U.PORTO

FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

MARINE MACROALGAE AS A SUSTAINABLE SOURCE OF PROTEIN AND BIOACTIVE PEPTIDES

ANA FILIPA GOMES CONCEIÇÃO
MASTER THESIS IN CHEMICAL ENGINEERING
PRESENTED TO THE FACULTY OF ENGINEERING
OF THE UNIVERSITY OF PORTO

Master in Chemical Engineering

Marine macroalgae as a sustainable source of protein and bioactive peptides

Master dissertation

of

Ana Filipa Gomes Conceição

Developed within the course of dissertation

held in

FEUP / LEPABE

Supervisor at FEUP: **Doutora Tânia Tavares**

Joint supervisor at FEUP: **Prof. Doutor F. Xavier Malcata**

October 2022

Acknowledgments

Coming to the end of this 5-year journey at FEUP, and culminating with this Master Thesis, I feel honored to have had the opportunity to meet so many remarkable people and be supported by so many others, to whom I extend my sincerest gratitude:

To Doutora Tânia Tavares, my supervisor, for teaching me so much over the past few months, for challenging, encouraging and motivating me. For sharing with me her excitement and passion for the work under development. For helping me in every step of the way, and for always being available and making me strive for more. Working alongside her throughout my Dissertation was a great privilege, and I must recognize how lucky I was to have had that opportunity.

To Prof. F. Xavier Malcata, my co-supervisor, for being an outstanding instructor and greatly inspiring me with his lectures, for all his invaluable help and incitement toward ambitious goals, for his careful revision of my dissertation, and above all for accepting me as his Dissertation student in the first place.

To the instructors who have strongly influenced me along my academic journey – Prof. João Campos, Prof. Ana Mafalda Ribeiro, Prof. Miguel Madeira, Prof. Margarida Bastos, Prof. João Bastos, Prof. Filipe Mergulhão, Prof. Manuel Simões, and so many others – for being challenging and passionate teachers, and for constantly pushing us to do better.

To Doutora Carla Ferreira, Mestre Sílvia Faia and Mestre Joana Rocha, for always offering me their advice, wisdom and kindness, and always being ready to help.

To Doutora Rebeca Cruz, for her help at the end stage of this Dissertation; and to Afonso Silva, for being a great laboratory partner.

To my dear friends – Eva, Beatriz, Hugo and Manuel, who hold such a special place in my heart, and Sara, André, Miguel, Moisés and Beata – for their friendship over the years, their guidance and support, and for always reassuring me that we were sailing through the storm together.

To João, Conceição and Diogo, for welcoming me and being my second family, for always cheering me on and for all the amazing times we have shared.

To Gonçalo, for his unwavering support and, most of all, for being my partner in life.

To my family - my parents, brother and grandfather - for their love, support, and for making me laugh in a year that was hard for all of us. None of it would have been possible without you.

Abstract

The United Nation's projection of the world population reaching 9.7 billion by 2050, aligned with the current political and socioeconomical framework, highlight the reoccurring theme of a food crisis, namely when it comes to protein availability. The need for novel protein sources is urgent, in order to maintain its consumption affordable and sustainable. Macroalgae have been suggested as alternative to traditional protein sources, due to their relatively high protein content and nutritional quality.

In order to explore the potential of green macroalga *Ulva sp.* proteins, an extraction process was optimized, and the resulting extract was subjected to hydrolysis and assessment of biological activity. The optimized extraction process consisted of a pre-treatment with EtOH for pigment removal, a successive extraction with H_2O in a cell homogenizer followed by a conventional extraction with NaOH. Chromatographic analysis of extracts showed that peptides were being obtained during the extraction process. Optimum hydrolysis conditions were determined by design of experiments (DOE) via a response surface methodology (RSM), considering reaction time and enzyme/substrate ratio (E/S) as processing factors, and angiotensin-converting enzyme (ACE) inhibitory activity and antioxidant capacity as response parameters.

The obtained experimental data was successfully fitted to a mathematical model. Under optimum processing conditions, the hydrolysates exhibited ACE-inhibitory activity characterized by IC_{50} of 92.3 ± 2.0 µg/mL and 66.8 ± 1.0 µg/mL, for the total and <3 kDa fractions, respectively. In terms of antioxidant capacities, results obtained through the ORAC method for total and <3 kDa fractions were 1.96 ± 0.04 $\mu mol_{Trolox\ equivalent}/mg_{hydrolysed\ protein}$ and 2.6 ± 0.06 $\mu mol_{Trolox\ equivalent}/mg_{hydrolysed\ protein}$, respectively. Regarding the ABTS and DPPH assays, hydrolysates attained values of 11.74 ± 0.19 $\mu mol_{Trolox\ equivalent}/g_{Alga\ DW}$ and 1.74 ± 0.06 $\mu mol_{Trolox\ equivalent}/g_{Alga\ DW}$, respectively.

This study unfolded the great potential of marine macroalga *Ulva sp.* to yield bioactive peptides with promising ACE-inhibitory activity – in principle suitable for use as functional ingredient toward nutraceutical and pharmaceutical applications.

Keywords: macroalgae, proteins, enzymatic hydrolysis, bioactive peptides, ACE-inhibitory activity, antioxidant capacity

Resumo

A projeção das Nações Unidas de que a população mundial atingirá os 9.7 mil milhões de pessoas em 2050, conjugada com o atual enquadramento político e socioeconómico, reforçam a preocupação global acerca de uma possível crise alimentar, especialmente no que que se refere à disponibilidade de proteína. A necessidade de novas fontes de proteína é urgente, para que o seu consumo se possa manter acessível e sustentável. As macroalgas surgem assim como alternativa às fontes de proteína tradicionais, devido ao seu alto conteúdo em proteína e qualidade nutricional.

De forma a explorar o potencial das proteínas da macroalga verde *Ulva sp.*, foi desenvolvido um processo de extração, e o extrato obtido foi sujeito a hidrólise enzimática e posterior avaliação de atividade biológica. O processo de extração otimizado consistiu num pré-tratamento com EtOH para remoção de pigmentos, uma extração sucessiva com H_2O num homogenizador celular e uma extração convencional com NaOH. A análise cromatográfica dos extratos demonstrou que o processo de extração conduziu a hidrólise parcial das proteínas. As condições ótimas de hidrólise foram determinadas através de um projeto experimental baseado no método de superfície de resposta (RSM), tomando tempo de reação e razão enzima/substrato como parâmetros processuais, e a atividades de inibição da enzima conversora da angiotensina (ACE) e capacidade antioxidante como parâmetros de resposta. Os dados experimentais obtidos foram estudados e ajustados com sucesso a um modelo matemático com significado estatístico. Sob condições ótimas, os hidrolisados apresentaram atividades de inibição da ACE caracterizadas por valores de IC_{50} de 92.3 ± 2.0 µg/mL e 66.8 ± 1.0 µg/mL, para as frações total e <3 kDa, respetivamente. Para as capacidades antioxidantes, os resultados obtidos pelo método ORAC para as frações total e <3 kDa foram de 1.96 ± 0.04 $\mu mol_{Trolox\ equivalente}/mg_{Proteína\ hidrolisada}$ e 2.60 ± 0.06 $\mu mol_{Trolox\ equivalente}/mg_{Proteína\ hidrolisada}$, respetivamente. Para os métodos ABTS e DPPH, os hidrolisados obtidos para as condições ótimas exibiram valores de 11.74 ± 0.19 $\mu mol_{Trolox\ equivalente}/g_{Alga\ DW}$ e 1.74 ± 0.06 $\mu mol_{Trolox\ equivalente}/g_{Alga\ DW}$, respetivamente.

O estudo desenvolvido demonstrou o grande potencial da alga marinha *Ulva sp.* para fornecer péptidos bioativos com atividade inibidora da enzima conversora da angiotensina, sendo em princípio apropriada para uso como ingrediente funcional em eventuais aplicações nutracêuticas e farmacêuticas.

Palavras-chave: macroalgas, proteínas, hidrólise enzimática, péptidos bioativos, atividade inibitória da enzima conversora da angiotensina, capacidade antioxidante

Declaration

I hereby declare, under word of honour, that this work is original, that all non-original contributions are indicated and that due reference is given to the author and source.

Ana Filipa Gomes Conceição

Ana Filipa Gomes Conceição, 26 de setembro de 2022

Index

1 Introduction 1

1.1 Framing and presentation of the work 1

1.2 Presentation of the company 2

1.3 Contribution of the author to the work 2

1.4 Organization of the dissertation 2

2 Context and State of the art 5

2.1 Protein shortage and the need for novel protein sources 5

2.2 Algae as an alternative protein source 6

2.2.1 Microalgae 6

2.2.2 Macroalgae 7

2.2.3 Strategies for protein extraction 8

2.3 Bioactive peptides 9

2.3.1 Production of bioactive peptides 9

2.3.2 Biological activities 10

3 Materials and Methods 12

3.1 Algae sample preparation and characterization 12

3.1.1 Amino acid profile determination 12

3.2 Protein extraction 13

3.2.1 EtOH pre-treatment 14

3.2.2 Precellys extraction 14

3.2.3 Conventional extraction 14

3.3 Preparation of selected extraction samples 14

3.3.1 Lyophilization 14

3.3.2 Filtration 14

3.4 Enzymatic hydrolysis 15

3.4.1 Experimental design, modeling and optimization by design of experiments (DOE) techniques 15

3.4.2 Performance of enzymatic hydrolysis 15

3.4.3 Degree of hydrolysis (DH) determination 16

3.5 Determination of biological activities 16

3.5.1 Antioxidant capacity determination 16

3.5.2 ACE-inhibitory activity determination 19
3.6 Protein content determination 20
3.7 Protein profile determination 20
3.8 Chlorophylls and carotenoids determination 21
3.9 Statistical analysis 21
4 Results and discussion 23
4.1 Amino acid profile of algae sample 23
4.2 Optimization of protein extraction 24
4.3 Experimental design, modelling and optimization of biological activities 30
4.4 Protein profile 36
4.5 Chlorophylls and carotenoids profile 38
5 Conclusions 39
6 Final assessment and future work 40
7 References 41
Appendix A 49
Appendix B 51

List of Figures

Figure 4.1. Appearance of supernatants after single and sequential extractions in Precellys homogenizer: a) from left to right: supernatant after first 5 mL extraction with EtOH, supernatant after second 5 mL extraction with EtOH and supernatant after 10 mL extraction with H_2O; b) combined supernatants of two 5 mL extractions with EtOH and two 5 mL extractions with H_2O; c) supernatant after 10 mL extraction with EtOH and 10 mL extraction with H_2O; d) supernatant after first 5 mL extraction with H_2O, supernatant after second 5 mL extraction with H_2O and supernatant after single 10 mL extraction with H_2O. 25

Figure 4.2. Flowchart for the optimized process of protein extraction from Ulva sp. 29

Figure 4.3. Variation of predicted responses for five parameters analysed: a) degree of hydrolysis, b) ACE-inhibitory activity and antioxidant capacity through c) ORAC, d) ABTS and e) DPPH assays, as a function of each term in the model – time and E/S ratio, obtained for Ulva sp. extract hydrolysed with alcalase from Bacillus licheniformis. 34

Figure 4.4. Chromatogram of samples after each extraction step: 1st and 2nd extractions with 5 mL H_2O in the Precellys homogenizer and conventional extraction with NaOH; and the extract obtained after hydrolysis at optimum conditions. 37

Figure 4.5. Chromatogram of sample after pre-treatment with EtOH for pigment removal: respective carotenoid and chlorophyll profile. 38

Figure A.1. Antioxidant capacity dependency on reaction time for the ABTS assay. a) Absorbance of Trolox standards (black dotted lines) and test sample (blue line) over time; b) Absorbance vs. Trolox standards concentration. 49

Figure A.2. Antioxidant capacity dependency on reaction time for the ABTS assay. a) Absorbance of Trolox standards (black dotted lines) and test sample (blue line) over time; b) Absorbance vs. Trolox standards concentration. 50

Figure B.1. Diagnostics graphs refering to DH, a) Normal plot of residuals; b) Residuals vs. Run; c) Residuals vs. Reaction Time; d) Residuals vs. E/S ratio; e) Residuals vs. Predicted and f) Cook's Distance. 52

Figure B.2. Diagnostics graphs refering to ln (IACE), a) Normal plot of residuals; b) Residuals vs. Run; c) Residuals vs. Reaction Time; d) Residuals vs. E/S ratio; e) Residuals vs. Predicted and f) Cook's Distance. 53

Figure B.3. Diagnostics graphs refering to ORAC, a) Normal plot of residuals; b) Residuals vs. Run; c) Residuals vs. Reaction Time; d) Residuals vs. E/S ratio; e) Residuals vs. Predicted and f) Cook's Distance. 54

Figure B.4. Diagnostics graphs refering to ABTS, a) Normal plot of residuals; b) Residuals vs. Run; c) Residuals vs. Reaction Time; d) Residuals vs. E/S ratio; e) Residuals vs. Predicted and f) Cook's Distance. 55

Figure B.5. Diagnostics graphs refering to DPPH, a) Normal plot of residuals; b) Residuals vs. Run; c) Residuals vs. Reaction Time; d) Residuals vs. E/S ratio; e) Residuals vs. Predicted and f) Cook's Distance. 56

List of Tables

Table 2.1. Composition (moisture, protein, lipids, carbohydrates and ash) of several macroalgae species of the Ulva genus. [35-39] 7

Table 4.1. Amino acid composition of Ulva sp. 23

Table 4.2. Extraction yield of each step, and total extraction yield, of single and sequential extractions performed in Precellys homogenizer with different solvents for Ulva sp. 24

Table 4.3. Extraction yield of each step, and total extraction yield, of conventional extractions performed with H_2O *over 90 minutes, following single and sequential Precellys extractions with EtOH, for Ulva sp.* 26

Table 4.4. Extraction yield of each step, and total extraction yield, of conventional extractions performed with NaOH and HCl over 90 minutes, following pre-treatment with EtOH, and/or single and sequential Precellys extractions with EtOH or H_2O*, and/or conventional extraction with* H_2O*, for Ulva sp.* 27

Table 4.5. Experimental design encompassing two processing parameters – time and E/S ratio, and results pertaining to 5 responses – degree of hydrolysis (DH), ACE-inhibitory activity and antioxidant capacity through 3 different assays: ORAC, ABTS and DPPH, obtained for Ulva sp. extract, hydrolysed with alcalase from Bacillus licheniformis. 31

Table 4.6. Best estimates of each term in the model – constant, time, T (linear and quadratic), E/S ratio, R (linear and quadratic) and interaction thereof (linear) and corresponding relevant statistics – R^2 *and p-value for the model and respective lack of fit, pertaining to five responses – degree of hydrolysis, antioxidant capacity (through the ORAC, ABTS and DPPH assays) and ACE-inhibitory activity, obtained for Ulva sp. extract, hydrolysed with alcalase from Bacillus licheniformis.* 32

Table 4.7. Predicted ACE-inhibitory activity and antioxidant capacity for Ulva sp. hydrolysates, and corresponding values for lower and upper bound of 95% confidence interval. 35

Table 4.8. ACE-inhibitory activity and antioxidant capacity for total and <3 kDa fractions of Ulva sp. hydrolysates at optimum conditions. 36

Notation and Glossary

Y_E	Extraction yield	% (m/m)
m_{P_E}	Mass of protein in the extract	mg
m_{Alga}	Mass of *Ulva sp.* used for extraction	mg
X	Quadratic polynomial model	
R	E/S ratio	% (v/v)
T	Reaction time	h
L_i	Amount of liberated amino acids in sample i	$mg_N/g_{protein}$
L_0	Amount of amino acids in the original substrate	$mg_N/g_{protein}$
L_{max}	Maximum amount of the specific amino acids in the substrate obtained after total hydrolysis	$mg_N/g_{protein}$
f_0	Initial fluorescence reading	Au
f_i	Fluorescence reading at a given time i	Au

Greek letters

β_0	Vertical intercept of the quadratic polynomial model
β_1	Linear coefficient of the quadratic polynomial model
β_2	Linear coefficient of the quadratic polynomial model
$\beta_{1,1}$	Quadratic coefficient of the quadratic polynomial model
$\beta_{2,2}$	Quadratic coefficient of the quadratic polynomial model
$\beta_{1,2}$	Interaction coefficient of the quadratic polynomial model
ε	Experimental error of the quadratic polynomial model

List of Acronyms

AA	Amino acid
AAPH	2,2'-azobis-2-methyl-propanimidamide dihydrochloride
ABTS	2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
ACE	Angiotensin converting enzyme
ANOVA	Analysis of variance
AUC	Area under the curve
BCA	Bicinchoninic acid
CCD	Central composite design
CIIMAR	Centro Interdisciplinar de Investigação Marinha e Ambiental
DH	Degree of hydrolysis
DPPH	2,2-diphenyl-1-picrylhydrazyl
DOE	Design of experiments
EC_{50}	Half maximal effective concentration
EFSA	European Food Safety Authority
E/S	Enzyme/substrate
FL	Fluorescein
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
LOD	Limit of detection
LOQ	Limit of quantification
MAE	Microwave-assisted extraction
MWCO	Molecular weight cut-off
ORAC	Oxygen radical absorbance capacity
PAD	Photodiode array detector
PEF	Pulsed electric field
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNBS	2,4,6-trinitrobenzenesulphonic acid
Trolox	(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
UAE	Ultrassound assisted extraction

1 Introduction

1.1 Framing and presentation of the work

Since the 20th century, a protein shortage has been predicted by several studies – indicating that food resources are not increasing at a rate compatible with that of the world population. Currently, the United Nations anticipates that the population will reach 9.7 billion by 2050, yet the number of people affected by hunger has steadily risen in the past few years. Although this is not a recent problem, it became more serious due to a number of recent events: COVID-19 pandemic, global war conflicts, and the persistent effect of climate change. The need for novel food sources, more specifically novel protein sources, has thus provided a strong impetus for current research, in attempts to ensure that protein consumption remains affordable and sustainable for all in the coming future.

Marine algae arise as a promising alternative to traditional protein sources – for exhibiting high protein contents with nutritional suitability. Aside from high growth and production rates, they do not compete with traditional crops for land space, and can even be cultured in wastewater treatment ponds. More specifically, macroalgae have the advantage of having been used in human diets for many years, being resistant to environmental stress, and exhibiting fast growth. Their protein contents can reach 47% for red algae and 26% for green algae – with the latter more appropriate for extraction, owing to a lower content of carbohydrates. Protein extraction from macroalgae can be performed by several methods, with interplay of solvents of chemical nature and physical factors being the most commonly used. Once extracted, seaweed proteins are valuable sources of biologically active compounds, with potential applications in nutraceuticals and pharmaceuticals.

Among those biologically active compounds emerge bioactive peptides – i.e. small protein fragments that could account for several biological activities. They are released from their parent protein through physical processes or enzymatic hydrolysis, and can exert angiotensin converting enzyme (ACE) inhibitory activity, antioxidant, anti-inflammatory, anticancer and antimicrobial activities, among others. Compared to synthetic drugs, such compounds have the advantages of low toxicity and poor accumulation in human tissues, thus entailing natural alternatives to traditional pharmaceuticals. Macroalgae have the potential to supply these compounds, either in the form of purified peptides, protein extracts, or as whole food.

This dissertation aimed at obtaining bioactive peptide extracts from macroalgae. The work focused, firstly, on the optimization of a protein extraction method for the green seaweed *Ulva sp.*, which evaluated how different solvents and extraction techniques influence total extraction yield, and how this parameter can be maximized while still attaining pigment

removal. The main scope was indeed related to the optimization of enzymatic hydrolysis, which was achieved through design of experiments (DOE) following a response surface methodology (RSM). The ultimate goal was to obtain bioactive peptides from said macroalga, and maximize their biological activities – namely ACE-inhibitory activity and antioxidant capacity, by manipulating parameters relevant for hydrolysis. Chromatographic techniques were used to characterize the macroalga amino acid content, as well as the protein and peptide profiles of the extracts.

1.2 Presentation of the company

The Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE) was founded in 1997, and is a research unit of the Faculty of Engineering of the University of Porto, and part of the Associate Laboratory in Chemical Engineering (ALICE). LEPABE looks at developing innovative processes and products to respond effectively to societal challenges, using chemical and biological engineering to improve quality of life. It is based on 3 pillars of development: Sustainability and Energy, Processes and Products and Environment and Health, always with a focus on research of excellence. In particular, the laboratory under Prof. Malcata's coordination focuses on metabolite production and recovery from micro and macroalgae, for either bulk or specialty uses – besides work on novel functional foods of probiotic nature.

1.3 Contribution of the author to the work

All the work detailed in this thesis was performed by its author. The handling of specific laboratory equipment was always performed with the support/under the supervision of the responsible Professor and/or technician.

1.4 Organization of the dissertation

This thesis is divided into 7 main chapters, for which a brief description follows.

Chapter 1 presents a short overview of the problem under study, the main methods used, and the main goals of the work developed. It provides an overall perspective on the subject of the dissertation, and sets the foundations for an understanding of the following chapters.

