TLC (Thin Layer Chromatography) technic was performed as a rapid and reliable way to ascertain biochemical differences between these three different macroalgae, screening the presence of Neutral Lipids (NL), Glycolipids (GL) and Phospholipids (PL) in the lipid content. TLC results are presented in table 2 as a presence (+) or absence (-) of similar spots. Qualitative assessment of NL was carried out by comparison using cholesterol acetate, cholesterol, β-carothene and phytol standards. The NL of *U. lactuca* and *Z.*

tournefortii presented spots with retention factors (Rf) similar to phytol. Unesterified sterols with an Rf similar to cholesterol was visible in all the macroalgae used in this work but esterified sterol and β-carothene were not detected. For GL fraction, a spot with the same Rf of digalactosyldiacylglycerol (DGDG) standard was found in *U. lactuca* but not on the other macroalgae. Concerning the phospholipid fraction, standards were L-α-Phosphatidylcholine (PC), L-α-Phosphatidylethanolamine (PE), 1-(3-sn-Phosphatidyl)-rac-glycerol sodium salt (PG), 1,2-Diacyl-sn-glycero-phospho-L-serine (PS), Cardiolipin sodium salt (CL) and L-α-Lysophosphatidylcholine (LPC). *U. lactuca* presented a similar Rf developed by CL and PC. *A. taxiformis* and *Z. tournefortii* demonstrated similar Rf with PE and PG standards. None of the macroalgae had similar Rf developed by PS and LPC standards.

Table 2 – Thin Layer Chromatography (TLC) performed in *Ulva lactuca*, *Zonaria tournefortii* and *Asparagopsis taxiformis*

	Standards	***U. lactuca***	***A. taxiformis***	***Z. tournefortii***
Neutral lipids	Cholesterol acetate	-	-	-
	Cholesterol	+	+	+
	β-carothene	-	-	-
	Phytol	+	-	+
Glycolipids	DGDG	+	-	-
Phospholipids	PC	+	-	-
	PE	-	+	+
	PG	-	+	+
	PS	-	-	-
	CL	+	-	-
	LPC	-	-	-

Lipid classes comprising the lipid extract performed according to Folch et al. (1957) methodology, using the LE (Lipophilic Extract) was performed with $CHCl_3$:MeOH (2:1). The table indicates the presence (+) or absence (-) of similar retention factor (Rf) spots of the working standards for each lipid classe. Digalactosyldiacylglycerol (DGDG); L-α-Phosphatidylcholine (PC); L-α-Phosphatidylethanolamine (PE); 1-(3-sn-Phosphatidyl)-rac-glycerol sodium salt (PG); 1,2-Diacyl-sn-glycero-phospho-L-serine (PS); Cardiolipin sodium salt (CL); L-α-Lysophosphatidylcholine (LPC).

2.8.4.2 Fatty acids (FA)

The results concerning FA with carbon chain from C13 to C24 are presented in table 3. Maximum value of total FA content was determined in *Z. tournefortii* (12.32 ± 1.28 $mg.g^{-1}$), followed by *U. lactuca* (7.54 ± 0.92 $mg.g^{-1}$) and *A. taxiformis* (6.01 ± 0.70 $mg.g^{-1}$). Total SFA were higher in *A. taxiformis* (5.65 ± 0.66 $mg.g^{-1}$), comprising about 94% of total FA, and the remaining being monounsaturated FA (MUFA). The majority of the SFA determined in *A. taxiformis* were palmitic (16:0) and myristic acid (14:0), accounting for almost 72 and 19% of total FA in this macroalgae. For *Z. tournefortii*, of the 12.32 ± 1.28 $mg.g^{-1}$ of FA, approximately 40% were SFA, 43% MUFA and 17% PUFA. As above, the majority of SFA were palmitic and myristic acid, 32 and 6% of total FA, respectively. The MUFA were mainly represented by monounsaturated palmitic (16:1) and stearic acid (18:1), accounting for almost 27 and 17% of total FA, respectively. Concerning the polyunsaturated ω3 and ω6 FA, these were mainly represented by stearidonic (18:4ω3), eicosapentaenoic (20:5ω3), docosapentaenoic (22:5ω3) and arachidonic (20:4ω6) acids, representing 5, 3 for the former and 1% for the last two PUFA, respectively. For *U. lactuca*, which contained 7.54 ± 0.92 $mg.g^{-1}$ of FA, approximately 54% were SFA, 30% MUFA and 16% PUFA. Again, palmitic acid represented the majority of SFA, accounting for 50% of total SFA in *U. lactuca*. The MUFA were mainly represented by the monounsaturated stearic (25%) and palmitic acid (5%). PUFA in *U. lactuca* were characterized primarily by α-linolenic, stearidonic, linoleic and docosapentaenoic acids, representing 6, 5, 4 and 1%, respectively, of total FA in this macroalgae. The ω6/ω3 ratio was determined and higher values were achieved in *U. lactuca* (0.71) and *Z. tournefortii* (0.13). It was not possible to calculate this ratio for *A. taxiformis*, due to undetermined values for PUFA.

Table 3 – Fatty acid composition performed by GC-MS in *Ulva lactuca*, *Zonaria tournefortii* and *Asparagopsis taxiformis*

	U. lactuca		***Z. tournefortii***		***A. taxiformis***	
	(mg/g)	% of TFA	(mg/g)	% of TFA	(mg/g)	% of TFA
14:00	0.07 ± 0.01	0.95 ± 0.02	0.76 ± 0.08	6.14 ± 0.20	1.14 ± 0.12	18.82 ± 0.18
16:00	3.76 ± 0.41	49.84 ± 1.00	3.97 ± 0.47	32.18 ± 0.57	4.32 ± 0.52	71.86 ± 0.25
18:00	0.07 ± 0	0.86 ± 0.02	0.09 ± 0.01	0.70 ± 0.03	0.14 ± 0.02	2.30 ± 0.02
16:01	0.41 ± 0.04	5.40 ± 0.04	3.30 ± 0.39	26.77 ± 0.47	0.04 ± 0.01	0.72 ± 0.02
18:01	1.86 ± 0.16	24.65 ± 0.07	2.03 ± 0.22	16.47 ± 0.29	0.32 ± 0.04	5.29 ± 0.07
18:2ω6	0.32 ± 0.02	4.23 ± 0.07	n.d.	n.d.	n.d.	n.d.
18:3ω3	0.43 ± 0.01	5.73 ± 0.35	n.d.	n.d.	n.d.	n.d.
18:4ω3	0.35 ± 0.02	4.60 ± 0.11	0.65 ± 0.06	5.24 ± 0.14	n.d.	n.d.
20:4ω6 (AA)	n.d.	n.d.	0.14 ± 0.01	1.17 ± 0.11	n.d.	n.d.
20:5ω3 (EPA)	n.d.	n.d.	0.32 ± 0.01	2.59 ± 0.28	n.d.	n.d.
22:5ω3	0.08 ± 0.01	1.11 ± 0.20	0.15 ± 0.01	1.23 ± 0.18	n.d.	n.d.
ω3	0.86 ± 0.03	11.44 ± 0.66	1.11 ± 0.06	9.05 ± 0.55	n.d.	n.d.
ω6	0.32 ± 0.02	4.23 ± 0.07	0.14 ± 0.01	1.17 ± 0.11	n.d.	n.d.
Σ SFA	4.09 ± 0.42	54.28 ± 0.70	4.95 ± 0.57	40.13 ± 0.64	5.65 ± 0.66	94.00 ± 0.06
Σ MUFA	2.26 ± 0.20	30.05 ± 0.08	5.33 ± 0.61	43.24 ± 0.43	0.36 ± 0.04	6.00 ± 0.006
Σ PUFA	1.18 ± 0.05	15.67 ± 0.72	2.04 ± 0.14	16.63 ± 0.94	n.d.	n.d.
ω3 HUFA	0.08 ± 0.01	1.11 ± 0.20	0.47 ± 0.02	3.81 ± 0.45	n.d.	n.d.
Total (mg/g)	7.54 ± 0.92	-	12.32 ± 1.28	-	6.01 ± 0.70	-
ω6/ω3 Ratio	0.71		0.13		n.d.	
IT	0.59		0.35		30.02	
IA	1.18		1.06		24.57	

Data presented are mean values ± standard deviation (SD) of FAME in $mg.g^{-1}$ of dry weight (DW) or in percentage (%) of total fatty acids (TFA). AA – arachidonic acid; EPA – eicosapentaenoic acid; ω – omega; SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; HUFA – High unsaturated fatty acids; n.d. – Not detected; IT – index of thrombogenicity; IA – index of atherogenicity.

