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Research and Characterization of Bioactive Compounds with Potential Pharmaceutical Application Produced by Microalgae and Cyanobacteria

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De acordo com o disposto no ponto n.º 2 do Art.º 31º do Decreto-Lei n.º 74/2006, de 24 de Março, aditado pelo Decreto-Lei n.º 230/2009, de 14 de Setembro, o autor esclarece que na elaboração desta tese foram incluídos dados das publicações abaixo indicadas, e declara ter participado activamente na concepção e execução das experiências que estiveram na origem dos mesmos, assim como na sua interpretação, discussão e redacção.

According to the relevant national legislation, the author clarifies that this thesis includes data from the publications listed below, and declares that he participated actively in the conception and execution of the experiments that produced such data, as well as in their interpretation, discussion and writing.

PUBLICAÇÕES / PUBLICATIONS

Papers

Amaro, H. M.; Barros, R.; Guedes, A. C.; Sousa-Pinto, I.; Malcata, F. X. (**2013**) Microalgal compounds modulate carcinogenesis in the gastrointestinal tract. Trends in Biotechnology 31:92-98. doi.org/10.1016/j.tibtech.2012.11.004

Amaro H. M., Fernandes F., Valentão P., Andrade P. B., Sousa-Pinto I., Malcata F. X. and Guedes A. C. (**2015**) Effect of Solvent System on Extractability of Lipidic Components of *Scenedesmus obliquus* (M2-1) and *Gloeothece* sp. on Antioxidant Scavenging Capacity Thereof. Mar. Drugs, 13(10), 6453-6471; doi: 10.3390/md13106453

Amaro H. M., Guedes A. C., Preto M. A. C., Sousa-Pinto I. and Malcata F. X. (**2016**) Carotenoid and fatty acid extraction from *Gloeothece* sp. via improved continuous pressurized solvent extraction system. Bioresource Technology – s*ubmitted*

Amaro H. M., Pagels F., Azevedo J., Sousa Pinto I., Malcata F. X., Guedes A. C. (**2016**) Fluorescent light vs. LED for *Gloeothece* sp. in biomass and high value-metabolite production – a promising approach from blue biotechnology?, Algal research – *submitted*

Book Chapters

Amaro H. M., Malcata, F. X. (**2011**) Carotenoids from microalgae and cyanobacteria: features, production and applications; In: Carotenoids: Properties, Effects and Diseases. Chapter 3. ISBN 978-1-61209-713-8

Guedes A. C., Amaro, H. M., Sousa-Pinto, I, Malcata, F. X. (**2014**) Bioactive carotenoids from microalgae, In: Bioactive Compounds from Marine Foods: Plant and Animal Sources. Chapter 7. John Wiley & Sons, 131-152. ISBN: 978-1-118-41284-8.

Amaro H. M., Sousa-Pinto, I., Malcata F. X. and Guedes A. C. (**2016**) Microalgal fatty acids – from harvesting till extraction. In: Microalgae-Based Biofuels and Bioproducts. Chapter 17. Editors: Gonzalez-Fernandez C. and Torre R.M., Elsevier, Amsterdam, Netherlands – *submitted*

Amaro H. M., Sousa-Pinto I., Malcata, F. X., Guedes A. C. (**2016**) Microalgae as source of pigments – extraction and purification methods. In: Marine Microorganisms Extraction and Analysis of Bioactive Compounds, Chapter 6. Taylor and Francis Group CRC, Florida, USA – *submitted*.

Amaro H. M., Sousa-Pinto I., Malcata, F. X., Guedes A. C. (**2016**) Cyanobacteria Compounds with Anticancer Properties. In: Cyanobacterial Biotechnology. Editors: Dr. Pankaj Goyal & Dr. Abhishek Chauhan – *submitted.*

Posters

Amaro H. M., Guedes A. C., Sousa-Pinto I. and Malcata F. X. "Extraction of lipid components from *Scenedesmus obliquus* biomass upon radical scavenging capacity" 1st EABA Conference and 8th International Algae Congress, 1-6 December **2014**, Florence, Italy.

Amaro H. M., Guedes A. C., Sousa-Pinto I. & Malcata F. X. " Influence of solvents on the extractability of lipids from *Gloeothece* sp., and effects on antioxidant scavenging capacity" 106th AOCS annual meeting and industry showcase, 3-6 May **2015**,Rosen Shingle Creek, Orlando, Florida, USA

Amaro H. M., Guedes A. C., Sousa-Pinto I..and Malcata F. X. Fluorescent vs. LED light for *Gloeothece* sp. biomass and PUFA production – a promising approach from blue biotechnology? 1-3 December **2015**, Lisbon, Portugal.

Pagels F., Amaro H. M., Sousa-Pinto I., Malcata F. X. and Guedes A. C. The influence of light quality in *Gloeothece* sp. biomass and carotenoid production – a new setup in algal biotechnology. IJUP 17-19 February **2016**, Porto, Portugal.

Amaro H. M., Pagels F., Sousa Pinto I., Malcata F. X. and Guedes A. C. LED light for microalgal carotenoids and antioxidant capacity increase - a profitable advance in blue biotechnology. Front. Mar. Sci. Conference Abstract: XIX Iberian Symposium on Marine Biology Studies 5-9 September **2016**. doi: 10.3389/conf.FMARS.2016.05.00213

Abstract

Microalgae constitute a diverse group of microscopic prokaryotic (cyanobacteria) and eukaryotic photosynthetic organisms; due their large diversity, along with their physiological and functional flexibility, they constitute as a valuable source of added-value bioproducts. Biotechnology of microalgae has accordingly gained growing importance in recent decades, with applications ranging from plain biomass production for food and feed, to valuable products for nutra- and pharmaceutical uses. Some of their bioproducts have indeed been identified as bioactive compounds, such as carotenoids and polyunsaturated fatty acids (PUFAs) – exhibiting a great potential for application in pharmaceutical formulations, based on their capacity to act as antioxidant, antiviral, anti-inflammatory, antimicrobial or antitumoural agents.

However, extraction costs of microalgal intracellular metabolites remain high; the downstream separation stages often account for 50%–80% of the total production costs, depending on the biochemical characteristics of the target metabolite and purity required for the intended use – thus limiting commercial exploitation. Therefore, the work developed along this thesis attempted to alleviate some of the aforementioned bottlenecks in microalga-based processes – namely intracellular concentration and extraction of bioactive compounds.

Microalgal extracts require characterization prior to concluding on their potential pharmaceutical application. In order to achieve this goal, efforts were developed to: I) ascertain the influence of several food GRAS (Generally Recognized As Safe) solvents upon recovery of bioactive extracts – rich in lipidic components (carotenoids and PUFA), and provide preliminary biochemical characterization thereof; II) optimize the extraction conditions of said bioactive components, in terms of temperature and pressure, using the solvent selected in I); and III) optimize the concentration of said bioactive components in the microalga cell itself, using light as a tool – so as to reduce the need for purification downstream.

The work developed along these lines supported the following major conclusions:

I) the food-GRAS solvents tested – ethanol, acetone, hexane:Isopropanol (3:2) (HI) and ethyl lactate, exhibited different rates of extraction of lipidic compounds in two microalgal models encompassing disparate cell complexity – *Scenedesmus obliquus* (eukaryote) and *Gloeothece* sp. (prokaryote). The antioxidant bioactivity of the extracts obtained – assessed via four distinct assays ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, $^{\bullet}NO$ and $O_2^{\bullet-}$), indicated that solvent nature appears critical upon the final antioxidant performance – probably due to the resulting carotenoid/PUFA balance. Ethanol and

Gloeothece sp. were thus selected for further optimization studies: ethanol for its good performance in extraction of both carotenoids and PUFA; and *Gloeothece* sp. for its ethanol extract being active against all radicals tested – further to its being a prokaryotic microalga poorly studied to date in this regard.

Wide experimental evidence has unfolded the direct role of some antioxidants toward prevention of appearance, and control of growth of certain cancer tumours. Therefore, the aforementioned extracts were tested for antitumor activities, departing from their strong antioxidant features; two gastric cancer cell lines in particular, i.e. AGS and MNK45, served as model to evaluate such a bioactivity. Although the study still is on processing, some preliminary studies revealed that all extracts were able to modulate cancer cell viability, particularly HI and ethyl lactate, that exhibited cell death and anti-proliferative effects upon the two gastric cancer cell lines.

II) temperature (T) and pressure (P) are effective in acceleration, and eventual improvement of bioactive compound extraction from microalgae. Hence, a laboratory-made continuous pressurized solvent extraction system (CPSE) was built, aimed at optimizing extraction of carotenoids and/or fatty acids from *Gloeothece* sp. Biomass amount in the extraction column, solvent flow rate, pressure, temperature and overall solvent volume – including extract fractioning and degree of solvent recirculation, were sequentially optimized as operation parameters. Carotenoids and fatty acids were identified by HPLC-DAD and GC-FID, respectively, while antioxidant capacity was assessed by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ methods. It was found that 60 °C and 180 bar were the best temperature and pressure, respectively, toward extraction of lipidic compounds. Collection as different volume fractions allowed one to obtain extracts with distinct characteristics, e.g. PUFA-rich, carotenoid-rich (particularly in terms of lutein) and/or antioxidant compound-rich. Recirculation of extract for up to 3 cycles (a cycle being defined as passage of a set volume of solvent through the biomass-containing column) led to a 1.7-fold increase in lutein and an 11-fold increase in β-carotene contents, as well as a 1.4-fold increase in antioxidant capacity; and a 7.4-fold increase in C18:2 n6 t was observed when using 5 cycles of recirculation. When compared to a conventional extraction method, ultrasound assisted extraction (UAE), our CPSE proved proved clearly superior in extraction yield.

III) light emitting diodes (LED) can be a valid alternative to fluorescent light for the associated decrease in cost, besides enhancement of biomass growth rate and added value-metabolite yield. Light plays a crucial role in several metabolic

pathways, especially when microalgae operate under strict photoautotrophy; the possibility of use of specific wavelengths when operating with LED is a key feature toward biomass production and metabolite synthesis. Different LED wavelengths – more specifically blue (B), red (R) and two combinations thereof (BR), were tested in terms of biomass productivity of *Gloeothece* sp. (P_X), as well as content of carotenoids and fatty acids (FA), and associated antioxidant capacity (AC). Combination with infrared (IR) LEDs was also tested, using the best performant visible LEDS. The B LEDs induced higher P_X and high FA, although R LEDs supported the best AC. The BR combination (40:60) promoted biomass richer in carotenoids, particularly lutein and β-carotene; extra combination of BR and R with IR enhanced P_X and FA, besides producing a change in carotenoid profile and AC over time.

All in all, *Gloeothece* sp. proved a quite promising source of bioactive compounds, namely carotenoids and PUFAs, due the antioxidant and antitumoural features found in extracts obtained therefrom. It is hoped that the work developed along this thesis will constitute a valid contribution to solve (some of the) bottlenecks found in microalga-based industrial processing – specifically with regard to increase in intracellular concentration and solvent-mediated extraction afterwards of bioactive compounds.

Resumo

As microalgas constituem um grupo diversificado de microorganismos fotossintéticos, podendo ser procariotas (cianobactérias) ou eucariotas. Devido à sua grande diversidade e flexibilidade, tanto fisiológica como funcional, constituem uma valiosa fonte de produtos de valor acrescentado. Consequentemente, a biotecnologia microalgal tem vindo ganhar importância ao longo das últimas décadas – sendo que a sua aplicação vai desde a simples produção de biomassa para alimentação, humana e animal, até à produção de metabolitos importantes para aplicação na indústria nutra- e farmacêutica. De facto, alguns desses metabolitos foram identificados como sendo bioativos, p.ex. carotenóides e ácidos gordos polinsaturados (PUFA); estes exibem potencial particular de aplicação em fórmulas farmacêuticas, devido à sua capacidade de actuação como agentes antioxidantes, antivirais, anti-inflamatórios, antimicrobianos e até mesmo antitumorais.

Contudo, os custos associados à extração de metabolitos intracelulares das microalgas permanecem normalmente elevados – e, dependendo das etapas necessárias ao seu processamento e da sua finalidade, podem mesmo atingir valores na ordem dos 50–80% do custo total de produção, limitando assim a sua viabilidade económica a larga escala.

Em face do exposto, o trabalho desenvolvido ao longo desta tese pretendeu ser um contributo para a resolução das referidas limitações na biotecnologia microalgal, nomeadamente em termos de extracção e concentração intracelular de compostos bioactivos.

Os extractos obtidos de microalgas necessitam de caracterização prévia com vista a aferir do seu potencial de aplicação farmacêutica. Nesse contexto, foram feitos estudos com base nas seguintes abordagens: I) averiguar a influência de diferentes solventes GRAS (geralmente reconhecidos como seguro) de grau alimentar na extracção optimizada de compostos bioactivos do tipo lipídicos (carotenóides e AGPI), assim como caracterizar bioquimicamente os extractos obtidos; II) optimizar as condições de extracção de tais componentes bioactivas, em termos de temperatura e pressão, usando o solvente seleccionado em i); e III) optimizar a concentração das componentes lipídicas na célula da microalga, usando a luz como ferramenta, com vista a minorar a purificação posterior dos compostos.

O trabalho desenvolvido nesta tese permitiu concluir que:

I) Os solventes GRAS de grau alimentar testados – etanol, acetona, hexano;isopropanol (3:2) e etil lactato, exibiram diferentes capacidades de

extracção de compostos lipídicos nas duas microalga modelo, as quais representam diferentes graus de complexidade celular – o *Scenedesmus obliquus* porquanto eucacariota, e a *Gloeothece* sp. porquanto procariota. Foi avaliada também a capacidade antioxidante dos extractos obtidos, recorrendo a quatro ensaios diferentes ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, $^{\bullet}NO$ e $O2^{\bullet-}$); os resultados revelaram que os solventes usados para extrair as componentes lipídicas constituem um factor crítico na prestação antioxidante dos extractos – tendo assim colocada a hipótese de tal prestação ser afectada pelo balanço de carotenóides e AGPIs.

O etanol e a *Gloeothece* sp. acabaram por ser seleccionados como solvente e microalga para estudos posteriores de optimização da extracção pelas seguintes razões: o etanol devido à sua alta performance na extracção de carotenóides e AGPIs, ao mesmo tempo que o extracto obtidos apresenta alta actividade antioxidante; e a *Gloeothece* sp. porque o seu extrato etanólico apresentou capacidade antioxidante contra todos os radicais testados – para além de esta microalga procariota se encontrar parcamente estudada até ao momento.

Evidências experimentais relatadas na literatura confirmam o benefício de alguns antioxidantes na prevenção e controlo de certos tumores. Com base nos resultados promissores obtidos referentes a capacidade antioxidante, os referidos extractos foram avaliados quanto à sua capacidade antitumoral – particularmente sobre duas linhas de cancro gástrico, AGS e MKN 45. Ensaios preliminares revelaram que todos os extractos foram capazes de modular a viabilidade celular nas referidas linhas de cancro, particularmente os extratos HI e etil lactato, que desempenharam também efeitos de indução de morte celular e anti-proliferativos sobre as referidas linhas.

II) A temperatura e a pressão são factores eficazes na aceleração e na melhoria da extracção de compostos bioactivos de microalgas. Em conformidade, foi construído um sistema de extracção contínua pressurizado (CPSE) à escala laboratorial, de maneira a melhorar a extracção de carotenóides e AGPIs de *Gloeothece* sp. – dada a sua evidente capacidade antioxidante. Foram testados parâmetros da extracção tais como quantidade de biomassa no interior da coluna de extracção, caudal/pressão do solvente, temperatura e volume de solvente – nomeadamente em termos de fraccionamento do volume total e número de passagens do solvente pela coluna de extracção, tendo-se procedido a optimização sequencial optimizadas usando etanol como solvente.

Além disso, os carotenóides e AGPIs contidos nos extratos foram identificados por HPLC-DAD e GS-FID, respectivamente, e apurada a sua capacidade antioxidante pelos métodos $ABTS^{\bullet+}$ e $DPPH^{\bullet}$.

De entre as condições estudadas, 60 ºC e 180 bar resultaram como as melhores condições de temperatura e pressão para extrair os compostos lipídicos de interesse. Além disso, várias fracções recolhidas mostraram que é possível obter extractos com diferentes características, p.ex. rico em AGPIs, rico em carotenóides (particularmente luteína) e/ou rico em compostos antioxidantes. A recirculação de uma fracção de solvente pela coluna de extracção em 3 ciclos (em que cada ciclo corresponde a uma passagem de solvente pela biomassa) permitiu aumentar em 1.7 vezes a concentração de luteína, cerca de 11 vezes a de β-caroteno, e até 1.4 vezes a capacidade antioxidante do extracto obtido. Por outro lado, usando 5 ciclos, verificou-se um aumento de 7.4 vezes na concentração de C18:2 (n-6) t. Em geral, o sistema CPSE desenvolvido mostrou ser capaz de atingir melhores rendimentos, quando comparado com um sistema convencional de extracção assistido por ultra-sons.

III) Os díodos emissores de luz (LED) são uma alternativa válida ao uso de luz fluorescente (FL) na produção de microalgas, diminuindo os custos inerentes à utilização ao mesmo tempo que aumentam a produção de biomassa e metabolitos de valor acrescentado. A luz tem um papel crucial na regulação de diversas vias metabólicas, particularmente quando as microalgas optam por um regime de foto-autotrofia estrita; o uso de comprimentos de onda específicos, facilmente obtidos recorrendo a LED, são um ponto-chave para a produção de biomassa e a modulação da sua composição bioquímica. Diferentes comprimentos de onda de LED – mais especificamente na zona do azul (A), vermelho (V) e duas combinações das duas, foram analisados em termos da produção de biomassa P(X) de *Gloeothece* sp., carotenóides e ácidos gordos (AG), assim como sobre a capacidade antioxidante (CA). Para além disso, no caso dos LEDs para os quais esta microalga atingiu melhores resultados, foi testado o efeito de um LED infravermelho (IF) sobre os parâmetros acima referidos. Os resultados mostraram que o A induziu um alto P(X) e um alto conteúdo em AG, em comparação com os outros LEDs testados. Por outro lado, culturas iluminadas por V exibiram os melhores resultados relativamente à CA. Uma combinação AV (40:60) deu origem a biomassa mais rica em carotenóides, particularmente luteína e β-caroteno. Por fim, a luz IV em conjugação com AV (40:60) e V aumentou a P(X) e o conteúdo em AG, alterando também o perfil de carotenóides produzidos ao longo do tempo, embora mantendo os valores máximos.

Em conclusão, a microalga *Gloeothece* sp. afigura-se como uma nova promissora fonte de compostos bioactivos, tais como carotenóides e AGPIs, devido à capacidade antioxidante e antitumoral detetada nos extratos obtidos. Espera-se que o trabalho desenvolvido ao longo desta tese possa constituir uma contribuição válida para aliviar algumas das limitações encontradas no processamento industrial com microalgas – em particular no que toca à concentração intracelular de compostos e à sua extracção via solvente.

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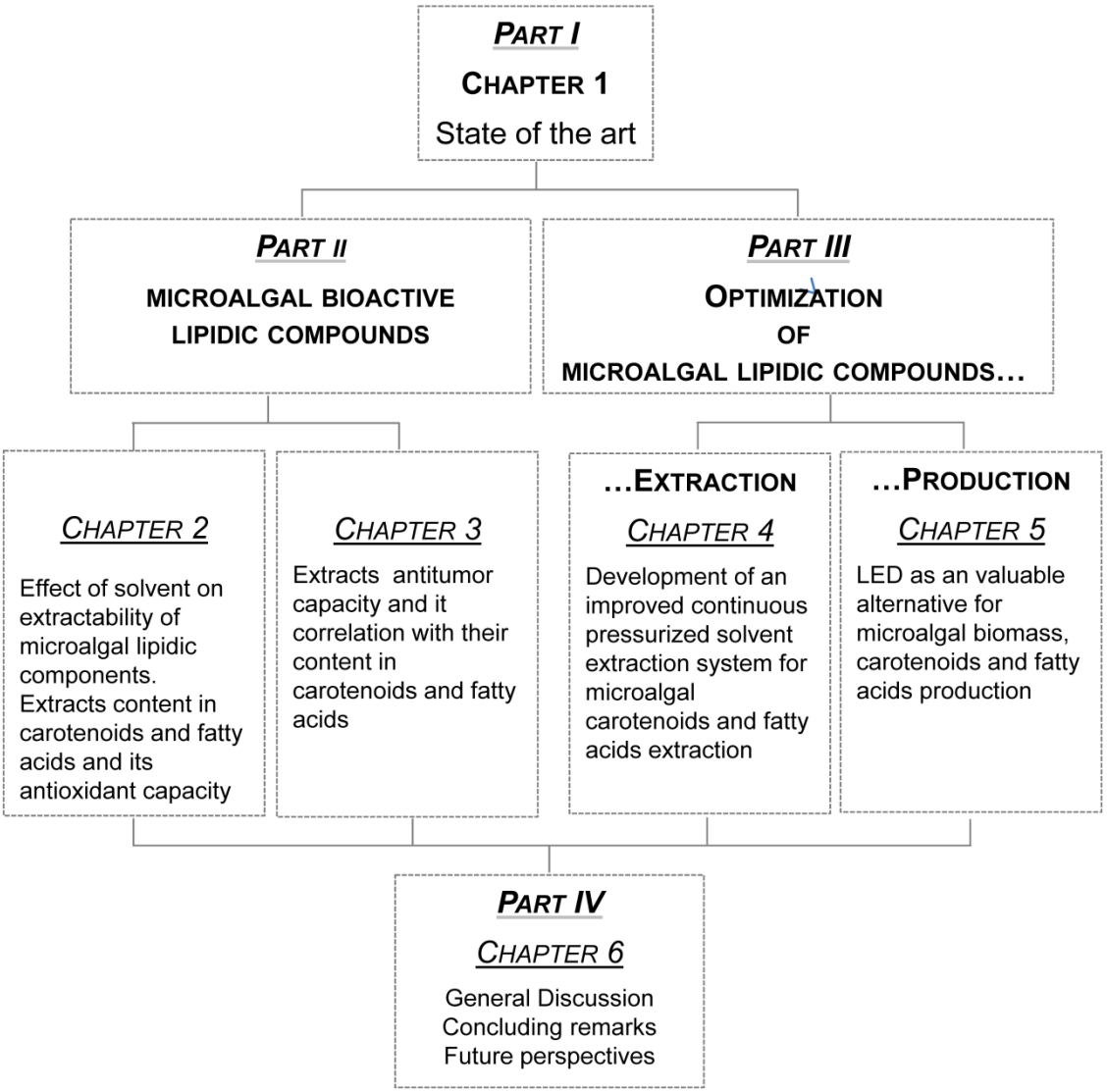
Table of contents

Outline of thesis structure

This thesis is composed by 4 main parts, and each of them is based on published or submitted papers and book chapters, written in the scope of the thesis.



PART I

CHAPTER 1 – **STATE OF THE ART** – this first part entails a literature review on bioactive microalgal metabolites and their health/pharmacological benefits, particularly in what concerns to microalgal lipidic components. Moreover, some factors that affect their production by microalgae, as well as their most reported extraction methods, will be reviewed. Accordingly, this chapter was based in the following publications:

- Bioactive carotenoids from microalgae (Book chapter published in: Bioactive Compounds from Marine Foods: Plant and Animal Sources, ISBN: 978-1-118-41284-8, 2013)

- Carotenoids from microalgae and cyanobacteria: features, production and applications (Book chapter published in: Carotenoids: Properties, Effects and Diseases. Chapter 3. ISBN 978-1-61209-713-8, 2011)

- Microalgal compounds modulate carcinogenesis in the gastrointestinal tract. (Review published in Trends in Biotechnology, 2013)

- Cyanobacteria Compounds with Anticancer Properties. (Book chapter accepted in: Cyanobacterial Biotechnology. Editors: Dr. Pankaj Goyal & Dr. Abhishek Chauhan)

- Microalgae as source of pigments – extraction and purification methods. (Book chapter accepted in: Marine Microorganisms Extraction and Analysis of Bioactive Compounds, chapter 6, Taylor and Francis Group CRC)

- Microalgal fatty acids – from harvesting till extraction. (Book chapter accepted in: Microalgae-Based Biofuels and Bioproducts. Editors: Gonzalez-Fernandez C. and Torre R.M., Elsevier, Amsterdam, Netherlands)

PART II

CHAPTER 2 – **EFFECT OF SOLVENT SYSTEM ON EXTRACTABILITY OF LIPIDIC COMPONENT OF *SCENEDESMUS OBLIQUUS* (M2-1) AND *GLOEOTHECE* SP. ON ANTIOXIDANT SCAVENGING CAPACITY THEREOF** – this chapter is an approach to optimization of lipidic microalgal component extraction. It is based on a classical extraction method that is intended to ascertain the influence of the several extraction solvents on microalga metabolites recovery (namely carotenoids and fatty acids), and its effect on the extracts bioactivity in terms of antioxidant capacity. Hence, this chapter was based in the following publication:

- Effect of Solvent System on Extractability of Lipidic Components of *Scenedesmus obliquus* (M2-1) and *Gloeothece* sp. on Antioxidant Scavenging Capacity Thereof. (paper published in Marine Drugs, doi: 10.3390/md13106453, 2015) by Amaro H.M., Fernandes F., Valentão P., Andrade P.B., Sousa-Pinto I., Malcata F.X. and Guedes A.C.