Chapter 2 presents a literature review related to the theme under study, providing context for the current work by quoting previously published results and setting a theoretical basis for the following chapters. In the first section of this chapter, the current problem of protein shortage is discussed, and the need for novel protein sources is explored. Next, the potential of algae as an alternative to traditional protein sources is assessed, and comparisons are drawn between microalgae and macroalgae. The third section approaches the topic of bioactive peptides, by

mentioning their potential as pharmaceutical and nutraceutical ingredients. The main production methods are briefly tackled, and insight into specific biological activities is given – namely ACE-inhibitory and antioxidant ones.

Chapter 3 presents all materials used in this work, as well as a detailed description of all procedures employed. A description of all protein extraction methods tested and how the final extract preparation was performed is provided. The methods used for enzymatic hydrolysis, protein content determination, and biological activity determination are also elucidated in a detailed manner. Finally, all chromatographic techniques employed are described to some length.

Chapter 4 contains all results obtained throughout the work developed, as well as detailed discussion and comparison to data available in the literature. The results obtained throughout the optimization of protein extraction are laid out, and the final extraction method is described. Then, the experimental results obtained from application of the experimental model are presented – namely degree of hydrolysis, ACE-inhibitory activity, and antioxidant capacity (evaluated through ORAC, ABTS and DPPH assays) of the samples. A model was proposed, and the parameters and responses to be included therein were decided upon the drawn conclusions. The validation of the model was then performed by repeating all analyses under optimum conditions. The extract and hydrolysate protein profiles were analyzed through chromatographic techniques; and the macroalga amino acid profile was determined. Finally, the carotenoids and chlorophylls extracted from the samples were quantitated.

Chapter 5 presents the main conclusions of the study.

Chapter 6 presents an assessment of the work developed, followed by suggestions for future work.

Chapter 7 lists all bibliographic references used in the preparation of this thesis.

2 Context and State of the art

2.1 Protein shortage and the need for novel protein sources

In 2015, the United Nations Member States adopted the 2030 Agenda for Sustainable Development, which includes 17 Sustainable Development Goals deemed as "*the blueprint to achieve a better and more sustainable future for all*". [1,2] Among them is hunger eradication [1], at a time when nearly 10% of people globally are affected by hunger, and 29.3% by moderate or severe food insecurity. [3] This goal seems unattainable if the global and political frameworks continue to randomly shift – knowing that climate variability and related shocks play a significant role. More recently, COVID-19 pandemic and growing conflicts in Asia and Africa, as well as the Ukraine war crisis seem to further undermine food security worldwide, and erase the progress attained so far. [4] Soaring food prices are also affecting the population worldwide, with the Russian invasion of Ukraine dramatically worsening already inflated food prices. [5] Its effects are particularly notorious in developing and emerging market countries – where the capacity to purchase basic food staples has been rapidly declining as a result of the uncertainty surrounding the global food supply; knowing that they were already being impacted by the ongoing climate changes. [5,6] Together with the United Nations projection of the world population reaching 9.7 billion by 2050 [7], one easily concludes on the importance of finding alternative and innovative solutions for the world current and most pressing nutritional problems.

Reports from as early as the 1960's predicted a future need in food supplies – not only food in general but more specifically protein [8,9], owing to the world population increasing at a rate above that of food resources. In 1971, A. Woodham suggested novel protein sources, such as synthetic protein and protein isolates from plants, to help overcome this issue; and highlighted the importance of educating the population on their value and ultimate necessity. [10] By 2050, it will be impossible for the global population to consume the amount and type of protein typical of the current diets in North America and Europe, in an affordable and sustainable manner. [11] Consumption of conventional protein, i.e. animal protein or animal-based food, has also been questioned over the past few years, due to its significant environmental impacts; livestock agriculture is indeed responsible for a large proportion of greenhouse gas emissions in the food system, as well as heavy water and land usage and degradation, thus making plant-based diets more popular over the years. [12] Therefore, the search for novel and emerging sources of protein is of extreme importance, with plant-derived protein (such as pulse grains), insects and algae being top contenders. [13]

The unique natural resources of each region may also play a significant role – in which alternative protein sources exist that are more favorable and suitable for exploration. Portugal holds an intrinsic and historical connection to the sea. With a coastline of about 2500 km [14], the ocean has always been an essential part of the country economic expansion – with an obvious great potential for renewable ocean energy, fishing and aquaculture, as well as logistics and transportation. [15] As a fundamental part of Portugal's marine biodiversity, algae have traditionally been used as fertilizer and raw material for the agar industry, which was once very prolific in the country. More recently, research has been conducted on macroalga cultivation, with several coastal species – like *Ulva sp.*, *Plocamium sp.* and *Sphaerococcus sp.* being cultivated in open sea [16]; further to the case study of ALGAplus, a pioneering company based in Ílhavo that cultivates native marine algae for application in food products, cosmetics and animal nutrition. [17]

2.2 Algae as an alternative protein source

Algae present a high nutritional value in terms of protein content, amino acid quality and nutritional acceptability. [18,19] They are potentially a viable and sustainable source of protein, with a protein content ranging from 9 to 47% (of dry matter) in macroalgae and up to 70% in microalgae. They also exhibit high growth and production rates, and do not require arable land, thus eliminating competition with traditional crops. They further entail high photosynthetic efficiency – meaning high biomass productivity and low water consumption (which is also a benefit of insect production for protein), relatively easy extraction processes and carbon-neutral emissions. [19] Algae can even be grown in wastewater treatment ponds [20,21], serving as biomass in effluent treatment and later as a food or biofuel source.

2.2.1 Microalgae

Microalgae encompass eukaryotic photosynthetic microorganisms and prokaryotic cyanobacteria, found in seawater and freshwater, which have the ability to perform photosynthesis and present alternative forms of metabolic growth. [22] With around 30 000 species identified and studied to date [23], such organisms have been used as food for centuries, with *Spirulina maxima* being used by ancient civilizations like the Aztecs in Mexico and Chad natives, and *Nostoc flagelliforme* serving as food for more than 2000 years in China. [24] Microalgae have been reported to contain similar amounts of protein to milk, soybean, egg and meat [25] - viewed as traditional protein sources, besides being rich in carbohydrates, lipids, vitamins and minerals essential for human nutrition. [26] However, commercialization of microalgal biomass has faced several challenges, namely in Europe, where regulations are strict and most products are *Spirulina*-based; however, other issues, such as low production capacity

and high cost, as well as the biomass's generally unappealing color and smell remain to be adequately addressed. [27]

2.2.2 Macroalgae

Macroalgae, or seaweeds, are photosynthetic multicellular organisms that inhabit marine littoral zones, in depths with sufficient light to drive photosynthesis. As their name indicates, they are macroscopic (as opposed to microalgae), and their morphology resembles that of land plants. They are considered extremely promising – not only as a potential protein source, but also as biorremediators and feedstock for renewable energy sources like biodiesel or biogas. This latter feature arises chiefly from their polysaccharide content. [28] They are divided into three main groups, based on their color: brown algae, or Phaeophyceae class; green algae, or Chlorophyceae class; and red algae, or Rhodophyceae class. [29,30] Regarding composition, red algae contain the highest amount of protein, with contents reaching 47% of dry weight. In comparison, green seaweed protein content ranges from 9 to 26%, and from 3 to 15% in brown algae. [31] On the other hand, red seaweed contains higher amounts of carbohydrates, which may turn the protein extraction process difficult; while green and brown algae exhibit a lower carbohydrate content. [32] Note that macroalgae composition may vary according to development stage or life cycle, as well as due to environmental conditions like light, salinity, temperature and pollution. [33]

Green algae entail a good compromise between protein and carbohydrate content; the genus *Ulva*, also known as sea lettuce, is one of the most prominent. It is found all around the globe, and it is relatively resistant to environmental changes. [34] Table 2.1 tabulates the composition of several *Ulva* species.

Table 2.1. Composition (moisture, protein, lipids, carbohydrates and ash) of several macroalgae species of the *Ulva* genus. [35-39]

	Protein (%)	Lipid (%)	Carbohydrates (%)	Ash (%)	Moisture (%)
Ulva meridionalis	24.7	6.5	44.9	8.6	-
Ulva lactuca	15.23	0.76	38.21	27.37	15.31
Ulva ohnoi	13.1	12.6	34.5	24.6	-
Ulva compressa	15.66	1.67	14.45	18.03	-
Ulva rigida	15.78	1.02	16.74	20.60	-
Ulva intestinalis	16.4	8.7	62.2	28.4	5.4
Ulva pertusa	16.1	7.4	52.2	28.6	6.0

As opposed to microalgae, seaweeds have been regularly used in the human diet for many years – with coastal populations traditionally using them not only for food, but also as disease remedy, animal feed and fertilizer, following direct harvest from the ocean. Consumption of seaweed

in countries like China, Japan, Korea and Indonesia is a regular practice, and thus part of their basic diet. [24] Consequently, the number of EFSA-approved macroalgae is higher than that of microalgae, aligned with a growing demand and the recent expansion of the seaweed market in the West. [40] Around 7.5 million tonnes of seaweed dry matter are produced annually around the world, compared to a mere 5000 tonnes of microalgal dry matter. [41] Macroalgae also grow faster than microalgae, and are less vulnerable to environmental stress. [42]

For these reasons, macroalgae appear as the most appealing and viable option to be explored as a substitute to conventional protein sources. In addition to being valuable sources of nutritious food components, they are also reservoirs of novel biologically active compounds – thus representing the ideal raw material to generate new marine protein-derived bioactive peptides. [43]

2.2.3 Strategies for protein extraction

The main challenge when extracting protein from algae is the complex and resistant nature of their cell wall; algal cells possess mainly intracellular proteins, so disruption of the cell wall is essential to obtain significant extraction yields. This often requires specific and aggressive extraction processes. [44]

Conventional extraction techniques generally resort to several solvents – such as distilled water, buffers and acid or alkaline solutions [45-47], which are often combined with physical methods, like osmotic shock, freezing/thawing, shearing and grinding, to bring about extraction. Such additional stress factors have been shown to significantly increase extraction yield in red algae. [48] Since different algal proteins are soluble in different solvents, extractions are often performed in sequential steps with different solvents, thus allowing extraction of different types of protein. [44]

Additionally, enzymes such as polysaccharidases have also been used as a means for cell disruption and increased protein availability, since the abundant polysaccharides often disturb protein extraction. [29,49]

More recently, a few novel protein extraction methods have been developed, aimed at overcoming the constraints of traditional processes – which are not only laborious and time-consuming, but quite often require more aggressive means of cell disruption. [29]

Ultrasound-assisted extraction (UAE) is based on acoustic cavitation, i.e. the formation of bubbles inside the cells that implode with increased pressure and temperature. UAE offers fast processing time and low solvent consumption, while reducing downstream processing. [29,50] Application of this method as pre-treatment led to dramatic results in brown alga *Ascophyllum nodosum*, with extraction yield increasing by 540%. [51] Microwave-assisted extraction (MAE) follows a similar mode of action, but it involves heating the material, thus leading to bubble

formation followed by rupture; its use in algae is limited, due to impaired function with dried samples. [50]

Other techniques have also been studied, such as pulsed electric field (PEF) extraction, and extraction with subcritical and supercritical fluids. Despite promising results and inherent sustainability, these technologies require high investment costs. [29]

2.3 Bioactive peptides

Bioactive peptides are small protein fragments that exhibit a positive effect at physiological level, possibly benefiting health. If part of a parent polypeptide chain, they prove essentially inactive. However, once released by enzymatic hydrolysis - whether during food processing or in the digestive tract after human consumption, their biological activity is activated. [52,53] Some of these compounds have been shown to possess anti-hypertensive, antioxidant, antimicrobial, immunomodulatory and antithrombotic activities, and act as opioids and appetite suppressors. [54] Bioactive peptides are generally made up of 2 to 20 aminoacids, and have a molecular weight below 6k Da – with their specific amino acid sequence heavily influencing biological activity. Even though some mechanisms have been elucidated, how the peptide structure influences its bioactivity remains to be fully elucidated. [52]

Bioactive peptides also offer several advantages compared to synthetic drugs, such as high activity and specificity, low toxicity and lower accumulation in human tissues. [55]

2.3.1 Production of bioactive peptides

Several processes can release bioactive peptides from their parent proteins, namely such physical processes as heat application; chemical processes – when acid or alkaline agents are applied to food products, and biological processes are nevertheless the most commonly applied. [56]

The classical approach to production of bioactive peptides is enzymatic hydrolysis – in which a food or other product, rich in protein, is subjected to hydrolysis, effected by a specific enzyme or a set of enzymes, at given temperature and pH for a determined period of time. Another critical parameter is enzyme concentration, or E/S ratio. There are several commercial enzyme preparations that contain specific amino acid residues and co-factors involved in protein breakdown. [57] Hydrolysis can be assessed via quantification of degree of hydrolysis (DH), which measures the percentage of peptide bonds broken during the hydrolytic process. [56] This approach is generally predictable, and easy to scale-up to an industrial level. [55] The peptides obtained differ depending on the enzyme used, so proteases that yield low molecular weight peptides – which have been shown to possess better activities [58,59], are normally preferred. In an industrial setting, where the hydrolytic process can last for a long time, pre-treatment may

also be applied to food products in order to prevent microbial contamination; for instance, Tavares *et al.* [60] suggested a pre-treatment of whey protein concentrate through a microfiltration membrane to reduce microbial contamination prior to hydrolysis, a relatively low-cost and easy to scale up solution.

A different approach to hydrolysis is use of bacterium, yeast or fungus cultures on the protein substrates; said microbes will hydrolyse the proteins via their enzymes, as part of metabolic growth. The extent of hydrolysis depends essentially on the strain used and fermentation time. [55] However, this is a much more time-consuming approach, and it does not offer accurate controllability of enzymatic hydrolysis.

Following the hydrolysis step, the peptide product is processed based on its physicochemical or structural properties – e.g. size and hydrophobicity, to enhance its bioactivity. Several purification methods can be applied, such as membrane ultrafiltration or chromatography techniques, via size exclusion or ion-exchange. [61]

A useful tool for optimization of enzymatic hydrolysis is RSM, aligned with proper DOE. This combined mathematical and statistical approach explores the relationships between several explanatory variables and one or more response variables, through performance of a sequence of experiments designed to obtain an optimal response. [62] RSM allows prediction of a response as exactly and precisely as possible, in points within the experimental domain where no experiments were performed; while maintaining a high efficiency regarding the process practical limitations [62]; in the case of enzymatic hydrolysis, reaction time and E/S ratio would consubstantiate such limitations, with an effect also upon final cost. This methodology has been successfully used for the optimization of enzymatic reactions elsewhere. [63-66]

2.3.2 Biological activities

ACE-inhibitory activity

ACE is a dipeptidyl carboxypeptidase responsible for vasoconstriction, having a very important role in blood pressure regulation; the enzyme catalyzes the reaction that converts angiotensin I to angiotensin II, thus resulting in vasoconstriction and increase in blood pressure. The inhibition of this enzyme results in lower hypertension, potentially preventing cardiovascular health issues. [67] Several antihypertensive drugs, like Captopril, are available in the market. However, they pose severe side effects that make them undesirable [68-70] – so a window of opportunity arises toward bioactive peptides that exhibit this kind of activity.

Multiple peptides with ACE inhibitory activity have been isolated from macroalgae – Fitzgerald *et al.* [71] and Furuta *et al.* [72] purified peptides from red alga *P. palmata*, through hydrolysis with papain and thermolysin, respectively – which exhibited potent ACE inhibition activity. Small peptides, with 2 and 3-amino acid sequences, isolated from *Ulva rigida* [73] and

peptides purified from *Ulva intestinalis* [74] also showed great potential; and many other studies involving macroalgae reported promising results, both *in vitro* and *in vivo* [75-79].

Several authors have suggested that ACE-inhibitory activity is influenced by C-terminal residues in peptides, with the ACE preferring di- and tripeptide inhibitors containing such hydrophobic residues as tryptophan, tyrosine, phenylalanine and proline. [80] Amino acids with a positive charge have also been shown to contribute to ACE inhibition. [81] However, few studies with bioactive peptides specifically originating from algae have attempted to relate their structure to some proposed mechanism of action.

Antioxidant capacity

Free radicals produced in oxidation reactions have been shown to cause damage to cells and tissues, owing to their participation in lipid peroxidation and protein and DNA modifications – thus promoting various health issues, like osteoarthritis, cancer, diabetes, and cardiovascular diseases. Compounds with antioxidant capacity have been shown to play an essential role in combating the body's oxidative stress, and can help prevent and control health problems, along with neurodegenerative diseases like Alzheimer's and Parkinson's. [82-84]

Usually, peptides exhibiting antioxidant capacity contain between 5 and 16 amino acid residues; their activity can be presented through various mechanisms of action. These compounds may act through free radical chain breaking, oxygen scavenging, metal chelation or inhibition of oxidative enzymes; and when present in a complex food product matrix, more than one of these mechanisms may be at play. [85]

Cian *et al.* [86] reported the antioxidant capacity of peptides obtained from red macroalga *Pyropia columbina*, which maintained and even increased its activity after *in vitro* gastrointestinal digestion. It has been claimed that peptides containing such amino acids as tyrosine, tryptophan, methionine, lysine, cysteine and histidine exhibit antioxidant capacity, as well as amino acids with aromatic residues, since they can donate protons to electron-deficient radicals. [87] Rajapakse *et al.* [88] also suggested that peptides containing histidine show antioxidant capacity due to the ability of its imidazole group to donate hydrogen, ion-chelate metal and/or trap lipid peroxyl radicals. However, the exact underlying mechanism relating antioxidant capacity to small peptides is not fully understood at present.

3 Materials and Methods

3.1 Algae sample preparation and characterization

Ulva sp. (a green seaweed) was chosen for this study due to its innate high protein content. It was harvested off the Portuguese coast in November 2021, and dried by CIIMAR. After drying, samples were portioned (roughly 200 g) and packed in plastic bags. Prior to extraction, samples were ground using a Knife Mill Grindomix GM 200 (Retsch, Haan, Germany) at 10,000 rpm for 20 seconds. A fine algal powder was obtained, ideal for the following cell disruption process and solvent extraction.

The alga contains 11.9% water, 27.4% ash, 0.2% total lipids, 24.0% total protein and 36.6% total carbohydrates – of which 32.4% are total dietary fiber - as determined by previous work by the research group.

3.1.1 Amino acid profile determination

Profiles were obtained by HPLC, performed with a Waters (Milford, Massachusetts, USA) Alliance HPLC Model 2695, equipped with a photodiode array detector (PAD) Model 2998 (Waters), a fluorescence detector 2475 (Waters) and a column heater (Waters).

The AccQ-Tag method was used for derivatization and chromatographic determination. The AccQ-Tag Reagent Kit was purchased from Waters. The reagent kit consists of Waters AccQ-Fluor Borate Buffer, Waters AccQ-Fluor Reagent Powder (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate – AQC), Waters AccQ-Fluor Reagent Diluent and Waters AccQ-Tag Eluent A (1:10). All amino acids were purchased from Sigma-Aldrich (Steinheim, Germany).

About 20 mg of *Ulva sp.* algal sample was weighed in a 4 mL glass crimp vial; 3 mL of hydrochloric acid solution HCl 6 M containing 0.5% (w/v) phenol was added, and vials were sealed under vacuum by passing nitrogen gas into the vials. Acid hydrolysis was performed at 110 ºC for 24 h, according to AOAC 982.30 (AOAC, 2000) as reference hydrolysis method; 0.2 mL from the resulting hydrolysate was taken and neutralized with NaOH 6 M, and the final volume was adjusted with borate buffer 0.1 M to 1 mL. Undissolved particles were separated by ultrafiltration at 13,000 rpm for 1 min. Derivatization of amino acids was performed with AccQ-Tag derivatization kit. Briefly, 10 µL of the sample was mixed with 10 µL of internal standard 50 µM (gamma-aminobutyric acid), and 60 µL borate buffer and 20 µL derivatization agent were then added to the mixture. The resulting derivatization of AAs was subjected to HPLC analysis, with an AccQ-Tag Amino Acids C18 Column (4 µm, 150 × 3.9 mm) (Waters, Ireland), a fluorescence detector set at λ_{Ex} = 250 nm and λ_{Em} = 395 nm (PMT = 100), a photodiode array set to spectrum scanning within the range of λ = 210–600 nm, and a column heater set to 37.0 ºC.

After injection (5 µL sample), a chromatographic separation was performed using a mixture of three eluents: a mobile phase A consisted of aqueous buffer, a mobile phase B consisted of ultrapure water and a mobile phase C consisted of acetonitrile. Elution was performed at 1 mL/min, and chromatographic separation was accomplished as follows: 0 min (A, 100 %), 0.5 min (A, 99 %; B, 1%), 18 min (A, 97.5 %; B, 2.5%), 30.5 min (A, 86.4 %; B, 5 %; C, 8.6 %), 31.5 min (A, 82.4 %; B, 9 %; C, 8.6 %), 42 min (A, 74.4 %; B, 17 %; C, 8.6 %), 44.5 min (B, 60 %; C, 40 %) and 47.5 min (A, 100 %); a total analysis time of 57.5 min resulted.