2.8.4.3 ATR-FTIR analysis

The three macroalgae studied in this work were assessed using ATR-FTIR analysis (figure 1), for comparison between them and to determine the peaks that characterize each of the species. Sixteen important bands were detected from 650 to 2,000 cm^{-1}, the transmittance transformed to absorbance and a comparison was carried out. At 790 cm^{-1} (A) and 846 cm^{-1} (B) two small peaks were distinguished which could represent the C-O-S vibration in the equatorial position and axial position, respectively. A strong IR band (C) at 1,020 cm^{-1} was detected, indicating a C-O-C bending of polysaccharides constituents. Another peak was detected at 1,050 cm^{-1} (D), representative of C-O-C skeletal vibration and at 1,080 cm^{-1} (E) a vibration of C-O-C structure of polysaccharides. Likewise, a shoulder at 1,161 cm^{-1} (F), was detected, corresponding to vibration of C-O-C of carbohydrates from polysaccharides. Furthermore, at 1,222 cm^{-1} (G) a medium intensity S=O vibration arouse and a shoulder at 1,256 cm^{-1} (H) which indicates the asymmetric O=S=O stretching vibration of sulphate esters with possibly some contribution of COH, CC and CO vibration. A medium intensity peak was visualized at 1,375 cm^{-1} (I), representative of symmetric deformations in CH_2 in proteins and $N(CH_3)_3$ methyl bending in lipids. At 1,434 cm^{-1} (J) a C-H bending vibration was detected and at 1,455 cm^{-1} (K) a scissoring vibration of CH_2 or/and an asymmetric bending vibration of CH_3 of fucose. Between 1,510 cm^{-1} (L) and 1,558 cm^{-1} (M) four peaks emerged and their presence is attributed to bending vibrations of N-H of protein amide II and stretching vibrations of C-N. Similarly, medium intensity peaks were identified at 1,622 cm^{-1} (N) and 1,648 cm^{-1} (O), representing the stretching vibrations of C=O of protein amide I. At 1,739 cm^{-1} (P), a small intensity peak was observed which corresponds to C=O vibration of aldehyde.

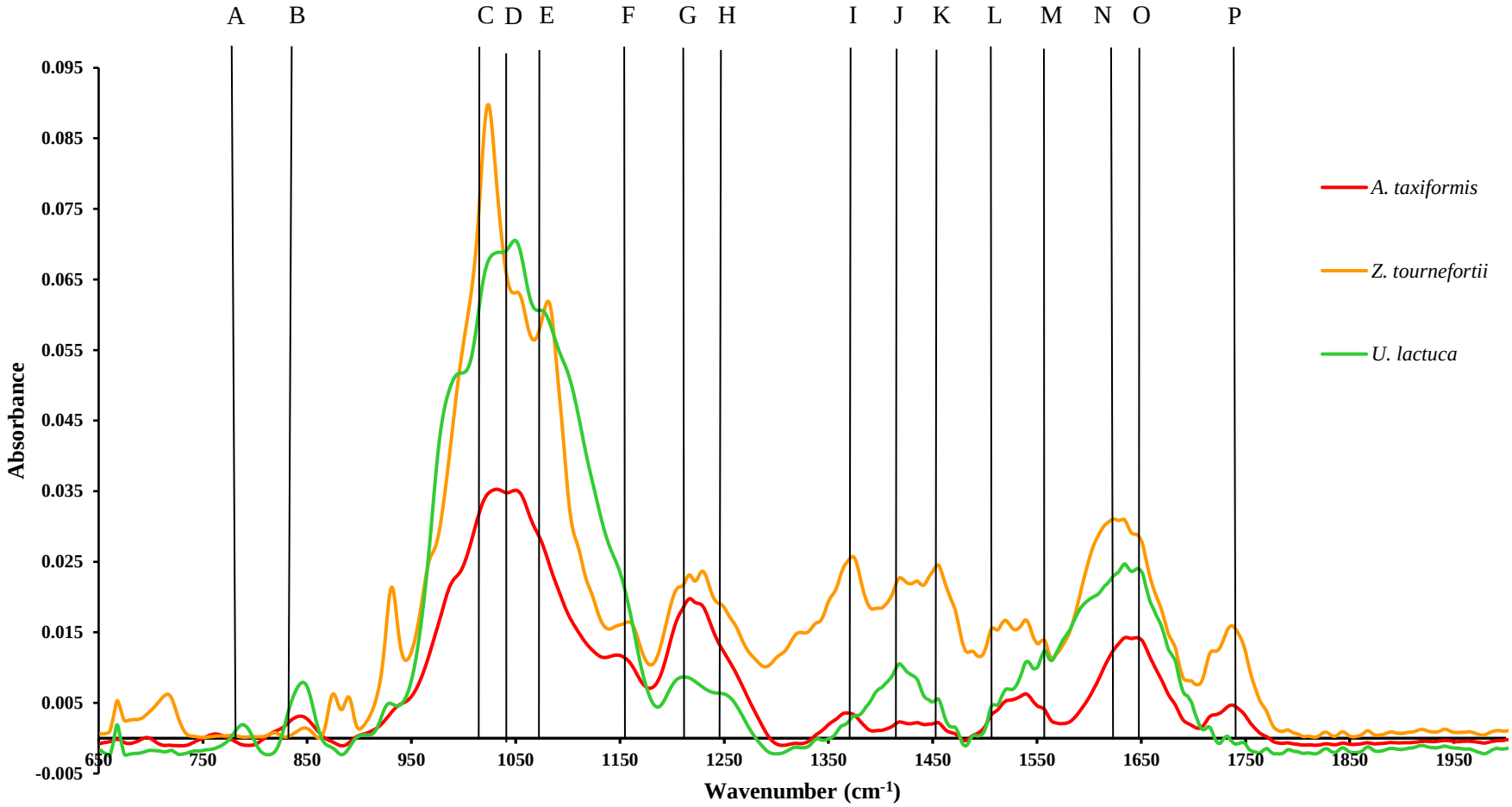

Figure 1 – ATR-FTIR spectra, plotting wavenumber (cm^{-1}) by Absorbance, for the macroalgae lyophilized with bands from A to P indicating important peaks.

2.8.4.4 Bioactivity

The anti-acetylcholinesterase (anti-AChE) and anti-butyrylcholinesterase (anti-BuChE) activities for the Lipophilic Extract (LE) extracts and the Ethanolic Extracts (EE) are presented in table 4. These extractions were compared to determine the difference in bioactivity when using extracts performed using chloroform and methanol (LE) or a scalable extractor (Timatic) that can be used in the industry with a more environmental friendly solvent, ethanol (EE). The results have shown that LE extracts were more active AChE inhibitors when compared with EE, as seen by the lower IC_{50} values and/or higher inhibition percentages at 150 μg/mL. The highest activity was found in *A. taxiformis* extracts, with IC_{50} = 8.92 ± 0.43 and 46.33 ± 6.02 μg/mL for LE and EE, respectively. Concerning BuChE inhibition, once again *A. taxiformis* was more active, with 13.96 ± 0.32 and 28.1 μg/mL for LE and EE, respectively. All of these extracts were double cholinesterase inhibitors, except for *U. lactuca* EE, and the high activity of *A. taxiformis* LE must be highlighted, with low IC_{50} values and in the case of BuChE inhibition, almost 4-fold more active than Donepezil standard, which is used in the treatment of Alzheimer's disease.

Table 4 – *In vitro* anti-cholinesterasic potential of macroalgae extracts

	AChE activity		**BuChE activity**	
	% inhibition (150 μg.mL^{-1})	IC_{50} (μg.mL^{-1})	% inhibition (150 μg.mL^{-1})	IC_{50} (μg.mL^{-1})
***U. lactuca* LE**	37,06 ± 1,06	>150	16,52 ± 4,72	>150
***U. lactuca* EE**	0	>150	0	>150
***Z. tournefortii* LE**	42,73 ± 1,70	>150	19,59 ± 5,55	>150
***Z. tournefortii* EE**	40,12 ± 4,61	>150	30,81 ± 3,26	>150
***A. taxiformis* LE**	100	8,92 ± 0,43	100	13,96 ± 0,32
***A. taxiformis* EE**	100	46,33 ± 6,02	100	28,10 ± 0,93
Donepezil	95,20 ± 0,40	0,01 ± 0,00	100	55,62 ± 3,47
Galantamine	98,40 ± 1,50	0,43 ± 0,09	37,80 ± 0,90	>150

For the percentage (%) of inhibition using the concentration of 150 μg.mL^{-1} of extract, data is presented as %. The IC_{50} results are presented as the concentration of extract needed, in μg.mL^{-1}, to inhibit 50% of the enzyme. LE (Lipophilic Extract) indicates that extract was performed with $CHCl_3$:MeOH (2:1). EE (Ethanolic Extract) indicates that the extract was performed with Timatic extractor using 96% ethanol. AChE – acetylcholinesterase; BuChE – Butyrylcholinesterase.