CHAPTER 3 – **ANTITUMOUR POTENTIAL OF LIPIDIC EXTRACTS FROM *GLOEOTHECE*** sp. – In sequence of the previous chapter, the lipidic extracts obtained with the different tested solvents were assessed for their antitumor capacity against two gastric cancer cell lines. Furthermore, its capacity was tentatively correlated with its content in carotenoids and fatty acids.

- Antitumoral potential of *Gloeothece* sp. lipidic extracts (paper still is in progress but will be submitted to Topical Collection "Bioactive Compounds from Marine Plankton" of Marine Drugs)

PART III

CHAPTER 4 – **CAROTENOID AND FATTY ACID EXTRACTION FROM *GLOEOTHECE* SP. VIA IMPROVED CONTINUOUS PRESSURIZED SOLVENT EXTRACTION SYSTEM** – this chapter is a sequel of the former studies. It aimed to develop an optimized extraction method to recover the so far proved bioactive microalgal lipidic compounds – carotenoids and fatty acids. Thus, an innovative improved continuous pressurized solvent extraction system was developed testing the most proper extraction conditions of temperature and pressure to extract specific compounds. This chapter was based in the following publication:

- Carotenoid and fatty acid extraction from *Gloeothece* sp. via an improved continuous pressurized solvent extraction system (paper submitted to Bioresource Technology, 2016)

CHAPTER 5 – **FLUORESCENT LIGHT VS. LED FOR *GLOEOTHECE* SP. IN BIOMASS AND HIGH VALUE-METABOLITE PRODUCTION – A PROMISING APPROACH FROM BLUE BIOTECHNOLOGY?** – This chapter focus on the optimization of microalgal biomass and its bioactive metabolites production, particularly carotenoids and fatty acids. LED, particularly blue, red and infra-red were tested as an alternative to common fluorescent light source used in microalga production. This chapter was based in the following publication:

- Fluorescent light vs. LED for *Gloeothece* sp. in biomass and high value-metabolite production – a promising approach from blue biotechnology? (paper submitted to Alga Research, 2016).

PART IV

CHAPTER 6 – General Discussion, concluding remarks and future perspectives. At last, conclusions and suggestions for further work were presented in this sixth part.

Figures List

PART III

CHAPTER 4

Tables List

PART III

CHAPTER 4.

CHAPTER 5

PART IV

CHAPTER 6

Abbreviations

$^1O_2$, singlet oxygen

AA, arachidonic acid, C20:4 n-6

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

AC, antioxidant compounds

ALA, α-linolenic acid, C18:3n-3

AMD, age-related macular degeneration

AP-1, pathway

ATCC, american type culture collection

B, blue

BG11, blue green medium

BR, blue:red

C, circulation

CL, cardiolipin

CNS, central nervous system

COX-2, Ciclo-oxigenase-2

CPSE, continuous pressurized solvent extraction

CVDs, cardiovascular diseases

$Cytb_6$ *f*, cytochrome

DAD, diode array detector

DHA, docosahexaenoic acid, C22:6n-3

DMH, dimethylhydrazine

DPPH, 2,2-diphenyl-1-picrylhydrazyl

DW, dry weight

EPA, eicosapentaenoic acid, C20:5n-3

F, fraction

FA, fatty acids

FAMES, fatty acid methyl esters

FL, fluorescent lamps

GAC, Green Analytical Chemistry

GC, gas chromatograph

GLA, γ-linoleic acid, C18:3n-6

GRAS, generally recognized as safe

H_2O_2, hydrogen peroxide

HAECs, Human aortic endothelial cells

HPLC, high-performance liquid chromatography

K-ras, mitogen-activated protein

LA, linoleic acid, C18:2n-6

LDL, low density lipoprotein

LED, light emitting diode

LHC, light harvesting complexes

NO, Nitric oxide radical

$O_2^{\bullet-}$, Superoxide radical

OD, optical density

OH, hydroxyl radical

OHM, optimal Haematococcus medium

$ONOO^-$, peroxynitrite

PAR, photosynthetic active radiation

Pfr, protein pigment active form

PKB, protein kinase B

PLE, pressurized fluid extraction

Pr, protein pigment inactive

PS I, photosystem I

PS II, photosystem II

PUFA, polyunsaturated fatty acids

P_X, biomass productivity

Q, flow rate

R, red

RNS, reactive nitrogen species

ROS, reactive species of oxygen

Rpm, rotation per minute

SFE, supercritical fluid extraction

SOR, singlet oxygen radicals

TAG; tri-acylglycerols

TBARS, thiobarbituric acid reactive substances

$\mathbf{t_d}$, duplication time

TE, trolox equivalents

TNF-α, Tumor necrosis factor alpha;

Tris-HCl, Tri-(hydroxymethyl)-aminomethane hydrochloride

UAE, ultrasound assisted extraction

UV, ultraviolet radiation

UVA, ultraviolet A radiation

$\mathbf{\mu_{max}}$, specific growth rate

IR, infrared

PART I

STATE OF THE ART

CHAPTER 1

State of the art

1.1 Introduction

Microalgae constitute a diverse group of microscopic prokaryotic (cyanobacteria) and eukaryotic photosynthetic organisms of paramount ecological importance. They indeed include some of the most efficient converters of solar energy to biomass; their only growth requirements are CO_2, a broth containing basic nutrients such as nitrogen and phosphorus, and a light source. Several environmental factors, e.g. temperature, salinity, light and nutrients, as well as culture time affect the growth and biochemical composition of microalgae as discussed by Guedes et al. (2010). Among them, light intensity and its spectrum have been reported to affect microalgal composition to extents that are species-specific (Fu et al., 2013, Guedes et al., 2010).

Microalgae are known to be rich sources of bioactive compounds, such as carotenoids and polyunsaturated fatty acids (PUFAs), with potential applications in pharmaceutical formulations – e.g. as antioxidant, anti-viral, anti-inflammatory, antimicrobial and antitumoral active principles. Furthermore, as photoautotrophs, their simple growth requirements make them particularly attractive for bioprocesses aimed at producing these high-added value compounds that are in large demand –e.g. as lutein, a known antioxidant produced by several microalga, with important anti-inflammatory and antitumoral roles.

In view of the diversity of added-value bioproducts produced by microalgae, together with their wide physiological and functional flexibility, it might be expected that microalgal cultures would be ideal “biofactories” – particularly due to their secondary metabolism that can be easily triggered by most forms of externally applied stress like e.g. excess or limitation of light. These characteristics of microalgae have attracted commercial interest due to their potential in valuable products and high biomass productivity. Large-scale cultivation experiments with biotechnological purposes were initiated in the USA, Japan and Israel in the early 1950s (Guedes and Malcata, 2011); in the last 50 years, microalgal biotechnology has achieved a range of applications, from traditional (extensive) biomass production for human and animal nutrition (including aquaculture), soil conditioning, bioremediation, and formulation of cosmetics and pharmaceuticals, to high-tech applications based on the manufacture of products for research and medical diagnostic (Guedes and Malcata, 2011).

However, a few limitations remain, owing to metabolic and processing constraints; this is the case of metabolite extraction, due the high costs associated. This has urged some of the research efforts described in this dissertation.

Therefore, a briefly state of the art is presented next covering a group of added-value bioproducts with health/pharmacological benefits, i.e. lipidic microalgal compounds –

carotenoids and PUFA. Moreover, some recent advances in their optimization in terms of extraction and production will be presented in the following sections.

1.2. Microalgal lipidic compounds and their health/pharmacological benefits

A particular interest has been received by lipidic components, such as carotenoids and PUFAs, due their great potential in industrial formulation of nutra- and pharmaceutical products. These families of microalgal lipidic components are two examples of microalgal molecules able to protect living organisms from oxidative damage (Marxen et al., 2007).

Based on their physicochemical characteristics, microalgal lipids can be divided into two major types: polar lipids, e.g. phospholipids and glycolipids; and neutral/non-polar lipids, e.g. mono-, di- and tri-acylglycerols (TAG) (Schuhmann et al., 2012). While polar lipids are important structural components of cell membranes and organelles, and can operate as signal molecules or their precursors; neutral (non-polar) lipids, such as tri-acylglycerols (TAG), are a most widespread group of compounds involved in catabolism for production of energy as required by the cell (Schuhmann et al., 2012). A considerable number of observations indicate that TAG possesses diverse functions in photoautotrophic organisms, like adaptation to environmental factors such as temperature, illumination or salinity. In many microalgal species, low light fluxes or nutrient deficiency induce TAG to accumulate as cytoplasmic oil bodies in the cells (Merzlyak et al., 2007). Therefore, conditions favourable for TAG biosynthesis are stressful for microalgae: they delay cell division and slow down culture growth (and thus biomass build-up), and this constitutes a major obstacle to attain high accumulation of algal biomass enriched by such valuable compounds. Finding solutions for this nontrivial task is thus very important for photobiotechnology involving microalga cultivation.

A simple type of lipids, which are bioactive as such, is free fatty acids; they are mainly composed of a 12-22 carbon chain with a methyl group at one end of the molecule (labelled as omega, ω) and a carboxyl group at the other end. They can be saturated or unsaturated, depending on presence of one or multiple double carbon–carbon bonds; in the case of the latter if the number of such double bonds is above unity, they are termed poli-unsaturated fatty acids (PUFA) (Rustan and Drevon, 2001), some examples are depicted in Fig. 1. According to the position of the first double bond in the structure, PUFAs can be classified into two major categories, namely ω-3 and ω-6 PUFA (or n3 or n6); they are essential cellular components that hold diverse biofunctions.

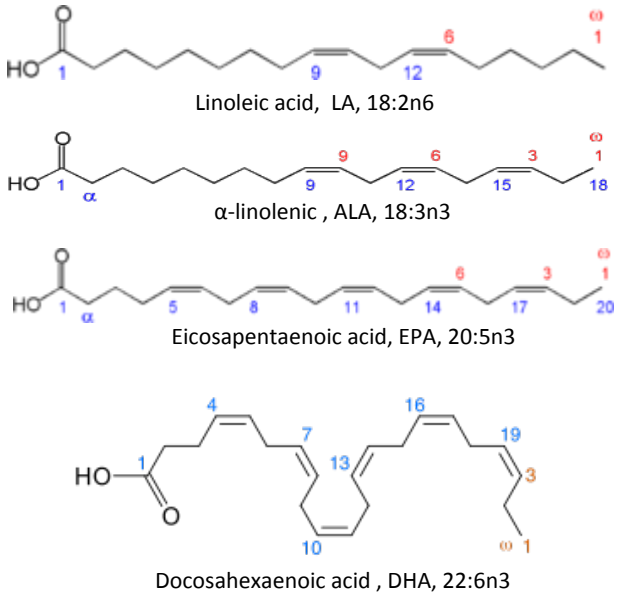


Fig. 1.1. Chemical structure of some poli-unsaturated fatty acids (PUFA) produced by microalgae.

PUFAs are found in animals, plants, fungi, microalgae and bacteria as a part (or included as moieties of more complex compounds) of membranes or storage organelles, typically in the form of glycolipids, phospholipids, sphingolipids and lipoproteins (Thelen and Ohlrogge, 2002).

Many species of microalgae contain PUFA in their polar lipids, but the content of these compounds is subject to stringent regulation within the cell (Makewicz et al. 1997; Cohen et al. 2011). Moreover, they can be found in neutral lipids, like linoleic (18:2n-6, LA), α-linolenic (18:3n-3, ALA), arachidonic (20:4n-6, AA), eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA), among others. They are considered valuable for humans, due to their physiological roles in cells as precursors and primary preventers of health conditions, e.g. as anti-inflammatory or neuroprotective agents (Guedes and Malcata, 2011). Moreover, AA, EPA, γ-linoleic acid (GLA) and DHA are also important structural components of membrane lipids and act as precursors of biologically active eicosanoids, such as prostaglandins and leukotrienes (Qi et al., 2002). It is known that AA and DHA play relevant roles in nervous tissues and blood vessels, besides being essential for pre- and post-natal development of the brain and the retina (van Goor et al., 2008). On the other hand, EPA and AA have been implicated in prevention of coronary heart diseases, hypertriglyceridemia, blood platelet aggregation, atherosclerosis, general inflammation and several carcinomas (Guil-Guerrero et al., 2000). Both EPA and DHA have as well been included in therapeutics to prevent dementia; the mechanisms that qualify such PUFAs as aids in primary prevention of those health conditions include their anti-atherogenic, anti-inflammatory , antioxidant, anti-amyloid and neuroprotective features (Lim et al., 2006, Mullen et al., 2010).

Presence of DHA in the diet of infants has been requested for full neurological development (Tonon et al., 2002). Dietary DHA showed to affect expression of several key genes involved in cholesterol metabolism, besides its role in prevention and treatment of such chronic diseases as coronary heart disease, hypertension, type II diabetes, ocular diseases, arthritis and cystic fibrosis (Kramer et al., 2003). Additionally, GLA and AA appear to play a relevant role in prevention of diabetes, reproductive disorders and a few skin diseases (Suresh and Das, 2003, Guedes and Malcata, 2011).

Studies have indicated that a balanced ω6/ω3 ratio in in diet decreases the doses required of some pharmaceutical medications; a decrease in ω6 intake concomitant with an increase in ω3 intake is accordingly recommended to assure homeostasis and regular health (Simopoulos, 2002).

Carotenoids are organic pigments found in the chloroplasts and chromoplasts of microalgae. They are classified into two classes: xanthophylls (which contain oxygen) and carotenes (which are purely hydrocarbons, and thus do not contain oxygen). All carotenoids are natural pigments derived from 5-carbon isoprene units that are enzymatically polymerized to form regular, highly conjugated 40-carbon structures (with up to 15 conjugated double bonds). One or both ends of the carbon backbone may be substituted by oxo (e.g. canthaxanthin), hydroxyl (e.g. lutein) or epoxy (e.g. astaxanthin) groups, at different positions, to form different xanthophylls (Del Campo et al. 2007), as depicted in Fig. 2. A distinction is usually made between primary and secondary carotenoids: the former (i.e. xanthophylls) are structural and functional components of the cellular photosynthetic apparatus, so they are essential for survival, while the latter encompass those produced to a large extent by microalgae (via carotenogenesis) but only in response to specific environmental stimuli (Guedes and Malcata, 2011). Carotenoids may be found naturally in microalgae, either in their free form or esterified with fatty acids.

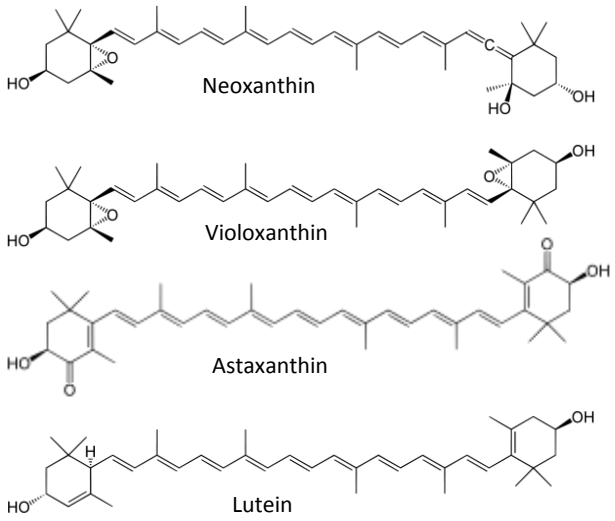


Fig. 1.2. Chemical structures of some xanthophylls found in microalgae.

Xanthophylls are relatively hydrophobic molecules, so they are typically associated with membranes and/or involved in non-covalent binding to specific proteins; they are often located in the thylakoid membrane, whereas secondary carotenoids are mainly found in lipid vesicles — in either the plastid stroma or the cytosol. Most xanthophylls in cyanobacteria and oxygenic photosynthetic bacteria are associated with chlorophyll-binding polypeptides of the photosynthetic apparatus (Grossman et al., 1995); however, in most green microalgae, carotenes and xanthophylls are synthesized and accumulate inside plastids. Conversely, secondary xanthophylls in a few green microalgae, such as astaxanthin in *Haematococcus* sp., accumulate in the cytoplasm; this raises the hypothesis of an extra-plastidic site of carotenoid biosynthesis in that genus. Alternatively, xanthophylls synthesized in the chloroplast may be exported and will eventually accumulate in the cytoplasm (Jin et al., 2003).

All xanthophylls synthesized by higher plants (e.g. violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, and lutein) can also be synthesized by green microalgae; however, the latter possess additional xanthophylls, such as loroxanthin, astaxanthin, and canthaxanthin – and diatoxanthin, diadinoxanthin and fucoxanthin can also be obtained from brown algae or diatoms (Jin et al., 2003).

At least 600 different carotenoids that play important biological functions in bacteria, algae, plants, and animals have been identified to date (Polivka and Sundstrom, 2004). Animals cannot endogenously synthesize carotenoids, so they must resort solely to their diet to obtain them. Of the 600+ carotenoids identified to date, around 40 can be found in common foods. However, selective uptake along the digestive tract leads to that only 14

carotenoids (and corresponding derivations) are eventually found in human plasma and tissues.

As emphasized before, carotenoids are essential constituents of the photosynthetic apparatus, primarily in the reaction centres of photosystems (or inserted in pigment–protein antenna complexes), where they act: (1) as accessory pigments for light-harvesting processes during photosynthesis, (2) as structural stabilizers for protein assembly in photosystems, and (3) as inhibitors of either photo- or free-radical oxidation caused by exposure to excess light (Zhang et al., 1999). The intrinsic antioxidant capacity of carotenoids constitutes the basis for their protective action against oxidative stress; however, not all biological activities claimed for carotenoids pertain to their ability to inactivate free radicals and reactive oxygen species (ROS) (Guedes and Malcata, 2011). Minor structural differences between said compounds account for variations in their biological activities (Sun and Yao, 2007); although most reports have dealt with the conjugated double bonds with regard to their role upon biological impact, few data are available on the role of hydroxyl groups (Cha et al. 2008).

As reviewed before, numerous epidemiological studies suggest that consumption of carotenoids correlates with a lower risk of contracting several types of degenerative disease in human beings (Guedes and Malcata, 2011). Most of these reports have actively focused on carotenoids from microalgal sources; the major fields, in terms of current or potential industrial applications, are food and health — and the antioxidant properties exhibited by this class of compounds constitutes the core interest.

Carotenoids provide direct photoprotection against ultraviolet (UV) light-induced photo-oxidation in the skin, while β-carotene modulates UVA-induced gene expression in human keratinocytes (Wertz et al., 2005, Aust et al., 2005). Astaxanthin is believed to play a key role in amelioration/prevention of several human pathological processes, such as skin UV-mediated photo-oxidation, inflammation, prostate and mammary carcinogenesis, ulcers caused by *Helicobacter pylori*, and age-related diseases (Guerin et al., 2003). Among the benefits of carotenoids for eye health, the occurrence of age-related macular degeneration (AMD) has been associated to lower levels of both zeaxanthin and lutein (xanthophylls) in the macula (Neelam et al., 2005); prospective epidemiological data show a 19 % lower risk of cataract in men who regularly take high doses of both xanthophylls (Meyer and Sekundo, 2005). Based on several medical and nutritional trials, authors have hypothesized that the antioxidant activity of carotenoids is likely the key factor in reducing incidence of many diseases mediated by light (Astley et al., 2004).

Considering the close relationship between antitumor and anti-inflammatory features and immune system improvement, these should not be viewed as independent systems – as long as the role of lipidic compounds, such as carotenoids and PUFA, in regulating these

mechanisms is strongly related to their antioxidant features. Although such properties associated with carotenoids are not restricted to those originated in microalgae, all carotenoids tested are also synthesized by microalgae.

Interest in compounds with antioxidant properties has indeed been on the rise, since such compounds can play favourable roles in human health. The evidence collected from a large number of studies has confirmed the positive effects of antioxidants (section 2.1) in prevention and control of growth of certain tumours (see Section 2.2). Therefore, bioactive compounds, such as carotenoids or PUFA, may hold at least two capacities within the set of antioxidant and antitumor capacities, as will be discussed below.

1.2.1. Antioxidant features of lipidic components

A definition of antioxidant is “any substance that delays, prevents or removes oxidative damage to a target molecule” (Halliwell, 2001). Concomitantly, such compounds may display antiviral and antimicrobial activities, as well as ability to chelate iron, inhibit enzymes, regulate gene expression, and improve endothelial function (Tabart et al., 2009).

Antioxidants are generally divided into two groups, depending on their mechanism of action: chain-breaking antioxidants and preventive antioxidants. The latter reduce the rate of chain initiation, while the former (that include carotenoids and PUFA) interfere with chain propagation.

Antioxidants may alternatively be classified as hydrogen-donating compounds, singlet oxygen quenchers, metal chelators, or oxygen scavengers. Singlet oxygen quenchers include carotenoids, PUFA, α-tocopherol and ascorbic acid; they can react with oxygen before other essential structures do, thus leading to useful and high quenching rates (Giao et al., 2010).

To realize the importance of antioxidant compounds, it is crucial to understand the process leading to their origin, oxidation. “Oxidation” denotes a set of common processes in nature, led by reactive species that are also involved in a variety of biological phenomena. Such processes occur when electrons are removed from an atom or a group of atoms, and may entail addition of oxygen atoms to, or removal of hydrogen atoms from the compound undergoing oxidation. Simultaneously, there is a corresponding reduction that involves addition of electrons to a distinct atom or group of atoms (Giao et al., 2010).

Chemical compounds capable of generating (potentially toxic) reactive species of oxygen (ROS) are referred to as “pro-oxidants”. In a normal cell, pro-oxidants and antioxidants are in balance, yet this balance may be shifted towards pro-oxidants when production of ROS increases to a sufficient level (e.g. following ingestion of certain chemical compounds or drugs), or when the cellular levels of antioxidants are somehow reduced. This

situation is widely termed “oxidative stress”, and may follow essentially two mechanisms: (1) the concentration of antioxidants is reduced or (2) the number of oxygen/nitrogen/carbon-based reactive species derived from activated phagocytes is increased — as happens with chronic inflammation (Somogyi et al., 2007).

However, ROS are unavoidable by-products in normal aerobic metabolism; in the human being, oxidation driven by ROS may lead to various detrimental phenomena, including pigment and protein damage and DNA decay or mutation – which may in turn cause several syndromes, such as cardiovascular diseases (CVDs), some forms of cancer, degenerative diseases, and ageing (Halliwell, 2001).

The antioxidant capacity of two lipid components, carotenoids and PUFA, will be briefly discussed next, with particularly emphasis on those (also) obtainable from microalgal sources.

1.2.1.1.Antioxidant capacity of carotenoids

Since carotenoids are potent biological antioxidants; they are able to absorb the excitation energy of singlet oxygen radicals (SOR) into their complex ringed chain, thus promoting energy dissipation while protecting tissues from putative chemical damage. Their radical scavenging capacity lessens tissue decay by reducing the decay of such molecules as DNA, proteins, and membrane lipids (Bai et al., 2005). A few processes brought about by SOR have also been linked to the ageing process, as well as to pathogenesis of several diseases, namely cancer, CVDs, atherosclerosis, rheumatoid arthritis, muscular dystrophy, cataracts, and several neurological disorders (Abe et al., 2007, Kim and Cha, 2010). For instance, lutein has been recommended (and even prescribed) for prevention of cancer and of diseases related to retinal degeneration (Granado-Lorencio et al., 2009). Another illustrative example is the decline of cognitive ability in Alzheimer’s disease, which is apparently caused by persistent oxidative stress in the brain (Mattson, 2004). Nakashima et al. (2009) reported that progression of cognitive impairment in transgenic mice could be prevented to a significant extent by feeding them with *Chlorella* sp. containing carotenoids (e.g. β-carotene and lutein).