The following amino acids were found and duly quantified in the samples: L-Alanine, L-Arginine, L-Asparagine, L-Aspartic acid, L-Cysteine, L-Glutamine, L-Glutamic acid, L-Glycine, L-Histidine, L-Isoleucine, L-Leucine, Lysine, Methionine, Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tyrosine, Valine. Amino acid calibration curves were constructed using a least squares linear regression model, via plotting the peak area ratios of the various amino acids and corresponding internal standard versus concentration of each analyte under study. Standard calibration curves were prepared using nine calibration points for amino acids (with a range covering from 5 to 200 µM of each analyte).

Each test was performed at least in three independent experiments. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the calibration curve parameters, where the LOD was equal to the calculated intercept of the linear regression (a) plus three times the Sy/x, and for LOQ ten times this value. The amino acid composition was reported as mg_{AA}/g_{Sample}.

3.2 Protein extraction

Protein extracts from *Ulva sp.* were obtained by a series of single and sequential extractions, performed with different solvents: deionized water (H_2O), ethanol 96% (EtOH), hydrochloric acid (HCl) 0.4 M, and sodium hydroxide (NaOH) 0.4 M. Different extraction methods were applied in tandem, to determine the optimal combination of extraction steps to maximize protein yield. All obtained extracts were stored at -75 ºC, until lyophilization or filtration. At least three replicates of each extract were prepared independently, and chemically analyzed also in triplicate for each method, unless stated otherwise.

The extraction yield (% mass weight), Y_E, was determined for each extraction as

$$Y_E = \frac{m_{P_E}}{m_{Alga}} \times 100\% \qquad 3.1$$

where m_{P_E} is the mass of protein in the obtained extract, determined through the BCA assay, and m_{Alga} is the mass of *Ulva sp.* used in the extraction.

3.2.1 EtOH pre-treatment

A mass of 250 mg of freeze-dried *Ulva sp.* was suspended in 10 mL of EtOH, and gently stirred to ensure that all biomass contacted with the solvent. After 10 min, the sample was centrifuged in a 1580R centrifuge (Gyrozen, Gimpo, South Korea), at 8000 rpm and 5 ºC for 10 min. The supernatant was concentrated under a N_2 gas stream and analyzed for pigment determination.

3.2.2 Precellys extraction

Extractions using a Precellys cell homogenizer (Bertin Technologies, France) were performed. The extraction mixture was composed of 250 mg of freeze-dried *Ulva sp.* or the pellet from the EtOH pre-treatment, 8 ceramic beads and 5 or 10 mL of solvent, in sequential (5 mL + 5 mL) or single (10 mL) steps, with 6 min and 45 s cycles at 8000 rpm (30 s homogenization with 45 s of stopping intervals in between). After each cell homogenization cycle, the sample was centrifuged at 8000 rpm and 5 ºC for 10 min. The supernatants were collected for further analysis, and the pellets used for the following extraction steps. Solvents tested for this extraction were H_2O, EtOH and HCl.

3.2.3 Conventional extraction

The pellet obtained from the Precellys extraction was suspended in 10 mL of solvent, and the suspension was gently stirred for 90 min at room temperature. Samples of 200 µL were collected every 10 min for further analysis. The suspension was then centrifuged at 8000 rpm and 5 ºC for 10 min, and the supernatant collected for analysis. Solvents tested for this extraction were H_2O, HCl and NaOH.

3.3 Preparation of selected extraction samples

3.3.1 Lyophilization

Supernatants obtained from the Precellys extraction with H_2O were lyophilized in a 6K Benchtop freeze dryer (VirTis, New York, USA) under vacuum, until samples became fully dehydrated.

3.3.2 Filtration

Supernatants obtained from conventional extraction with NaOH were ultrafiltered using a Macrosep Advance Centrifugal device (Pall Life Sciences Ireland, Cork, Ireland); a molecular weight cut-off (MWCO) of 1 kDa was employed. A diafiltration procedure was applied for desalting, buffer exchange and sample concentration. First, 20 mL of the supernatant was placed in the device, and centrifuged in a 5810 R centrifuge (Eppendorf, Hamburg, Germany) at 4000 rpm and 5 ºC for 60 min for volume reduction. The volume was then reconstituted with ultrapure water, and centrifugation was performed under the same conditions. This washing procedure was repeated 5 to 7 times for each sample, to ensure final adequate pH and salt

content. For each time, the filtrate was discarded. The sample was concentrated until the desired volume at the final concentration step.

3.4 Enzymatic hydrolysis

3.4.1 Experimental design, modeling and optimization by design of experiments (DOE) techniques

Hydrolysis of *Ulva sp.* protein extracts was optimized using RSM, more specifically a central composite design (CCD), developed using Design Expert Version 13 (StatEase, Minneapolis, MN, EUA). The parameters to be optimized, namely E/S ratio and reaction time, were studied for their effects upon degree of hydrolysis (DH), antioxidant capacity and ACE-inhibitory activity of the corresponding hydrolysates. The CCD consisted of a complete 2^2 factorial design, with 13 independent experiments: 4 were accounted for by two levels (-1 and $+1$); another 4 were axial points (at a normalized distance of $\pm\sqrt{2}$); and the remaining 5 corresponded to center points (used as variance estimators, at nil coordinate). All experiments were run in random order. This design allowed five distinct levels to be tested: 0.09, 0.50, 1.50, 2.50 and 2.91, for the E/S ratio; and 0, 1, 3.5, 6 and 7 h, for the reaction time.

The quadratic polynomial model proposed for each response variable, X, took the form:

$$X = \beta_0 + \beta_1 R + \beta_2 T + \beta_{1,1} R^2 + \beta_{2,2} T^2 + \beta_{1,2} RT + \varepsilon \quad (3.2)$$

where R denotes the E/S ratio and T the reaction time; β_0 is the vertical intercept; β_1 and β_2 are linear coefficients, $\beta_{1,1}$ and $\beta_{2,2}$ are quadratic coefficients, and $\beta_{1,2}$ is the interaction coefficient; and ε represents the experimental error.

3.4.2 Performance of enzymatic hydrolysis

Ulva sp. protein extracts were submitted to hydrolysis brought about by alcalase from *Bacillus licheniformis* (Merck KGaA, Darmstadt, Germany), using the best pH and temperature conditions as recommended by the manufacturer, namely a pH ranging from 6 to 8.5 and an optimal temperature of 55 ºC.

Substrate solutions were prepared by mixing the lyophilized powder from the Precellys extraction with H_2O, with the filtrate from the conventional extraction with NaOH. The pH of the resulting solution was adjusted to 7.5 with 5 M HCl. The E/S ratio for each experiment was expressed on a volume basis, knowing that 5 mL of substrate was used for each experiment. The substrate mixture was incubated at 55 ºC for 7 h, and samples were taken at 0, 1, 3.5, 6 and 7 h; the reaction was stopped by heating at 99 ºC for 10 min. The hydrolysates were centrifuged at 8000 rpm and 5 ºC for 15 min, and the supernatants were frozen at -20 ºC until used.

The same procedure was followed when the manipulated parameters were at their optimum levels.

3.4.3 Degree of hydrolysis (DH) determination

The DH was quantified by measuring the increase in free amino groups, using a picrylsulfonic acid, TNBS (Sigma Aldrich, Missouri, USA) solution, according to McKellar's method. [89] Each sample (0.050 mL) was mixed with 0.5 mL of 1M potassium borate buffer at pH 9.2 and 0.2 mL of 0.015 %(w/v) of TNBS, and incubated in the dark at 25°C for 30 min; then, 0.2 mL of 2 M Na_2HPO_4 (Sigma Aldrich), containing 18 mM Na_2SO_3 was added to quench the reaction, and absorbance was measured spectrophotometrically at 420 nm in a UV-1800 UV Spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was prepared with L-Leucine (Merck KGaA) covering the range 0 – 2.065 mM, and absorbance was converted to $\mu mol_{free\ amino\ groups}$/mL using said curve. The total number of amino groups in a sample was determined by complete hydrolysis using 6 M HCl at 98 °C for 24 h. The percent DH values were calculated using the following formula [90]:

$$DH\ (\%) = \frac{L_i - L_0}{L_{max} - L_0} \times 100\% \qquad 3.3$$

where L_i is the amount of liberated amino acids in sample i, L_0 is the amount of amino acids in the original substrate (blank) and L_{max} is the maximum amount of the specific amino acids in the substrate obtained after hydrolysis.

3.5 Determination of biological activities

3.5.1 Antioxidant capacity determination

The antioxidant capacity of samples was evaluated using three distinct assays: oxygen radical absorbance capacity (ORAC) assay, which measures the scavenging activity of a compound against peroxyl radicals [91], improved with the use of fluorescein (ORAC-FL); ABTS assay, which uses 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{\bullet+}$); and DPPH assay, which assesses scavenging activity of samples using 2,2-diphenyl-1-picrylhydrazyl radicals ($DPPH^{\bullet}$). [92]

ORAC-FL assay

Working solutions

A stock solution of 1.17 mM fluorescein (FL) sodium salt (Sigma Aldrich) was prepared and preserved at 4 ºC for a month. A 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) (Cayman Chemical Company, Michigan, USA) 46.6 mM solution was prepared daily with 75 mM

phosphate buffer at pH 7.4, and FL was diluted to 0.117 µM from the stock solution with phosphate buffer.

Standard solutions

A stock solution of (±)-6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) (Sigma Aldrich) 1 mM in 1 mL of methanol was prepared and diluted in phosphate buffer 75 mM to a final volume of 50 mL. A T0 solution (0.1 mM) was prepared from the previous solution in the same buffer. The standard solutions were prepared from the T0 solution, with concentrations between 0 and 100 µM in phosphate buffer 75 mM at pH 7.4.

Assay execution

In black ninety six-well microplates (BRAND, Wertheim, Germany), 20 µL of standard solution or hydrolysate sample (diluted between 1:100 and 1:400) and 120 µL of FL solution was loaded into each well. A control was prepared with 20 µL of phosphate buffer instead of sample. The mixture was incubated at 37 ºC for 10 min; 60 µL of AAPH solution was added and the reaction was carried out at 37 ºC for 80 min, with fluorescence being recorded every minute. The microplate was automatically shaken before each reading cycle. The antioxidant capacity of samples was measured in triplicate in a FLUOstar® Omega microplate reader (BMG Labtech, Germany), with 485 nm and 520 nm excitation and emission filters, respectively.

Results

The antioxidant capacity of samples was calculated as proposed by Hernández-Ledesma *et al.*[91] Fluorescence measurements were normalized by the curve of the blank (absence of antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as indicated in Equation 3.3, where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at a given time i.

$$AUC = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0} \qquad 3.4$$

The net AUC of a sample was calculated by subtracting the blank AUC to sample i AUC. The regression equation between net AUC and antioxidant concentration was then calculated. The slope of the equation was used to calculate the ORAC-FL value by comparing it to the Trolox curve obtained for each assay. Final ORAC-FL values were expressed as $\mu mol_{Trolox\ equivalent}/mg_{hydrolysed\ protein}$.

Additionally, the EC_{50} (µg/mL) value of samples for the ORAC-FL assay was determined using software GraphPad Prism 8.0.2 (GraphPad Software, San Diego, California, USA) by resorting to a nonlinear regression of protein concentration *vs.* normalized response (%) plot, using a dose-

response stimulation model. Normalized response was calculated for each sample as proposed by Suriyatem *et al.* [93]:

$$Normalized\ response\ (\%) = \frac{AUC_{Control} - AUC_{Sample\ i}}{AUC_{Control}} \times 100\% \qquad 3.5$$

where $AUC_{Control}$ and $AUC_{Sample\ i}$ are the absorbance of the control and sample i, respectively, after the blank was subtracted.

ABTS assay

Working solutions

A solution of $ABTS^{\bullet +}$ radical cation was produced via reaction of potassium persulfate 2.45 mM with a stock solution of ABTS 7 mM at equal volumes. This solution was left in the dark overnight at room temperature. The solution was then diluted to assure a working absorbance of 0.700 ± 0.020 at 734 nm.

Standard solutions

A stock solution of Trolox 1 mM in 1 mL of methanol (MetOH) was prepared and diluted in acetate buffer 50 mM pH 4.6 to a final volume of 50 mL. A T0 solution (0.1 mM) was prepared from the previous solution in the same buffer. The standard solutions were prepared from the T0 solution, with concentrations between 0 and 100 µM in acetate buffer 50 mM pH 4.6.

Assay execution

In a standard microplate, 30 µL of standard solutions or hydrolysate samples and 170 µL of ABTS solution in acetate buffer were added to each well. A control was prepared with 30 µL of acetate buffer, instead of standards or samples to evaluate the absorbance of ABTS in the absence of antioxidant species. The microplate was incubated for 3.5 hours at room temperature, as per the results obtained from the kinetic matching study (Appendix A). Absorbance was then measured at 734 nm in a FLUOstar® Omega microplate reader.

Results

The antioxidant capacity of samples was calculated by comparison to the Trolox calibration curve, with final ABTS values being expressed as $\mu mol_{Trolox\ equivalent}/\ mg_{hydrolysed\ protein}$ or $\mu mol_{Trolox\ equivalent}/\ g_{Alga\ DW}$.

Additionally, the EC_{50} (µg/mL) value of samples for the ABTS assay was determined as described above for the ORAC-FL assay, with normalized response (%) being calculated as:

$$Normalized\ response\ (\%) = \frac{Abs_{Control} - Abs_{Sample\ i}}{Abs_{Control}} \times 100\% \qquad 3.6$$

where $Abs_{Control}$ and $Abs_{Sample\ i}$ are the absorbance of the control and sample i, respectively.

DPPH assay

Working solutions

A solution of 0.15 mM $DPPH^{\bullet}$ radicals in methanol was produced and kept in the dark at room temperature until use. The solution was then diluted to assure a working absorbance between 0.800 ± 0.020 at 517 nm.

Standard solutions

A stock solution of Trolox 1 mM in 1 mL of MetOH was prepared and diluted in the same solvent to a final volume of 50 mL. A T0 solution (0.1 mM) was prepared from the previous solution in the same buffer. The standard solutions were prepared from the T0 solution with concentrations between 0 and 100 µM in ultrapure water.

Assay execution

DPPH radical scavenging activity was assessed by mixing 100 µL of standard solution or hydrolysate sample with 100 µL of DPPH solution in the microplate well. The mixture was incubated for 30 minutes at room temperature, following the results obtained from the kinetic matching study (Appendix A). The sample absorbance was then measured at 517 nm in a FLUOstar® Omega microplate reader.

Results

Antioxidant capacity of samples was calculated by comparison to the Trolox calibration curve, with final DPPH values being expressed as $\mu mol_{Trolox\ equivalent}$/ $mg_{hydrolysed\ protein}$ or $\mu mol_{Trolox\ equivalent}$/ $g_{Alga\ DW}$.

The EC_{50} (µg/mL) value of samples for the DPPH assay was also determined as described above for the ABTS assay.

3.5.2 ACE-inhibitory activity determination

The ACE-inhibitory activity of samples was determined according to Vermeirssen *et al.* [94]

Extraction and Preparation of ACE from rabbit lung acetone powder

Rabbit lung acetone powder (Sigma-Aldrich) was mixed in 10 mL of 150 mM Tris-base buffer containing 5% (v/v) glycerol at pH 8.3, using gentle magnetic stirring at 4 ºC overnight. The

extract solution was then centrifuged at 14000 rpm and 4 °C, until a minimal deposit was obtained. The supernatant containing ACE activity was retained and stored at -20 °C (≈ 2000 units ACE activity/L).

Working solutions

ACE stock solution was diluted 1:5 with 150 mM Tris-base buffer at pH 8.3, containing 1 µM $ZnCl_2$ 0.1 mM; 0.45 mM fluorescent substrate was prepared by dissolving Abz-Gly-Phe(NO_2)-Pro (BACHEM, Bubendorf, Switzerland) in 150 mM Tris-base buffer at pH 8.3, containing 1.125 M NaCl.

Assay execution

In a black ninety-six-well microplate, 40 µL of sample was mixed with 40 µL of the enzyme. The enzymatic reaction was initiated by adding 160 µL of 0.45 mM fluorescent substrate. A blank was also prepared, containing 80 µL of ultrapure water; as well as a control, containing 40 µL of ultrapure water and 40 µL of enzyme, and blank samples, containing 40 µL of sample and 40 µL of ultrapure water. After adding the fluorescent substrate, the microplate was shaken and incubated at 37 °C for 30 minutes. The fluorescence was measured at 0 and 30 minutes in a FLUOstar® Omega microplate reader, at excitation and emission wavelengths of 350 nm and 420 nm, respectively.

Results

The ACE-inhibitory activity of samples was expressed as IC_{50} (µg/mL) values, determined using software GraphPad Prism 8.0.2, by plotting a nonlinear regression of protein concentration *vs.* normalized response (%) using a dose-response inhibition model.

3.6 Protein content determination

The protein concentration for each sample was quantified using bicinchoninic acid (BCA) based on the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, USA), using bovine serum albumin (BSA) as standard. The microplate procedure was applied as suggested by the manufacturer, and absorbance was measured at 562 nm on a FLUOstar® Omega microplate reader.

3.7 Protein profile determination

Chromatographic separations were performed in the HPLC equipment described in Section 3.1.1, with an XBridge Protein BEH SEC, 450 Å (3.5 µm 7.8 x 150 mm) (Waters) column, following the suggested protein separation protocol; 100 mM sodium phosphate at pH 6.8 was accordingly used as mobile phase, at 0.86 mL/min at room temperature. Samples were preserved at 4 °C. The calibration standard (BEH450 SEC Protein Standard Mix, Waters) was used for qualitative

analysis of sample compounds. The injection volume for calibration standard and sample extracts was 20 µL; for each run and elution profile, detection was at 280 nm.

3.8 Chlorophylls and carotenoids determination

The extract obtained from the pre-treatment with EtOH was analysed in the HPLC equipment described in Section 3.1.1. In that order, chromatographic separations were performed in the HPLC equipment described, with a 4 x 250 mm Purospher Star RP-18e (5 µm) column (Merck), and mobile phase of ethyl acetate and acetonitrile:water (9:1). The sample was eluted over 55 min at 1 mL/min, a column pressure of 3000 bar, and a temperature of 25 °C ± 2 °C. Spectral data from all peaks were collected in the range from 250 to 750 nm. The compounds were identified by comparing retention times and UV-visible spectrum with those of the chromatographic standards. Standards (HPLC grade) were purchased from Extrasynthese (apo-carotenal, lutein, zeaxanthin, and ß-carotene). Standard calibration curves were prepared using seven calibration points for each analyte. Each test was performed at least in three independent experiments. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the calibration curve parameters, where the LOD was equal to the calculated intercept of the linear regression (a) plus three times the Sy/x and for LOQ ten times this value. The results were expressed in milligrams by grams of dry algae (mg/g_{DW}).

3.9 Statistical analysis

The experimental data were analyzed using software GraphPad Prism 8.0.2 (GraphPad Software, San Diego, California, USA). A Shapiro-Wilk test was performed to assess the normality of the data, considering a significance level of 0.05. To assess significant differences among results of extraction yields, degree of hydrolysis, antioxidant capacity and ACE-inhibition capacity, a one-way ANOVA with Tukey's multiple comparison tests was performed, at a confidence level of 95%. When an ANOVA analysis was not possible due to lack of sample replicates, a Student's *t*-test was performed, at the same confidence level.

4 Results and discussion

4.1 Amino acid profile of algae sample

Determination of the amino acid composition of *Ulva sp.* is of the uttermost interest to conclude on whether that macroalga is suitable for human nutrition – namely if it contains essential amino acids, which the human body is not capable of synthesizing and must thus be consumed as part of the diet. [95]

The results of the amino acid composition analysis for *Ulva sp.* are summarized in Table 4.1. The most abundant amino acid is glutamine (13.25 ± 0.68 mg_{AA}/g_{Alga}), followed by alanine (9.97 ± 1.03 mg_{AA}/g_{Alga}) – which are non-essential amino acids. Leucine is the most abundant essential amino acid, at 8.47 ± 1.55 mg_{AA}/g_{Alga}. Hence, this alga contains 6 of the 10 essential amino acids. These results lie under those reported in the literature [96-100], with less abundant amino acids not being detected by the method applied here. Considering the amino acid requirements in an adequate diet [101], this macroalga would be an appropriate protein source to be included in a human diet; it can be used as a better substitute for animal protein when compared to plant-based protein.

Table 4.1. Amino acid composition of *Ulva sp.*

Amino acids	Content (mg_{AA}/g_{Alga})
Alanine	9.97 ± 1.03
Arginine	8.20 ± 0.80
Asparagine	N.D.
Aspartic acid	9.47 ± 1.25
Cysteine	N.D.
Glutamic acid	8.48 ± 1.41
Glutamine	13.25 ± 0.68
Glycine	6.17 ± 1.31
Histidine[a]	N.D.
Isoleucine[a]	5.66 ± 0.94
Leucine[a]	8.47 ± 1.55
Lysine[a]	N.D.
Methionine[a]	3.63 ± 1.37
Phenylalanine[a]	7.75 ± 1.59
Proline	3.13 ± 0.32
Serine	2.38 ± 0.54
Threonine[a]	4.61 ± 0.90
Tyrosine	8.06 ± 1.18
Valine[a]	6.77 ± 1.25

N.D. – Not detected.
[1]Values expressed as mean ± standard deviation (SD).
[a]Essential amino acids

4.2 Optimization of protein extraction

Several extracts were obtained, as a result of applying single and sequential extraction methods, as described on Section 3.2. The results obtained for the first extraction method tested, using a Precellys homogenizer, are presented in Table 4.2.