The cytotoxicity in A549 adenocarcinomic human alveolar basal epithelial cells was also assessed, with results presented in table 5. The IC_{50} results ranged between 98.02 ± 0.23 μg.mL^{-1} and 140.42 ± 4.99 μg.mL^{-1}, for *A. taxiformis* LE and EE extracts, respectively.

Table 5 – Cytotoxicity effect of macroalgae extracts against A549 tumour cell line

	% toxicity (200 μg.mL^{-1})	IC_{50} (μg.mL^{-1})
***U. lactuca* LE**	0	>200
***U. lactuca* EE**	90.15 ± 6.96	140.42 ± 4.99
***Z. tournefortii* LE**	n.d.	n.d.
***Z. tournefortii* EE**	90,41 ± 1,52	137,64 ± 7,89
***A. taxiformis* LE**	100	98.02 ± 0.23
***A. taxiformis* EE**	20.14 ± 5.65	>200
Colchicine	71,69 ± 0,96	2,78 ± 0,71
Paclitaxel	97,44 ± 0,66	5,96 ± 0,98

The IC_{50} results are presented as the concentration of extract needed, in μg.mL^{-1}, to inhibit tumor cell viability by 50%. LE (Lipophilic Extract) performed with $CHCl_3$:MeOH (2:1). EE (Ethanolic Extract) performed with Timatic extractor using 96% ethanol. n.d. – not determined

2.8.5 Discussion

2.8.5.1 Total Lipids

The macroalgae analyzed in this work have a low lipid content, which is in agreement with previous works (Paiva et al. 2014; Kendel et al. 2015). *U. lactuca* lipid content was 1.33 ± 0.03 g/ 100g dw, which is in the middle of the values range reported by Santos et al. (2015), who obtained 0.3 g/ 100g dw *U. lactuca* produced by aquaculture, and by Khotimchenko et al. (2002), who reported 3.06 g/ 100g dw in *U. lactuca* collected in Bodega Bay, California. Therefore lipid content can be extremely variable in this species, possibly according to environmental differences. Ragonese et al. (2014) also evaluated *A. taxiformis* collected in the Sicilian coast and determined a lipid yield of 12.22 g/ 100g dw, roughly twice that we have determined (6.08 ± 0.45 g/ 100g dw). The same author analyzed *D. dichotoma* and the lipid content was found to be less than *Z. tournefortii*, belonging to the same family, these macroalgae contain 6.27 g/ 100g dw and 9.66 ± 0.30 g/ 100g dw, respectively. Burreson et al. (1976) determined 42 different components in the essential oil of *A. taxiformis*, collected in Hawaii and the bromoform $CHBr_3$, used in the past as a sedative, is the major constituent. It was also stated that although being the favorite edible macroalgae in Hawaii archipelago, the existence of haloforms and halogenated compounds in its essential oil could mean that this macroalgae could be poisonous when consumed. It was not possible to compare results concerning lipid classes with other published works, since few reports exist assessing these lipid groups in these macroalgae. The most similar work is that of Kendel et al. (2015), who evaluated *U. armoricana* and determined that NL was 55.60% (ours 50.63%), GL was 29.10% (ours 16.11%) and PL is 15.30% (ours 35.11%). Comparing these results, we can determine that these lipid classes yield varies significantly, even for macroalgae of the same genus.

The TLC analysis, performed for each lipid class revealed that *U. lactuca* lipid composition could contain phytol and a type of cholesterol compound in its NL, also revealed the same spots Rf as DGDG in its GL and developed similar Rf to cardiolipin and L-α-phosphatidylcholine in its PL. Phytol is a remarkable compound due to its use as a precursor of industrial synthesis of vitamin E and K (Kendel et al. 2015). The

Z. tournefortti also revealed in its NL classes similar Rf presented by phytol and cholesterol and the PL classes contain similar Rf presented by L-α-phosphatidylethanolamine and 1-(3-sn-phosphatidyl)-rac-glycerol. The *A. taxiformis* revealed similar spots with an Rf similar to cholesterol in its NL fraction and spots with similar Rf presented by phospholipids in *Z. tournefortii* PL fraction. Ragonese et al. (2014) assessed these classes in 8 macroalgae (2 green, 2 red and 4 brown) and determined that *U. rigida* revealed also analogous spots with an Rf similar to L-α-phosphatidylcholine. The same author determined that some species of red and brown macroalgae also present spots with the same Rf as presented by L-α-phosphatidylethanolamine and 1-(3sn-phosphatidyl)-rac-glycerol.

2.8.5.2 Fatty acids (FA)

The FA yield in this work was higher in *Z. tournefortii* (1.23% dw), followed by *U. lactuca* (0.75% dw) and *A. taxiformis* (0.6% dw). Schmid et al. (2018) evaluated 61 species of macroalgae collected along the Southern Australian shore and also determined the TFA for the *Dictyopteris muelleri*, from the same family that *Z. tournefortii* (*Dictyotaceae*). The TFA varied from 1.6% to 4.8%, for *Z. turneriana* and *Dictyopteris muelleri*, respectively. This author also determined for *Ulva* a variation between 0.5% to 1.9% of TFA and for the *Ptilonia australasica* and *Delisea pulchra*, belonging to the same family as *A. taxiformis* (*Bonnemaisoniaceae*), 0.9% and 2.2%, respectively. Our values agree with or were close to the reported values, with some discrepancy due to environmental factors such as availability of nutrients in seawater, light availability, herbivory pressure, seasonal period of harvest, geographical location, among others.

Highest amount of SFA was detected in the rhodophyta *A. taxiformis*, 94% of total FA of which 72% is comprised by palmitic acid (C16:0). This result does not agree with of Mellouk et al. (2017), who reported 23.7% of SFA and 67.8% of Unsaturated Fatty Acids (UFA) for the same species, from the Mediterranean, near the Algerian coast. This enormous difference is not easy to explain, although it is probably due to environmental differences between the Mediterranean and the open Atlantic Ocean, such as water temperature and hydrodynamism. It also raises the need to assess nutritional profiles of macroalgae collected in different locations and possibly also at different phases of the life cycle. Gressler et al. (2010) determined that SFA varied from 51 to 78% and palmitic acid is the most representative SFA in four species of red macroalgae, collected in the intertidal zone of Anchieta, Espírito Santo State, Brazil,. SFA composition of *Z. tournefortii*, (40%) and *Dictyopteris muelleri* (39%) was similar (Schmid et al. 2018). The FA composition of the chlorophyte *U. lactuca* subjected to different environments was analysed. For instance, Cardoso et al. (2017) evaluated this macroalgae cultivated in fish ponds and demonstrated that SFA were lower (38%) when comparing with our results (54%) with palmitic acid (16:0) accounting for 19% and 50% of total FA, respectively. Mai et al. (1994), assessed the nutritional quality of *U. lactuca* for the nutrition of two species of abalone pointing out that palmitic acid (24%) is a major component of FA fraction in *U. lactuca*. The assessment of *U. lactuca* collected by Verma et al. (2017) from the intertidal zone, in Port Okha (Gujarat), India, also revealed high yield of this SFA (31%). Kendel et al. (2015) evaluated two macroalgae, *Ulva armoricana* and *Solieria chordalis* from Brittany (France) and determined that palmitic acid was predominant in these macroalgae. Furthermore, Sánchez-Machado et al. (2004), evaluated 5 edible macroalgae, *Himanthalia elongata*, *Laminaria ochroleuca*, *Undaria pinnatifida*, *Palmaria* sp. and *Porphyra* sp., along the northwest Iberian Coast, whereas the SFA varies from 20.39 to 64.95%, and palmitic acid being again the major FA, with values between 16.51 till 63.19% of total FA.

The highest values concerning MUFA was detected in the *Z. tournefortii* (43.27%), followed by *U. lactuca* (30.04%) and *A. taxiformis* (6.01%). The most prominent FA among MUFA changed between species, since in *U. lactuca* and *A. taxiformis* was the stearic acid (C18:1), with 24.65 % and 5.29 %, respectively, whilst in *Z. tournefortii*, the major concentration of MUFA was palmitic acid (C16:1), corresponding to 26.80 % of total FA. Cardoso et al. (2017) determined to be the most representative MUFA (16.6%) in *U. lactuca*, collected from fish ponds, the stearic acid (C18:1) with 7.7% of total FA. Dawczynski et al. (2007) determined that MUFA content varies between 7.8% and 20.7% in 4 dried edible macroalgae *Porphyra* sp., *Undaria pinnatifida*, *Laminaria* sp. and *Halymenia fusiforme*, with stearic acid (C18:1) being the most representative, varying from 5.95 to 15.3% of total FA.