A list of microalgae rich in carotenoids that have already been recognized as strong antioxidants is given in Table 1. The antioxidant effects of carotenoids are dependent on the number of conjugated double bonds, the chain structure, and the specific functional groups — as detailed in the following sections. Unfortunately, there are contradictory results pertaining to lutein (and its esterified derivatives) with regard to antioxidant effects: although esterification of lutein with fatty acid increased its stability against heat and light, no effect was found upon its antioxidant capacity (Subagio and Morita, 2001).

Table 1.1. Carotenoids produced by microalga with proven antioxidant capacity.

Carotenoid	Microalga source	Reference
Asthaxanthin	*Haematococcus pluvialis* *Chlorella vulgaris*	(Mendes et al., 2003, Plaza et al., 2009, Guedes and Malcata, 2011)
Cantaxanthin	*Haematococcus pluvialis* *Chlorella vulgaris*	
Lutein	*Chlorella pyrenoidosa* *Haematococcus pluvialis* *Scenedesmus obliquus*	(Wu et al., 2007, Guedes and Malcata, 2011)
Violaxanthin	*Chlorella pyrenoidosa*	
β-carotene	*Dunaliella salina*	(Mendes et al., 2003, Plaza et al., 2008, Guedes and Malcata, 2011)

Singlet oxygen ($^1O_2$) is effectively quenched by xanthophylls; lutein is an efficient quencher of triplet chlorophyll, while β-xanthophylls (zeaxanthin and neoxanthin) serve as quenchers to $^1O_2$ (Dall'Osto et al., 2007). Under high light intensity, violaxanthin is converted to zeaxanthin via enzymatic removal of epoxy groups from violaxanthin; its conversion to zeaxanthin is catalyzed by violaxanthin de-epoxidase, while the reverse conversion is effected by zeaxanthin epoxidase (Pospisil, 2012).

Antioxidants (such as astaxanthin) provide a broad, “upstream” approach that quenches ROS/reactive nitrogen species (RNS) or free radical chain-breaking; consequently, antioxidants appear to be an appropriate therapeutic option, as epidemiologic, dietary, and *in vivo* animal model data suggest (Giao et al., 2010). However, lutein is more effective than β-carotene in inhibiting auto-oxidation of cellular lipids and in protecting against oxidant-induced cell damage (Lakshminarayana et al. 2010). For instance, (Manabe et al., 2008) have indicated that astaxanthin can scavenge ROS in high-glucose-treated mesangial cells. Other studies have revealed that this compound can also convey antioxidative protection in diabetic animals (Marin et al. 2011).

1.2.1.2. Antioxidant scavenging capacity of PUFA

As mentioned previously, EPA and DHA have been claimed as therapeutical agents to prevent several diseases, and some of the mechanisms are related to their antioxidant properties (Mazza et al., 2007).

Common knowledge on fatty acids has it that the higher degree of unsaturation promotes a higher susceptibility to oxidation, but several pieces of evidence indicate that this

assumption does not always hold true. As an example, when individual fatty acids are oxidized in an aqueous environment, the generation of peroxidation products does not reflect their degree of unsaturation (Visioli et al., 1998). Maziere et al. (Maziere et al., 1998) compared the effects of omega 6 and omega 3 fatty acids, incorporated into endothelial cells, with regard to cellular ability to oxidize low density lipoprotein (LDL). They reported that ω-3 fatty acids lowered TBARS production (thiobarbituric acid reactive substances), by-products of lipid peroxidation), superoxide anion secretion, and LDL peroxidation as compared with ω-6. One explanation proposed by the authors was that the ω-3 series, due to their double bond positions, are less susceptible to oxidative damage than the ω-6 series

Free radical-scavenging potential of PUFA and the production of reactive oxygen/nitrogen (ROS/RNS) species by human aortic endothelial cells (HAECs) supplemented with different fatty acids, was investigated (Richard et al., 2008). It was found that fatty acid micelles scavenged superoxide in an unsaturation-dependent manner, and highlighted EPA as the most effective. Additionally, they reinforced the idea that supplementation of HAEC with polyunsaturated fatty acids of the ω-3 series results in lower formation of ROS, as compared to cells supplemented with saturates, mono-, or poly-unsaturates of the ω-6 series. Hence, they claimed that fatty acids might diminish inflammation and, in turn, the risk of atherosclerosis and cardiovascular disease.

More recently, Mas et al. (2010) found that DHA can reduce oxidative stress by reducing the levels of F2-isoprostanes; these ω-3 fatty acids reduced the oxidative stress, which is likely related to their anti-inflammatory action and reduction in leukocyte activity (Mas et al., 2010). These findings further backup supplementation of one's diet with ω-3 fatty acids aimed at preventing said diseases.

Microalgae are an important source of long-chain fatty acids, mainly PUFA, as described before in many studies (Plaza et al., 2008, Guedes et al., 2011a).

Although scarce studies have focused specifically on antioxidants properties of microalgal PUFA, a correlation of microalgal extracts rich in PUFA as having antioxidant capacity has been proposed (Amaro et al., 2015, Maadane et al., 2015).

1.2.1.Antitumor features of microalgal lipidic components

Despite significant progress in prevention, diagnosis, and development over the last quarter of a century, cancer still represents the second highest cause of mortality in developed countries, after CVDs. There are 3.2 million new cases of cancer every year; in 2008, 1.7 million deaths were associated with cancer. The most common forms are colorectal (13.6 %), breast (13.1 %), lung (12.2 %), and prostate (11.9 %) cancers. The main

causes of death are lung (19.9 %), colorectal (12.3 %), breast (7.5 %), and stomach (6.8 %) cancers (Ferlay et al., 2010).

In said disease, cells divide and grow uncontrollably due an imbalance in the rate of cell proliferation and apoptosis, thus leading to formation of malignant tumours that can invade nearby parts of the body and spread to more distant parts of the body through the lymphatic system or the bloodstream.

Tumour suppression and/or cell death is thus crucial in cancer therapy. The fundamental mechanisms of the former often involve inhibition of tumour cell-mediated protease activity, attenuation of tumour-induced angiogenesis, promotion of cell cycle arrest, induction of apoptosis, and immunostimulation (Amaro et al., 2013). On the other hand, cell death entails mainly apoptosis, necrosis or autophagy phenomena.

Comparing cell death mechanisms in neoplastic cells, occurrence of apoptosis is preferred. It involves action of proteins from different families, such as Bcl-2, Bax, and caspases 3, 6, 7, 8, and 9; these are engaged in complex signal transduction pathways, and may affect tumour growth at one or more stages of carcinogenesis (Amaro et al., 2013). Conversely, cell necrosis is difficult to prevent and always develops an inflammatory response and death of surrounding cells (Han et al., 2008).

Autophagy, a mechanism that disassembles unnecessary or dysfunctional cellular components, and also described as a mechanism of cell death, is likewise indicated as a cancer therapeutic target. However, it has a dual effect since maintaining cell survival can promote growth of established tumours (Costa et al., 2012, Amaro et al., 2013). Several anticancer drugs work as apoptotic modulators, in order to eliminate silent and cleanly the unwanted cell (Costa et al., 2012).

Until now, cancer research has focused on the search for curative treatments; however, these treatments normally entail side effects (Sangeetha et al., 2014). A few studies have aimed at developing preventive strategies for the control of various cancers. Hence, discovery of new drugs that are more active, more selective, and less toxic – but which limit deleterious side effects and tumour multidrug resistance, will obviously constitute a challenge in coming years (Pasquet et al., 2011).

Chemoprevention is an old concept that consists on use of drugs, vitamins, or nutritional supplements to reduce the risk of developing or having a recurrence of cancer. Considering the important role of inflammation in the origin and evolution of a variety of tumours, the interest in chemoprevention has markedly increased in the last years (Sporn, 2011). Carcinogenesis of common epithelial tumours, including lung, colon, pancreas, ovary, skin, prostate and breast that are responsible for most deaths, is a slow process that could start twenty years before the first symptoms appear. This long period is suitable for use of chemopreventive strategies that block the development of invasive and/or metastatic

disease. Toward these goals, cancer chemoprevention resorts to natural, synthetic or biological substances to reverse, suppress or prevent either the initial phase of carcinogenesis or the progression of neoplastic cells to cancer (Demaria et al., 2010).

Some of the aforementioned preventive features have been assigned to antioxidant micronutrients, including those belonging to the carotenoid and PUFA group (Sun and Yao, 2007). Their isolation from the marine environment has generated interest among many groups – including purification of original compounds and understanding their biological activity, as well as in identifying their pharmacological targets (Pasquet et al., 2011).

Additionally, more than 50 % of the marine prokaryotic microalga are potentially exploitable for extracting bioactive substances that are effective in killing cancer cells by inducing apoptotic death (Sangeetha et al., 2014). Moreover, an increasing number of marine cyanobacterial compounds are found to promote cell cycle arrest by targeting tubulin or actin filaments in eukaryotic cells, thus making them an attractive source of natural products as anticancer agents (Sangeetha et al., 2014). Until now, a considerable number of cyanobacteria compounds have shown antitumor capacity towards several human cancer cell lines. In a simplified approach, they can be grouped according to class of compound (lipopeptides, peptides, fatty acids, macrolides and amides).

Furthermore, studies on human cells with bio-guided fractionation of microalgal extracts have proven that many pigments, beyond their ecological function as light-harvesting molecules, act as potent bioactive compounds against cancer cells – and may thus hold great potential in the prevention and treatment of cancers (Folmer et al., 2010). In particular, carotenoids have received increasing attention because of the decreased incidence of cancers associated with their dietary consumption via fruits and vegetables (Nishino et al., 2000, Nishino et al., 2009). Microalgae have a high carotenoid content, and may thus be of great interest as functional foods for prevention of cancer, or even as a source of pure carotenoids.

Clinical trials have demonstrated that phytomedicine (including phycomedicine) is effective in treating pathologies related to vascularization and cell proliferation in prostate hyperplasia, especially using carotenoids (Tanaka et al., 2012). Its underlying activity rests on several mechanisms, including enzyme activity (as in the case of topoisomerases), prevention of oxidative damage, immune modulation, hormone and growth-factor signalling, regulatory mechanisms of cell-cycle progression, cell differentiation, and apoptosis (Tanaka et al., 2012, Wu et al., 2010).

1.2.1.1. Antitumor features of carotenoids

Microalgal carotenoids, mainly β-carotene, astaxanthin, and lutein, have been consistently suggested as antitumor agents (see Table 2). Other carotenoids, however, also found in microalgae, have also been found to possess antitumor capacity (see Table 3). Selected possibilities will be briefly discussed in this section, on a compound-by-compound basis.

Table 1.2. Carotenoids produced by microalga with antitumor capacity tested on cancer cell lines.

Carotenoid	Action	Type of cancer	Microalga source	Reference
Asthaxanthin	Apoptosis induction Growth inhibition Proliferation inhibition	HCT-116 (colon cancer) HepG2 (hepatic cancer) MCF-7 (breast cancer)	*Haematococcus pluvialis*	(Tanaka et al., 2012)
Violaxanthin	Proliferation inhibition	AGS (stomach) MCF-7 (breast) HeLa (cervical) DLD (colon) Hep-G2 (liver)	*Chlorella ellipsoidea*	(Soontornchaiboon et al., 2012)
Violaxanthin + antheraxanthin + zeaxanthin	Proliferation inhibition Apoptosis induction	HCT116 (Colon)		(Cha et al., 2008)
Diadinochrome A, B, Diatoxanthin/cynthiaxanthin	Cytotoxic effect	HeLa cells	*Peridinium bipes*	(Guedes et al., 2011)
Fucoxanthin	Proliferation inhibition	A549 (bronchopulmonar) NSCLC-N6 (bronchopulmonar) SRA 01/04 (epithelial)	*Odontella aurita,* *Chaetoseros* sp. *Isochrisys* aff. *galbana*	(Moreau et al., 2006)

Table 1.3. Carotenoids produced by microalgae with proven antitumor capacity and produced by microalgae.

Carotenoid	Action	Type of cancer	Microalga source	Reference
β-Carotene	Tumor inhibition	Hepatic cancer	*Dunaliella salina*	(Mukherjee et al., 2011)
α-Carotene	Proliferation inhibition	Skin, lung, liver and colon Neuroblastoma	*D.salina*	(Nishino et al., 2002, Murakoshi et al., 1989)
Lutein	Growth inhibition Proliferation inhibition Chemoprotective effect	Prostate Mammary tumor Colon cancer Mouth epithelial cancer	*Muriellopsis* sp., *Scenedesmus almeriensis*, *Chlorella protothecoide*, C. *zofingiensis*, C. *citriforme*, *Neospongiococcus gelatinosum*	(Narisawa et al., 1996b, Lakshminarayana et al., 2010a, Reynoso-Camacho et al., 2011b, Fernández-Sevilla et al., 2010)

1.2.2.1.1. Antitumor action by α-and β-carotene

The discovery in the 1970s of β-carotene as a possible anticancer agent opened a new route in the field of cancer chemoprevention. The deficiency of β-carotene (along with other antioxidants) associated with some cancers suggests that a low-antioxidant diet may allow neoplastic changes to continue. A cancer-protective role for β-carotene has been claimed, and an apparent primary mechanism as antioxidant has been suggested – relying on oxidative tissue damage (Nishino et al., 2000). In fact, a study involving the population of Linxian, China – known for the highest rate of incidence of esophageal/gastric cancer in the world, has confirmed that supplementation with β-carotene, vitamin E, and selenium substantially reduces the risk of developing this type of cancer (Liu et al., 1998, Clerici et al., 2004). At the same time, diets with a low serum β-carotene level have also been unfolded as a risk factor for various other forms of cancer, such as leukemia, lymphoma, and central nervous system (CNS), bone, and renal cancers. β-carotene has been reported to play an anticarcinogenic role against hepatocarcinogenesis in rats (Chattopadhyay et al., 2004).

The anticancer activity of β-carotene appears to be more effective in long-term trials when administered for a prolonged period rather than in mere initiation or promotional stages; this was demonstrated in a study involving 2-acetylaminofluorine-induced hepatocarcinogenesis. Hence, β-carotene may be better suited for use as a prophylactic than chemopreventive agent; moreover, pre-neoplastic lesions induced by diethylnitrosamine in resistant rat hepatocytes were shown to decrease when β-carotene was present, to a statistically significant extent (Bishayee et al., 2000). In general, the anticancer potential of β-carotene relies upon its free radical scavenging nature coupled with its immediate involvement in trapping singlet oxygen; this provides an overall reducing environment in the hepatic tissues on long-term exposure to β-carotene (Mukherjee et al., 2011).

However, administration of large doses of synthetic β-carotene was found to have no effect on mesothelioma and lung cancer in human subjects (De Klerk et al. 1998). β-Carotene is one of the most efficient known substances in quenching the excitation energy of $^1O_2$ and in trapping certain organic free radicals (thus preventing oxidative tissue damage); its capacity to enhance gap-junctional communication and inhibit lipid peroxidation in chemically induced neoplastic transformation in 10T1/2 cells, and to act as a chain-breaking antioxidant in the lipid phase via neutralization of peroxy radicals appear to be the underlying mechanism in controlling cancer growth (Black and Gerguis, 2003, Yang et al., 2004).

The microalga *Dunaliella salina* (Teo.) is well known for accumulating β-carotene (β, β-carotene) when subjected to growth-limiting conditions (e.g. exposure to high irradiances). However, some of the detectable carotenoids in the human body have more potent activity than β-carotene in suppressing the process of carcinogenesis, including α-carotene (Orset and Young, 1999). In addition, α-carotene (β, ε-carotene) may be synthesized and then accumulated by *D. salina* under specific growing conditions. It appears that α-carotene inhibits proliferation by inducing G1 arrest in the cell cycle of human neuroblastoma (cell line GOTO), in a dose- and time-dependent manner (Orset and Young, 1999). Moreover, this carotenoid shows a 10-fold greater inhibitory activity than β-carotene in suppressing tumours in the skin, lung, liver, and colon (Nishino et al., 2009).

A particularly relevant note is that β-carotene of natural origin (e.g. microalgae) is preferred by the health market due their mixture of trans- and cis-isomers, which are hardly obtained via chemical synthesis; the latter exhibit better anticancer features (Guedes and Malcata, 2011).

1.2.2.1.2. Antitumor action by lutein and zeaxanthin

Lutein and zeaxanthin are stereoisomers that belong to the xanthophyll family. Several reports have revealed an inverse correlation between lutein or zeaxanthin intake and cancer occurrence. Lutein can significantly inhibit growth of androgen-dependent and androgen-independent prostate cancer cell lines *in vitro*, and prevent colon carcinogenesis *in vivo* (Narisawa et al., 1996a); it also plays a role upon inhibition of proliferation of human mouth epithelial cancer line KB (Sun Zhen, 2006) (Table 3).

A few *in vivo* studies involving mice have unfolded the potential of dietary lutein as an antitumor agent; indeed it was demonstrated that a diet containing lutein reduced the growth of (transplantable) mammary tumor and enhanced lymphocyte proliferation (Lakshminarayana et al., 2010b). Furthermore, Reynoso-Camacho et al. (2011) verified an additional chemoprotective effect of lutein against colon cancer induced by DMH (dimethylhydrazine), via modulation of proteins involved in the regulation of cellular proliferation or differentiation; in other words, they increased the expression of mitogen-

activated protein (K-ras), protein kinase B (pKB), and β-catenin proteins. Lutein fed at 0.002 % of the diet, before (prevention) and after (treatment) DMH administration, decreased the number of tumours by 55 and 32 %, respectively. Lutein significantly decreased the expression of K-ras (25 and 39 %), β-catenin (28 and 26 %), and pPKB (32 and 26 %) in tumours during prevention and treatment, respectively (Reynoso-Camacho et al., 2011a).

Concerning zeaxanthin alone, it appears to play an important role upon progression of melanoma by inhibiting the migration of skin fibroblasts (cells involved in melanoma growth and progression) at several stages (Wu et al., 2010).

1.2.2.1.3. Antitumor action by violaxanthin

Violaxanthin is another carotenoid that shows strong antiproliferative activity *in vitro* against human mammary cancer cells (MCF-7), thus suggesting that violaxanthin and derivatives thereof obtained via pharmacomodulation should be considered as tentative new drugs for treatment of breast cancer (Yang et al., 2004). Violaxanthin extracts from *C. ellipsoidea* possess a strong antiproliferative and pro-apoptotic activity against HCT116 human colon cancer cells (Sheu et al., 2008). Further studies are, nevertheless, needed to define the pharmacological mechanisms involved in its antiproliferative activity in human cancer cells.

1.2.2.2. Antitumor action by PUFA

As seen before, PUFAs can be classified into two major categories, ω-3 and ω-6. Between these two different classes of PUFAs, there is a great deal of variation in bioactivities – namely those that are cancer-related. For example, ω-3s, such as EPA and DHA, have been associated with cancer suppression, while ω-6s, particularly AA, is generally associated to cancer promotion (Yang et al., 2004, Xu and Qian, 2014). Hence, a high intake of ω-6s was found to correlate with a high risk of breast, prostate, and colon cancer incidence in many animal and human studies, and the ratio of ω-6s to ω-3s was suggested to be a predictor for cancer progression (Williams et al., 2011).

However, it was reported that other ω-6s, such as linoleic acid (LA), GLA, and dihomo-γ-linolenic acid (DGLA), may possess anticancer effects, and there is experimental evidence that LA can be involved in both pro- and anti-cancer activities. For example, studies indicate that LA stimulates cell proliferation in the human breast cancer cell line BT-474 and lung cancer cell line A549 *in vitro*, and promotes colon and prostate tumorigenesis and tumour growth in animal models (Sauer et al., 2007). However, others claim that a high dose of LA inhibits proliferation of the colon cancer cell line Caco-2, and shows a protective effect against cancer development (Horrobin and Ziboh, 1997, Dommels et al., 2003).

Other studies also demonstrate that GLA is associated with anticancer activities either *in vitro* as *in vivo*. GLA inhibited cell growth of the human neuroblastoma cell lines GOTO, SK-N-DZ, NKP, and NCG, a rat C6 glioma cell line, and the rat carcinosarcoma cell line LLC-WRC256 *in vitro* (Fujiwara et al. 1998). Additionally, a dietary supplement of GLA also reduced tumor growth in an implanted WRC256 using a rat model (Colquhoun et al. 2002). More interestingly, GLA-induced cytotoxicity was shown to exhibit high selectivity toward cancer cells, with no significant effect on normal cell growth. For instance, a series of studies suggested that 3–7 days of incubation with GLA could selectively induce cell death in various human cancer cell lines, including the human breast cancer cell ZR-75–1, the lung cancer cell A549, and the prostatic cancer cell PC-3, without affecting normal cell growth (Das, 2006).

Lipid peroxides derived from docosahexaenoic acid (DHA), a PUFA rather abundant in microalgae, are generally regarded as toxic to cells. They exhibit anticancer effects that materialize in the mitochondria and nucleus. The mitochondria is considered a universal sensor of cell stress: it respond to stress by undergoing functional and/or structural changes leading to induction of apoptosis. PUFAs, such as DHA, are rapidly incorporated into the mitochondria, where they cause several changes that eventually promote cell death. Those changes encompass alteration of mitochondrial membrane properties and related functions in rat colonocytes, human colonic tumour cell line HT29, Walker 256 rat carcinosarcoma, and T24 and Hep2 cancer cells (Siddiq and Dembitsky, 2008).

Experimental evidence suggests that DHA accumulates preferentially in mitochondrial cardiolipin (CL) that is present in the inner membrane and at intermembrane contact sites (McMillin and Dowhan, 2002).

As review before (Amaro et al., 2013) DHA in CL is apparently susceptible to ROS generated through oxidative phosphorylation. An increase in mitochondrial Ca^{2+} due to changes in the cytosolic levels of this cation probably results in enhanced ROS generation and modification of membrane topography – both favouring CL peroxidation; cytochrome C catalyses that phenomenon. When peroxidation occurs, mitochondrial CL levels decrease, affecting the activity of CL-dependent proteins involved in energy transduction, and causing a drop in mitochondrial membrane potential – which in turn initiates apoptosis. Moreover, DHA-induced cell death and fragmentation apparently take place in parallel with activation of ERK, c-Jun N terminal kinase (JNK), and actuator protein 1 (AP-1) in gastric carcinoma AGS cells (Lee et al., 2009). DHA was able to increase the intracellular levels of p53, cytochrome C, and Bax in gastric cancer cells, thereby exerting anticancer effects via decreasing their proliferation and inhibiting cell cycle progression (Albino. et al. 2000).

In DHA-treated gastric cancer cells, AP-1 regulates apoptosis-associated gene expression – determined by the relative levels of p53, cytochrome c, and Bax metabolites.

DHA-induced DNA fragmentation and increases in the three metabolites were suppressed by inhibiting AP-1 transactivation. Hence, DHA can inhibit cancer cell proliferation and induce apoptosis through the AP-1 pathway in human gastric cancer cells (Lee et al., 2009). Despite these pieces of evidence in support of DHA from microalgae (or otherwise) as a beneficial nutrient for cancer treatment, further studies are required to shed light on the anticancer action of DHA on specific cancer cells, including its putative side-effects. Furthermore, DHA demonstrated a synergistic effect with fluorouracil – meaning that the dose of fluorouracil used to treat human gastric carcinoma may be decreased without compromising its therapeutic effect (Albino et al., 2000).

In recent years, microalgae have become a good alternative source of PUFAs, e.g. with EPA, DHA and ALA being the most representative; species of microalgae rich in those PUFA are *Tetraselmis* sp. and *Nannochloropsis oculata, Pavlova lutheri*, *Arthrospira platensis* and *Chlorella* sp. (Guedes et al., 2011a, Martins et al., 2013).