Table 4.2. Extraction yield of each step, and total extraction yield, of single and sequential extractions performed in Precellys homogenizer with different solvents for *Ulva sp.*

	Extraction yield (%)[1]	Total extraction yield (%)[1]
H_2O 1st 5 mL H_2O 2nd 5 mL	3.45 ± 0.36 2.00 ± 0.12	5.45 ± 0.38
H_2O 10 mL	3.41 ± 0.04	3.41 ± 0.04
EtOH 1st 5 mL EtOH 2nd 5 mL	0.34 ± 0.03 0.16 ± 0.02	0.50 ± 0.04
EtOH 10 mL	0.29 ± 0.01	0.29 ± 0.01
EtOH 1st 5 mL EtOH 2nd 5 mL H_2O 1st 5 mL H_2O 2nd 5 mL	0.34 ± 0.03 0.16 ± 0.02 2.53 ± 0.03 0.75 ± 0.08	3.78 ± 0.09
EtOH 1st 5 mL EtOH 2nd 5 mL H_2O 10 mL	0.34 ± 0.03 0.16 ± 0.02 2.42 ± 0.04	2.92 ± 0.05
EtOH 10 mL H_2O 1st 5 mL H_2O 2nd 5 mL	0.29 ± 0.01 2.37 ± 0.18 0.68 ± 0.03	3.34 ± 0.18
EtOH 10 mL H_2O 10 mL	0.29 ± 0.01 2.39 ± 0.16	2.68 ± 0.16
HCl 1st 5 mL HCl 2nd 5 mL	1.79 ± 0.11 0.63 ± 0.08	2.42 ± 0.14

[1]Values expressed as mean ± standard deviation (SD).

Yields vary depending on the solvent used, with H_2O standing out with the highest values for extraction yield, whether as single solvent used or following EtOH. A sequential 5 mL extraction with H_2O exhibits the highest extraction yield at 5.45 ± 0.38 %, i.e. over 1.5-fold the yield obtained for the single extraction with H_2O using the same overall volume (3.41 ± 0.04%). EtOH follows that same trend, although it presents the lowest extraction yields, over 10-fold lower than H_2O in single 10 mL extraction and sequential 5 mL extractions. In the case of HCl, only sequential 5 mL extractions were tested; as for the total yield obtained (2.42 ± 0.14%), although higher than for EtOH, it is still 2.3-fold lower than when H_2O was used.

When EtOH was used in combination with H_2O, the total extraction yield was significantly lower ($p > 0.05$) than when H_2O was used alone - 3.78 ± 0.09% and 3.34 ± 0.18% *vs.* 5.45 ± 0.38% for

sequential 5 mL extractions with H_2O, and 2.92 ± 0.05% and 2.68 ± 0.16% *vs.* 3.41 ± 0.04% for single 10 mL extraction with H_2O; this unfolds a possible interference from EtOH upon protein availability in the following steps. However, supernatants obtained from these extractions using EtOH stood out for their color when compared to extractions using H_2O. As pictured in Figure 4.1 a) to c), extractions performed in the Precellys homogenizer with EtOH produce supernatants with a deep green color, with the following extraction with H_2O being completely transparent. Conversely, single and sequential extractions using solely H_2O always result in green supernatants with a cloudy appearance, as apparent in Figure 4.1 d). These results indicate that EtOH efficiently removes pigments from the macroalga cells.

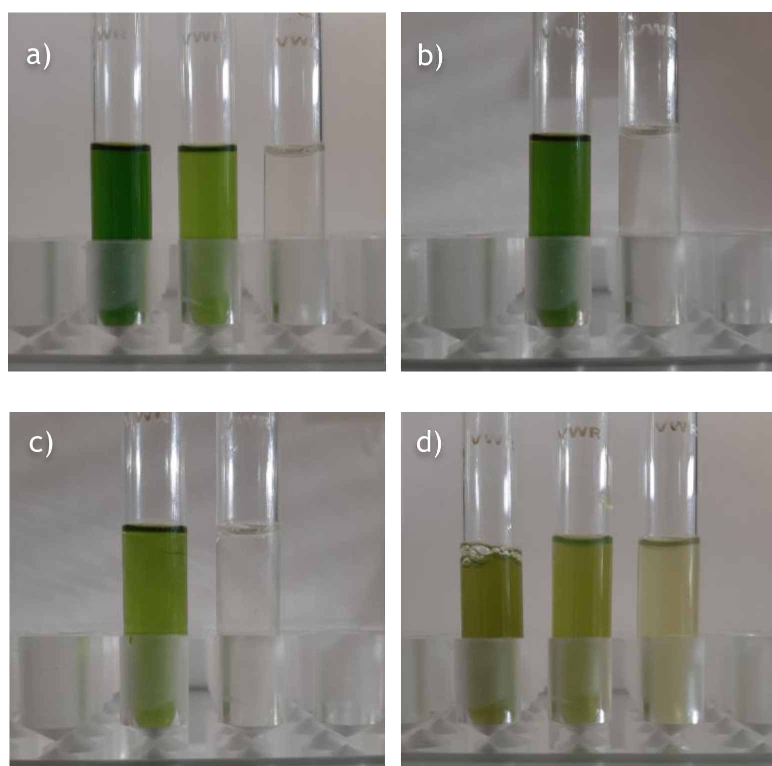


Figure 4.1. Appearance of supernatants after single and sequential extractions in Precellys homogenizer: a) from left to right: supernatant after first 5 mL extraction with EtOH, supernatant after second 5 mL extraction with EtOH and supernatant after 10 mL extraction with H_2O; b) combined supernatants of two 5 mL extractions with EtOH and two 5 mL extractions with H_2O; c) supernatant after 10 mL extraction with EtOH and 10 mL extraction with H_2O; d) supernatant after first 5 mL extraction with H_2O, supernatant after second 5 mL extraction with H_2O and supernatant after single 10 mL extraction with H_2O.

Discoloration of macroalgae after immersion in alcohols has been reported elsewhere [102]; and it is known that use of organic solvents, such as EtOH, results in extraction of intracellular chlorophylls. [103] The solvent acts by dissolving lipids and lipoproteins in the cell and chloroplast membranes, thus allowing its permeation through the cell and chloroplasts to extract the pigments. [104] It has been shown that cell disruption significantly increases pigment recovery [105,106], whether via mechanical homogenization (as applied here), sonication or other methods. The organic solvent used, as well as temperature and pressure applied, also influence

pigment extraction yield. [107,108] However, ethanol at high concentrations can destabilize proteins and cause protein denaturation [109], thus altering their structure via solvent polarity changes. [110]

Although the extractions with H_2O following application of EtOH resulted in a total extraction yield lower than that of using plain H_2O, the final extract obtained is colorless – which is preferable in view of the extract's future applications. A conventional extraction with H_2O, over 90 minutes, was also tested, to obtain a higher extraction yield than using the Precellys homogenizer, while still maintaining the extent of color removal. The corresponding results are depicted in Table 4.3, showing that the conventional extraction method with H_2O produces extraction yields similar to those by the Precellys method using the same solvent. Time of extraction does not play a critical role in its efficacy, since no statistical difference ($p > 0.05$) was found between extraction yields after 30 minutes for both cases.

Table 4.3. Extraction yield of each step, and total extraction yield, of conventional extractions performed with H_2O over 90 minutes, following single and sequential Precellys extractions with EtOH, for *Ulva sp.*

	Extraction yield (%)[1]	**Total extraction yield (%)[1]**
EtOH 1st 5 mL	0.34 ± 0.03	3.35 ± 0.08
EtOH 2nd 5 mL	0.16 ± 0.02	
H_2O 90 min	2.85 ± 0.07	
EtOH 10 mL	0.29 ± 0.01	3.53 ± 0.19
H_2O 90 min	3.24 ± 0.19	

[1]Values expressed as mean ± standard deviation (SD).

In attempts to maximize extraction yield with an additional extraction step, acid and alkaline solvents were also tested with a conventional extraction method – since these were found to be efficient in extracting protein from brown seaweed *A. nodosum* [51] and red macroalga *P. palmata*. [111] Fleurence *et al.* [45] also reported 3-fold and 2.6-fold increases in protein extraction from *Ulva rigida* and *Ulva rotundata*, respectively, when NaOH was used upon extraction with H_2O, as compared to a procedure applying only H_2O; and *Serot et al.* [47] optimized protein extraction in *Ulva sp.* using the same alkaline solvent. A solvent bath pre-treatment with EtOH, without cell disruption, was also analyzed, to achieve noticeable pigment removal – while obtaining protein from *Ulva sp.* in its native state, or as unaltered as possible, for the following hydrolysis step. The results obtained for the extraction yield at each step of the sequential extractions, as well as the total extraction yield for each overall process are depicted in Table 4.4.

Even after the color removal observed in the extraction steps involving EtOH, extraction with NaOH led to additional color removal, with the supernatant exhibiting a green color; while extraction steps with HCl resulted in dark brown supernatants.

Table 4.4. Extraction yield of each step, and total extraction yield, of conventional extractions performed with NaOH and HCl over 90 minutes, following pre-treatment with EtOH, and/or single and sequential Precellys extractions with EtOH or H_2O, and/or conventional extraction with H_2O, for *Ulva sp.*

	Extraction yield (%)[1]	Total extraction yield (%)[1]
EtOH 1st 5 mL	0.34 ± 0.03	8.01 ± 0.46
EtOH 2nd 5 mL	0.16 ± 0.02	
H_2O	2.85 ± 0.07	
NaOH	4.66 ± 0.45	
EtOH 10 mL	0.29 ± 0.01	7.94 ± 0.24
H_2O	3.24 ± 0.19	
NaOH	4.41 ± 0.14	
H_2O 1st 5 mL	3.45 ± 0.36	11.12 ± 0.50 [a]
H_2O 2nd 5 mL	2.00 ± 0.12	
NaOH	5.67 ± 0.33	
H_2O 10 mL	3.41 ± 0.04	8.40 ± 0.15
NaOH	4.99 ± 0.14	
EtOH bath	-	9.94 ± 0.41 [b]
H_2O 1st 5 mL	2.76 ± 0.11	
H_2O 2nd 5 mL	1.16 ± 0.08	
NaOH	6.02 ± 0.39	
H_2O 1st 5 mL	3.45 ± 0.36	5.52 ± 0.38
H_2O 2nd 5 mL	2.00 ± 0.12	
HCl	0.07 ± 0.05	
H_2O 1st 5 mL	3.45 ± 0.36	8.87 ± 0.41
H_2O 2nd 5 mL	2.00 ± 0.12	
HCl	0.07 ± 0.05	
NaOH	3.35 ± 0.15	
HCl 1st 5 mL	1.79 ± 0.11	11.14 ± 0.17 [a]
HCl 2nd 5 mL	0.63 ± 0.08	
NaOH	8.72 ± 0.11	
EtOH	-	9.59 ± 0.26 [b]
HCl 1st 5 mL + 2nd 5 mL	1.51 ± 0.22	
NaOH	8.08 ± 0.14	

[1]Values expressed as mean ± standard deviation (SD).
[a,b] No significant difference ($p > 0.05$) between values (one-way ANOVA, Tukey's multiple comparisons test)

From the various sequential methods applied, H_2O and HCl combined with NaOH yielded the best results regarding protein extraction - 11.12 ± 0.50% and 11.14 ± 0.17%, respectively, with extraction yield slightly decreasing to 9.94 ± 0.41% and 9.59 ± 0.26% when *Ulva sp.* was pre-treated with EtOH. It can be said that the choice of the first solvent is irrelevant toward the final value of total extraction yield, since there is no difference ($p > 0.05$) between total extraction yields in the cases where H_2O or HCl are applied as a tandem with NaOH.

Unpublished results from the research group showed that use of HCl as solvent for extraction poses problems in subsequent steps of the process, namely when treated supernatants are to be subjected to lyophilization. Since lyophilization was not an option here, supernatants would have to be filtered, thus adding a time-consuming and tedious step to the process. This was the deciding factor for electing H_2O as solvent for use in the sequential extraction in the Precellys homogenizer, since the supernatant could easily be lyophilized. Additionally, H_2O is known as the universal solvent, being a cheaper and far less polluting solvent alternative than HCl.

Statistical analysis of results from the conventional extraction with NaOH over 90 minutes revealed no statistical difference ($p > 0.01$) between extraction yields after 60 minutes – meaning that this amount of time is enough to obtain the maximum possible quantity of protein extracted.

Pre-treatment with EtOH was also included in the final method; although it entails a slightly lower extraction yield, it serves an essential purpose in the final appearance of the extract – by initially removing a substantial amount of pigment from *Ulva sp.* Given that extraction with NaOH also causes pigment removal, the final extract would present a very dark color without pre-treatment with EtOH – which would not be appealing for future applications, and would thus probably call for an additional color removal step at industrial settings.

The final optimized approach for protein extraction from *Ulva sp.*, illustrated in Figure 4.2, consisted of a pre-treatment with EtOH for pigment removal, followed by sequential 5 mL extractions with H_2O in a Precellys homogenizer, and finally a conventional extraction with NaOH for 60 minutes. Supernatants from the extractions were appropriately treated and produced an extract suitable for the hydrolysis step.

Protein contents of 7 to 25% for *Ulva sp.* have been reported in the literature [100,112], with accurate values depending on season and environmental growth conditions. [113] Considering a protein content of 24% for the *Ulva sp.* used, as indicated by previous work performed by the research group (Section 3.1), the selected method permits extraction of 41.43% of the alga total protein. This result is slightly higher than the ones obtained by Fleurence *et al.* [45] for *Ulva rigida* and *Ulva rotundata* of 26.8 ± 1.3% and 36.1 ± 1.4%, respectively, for a process that included extraction with H_2O followed by NaOH – although mechanical homogenization was not applied. Extraction of algal proteins is critically dependent upon disruption of the cell wall, so extraction of intracellular proteins is often improved by applying such stress factors as osmotic shock, shearing or grinding. [44] Juul *et al.* [46] applied a similar method to *Ulva fenestrata*, but included homogenization in a mixer – which resulted in a total extraction yield of 8.95 ± 0.79%, similar to the value obtained in this study.

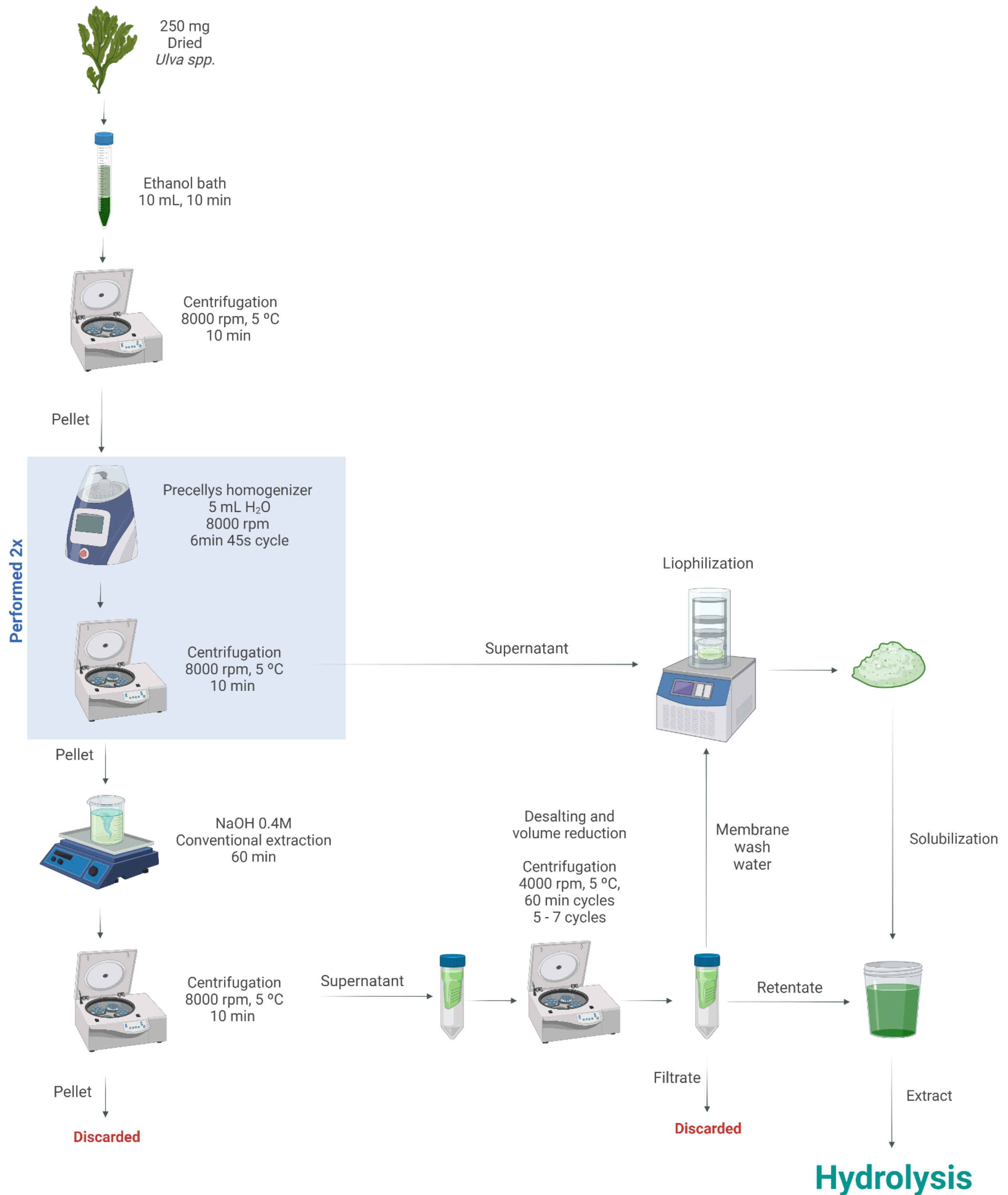


Figure 4.2. Flowchart for the optimized process of protein extraction from *Ulva sp*.

4.3 Experimental design, modelling and optimization of biological activities

Using statistically designed experiments covering a wide range of conditions for time of hydrolysis and E/S ratio combinations – but still taking practical industrial constraints into account for relating to putative (and eventual) scale-up of the process, 13 hydrolysis experiments were proposed. Once the experiments were performed, the resulting hydrolysates were tested for DH, ACE-inhibitory activity and antioxidant capacity; the obtained results are presented in Table 4.5, with relevant statistics presented in Table 4.6.

Regarding antioxidant capacity, it is worth noting that the measured antioxidant capacity of a sample depends on the free radical generator used in the measurement; hence, it should not be expected that similar values be obtained for the different assays performed. [114]

The statistical significance of the various terms in the polynomial model, fitted by multiple linear regression to the data, was duly analyzed to assure maintenance of significant model terms and avoid significant lack of fit. Several diagnostics graphs, which are presented in Appendix B, were also analyzed; this prompted application of data transformation to ACE-inhibition activity results. A natural log transformation was applied to that specific dataset prior to process optimization so as to stabilize variance, as advised elsewhere. [115,116]

As can be observed in Figure 4.3, DH varies linearly with the two processing parameters. However, the same cannot be strictly said for ACE-inhibitory or antioxidant capacity of samples. Regarding ACE-inhibitory activity, results are well fitted by the quadratic model. The same can be claimed for the ORAC assay, regarding antioxidant capacity, with the values obtained also well fitted by the quadratic model – and showing high correlation with DH. Upon inspection of Table 4.6, one concludes that: the determination coefficient (R^2) was 0.86 for ACE-inhibitory activity, 0.85 for DH and 0.85 for antioxidant capacity (ORAC method); and all presented a non-significant lack of fit. These results prove that the model is statistically appropriate to describe DH, ACE-inhibitory activity and antioxidant capacity (ORAC assay).

In contrast, the ABTS and DPPH assays presented R^2 values lower than 0.70 and significant lack of fit, so it became impossible to fit any model on statistically sound grounds. In other words, the model would not be a good predictor of either of these responses.

Upon analysis of the results presented in Table 4.5, values of $\mu mol_{Trolox\ equivalent}/mg_{hydrolysed\ protein}$ obtained for all samples through the ABTS and DPPH assays are not statistically different ($p > 0.05$) – thus implying that all samples exhibit the same antioxidant capacity, independent of DH. At this point, it is important to mention that one goal of this work was to obtain a protein concentrate designed for hydrolysis afterward. However, these results suggest that the obtained extract is not composed solely of protein, so its activity may not be fully attributed

Table 4.5. Experimental design encompassing two processing parameters – time and E/S ratio, and results pertaining to 5 responses – degree of hydrolysis (DH), ACE-inhibitory activity and antioxidant capacity through 3 different assays: ORAC, ABTS and DPPH, obtained for Ulva sp. extract, hydrolysed with alcalase from *Bacillus licheniformis*.