The highest content of PUFA was determined in *Z. tournefortii*, 2.04 ± 0.14 $mg.g^{-1}$ dw (16.58% of TFA), followed by *U. lactuca* with 1.18 ± 0.05 $mg.g^{-1}$ dw (15.62% of TFA), whilst no PUFA was detected in *A. taxiformis*. Sánchez-Machado et al. (2004) determined that PUFA content varies between 16.10 and 69.11% in edible macroalgae, with our values close to the lowest assessed content. The most representative PUFA was determined to be species dependent. In *U. lactuca* the α-linolenic acid (18:3ω3-ALA), 0.43 ± 0.01 $mg.g^{-1}$ dw (5.71 % of TFA) and *Z. tournefortii* the stearidonic acid (18:4ω3), 0.65 ± 0.06 $mg.g^{-1}$ dw (5.24 % of TFA) are the main PUFA components. According to several scientific reports, the most representative PUFA in seaweed appears to be variable. Cardoso et al. (2017) and Wahbeh (1997) refer that linoleic acid (18:2ω6) has the principal PUFA in *U. lactuca*, composing 9.5 and 9.7%, respectively. On the other hand, Mai et al. (1994) determined that stearidonic acid (18:4ω3) was the most representative PUFA in *U. lactuca* (14.8%). Verma et al. (2017), studying this macroalgae, determined that α-linolenic acid was the major PUFA, coinciding with our finding, but with higher 14.31% in the TFA. The eicosanoid precursor, arachidonic acid (20:4ω6-AA), 0.14 ± 0.01 $mg.g^{-1}$ dw (1.16 % of TFA) and eicosapentaenoic acid (20:5ω3-EPA), having 0.32 ± 0.01 $mg.g^{-1}$ dw (2.57% of TFA) was only determined in *Z. tournefortii*. Sánchez-Machado et al. (2004) also demonstrated that the most representative PUFA is also macroalgae dependent, showing the arachidonic acid (20:4ω6-AA) the highest content in the *Porphyra* sp. (6.80%), *H. elongata* (10.69%) and *L. ochroleuca* (14.20%). The stearidonic acid (18:4ω3) is present in the *U. pinnatifida* with 22.60% and the eicosapentaenoic acid (20:5ω3-EPA) in the *Palmaria* sp. with 24.05%. These different observations allow us to conclude that the major macroalgae FA content is seasonal dependent, and will vary depending on the geographical location and ecosystem dynamics. This conclusion is supported by dependent Nelson et al. (2002) and Polat and Ozogul (2013).

The ω6/ω3 ratio in *U. lactuca* were 0.71 and in *Z. tournefortii* 0.13, with no amount of PUFA detected in *A. taxiformis*. Sánchez-Machado et al. (2004) determined that this ratio varies from 0.13 to 1.21 in 5 edible macroalgae, Cardoso et al. (2017) determined a 0.86 ratio for *U. lactuca* grown in fish aquaculture ponds and Schmid et al. (2018) establish a 0.8 ratio for *Z. turneriana* collected in the Australian southern shore. It is currently accepted that human beings evolved with a ω6/ω3 ratio diet of approximately 1, but nowadays the western diets are between 15/1 to 16.7/1 ratio, whereas a maximum of 4/1 ratio is considered suitable to maintain a healthy status (Simopoulos 2002). Elevated ω6/ω3 ratio increase the prostanoid thromboxane (TXA_2) and inhibit prostacyclin (PGI_2) production which is linked to the increasing risk of thrombosis (Ulbricht and Southgate 1991). Moreover, Simopoulos (2002) described in his work that a ω6 rich diet could increase blood viscosity, vasospasm, vasoconstriction, when a higher consumption of ω3 helps the relaxation of large arteries and vessels, due to an increase of nitric oxide, an endothelium-derived

relaxing factor (EDRF), thus decreasing the atherogenicity effect. Ulbricht and Southgate (1991) developed two interesting equations, one for calculating an index of thrombogenicity (IT) and an index of atherogenicity (IA), where values indicate healthier results. For the macroalgae included in this work, the IT was 0.35 for *Z. tournefortii*, 0.59 for *U. lactuca* and 30.02 for *A. taxiformis*. These results indicate an anti-thrombogenic effect of *Z. tournefortii* and *U. lactuca*, but due to the absence of PUFA in *A. taxiformis*, this macroalgae appears unsuitable for this purpose, in addition to concerns that point out to the toxicity of halogenated compounds in this Rhodophyta (e.g., Li et al., 2016). For IA, the values were 1.06 for *Z. tournefortii*, 1.18 for *U. lactuca* and 24.57 for *A. taxiformis*. These results evidence an anti-atherogenicity effect of *Z. tournefortii* and *U. lactuca*, while *A. taxiformis* is confirmed as unsuitable for this purpose. Vizetto-Duarte et al. (2015) determined these indices in six different *Cystoseira* species and determined IT values varying between 0.54 and 1.61, and IA between 0.67 and1.94. Belattmania et al. (2018) also analyzed brown macroalgae from Morocco and determined IT to vary between 0.04 to 0.25 and IA from 0.55 to 1.35.

These results strongly enforce the possibility of using *U. lactuca* and *Z. tournefortii* lipids to enhance the nutraceutical characteristics of food products and to develop macroalgae based supplements to improve or maintain health. Considering the above, including *U. lactuca* and *Z. tournefortii* in human nutrition could be a means to reduce the ω6/ω3 ratio.

2.8.5.3 ATR-FTIR

This technique is extensively used for the recognition and characterization of numerous types of molecules, using the fact that it delivers an exclusive signature to each compound. The transmittance variance detected by the equipment in each wavenumber is due to the relative mass and geometry of the atoms in the sample. The conformation of the molecules enables resonance between vibrations that will modulate the spectra (Derenne et al. 2014). The assessment of our samples permitted to obtain distinguishable ATR-FTIR "fingerprints" and two small peaks, most prominent in *U. lactuca*, with 790 cm^{-1} (A) and 846 cm^{-1} (B), according to Robic et al. (2009), indicate the presence of ulvans. These complex polysaccharides are integrated in the cell wall and due to their unusual composition and structure, considered a functional biopolymer (Lahaye and Robic 2007). Three well distinguished peaks and one shoulder located between 1,000 and 1,200 cm^{-1} (C, D, E and F) indicates the vibration of sugar rings, overlapping with stretching vibrations of C-OH side groups and the C-O-C glycosides bonds. According to Robic et al. (2009) ulvan extracts present strong absorption at 1,055 cm^{-1}, close to our D peak at 1,050 cm^{-1}. This is well distinguished for *U. lactuca*, due to C-O stretching in rhamnose and glucuronic acid. A medium intensity peak at 1,222 cm^{-1} (G) and 1,256 cm^{-1} (H) was detected in the three macroalgae, indicating polysaccharides with some degree of sulfation. Generally, sample sulfation intensity is detected between 1,210 and 1,260 cm^{-1} (Gómez-Ordóñez and Rupérez 2011). In green macroalgae this would be represented by ulvans, in red macroalgae by carrageenans and for brown macroalgae by fucoidan. These polysaccharides are valuable for their intrinsic rheological qualities (carrageenans) or for their bioactive properties (ulvans and fucoidan). For example, fucoidan, a fucose-enriched sulfated polysaccharide with a high degree of complexity, is described as a compound with anti-inflammatory, anticoagulant, antithrombotic and antioxidant properties (Senthilkumar et al. 2013) and is exploited as an additive in some

products for health food, drinks and cosmetics (Pádua et al. 2015). These biological properties will vary, depending on the density and specific positions of these sulfated groups (Berteau and Mulloy 2003).