1.3. Lipidic components extraction

One of the major bottlenecks in obtaining molecules from microalgae is the difficulty of extracting some metabolites, which can compromise high-throughput screening analyses. Development of extraction techniques for microalgae has become a field of growing interest for the scientific community. Most techniques developed are intended for heat-stable molecules, and thus seldom suitable for high-throughput screening for sensitive molecules. Concerning the latter, a few studies have been carried out to extract natural bioactive products from microorganisms since the 1980s. Many extraction techniques have accordingly been developed (Serive et al., 2012). Pigment studies, in particular, have been steadily increasing since the early 1970s, both in the field of oceanography (Szymczak-Żyła et al., 2011) and for industrial applications (Hejazi et al. 2002, Machmudah et al. 2006). These techniques are often suitable for molecule purification from large amounts of biomass (Hosikian et al., 2010), but not for high-throughput screening purposes.

Microalgae species are characterized by a huge biodiversity. This biodiversity includes thick-walled green or red algae, silicified diatoms, cyanobacteria with multi-layered walls, red algae with wall-bound exopolysaccharides and armoured dinoflagellates, which need to be broken before extraction is possible (Serive et al., 2012). Another important requirement is easy solubilisation of molecules of a wide polarity range. At laboratory scale, it is tempting to use strong solvents to extract target molecules. However, acetone, chloroform, dimethyl acetamide, dimethyl formamide, dimethyl sulfoxide and methanol are unsuitable at industrial scale, due to safety considerations (low lethal dose, carcinogenic, harmful, irritant or toxic) (Serive et al., 2012). Eco-friendly approaches may be used instead, via mild solvents that

would limit health risks while facilitating scale-up. The extraction method needs to be quick, simple to use and requiring no heavy equipment. The methods most widely applied for carotenoid extraction use classical solvent extraction, pressurized fluid extraction (PLE) and supercritical fluid extraction (SFE) – and will be discussed in terms of potential of applicability and limitations.

1.3.1Classical solvent extraction of lipidic components

1.3.1.1. Carotenoids classical solvent extraction

In classical solvent extraction, several different methods are employed depending on the carotenoid – see Table 4. For carotenoids, such as astaxanthin, classic extraction methods may include use of organic solvents, preceded by a breakdown pre-treatment of cells (cryogenic grinding and acid/base treatment), enzyme lysis (kitalase, cellulose and abalone acetone powder, mainly β-glucoronidase), mechanical disruption and spray-drying (Sarada et al., 2002, Kang and Sim, 2007).

These methods have been comprehensively tried – Sarada et al. (2002) tested the extractability of carotenoids from *H. pluvialis* with hydrochloric acid (2N) for 10 min at 70°C, followed by acetone extraction for 1 h – thus extracting 87 % (w/w) of astaxanthin without affecting its composition. Later, Kang & Sim (2007) developed a two-stage solvent procedure with dodecane and methanol to extract free astaxanthin from *H. pluvialis* cells, by mixing the solvents with the culture broth, followed by settling of the mixture for 48 h. The dodecane extract was separated from the cell debris, placed in another tank and mixed with NaOH in methanol (0.02 M), at a volume ratio of 1:1 (to promote saponification of astaxanthin esters to free form). Then, the tank was kept in darkness at 4 °C (12h) to support astaxanthin extraction toward the methanol phase. The results indicated a total recovery yield of free astaxanthin over 85 % Dry-Weight (DW.)

However, some disruption methods have a negative effect upon carotenoid recovery. For example, Zhao et al. (2006) found that methods like microwave and ultrasound induce instability of synthetic astaxanthin, thus inducing its conversion to other astaxanthin isomers – while ultrasound showed to degrade this pigment into colorless compounds, due the cavitation produced in the solvent from propagation of ultrasonic waves.

Table 1.4. Microalgal carotenoid classical solvent extraction.

Carotenoids	Microalgae source	Extraction/Purification method	Yield/Extraction efficiency	Reference
Astaxanthin	*Chlorococcum* sp.	Methanol:dichloromethane 1:3 (v/v). Cells disrupted by French Pressure at 110MPa. Saponification in darkness (50 mg NaOH in 100 ml methanol).	Yield: 7.09 mg/g $_{DW}$	(Ma and Chen, 2001)
	Haematococcus pluvialis	Cell acid digestion with HCl 2M. Acetone extraction at 70°C for 1h.	Efficiency: 87 %	(Sarada et al., 2002)
		Dodecane mixing for 48h. Saponification with methanolic NaOH (0.02M). Sedimentation in darkness at 4°C, 12 h.	Efficiency: 85 %	(Kang and Sim, 2007)
		Hexane:acetone: ethyl alcohol (100:70:70 %v/v) extraction.	N/A	(Domínguez-Bocanegra et al., 2004)
		DMSO extraction at 55°C, vortex 30s	N/A	(Orosa et al., 2005)
		Dodecane :methanol (1:1) (v/v), 2-step procedure, and addition of NaOH at 25 ºC	Efficiency: 85 %	(Mäki-Arvela et al., 2014)
β-carotene	*Dunaliella salina*	Hexane/acetone/ EtOH (2:1:1) (v/v) at 25 ºC for 24 h Followed by KOH saponification	Efficiency: 90.42 %	(Hu et al., 2008)
Carotenoids	*D. salina*	DMF extraction at 25 ºC for 3 min of sonification. Storage at 4 ºC.	Yield: 27.7 mg/g	(Macias-Sanchez et al., 2009)
Lutein	*Scenedesmus obliquus*	Bead beater pretreatment, extraction with diethyl ether at 25 ºC in S/R ratio, 2-5 extraction steps.	Efficiency: 99 % Yield: 2.05 mg/g	(Chan et al., 2013)
Fucoxanthin	*Phaeodactylum trocornutum*	Freeze-dried cells, extraction with ethanol during 60 min.	Yield: 15.33 %	(Kim et al., 2012)

1.3.1.2. Lipids classical solvent extraction

For an ideal lipid extraction, the organic solvents should preferably be volatile for low-energy distillation from the crude lipids (Medina et al., 1998).

Chloroform:methanol 1:2 (v/v) is the organic solvent mixture most frequently used for lipid extraction from any living tissue. Using this organic solvent system, originally developed by Folch et al. (1951), residual endogenous water in the microalgal cells acts as a ternary component that enables quantitative extraction of both neutral and polar lipids (Folch et al., 1957). This method does not require complete drying of microalgal biomass. Once the cell debris is removed, more chloroform and water are added to induce biphasic partitioning. The lower organic phase (chloroform with some methanol) contains most lipids (both neutral and polar), while the upper aqueous phase (water with some methanol) encompasses most non-

lipids (proteins and carbohydrates) (Medina et al., 1998). Chloroform, however, is highly toxic and its usage is undesirable.

Therefore, a hexane:isopropanol (3:2 (v/v)) mixture has been suggested as a low-toxicity substitute to chloroform:methanol system (Halim et al., 2011). The mixture works in a similar fashion to the chloroform:methanol system. Upon biphasic separation, the upper organic phase (hexane with some isopropanol) contains most lipids (both neutral and polar), while the lower aqueous phase (water with some isopropanol) contains most non-lipids (proteins and carbohydrates). When evaluated for microalgal lipid extraction, the hexane:isopropanol mixture was found more selective towards neutral lipids compared to chloroform:methanol (Halim et al., 2011).

Guckert et al. (1988) found a neutral lipid selectivity by hexane:isopropanol mixture, and its inability to extract the polar lipid constituents of microalgal membranes (chloroplast membranes contain glycolipids and cell membranes contain phospholipids) (Guckert and Cooksey, 1990). The hexane:isopropanol system, however, yielded a surprisingly low total lipid recovery when applied to *Botryococcus braunii* (Lee et al., 1998).

Nagle and Lemke (1990) have evaluated the efficiencies of three organic solvents (butanol, hexane:2-propanol mixture and ethanol) in extracting crude lipids from *Chaetoceros muelleri*, and compared them to a control water:methanol:chloroform mixture(Nagle and Lemke, 1990). Even though the control polar/non-polar mixture was found to be the most effective organic solvent system (assigned an arbitrary extraction efficiency of 100 %), butanol (with an average extraction efficiency of 94 %) was found to be highly promising with a final total lipid yield consistently higher than hexane:2-propanol mixture or ethanol; and it showed lower sensitivity to changes in extraction procedure – an essential attribute to scale-up the procedure. Due to its tendency to inactivate many phosphatidases and lipases, the use of isopropanol-containing organic solvent mixture was recommended to extract lipids from unicellular microalgal species that produces lipid degradative enzymes (Halim et al., 2011).

Some extraction techniques could be combined with polar extraction solvents to enhance the kinetics of lipid extraction, through fast cell disruption structures such as Soxhlet extraction, microwave-assisted extraction, ultrasound-assisted extraction, extraction via pulsed electric field, bead-beating-assisted extraction, and others. Advantages and disadvantages will be briefly discussed next.

Some laboratory-scale organic solvent lipid extractions methods resort to use Soxhlet apparatus, but this batch extraction is limited by the lipid mass transfer equilibrium. In order to overcome this limitation, the continuous process requires a large amount of organic solvent so it becomes too expensive. Soxhlet ingenious extraction endorses cycles of solvent evaporation and condensation through microalgal biomass, and continuously replenishes

cells with fresh organic solvent (hence circumventing equilibrium limitation) while simultaneously minimizing solvent consumption; despite its advantageous design in avoiding equilibrium limitation, the Soxhlet apparatus suffers from the high energy requirement of continuous distillation (Luque de Castro and García-Ayuso, 1998).

However, independent studies by Guckert et al. (1988) and Halim et al. (2011) confirmed the superior efficacy of Soxhlet extraction when compared to batch extraction. Among the three systems texted by Guckert et al. (1988) to extract lipids from *Chlorella* sp., Soxhlet extraction using a methylene chloride:methanol 2:1 (v/v) mixture attained the highest final total lipid yield; the final total lipid recovered was ca. 11.9 % in terms of dry microalgal weight. Moreover, Halim et al. (2011) found Soxhlet operation of hexane extraction to be significantly more efficient than its batch counterpart when used to extract lipids from *Chlorococcum* sp. – with a final total lipid yield of batch extraction of 0.015 $g.g_{DW}^{-1}$, and a final total lipid yield of Soxhlet extraction of 0.057 $g.g_{DW}^{-1}$.

Despite its improved total lipid recovery, Soxhlet extraction potentially suffer from lipid degradation resulting from use of high temperature throughout the process – particularly in the case of PUFAs, more labile to thermal degradation (Guckert and Cooksey, 1990).

Microwave-assisted organic solvent extraction resorts to electromagnetic radiation, within a specific frequency range, to deliver large amount of thermal energy to the microalgal cells (Balasubramanian et al., 2011). When the cells receive this energy, local internal superheating occurs, leading to instantaneous temperature rise within the matrices and rapid pressure effects on the cell wall/membrane structure. Cell structures are immediately ruptured, thus forcing cell constituents to spill out. This effective release of cell materials facilitates diffusion of microalgal lipids into the extracting organic solvent. Microwave-assisted heating is substantially more rapid than conventional heating, as heat is delivered via radiation rather than convection and conduction. Microwave-assisted hexane extractions were found to lead to higher oil yields compared to conventionally water-heated hexane extraction control methods, at all extraction temperatures and times. While the microwave system extracted 76-77 % of total recoverable oil within 20-30 min at 95 °C, the water-heated hexane only extracted 43-47 %.

1.3.2. Pressurized fluid extraction

1.3.2.1. Pressurized fluid extraction of carotenoids

Pressurized fluid extraction (PFE) or Pressurized Liquid Extraction (PLE) is a method that operates with conventional solvents; mainly due to the use of elevated temperatures and pressures, it increases compound solubility. Unlike extraction at room temperature, pressure keeps the solvent in its liquid state, even if temperatures above the boiling point are applied. Additionally, pressure favors penetration of the solvent into the biological matrix. This is specifically beneficial for microalgal cells with thick cell walls. The PFE device includes an extraction cell (1 up to 100 mL) maintained within 80-200°C, into which a solvent is pumped and maintained at 10-20 MPa for a few minutes. The extract is then pushed into a collection vial by a second volume of solvent, and finally the whole solvent is pushed with an inert gas flow (Camel, 2001).

PFE show higher (or equal) extraction efficiencies as compared to traditional solvent extraction, while maintaining the integrity of chemical components (Denery et al., 2004). High pressure typically shortens the extraction time and the amount of solvent used. Pressurized fluid extraction has been actively studied (Denery et al., 2004, Plaza et al., 2008, Koo et al., 2012, Jaime et al., 2010) Pressurized liquid extraction of algae has been preferred due to specific benefits, and is a powerful tool in the nutraceutical industry: possibility to avoid excessive heat, oxygen and light that cause degradation of sensitive compounds (Plaza et al., 2010), lower amount of solvent needed (Jaime et al., 2010), higher selectivity compared with Soxhlet and ultrasound-assisted extraction (Koo et al., 2012), and shorter time needed for extraction (Jaime et al., 2010). Several solvents have been investigated for pressurized liquid extraction of algae, such as ethanol, 2-propanol, hexane, petroleum ether and water. Ethanol has been one of the best solvents, giving both high yields (Koo et al., 2012) and extracts with high antioxidative capacity (Herrero et al., 2005) – as can be grasped in Table 5.

Pressurized extraction of zeaxanthin has been investigated with *Chlorella ellipsoidea* under pressurized conditions with different solvents, such as hexane, ethanol and 2-propanol, and with *Chlorella vulgaris* using ethanol as solvent (Koo et al., 2012, Chan et al., 2013). Use of hexane and 2-propanol as solvent requires higher temperatures when compared to ethanol (Koo et al., 2012). The high extraction efficiency of ethanol was also observed in the extraction of carotenoids from *Phormidium* in the temperature range 50-100 °C at 10.3 MPa, compared to those achieved with water or hexane (Rodriguez-Meizoso et al., 2008). Ethanol was also an efficient solvent for extraction of astaxanthin from *Haematococcus pluvialis* at 10.3 MPa and 200 °C. When non-polar hexane was applied, low

efficiency was achieved due to the polar nature of zeaxanthin. It has been claimed that zeaxanthin is hardly soluble in hexane and petroleum ether (Mäki-Arvela et al., 2014).

When comparing different solvents toward extraction of carotenoids from *Spirulina platensis* at 10.3 MPa in hexane, petroleum ether, ethanol and water, the highest yields were attained in ethanol (19.7 wt%), followed by water (10.12 wt%), hexane (4.3 wt%) and petroleum ether (4.0 wt%) (Herrero et al., 2005). On the other hand, the use of polar solvents favors extracts in terms of antioxidant capacity, as is the case of ethanol. Similarly to the work by Herrero et al. (2005), ethanol gave higher extraction yields compared with hexane during extraction of *Haematococcus pluvialis* within the temperature range 50-200 °C at 10.3 MPa.

Table 1.5. Optimal conditions for pressurized liquid extraction of carotenoids from microalgae.

Carotenoid	Microalga source	Solvent system	Processing conditions	Efficiency/ yield (%)	References
Lutein	*Synechocystis* sp.	Ethanol	T (ºC): 100 P (MPa): 10.3 T (min): 20	Yield: 2.04 mg/g	(Plaza et al., 2009)
	Chlorella vulgaris	20 ml ethanol for 0.5 g microalga	T (ºC): 160 P (MPa): 10.3 T (min): 30	Yield: 3.78 mg/g	(Chan et al., 2013)
Zeaxanthin	*Chlorella*	Jet mill treated cell extracted with EtOH, 6 % KOH	T (ºC): 50 P (MPa): 3 T (min):	Efficiency:1.46	(Shibata et al., 2004)
	Chlorella ellipsoidea	Ethanol	T (ºC): 115.4 P (MPa): 10.3 T (min): 23.3	Yield: 4.28 mg/g:	(Koo et al., 2012)
	Synechocystis sp.	Ethanol	T (ºC): 100 (MPa) P (MPa) 10.3 T (min): 20	Yield:1.64 mg/g	(Plaza et al., 2010)

As emphasized before, pressurized liquid extraction shortens extraction time. Typically, a longer extraction time leads to a higher extraction yield; however, in some cases, e.g. extraction of lutein, lower yields were achieved with longer extraction times, since lutein was less thermolabile than astaxanthin (Denery et al., 2004). Short extraction time (just 10 min) gave high extraction yield of fucoxanthin from *Phaeodactylum tricornutum* at 100 °C in ethanol – whereas comparable methods led to about the same yields either at room temperature with ultrasound-assisted extraction or in Soxhlet extraction at 80 °C for 30 min (Kim et al., 2012).

Temperature can either be dominant, or exhibit a minor effect. However, some combinations of temperature and time can promote side reactions, e.g. pheophorbide

formation from chlorophyll *a* (Koo et al., 2012). The most efficient solvent for extraction of zeaxanthin is ethanol, with an optimum temperature for extraction of 115.4 °C, whereas extraction in hexane produces a lower amount of zeaxanthin. When comparing ultrasound-assisted extraction with pressurized extraction of carotenoids at high temperature, a benefit claimed for zeaxanthin extraction via the pressurized system is the lower liquid viscosity (Koo et al., 2012). One drawback of using higher extraction temperatures, namely in extraction of lutein and β-carotene from *Chlorella vulgaris*, is formation of pheophorbide from chlorophyll *a*. This formation increases with extraction time, and the highest pheophorbide formation was observed at 60 °C – but decreased with increasing temperature, due to deactivation of chlorophyllase at high temperature. Pheophorbide is a Mg^{2+}-free chlorophyll that may cause dermatitis in human skin and food poisoning above 1.6 mg g^{-1} (Mäki-Arvela et al., 2014). Temperature exhibited only a minor effect upon astaxanthin yields during pressurized liquid extraction of *Haematococcus pluvialis* performed in acetone at 10.3 MPa, within 20–100 °C for 5 min, using three extraction cycles (Denery et al., 2004). The results revealed that astaxanthin yield was nearly unaffected by temperature, whereas a slight decrease of lutein was observed at higher temperature (Denery et al., 2004). Furthermore, no *trans*- to *cis*-isomerization of astaxanthin occurred at 40 °C and 10.3 MPa. It was finally stated that the pressurized liquid extraction required only half the amount of solvent and 20 min extraction time, when compared to the traditional extraction time of 90 min.

1.3.2.1. Pressurized fluid extraction in PUFA extraction

PLE extraction for microalga PUFA recovery has been seldom reported, yet there is evidence that an extract rich in fatty acids was obtained by this technique from *Phormidium* sp.(Rodriguez-Meizoso et al., 2008). Pieber et al. (2012) also found that ethanol was very effective in EPA extraction via a PLE system from *Nannochloropsis oculata* biomass, achieving yields of 16.7 ± 0.6 biomass% in terms of fatty acids and 3.7 ± 0.1 mass %, particularly EPA (Pieber et al., 2012).

1.3.2. Supercritical fluid extraction

Supercritical Fluid Extraction (SFE) is a relatively rapid extraction process due the low viscosities and high diffusivities that characterize supercritical fluids. Supercritical CO_2 exhibits indeed a relatively lower viscosity and higher diffusivity – ca. 10^{-4} $cm^2.s^{-1}$, whereas liquid solvents are characterized by ca. 10^{-5} $cm^2.s^{-1}$, so it can penetrate porous solid materials more effectively than liquid solvents would, and consequently render mass transfer much faster. For instance, for a given level of recovery, the extraction time may be reduced from hours or even days in liquid-solid extraction to just a few tens of minutes in SFE (Brühl and

Matthäus, 1999). On the other hand, extraction can be made selective by controlling the density of the medium; whereas the material extracted can be recovered by simply depressurizing, thus allowing the supercritical fluid to return to its gaseous form while leaving no (or very little) residual solvent in the solute precipitate (Bravi et al., 2007). In SFE, the physicochemical properties of a given fluid – viz. density, diffusivity, dielectric constant and viscosity, can indeed be easily controlled by changing the operating pressure and/or temperature, as long as the phase boundaries are not crossed (Bravi et al., 2007). Supercritical CO_2 ($SCCO_2$) has so far been the most employed supercritical fluid – because it is non-flammable, non-toxic, inexpensive and relatively inert from a chemical point of view. On the other hand, due to its moderate critical temperature (31.1 ºC), extraction of thermolabile compounds can take place without significant thermal degradation (Mendes et al., 2003). Moreover, addition of a small amount a co-solvent with some polarity may increase the solvation power of CO_2; for example, addition of 1-10 % of ethanol to CO_2 expands the extraction range so as to include several polar solutes (Bravi et al., 2007).

There are several advantages (but also drawbacks) in using supercritical CO_2 relative to classical organic solvents. Despite its properties that make it suitable for extracting thermally labile and non-polar bioactive compounds, it performs much worse in terms of polar molecules; the decreases in processing time and the increases in yield are outrun by its presence complicating the associated thermodynamics and increasing capital costs (Guedes et al., 2013). On the other hand, use of high purity, SFE-grade CO_2 is not required; however, impurities and moisture in industrial operation may accumulate, and eventually interfere with further operation.

So far, high installation costs of the extraction pressure vessel, coupled with unfavorable energy requirements for fluid compression and heating remain the primary obstacles for scaling-up $SCCO_2$ extraction.

1.3.2.1. Supercritical fluid extraction of carotenoids

Supercritical extraction with CO_2 or ethane has been applied to carotenoid separation, due to its high selectivity and safety toward thermolabile carotenoids (Jaime et al., 2010). In some cases, supercritical CO_2 extraction gives low yields, e.g. in astaxanthin extraction, so use of ethanol as a co-solvent is recommended. However, in supercritical extraction of β-carotene, ethane or ethylene have been successfully demonstrated as effective – since β-carotene has higher solubility in these hydrocarbons than in CO_2 (Guedes et al., 2013).

The extraction for carotenoids entails three different stages: i) in the beginning, the extraction is linear with time – thus indicating a constant rate, which is caused by either solubility equilibrium or a constant mass transfer resistance; ii) extraction rate declines

because most of the carotenoids have already been stripped from the solid–liquid interface; and iii) in the final region, the extraction rate is very low due to the need for solvent to diffuse into the algae matrix for residual extraction. The addition of ethanol as a co-solvent can enhance permeabilization and cell rupture and/or matrix swelling, thus facilitating internal mass transfer (Macias-Sanchez et al., 2009).

The effects of pressure and temperature upon supercritical extraction of carotenoids are interrelated; when increasing temperature at relatively low pressure, extraction yield is lowered due the lower density of CO_2 – which also lowers solubility of carotenoids in the solvent, as observed at 70 °C and 30 MPa in supercritical CO_2 (Kitada et al., 2009). Otherwise, extraction yields are increased with increasing pressure and temperature, if the carotenoid is sufficiently thermoresistant. Note that solute properties, such as thermal and chemical stability, as well as polarity and solubility also affect extraction efficiency (Cardoso et al., 2012).

At high pressures, the carotenoid yield typically increases with increasing of CO_2 pressure and temperature (Machmudah et al., 2006). Solubility and vapor pressure of solute are important parameters in determining extraction efficiency. Furthermore, solvent viscosity decreases with increasing temperature. High temperature enhanced the yield of astaxanthin from *Haematococcus pluvialis* with supercritical CO_2, when changing temperature from 40 to 70 °C (Aravena et al. 2012) – see Table 6. This result was explained by the increase in vapour pressure of the solute, thus facilitating mass transfer into the CO_2 phase.

Similar results were also achieved by Machmudah et al. (2006)- high yields of astaxanthin from *Haematococcus pluvialis* were attained with pure CO_2 at relatively high temperatures, 60-80 °C, and pressures of ca. 5 MPa – i.e. similar to those found by Aravena et al. (2012) when starting from dry alga powder.