				ACE-inhibitory Activity[1]	Antioxidant Capacity[1]					
					ORAC assay		ABTS assay		DPPH assay	
Ex	Time (h)	E/S	DH (%)[1]	IC_{50} (µg/mL)	EC_{50} (µg/mL)	$µmol_{Trolox\ equivalent}/mg_{hydrolysed\ protein}$	EC_{50} (µg/mL)	$µmol_{Trolox\ equivalent}/mg_{hydrolysed\ protein}$	EC_{50} (µg/mL)	$µmol_{Trolox\ equivalent}/mg_{hydrolysed\ protein}$
1	1	0.5	3.79 ± 0.09	225.6 ± 6.02	97.7 ± 17.9	1.25 ± 0.12	196.5 ± 14.2	0.345 ± 0.023	646.6 ± 48.1	0.052 ± 0.005
2[a]	3.5	1.5	40.22 ± 3.71	62.4 ± 3.68	63.7 ± 5.8	2.04 ± 0.17	159.3 ± 16.2	0.381 ± 0.012	599.3 ± 44.7	0.054 ± 0.008
3	0	1.5	0.0 ± 0.0	462.5 ± 32.60	122.2 ± 34.0	0.98 ± 0.10	- [b]	0.350 ± 0.034	955.8 ± 55.6	0.033 ± 0.005
4[a]	3.5	1.5	31.31 ± 1.84	75.4 ± 3.32	63.1 ± 4.4	2.30 ± 0.22	186.0 ± 19.9	0.328 ± 0.027	560.6 ± 31.1	0.056 ± 0.010
5	3.5	2.9	51.33 ± 2.56	72.0 ± 3.53	58.7 ± 4.8	2.35 ± 0.09	130.8 ± 23.4	0.388 ± 0.014	576.4 ± 68.1	0.060 ± 0.012
6	1	2.5	29.26 ± 0.68	117.7 ± 4.96	65.9 ± 11.3	2.24 ± 0.04	168.3 ± 12.1	0.342 ± 0.032	512.7 ± 97.5	0.060 ± 0.015
7[a]	3.5	1.5	36.67 ± 2.52	119.8 ± 3.96	133.8 ± 29.6	1.83 ± 0.16	165.9 ± 16.0	0.331 ± 0.031	535.3 ± 76.5	0.060 ± 0.014
8	7	1.5	51.79 ± 4.62	94.0 ± 2.74	101.1 ± 24.5	1.84 ± 0.20	187.7 ± 11.3	0.348 ± 0.028	540.6 ± 41.7	0.061 ± 0.014
9	6	0.5	31.84 ± 4.34	200.6 ± 6.98	95.6 ± 29.7	1.80 ± 0.27	- [b]	0.375 ± 0.030	459.6 ± 20.1	0.068 ± 0.013
10	6	2.5	48.23 ± 5.34	88.9 ± 2.11	77.5 ± 11.7	2.25 ± 0.18	156.5 ± 16.5	0.369 ± 0.028	412.5 ± 23.1	0.061 ± 0.017
11[a]	3.5	1.5	44.22 ± 8.62	70.7 ± 3.60	99.1 ± 11.3	1.98 ± 0.27	215.3 ± 36.7	0.319 ± 0.029	462.6 ± 30.0	0.061 ± 0.015
12[a]	3.5	1.5	31.38 ± 2.32	63.7 ± 2.75	88.8 ± 15.9	2.19 ± 0.21	- [b]	0.326 ± 0.037	530.1 ± 22.1	0.054 ± 0.012
13	3.5	0.09	8.79 ± 2.04	355.7 ± 13.93	108.4 ± 19.3	1.22 ± 0.17	- [b]	0.340 ± 0.011	481.3 ± 50.1	0.065 ± 0.014

[1]Values expressed as mean ± standard deviation (SD).
[a] Central point of the design.
[b] Data did not allow construction of an appropriate non-linear regression.

Table 4.6. Best estimates of each term in the model – constant, time, T (linear and quadratic), E/S ratio, R (linear and quadratic) and interaction thereof (linear) and corresponding relevant statistics – R^2 and p-value for the model and respective lack of fit, pertaining to five responses – degree of hydrolysis, antioxidant capacity (through the ORAC, ABTS and DPPH assays) and ACE-inhibitory activity, obtained for Ulva sp. extract, hydrolysed with alcalase from *Bacillus licheniformis*.

TERM	DH (%)	ACE-inhibitory Activity	Antioxidant Capacity		
			ORAC assay	ABTS assay	DPPH assay
Constant	31.30	4.29	2.07	0.3300	0.0570
T	14.70	-0.29	0.22	0.0068	0.0069
R	12.75	-0.46	0.38	0.0074	-0.0008
T x T	-	0.43	-0.26	0.0063	-0.0038
T x R	-	-0.07	-0.14	-0.0007	-0.0037
R x R	-	0.40	-0.07	0.0138	0.0042
STATISTIC					
R^2	0.85	0.86	0.85	0.38	0.63
Model (p-value)	< 0.0001[a]	0.007[a]	0.007[a]	0.54[b]	0.02[b]
Lack of fit (p-value)	0.24[b]	0.57[b]	0.23[b]	0.63[b]	0.11[b]

[a] Significant at a 99% confidence level.
[b] Not significant at a 99% confidence level.

to proteins or peptides in the extract. *Ulva sp.* has indeed a polysaccharide content of ca. 50% and a protein content of ca. 10% [117], so the former will likely constitute the majority in the extract; besides minerals and phytochemicals, such as pigments, that might be bound to polysaccharides or proteins. [118] This realization suggests that the antioxidant capacity displayed in ABTS and DPPH assays is not coming from the peptides being generated through hydrolysis, since samples hydrolysed to higher extents did not exhibit higher activities; but rather from other components in the extract, most likely polysaccharides, as they remain essentially unaltered upon hydrolysis. On the other hand, results obtained for the ORAC assay unfold differences among samples – thus proving that this assay is the most appropriate for measurement of antioxidant capacity of peptides.

DH is a helpful parameter if the results obtained in biological activities are a consequence of extensive hydrolysis, or due to some intrinsic properties of the substrate or enzyme specificity - since antihypertensive activity and antioxidant capacity have been shown to considerably depend on DH of protein substrates. [119] In this case, DH is (as expected) highly correlated with reaction time and E/S ratio – as proven in Table 4.5 by the two highest values for DH being 51.33 ± 2.56% (for the highest E/S ratio tested) and 51.79 ± 4.62% (for the longest reaction time tested).

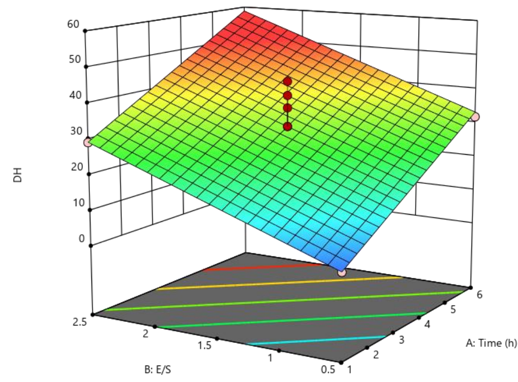
However, DH was used merely as an indicator of proper hydrolysis, rather than a processing parameter – since it does not directly relate to higher biological activity of samples. Additionally, reaction time and E/S ratio are processing parameters that can easily be manipulated *a priori* – unlike DH that would require feedback control and monitoring before startup in an industrial setting.

ACE-inhibitory activity response is plotted in Figure 4.3 b); the highest IC_{50} value recorded is 62.4 ± 3.68 μg/mL, for one of the design's central points. This is a promising result when compared to other reports on ACE-inhibitory activity of macroalga protein hydrolysates. Values of IC_{50} of 262 μg/mL were reported for protein hydrolysates from red alga *Mazzaella japonica* [120], as well as 86 μg/mL [121], 183 μg/mL [74] and 483 μg/mL [73] for green macroalgae *Undaria sp.*, *Ulva intestinalis* and *Ulva rigida*, respectively. For brown macroalgae, a value of IC_{50} for *L. digitata* of 590 μg/mL was also reported. [121]

Several studies analyze the ACE-inhibitory activity of specific peptides derived from algae, with IC_{50} values ranging from 20 to 57.2 μg/mL for green alga *Ulva rigida* and red alga *Bangia fuscopurpurea*, respectively [122] – while comparing it to the standard antihypertensive drug Captopril. However, this study deals only with optimization of crude protein hydrolysates, rather than pure peptides – so comparisons can only be drawn to other alga hydrolysates, and not isolated pure peptides (which exhibit much higher ACE-inhibiting activity).

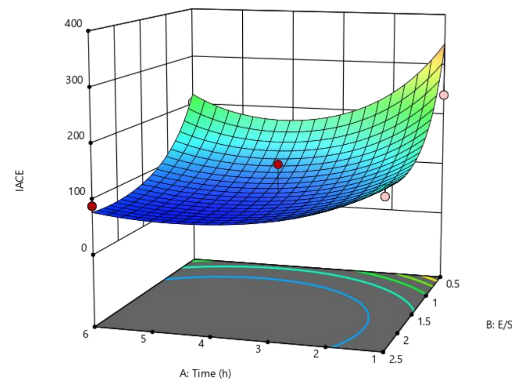
DEGREE OF HYDROLYSIS

a)



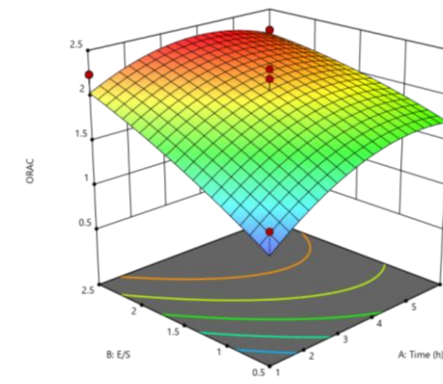
ACE-INHIBITORY ACTIVITY

b)



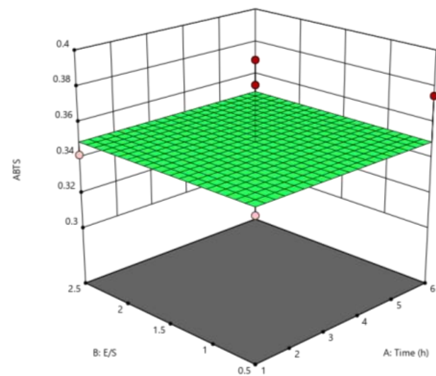
ANTIOXIDANT CAPACITY – ORAC ASSAY

c)



ANTIOXIDANT CAPACITY – ABTS ASSAY

d)



ANTIOXIDANT CAPACITY – DPPH ASSAY

e)

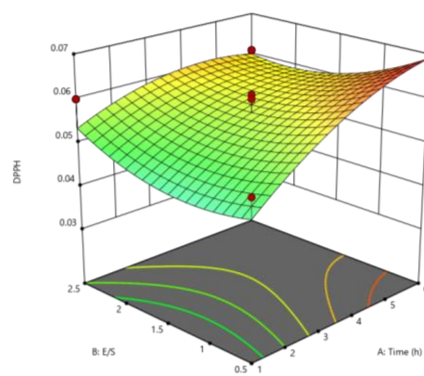


Figure 4.3. Variation of predicted responses for five parameters analysed: a) degree of hydrolysis, b) ACE-inhibitory activity and antioxidant capacity through c) ORAC, d) ABTS and e) DPPH assays, as a function of each term in the model – time and E/S ratio, obtained for *Ulva sp.* extract hydrolysed with alcalase from *Bacillus licheniformis*.

Upon the foregoing analysis – and in light of previous observations, ABTS and DPPH responses were not included in the final model, so optimization was performed only with regard to ACE-inhibitory activity and antioxidant capacity through the ORAC assay.

The optimal processing conditions, and the corresponding prediction by the ACE-inhibitory activity and antioxidant capacity model through the ORAC assay were then found by multiple response optimization of the Design Expert software. The ACE-inhibitory activity was minimized – since it was expressed in IC_{50}, and antioxidant capacity was maximized; both optima were determined taking into account the design point with the highest predicted desirability. The best reaction time was 4.3 h and the optimum E/S ratio was 2.4, with predicted values for ACE-inhibitory activity and antioxidant capacity (through the ORAC assay) depicted in Table 4.7.

Table 4.7. Predicted ACE-inhibitory activity and antioxidant capacity for *Ulva sp.* hydrolysates, and corresponding values for lower and upper bound of 95% confidence interval.

Activity	Predicted Mean[1]	95% Confidence Interval	
		Lower Bound	Upper Bound
ACE-inhibitory[a]	64.94 ± 21.72	44.37	95.04
Antioxidant capacity[b]	2.35 ± 0.23	2.09	2.618

[1]Values expressed as mean ± standard deviation (SD).
[a] IC_{50} (µg/mL)
[b] obtained by ORAC method ($\mu mol_{Trolox\ equivalent}$/ $mg_{hydrolysed\ protein}$)

The model was then validated, by experimentally testing the optimum conditions and analyzing the obtained samples for ACE-inhibitory activity and antioxidant capacity. ABTS and DPPH assays were accordingly performed, but the results were expressed as $\mu mol_{Trolox\ equivalent}$/ $g_{Alga\ DW}$ because it was concluded that the antioxidant capacity exhibited by these assays was not due to peptides present in the hydrolysates. Since the molecular weight of ACE-inhibitory peptides is usually below 3 kDa, hydrolysates were submitted to ultrafiltration through a hydrophilic 3 kDa cut-off membrane – and biological activities were determined for the total fraction and the <3 kDa fraction.

Hydrolysates produced following application of optimum conditions were characterized by a DH value of 36.12 ± 1.64%. The results pertaining to biological activities of the total and <3 kDa fractions are depicted in Table 4.8. The actual experimental optima for ACE-inhibitory activity results lied within the 95% confidence interval of the values theoretically estimated as optima via the model – so our model proved adequate to describe the experimental data.

The results obtained for the ABTS and DPPH assays, expressed as a function of alga dry weight, are overall consistent with literature results pertaining to other macroalgae – for instance, Vega *et al.* [123] reported maximum values of 14 $\mu mol_{Trolox\ equivalent}/g_{DW}$ in ABTS assay, and

4 $\mu mol_{Trolox\ equivalent}/g_{DW}$ in DPPH assay for the red alga *Osmundea pinnatifida*. For all algae tested, the ABTS assay in the same study reached higher values than the DPPH assay - a deed also found in this study. Enzymatic extracts of several macroalgae exhibited EC_{50} values for DPPH radical scavenging activity ranging from 0.05 ± 0.003 to 2.0 ± 0.1 mg/mL, as a function of extract concentration, with *C. cornuta* and *C. dasyphylla* exhibiting the highest antioxidant capacity; while *U. lactuca* showed the lowest. EC_{50} values also changed significantly, depending on the enzyme used for hydrolysis. [124]

Table 4.8. ACE-inhibitory activity and antioxidant capacity for total and <3 kDa fractions of *Ulva sp.* hydrolysates at optimum conditions.

	ACE-inhibitory activity[1]	Antioxidant capacity[1]		
		ORAC assay	ABTS assay	DPPH assay
Total	92.3 ± 2.0	1.96 ± 0.04[a] 68.8 ± 5.2[b]	11.74 ± 0.19[c] 6.43 ± 0.43[d]	1.74 ± 0.06[c] 19.53 ± 2.92[d]
<3 kDa	66.8 ± 1.0	2.60 ± 0.06[a] 58.7 ± 1.8[b]	-	-

[1]Values expressed as mean ± standard deviation (SD).
[a] $\mu mol_{Trolox\ equivalent}/\ mg_{hydrolysed\ protein}$
[b] EC_{50} (µg/mL) referring to protein concentration.
[c] $\mu mol_{Trolox\ equivalent}/\ g_{Alga\ DW}$
[d] EC_{50} (mg/mL) referring to alga concentration.

As expected, <3 kDa fractions presented better activity values than total fractions, with the most notorious difference found for ACE-inhibitory activity. This corroborates the claim made above that peptides responsible for this specific biological activity usually have a molecular weight below 3 kDa. However, considering process scale-up and future industrial applications, further analysis should be performed to confirm whether the difference between the total and <3 kDa fraction is significant enough to justify incurring in the additional purification cost.

The performance of *Ulva sp.* hydrolysates in terms of the described biological activities would also require *in vivo* tests; since it has been reported that bioactive peptide activity can be significantly reduced upon gastro-intestinal digestion. [125]

4.4 Protein profile

Extracts obtained from the Precellys extraction with H_2O, after pre-treatment with EtOH, and the conventional extraction with NaOH, as well as the final extract obtained for optimum conditions were analyzed by HPLC; the corresponding protein chromatographic profiles are depicted in Figure 4.4.

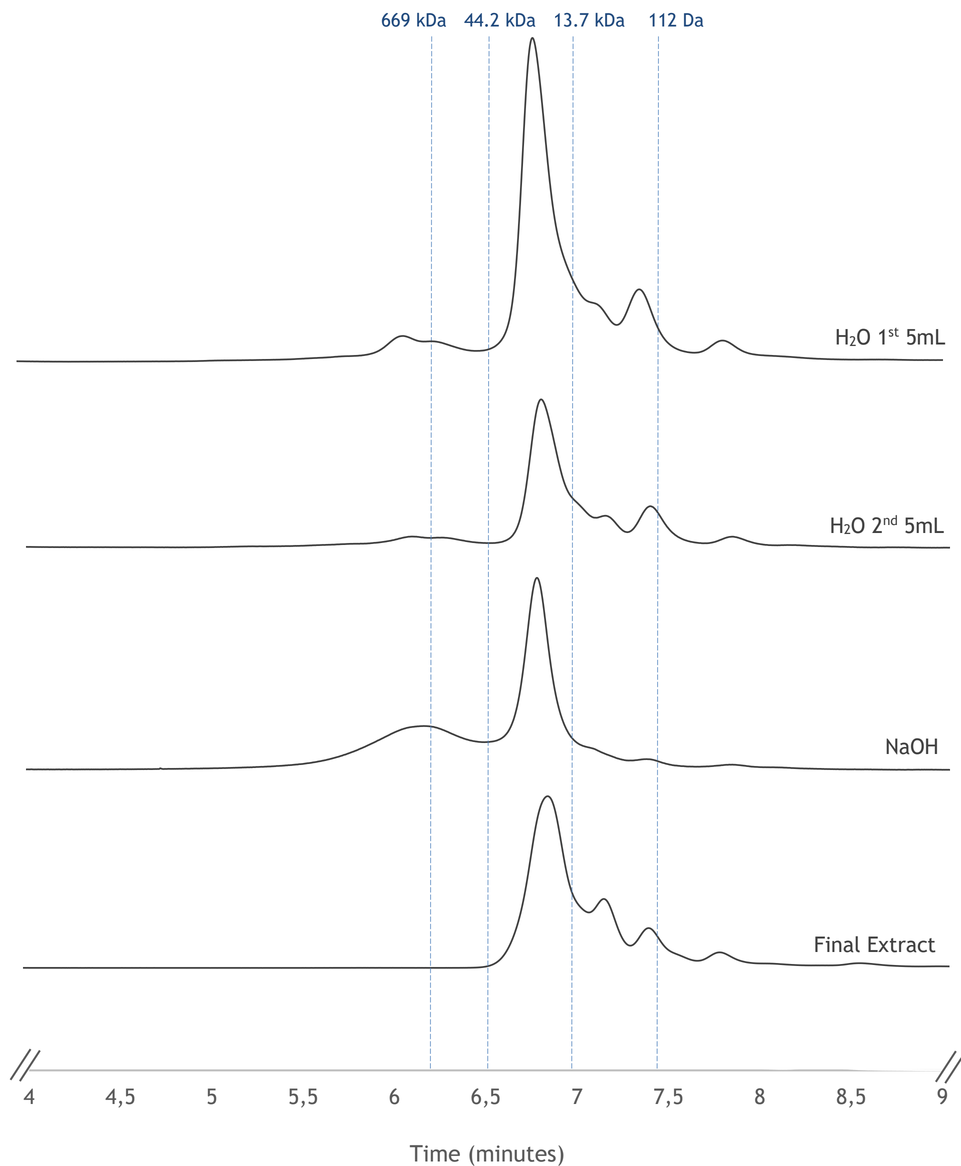


Figure 4.4. Chromatogram of samples after each extraction step: 1st and 2nd extractions with 5 mL H_2O in the Precellys homogenizer and conventional extraction with NaOH; and the extract obtained after hydrolysis at optimum conditions.

The extract protein profile exhibits a small peak, between 669 kDa and 44.2 kDa, indicative of the presence of large proteins in the samples. A more prominent and defined peak appears as well, representing proteins with sizes between 44.2 kDa and 13.7 kDa. Such peak, and the following smaller ones, indicate that the extracts are mainly composed of small proteins and macropeptides. *Ulva sp.* original proteins are being hydrolysed during the extraction process – which may have led to better than expected results for biological activities, namely regarding ACE-inhibition. These results are in agreement with the ones obtained by Kazir *et al.* [100]; several *Ulva sp.* protein extracts – obtained through several different methods, were analyzed by SDS-PAGE, and all extracts displayed bands with molecular weight of 10 000-12 000 Da. Another study analyzed the protein profile of *Ulva sp.* extracts using SDS-PAGE, and concluded that the profile changes with the season – with some protein bands being expressed only during

early winter. In all profiles obtained, a band below the 22 000 Da casein standard was present, thus indicating presence of small proteins. [113]

The chromatogram of the extract obtained after hydrolysis has the same large peak appearing at the same retention time as the other samples. However, this sample presents two peaks between 13.7 kDa and 112 Da, and a small peak after 112 Da, corresponding to low molecular weight peptides.