2.8.5.4 Bioactivity

Anti-acetylcholinesterasic activity was higher for LE when compared with EE, which can be seen by the lower IC_{50} results and/or the higher % inhibition at the highest concentration tested. These results suggest that the molecules responsible for this effect are either lipids or similar compounds. The macroalgae *A. taxiformis* stands out, presenting 100% inhibition using 150 $\mu g.mL^{-1}$ for both types of extract and the lowest IC_{50} results (8.92 ± 0.43 $\mu g.mL^{-1}$) using LE extract. The EE of this macroalgae also delivered good results, presenting an IC_{50} of 46.33 ± 6.02 $\mu g.mL^{-1}$. For comparison purposes, two widely used drugs in Alzheimer's patients were used in this work. Donepezil, a piperidine derivative and Galantamine, an alkaloid. Although the activity presented by these compounds was much stronger, it must be pointed out that the activities of the LE and EE *A. taxiformis* extracts, which are mixtures containing a high variety of compounds, are nonetheless high enough to justify a purification to identify the active molecules. These results are far higher than those presented by Stirk et al. (2007), who evaluated 7 macroalgae species from the east coast of South Africa determining the IC_{50} results for AChE inhibitory activity. The best result obtained by those authors (IC_{50} of 4800 $\mu g.mL^{-1}$ for *Dictyota humifusa*) is more than 500 times less active than the *A. taxiformis* EE presented herein. The most interesting result reported by Stirk et al. (2007) is the effect of seasonality on the activity, which may be due to differences in environmental pressures such as predation along the year cycle. The work of these authors point out the need to carry out studies determining the best phase of the algal cycle to collect biomass with significant bioactivity.

Most of the extracts tested also inhibited Butyrylcholinesterase, and once again *A. taxiformis* was most prominent, with LE approximately two-fold more active than EE. In addition, the dual inhibitory activity of AChE and BuChE is quite positive, since there is evidence that compounds with this feature can intensify the effectiveness of AD treatment, possibly by also reducing the formation of β-amyloid plaques(Giacobini 2003).

Overall, the results obtained further reinforce the potential of *A. taxiformis* extracts for further studies to inhibit these enzymes, since they are much stronger than results reported by other authors for macroalgae extracts. The *Cystoseira* species extracts (Custódio et al 2016) have a dual AChE and BuChe inhibition, but at much higher concentrations (between 1,000 and 10,000 μg/mL) than the ones used herein (0.293 and 150 μg/mL). What is mentioned above for AChE inhibition is also true for BuChE, i.e., that the compounds with higher anti-cholinesterasic activity are probably lipids or compounds with a similar polarity, since LE extracts were consistently better inhibitors of both enzymes. To avoid the use of chloroform, the ethanolic extraction (EE) could be optimized to effectively extract these compounds, varying the time and number of compression and decompression cycles. These parameters enable the variable pressure for solvent recirculation through the sample. Solvent mixtures could also be applied, adding ethyl acetate, an extensively used solvent in the food industry to decrease polarity. This effort is necessary to develop "greener" methodologies to produce extracts comprising of apolar extracts that could perform equally, efficient reducing the usage of toxic solvents, known to be harmful to the environment.

The cytotoxicity against A549 tumour cell line does not present a clear activity pattern as in the case of anti-cholinesterasic activities. The *A. taxiformis* LE, *Z. tournefortii* EE and *U. lactuca* EE were the most active extracts, with IC_{50} values of 98.02±0.23, 137.64±7.89 and 140.42±4.99 $\mu g.mL^{-1}$, respectively. The *A. taxiformis* LE again exhibited the best results, highlighting the bioactive potential of this macroalgae and extract for further compound purification. These values are comparable with others reports of activity against this cell line, such as the IC_{50} of 110 $\mu g.mL^{-1}$ obtained by Dellai et al. (2013), using *Laurencia obtusa* MeOH:$CHCl_3$ extracts. Kang et al. (2017) obtain an IC_{50} of 50$\mu g.mL^{-1}$ against A549 cells, inducing apoptosis, using *Gracilariopsis lemaneiformis*. However this result corresponds to purified polysaccharides and is therefore not comparable with the extracts presented here, since they were extracted with distilled water. Additionally, an *Ecklonia cava* ethyl acetate extract displayed *in vitro* metastatic activity against A549 cells, although it did not affect cell viability (Lee et al. 2011). These results suggest is that aqueous and fractions of intermediate polarity may also have anticancer potential and should also be investigated. Concerning the results presented herein, the extracts and / or isolated compounds isolated thereof will be tested against other cell lines, namely obtained from non-tumour origin, and the mechanism of toxicity will be assessed. However, in a preliminary study, cytotoxicity against a cell line is a good indicator of potential for further studies using a bioassay guided approach.

2.8.6 Conclusion

The macroalgae analysed in the present work present interesting characteristics that justify further research. *U. lactuca* and *Z. tournefortii* have qualities that point out their interest in nutrition, such as their low lipid content and favourable IT and IA values. The presence of polysaccharides such as ulvans or fucoidans with some degree of sulfation, as indicated by ATR-FTIR results, also points out the interest of these two macroalgae as staple food, if not for human consumption, a least in animal feed. Concerning *A. taxiformis*, the high percentage of SFA, the extremely high IA and IT values and the studies that point out to the toxicity of its halogenated compounds do not recommend it as an edible macroalgae, but it displays biological activities that indicate its pharmaceutical interest. The low IC_{50} values for anticholinesterasic activity, particularly those for the LE extract, and the fact that both *A. taxiformis* extracts present dual AChE and BuChE inhibition, are good indicators of the possibility of finding a good candidate scaffold for an anti-Alzheimer Disease drug.

2.8.7 References

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Chapter 3

3 Discussion, Conclusion and Future prespectives

3.1 Discussion

The main objective of this thesis was to evaluate the use of marine macroalgae, collected in the Madeira Archipelago, for the industrial production of biorefinery-derived biofunctional products. The biorefinery concept can be linked to high efficiency fractionation of the biomass, with production of valuable biofunctional compounds. It represents a sustainable multi-step process, transforming biomass into various marketable value-added products. Several strategies for numerous industrial crops or biomass applications were already developed. Chemical production or extraction using macroalgae as feedstock is mainly focused on single products, such as the extraction and purification of hydrocolloids, polysaccharides, pigments, proteins and biofuels production, discarding the remaining biomass. For a profitable industry, sustainable strategies and cascade processing with an efficient disintegration of the biomass are needed to obtain valuable biocompounds. Also, the increasing need for higher quantities and standardized biomass, enables the necessity to cultivate macroalgae, reducing the influence of environmental conditions of marine ecosystems on raw material. The first scientific article presented in this work, sub-chapter 2.1., refers this possibility and is entitled "Sampling, structure identification, cultivation and accessions maintenance of three seaweeds from Madeira Archipelago". It was possible to identify the preferable sites and conditions in which *Ulva lactuca*, *Asparagopsis taxiformis* and *Zonaria tournefortii* grows. This is critical information when trying to replicate these conditions in the laboratory to assess the establishment and growth conditions. For these three different species, spring was the preferable season to specimens sampling and study. Furthermore, it was possible to study the microscopic structures, evolved in the sexual or asexual reproduction, during species life cycle. For instance, in *U. lactuca* the blade is composed by identical individual cells, showing no differentiation, being considered a complex single cellular colony, due to the absence of plasmodesmata, microscopic conduits, which enable communication and metabolite transport between cells. Considering a protoplast protocol, these individual cells could be separated and each one can develop a fully matured individual. For *A. taxiformis*, the presence and formation of the tetrasporophyte state, known as the "*Falkenbergia hillebrandii*" was detected in Madeira's populations. This structure has the ability to produce spores that could develop into full mature gametophyte individuals. The brown macroalga *Z. tournefortii* is an isomorphic species, which gametophyte and sporophyte cannot be distinguish, at naked eye. Male and female gametophytes releases their gametes to the environment to enable fecundation, when this happens, a new sporophyte organism arise. Adult and mature sporophyte, releases spores that start gametophyte generation. This information was used to setup cultivation trials, aiming to maintain species samples *in vitro* to enable the production of viable propagules to maintain the macroalgae accessions. These structures transferrence from *in vitro* to field tanks or open sea offshore for seedling and aquaculture production is the next step that should be solve in perspective. The properly assessment of the development and maintenance of these structures was realized, testing several cultivation media to determine the optimal growth conditions, with the selection of F/2 medium. Also, different techniques were applied to produce the propagules, namely using solid and/or liquid media, with preference given to the last one. In order to oblige these organisms to release the propagation structures, stress conditions were used to enforce it, after which the seawater was used to test the germination. This procedure was particularly useful to release the spores from the tetrasporophyte "*Falkenbergia hillebrandii*", which we consider to be the most promising way to maintain the *ex situ A.*

taxiformis culture. Spores release and propagules culture for this macroalgae was obtain. Although the culture contamination by diatomaceas should be overcame in order to long term maintaining of macroalgae seedings. Axenic cultures were difficult to obtain at this stage due to the difficulties in spores' separation from sources of contamination. The imposition of stress conditions was also tested in the case of *Z. tournefortii*, through the maintenance of the sporophyte, during 24 hours, in dark conditions. In total 7 trials were performed whithout success on the release or germination of the reproductive structures. This could be attributed to specific timings that this macroalgae has to produce spores or specific lunar cycles that enable its release. An additional study of species life cycle in wild was required. Trials for *U. lactuca* included the cutting of algal blade in small pieces and its cultivation in natural seawater, enriched with F/2 medium. It was observed that some small pieces would have a vegetative growth and some spores were released during the process, with the development of new matured organisms. However, this procedure requires further research to improve the conditions, which enable specimen's maturity and sporulation, being these processes highly species specific. New biotechnological approaches like protoplast production, could enable the production of viable protoplasts, individual cells, obtained from the macroalgae thallus, after its digestion by a mix of lytic enzymes, used to remove the polysaccharide cell wall. This approach, applied to certain macroalgae species, allows to substitute the production of spores or zygotes as the main source of propagules. The difficulties in this procedure were related with the optimization of the lysis procedure. Since protoplasts yield were very low and in the further cultivation tests, propagation was not observed. Moreover, it was possible to maintain the three macroalgae under cultivation, during months, as viable germplasm accession in the bank, requiring its recultivation. Nevertheless, this result seems to us very important, since it allows to perform germination testing for longer time periods, as well as to maintain an *ex situ* collection of macroalgae for research and cultivation purposes.