Different optimum temperatures and pressures were obtained for extraction of β-carotene and zeaxanthin from *Synechococcus* sp., due to the fact that β-carotene is non-polar – whereas zeaxanthin has two hydroxyl groups; hence, different temperature and pressure optima for their extraction have been reported (Cardoso et al., 2012). β-carotene yield from *Synechococcus* sp. was largest at the highest temperature and pressure studied, i.e. 60 °C and 40 MPa, respectively, when using pure CO_2 as solvent with ethanol (Cardoso et al., 2012). On the other hand, the highest extraction efficiency for zeaxanthin was achieved with CO_2 at the highest temperature and lowest pressure, 60 °C and 20 MPa, respectively (Cardoso et al., 2012). Extraction of β-carotene from *Dunaliella salina* has also been demonstrated in supercritical ethane or ethylene (Mendes et al., 2003). Typically, extraction yields increase with increasing density of ethane or ethylene, and ca. 59 wt% yield of β-carotene was attained within 20 min. On the other hand, the carotenoid yields were quite low at relatively low CO_2 pressure and high temperature, due to the decrease in density and

solubility of carotenoid (Mendes et al., 2003, Kitada et al., 2009). This effect was apparent in the final yield of lutein using 40 MPa at 70 °C; it was lower at 40 MPa than 30 MPa. The solubility of lutein was, however, higher at 40 MPa compared to 30 MPa, thus unfolding diffusion limitations. Furthermore, the initial extraction rates were the same at 30 MPa within 60-80 °C. This result was justified by the fact that lutein solubility at 80 °C was the rate-limiting factor, because the density of supercritical CO_2 decreases with increasing temperature at constant pressure (Kitada et al., 2009). Lutein extraction rate and final lutein recovery after 2 h were similarly very low for *Chlorella vulgaris* at 40 MPa and 80 °C, whereas at 40 °C lutein recovery was much higher, 0.6 % and 1.6 %, respectively (Ruen-ngam et al., 2012). In the work by Mendes et al. (2003), a lower carotenoid yield was achieved at 55 °C than 40 °C, and 20 MPa. The results showed an analogous trend of low solubilities of astaxanthin at three different temperatures, and low CO_2 pressure. Analogously, at pressures close to the critical pressure of CO_2, a temperature increase lowers the recovery degree of astaxanthin – since solubility of astaxanthin decreases with increasing temperature, due to a decrease in density (Machmudah et al., 2006). In addition, only a slight increase in astaxanthin extraction efficiency from *Haematococcus pluvialis* was observed at 30 MPa of CO_2, when increasing the extraction temperature from 40 °C to 60 °C.

In some cases, carotenoid yields also decrease with increasing pressure. Supercritical CO_2 has been investigated in carotenoid extraction from *Dunaliella salina* (Macias-Sanchez et al., 2009) – see Table 6. For instance, carotenoid yield exhibited a maximum at 40 MPa and 60 °C, whereas at a higher pressure, 50 MPa, the yield of carotenoids was much lower. This result was explained by the fact that diffusivity of solvent increases with increasing temperature, while density of CO_2 decreases. Furthermore, vapour pressure of pigments increased as well (Macías-Sánchez et al., 2009).

Selectivity for carotenoid extraction is high in microalga extraction with pure CO_2, but its being very non-polar limits the yields of such relatively polar carotenoids as astaxanthin (Cardoso et al., 2012, Mendes et al., 2003, Macias-Sanchez et al., 2009). In some cases, the solubility of *cis*- versus *trans*- isomers of carotenoid is different in pure CO_2, thus favoring faster extraction of the other isomer (Mendes et al., 2003)

Supercritical extraction of lutein with 30 MPa CO_2 at 60 °C results in relatively low yield, 0.5 $mg.g^{-1}$, but maximum selectivity – whereas in the presence of ethanol the yield of lutein was 3 $mg.g^{-1}$, while ca. 9 $mg.g^{-1}$ chlorophylls were extracted under the same conditions (Kitada et al., 2009) as per Table 6 . Supercritical extraction with CO_2 is also very selective toward β-carotene (Cardoso et al., 2012). It was demonstrated that the supercritical extraction of carotenoids gave rise to both carotenoids and chlorophylls, but it was about 18-fold more selective for carotenoid extraction than achieved with ultrasound-assisted extraction with methanol (Macias-Sanchez et al., 2009).

The solubility difference between *cis*- and *trans* isomers of β-carotene in supercritical CO_2 facilitates selective production of the former in supercritical CO_2, since solubility of *cis*-β-carotene is higher than *trans*-β-carotene in CO_2 (Mendes et al., 2003). It is also known that the *cis*-form is more easily absorbed by the human body than its *trans*-isomer, thus emphasizing the importance of selective recovery of *cis*-β-carotene. When comparing the extraction efficiency of acetone with the efficiency of supercritical CO_2 extraction, a two-fold enhancement of the *cis*/*trans* ratio was obtained for extraction of β-carotene from *Dunaliella salina* using supercritical CO_2 as solvent.

Table 1.6. Optimal conditions of supercritical fluid extraction for pigments from microalgae.

Carotenoid	Microalga source	Processing conditions	Achievements	Reference
Astaxanthin		EtOH/10 % soybean oil extraction at 70 ºC, 4Mpa	Efficiency: 36 %	(Krichnavaruk et al., 2008)
	Haematococcus pluvialis	$SCCO_2$ at 55 MPa and 70 ºC.	Total extracted: 21.8 %, Amount extracted: 77.9 %. AX content in the extract: 12.3 %	(Machmudah et al., 2006)
		CXE at 7 MPa and 45 °C with 50 % w/w EtOH content in CO_2	Extraction yield: 333.1 $mg/g_{DW.}$ AX content: 62.57 $mg/g_{DW.}$ AX recovery: 124.2 % w/w	(Reyes et al., 2014)
	Monoraphidium sp.	$SCCO_2$ with acid treatment, EtOH as co-solvent at 20 MPa and 60 °C for 1 h	AX yield: 2.45 mg/g_{DW}	(Fujii, 2012)
Total carotenoids	*Scenedesmus almeriensis*	SFE at 40 MPa and 60 °C	12.17 ± 0.24 $\mu g/mg_{DW}$	(Macías-Sánchez et al., 2010)
Lutein	*Sc. almeriensis*	$SCCO_2$ at 30 MPa and 39 ºC for 300 min	0.0236 $mg_{pigments}/g_{DW}$	(Macias-Sanchez et al., 2009)
	Scenedesmus obliquus	$SCCO_2$ at 25 MPa, 40 ºC with a CO_2 flow of 2 $g.min^{-1}$	0,028 mg/g_{DW}	(Guedes et al., 2013)

AX- Astaxanthin; CXE- CO2-expanded ethanol extraction; EtOH- ethanol; MeOH- methanol

As stated before, due to the fact of $SCCO_2$ is a very non-polar solvent and xanthophylls (e.g. lutein and astaxanthin) have low solubility, addition of ethanol aids in the extraction of hydroxyl-containing carotenoids; however, extraction selectivity towards one specific carotenoid is reduced when compared with pure CO_2 (Kitada et al., 2009). Lutein extraction from *Scenedesmus* sp. was found very efficient with CO_2 and ethanol as co-solvent (Guedes et al., 2013). Lutein yield from *Scenedesmus* sp. increased with increasing CO_2 pressure at 47.5 °C, with the maximum recovery being only 3.1 % at 40 MPa (Yen et al., 2012). When ethanol was used as entrainer, the yield increased with increasing molar fraction of ethanol up to 62.2 %, using 40 mol% ethanol at 40 MPa and 70 °C. Therefore, supercritical CO_2 extraction of spray-dried *Scenedesmus* sp. was not feasible without ethanol as co-solvent. Furthermore, an optimum ethanol concentration in $SCCO_2$ gives the highest carotenoid yield (Yen et al., 2012). The optimum ethanol concentration in the extraction of lutein from *Scenedesmus* sp. was 20 mol %, thus yielding 76.65 % recovery at 40 MPa and 70 °C, whereas it was only 5 % for astaxanthin extraction from *Haematococcus pluvialis* – with CO_2

giving the highest astaxanthin yield, of 77.9 %, at 70 °C and 40 MPa within 240 min (Yen et al., 2012, Machmudah et al., 2006). In the presence of ethanol as co-solvent, the increase of temperature can in some cases have a negative effect – e.g. in supercritical CO_2 extraction of astaxanthin from *Haematococcus pluvialis* (Machmudah et al., 2006). The rationale for these results may lie on the isomerization of astaxanthin in ethanol favoring oxidation (Bustamante et al. 2008). Different optimum temperatures and pressures were also found for extraction of β-carotene and zeaxanthin from *Synechococcus* sp., due to their different solubilities in CO_2–ethanol mixture. The optimum temperature for extraction of β-carotene was 40 °C at 20 MPa CO_2 and 5 vol % ethanol, whereas the optimum temperature and pressure were 60 °C and 20 MPa, respectively, for zeaxanthin (Cardoso et al., 2012).

Another promising method for supercritical CO_2 is to use vegetable oil as a co-solvent. The benefits of this method are higher solubility of e.g. astaxanthin in soybean oil–CO_2 mixture compared to that in pure CO_2, and the possibility of avoiding the subsequent separation step of the co-solvent – since the carotenoid can remain in vegetable oil products (Krichnavaruk et al., 2008). This method has been utilized in the preparation of astaxanthin extracted from *Haematococcus pluvialis* with supercritical CO_2 using vegetable oils – see Table 7 (Krichnavaruk et al., 2008) The optimum amount of soybean co-solvent was 10 %, giving 36 % extraction efficiency for astaxanthin at 70 °C and 40 MPa CO_2.

1.3.3.2. Supercritical fluid extraction in PUFA extraction

Many algae and microalgae are rich in polyunsaturated fatty acids that can be extracted by SFE; however, fatty acid extraction has been studied to a lesser extent than carotenoids (Herrero et al., 2006).

Almost two decades ago, Cheung (1999) studied the effect of extraction conditions to obtain fatty acids, particularly ω-3 from non-conventional algae, *Hypnea charoides*, using supercritical CO_2, with temperature ranging from 40 to 50 ºC and pressure from 241 and 379 bar (Cheung, 1999). Although, in general, the lipid recovery increased along a pressure and temperature increase, the ratio of unsaturated fatty acids was also increased (Herrero et al., 2005).

Concerning the extraction of ω-3 fatty acids, their solubility was shown to depend on their chain length in the case of *Botrycoccus braunii* cells. It was observed that solubility of these type of compounds in CO_2 increased with pressure; at 300 bar, the best conditions were attained, also in terms of extraction rate.

Another microalga species tested in term of fatty acids extraction by SFE was *Arthospira* (Spirulina) *maxima (Mendes et al., 2003)*. This microalga produces high amounts of GLA, and was tested for said PUFA recovery with pure CO_2 – with CO_2 plus ethanol as co-

solvent, and with traditional organic solvent extraction. Although both CO_2 and n-hexane provided similar extraction yields, CO_2 allowed a higher recovery of GLA. Moreover, maximum extraction yield was obtained using CO_2 with 10 % of ethanol as modifier, and performing the extraction at 350 bar and 60 ºC. The same was observed in other species of this cyanobacteria, *Arthospira platensis*, as studied by Qiuhui (1999) (Albino et al., 2000, Qiuhui, 1999) with regard to GLA extraction. The maximum extraction yield was obtained at 350 bar, in good agreement with Mendes et al. (2003). The temperature was set at 40 ºC, while the CO_2 flow rate was fixed at 24 $Kg.h^{-1}$ for 4 hours.

Although SFE is an effective method for microalgal fatty acids extraction, there is a limitation in its application – the moisture content of the original feedstock. High moisture content reduces the contact time between solvent and feedstock. Microalgal biomass then acquires a thick consistency – with moisture acting as barrier against diffusion of CO_2 and of lipids off the cells, so samples are usually dried prior to SFE (Sahena et al., 2009). Although applied with success to *Arthrospira maxima*, *Arthrospira platen*sis, *Botryococcus braunii*, *Chlorella vulgaris*, *Ochronomas danica*, *Skeletonema costatum* and *Isochrysis galbana*, only limited information exists on the kinetics of the underlying process, and the influence of operating conditions upon the fatty acid composition of the final lipid extracts (Pereira et al., 2013). Its main disadvantage is being expensive, and requiring complex instrumentation and powerful pumping facilities.

1.4. Optimization of lipidic components production by microalgae

Several environmental factors (e.g. temperature, pH and light) not only affect photosynthesis and growth rate of microalgae, but also influence the activity of cellular metabolism and composition (Guedes and Malcata, 2011). Hence, selection and control of the most appropriate parameters allow redirection of metabolism towards the target compounds, turning the concept of microalgae as “biofactories” to a much more effective one – particularly if added-value bioproducts are intended.

1.4.1. Temperature

Temperature is perhaps one of the most important environmental factors that influence algal growth rate, cell size, biochemical composition and nutrient requirements. Below optimal growth temperatures, growth rate (μ) increases with increasing temperature, but declines markedly above the species- or strain-specific optimum (Juneja et al., 2013). Growth at temperature optima results in minimal cell size, while the efficiency of carbon and nitrogen utilization decreases at non-optimal temperatures (Juneja et al., 2013). Temperature

may also play a key role in photoinhibition, which is known to strongly impact algal growth rate.

It was observed that temperature has a major effect upon fatty acids synthesized by microalgae; many microalgal species respond indeed to a decrease in temperature by increasing the ratio of unsaturated to saturated fatty acids; however, this this feature is a species-dependent trait, with no overall consistent relationship between temperature and fatty acid unsaturation (Thompson et al., 1992).

High temperature also favours accumulation of lutein, as is the case with other carotenoids (e.g. β-carotene); this was observed in *Dunaliella* sp., and was reviewed by (Guedes and Malcata, 2011). Such temperatures are close to the edge of causing environmental stress; hence, the operational window is narrow, because further temperature increases would be harmful and eventually cause decreases in biomass productivity.

1.4.2. pH

In microalgal cultivation, pH is one of the most important factors – since it controls solubility and availability of CO_2 and other essential nutrients, so it can have a significant impact upon algal metabolism (Juneja et al., 2013). On the other hand, it can rise significantly in algal cultures due the uptake of inorganic carbon by microalgae (Juneja et al., 2013). Usually, maximum algal growth occurs around neutral pH; a higher pH may limit the availability of carbon from CO_2, thus suppressing growth and lowering the affinity of algae to free CO_2 (Azov, 1982). Alkaline pH indirectly results in an increase of triglyceride accumulation, but a decrease in membrane-associated polar lipids due to cell cycle inhibition. Membrane lipids in *Chlorella* were observed to be less unsaturated under conditions of alkaline pH (Juneja et al., 2013). Acidic conditions may instead alter nutrient uptake, or induce metal toxicity and thus affect algal growth (Gensemer et al., 1993).

However, *Chlamydomonas* sp. and *Pinnilaria braunii* var. *amplicephala* (an acidophilic diatom) were able to accumulate storage lipids such as triacylglycerides under highly acidic conditions (pH 1) (Poerschmann et al., 2004). It was also observed that acidic conditions promoted an increase in saturated fatty acid content, which reduces membrane fluidity and inhibits high proton concentrations (Poerschmann et al., 2004). Such adaptation was also reported in a *Chlamydomonas* sp. – for which total fatty acid content slightly increased from 2 % at pH 7, to 2.4 % at pH 2.7 (Poerschmann et al., 2004).

In terms of carotenoid production, available data show that pH does not exert a relevant role in their production; in fact, the best productivities are normally attained at the optimum pH for biomass production (Guedes et al., 2011c).

1.4.3. Light

Light is the most important factor influencing growth of a photosynthetic microorganism – and represents the main source of energy for microalgae. Irrespective of microalgal production system, the light source, intensity and spectrum are critical upon performance of phototrophic growth (Mata et al., 2010).

Sun is the universal light source, but is only available in outdoor microalgae cultivation and is restricted to the daylight period. On the other hand, sunlight is composed by a wide spectrum – but only the visible part contains photosynthetic active radiation (PAR), which ranges from violet (380 nm) to the far red at (750 nm). For photosynthetic organisms like microalgae, only a restricted range is harvested, namely blue (about 400 nm) and red (around 700 nm).

Light harvesting by photosynthetic organisms occurs due presence of three major classes of pigments: chlorophylls (Chl), carotenoids and phycobilins, which are arranged in light harvesting complexes (LHC). All types of LHC are composed by a core and a reaction center pigment (Chl *a*), and light-harvesting antennae composed by such other pigments as subtypes of Chl and carotenoids or phycobilins (in the case of prokaryotic microalga) – composing the so-called accessory (antennae) pigments that allow extension of the range of light absorption (Masojídek et al., 2007).

Although blue and red lights are the most effectively absorbed by photosynthetic pigments, photoregulation of microalgae is not limited to the photosynthetic apparatus; several photoreceptors, i.e. pigments that absorb light and transduce light signals, are also actively involved in triggering various light responses, which are independent of the photosynthetic apparatus (Masojídek et al., 2007). For instance, blue light has been proven to influence gene expression and several metabolic pathways in microalgae and plants, like endogenous breakdown of carbohydrate reserves (Schulze et al., 2016), and to induce nitrate and nitrite uptake. Moreover, red and far-red lights appeared to affect growth, cell size, and photosynthesis rate in microalgae (Lee and Palsson, 1996).

Following selection of the most adequate light source, it is possible to manipulate the microalgal biomass toward maximum biomass production, as well its content of high value metabolites for specific uses (Schulze et al.).

Fluorescent lightening is the most common light source employed in indoor microalga cultivation; as happens with sunlight, it possesses a wide light spectrum that makes it an energetically inefficient light source, and besides its high energy cost also leads to unwanted heat generation (Schulze et al., 2016). A new technology for microalga culture illumination has arisen in recent years to overcome these limitations – light emitting diodes (LEDs). Their advantages include longer life-expectancy, lower heat generation, and higher conversion

efficiency – further to narrow light emission spectra, between 20 and 30 nm of amplitude, which can be matched to photosynthetic needs (Chen et al., 2011).

Light intensity also affects growth and cellular composition of algae. An excessive supply of light above saturating limits is known to cause photoinhibition; it leads to disruption of the chloroplast lamellae, and inactivation of enzymes involved in carbon dioxide fixation (Juneja et al., 2013). It was observed that the growth rate of *Dunaliella viridis* decreased to 63 % following an increase in light intensity from 700 to 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Gordillo et al., 1998). Moreover light intensity affects the cellular composition of microalgae. *Dunaliela tertiolecta* subjected to high light intensity, up to saturation, exhibited a decrease in protein content and an increase in lipid fraction (Juneja et al., 2013).

Conversely, low light intensities led to an increase in the rate of protein synthesis by *Phaeodactylum tricornutum (Juneja et al., 2013)*. In absence of light, an increase in total lipid content of *D. virdis* was observed – but a reduced content in triglycerides, free fatty acids, free alcohols and sterols arose (Smith et al., 1993). As reviewed by Juneja et al. (2013) regarding *Nannochloropsis* sp., detrimental effects of low light intensities have been observed: 40 % of the total lipids were found to be galactolipids, and 26 % were found to be triacylglycerols. In the same system, however, high light conditions resulted in increased synthesis of triacylglycerol, with concomitant reduction in galactolipid synthesis (Juneja et al., 2013).

Strong light, in general, leads to oxidative damage of PUFA. Numerous studies have suggested that cellular lipid content and PUFA levels decrease with increase in light intensity (Renaud et al., 1991, Juneja et al., 2013). Conversely, *Nannochloropsis* cells under low light conditions were characterized by high lipid content and high proportions of EPA (Sukenik et al., 1989). There are contradictory claims regarding PUFA levels versus increasing light intensity (Molina Grima et al. 1999). Such differences in response to environmental conditions by different microalgae may be related to difference in their metabolic pathways. Increase in oxygen-mediated lipid desaturation could be one reason for the observed increase in PUFA levels under conditions of higher light intensity (Fernández-Sevilla et al., 2010).

As seen above – due to the carotenoid role in light harvesting, particularly under stress conditions, it is easy to conclude that their production is highly affected by light intensity. Excess of photo-oxidation caused by high light irradiance does apparently trigger synthesis of carotenoids, as part of a cellular strategy aimed at cell protection against oxidative damage (Fu et al., 2013). In particular, flashing light showed to increase the rate of some carotenoid concentrations, such as astaxanthin in *H. pluvialis* (Hu, 2007). Furthermore, high irradiance provides more energy for photosynthetic fixation of carbon, which leads to a higher rate of astaxanthin synthesis; this may be further enhanced by raising the C/N ratio (Juneja

et al., 2013). In *D. salina*, it has been widely accepted that light intensity is a key stimulus for β-carotene overproduction.

Unlike fatty acids, biosynthesis of carotenoids is complex and coordinated with biogenesis of chlorophylls and proteins of the photosynthetic apparatus (Fu et al., 2013); the light spectrum influences their production, but the underlying mechanism is not fully clear yet.

Some studies have related blue light to induction of production of astaxhantin in *H. pluvialis* (Katsuda et al., 2004); others showed that red light induces production of a larger pool of xanthophylls and Chl *a* in *Phaeodactylum tricornutum* (Schellenberger Costa et al., 2013). However, others authors claim that a higher carotenoid/chlorophyll ratio is obtained under red light when compared to blue or green light in *Botryococcus braunii* Bot-144 cultures (Baba et al., 2012). In *Dunaliella salina*, red light supplemented with blue one increase accumulation of β-carotene and lutein (Fu et al. 2013).

1.5. Thesis aims

So, the main objective of this thesis was the study and characterization of bioactive compounds with potential pharmaceutical application produced by microalgae. To achieve this goal, efforts founded in resolution of biotechnological bottlenecks were founded in the following approaches:

I) ascertain the solvent influence on optimized recovery of bioactive extracts rich in lipidic components (carotenoids and PUFA) and their characterization;

II) optimize the extraction conditions of extraction of said bioactive components in terms of temperature and pressure, using the solvent selected in I);

III) optimize concentration of lipidic components in the microalga cell in order to minimize further purification processes, using light quality as a tool.

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PART II

MICROALGAL BIOACTIVE LIPIDIC COMPONENTS

CHAPTER 2

Effect of Solvent System on Extractability of Lipidic Components of Scenedesmus obliquus *(M2-1) and* Gloeothece *sp. toward Antioxidant Scavenging Capacity thereof*

Effect of solvent system on extractability of lipidic components of *Scenedesmus obliquus* (M2-1) and *Gloeothece* sp. on antioxidant scavenging capacity thereof

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Abstract: Microalgae are well known for their biotechnological potential, namely with regard to bioactive lipidic components – especially carotenoids and PUFA, well-known for therapeutic applications based on their antioxidant capacity. The aim of this work was to evaluate the influence of four distinct food-grade solvents upon extractability of specific lipidic components, and on the antioxidant capacity exhibited thereby against both synthetic ($DPPH^{\bullet}$ and $ABTS^{+\bullet}$) and biological reactive species ($O_2^{-\bullet}$ and $NO^{-\bullet}$). An eukaryotic microalga (*Scenedesmus obliquus* (M2-1)) and a prokaryotic one (*Gloeothece* sp.) were used as case studies. Concerning total antioxidant capacity, the hexane:isopropanol (3:2) and acetone extracts of *Sc*. o*bliquus* (M2-1) were the most effective against $DPPH^{\bullet}$ and $ABTS^{+\bullet}$, respectively. *Gloeothece* sp. ethanol extracts were the most interesting scavengers of $O_2^{\bullet-}$, probably due their high content in linolenic acid. On the other hand, acetone and hexane:isopropanol (3:2) extracts were the most interesting ones regarding the $NO^{\bullet-}$ assay. The acetone extract exhibited the best results for the ABTS assay, likely associated to its content in carotenoids, in both microalgae. Otherwise, ethanol stood out in terms of PUFA extraction. Therefore, the profile of lipidic components extracted is critical upon the antioxidant performance – which appears to hinge, in particular, on the balance between carotenoids and PUFAs.

Keywords: *carotenoid; PUFA; extract; microalga; cyanobacteria; $ABTS^{+\bullet}$; $DPPH^{\bullet}$; superoxide ($O_2^{-\bullet}$) assay; nitric oxide (•NO) assay*

2.1. Introduction

Reactive oxygen species (ROS) occur naturally as by-products of aerobic metabolism. In microalgae under non-stress conditions, the production and scavenging of ROS remain in equilibrium [1]. However, several environmental stress factors, such as pollution, drought, high temperature, excessive light intensity, and nutritional limitation may increase the production of ROS, thus inducing oxidative stress. The formation of these unstable, yet very reactive radicals, can trigger human diseases – e.g. cancer and cardiovascular diseases, owing to the damage caused in proteins, DNA, and lipids [1,2].