4.5 Chlorophylls and carotenoids profile

From analysis of the chlorophyll and carotenoid profile of *Ulva sp.*, a total of 4 carotenoids were identified, including ß-carotene and xantophylls violaxanthin, neoxanthin and lutein; besides non-identified chlorophylls. These carotenoids are the ones usually present in *Ulva sp.*, as reported in the literature. [126] The corresponding chromatogram is conveyed by Figure 4.5.

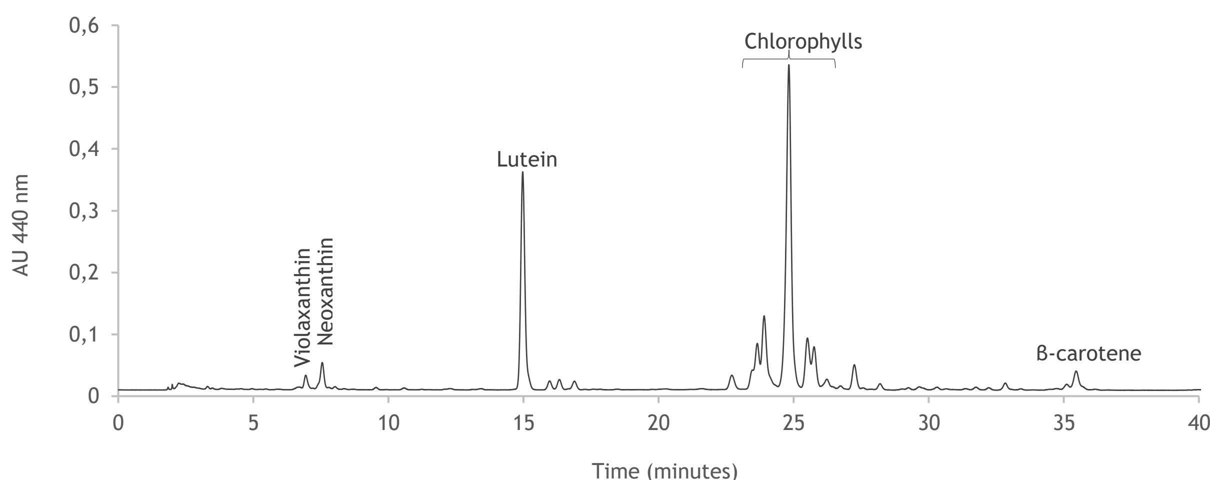


Figure 4.5. Chromatogram of sample after pre-treatment with EtOH for pigment removal: respective carotenoid and chlorophyll profile.

Carotenoids can be quite beneficial for human health – from lutein and zeaxanthin in human eyes, responsible for the filtering of the high-energy wavelengths of blue light (reducing oxidative stress on the retina), to playing a role as pro-vitamin A and antioxidant. Based on these benefits, carotenoids are sought for use in nutraceutical and pharmaceutical formulations. [127] Being a by-product of the protein extraction process developed in this work, said pigments could be concentrated and purified, toward a carotenoid-rich extract with great potential. [128] A closed loop biorefinery approach could even be studied in the future, as it has been suggested for microalgae [129,130], with the algae being used for wastewater treatment (while producing biogas) and the resulting biomass used as feedstock for the generation of secondary products, such as proteins, bioactive peptides, carbohydrates, lipids and carotenoids, for applications in feed or food supplements, bioenergy and high-value products such as cosmetics and pharmaceuticals.

5 Conclusions

The main goal of this work was to obtain a protein hydrolysate from marine macroalga *Ulva* sp., bearing potentially relevant biological activities – suitable to eventually serve as nutraceutical or pharmaceutical ingredient. Several extraction methods were accordingly studied; the best compromise between total extraction yield and pigment removal was obtained with a sequential extraction process: pre-treatment of 250 mg of alga biomass with 10 mL of EtOH, followed by two sequential 5 mL-extractions with H_2O in a Precellys homogenizer, and a final step of conventional extraction with NaOH over 60 minutes. This method led to a total extraction yield of 9.94 ± 0.41%, thus accounting for almost 50% of the algal total protein. The extract was then subjected to hydrolysis with alcalase, with optimum hydrolysis conditions determined by RSM.

The determined optimum hydrolysis conditions for maximum ACE-inhibitory activity and antioxidant capacity were attained upon a reaction time of 4.3 h and an E/S ratio of 2.4. The resulting hydrolysates exhibited ACE-inhibitory activity characterized by an IC_{50} of 92.3 ± 2.0 and 66.8 ± 1.0, for the total and <3 kDa fractions, respectively. In terms of antioxidant capacity, results were referred to hydrolysed protein for the ORAC method. Conversely, ABTS and DPPH results were expressed per unit amount of algal dry weight. Results obtained through the ORAC method for total and <3 kDa fractions were 1.96 ± 0.04 $\mu mol_{Trolox\ equivalent}/mg_{hydrolysed\ protein}$ and 2.60 ± 0.06 $\mu mol_{Trolox\ equivalent}/mg_{hydrolysed\ protein}$, respectively. Regarding the ABTS and DPPH assays, the hydrolysates attained values of 11.74 ± 0.19 $\mu mol_{Trolox\ equivalent}/g_{Alga\ DW}$ and 1.74 ± 0.06 $\mu mol_{Trolox\ equivalent}/g_{Alga\ DW}$, respectively. The results obtained for ACE-inhibitory activity are promising, since they lie significantly above those described in the literature for similar protein hydrolysates of macroalgae. On the other hand, the antioxidant capacity was relatively low per the ORAC assay; while results obtained via DPPH and ABTS methods were in line with those reported in the literature for algal extracts.

The obtained extract showed significant results as an ACE-inhibitor. Containing 6 out of the 10 essential amino acids, it proves to have great promise as a food product or to be included in food formulations. Additionally, it also contains value-added products, such as carotenoids, that may also exhibit bioactivity. Overall, it is a complete product that shows potential be used in nutraceutical, pharmaceutical and food applications.

6 Final assessment and future work

The work developed for this Master thesis allowed for the development of a method for protein extraction from green seaweed *Ulva sp.*, that proved efficient in producing extracts with significant biological activity following hydrolysis. Although a good deal of data has been generated, there is still a considerable amount of work to be done before the methodologies herein developed can be applied in an industrial setting for food or pharmaceutical production.

Concerning the optimized protein extraction method, it would be important to implement an additional step for polysaccharide extraction and separation, thus allowing the process to yield a proper protein concentrate. The pigment removal method applied here should also be studied further, with more efficient processes tested that would allow for total color removal. Moreover, and on a later stage of the process, the scalability and economic feasibility of the process should the thoroughly analyzed.

Regarding hydrolysis, detailed studies on the effect of other enzymes in the process are welcome. For industrial applications, the immobilization of the hydrolytic enzyme should be tested, for recyclability and cost-effectiveness. Additionally, other biological activities could be tested, namely anti-inflammatory and anti-microbial. The study of mechanisms underlying the extracts' biological activities would also be of uttermost interest, providing insight into a little-known research area.

Most importantly, the *in vivo* activity of extracts or pure peptides obtained should be analyzed – check if activity is maintained after gastro-intestinal digestion and after that, verify their bioavailability in the bloodstream. Furthermore, the performance of the extracts should also be tested in actual food systems, and their delivery method hypothesized (for example, through microencapsulation).

7 References

[1] **United Nations, Department of Economic and Social Affair - Sustainable Development**. *Do you know all 17 SDGs?* Retrieved from https://sdgs.un.org/goals#goals

[2] **United Nations**. *Sustainable Development Goals*. Retrieved from https://www.un.org/sustainabledevelopment/sustainable-development-goals/

[3] **WFP, W., & UNICEF.** (2022). The State of Food Security and Nutrition in the World 2022.

[4] **United Nations.** (2022). *The Sustainable Development Goals Report 2022*

[5] Bankova, D., Kumar Dutta, P., & Ovaska, M. (2020, May 30 2022). The war in Ukraine is fuelling a global food crisis. *Reuters*.

[6] The War in Ukraine Stokes Global Food Inflation. (2022, 08 June 2022). *Goldman Sachs*.

[7] **United Nations - Department of Economic and Social Affairs** (2022). *World Population Prospects 2022 - Summary of Results*

[8] Aylward, F. (1962). *An assessment of the world's protein needs and supplies*. Paper presented at the The Better Use of the World's Fauna for Food, Symp. Inst. Biol.

[9] **Sukhatme, P.** (1961). The world's hunger and future needs in food supplies. *Journal of the Royal Statistical Society: Series A (General), 124*(4), 463-508.

[10] **Woodham, A.** (1971). The world protein shortage: prevention and cure. *World review of nutrition and dietetics, 13*, 1-42.

[11] Sweet, L. (2019). How can we produce enough protein to feed 10 billion people? *World Economic Forum*.

[12] **Espinosa-Marron, A., Adams, K., Sinno, L., Cantu-Aldana, A., Tamez, M., Marrero, A., *et al*.** Environmental Impact of Animal-Based Food Production and the Feasibility of a Shift Toward Sustainable Plant-Based Diets in the United States. *Frontiers in Sustainability*, 19.

[13] **Henchion, M., Hayes, M., Mullen, A. M., Fenelon, M., & Tiwari, B.** (2017). Future protein supply and demand: strategies and factors influencing a sustainable equilibrium. *Foods, 6*(7), 53.

[14] **Atmosfera, I. P. d. M. e. d.** (2018). Diretiva-Quadro "Estratégia Marinha". Descritor 7 - Condições Hidrográficas. Relatório do segundo ciclo de avaliação do bom estado ambiental das águas marinhas nas subdivisões do Continente e da Plataforma Continental Estendida.

[15] **República Portuguesa.** (2021). *National Ocean Strategy 2021-2030*

[16] **Pereira, L.** (2008). As algas marinhas e respectivas utilidades. *Monografias, 913*, 1-19.

[17] **Algaplus**. Retrieved from https://www.algaplus.pt/

[18] **Chronakis, I. S., & Madsen, M.** (2011). Algal proteins. In *Handbook of food proteins* (pp. 353-394): Elsevier.

[19] **Geada, P., Moreira, C., Silva, M., Nunes, R., Madureira, L., Rocha, C. M., *et al*.** (2021). Algal proteins: Production strategies and nutritional and functional properties. *Bioresource Technology, 332*, 125125.

[20] **Craggs, R., Sutherland, D., & Campbell, H.** (2012). Hectare-scale demonstration of high rate algal ponds for enhanced wastewater treatment and biofuel production. *Journal of Applied Phycology, 24*(3), 329-337.

[21] **Tango, M. D., Calijuri, M. L., Assemany, P. P., & do Couto, E. d. A.** (2018). Microalgae cultivation in agro-industrial effluents for biodiesel application: effects of the availability of nutrients. *Water Science and Technology, 78*(1), 57-68.

[22] **Vale, M. A., Ferreira, A., Pires, J. C., & Gonçalves, A. L.** (2020). CO2 capture using microalgae. In *Advances in Carbon Capture* (pp. 381-405): Elsevier.

[23] **Richmond, A.** (2008). *Handbook of microalgal culture: biotechnology and applied phycology*: John Wiley & Sons.

[24] **Sánchez, J., Curt, M. D., Robert, N., & Fernández, J.** (2019). Biomass resources. In *The role of bioenergy in the bioeconomy* (pp. 25-111): Elsevier.

[25] **Koyande, A. K., Chew, K. W., Rambabu, K., Tao, Y., Chu, D.-T., & Show, P.-L.** (2019). Microalgae: A potential alternative to health supplementation for humans. *Food Science and Human Wellness, 8*(1), 16-24.

[26] **Sathasivam, R., Radhakrishnan, R., Hashem, A., & Abd_Allah, E. F.** (2019). Microalgae metabolites: A rich source for food and medicine. *Saudi journal of biological sciences, 26*(4), 709-722.

[27] **Lafarga, T.** (2019). Effect of microalgal biomass incorporation into foods: Nutritional and sensorial attributes of the end products. *Algal Research, 41*, 101566.

[28] **Shackira, A., Jazeel, K., & Puthur, J. T.** (2021). Phycoremediation and phytoremediation: Promising tools of green remediation. In *Sustainable Environmental Clean-up* (pp. 273-293): Elsevier.

[29] **Bleakley, S., & Hayes, M.** (2017). Algal proteins: extraction, application, and challenges concerning production. *Foods, 6*(5), 33.

[30] **Serrano, O., Kelleway, J. J., Lovelock, C., & Lavery, P. S.** (2019). Conservation of blue carbon ecosystems for climate change mitigation and adaptation. In *Coastal wetlands* (pp. 965-996): Elsevier.

[31] **Fleurence, J., Morançais, M., & Dumay, J.** (2018). Seaweed proteins. In *Proteins in food processing* (pp. 245-262): Elsevier.

[32] **Panggabean, J. A., Adiguna, S. b. P., Rahmawati, S. I., Ahmadi, P., Zainuddin, E. N., Bayu, A., *et al*.** (2022). Antiviral Activities of Algal-Based Sulfated Polysaccharides. *Molecules, 27*(4), 1178.

[33] **Pliego-Cortés, H., Wijesekara, I., Lang, M., Bourgougnon, N., & Bedoux, G.** (2020). Current knowledge and challenges in extraction, characterization and bioactivity of seaweed protein and seaweed-derived proteins. *Advances in botanical research, 95*, 289-326.

[34] **Vázquez-Rodríguez, J. A., & Amaya-Guerra, C. A.** (2016). Ulva genus as alternative crop: Nutritional and functional properties. In *Alternative Crops and Cropping Systems* (pp. 29-44): IntechOpen.

[35] **Tsubaki, S., Oono, K., Hiraoka, M., Ueda, T., Onda, A., Yanagisawa, K., *et al*.** (2014). Hydrolysis of green-tide forming Ulva spp. by microwave irradiation with polyoxometalate clusters. *Green Chemistry, 16*(4), 2227-2233.

[36] **El-din, S., & Hassan, S. M.** (2016). The promotive effect of different concentrations of marine algae on spinach plants (Spinacia oleracea L.). *Egypt J. Hort, 43*, 109-122.

[37] Sinurat, E., Kusumawaty, R., & Fransiska, D. (2021). *Effect different sampling times of Ulva sp on polysaccharide sulfate content*. Paper presented at the Journal of Physics: Conference Series.

[38] **Benjama, O., & Masniyom, P.** (2011). Nutritional composition and physicochemical properties of two green seaweeds (Ulva pertusa and U. intestinalis) from the Pattani Bay in Southern Thailand. *Sonklanakarin Journal of Science and Technology, 33*(5), 575.

[39] **Paiva, L., Lima, E., Neto, A. I., Marcone, M., & Baptista, J.** (2017). Nutritional and functional bioactivity value of selected Azorean macroalgae: Ulva compressa, Ulva rigida, Gelidium microdon, and Pterocladiella capillacea. *Journal of Food Science, 82*(7), 1757-1764.

[40] **Banach, J., Hoek-van den Hil, E., & van der Fels-Klerx, H.** (2020). Food safety hazards in the European seaweed chain. *Comprehensive reviews in food science and food safety, 19*(2), 332-364.

[41] **Pulz, O., & Gross, W.** (2004). Valuable products from biotechnology of microalgae. *Applied microbiology and biotechnology, 65*(6), 635-648.

[42] **Chojnacka, K., Wieczorek, P. P., Schroeder, G., & Michalak, I.** (2018). *Algae biomass: Characteristics and applications: Towards algae-based products* (Vol. 8): Springer.

[43] **Harnedy, P. A., & FitzGerald, R. J.** (2011). Bioactive proteins, peptides, and amino acids from macroalgae 1. *Journal of Phycology, 47*(2), 218-232.

[44] **Gordalina, M., Pinheiro, H. M., Mateus, M., da Fonseca, M. M. R., & Cesário, M. T.** (2021). Macroalgae as protein sources—a review on protein bioactivity, extraction, purification and characterization. *Applied Sciences, 11*(17), 7969.

[45] **Fleurence, J., Le Coeur, C., Mabeau, S., Maurice, M., & Landrein, A.** (1995). Comparison of different extractive procedures for proteins from the edible seaweeds Ulva rigida and Ulva rotundata. *Journal of Applied Phycology, 7*(6), 577-582.

[46] **Juul, L., Danielsen, M., Nebel, C., Steinhagen, S., Bruhn, A., Jensen, S., *et al*.** (2021). Ulva fenestrata protein-Comparison of three extraction methods with respect to protein yield and protein quality. *Algal Research, 60*, 102496.

[47] **Serot, T., Courcoux, P., & Guillemineau, F.** (1994). Extraction and partial characterization of protein from the green algae Ulva sp. *Sciences des aliments, 14*(3), 301-309.

[48] **Barbarino, E., & Lourenço, S. O.** (2005). An evaluation of methods for extraction and quantification of protein from marine macro-and microalgae. *Journal of Applied Phycology, 17*(5), 447-460.

[49] **Fleurence, J., Massiani, L., Guyader, O., & Mabeau, S.** (1995). Use of enzymatic cell wall degradation for improvement of protein extraction from Chondrus crispus, Gracilaria verrucosa and Palmaria palmata. *Journal of Applied Phycology, 7*(4), 393-397.

[50] **Chemat, F., & Khan, M. K.** (2011). Applications of ultrasound in food technology: processing, preservation and extraction. *Ultrasonics sonochemistry, 18*(4), 813-835.

[51] **Kadam, S. U., Álvarez, C., Tiwari, B. K., & O'Donnell, C. P.** (2017). Extraction and characterization of protein from Irish brown seaweed Ascophyllum nodosum. *Food Research International, 99*, 1021-1027.

[52] **Sánchez, A., & Vázquez, A.** (2017). Bioactive peptides: A review. *Food Quality and Safety, 1*(1), 29-46.

[53] **Dziuba, M., & Darewicz, M.** (2007). Food proteins as precursors of bioactive peptides—classification into families. *Food Science and Technology International, 13*(6), 393-404.

[54] **Korhonen, H., & Pihlanto, A.** (2006). Bioactive peptides: production and functionality. *International Dairy Journal, 16*(9), 945-960.

[55] **Daliri, E. B.-M., Oh, D. H., & Lee, B. H.** (2017). Bioactive peptides. *Foods, 6*(5), 32.

[56] **Moura, J. J., Moura, I., & Maia, L. B.** (2021). *Enzymes for Solving Humankind's Problems*: Springer.

[57] **Rawlings, N. D., Waller, M., Barrett, A. J., & Bateman, A.** (2014). MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic acids research, 42*(D1), D503-D509.

[58] **Fernández-Musoles, R., Manzanares, P., Burguete, M. C., Alborch, E., & Salom, J. B.** (2013). In vivo angiotensin I-converting enzyme inhibition by long-term intake of antihypertensive lactoferrin hydrolysate in spontaneously hypertensive rats. *Food Research International, 54*(1), 627-632.

[59] **García-Tejedor, A., Sanchez-Rivera, L., Castelló-Ruiz, M., Recio, I., Salom, J. B., & Manzanares, P.** (2014). Novel antihypertensive lactoferrin-derived peptides produced by Kluyveromyces marxianus: gastrointestinal stability profile and in vivo angiotensin I-converting enzyme (ACE) inhibition. *Journal of Agricultural and Food Chemistry, 62*(7), 1609-1616.

[60] **Tavares, T. G., Amorim, M., Gomes, D., Pintado, M. E., Pereira, C. D., & Malcata, F. X.** (2012). Manufacture of bioactive peptide-rich concentrates from Whey: Characterization of pilot process. *Journal of Food Engineering, 110*(4), 547-552.

[61] **Udenigwe, C. C., & Aluko, R. E.** (2012). Food protein-derived bioactive peptides: production, processing, and potential health benefits. *Journal of Food Science, 77*(1), R11-R24.

[62] **Sarabia, L., & Ortiz, M.** (2009). Response surface methodology, vol. 1. In: Elsevier, Amsterdam.

[63] **Agarwal, R. K., & Bosco, S.** (2014). Optimization of Viscozyme-L assisted extraction of coconut milk and Virgin Coconut Oil. *Asian Journal of Dairy & Food Research*.

[64] **del Mar Contreras, M., Hernández-Ledesma, B., Amigo, L., Martín-Álvarez, P. J., & Recio, I.** (2011). Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: Optimization by response surface methodology. *LWT-Food Science and Technology, 44*(1), 9-15.

[65] **Guan, X., & Yao, H.** (2008). Optimization of Viscozyme L-assisted extraction of oat bran protein using response surface methodology. *Food chemistry, 106*(1), 345-351.

[66] **See, S., Hoo, L., & Babji, A.** (2011). Optimization of enzymatic hydrolysis of Salmon (Salmo salar) skin by Alcalase. *International Food Research Journal, 18*(4), 1359.

[67] **Möller, N. P., Scholz-Ahrens, K. E., Roos, N., & Schrezenmeir, J.** (2008). Bioactive peptides and proteins from foods: indication for health effects. *European journal of nutrition, 47*(4), 171-182.