A biochemical analysis was performed for the previous referred macroalgae and also for additional number of species. Since a prospection and gathering of macroalgae was realized in different locations of the Madeira Archipelago, with the ultimate goal to evaluate viable sources of valuable biomass for future biorefinery efforts. Part of these work was published in Nunes et al. (2017) and discusses the biochemical composition and antioxidant properties of seven seaweeds from Madeira Archipelago. A first understanding of the macroalgae species found in the intertidal and subtidal shore of the south coastline of Madeira Island, was obtained. These seaweeds are considered staple food only in a few countries and regions, but this raw material has the potential to be introduced as an ingredient in various foods, increasing its nutritional quality and helping some food products to meet the recommended dose intake (RDI). The red macroalga *A. taxiformis*, known as "limu kohu" in Hawaii, is consumed as a staple food in this archipelago and belongs to their traditional diet. We have determined that this macroalgae is a prominent source of protein, due to its higher content among the studied species, 17.55 g/100g dw. Although this statement should be considered with caution, due to the potentially presence of toxic halogenated compounds in these macroalgae. The analysis of the green macroalgae, *U. lactuca*, allowed for food consumption in Europe, showed higher content of fiber and matricial polysaccharides, 54.04 g/100 dw, as well as the highest content of a specific family of antioxidants, flavonols (604.77 mg QE/100g dw). The brown macroalgae *Z. tournefortii* was noticeable as a source of fat (12.04 g/100g dw), pigments and antioxidant compounds such as chlorophyll *a* (244.25 mg/100g dw), total carotenoids (297.77 mg/100g dw) and total phenolic compounds (2154.77 mg GAE/100g dw). *Z. tournefortii* showed the highest results for FRAP (6078.29 mg

AAE/100g dw), FIC (77.93%), FRSA (3927.83 mg AAE/100g dw) and β-CB tests (95.03%), being this macroalgae a preferable source to integrate in the study of antioxidant extracts. Statistical analysis performed in this work evidenced that fat has a high correlation with several antioxidant parameters and activity suggesting that the biochemical compounds that provide part of the antioxidant capabilities have lipophilic nature. This means that when performing fat extracts, a high degree of antioxidant activity is to be expected and these extracts could be integrated in food and beverage products to test their inclusion as natural antioxidant agents.

The biochemical study of attached macroalgae from the Madeira Archipelago and beach-cast macroalgae from the Canary Islands includes an additional fifteen species from Madeira and two beach-casts macroalgae. This work, accepted for publication in *Botanica Marina Journal*, uses statistical modelling to determine correlations between the biochemical parameters, helping to predict how these fluctuate. Also, same species collected from different sites show dissimilar biochemical contents. For example, *A. taxiformis* collected in Porto Santo Island showed from 1.36 to 2.11 times higher mineral content when compared with the samples of the same species collected in Madeira Island. The mineral content shows variability due to several exogenous environmental factors such as the concentration of minerals, seawater pH and temperature. The green macroalgae *Ulva intestinalis*, collected in offshore cages of the *Sparus aurata* fish farm shows to be rich in protein (16.85 g/100g dw) and a good source of total carbohydrates (41.81 g/100g dw). We can hypothesize that high protein content in this seaweed is a result from the high availability of nutrients dissolved in the surrounding waters, discharged or unused by the fish farm facility. This eutrophication process can be substantially reduced with the incorporation of macroalgae and fish productions in the same facility. The macroalgae increases its growth rate and protein content as a consequence. This is a good strategy to reduce the environmental impact of fish aquaculture expansion in Madeira Archipelago and enables the development of a biomass resource that has a market potential. Beach-cast macroalgae showed a high polysaccharide content (34.04 and 48.27 g/100g dw), likely related to the species composition of these biomasses. Specific biochemical pathways and habitat features force these organisms to biosynthesize different contents and types of polysaccharides, enabling content fluctuations. *Cystoseira humilis* and *Cystoseira usneoides* were found to be a prominent source of phenolic compounds, 33.14 mg GAE/mg dw and 35.20 mg GAE/mg dw, respectively, synthesized as protection against marine grazers, epiphytes, pathogens and photoprotection. Brown macroalgae have higher content of these compounds due to enzymatic cross-linking of alginates by phenols used in the strengthening of the cell wall, influenced by the habitat conditions. *Halopithys incurva*, a red macroalga and beach-cast macroalgae were determined to be rich sources of phycobilins, water-soluble macromolecules used as natural dye in food products. *Sargassum vulgare*, a brown macroalga, was found to be a rich source of fucoxanthin, 1.19 mg/g dw, an important marine xanthophyll with antioxidant capability. Specific environmental conditions can trigger adaptation mechanisms, enabling strategic metabolic pathways, which leads to an increase in carotenoid production. Statistical analysis shows correlations between several biochemical parameters. Fifteen significant correlations at 1% and five at 5% were detected. Between those, three significant correlations at 1%, with the highest Pearson coefficient value were determined between five parameters (carbohydrates, chlorophyll *a*, fucoxanthin, TCC and total minerals). Carbohydrates were found to be negatively correlate with the mineral content. Additionally, fucoxanthin was found to have a positive correlation with TCC. This could be attributed to the fact that fucoxanthin contributes to the total amount

of carotenoids in macroalgae resources. Furthermore, the two samples of beach-cast macroalgae were statistically different from each other in terms of their phycobilin, total mineral and TPC content. This demonstrates that if this bioresource is to be considered for biocompound extraction or other economic use, a deeper study is needed to understand the compositional variability of these resources throughout the year, to improve its industrial integration. These biochemical evaluations were important since some of the macroalgae species, introduced in this work as also the beach-cast macroalgae were, for the first time, analysed its biochemical composition and hypothesized its possible industrial applications. Moreover, two species of macroalgae collected in the offshore fish farms were evaluated, providing a valuable insight to reduce eutrophication in these sites and use these species for food, supplements or extract bioactive compounds with a high market value.

The development of new methodologies and techniques were essential in this work to analytically quantify the iodine and fucoxanthin content in macroalgae samples. The publication Nunes et al. (2019a) published our validation of a spectrophotometric method for a rapid iodine analysis in algae and seaweed casts, where linearity, sensitivity, accuracy, precision and matrix effect were assessed. A rapid method for iodine measurements, using a common equipment is requested by industry. Method validation consisted in the iodine content determination in nine macroalgae samples, using inductively coupled plasma mass spectrometry (ICP-MS) and UV-Vis spectrophotometric analysis. The results of both techniques were plotted against each other obtaining a R^2=0.91 and showing an excellent correlation. Additionally, the iodine content was determined in twenty-five macroalgae samples, comprising 5 green, 9 red, 11 brown macroalgae and 12 samples of beach-cast macroalgae. The highest iodine content was determined in red macroalgae, more specifically in *Asparagopsis taxiformis* collected in Madeira Island. Also, this work suggests the use of these beach-cast macroalgae as an iodine source that can be applied in soil for crops biofortification and animal feed to overcome iodine soil depletion. This validated method for iodine content in macroalgae and beach-cast macroalgae is fast, reliable and could be implemented in any laboratory with minimal budget. Furthermore, for some of the macroalgae species, this was the first report of its iodine composition, increasing the knowledge of its mineral composition.