Photosynthetic organisms, like microalgae, are able to counteract the aforementioned negative effects *via* a number of enzymatic and non-enzymatic mechanisms [1]. Lipidic components, such as carotenoids and polyunsaturated fatty acids (PUFA), are but two examples of non-enzymatic classes of molecules able to protect the organism from oxidative damage [2,3]. A particular interest has been received by those two families of compounds due their great potential in industrial formulation of nutra- and pharmaceutical products [4]. PUFA, found in microalgae as components of polar and neutral lipids, include linoleic (18:2), α-linolenic (18:3), arachidonic (20:4), eicosapentaenoic (20:5) and docosahexaenoic (22:6), among others; they are valuable for humans due to their physiological roles in cells – as precursors and primary preventers of health conditions, e.g. as anti-inflammatory or neuroprotective agents [6,7]. Besides being excellent singlet oxygen scavengers suitable for use as food colorants, carotenoids may be employed as dietary supplements in cosmetics and nutraceuticals [8]. In particular, lutein has proven to alleviate cardiovascular diseases, some types of cancer and degenerative human diseases [9]. Hence, combined extraction of those lipidic compounds appears crucial in attempts to maximize their extra added value in nutra- and pharmaceutical formulations.

The mode of recovery of functional ingredients from natural matrices should be carefully addressed. There is in fact a need to combine appropriate, selective, cost-effective, and environment-friendly extraction procedures with legal requirements regarding use of food-grade solvents and associated processes. Extraction costs of microalgal intracellular metabolites are normally high; the downstream separation stages may account for 50–80% of the total production costs [10]. Despite the worldwide increasing interest on lipidic components from microalgae, there is no optimum standardized method for their extraction. It has been established that efficient extraction of lipids is strongly dependent on the polarity of

the organic solvent or solvent mixture employed [11]; however, other issues such as location of compound inside the cell have to be addressed, depending on cell structure complexity.

Based on their physicochemical characteristics, microalgal lipids can be divided into two major types: polar lipids, e.g. phospholipids and glycolipids; and neutral/non-polar lipids, e.g. mono-, di- and tri-acylglycerols (TAG) and carotenoids [11,12]. Polar lipids are important structural components of cell membranes and organelles, where they apparently operate as signal molecules (or precursors thereof). Among non-polar lipids, TAG are the most widespread group of compounds aimed at storage – and are accumulated as cytoplasmic oil bodies [7].

Carotenoids are hydrophobic molecules that, depending on their role, can be divided in two categories – primary and secondary ones. Primary carotenoids – including β-carotene and such xanthophylls as lutein, neoxanthin, violaxanthin, antheraxanthin, and zeaxanthin (in *Chlorophyta*), are contained within the non-polar 'pouches' of the thylakoid membrane, and are pigment-protein complexes of the photosynthetic apparatus; hence, they essentially do not interact with the hydrophilic environment [13]. Secondary carotenoids, like astaxanthin, are often esterified by fatty acids and accumulated in ester form – being present in oil bodies and plastoglobuli [13].

Neutral lipids are extracted with relatively non-polar solvents, e.g. hexane, whereas membrane-associated lipids are more polar, thus demanding such polar solvents as ethanol or methanol to disrupt hydrogen bonds and electrostatic forces.

The efficiency of extraction of lipids is highly dependent on polarity of the organic solvent or solvent mixture used. In general, solvent mixtures containing a polar and a non-polar component are able to extract a greater amount of lipids [12]. Hexane/isopropanol (3:2) has accordingly proven to be one of the best non-halogenated solvent mixtures to extract fatty acids in *Isochrysis galbana* [13]. By the same token, most extraction methods suitable for carotenoids resort to such organic solvents as hexane, ethanol, isopropanol, acetone, methanol, benzene and petroleum ether [14,15]. Although carotenoids can be polar (e.g. lutein) and nonpolar (e.g. β-carotene or carotenoids in ester form), the former are easily dissolved in polar solvents (e.g. acetone), while the latter are easily dissolved in nonpolar solvents (e.g. petroleum ether or hexane) [16].

Therefore, food GRAS (Generally Recognized As Safe) solvents with lower environmental impact and toxicity were selected for this work. Ethyl lactate was chosen as alternative to ethyl acetate and halogenated solvents. It is environment-friendly and fully biodegradable into CO_2 and water. Its use has been approved for food products by U.S. Food and Drug Administration, and its miscibility with both hydrophilic and hydrophobic compounds make it

appropriate to extract a diverse range of metabolites, namely carotenoids (in their stereoisomeric forms) and PUFA [17]. Ethanol and isopropanol, two short chain alcohols, have been proposed as alternative extracting solvents due to their greater safety and lack of regulatory problems, namely for extraction of carotenoids [8].

In attempts to cover a large range of polarities consistent with the various lipidic components of interest in microalgae, the next five food grade solvents were selected based on literature searches including data on their relative polarities: hexane, 0.009; acetone, 0.355; ethyl lactate, 0.460; isopropanol, 0.617; and ethanol, 0.654. Experimentation was conducted with plain ethanol, plain acetone, a mixture of hexane/isopropanol (3:2) (v/v) and plain ethyl lactate.

Due to absence of a standard extraction method for lipidic components, our motivation was to investigate the potential impact of the aforementioned food grade solvents upon extraction, and assess the bioactivity potential of the extracts afterwards. The target compounds were carotenoids and PUFA, and the tested species were representative of two levels of cell complexity, i.e. *Gloeothece* sp. (prokaryote) and *Scenedesmus obliquus* (M2-1) (eukaryote). The antioxidant scavenging capacity was measured by four distinct assays: total activity ($ABTS^{+\bullet}$ and $DPPH^{\bullet}$ radicals), and superoxide ($O_2^{-\bullet}$) and nitric oxide (•NO) radicals. Our findings may be useful in efforts to design more selective extraction protocols, and further incorporation of the extract obtained in food or cosmetic formulation based on the antioxidant potential attained.

2.2. Experimental Section

2.2.1. Microorganism source and growth conditions

Sc. obliquus (M2-1) strain was previously isolated from Portuguese aquaculture biofilters, and cultivated using Optimal Haematococcus Medium (OHM) [41]. This species was selected due to its high antioxidant capacity [38]. *Gloeothece* sp. (ATCC 27152) was purchased from ATCC — American Type Culture Collection (USA), and cultivated using Blue Green Medium (BG11) [42]. For each 4 L batch biomass production, a pre-inoculum with an initial optical density of 0.1 (at 560 nm or 680 nm for *Gloeothece* sp.) was cultivated for 10 days in 800 mL of buffered OHM or BG11 medium, with Tri-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) aimed at maintaining a constant pH of 8. This pre-inoculum ensured that the microalga is at exponential growth phase by the time of inoculation. A continuous illumination with fluorescent BIOLUX lamps, with intensity of 250 $\mu mol_{photon}.m^{-2}.s^{-1}$, was provided, as well as air bubbling at a flow rate of 0.5 $L.min^{-1}$.

2.2.2. Biomass quantification

2.2.2.1. Optimization of culture time

In order to choose the harvesting day yielding the best antioxidant potential, growth curves and associated antioxidant activity were obtained for both *Gloeothece* sp. and *Sc. obliquus*. Microalga cultures were accordingly settled in triplicate, samples were taken over time, and assayed (in duplicate) for optical density (OD) and dry weight (DW). The OD was measured spectrophotometrically at 560 and 680 nm for *Sc. obliquus*, and 680 nm for *Gloeothece* sp. (UV–Vis mini 1800, Shimadzu, Japan); these wavelengths correspond to the maximum and minimum culture absorption peaks. On the other hand, DW was determined by first filtering a volume of culture through preconditioned GF/C glass fiber filters (Whatman, UK), and drying at 100 ºC to constant weight. For the antioxidant capacity assessment, the procedure followed has been reported elsewhere [40].

2.2.2.2. Biomass production

Following the optimization in section *2.1.,* the biomass production was performed as described in section *3.1.* during 14 days. It was then collected by centrifugation at 4000 rpm for 10 min, freeze-dried and stored under nitrogen at -20 °C prior to analysis.

2.2.3. Lipidic component extraction

To evaluate the influence of solvents in lipid extractability, four different solvents/mixtures were tested: ethanol (99.6% purity), acetone (99.6% purity), a mixture (3:2) of hexane/isopropanol (99.6 and 99.8% purity respectively), and ethyl lactate (97% purity). Each extraction was performed in triplicate, in a triple stage extraction at a ratio of 1:60 (w_{DW}/v), at 40 ºC and 250 rpm for 20 min. To remove cells debris, extracts were then centrifuged at 20000 rpm for 10 min and filtered by 0.45 µm pore size. Extracts were stored under nitrogen, at -20 ºC in the dark, prior to analyses.

2.2.4. Antioxidant scavenging capacity assessment of extracts

The antioxidant scavenging activity was ascertained via four different assays. Two synthetic reactive species that measure the total activity ($DPPH^{\bullet-}$ and $ABTS^{\bullet+}$), and two biological reactive species ($O_2^{-\bullet}$ and •NO). $DPPH^{\bullet}$, $O_2^{\bullet-}$ and $^{\bullet}NO$ microassays were monitored spectrophotometrically in a Multiskan Ascent plate reader (Thermo, Electron Corporation), and $ABTS^{\bullet+}$ assay was performed in a spectrophotometer (Shimadzu). Antioxidant scavenging capacity was compared based on their IC_{50} and IC_{25} values. IC50 value is

defined as the concentration of an extract required to achieve half maximal inhibition of radicals, a parameter that is indicative of antioxidant capacity. IC values were calculated using GraphPad Prism (Version 5.0, 2007), via interpolation of dose-response curves obtained by plotting variation of radical scavenging % inhibition (average ± standard deviation) as a function of extract concentration ($mg.mL^{-1}$) for each radical assay tested.

2.2.4.1. $ABTS^{\bullet+}$ scavenging capacity

Extracts, obtained as described above, were evaporated and the residue re-suspended in ethanol:water 50:50 v/v to a final concentration of 10 $mg.mL^{-1}$. A dilution series was prepared (in triplicate), with concentrations ranging from 0.312 to 10 $mg.mL^{-1}$, in order to assess the IC_{50} values. The radical-scavenging capacity of the extracts was assessed via the $ABTS^{\bullet+}$ radical cation ($ABTS^{\bullet+}$) assay (in triplicate) – following the method described elsewhere [43,44], and recently refined by Guedes *et al.* [40]. For determination of evolution of total antioxidant capacity for both microalgae species, the results were expressed as Trolox Equivalent – TE, per unit of biomass, as given by dry weight, DW – where 1 TE unit is the mass of trolox possessing an equivalent antioxidant power.

2.2.4.2. $DPPH^{\bullet}$ scavenging capacity

Each extract was evaporated, and the residue resuspended in methanol to a final concentration of 10 $mg.mL^{-1}$. In order to obtain the IC_{50} and IC_{25}, a dilution series was prepared (in triplicate), with concentrations ranging from 0.312 to 10 $mg.mL^{-1}$, and tested in a 96-well plate. The plates were incubated for 30 min at room temperature, after addition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanol, and the scavenging reaction was monitored 515 nm as described by Ferreres *et al.* [24].

2.2.4.3. Superoxide radical ($O_2^{\bullet-}$) scavenging capacity

Each evaporated extract was re-suspended in phosphate buffer (100 mM, pH 7.4) with 20% DMSO. A dilution series was generated, ranging from 9.8 $\mu g.mL^{-1}$ to 10 $mg.mL^{-1}$, and tested in a 96-well plate. The superoxide radical induced by reduction of NBT was monitored spectrophotometrically, in kinetic function, at 562 nm. Superoxide radicals were generated by the NADH/PMS system as reported previously [24].

2.2.4.4. Nitric oxide radical (•NO) scavenging capacity

Each evaporated extract was re-suspended in phosphate buffer with 20% DMSO, and diluted in a range series from 4.9 $\mu g\ mL^{-1}$ to 2.5 $mg\ mL^{-1}$. Samples (in triplicate) were then

incubated with sodium nitroprusside, for 60 min at room temperature, in the light. Griess reagent was added afterwards, and the chromophore reaction was undertaken in the dark for 10 min, with absorbance read at 562 nm [24].

2.2.5. Chemical characterization of extracts

2.2.5.1. Determination of polyunsaturated fatty acids profile

Fatty acid methyl esters were produced for each extract obtained in section *3.3*. by direct transesterification – according to the acidic method described by Lepage and Roy [44], after modifications introduced by Cohen *et al.* [45], using heptadecanoic (C17:0) acid as internal standard and acetyl chloride as catalyst. Esters were analysed in a GC ThermoFinnigan Model gas chromatograph, using a flame ionization detector, and quantified with the program Chroma Card data system (2003). A silica CP-WAX 52 CB (Chrompac cp 7723) column was used, and helium was employed as carrier gas in splitless mode. Injector and detector were maintained at 260 and 280 °C, respectively, and the oven heating program consisted of a linear increase of column temperature from 150 to 260 °C, at a rate of 1 °C.min^{-1}. Chromatographic grade standards of fatty acids in methyl ester form (Sigma) were used for tentative identification, based on comparison of retention times: myristoleic, palmitoleic, petroselinic, oleic, elaidic, *cis*-vaccenic, linoleic, linolelaidic, linolenic, *cis*-11-eicosenoic, arachidonic, erucic, *cis*-4,7,10,13,16,19-docosahexanoic and nervonic. The average of the results from the aforementioned chemical assays were used as a datum point.

2.2.5.2. Determination of carotenoids profile

Carotenoids in each extract were tentatively identified, and then quantified by an HPLC-DAD method. Solvent was evaporated in a rotavapor, and the residue re-suspended in methanol LiChrosolv (Merck 99,9% purity) to a final concentration of 20 mg.mL^{-1}.

A Gilson HPLC-DAD with UV-visible photodiode array detector was employed to resolve, detect and identify the various chemical compounds of interest in each extract. The stationary-phase was a C30 YMC column – 5 µm, 250 x 4.6mm (YMC, Japan), maintained at room temperature, according to a previously described procedure [46] with modifications. The mobile phase consisted of two solvents: methanol (Merck) (A) and *tert*-butyl methyl ether (Sigma-Aldrich) (B), starting with 95% A and using a gradient to obtain 70% by 30 min, 50% by 50 min, 0% at 65 min, and 95% by 68 min. The injection volume was 20 µl, and the flow rate 0.9 mL.min^{-1}. Spectral data from all peaks were collected in the range 200-700 nm, and

chromatograms recorded at 450 nm. Data were processed on Unipoint System software (Gilson Medical Electronics, Villiers le Bel, France).

Carotenoids were identified by comparing their elution order and UV-Vis spectra with chromatographic HPLC-grade standards under identical conditions – lutein, zeaxanthin, β-carotene, fucoxanthin, astaxanthin (Sigma-Aldrich - St. Louis MO, USA), β-cryptoxanthin (Extrasynthese - Genay, France), astaxanthin, violaxanthin, neoxanthin, anteraxantina, lycopene, ε-carotene, γ-carotene and α-carotene (CaroteNature Lupsingen, Switzerland).

2.2.6 Statistical analyses

The experimental data were analysed using GraphPad Prism v. 5.0. A first diagnostic unfolded a non-normal distribution of the data, so 1-way ANOVA with Tukey's multicomparison test was used to assess variances between PUFA and carotenoid content, for the various solvents tested. Since each datum point had been replicated, a representative measure of variability was available in all cases to support said statistical analyses.

2.3. Results and Discussion

2.3.1. Microalgae production and harvesting

Microalgae species were selected based on earlier studies by Guedes *et al.* [18]. They found that intracellular extracts of *Sc. obliquus* (strain M2-1) possess a high antioxidant capacity when compared with other strains of *Scenedesmus*. Moreover, the scavenging activity correlated well with protective effects against DNA oxidative damage, with no mutagenic effects. It was also found that the maximum production of antioxidant compounds took place in the plain exponential phase, coinciding with the maximum peak production of lutein and β-carotene – thus suggesting a correlation between antioxidant capacity and presence of those carotenoids. Additionally, *Sc*. *obliquus* (M2-1) showed to have high content in PUFA, namely linoleic acid C18:2 (n-6) [6,18]. In the same study, *Gloeothece* sp. revealed to possess antioxidant potential and an interesting profile of PUFA [6,18]. The growth conditions selected for biomass production were 25 ºC and pH 8, based in an earlier study [19].

In order to unfold best antioxidant potential of each microalga, the culture time was selected based on growth curves and evolution in total antioxidant capacity (Fig. 1).

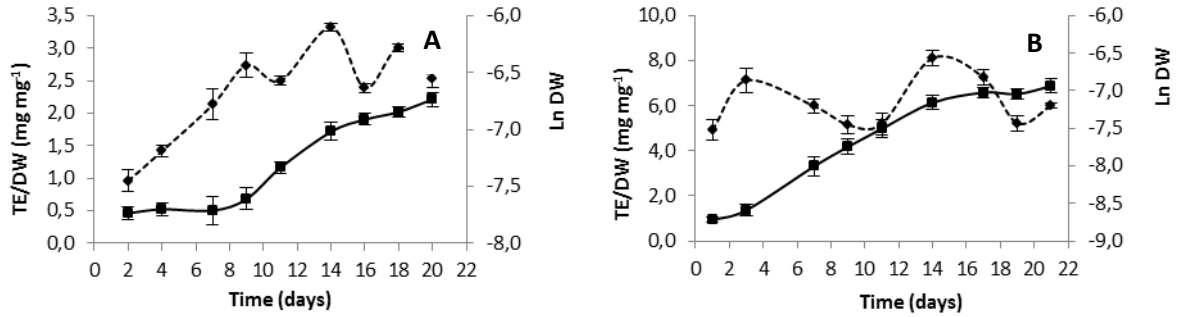

Fig. 1. Variation in time of biomass expressed as natural logarithm of dry weight (Ln DW) (average ± standard deviation) (—), and variation of intracellular extract antioxidant capacity expressed as ratio of trolox equivalent (TE) antioxidant capacity to dry weight, DW (average ± standard deviation) (- - -), for *Gloeothece* sp. (**A**) and *Sc. obliquus* (M2-1) (**B**).

Inspection of Fig. 1 indicates a maximum antioxidant intracellular capacity of both species in the intermediate exponential phase by 14 days of growth; hence, this was established as biomass harvesting day for subsequent use in lipidic extraction assays.

2.3.2. Extracts characterization

The principles underlying organic solvent extraction of microalgal lipidic compounds are anchored on the basic chemistry concept of 'like dissolving like'. Due to the interactions between their long hydrophobic fatty acid chains, neutral lipids – such as TAG and carotenoids [11], contribute to weak van der Waals attractions between one another, leading to formation of globules in the cytoplasm [11].

A 5-step protocol for organic solvent extraction has been proposed by Halim *et al.* [20], applicable to either non-polar or polar solvents. When a microalgal cell is exposed to a non-polar organic solvent, such as hexane: 1) the organic solvent penetrates through the cell membrane into the cytoplasm; 2) interacts with the neutral lipids via alike van der Waals forces; 3) an organic solvent-lipids complex is formed; 4) driven by a concentration gradient, the lipid complex diffuses across the cell membrane; and 5) said complex eventually crosses the static organic solvent film surrounding the cell into the bulk organic solvent. As a result, the neutral lipids are extracted out of the cells and remain dissolved in the non-polar organic solvent. A static organic solvent film is formed because the interaction between organic solvent and cell wall remains undisturbed for every rate of solvent flow or agitation. Some neutral lipids are, however, found in the cytoplasm complexed with polar lipids; such complexes are strongly linked *via* hydrogen bonds to proteins in the cell membrane. The van der Waals interactions between non-polar organic solvent and neutral lipids in the complex

are insufficient to disrupt the membrane-based lipid-protein associations. Conversely, polar organic solvents (e.g. ethanol, isopropanol or acetone) can disrupt the lipid–protein associations by forming hydrogen bonds with the polar lipids in the complex [11].

The mechanism of extraction of membrane-associated lipids by the mixture of non-polar/polar organic solvent follows the same major principles, except for minor differences arising from the solvent nature: 1) the organic solvent (both non-polar and polar) penetrates the cell membrane into the cytoplasm; 2) the solvent then interacts with the lipid complex – the non-polar organic solvent surrounds the lipid complex and engages in van der Waals associations with the neutral lipids of the complex, while the polar organic solvent surrounds the lipid complex and forms hydrogen bonds with the polar lipids in the complex, strong enough to counteract the lipid–protein associations binding the lipid complex to the cell membrane; 3) an organic solvent-lipid complex is formed, and dissociates away from the cell membrane; 4) the organic solvent-lipid complex diffuses across the cell membrane; and 5) said entity crosses the static organic solvent film surrounding the cell into the bulk organic solvent. Consequently, addition of a polar organic solvent to a non-polar organic solvent facilitates extraction of membrane-associated neutral lipid complexes. However, the process inevitably leads to co-extraction of polar lipids [11].

In this regard, it is expected that the intracellular location of a given compound affects its extractability by distinct solvents. Resorting to the solvents chosen, it was possible to produce extracts with different composition and, consequently, distinct antioxidant capacity, as discussed next.

2.3.2.1. Antioxidant capacity

Numerous methods are used to assess the antioxidant capacity of natural compounds in biological systems. Two free radical scavenging methods commonly used involve $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, yet both such radicals are foreign to biological systems. $ABTS^{+\bullet}$ assays measures the relative ability of an antioxidant to scavenge the $ABTS^{+\bullet}$ generated in aqueous and organic solvents, as in ethanol:water 50:50 (v/v). Conversely, $DPPH^{\bullet}$ is widely used to determine antiradical/antioxidant capacities, but acts only upon species generated in a methanol phase. Comparatively, $ABTS^{+\bullet}$ is more stable, so it can be used at different pH levels. $DPPH^{\bullet}$ may also suffer from colour interference, for instance in the case of anthocyanins or carotenoids, which leads to underestimation of antioxidant capacity; moreover, it was reported that this method may be more sensitive to phenolic antioxidants over time [20,21]. Therefore, there is some controversy in the applicability of these assay for carotenoid antioxidant capacity assessment [4,21,22]. In a report by Müller *et al.* [22], when

comparing several methods to evaluate antioxidant capacity of carotenoids, $DPPH^{\bullet}$ did not show any scavenging capacity. However, this method seems appropriate to measure antioxidant capacity of poly-unsaturated fatty acids, as is the case of conjugated linoleic acid [23]. Therefore, to avoid a misinterpretation of the total antiradical capacity of extracts, both $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ assays were performed – thus allowing consistent confirmation of the relation between biochemical profile and results of said antioxidant assays (as described in the following sections).

Nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$) are two of the six major reactive oxygen species causing oxidative damage in the human body [4]. The former is a short-lived free radical generated endogenously, involved in different physiological functions [24]. It interacts with lipids, DNA and proteins, *via* direct oxidative reactions or *via* indirect radical-mediated mechanisms. Hence, any antioxidant scavenging capacity against this radical may unfold a similar capacity *in vivo*, and a potential to prevent such diseases as chronic inflammatory diseases, cancer or neurodegenerative disorders [25]. On the other hand, superoxide radical is the first product of oxygen univalent reduction. Its biological significance derives from its ability to generate other more reactive species, like hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($ONOO^{-}$), and induce major damages *in vivo* [26].

All extracts of both microalgae acted as scavengers of $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $O_2^{-\bullet}$ and $^{\bullet}NO$, in a concentration-dependent manner, with topical exceptions. Data can be compared through calculation of inhibitory concentration (IC) values, as acquired by plotting inhibitory scavenging percentages for various extract concentrations. Extracts from the two microalgae exhibited a distinct behaviour for each scavenging assay (Table 1).

Table 2.1. Comparison of antioxidant capacity of *Gloeothece* sp. and *Sc. obliquus* (M2-1) extracts, in terms of IC (μg.mL^{-1}) toward radicals ABTS$^{+\bullet}$, DPPH$^{\bullet}$, •NO and $O_2^{-\bullet}$.