[68] **Quan, A.** (2006). Fetopathy associated with exposure to angiotensin converting enzyme inhibitors and angiotensin receptor antagonists. *Early human development, 82*(1), 23-28.

[69] **Laube, G. F., Kemper, M. J., Schubiger, G., & Neuhaus, T. J.** (2007). Angiotensin-converting enzyme inhibitor fetopathy: long-term outcome. *Archives of Disease in Childhood-Fetal and Neonatal Edition, 92*(5), F402-F403.

[70] **Pryde, P. G., Sedman, A. B., Nugent, C. E., & Barr, M.** (1993). Angiotensin-converting enzyme inhibitor fetopathy. *Journal of the American Society of Nephrology, 3*(9), 1575-1582.

[71] **Fitzgerald, C. n., Mora-Soler, L., Gallagher, E., O'Connor, P., Prieto, J., Soler-Vila, A., *et al*.** (2012). Isolation and characterization of bioactive pro-peptides with in vitro renin inhibitory activities from the macroalga Palmaria palmata. *Journal of Agricultural and Food Chemistry, 60*(30), 7421-7427.

[72] **Furuta, T., Miyabe, Y., Yasui, H., Kinoshita, Y., & Kishimura, H.** (2016). Angiotensin I converting enzyme inhibitory peptides derived from phycobiliproteins of dulse Palmaria palmata. *Marine drugs, 14*(2), 32.

[73] **Paiva, L., Lima, E., Neto, A. I., & Baptista, J.** (2016). Isolation and characterization of angiotensin I-converting enzyme (ACE) inhibitory peptides from Ulva rigida C. Agardh protein hydrolysate. *Journal of Functional Foods, 26*, 65-76.

[74] **Sun, S., Xu, X., Sun, X., Zhang, X., Chen, X., & Xu, N.** (2019). Preparation and identification of ACE inhibitory peptides from the marine macroalga Ulva intestinalis. *Marine drugs, 17*(3), 179.

[75] **Pan, S., Wang, S., Jing, L., & Yao, D.** (2016). Purification and characterisation of a novel angiotensin-I converting enzyme (ACE)-inhibitory peptide derived from the enzymatic hydrolysate of Enteromorpha clathrata protein. *Food chemistry, 211*, 423-430.

[76] **Cao, D., Lv, X., Xu, X., Yu, H., Sun, X., & Xu, N.** (2017). Purification and identification of a novel ACE inhibitory peptide from marine alga Gracilariopsis lemaneiformis protein hydrolysate. *European Food Research and Technology, 243*(10), 1829-1837.

[77] **Joel, C. H., Sutopo, C. C., Prajitno, A., Su, J.-H., & Hsu, J.-L.** (2018). Screening of angiotensin-I converting enzyme inhibitory peptides derived from Caulerpa lentillifera. *Molecules, 23*(11), 3005.

[78] **Deng, Z., Liu, Y., Wang, J., Wu, S., Geng, L., Sui, Z., *et al*.** (2018). Antihypertensive effects of two novel angiotensin I-converting enzyme (ACE) inhibitory peptides from Gracilariopsis lemaneiformis (Rhodophyta) in spontaneously hypertensive rats (SHRs). *Marine drugs, 16*(9), 299.

[79] **Wu, H., Xu, N., Sun, X., Yu, H., & Zhou, C.** (2015). Hydrolysis and purification of ACE inhibitory peptides from the marine microalga Isochrysis galbana. *Journal of Applied Phycology, 27*(1), 351-361.

[80] **Haque, E., & Chand, R.** (2008). Antihypertensive and antimicrobial bioactive peptides from milk proteins. *European Food Research and Technology, 227*(1), 7-15.

[81] **Vermeirssen, V., Van Camp, J., & Verstraete, W.** (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition, 92*(3), 357-366.

[82] **Suetsuna, K., Ukeda, H., & Ochi, H.** (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *The Journal of nutritional biochemistry, 11*(3), 128-131.

[83] **Smith, M. A., Rottkamp, C. A., Nunomura, A., Raina, A. K., & Perry, G.** (2000). Oxidative stress in Alzheimer's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1502*(1), 139-144.

[84] **Bolton, J. L., Trush, M. A., Penning, T. M., Dryhurst, G., & Monks, T. J.** (2000). Role of quinones in toxicology. *Chemical research in toxicology, 13*(3), 135-160.

[85] **Frankel, E. N., & Meyer, A. S.** (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture, 80*(13), 1925-1941.

[86] **Cian, R. E., Garzón, A. G., Ancona, D. B., Guerrero, L. C., & Drago, S. R.** (2015). Hydrolyzates from Pyropia columbina seaweed have antiplatelet aggregation, antioxidant and ACE I inhibitory peptides which maintain bioactivity after simulated gastrointestinal digestion. *LWT-Food Science and Technology, 64*(2), 881-888.

[87] **Wang, W., & De Mejia, E. G.** (2005). A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. *Comprehensive reviews in food science and food safety, 4*(4), 63-78.

[88] **Rajapakse, N., Mendis, E., Jung, W.-K., Je, J.-Y., & Kim, S.-K.** (2005). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Research International, 38*(2), 175-182.

[89] **McKellar, R. C.** (1981). Development of Off-Flavors in Ultra-High Temperature and Pasteurized Milk as a Function of Proteolysis1. *Journal of Dairy Science, 64*(11), 2138-2145.

[90] **Baek, H., & Cadwallader, K.** (1995). Enzymatic hydrolysis of crayfish processing by-products. *Journal of Food Science, 60*(5), 929-935.

[91] **Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L.** (2005). Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry, 53*(3), 588-593.

[92] **Magalhães, L., Ramos, I., Reis, S., & Segundo, M.** (2014). Antioxidant profile of commercial oenological tannins determined by multiple chemical assays. *Australian journal of grape and wine research, 20*(1), 72-79.

[93] **Suriyatem, R., Auras, R. A., Intipunya, P., & Rachtanapun, P.** (2017). Predictive mathematical modeling for EC50 calculation of antioxidant activity and antibacterial ability of Thai bee products. *Journal of Applied Pharmaceutical Science, 7*(9), 122-133.

[94] **Vermeirssen, V., Van Camp, J., & Verstraete, W.** (2002). Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. *Journal of biochemical and biophysical methods, 51*(1), 75-87.

[95] **Nollet, L. M., & Toldrá, F.** (2012). *Handbook of analysis of active compounds in functional foods*: CRC Press.

[96] **Gajaria, T. K., Suthar, P., Baghel, R. S., Balar, N. B., Sharnagat, P., Mantri, V. A., *et al.*** (2017). Integration of protein extraction with a stream of byproducts from marine macroalgae: a model forms the basis for marine bioeconomy. *Bioresource Technology, 243*, 867-873.

[97] **Shuuluka, D., Bolton, J. J., & Anderson, R. J.** (2013). Protein content, amino acid composition and nitrogen-to-protein conversion factors of Ulva rigida and Ulva capensis from natural populations and Ulva lactuca from an aquaculture system, in South Africa. *Journal of Applied Phycology, 25*(2), 677-685.

[98] **Yaich, H., Garna, H., Besbes, S., Paquot, M., Blecker, C., & Attia, H.** (2011). Chemical composition and functional properties of Ulva lactuca seaweed collected in Tunisia. *Food chemistry, 128*(4), 895-901.

[99] **Moustafa, Y., & Eladel, H.** (2016). Amino acids profile of Ulva sp. macroalgae collected from Alexandria coast in Egypt: a potential food resource. *Poi. J. Bot. Micr. Res, 1*, 15-22.

[100] **Kazir, M., Abuhassira, Y., Robin, A., Nahor, O., Luo, J., Israel, A., *et al.*** (2019). Extraction of proteins from two marine macroalgae, Ulva sp. and Gracilaria sp., for food application, and evaluating digestibility, amino acid composition and antioxidant properties of the protein concentrates. *Food Hydrocolloids, 87*, 194-203.

[101] **Council, N. R.** (1989). Protein and amino acids. In *Recommended Dietary Allowances: 10th Edition*: National Academies Press (US).

[102] **Kientz, B., Thabard, M., Cragg, S. M., Pope, J., & Hellio, C.** (2011). A new method for removing microflora from macroalgal surfaces: an important step for natural product discovery.

[103] **Hosikian, A., Lim, S., Halim, R., & Danquah, M. K.** (2010). Chlorophyll extraction from microalgae: a review on the process engineering aspects. *International journal of chemical engineering, 2010*.

[104] **Wright, S., Jeffrey, S., & Mantoura, R.** (2005). *Phytoplankton pigments in oceanography: guidelines to modern methods*: Unesco Pub. Paris, France.

[105] **Simon, D., & Helliwell, S.** (1998). Extraction and quantification of chlorophyll a from freshwater green algae. *Water research, 32*(7), 2220-2223.

[106] **Schumann, R., Häubner, N., Klausch, S., & Karsten, U.** (2005). Chlorophyll extraction methods for the quantification of green microalgae colonizing building facades. *International Biodeterioration & Biodegradation, 55*(3), 213-222.

[107] **Sartory, D., & Grobbelaar, J.** (1984). Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis. *Hydrobiologia, 114*(3), 177-187.

[108] **Macías-Sánchez, M., Mantell, C., Rodriguez, M., de la, De La Ossa, E. M., Lubián, L., & Montero, O.** (2009). Comparison of supercritical fluid and ultrasound-assisted extraction of carotenoids and chlorophyll a from Dunaliella salina. *Talanta, 77*(3), 948-952.

[109] **Kundu, S., Aswal, V., & Kohlbrecher, J.** (2017). Effect of ethanol on structures and interactions among globular proteins. *Chemical Physics Letters, 670*, 71-76.

[110] **Dufour, E., & Haertl´, T.** (1990). Alcohol-induced changes of ß-lactoglobulin-retinol-binding stoichiometry. *Protein Engineering, Design and Selection, 4*(2), 185-190.

[111] **Harnedy, P. A., & FitzGerald, R. J.** (2013). Extraction of protein from the macroalga Palmaria palmata. *LWT-Food Science and Technology, 51*(1), 375-382.

[112] **Chronakis, I. S.** (2000). Biosolar proteins from aquatic algae. In *Developments in food science* (Vol. 41, pp. 39-75): Elsevier.

[113] **Wijesekara, I., Lang, M., Marty, C., Gemin, M.-P., Boulho, R., Douzenel, P., *et al*.** (2017). Different extraction procedures and analysis of protein from Ulva sp. in Brittany, France. *Journal of Applied Phycology, 29*(5), 2503-2511.

[114] **Zulueta, A., Esteve, M. J., & Frígola, A.** (2009). ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food chemistry, 114*(1), 310-316.

[115] **Myers, R. H., Montgomery, D. C., & Anderson-Cook, C. M.** (2016). *Response surface methodology: process and product optimization using designed experiments*: John Wiley & Sons.

[116] **Box, G. E., & Draper, N. R.** (2007). *Response surfaces, mixtures, and ridge analyses*: John Wiley & Sons.

[117] Jatmiko, T., Prasetyo, D., Poeloengasih, C., & Khasanah, Y. (2019). *Nutritional Evaluation of Ulva sp. from Sepanjang Coast, Gunungkidul, Indonesia*. Paper presented at the IOP Conference Series: Earth and Environmental Science.

[118] **McManus, J. P., Davis, K. G., Beart, J. E., Gaffney, S. H., Lilley, T. H., & Haslam, E.** (1985). Polyphenol interactions. Part 1. Introduction; some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *Journal of the Chemical Society, Perkin Transactions* 2(9), 1429-1438.

[119] **Tavares, T., Contreras, M., Amorim, M., Martín-Álvarez, P., Pintado, M., Recio, I., *et al*.** (2011). Optimisation, by response surface methodology, of degree of hydrolysis and antioxidant and ACE-inhibitory activities of whey protein hydrolysates obtained with cardoon extract. *International Dairy Journal, 21*(12), 926-933.

[120] **Kumagai, Y., Kitade, Y., Kobayashi, M., Watanabe, K., Kurita, H., Takeda, H., *et al*.** (2020). Identification of ACE inhibitory peptides from red alga Mazzaella japonica. *European Food Research and Technology, 246*(11), 2225-2231.

[121] **Purcell, D., Packer, M. A., & Hayes, M.** (2022). Angiotensin-I-Converting Enzyme Inhibitory Activity of Protein Hydrolysates Generated from the Macroalga Laminaria digitata (Hudson) JV Lamouroux 1813. *Foods, 11*(12), 1792.

[122] **Amorim, A. P., Silva, G. H., Brandao, R. M., Porto, A. L. F., & Bezerra, R. P.** (2022). Algae as a source of peptides inhibitors of the angiotensin-converting enzyme: a systematic review. *Anais da Academia Brasileira de Ciências, 94*.

[123] **Vega, J., Bonomi-Barufi, J., Gómez-Pinchetti, J. L., & Figueroa, F. L.** (2020). Cyanobacteria and red macroalgae as potential sources of antioxidants and UV radiation-absorbing compounds for cosmeceutical applications. *Marine drugs, 18*(12), 659.

[124] **Habeebullah, S. F. K., Alagarsamy, S., Sattari, Z., Al-Haddad, S., Fakhraldeen, S., Al-Ghunaim, A., *et al*.** (2021). Effect of enzymatic hydrolysis on the antioxidant activity of red and green seaweeds and characterization of the active extracts. *Journal of the American Oil Chemists' Society, 98*(2), 185-200.

[125] **Jahandideh, F., Chakrabarti, S., Davidge, S. T., & Wu, J.** (2016). Antioxidant peptides identified from ovotransferrin by the ORAC method did not show anti-inflammatory and antioxidant activities in endothelial cells. *Journal of Agricultural and Food Chemistry, 64*(1), 113-119.

[126] **Eismann, A. I., Reis, R. P., da Silva, A. F., & Cavalcanti, D. N.** (2020). Ulva spp. carotenoids: Responses to environmental conditions. *Algal Research, 48*, 101916.

[127] **Ngamwonglumlert, L., Devahastin, S., & Food, A.** (2019). Carotenoids. *Encyclopedia of food chemistry, 1*, 40-52.

[128] **Pinho, L. S., de Lima, P. M., de Sá, S. H. G., Chen, D., Campanella, O. H., da Costa Rodrigues, C. E., *et al*.** (2022). Encapsulation of Rich-Carotenoids Extract from Guaraná (Paullinia cupana) Byproduct by a Combination of Spray Drying and Spray Chilling. *Foods, 11*(17), 2557.

[129] **Mohan, S. V., Hemalatha, M., Chakraborty, D., Chatterjee, S., Ranadheer, P., & Kona, R.** (2020). Algal biorefinery models with self-sustainable closed loop approach: Trends and prospective for blue-bioeconomy. *Bioresource Technology, 295*, 122128.

[130] **Fabris, M., Abbriano, R. M., Pernice, M., Sutherland, D. L., Commault, A. S., Hall, C. C., *et al*.** (2020). Emerging technologies in algal biotechnology: toward the establishment of a sustainable, algae-based bioeconomy. *Frontiers in plant science, 11*, 279.

[131] **Magalhães, L. M., Barreiros, L., Maia, M. A., Reis, S., & Segundo, M. A.** (2012). Rapid assessment of endpoint antioxidant capacity of red wines through microchemical methods using a kinetic matching approach. *Talanta, 97*, 473-483.

Appendix A

Antioxidant capacity determination assays use standard compounds, usually Trolox (as used in this work), and the antioxidant capacity of samples is expressed as equivalents of that given standard. Assays are, for that reason, usually performed in the reaction time referring to that standard compound. However, the reaction kinetics presented presented by these standards is often different from that exhibited by other products and/or matrices. In order to obtain reliable results for total antioxidant capacity of a sample, absorbance measurements should be taken at the reaction's endpoint condition of the sample. For this reason, a kinetic matching approach was implemented, as proposed by Magalhães *et al.* [131], to assess the endpoint of the oxidation reaction for the algal samples. This approach was performed for the ABTS and DPPH assays, and the obtained results are presented in Figures A.1 and A.2, respectively. Assays were performed as described in Section 3 with a single test sample, and absorbance was recorded every minute for 400 minutes and 200 minutes, for ABTS and DPPH assays, respectively.

Upon analysis of the obtained results, one can verify that when the absorbance values determined for the sample after 30 and 300 minutes (ABTS assay) are interpolated in the Trolox calibration curve determined at the same reaction time, the antioxidant capacity values increase as the reaction time increases. This proves the need for the oxidation kinetics of samples be first examined and measurements be taken at a time when the reaction has reached the endpoint conditions (when constant absorbance values are verified). It was then concluded that the endpoint antioxidant capacity of samples was given after 300 minutes for the ABTS assay, and after 30 minutes for the DPPH assay.

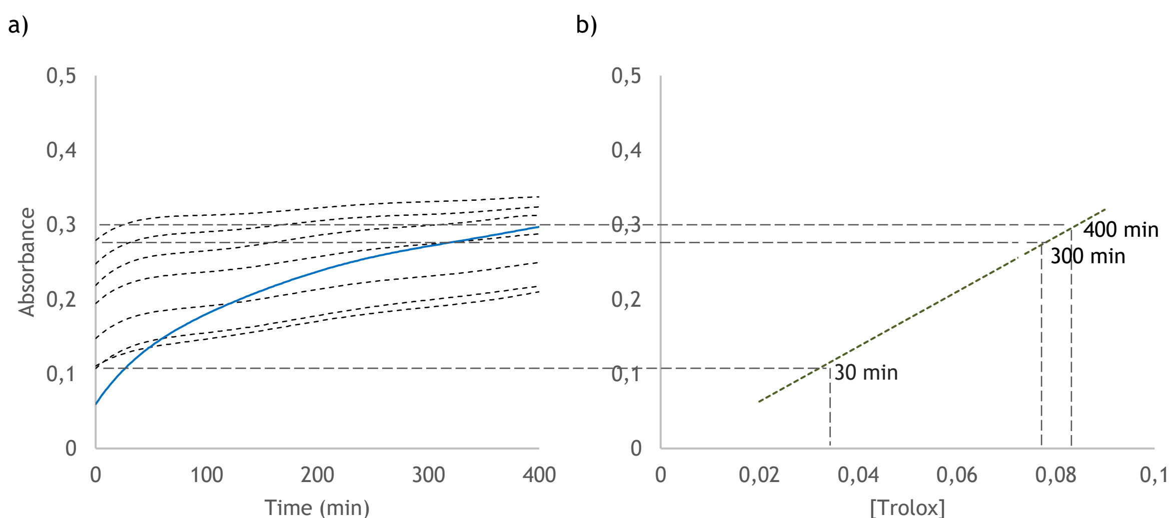


Figure A.1. Antioxidant capacity dependency on reaction time for the ABTS assay. a) Absorbance of Trolox standards (black dotted lines) and test sample (blue line) over time; b) Absorbance *vs.* Trolox standards concentration.

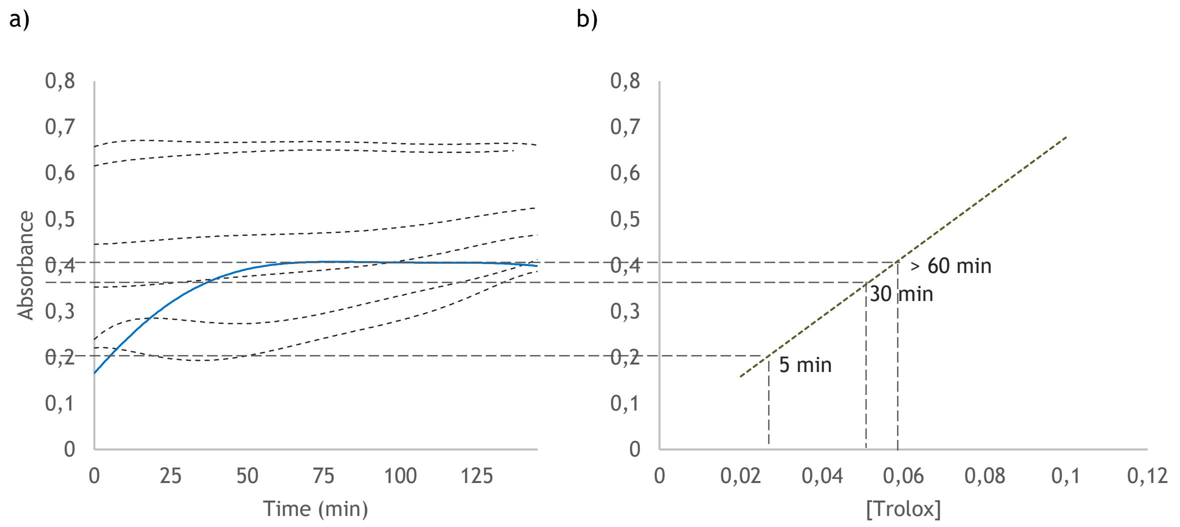


Figure A.2. Antioxidant capacity dependency on reaction time for the ABTS assay. a) Absorbance of Trolox standards (black dotted lines) and test sample (blue line) over time; b) Absorbance *vs.* Trolox standards concentration.

Appendix B

Diagnostics graphs were obtained through the Design Expert software for each response parameter analyzed – DH, ORAC assay, ABTS assay and DPPH assay, as well as the transformed data for ACE inhibitory activity – ln (IACE). Graphs are plotted in Figures B.1 to B.5.