Furthermore, a new methodology was developed for the measurement of fucoxanthin content in algae samples (Nunes et al. 2019b). The methodology consisted in the application of vortex-assisted solid-liquid microextraction using high-performance liquid chromatography with photodiode array detection. The method validation consisted in the use of linearity, sensitivity, matrix effects and evaluation of the selectivity, precision and accuracy, after determining the optimal conditions for fucoxanthin extraction. The statistical analysis, using Response Surface Methodology (RSM), was used to maximize the fucoxanthin micro-extraction prior to the HPLC analysis. Fucoxanthin was successfully quantified in 22 brown algae samples, with the highest content determined in *Z. tournefortii*, 852 µg/g. The fucoxanthin extracts were found to be stable for 10 days at 10ºC, which was a great result since simultaneous extraction of multiple samples could be performed and stocked before HPLC analysis. This methodology enables to accurate assess the fucoxanthin content in macroalgae biomass, using small quantities of biomass, allowing sample shipping to dedicated laboratories to perform several extractions at once, reducing extraction time. The use of ethanol in micro-quantities to extract this biocompound is preferable since this solvent is consider the "greener" solvent extraction option. This methodology allows the analysis of 50 individual vials in a 3 day period. Moreover, it is interesting to notice that same species revealed different concentration levels at

different locations (*D. dichotoma*) and collection dates (*Z. tournefortii*). The beach-cast macroalgae, sampled from "Playa de Las Canteras", Gran Canaria shown the lowest fucoxanthin content, which indicates that higher biomass yield are needed for purification strategies associated with a biorefinary implementation.

In this thesis, the biorefinery assessment, involved the red macroalgae *A. taxiformis* and the brown macroalgae *Z. tournefortii*. The biorefinery approach for the 1st macroalgae (Nunes et al. 2018) investigates two methods and four solvents for the development of *A. taxiformis* extracts and its iodine composition integrating a posterior downstream strategy for the remaining residue. This work demonstrated that iodine content in these bioactive extracts are in higher concentration when performing the extraction with ethanol, independently of the method used. The determinion of iodine content in macroalgae extracts are of extreme importance, when considering the use of these extracts for human supplementation. Iodine is an essential element for human metabolism and physiology due to its integration in the composition of thyroid hormones T3 (3,5,3-triiodothyronine) and T4 (thyroxine or 3,5,3,5-tetraiodothyronine). Furthermore, ethanol extracts were preferred than the methanol, water and ethyl acetate extracts, due to its high yield of total phenolic compounds, chlorophyll *a* and total carotenoids, showing a good antioxidant activity. An efficient downstream extraction from the final biorefinery residue was implemented, allowing to obtain lipids, carrageenan and cellulose. The ATR-FTIR analysis of these extracts and its comparison with a direct extraction of the same compound shows very similar results of these compounds content. This demonstrates that the downstream extraction maintains the compound quality in a successful biorefinary implementation, reducing waste and increasing profit.

The macroalgae biorefinary concerning *Zonaria tournefortii* (Nunes et al. 2019c) performed ethanol-derived bioactive extracts, using a Timatic extractor. This work assessed the statistical variability of the matrix extraction using a response surface methodology (RSM). The statistical analysis was performed, with assistance of the Box-Behnken design (BBD), to assess and optimize the extract production. The BBD model employed enable us to predict the biocompounds in the extract, modelling the independent variables. The prediction of the bioactive compounds included extract yield (Yield), total chlorophyll content (TChlC), total carotenoids content (TCC), total fucoxanthin content (TFxC), total phenolic content (TPC) and total flavonoids content (TFC). Also, it was integrated two dependent variables for antioxidant assessment, free radical-scavenging assay (DPPH) and reducing activity (RA). Higher extract yield could be achieved, performing the extraction exclusively under higher sonication time or alternatively increasing the number of cycles. The reducing activity (RA) is also higher when the ethanol percentage is equal to 50%. This means that when targeting an extract with a higher antioxidant capacity with this specific activity, a lower percentage of ethanol would be preferable, reducing ethanol costs. For the extraction of the remaining biocompounds, the highest ethanol percentage (96%) is preferable. The extraction cycles could be significantly diminished for almost all the biocompounds studied, except for DPPH and TChlC, reducing the time of extraction. Furthermore, the previous sonication performed to the biomass with the correspondent extract solvent could be neglected, when the target is TCC and TPC or reduced to 8 or 13 min when higher DPPH and RA activity are needed, respectively. The optimization of the conditions of extract production, enable us to target its specific features, reducing the costs, time of extraction and equipment utilization. Plotting all independent variables, in order to determine the best conditions of the overall extraction, these were found to be 96% ethanol, 14.4 cycles and 20 min sonication,

with desirability value reaching 0.72 among a maximum value of 1.0. After initial extraction, the residue was subjected to a downstream extraction, firstly isolating crude fucoidans and secondly cellulose. Both compounds shown a similar ATR-FTIR spectrum of the same compound, extracted directly from the biomass. This biorefinery effort was applied to create a possibility to overcome an industrial problem, which is the high input of biomass, large discharge of residue and low profitability, using an underused macroalgae.

The composition, anti-cholinesterase activity and *in vitro* cytotoxicity to A549 tumor cell line of extracts of the fatty acid fraction of 3 macroalgae were analysed and published (Nunes et al. 2019d). These analyses were realized, using TLC screening, ATR-FTIR and bioassays for cytotoxicity. The possibility to use these extracts as a drug, either against Alzheimer's disease or antitumor activity was studied, through anti-cholinesterase activity or its cytotoxic effect on A549 tumor cell line. Fatty acids (FAs) composition were higher in *Z. tournefortii*, showing the highest MUFAs and PUFAs content. The best ω6/ω3 ratio was found in *U. lactuca*, a green macroalgae. These results strongly enforce the possibility of using *U. lactuca* and *Z. tournefortii* lipids to enhance the nutraceutical characteristics of food products and to develop macroalgae based supplements to improve or maintain health. Extracts from the red macroalgae *A. taxiformis* were found to inhibit Butyrylcholinesterase and Acetylcholinesterase which dual inhibitory activity is quite positive, since there is evidence that compounds with this feature can intensify the effectiveness of Alzheimer disease treatment, possibly by also reducing the formation of β-amyloid plaques. Overall, the results obtained further reinforce the potential of *A. taxiformis* extracts for further studies, since they were found to be a prominent biomass and present higher capability than the extracts from other macroalgae published by other authors. The cytotoxicity against A549 tumour cell line does not present a clear activity pattern, depending in the methodology and solvent used for the extract production. The lipophilic extract of *A. taxiformis* exhibited the best results, even when comparing with its ethanolic extract, highlighting the bioactive potential of this macroalgae and extract for further compound purification. Concerning these results, the extracts should be tested against other cell lines, namely obtained from non-tumour origin, and the mechanism of toxicity assessed. However, the preliminary study of the cytotoxicity against a cell line is a good indication of its potential for further studies using a bioassay guided approach.

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3.2 Conclusion

As final conclusions, this work effectively prospected and evaluated the biochemical composition of 20 different marine macroalgae. This is the first effort to increase our knowledge about the biochemical composition, nutritional value, antioxidant potential and activity of this biomass, collected in Madeira archipelago. *A. taxiformis*, a red macroalgae existing in Madeira and Porto Santo islands, showed the highest content of protein and significant quantities of important nutritional parameters. Furthermore, *H. incurva*, also a red macroalgae, collected in Porto Santo island, was found to contain a good concentration of phycobilins, which when extracted with lower purity index, it can integrate as a natural dye and provide beneficial bioactivity in food products. *Z. tournefortii* and *D. dichotoma*, these understudied brown seaweeds, were also determined as an important biomass, since they contain high content of fat and antioxidant components. These macroalgae could be used to perform antioxidant extractions and purification for food applications. The brown macroalgae *Z. tournefortii*, *D. dichotoma, D. polypodioides* and *S. vulgare* were found to be potential sources of fucoxanthin, a valuable xanthophyll which acts as a potent antioxidant. These can be envisioned for the production of rich fucoxanthin extracts using "Green Chemistry" techniques, supplements or additives for food products. The macroalgae *U. intestinalis* demonstrated potential as a food source, since protein and carbohydrates values were interesting and coupling with the fact that this seaweed is permitted for food consumption in the European Union, harvested in the offshore seabream aquaculture in Madeira Island. This macroalgae has the potential to be harvested to upscale its production in an integrated aquaculture system, increasing profitability and reducing nitrate emissions to the environment. The biochemical evaluation of these bioresources has the outmost importance since we can determine the potential applications and delineate strategies, appropriate to each resource. The performed report on collection, structure identification, cultivation and germplasm bank maintenance enabled us to determine the best prospection areas and periods for three selected macroalgae. Structure analysis permitted us to study the phenotype of *U. lactuca*, *Z. tournefortii* and *A. taxiformis* and identify the development of reproductive structures when collecting these species. The cultivation experiments showed that the use of agar as an artificial substrate and artificial seawater were not suitable for cultivation tests since, when using, did not come clear if difficulties were from the use of these variations. Furthermore, the F/2 medium was considered the most usable in cultivation tests since it could be kept refrigerated for long periods of time, easy to perform and produce better cultivation results. Cultivation tests performed on *U. lactuca* vegetative growth and propagules were successfully achieved, after some optimization, although when performing protoplast production methods, these were limited and further optimization is necessary to achieve higher values, regarding the number of existing protoplasts, after lysis procedure. The reproductive structures, produced from *A. taxiformis* tetrasporophyte "*Falkenbergia hillebrandii*" were achieved although optimization is necessary to achieve axenic conditions. For *Z. tournefortii*, spore production was not possible and this fact could be due to specific collection periods for this macroalgae or spore release which could be intrinsically connected to lunar cycles. Furthermore, whole macroalgae was successfully maintained under controlled conditions, when these were brought attached to its substrate.