		Antioxidant activity (μg.mL^{-1})						
		ABTS$^{\bullet+}$	DPPH$^{\bullet}$		$^{\bullet}$NO		$O_2^{\bullet-}$	
	Solvent	IC_{50}	IC_{50}	IC_{25}	IC_{50}	IC_{25}	IC_{50}	IC_{25}
Gloeothece sp.	Ethanol	75	**629**	**274**	-	23	**247**	**54**
	Ethyl lactate	129	-	927	82	25	-	-
	Acetone	**63**	850	310	**22**	**6**	1394	278
	HI (3:2)	276	-	789	**25**	**7**	1183	357
Scenedesmus obliquus (M2-1)	Ethanol	87	-	633	-	**15**	637	416
	Ethyl lactate	195	878	261	-	-	**520**	**300**
	Acetone	**41**	-	488	-	-	826	620
	HI (3:2)	648	**412**	**194**	60	20	1236	513

HI - Hexane: isopropalnol (3:2) v/v.

Regarding ABTS$^{\bullet+}$, acetonic extracts of both *Gloeothece* sp. and *Sc. obliquus* (M2-1) attained the best IC_{50} values: 63 and 41 μg.mL^{-1}, respectively. On other hand, the most active in scavenging DPPH$^{\bullet}$ were the hexane:isopranol (3:2) extract of *S. obliquus* and the ethanol extract of *Gloeothece* sp. (IC_{25} of 194 and 274 μg.mL^{-1}, respectively). Therefore, with regard to synthetic reactive species, *S. obliquus* (M2-1) conveyed better results compared to *Gloeothece* sp; however, the other three extracts of the latter displayed the best results in the assay against ABTS$^{\bullet+}$.

In what concerns reactive species with biological significance, acetone and hexane:isopranol (3:2) extracts of *Gloeothece* sp. have strong activity against $^{\bullet}$NO – but being quite similar (IC_{25} values of 6 and 7 μg.mL^{-1}, respectively). On other hand, only the ethanol and hexane:isopranol (3:2) extracts of *Sc. obliquus* (M2-1) exhibited antioxidant capacity against this reactive nitrogen species (IC_{25} values of 15 and 20 μg.mL^{-1}, respectively). Ethanol extracts of *Gloeothece* sp. and ethyl lactate extracts of *Sc. obliquus* (M2-1) exhibited the best activities against $O_2^{\bullet-}$, described by IC_{25} of 54 and 300 μg.mL^{-1}. It is thus possible to conclude that each solvent system exerts a different scavenging activity because of its composition. In order to establish some relationship between the observed activity and the lipidic composition, carotenoids and PUFA were duly quantified.

2.3.2.2. Lipidic composition

As explained above, solvent polarity plays an important role on extractability of lipidic compounds, due to the basic chemistry concept of 'like dissolving like'. Moreover, it is important to remember that the cell location of the lipidic component is crucial for extraction, because the solvent needs to reach the compound in the cell.

As stated before, xanthophylls are relatively hydrophobic molecules, typically associated with membranes and/or involved in non-covalent binding to specific proteins. Primary carotenoids are structural and functional components of the photosynthetic apparatus, typically confined to the thylakoid membrane complex – with proteins being disrupted only by polar organic solvents able to form hydrogen bonds [14,27]. Secondary carotenoids are produced in large quantities by microalgal cells, only after exposure to specific environmental stimuli (carotenogenesis), being usually found in lipid vesicles — in either the plastid stroma or the cytosol [28].

In prokaryotic microalgae, such as *Gloeothece* sp., most xanthophylls are associated with chlorophyll-binding polypeptides of the photosynthetic apparatus [29]. In most green microalgae, carotenes and xanthophylls are synthesized within plastids, accumulating therein only. However, secondary xanthophylls in some green microalgae accumulate in the cytoplasm, which raises the possibility of an extra-plastidic site for carotenoid biosynthesis. Alternatively, xanthophylls synthesized in the chloroplast may be exported, and consequently accumulate in the cytoplasm – so they may be found in essentially all cellular compartments [29].

Prokaryotes and eukaryotes exhibit several structural differences of their cell wall, in terms of mechanical barrier. As happens with several other members of the *Chlorococcales* family, the trilaminar structure of the outer wall layers of eukaryotic *Scenedesmus* species is composed of cellulose in the inner wall layers, and insoluble, acetolysis-resistant, lipid-containing biopolymers – termed algaenans, located in the trilaminar outer layer, thus contributing to cell wall rigidity [30,31]. Furthermore, prokaryotic *Gloeothece* species holds a typical Gram-negative cell wall, mainly of polysaccharide nature, which differs in thickness and consistency [32].

Besides solvent polarity, the cell structural complexity, including cell location of metabolites, of the two microalga under scrutiny affects lipidic component extractability. However, it is possible to propose a correlation between affinity of carotenoids for acetone and PUFA for ethanol (Tables 2 and 3). At a first glance, *Gloeothece* sp. extracts entail higher variety of carotenoids and higher total amount of PUFAs than their *Sc*. *obliquus* (M2-

1) counterparts. Species belonging to the *Scenedesmus* genus possess particularly resistant cell walls, so extraction of carotenoids and fatty acids becomes notoriously difficult [33].

Acetone is a solvent widely used in pigment extraction, as it extracts most photosynthetic pigments with a wide range of polarity [28,34,35]. Our results indicate that acetonic extracts are the richest in carotenoids, particularly lutein. In *Gloeothece* sp., the lutein content corresponds to ca. 78% of the total quantified carotenoids (1.424 ± 0.079 $\mu g_{lutein}.g_{DW}^{-1}$ – see Table 2), and in *Sc. obliquus* (M2-1) to ca. 47% (1.392 ± 0.034 $\mu g_{lutein}.g_{DW}^{-1}$ – see Table 3). Conversely, violaxanthin and neoxanthin possess a significant expression in acetonic extract of *Sc. obliquus* (M2-1), 22.7 and 25.5% of the total quantified carotenoids, respectively (Table 3). However, acetone is not selective only for carotenoids, since PUFA are also extracted. In acetonic extract of *Sc. obliquus* (M2-1), the content of PUFA ranges from 50% in the case of oleic acid, to 71% of linoleic acid in the ethanol extract – and linolenic acid is even more concentrated in the acetonic extract (Table 3). This provides evidence for the dependence of the solvent ability to extract the feedstock species, as emphasized before [14].

Ethanol affinity for PUFA is clear; for example, it extracts 3-7.8-fold more linolenic acid from *Gloeothece* sp. than the other solvents (Table 2). Ethanol is also able to extract 10-fold more linolelaidic acid from both *Gloeothece* sp. and *Sc. obliquus* (M2-1) than ethyl lactate (Table 2). Ethanol can extract carotenoids as well, but at a lower rate; for instance, *Gloeothece* sp. ethanol extract contains 1.5-3 fold less carotenoids than its acetonic counterpart, although an exception occurs in what concerns to violaxanthin that is extracted to 3-fold an extent than with acetone (Table 2).

Ethanol has a different behaviour in extracting carotenoids from *Sc*. *obliquus* (M2-1), as it extracts 3-fold less lutein and 1.7- fold less neoxanthin. Due to its lower affinity for carotenoids, it was not possible to quantify the remaining carotenoids.

Ethyl lactate has been proposed to extract carotenoids, particularly lutein, from plant material [17]; however, its performance in the microalgae under the processing conditions used is less than expected, in view of the low level of extraction of carotenoids. nevertheless, ethyl lactate showed some selectivity for lutein in both species (Tables 2 and 3). Ethyl lactate was able to extract PUFA as γ- linolenic acid from *Spirulina* sp. [36]. Ethyl lactate indeed extracted 6.185 ± 0.265 $mg_{FA}.g_{DW}^{-1}$ from *Gloeothece* sp., 55% of that corresponding to linolenic acid; furthermore, it was the only solvent that extracted linolelaidic acid to detectable levels (Table 2). Converselys, ethyl lactate performance toward PUFA extraction from *Sc*. *obliquus* (M2-1) rated the poorest – see Table 3.

Previous studies have proven that the hexane:isopropanol (3:2) mixture is one of the best non-halogenated solvent mixtures to extract fatty acids [14]. However, it only led to a reasonable result regarding extraction of oleic and *cis*-vaccenic acid from *Gloeothece* sp. (Table 2) – and, surprisingly, of the xanthophyll violaxanthin. With respect to *Sc*. *obliquus* (M2-1), this solvent extracted 1.849 ± 0.156 $mg_{FA}.g_{DW}^{-1}$ of total PUFA (Table 3). In addition to carotenoids and PUFA, hexane: isopropanol (3:2) has been claimed to extract more non-lipids (e.g. proteins, carbohydrates) than plain hexane, due to the polar nature of isopropanol [14] – which may have contributed to the low recovery of PUFA and carotenoids.

Table 2.2. *Gloeothece* sp. extracts lipidic profile in terms of carotenoids ($\mu g_{carotenoid}/g_{DW}$) and PUFA ($mg_{FA}/g_{DW}$) (average ± standard deviation).

	Carotenoids ($\mu g_{carotenoid}\ g_{DW}^{-1}$)						**PUFA ($mg_{FA}\ g_{DW}^{-1}$)**					
Solvent	Violaxanthin	Neoxanthin	Lutein	α-Carotene	β-Carotene	**Total carotenoids**	Oleic	*cis*-Vaccenic	Linoleic	Linolelaidic	Linolenic	**Total PUFA**
Ethanol	0.181 ±0.004	0.114 ± 0.004	0.822 ± 0.021[a]	0.018 ± 0.001	0.122 ± 0.006	1.258 ± 0.022[b]	0.771 ± 0.064[c]	-	**2.250 ± 0.198**	-	**10.100 ± 0.212**	**13.219± 0.233**
Ethyl	0.067 ± 0.002	0.043 ± 0.001	0.424 ± 0.030	-_	0.050 ± 0.002	0.584 ± 0.031	1.007 ± 0.192	0.264 ± 0.074	1.267 ± 0.200	0.201 ± 0.046	3.406 ± 0.111	6.185 ± 0.265
Acetone	0.058 ± 0.005	**0.180 ± 0.013**	**1.424 ± 0.079**	**0.057 ± 0.004**	**0.251 ± 0.004**	**1.806 ± 0.080**	0.773 ± 0.054[c]	-	0.255 ± 0.30	-	1.286 ± 0.064	2.317 ± 0.106
HI (3:2)	**0.220 ± 0.008**	0.086 ± 0.004	0.868 ± 0.015[a]	**0.056 ± 0.003**	0.067 ± 0.002	1.301 ± 0.014[b]	**1.352 ± 0.032**	**0.689 ± 0.038**	0.538 ± 0.098	-	2.631 ± 0.119	5.216 ± 0.126

[a-c] Means within the same column, without a common superscript, are significantly different (P<.05). HI - Hexane: isopropanol (3:2) v/v.

Table 2.3. *Sc. obliquus* (M2-1) extracts lipidic profile in terms of carotenoids ($\mu g_{carotenoid}/g_{DW}$) and PUFA ($mg_{FA}/g_{DW}$) (average ± standard deviation).

	Carotenoids ($\mu g_{carotenoid}\ g_{DW}^{-1}$)						**PUFA ($mg_{FA}\ g_{DW}^{-1}$)**				
Solvent	Violaxanthin	Neoxanthin	Lutein	α-Carotene	β-Carotene	**Total carotenoids**	Oleic	Linoleic	Linolelaidic	Linolenic	**Total PUFA**
Ethanol	-	0.439 ± 0.019	0.464 ± 0.011a	-	-	0.904 ± 0.019	**0.889 ± 0.060**	**1.045 ± 0.097**	**1.045 ± 0.097**	0.932 ± 0.088	**2.888 ± 0.078**
Ethyl	-	-	0.156 ± 0.012	-	-	0.156 ± 0.012	0.320 ± 0.070	0.465 ± 0.012	0.147 ± 0.021	0.522 ± 0.078	1.454 ± 0.073
Acetone	**0.674 ± 0.057**	**0.759 ± 0.053**	**1.392 ± 0.034**	0.022 ± 0.011	0.100 ± 0.004	**2.970 ± 0.068**	0.427 ± .076c	0.752 ± 0.22a	-	**1.199 ± 0.089**	2.381 ± 0.122
HI (2:1)	0.020 ± 0.001	0.357 ± 0.009	0.420 ± 0.034a	-	-	0.797 ± 0.030	0.518 ± 0.055c	0.734 ± 0.075a	-	0.577 ± 0.049	1.849 ± 0.156

[a-c] Means within the same column, without a common superscript, are significantly different (P<0.05). HI - Hexane: isopropanol (3:2) v/v

2.3.3. Relation of antioxidant capacity with carotenoid and PUFA contents

There is a number of reports on the evaluation of antioxidant capacity in prokaryotic and eukaryotic microalgae compounds from lipophilic and hydrophilic nature [19,37,38], but most of them have not performed antioxidant scavenging assays in lipid-rich extracts. An important and well-known class of antioxidants from microalgae are carotenoids, and they are already produced to commercial scale (e.g. astaxanthin from *Haematococcus* sp. and β-carotene from *Dunaliella* sp.) for use as additive in food and feed, as well as in cosmetics and as food supplements [39]. Flavonoids, sterol, reducing sugars and tannins may also exert antiradical or antioxidant capacities in alcoholic extracts [21]. Their co-extraction may provide an explanation for some unexpected results of antioxidant capacity obtained with ethyl lactate and hexane:isopropanol (3:2) extracts from *Sc*. *obliquus* (M2-1). One should take into account that synergic or antagonic interactions may occur between the compounds found in an extract. Hence, high amounts of a known antioxidant compound do not necessarily imply a high antioxidant activity, in view of the crude nature of the extracts obtained.

The $ABTS^{\bullet+}$ assay was used before to evaluate the antioxidant capacity of carotenoid-rich extracts (namely in lutein and β-carotene [40]. Upon inspection of Tables 1, 2 and 3, it is possible to draw a few conclusions: acetonic extracts of both microalgae species attained the best IC_{50} values in this assay, and they contain the highest levels of carotenoids, namely lutein and β-carotene. IC_{50} values found for ethyl lactate extracts and its selectivity to lutein suggest that this xanthophyll may be responsible for the main antioxidant capacity of these extracts.

With regard to the results in Table 1, 2 and 3, one realizes that is not always possible to propose a correlation between carotenoids content and antiradical capacity; this is supported by some studies revealing that $DPPH^{\bullet}$ does not detect carotenoids antioxidant capacity [4,21,22]. Furthermore, this assay was used to quantify the antioxidant capacity of conjugated linoleic acid [23]. Nevertheless, one concludes that ethanol extract of *Gloeothece* sp. is particularly rich in linoleic and linolenic acids that, besides lutein, may contribute to the best IC_{25} values attained against $O_2^{\bullet-}$ [23].

Ethanolic and acetonic extracts from *Gloeothece* sp. seem interesting from an antioxidant point of view. In terms of scavenging capacity, the ethanolic extract attained the best results against $DPPH^{\bullet}$ and $O_2^{\bullet-}$, while acetonic was the most effective against $ABTS^{\bullet+}$ and $^{\bullet}NO$. These extracts have distinct contents of carotenoids and PUFA, which may explain the paired results. Ethanol extract is indeed richer in PUFA (13.219 ± 0.233 $mg_{FA}.g_{DW}^{-1}$ – 76.4% corresponding to linolenic acid and 17% of linoleic acid) than in carotenoids (1.258 ± 0.022 $\mu g_{carotenoid}.g_{DW}^{-1}$ – 65.3% of lutein and 9.7% β-carotene); and acetonic extract is richer in carotenoids (1.806 ± 0.080 $\mu g_{carotenoid}.g_{DW}^{-1}$ – 78.8% of lutein and 13.9% of β-carotene) than

in PUFA (2.317 ± 0.106 $mg_{FA}.g_{DW}^{-1}$ – 55.5% of linolenic acid and 11% linoleic acid). This pattern was not observed in *Sc. obliquus* (M2-1) extracts; in fact, each extract exhibited a specific antioxidant activity. The acetonic extract was the most interesting in the $ABTS^{\bullet+}$ assay, maybe due to its distinctive content in lutein (1.392 ± 0.034 $\mu g_{carotenoid}.g_{DW}^{-1}$ – 46.8% of total carotenoids). On the other hand, the hexane:isopropanol (3:2) extract exhibited a great activity in the $DPPH^{\bullet}$ assay and ethanolic extract in the $^{\bullet}NO$ assay, but these two extracts have 3-fold less carotenoids than the acetonic extract – although PUFA rank within the same magnitude. Ethyl lactate exhibited the best IC_{25} in $O_2^{\bullet-}$ assay (300 $\mu g.mL^{-1}$) between *Sc*. *obliquus* (M2-1) extracts, perhaps due to the great affinity of this solvent to lutein, which may exert an influence on its antioxidant activity.

2.4. Conclusions

Concerning total antioxidant capacity, ethanol *Gloeothece* sp. extracts performed best in $DPPH^{\bullet}$ and $O_2^{\bullet-}$ assays, possibly due to its content in PUFA (76.4% of linolenic acid) and carotenoids (65.3% of lutein and 9.7% of β- carotene). Similarly, the acetonic extract attained good results in $ABTS^{\bullet+}$ and •NO assays, and probably for the same reasons, i.e. its content in carotenoids (78.8% of lutein and 13.9% of β-carotene) and PUFA (55.5% of linolenic acid and 11% of linoleic acid). *Gloeothece* sp. is a prokaryotic microalga poorly studied so far, so the findings of this study may justify further exploitation of its antioxidant potential – once it appears promising toward nutraceutical formulations.

Sc. *obliquus* (M2-1) also seems to be a promising source of antioxidant-rich extracts. The acetone extract exhibited the best antioxidant capacity in $ABTS^{\bullet+}$ assay, likely associated to its content in carotenoids, 47% of which is lutein. Note that the hexane:isopropanol (3:2) extract also exhibited the best result of antioxidant capacity in the $DPPH^{\bullet}$ assay.

Solvents used in extraction of lipidic components seem to be critical upon antioxidant performance – which appears to hinge, in particular, on the balance between carotenoids and PUFAs. However, further studies are warranted to confirm whether said compounds are by themselves responsible for the good performance recorded in antioxidant assays, or some form of interaction/synergism exists between them.

In terms of lipidic components extraction, acetone is the most suitable to extract carotenoids at large, and ethanol stands out in PUFA extraction regardless of microalga species.

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CHAPTER 3

Antitumour potential of lipidic extracts from Gloeothece sp.

Antitumour potential of lipidic extracts from *Gloeothece* sp.

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THIS MANUSCRIPT STILL IN PROCESS.

Abstract Cancer ranks as one of the top causes of death. However some strategies based on chemoprevention are able to block the cancer development, indeed some antioxidant compounds, such as carotenoids and poli-unsaturated fatty acids (PUFA) were identified as chemoprevention agents. Microalgae are photosynthetic organism than naturally produce these lipidic compounds under certain conditions.

The main goal of this study is to ascertain if lipidic microalgal extracts with previous proved antioxidant capacity, are able to exert antitumoural capacity against gastric cancer, and how it may be related to their concentration in carotenoids and PUFA.

Thus, extracts from *Gloeothece* sp. biomass were obtained with several solvents, such as ethanol, acetone, hexane:isopropanol (3:2) (HI) and ethyl lactate and then tested for their ability to modulate two gastric cancer cell lines, AGS and MKN45.

First, the capacity of extracts to modulate cancer cell lines viability was ascertained by Sulforhodamine B assay, and established the inhibitory concentrations (IC_{50}) of each extract. It was found that extracts exhibited effects in a concentration- dependent manner; however HI attained the lowest IC_{50} for both cell lines.

At the same time, extracts biochemical composition in terms of carotenoid and PUFA, assessed by HPLC-DAD and GC-FID, revealed that each extract entailed a unique profile in carotenoids and PUFA.

This study still is in processing, so all results in term of extracts antitumor features still are preliminary. However, evaluating extracts cell death features (by TUNEL assay) it was found a capacity of HI and ethyl lactate extracts to induce cell death in both in AGS and MKN45 lines. Also same extracts revealed anti-proliferative effects in the tested gastric cancer cell lines.

Key words: *lutein, β-carotene, C18:3n3, C18:2n6*

3.1. Introduction

Nowadays, cancer constitutes one of the leading causes of death. Carcinogenesis of common epithelial tumours, like stomach, is a slow process that could start twenty years before the first symptoms appear. This long period is very suitable for using chemopreventive strategies that block the development of invasive and/or metastatic disease. Cancer research has been mainly focused on the search for curative treatments, and few studies have aimed to develop preventive strategies that can be useful in the long period of tumour development. In this regard, cancer chemoprevention with the use of natural, synthetic or biological substances may be able to suppress or prevent either the initial phase of carcinogenesis or the progression of neoplastic cells to cancer (Talero et al. 2015, Castro-Puyana et al. 2016).

Therefore, search of bioactive compounds from natural sources with health benefits is in high demand being at present an intense field of research aiming to develop new functional foods and/or nutraceuticals.

Among all the natural sources, microalgae have raised an enormous interest. Extensive screening of marine microalgae has led to the isolation and chemical determination of over 15,000 bioactive compounds such as fatty acids, sterols, phenolic compounds, terpenes, enzymes, polysaccharides, alkaloids, toxins and carotenoids (Pasquet et al. 2011). Indeed, these microorganisms possess an extra biotechnological advantage. Due to their huge metabolic plasticity, they can be used as natural bioreactors to synthesise compounds with health benefits, namely carotenoids and polyunsaturated fatty acids (Guedes, Amaro and Malcata 2011, Castro-Puyana et al. 2016).

Major carotenoids with antioxidant capacity have been extensively evaluated with regards to their cancer chemopreventive ability. Some of them, like β- carotene, lutein, violaxanthin, zeaxanthin, and fucoxanthin, have exhibited anti-proliferative capacity against different cancer cells (Tanaka, Shnimizu and Moriwaki 2012, Talero et al. 2015). These carotenoids, or ethanolic extracts rich in carotenoids, have been isolated from microalgae species such as *Chaetoceros calcitrans*, *Nannochloropsis oculata* or *Dunaliella tertiolecta, Muriellopsis* sp, *Scenedesmus almeriensis*, *Chlorella protothecoide*, *C. zofingiensis*, *C. citriforme* and *Neospongiococcus gelatinosum* (Cha, Koo and Lee 2008, Guedes, Amaro and Malcata 2011, Pasquet et al. 2011, Castro-Puyana et al. 2016, Sheu et al. 2008, Amaro et al. 2013). Evidences state that β-carotene may act against some cancer cell lines due its antioxidant function (Talero et al. 2015), and that this capacity may be due to its growth inhibitory and pro-apoctotic effects (Palozza et al. 2005). Studies with animal models of colon carcinogenesis reinforced these results, with dietary supplementation with β-carotene having anticancer effects (Choi et al. 2006). These effects have been shown to be dose-dependent,

with the highest doses being harmful and having a proliferative effect on cancer cells (Talero et al. 2015, Raju et al. 2005).

Reports suggest that another carotenoid, lutein may also present anticarcinogenic actions due to its ability to interact with the mutagens 1-nitropyrene and aflatoxin B1 (AFB1) and for stimulating certain genes involved in T-cell transformations activated by mitogens, cytokines and antigens (Gonzalez de Mejia, Ramos-Gomez and Loarca-Pina 1997, Tanaka et al. 2012, Park et al. 1999).

Likewise, polyunsaturated fatty acids (PUFA) were shown to possess anticancer potential on several *in vitro* studies (Das 2007). Cis-unsaturated fatty acids such as γ-linolenic acid (GLA), arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) were shown to selectively kill tumour cells without harming the normal cells (Das 2007, Das and Madhavi 2011). Oleic acid (OA), linoleic acid (LA), α-linolenic acid (ALA) have also been reported as having inhibited growth of methylcholanthrene-induced sarcoma cells (Ramesh and Das 1998, Das 1991). These facts may have contributed to the idea that high intake of n-3 PUFA has tumoural inhibitory effects (Sauer, Dauchy and Blask 2000).

Hence, extraction of microalgal lipidic components, carotenoids and PUFA, appears to be crucial in the attempt to maximize their added value for further nutra- and pharmaceutical formulations. This makes it critical to combine an appropriate, selective, cost-effective, and environmental-friendly extraction procedures with legal requirements regarding use of food-grade solvents and processes (Amaro et al. 2015). Therefore, food Generally Recognized as Safe (GRAS) solvents with lower toxicity should be selected to obtain extracts.