The normal probability plot, shown in Figures B.1-5 a), indicates if the data's residuals follow a normal distribution – data should be approximately linear with some moderate scatter. The Residuals *vs.* Run, plotted in Figures B.1-5 b), shows how lurking variables may have influenced response during the experiment. This graph should show a random scatter within the defined red lines. Plotted in Figures B.1-5 c) and d) are Residuals *vs.* Factor graphs (i.e., Reaction Time and/or E/S ratio), which allows to check for variance remaining after the model has been fit and to confirm if variance is stable at different factor settings. The Residuals *vs.* Predicted Plot, Figures B.1-5 e), is useful for outlier examination (runs with residuals outside the plot's red lines). If an outlier is observed, the choice of model should be analyzed. The Cook's Distance plot, in Figures B.1-5 f), measures how much the model regression changes if a case/experiment is deleted. Large values should be analyzed for recording or calculation errors, the wrong model choice or a design point far from the remaining ones.

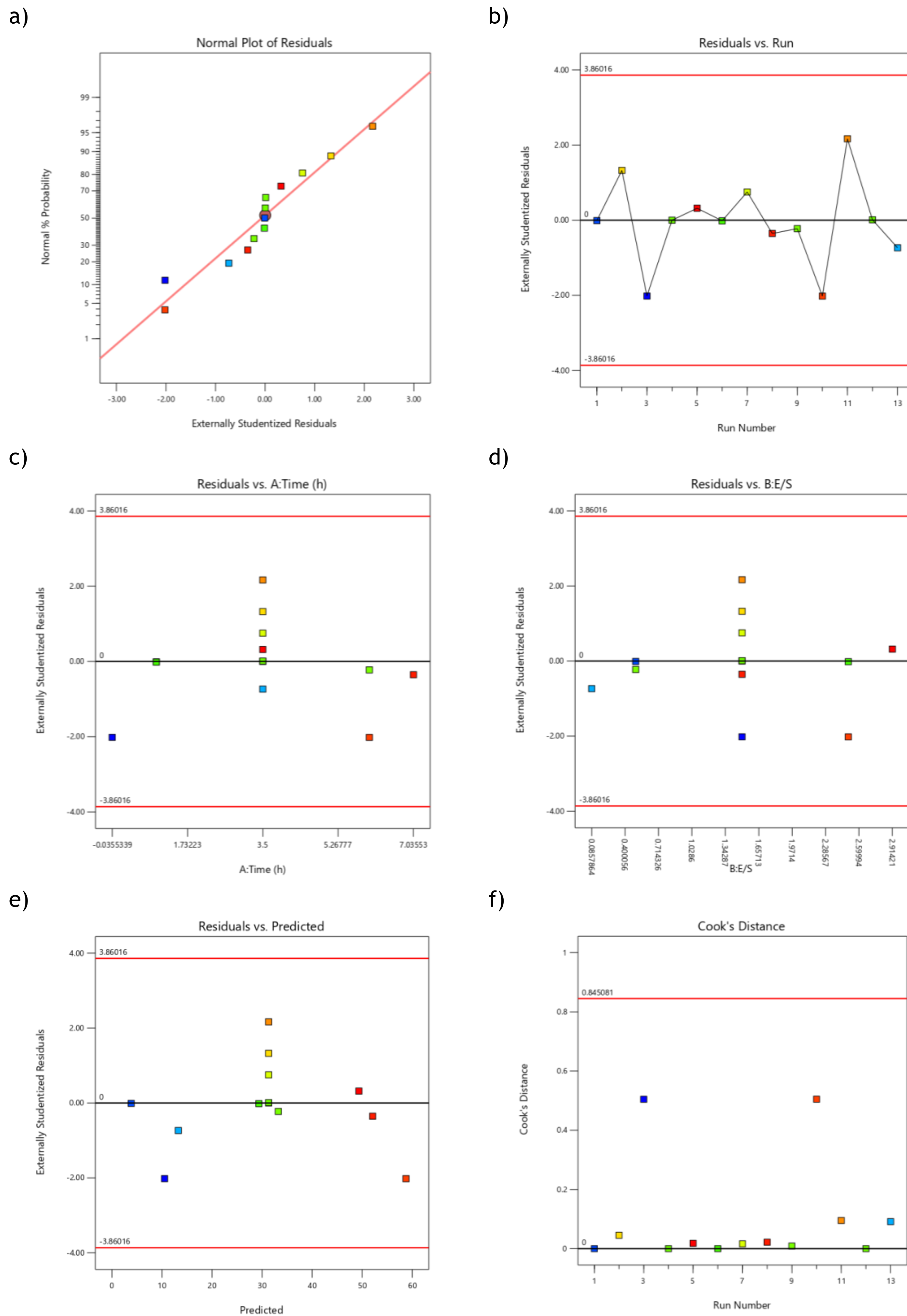


Figure B.1. Diagnostics graphs refering to DH, a) Normal plot of residuals; b) Residuals vs. Run; c) Residuals vs. Reaction Time; d) Residuals vs. E/S ratio; e) Residuals vs. Predicted and f) Cook's Distance.

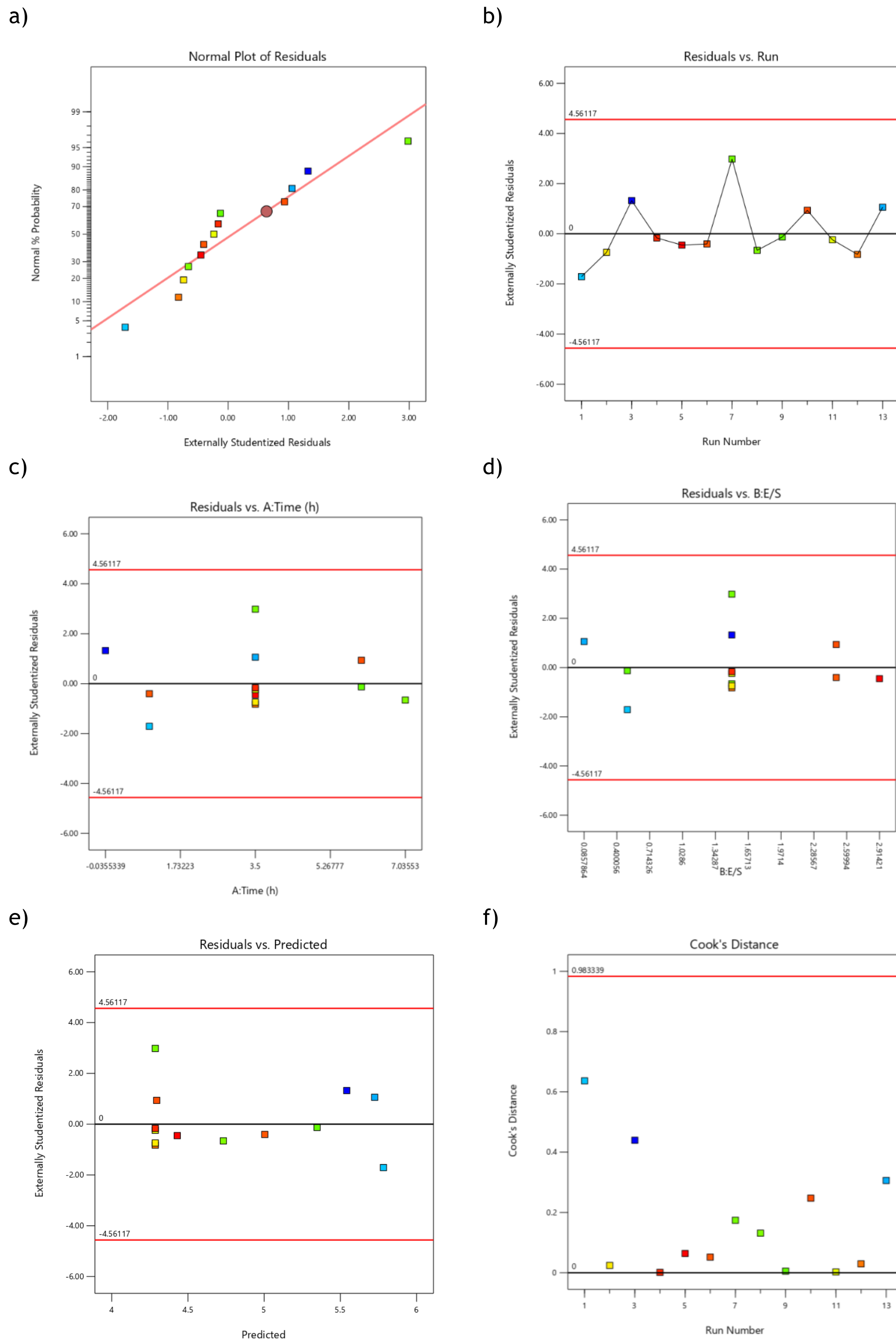


Figure B.2. Diagnostics graphs refering to ln (IACE), a) Normal plot of residuals; b) Residuals vs. Run; c) Residuals vs. Reaction Time; d) Residuals vs. E/S ratio; e) Residuals vs. Predicted and f) Cook's Distance.

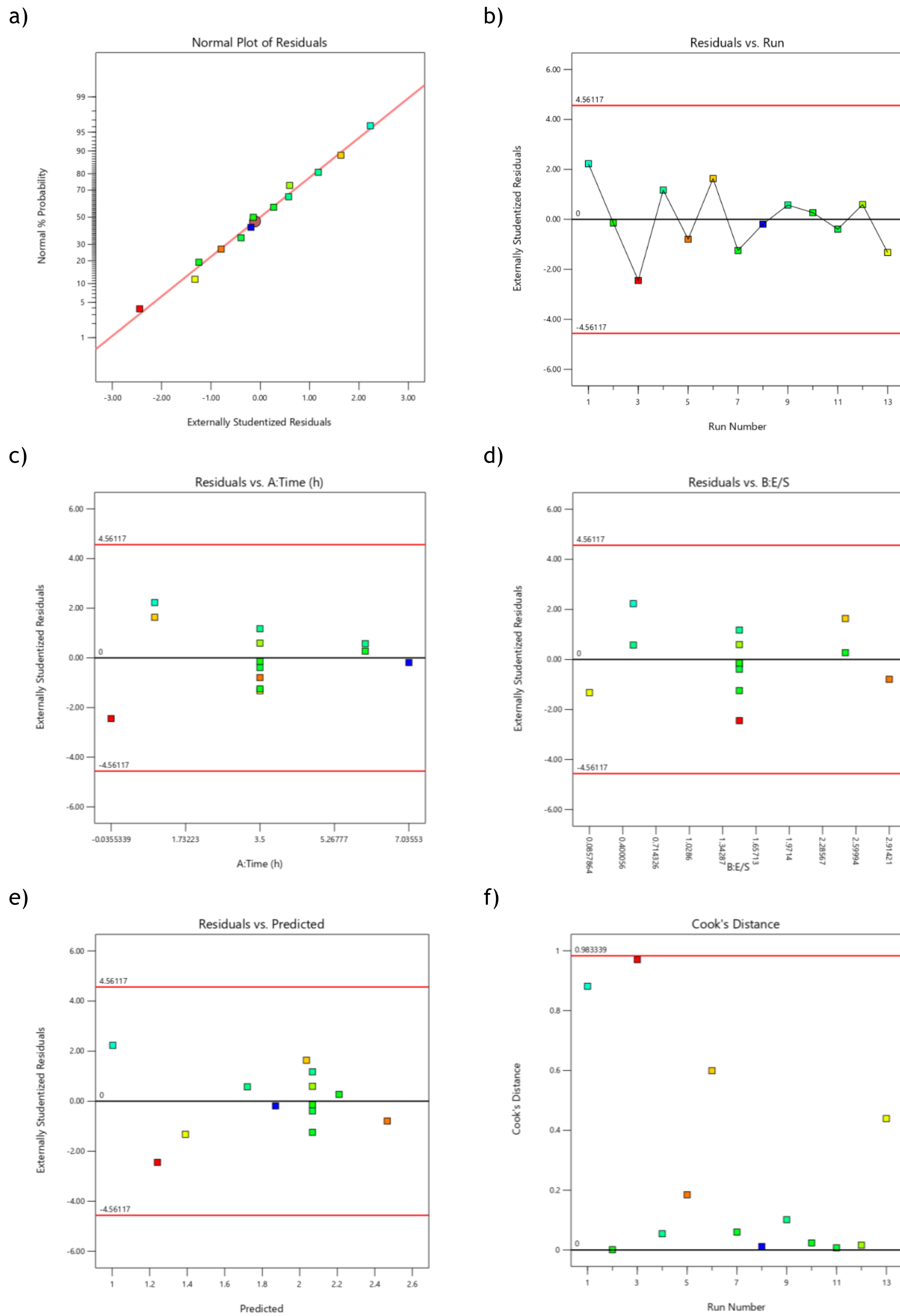


Figure B.3. Diagnostics graphs refering to ORAC, a) Normal plot of residuals; b) Residuals vs. Run; c) Residuals vs. Reaction Time; d) Residuals vs. E/S ratio; e) Residuals vs. Predicted and f) Cook’s Distance.

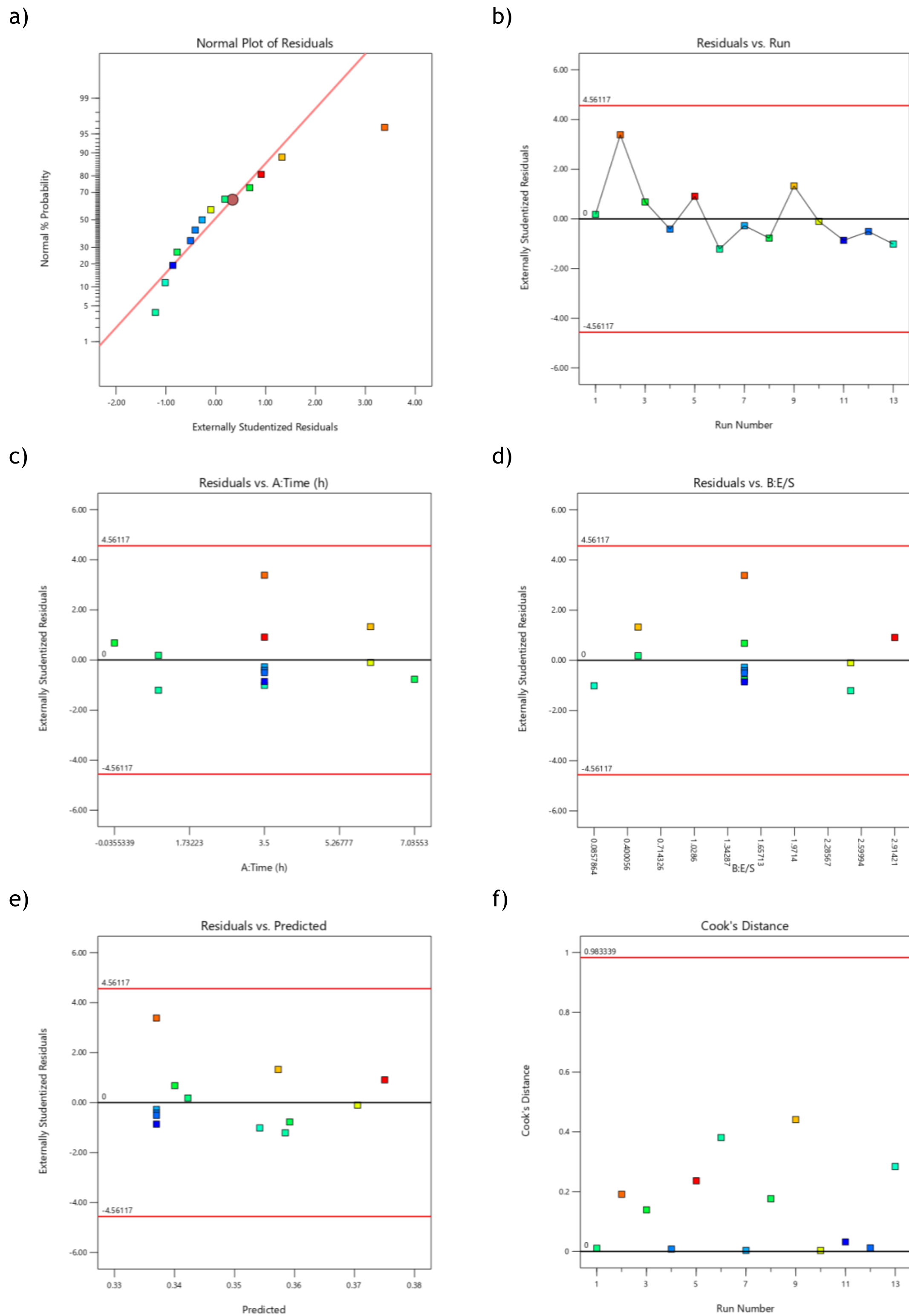


Figure B.4. Diagnostics graphs refering to ABTS, a) Normal plot of residuals; b) Residuals vs. Run; c) Residuals vs. Reaction Time; d) Residuals vs. E/S ratio; e) Residuals vs. Predicted and f) Cook's Distance.

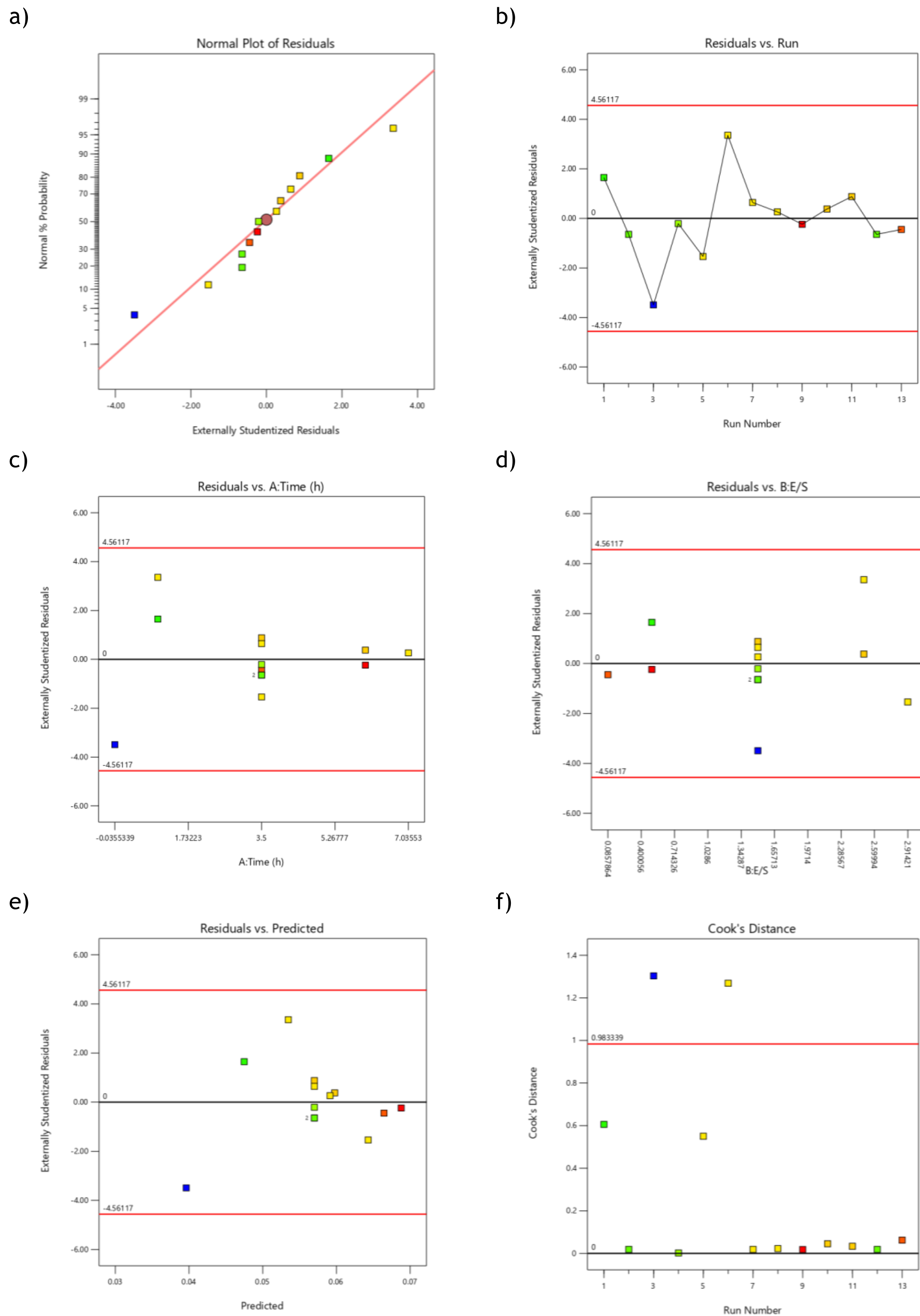


Figure B.5. Diagnostics graphs refering to DPPH, a) Normal plot of residuals; b) Residuals vs. Run; c) Residuals vs. Reaction Time; d) Residuals vs. E/S ratio; e) Residuals vs. Predicted and f) Cook's Distance.