Two methodologies were properly validated, iodine and fucoxanthin quantification, since these parameters are of extreme importance when considering marine macroalgae as a food source, food supplement or as an initial biomass for antioxidant extraction, in the case of fucoxanthin. Iodine yield is an important nutraceutical factor for humans, since this mineral is becoming scarce in terrestrial food due to

lixiviation processes and intensive cropping. Consequently, macroalgae could become a natural supplement of this essential mineral, preventing iodine deficiency related disorders. Iodine content varies greatly between species which can be considered as a positive remark, since it allows targeting seaweeds with a specific iodine content for precise applications. Results for *A. taxiformis*, a red macroalgae, suggest that is an iodine rich source, reaching values similar to a brown macroalgae, known as Kelp, which are considered the richest sources of this mineral. With this work, it was evidenced the potential of using these resources to produce bioactive extracts that could function directly or indirectly for iodine supplementation. These could be used for human consumption, applied in irrigated crops or even in animal production but further studys are necessary to investigate the toxicity level of this macroalgae and its extracts due to the halogenated compounds in its composition. Also, the prevalent awareness of iodine consumption, an important micronutrient. and its potential to integrate in functional foods are of great importance to prevent life threatening diseases, using local products or unused biomass. Furthermore, the developed methodology demonstrated that this spectrophotometric method is still a reliable technique for rapid determination of iodine content in algae samples. The validated methodology of fucoxanthin quantification in brown macroalgae was delineated to be a simple and reliable analytical method, using reduced amounts of sample and extraction solvent, using RP-HPLC-PDA technic analysis, which takes 30 min. CCD was crucial to optimize the fucoxanthin extraction yield, pointing out that 25 mg of sample with 300 µL of ethanol and vortexed for 15 min are the best experimental combination for the proposed sample preparation procedure. The ethanolic extracts produced were considered stable for 10 days when kept at 10 ºC and light protected, allowing the simultaneous extraction of multiple samples and stock them before HPLC analysis. This method proved to be an accurate tool for the evaluation of fucoxanthin concentration in macroalgae, as it was demonstrated by the analysis of 22 samples.

Macroalgae *A. taxiformis* and *Z. tournefortii* were considered suitable to integrate in a research study, to determine the ability of these resources to integrate in a biorefinary strategy, to extract several valuable products. It was effectively assessed the nutraceutical potential of *A. taxiformis* extracts, rich in iodine, performing 2 different methodologies and 4 solvents, permitted in the food industry, which ethanol was determined as the most prominent. Furthermore, it was developed a downstream strategy, to purify valuable compounds from the remaining residue such as lipids, carrageenans and cellulose, suggesting some applicability of this resource. ATR-FTIR analysis enabled a comparison between these compounds, extracted directly from whole seaweed *A. taxiformis* and those extracted from the biomass residue, were found to be similar. The biorefinary strategy for the brown macroalgae, *Z. tournefortii*, was conducted with the Timatic extractor equipment, using statistical analysis to produce ethanol derived extracts rich in fucoxanthin and high in antioxidant activity, predicting the potential to be used as a nutraceutical. Moreover, a cascade approach was implemented, which extractability and purification potential were evaluated for fucoidan and cellulose, purified from the remaining biomass residue. ATR-FTIR technic was performed in these two works and it was considered a valuable technic to determine variations in purity and to produce a fingerprint signature for these compounds. Furthermore, it was assessed the lipid composition and determined its fatty acid content, TLC screening, ATR-FTIR analysis, anti-cholinesterase activity and *in vitro* cytotoxicity to A549 tumor cell line of extracts for *U. lactuca*, *Z. tournefortii* and *A. taxiformis*. Green and brown macroalgae *U. lactuca* and *Z. tournefortii*, respectively, were found to possess some particular qualities which could interest in terms of nutrition, such as their low lipid content and

favourable IT and IA values. The existence of sulphated polysaccharides such as ulvans and fucoidans, determined by ATR-FTIR analysis, suggested some interesting uses as a staple food for human consumption or animal feed. The red macroalgae *A. taxiformis* developed low IC_{50} values for anticholinesterasic activity, showing to be a good candidate for an anti-Alzheimer Disease drug.

Macroalgae presents great potential for multi-products extraction, increasing sustainability of these resources, developing new extraction strategies, producing valuable marketable products and reducing waste, improving residue management and mitigating the environmental impacts. Several macroalgae species could be integrated in the biorefinary approach, since enough research is provided along with the development of marketable products, adapting this developing industry to the local conditions. Numerous economic appealing products can be extracted, due to the singular properties of macroalgae. Their production does not occupy land area, which reduces the deforestation pressure. The development of new cost-effective bioprocessing routes and employment of eco-friendly downstream processes in the biorefinary complex, could be the key factor for global successful implementation.

3.3 Future perspectives

Further research is advisable, concerning marine macroalgae from Madeira archipelago. Prospection work should be continued, since more species of macroalgae are certainly present in these coastal areas. Also, when relying in a few species of macroalgae for the manufacture of marketable products, a year around study should be carried out to determine biochemical fluctuations which are inherent to a particular species and collection area. Furthermore, an ecological work should be performed to determine the biomass availability throw-out the year, identifying these areas and determining the impact of collecting these macroalgae, when harvesting for industrial purposes. Cultivation tests should be continued to improve protoplast production, using *U. lactuca*, which could represent a reliable source of propagules, for industrial production of this specie inland and offshore. For *A. taxiformis*, deeper studies should also be carried to obtain spores from the tetrasporophyte phase and achieve axenic cultures of viable propagules. *Z. tournefortii* needs a deeper study of its life cycle throw-out the year, so it would be possible to understand the exact conditions which spore release occurs. Since this specie was found to contain high content of fat and antioxidant components and its extract exhibit high antioxidant activity, these extracts should be further studied in other bioactive activities to determine its full potential as a nutraceutical biomass and perform deeper analytical analysis to verify its composition. Additionally, the brown seaweed *S. vulgare*, determined in this work to be a promising source of fucoxanthin, an important antioxidant, deserves further study on the extraction of this xanthophyll, probably using "Green Chemistry" techniques, which could be used as a nutraceutical and implemented in functional foods. The red macroalgae *H. incurva* needs additional research to its phycobilins pigments, in order to determine the potential of this resource to provide these compounds, which could be used as natural dyes in the food industry and determine its beneficial bioactivity in functional foods. Concerning the biorefinary strategies, described in this work for *A. taxiformis*, a deeper analysis is needed for the initial extract and its possible integration in the market as a food supplement or as an additive for the functional food industry, such as toxicity level, side effects and iodine assimilation in the human body. For the downstream products, lipids could be further studied to determine their fatty acids composition, carrageenan studied in their gelling properties, purity and bioactivity. Cellulose could be the precursor from low to high end products, since this could be the starting material to produce paper, bioethanol or micro and nano-crystalline cellulose, a multifunctional ingredient in the food and pharmaceutical industry. Concerning the manufacture of lipid extracts, due to the low IC_{50} values for anticholinesterasic activity, demonstrated by the *A. taxiformis*, further research in this field should be carried, since it is predicted to be a good candidate to develop an anti-Alzheimer Disease drug. For the brown macroalgae, *Z. tournefortii*, the initial extracts should be tested also for their toxicity and beneficial antioxidant and bioactive activities, when integrating these extracts in the nutraceutical or food industry. Likewise, fucoidan, the first downstream product, should be further assessed in bioactivity assays, which can provide valuable information, providing beneficial outcomes to its consumers, depending on the sulfation degree.