Gloeothece sp. is a prokaryotic colonial microalga scarcely studied. Screening programs for discovery of bioactive compounds from prokaryotic microalgae have shown that cyanobacteria represent an untapped bioresource for a diverse range of secondary metabolites (Prasanna et al. 2010). Recent advances show that its extracts are rich in lipidic components with proved antioxidant capacity (Amaro et al. 2015), thus, this microalga was selected for this study as an enforcement to find new sources or bioactive compounds.

3.2. Material and methods

3.2.1. Microorganism source and growth conditions

Gloeothece sp. (ATCC 27152) was acquired from ATCC — American Type Culture Collection (USA), and cultivated using Blue Green Medium (BG11) (Stanier et al. 1971). For each 4 L batch biomass production, a pre-inoculum with an initial optical density of 0.1 (at

560 nm or 680 nm for *Gloeothece* sp.) was cultivated for 10 days in 800 mL of buffered BG11 medium, with Tri-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) aimed at maintaining a constant pH of 8. This pre-inoculum ensured that the microalga is at exponential growth phase by the time of inoculation. A continuous illumination with fluorescent BIOLUX lamps, with intensity of 250 $\mu mol_{photon}.m^{-2}.s^{-1}$, was guaranteed, as well as air bubbling at a flow rate of 0.5 $L.min^{-1}$.

The biomass production was performed during 14 days, collected by centrifugation at 4000 rpm for 10 min, freeze-dried and stored under nitrogen.

3.2.2. Extract preparation

Gloeothece sp. extracts were obtained from 200 mg of lyophilized biomass with four food grade solvents: ethanol (99.6% purity), acetone (99.6% purity), a mixture (3:2) of hexane/isopropanol (99.6 and 99.8% purity respectively) and ethyl lactate (97% purity). Each extraction was performed in triplicate, in a triple stage extraction at a ratio of 1:60 (DW/V), at 40 ºC and 250 rpm for 20 min, as performed before (Amaro et al. 2015) . To remove cells debris, extracts were then centrifuged at 2000 rpm for 10 min and filtered by 0.45 µm pore size. Solvent were removed by rotavapor and extracts stored under nitrogen, at -20 °C in the dark prior to analyses.

3.2.3. Anticancer effects of lipid crude extracts

3.2.3.1. Cell culture

Human gastric carcinoma cell lines MKN45 (obtained from the Japanese Cancer Research Bank; Tsukuba, Japan) and AGS (obtained from ATCC, USA) were maintained in RPMI1640 (Invitrogen, Thermo Fisher Scientific, Waltham, MA) supplemented with 10 % FBS (Lonza, Basel, Switzerland) and kept at 37 ºC in a humidified 5 % CO_2 incubator.

3.2.3.2. Cancer cell viability Sulforhodamine B assay

MKN45 and AGS ($1x10^4$) were seeded in 96-wells plates and treated for 48 h with different concentrations of microalgal extracts (0 to 550 µg/mL whenever possible) or DMSO (AppliChem, Darmstadt, Germany) as treatment control (0.05 % v/v). Then the cells were fixed by adding 50 µL of 50 % cold trichloroacetic acid (Merck Millipore, Kenilworth, NJ, USA) to each well and incubating the plates at 4ºC for 1 hour. After the fixation step, the plates were washed three times with deionized water and allowed to dry at room temperature. The cells were then stained with 50 µL of 4 % sulforhodamine B (Sigma-Aldrich, St. Louis MO, USA) (SRB) in 1 % acetic acid (Mallinckrodt Baker, Deventer, Holland) for 30 min and

thereafter washed three times with deionized water. Once the plates were dry, the cells were solubilized with 100 µL of 10 mM unbuffered Tris Base (Sigma-Aldrich, St. Louis MO, USA) and the optical density at 510 nm was measured using the fluorimeter SynergyTM 4 Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA).

3.2.3.3. Cancer cell death TUNEL assay

MKN45 and AGS were cultured in 6-well plates ($7.5x10^5$) and treated with the microalgal extracts at the IC50 for 48 h using DMSO (AppliChem, Darmstadt, Germany) as control treatment. Cells were washed, trypsinized and the pellet obtained was fixed in 3 mL of ice-cold methanol for 15 min. After that time, cells were washed and resuspended in 500 µL of PBS. Incubation with TUNEL reaction mix (1:9:10 in relation to the Dilution Buffer reagent, according to manufacture instructions, In Situ Cell Death Detection Kit Fluorescein, Roche, Mannheim, Germany) occurred for 1 h at 37 ºC in the dark after which data was acquired using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose CA, USA).

3.2.3.4. Cancer proliferative assay

MKN45 and AGS were cultured in 6-well plates ($7.5x10^5$) and treated with the microalgal extracts at the IC50 for 48 h using DMSO (AppliChem, Darmstadt, Germany) as control treatment. 5-bromo-2'-deoxyuridine (BrdU) (BrdU labelling and detection kit 1, Roche, Mannheim, Germany) was incorporated in the cell culture medium at the ratio of 1:1000, for 1 h at 37 ºC. Immediately after incubation, the cells were harvested, washed with PBS, fixed in 1 mL of ice-cold methanol for 30 min, washed again and resuspended in 500 µL of PBS. This was followed by the incubation with 1 mL of HCl (Mallinckrodt Baker, Deventer, Holland) 4 M for 20 min, two washing steps with PBS, a blocking step (PBS containing 0.5 % Tween 20 and 0.05 % BSA) and finally the incubation step with the primary antibody against BrdU (1:20, Bu20a, Dako, Glostrup, Denmark) for 1 h at room temperature. Afterwards, the cells were further washed with PBS and incubated with the secondary antibody labelled with FITC (1:200, polyclonal rabbit anti-mouse, Dako, Glostrup, Denmark) for 30 min, washed two times and suspended in 500 µL of Data acquisition was performed using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose CA).

3.2.4. Chemical characterization of extracts

3.2.4.1. Determination of polyunsaturated fatty acids profile

Extracts resuspended in DMSO at the IC_{50} values of concentration ($\mu g.mL^{-1}$) were lyophilized and the residue submitted to direct transesterification in order to produce fatty

acid methyl esters according to the acidic method described by Lepage and Roy (Lepage and Roy 1984), after modifications introduced by Cohen *et al.* (Cohen, Vonshak and Richmond 1988). Heptadecanoic (C17:0) acid was used as internal standard and acetyl chloride as catalyst. Esters were analysed in a GC Varian Chromapack CP-3800 gas chromatograph (GC), using a flame ionization detector, and quantified with the software Varian Star Chromatography WorkStation (Version 5.50). A silica CP-WAX 52 CB (Agilent) column was used, and helium was employed as carrier gas in splitless mode. Injector and detector were maintained at 260 and 280 °C, respectively, and the oven heating program was as described in Table 1. Chromatographic grade standards of fatty acids in methyl ester form CRM47885 (Supelco) were used for tentative identification, based on retention times: C13:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1 n9-cis + trans, C18:2n6, C18:2n6 c, C18: n6, C18:3n3, C20:0, C20:1, C20:5n3, C21:0, C22:0, C22:2, C22:1n9. The mean of the results from the aforementioned chemical assays was used as a datum point.

Table 3.1. Oven heating program for fatty acid identification.

T (ºC)	Rate (ºC.min^{-1})	Holding time (min)	Time (min)
100	-	5	5
180	6	0	18.33
200	2	0	28.33
205	0.5	0	38.33
230	1	0	63.33
233	0.5	0	69.33

3.2.4.2. Extracts carotenoids content

To ascertain the extracts carotenoids content high-performance liquid chromatography (HPLC) was employed as analytical technique as used before (Guedes eta al., 2011c). Extracts as obtained in *2.2. section* were resuspended in DMSO at the values of IC_{50} and then freeze-died and then resuspendend in injection method' solvent. β-apo-carotenol (Sigma) was used as internal standard. The carotenoid profile was produced in a Merck-Hitachi HPLC system, equipped with a Diode Array Detector (DAD) Merck-Hitachi L-7450, to resolve, detect and identify the various chemical compounds of interest. The absorption spectra were recorded between 270 and 550 nm. The stationary-phase was a 4 x 250 mm Purospher Star RP-18e (5µm) column (Merck). The mobile-phase was constituted by solvent A—ethyl acetate, and solvent B—acetonitrile/water at 9:1 (v/v), both from VWR, at various volumetric ratios along elution time, for an overall flow rate of 1 mL min-1. The following gradient was used: 0–31 min (0–60% A); 31–46 min (60% A); 46–51 min (60–100% A); 51–55 min (100% A); 55–60 min (100-0% A); and 60–65 min (0% A). The elution times of the chromatographic standards were: lutein 14.4 min, and β-carotene 34.4 min. Standards were purchased in CarotNature, Lutein (No. 0133, Xanthophyll, (3R,3'R,6'R)-β,ε-Carotene-3,3'-diol with 5% Zeaxanthin and purity of 96%), β-carotene (No. 0003, β, β -Carotene) with 96% purity) and β-apo-carotenol (No. 0482, 8'-Apo- β -caroten-8'-al) with 97%, purity). Identification was by comparison of retention times and UV–visible photo-diode array spectra, following a procedure a procedure detailed elsewhere (Guedes et al. 2011a).

3.3. Results and discussion

Cancer is characterized by an uncontrollable imbalance between the rate of cell proliferation and apoptosis, making it crucial to find a therapy capable to restore this balance, by reducing cancer cell growth and/or promoting cell death.

AGS and MKN45 are the two most studied human gastric adenocarcinoma cell lines (Ran et al. 2004, Bargiela-Iparraguirre et al. 2016)

Due to the evidences that supports the capacity of carotenoids and PUFA to exert antitumoural effect, the capacity of microalgal extracts rich in these families of compounds, obtained from *Gloeothece* sp. biomass, were tested for their ability to promote cancer cells death or to supress their proliferation.

However, this work still is on-going, so, some results are purely indicative as it will be stated.

3.3.1. Cancer cell viability evaluation by Sulforhodamine B assay

The effect of microalgal extracts in *in vitro* cancer cell viability upon AGS and MNK45 gastric cancer cell lines was established by Sulforhodamine B assay (SRB). This test uses the protein-binding dye SRB to indirectly assess cell growth (Vichai and Kirtikara 2006, Azevedo et al. 2013).

Extracts were resuspended in a universal solvent, DMSO, which is cytotoxic from certain concentration on. Therefore, several concentrations of this solvent were tested and a percentage of 0.25 was found not to exert any effect upon cells (data not shown). Thereafter, extracts were resuspended to a final concentration as not to exceed 0.25% DMSO in the cell growth medium.

However, DMSO presented a different solvent capacity to each dry extract. Therefore, maximum extract concentrations tested was 400, 380, 440 and 550 $\mu g.mL^{-1}$, for acetone, ethanol, HI and ethyl lactate dry extracts, respectively.

For each extract, a dose response curve was established using the two different cell lines, allowing the determination of extracts' concentration causing a cell growth inhibition of 50%, as shown in Table 2.

Regarding the results obtained (Table 2), HI extract particularly outstands due to the lowest IC_{50} values achieved for both cancer cell lines, reaching values of at least 5-fold lower when compared to the other extracts. Acetone extract also seemed to possess a potent effect upon AGS cell viability, although not so strong as HI.

Table 3.2. IC_{50} ($\mu g.mL^{-1}$) values of *Gloeothece* sp. extracts on cell viability in sulforhodamine B assay for the gastric cancer cell lines AGS and MKN45.

	IC_{50} ($\mu g.mL^{-1}$)	
	AGS	**MKN 45**
Ethanol	241,0 ± 22,5	262,8 ± 47,7
Acetone	114,4 ± 6,4	290,8 ± 20,3
Hexane:Isopropanol 3:2 (v/v)	**23,2 ± 1,9**	**41,8 ± 3,7**
Ethyl lactate	209,3 ± 11,0	266,9 ± 2,8

Hence, IC_{50} values discovered for each extract in each cell line were used to perform the cancer cell death and proliferation assay.

3.3.4. Biochemical characterization of extracts

One goal of this study was to find a possible correlation between antitumoural extracts capacity with their content in PUFA and carotenoids.

Thus, fatty acids and carotenoids content was determined, by GC-FID and HPLC-DAD respectively, for the extracts concentration responsible for the IC50 in each cell line. As expected, due to the results obtained before in Chapter 2, solvents exhibit different capacities in *Gloeothece* sp. lipidic components extractability. Thus, obtained extracts revealed to hold a unique profile in carotenoids an FA.

In terms of FA content, the ethyl lactate extract stands out due to the high content on palmitic acid (PA, C16:0) – c.a. 70 % of total FA. On other hand, acetone extract possess almost all fatty acids in the same order of amount, only with the exception of C 18:3n6 (GLA) which its content is very low. Ethanol extract showed to contain high amounts of C18.3n3, median concentrations of C18:1 n9 and C18:2 n6 t, and traces amounts of PA. In the opposite, HI extracts contain all identified fatty acid but in a very low content compared with other extracts.

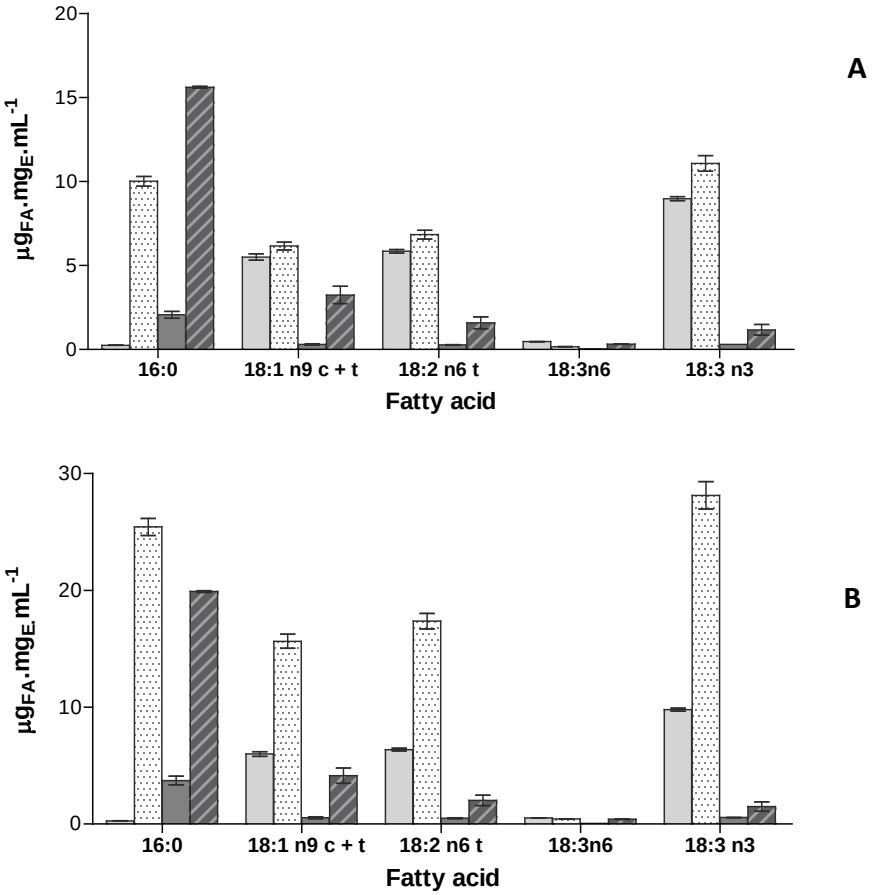

Fig. 3.1. Fatty acids content in each extract ▢ Ethanol, ⁘ Acetone, ■ Hexane:isopropanol (HI) (3:2) and ▨ Ethyl lactate in each IC_{50} values of concentration ($mg_{FA}.mg_E.mL^{-1}$) used to test anticancer effects of lipid crude extracts against A) AGS cells and B) MNK45 cells.

In terms of carotenoids concentrations, results show that although lutein is the most abundant carotenoid, its concentration is very different in each extract (Fig. 2). Lutein attains higher concentration in acetone extracts followed by ethanol, HI and ethyl lactate. β-carotene seems to be the second most extracted compound, however, in contrast to lutein, it was not present in the ethyl lactate extract. Neoxanthin was the carotenoid present at lowest concentration, and it was detected neither in HI nor in ethyl lactate extracts.

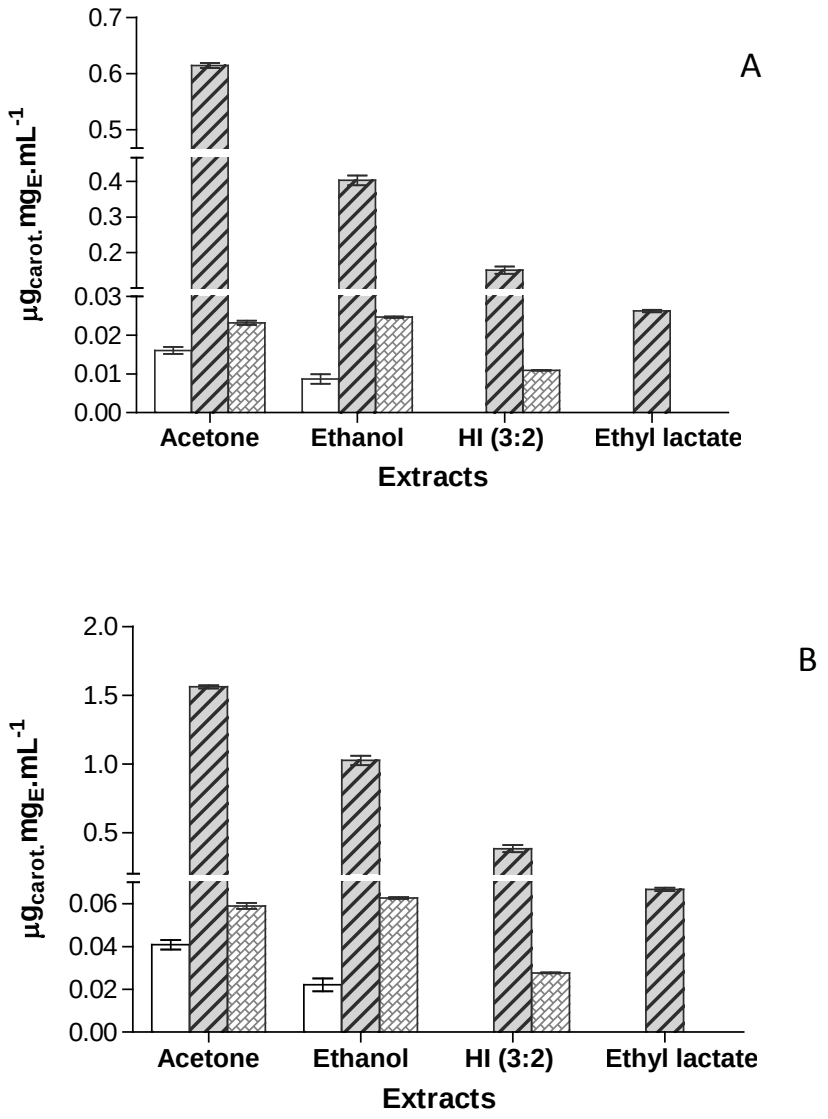

Fig. 2- Carotenoids content ☐ Neoxanthin, ▨ Lutein, ▩ β-carotene in each IC_{50} values of concentration ($mg_{carot}.mg_{E}.mL^{-1}$) used to test anticancer effects of lipid crude extracts against A) AGS cells and B) MNK45 cells.

3.3.2. Antitumor features of microalgal extracts

3.3.3.1.Cancer cell death TUNEL assay

TUNEL is a common method for detecting DNA fragmentation that may result from cell death, either by apoptotis or necrosis (Elmore 2007).

Induction of DNA fragmentation in AGS and MKN45 cells treated with IC_{50} of extracts, after 48 h of treatment, was examined via TUNEL detection assay. A preliminary assay (data

not show) revealed that the most promising extracts promoting cell death were HI and ethyl lactate, thus, so far, only these two extracts were used in subsequent studies.

Currently, this assay is still running, however, preliminary results (Fig. 3), indicated that both extracts at IC_{50}, although in a low rate, are able to induce cell death in both cell lines. Additionally, it seems that MKN cell line is more resistant to induced cell death than AGS tumour cells. Likewise, data so far suggests a higher capacity of cell death induction of ethyl lactate than HI extracts.

However, due to the low number of replica performed so far, is not possible to achieve any statistical conclusion.

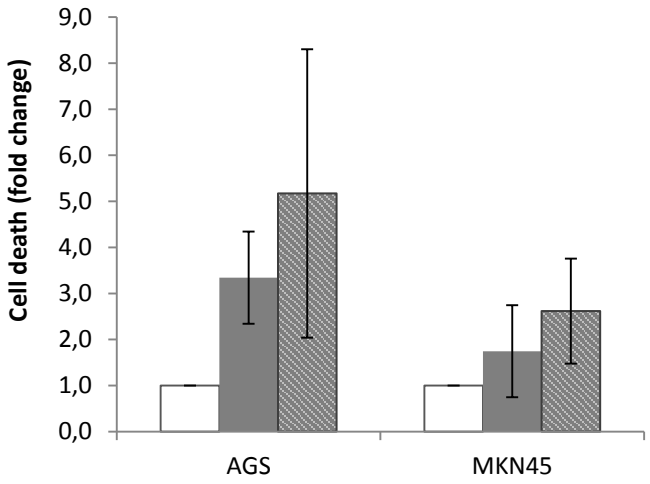

Fig. 3.3. AGS an MKN45 cell death, quantified by fold change), induced by ■ Hexane:isopropanol (HI) (3:2) and ▨ Ethyl lactate extracts using □ DMSO as negative control.

3.3.3.2. Cancer cell proliferation assay

This assay is based in cell label-incorporation into their replicating DNA of BrdU that can be further detected by immunofluorescence. For a quantative approach, samples were analysed by flow cytometry. Hence, in comparison to a negative control it is possible to infer the effect of a pro/anti-proliferative compound when incubated with a cancer cell line.

Thus, anti-proliferative features of IC_{50} HI and ethyl lactate extracts were ascertained in AGS and MK45 cell lines.

Currently, this assay is still running, but preliminary results, obtained so far are depicted in Fig. 4.

Results appear to reveal that HI and ethyl lactate extracts have the capacity to reduce cancer cell proliferation, in comparison to the control performed with DMSO. In AGS cells, both extracts at IC_{50} seemed to reduce cell proliferation in 1.8-fold. Similarly, in MKN 45

proliferation appear to be reduced in 1.6 and 1.3-fold in the presence on HI and ethyl lactate extracts, respectively.

However, at this stage, it was not possible to apply any statistical analysis of the data as the results presented are from a single experiment. More replicas are now needed to achieve any concrete assumption.

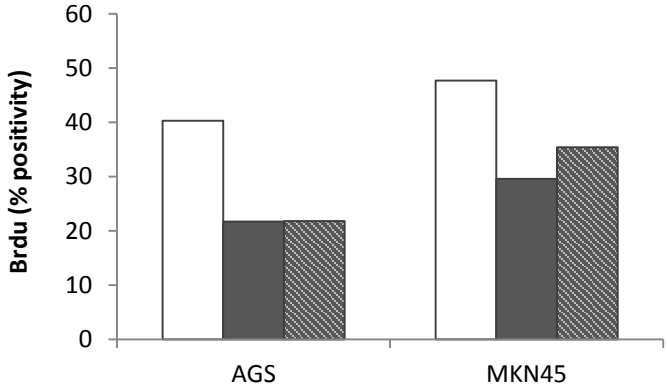

Fig. 3.4. Flow cytometric analysis of cell proliferation percentage (%) of AGS and MKN 45 treated with IC_{50} of ■ Hexane:isopropanol (HI) (3:2) and ▨ Ethyl lactate extracts, using □ DMSO as negative control.

3.3.4. Correlation between extracts antitumor features and their biochemical profile

Several studies have suggested carotenoids as antitumor agents, exerting effects on cancer cell either by i) tumour inhibition promoted by β-Carotene upon hepatic cancer cells, and ii) growth inhibition endorsed by lutein in prostate, mammary, and in colon cancer, as reviewed before (Guedes et al. 2011).

However, to the moment, there are not studies relating carotenoids' antitumor effects upon AGS or MKN45 gastric cancer cell lines.

Concerning fatty acids, particularly the poli-unsaturated belonging to class of ω-3s and ω-6s, there is a great deal of variation in terms of antitumor capacity. Some studies support that ω-3s, such as EPA and DHA, are associated with cancer suppression, while ω-6s, particularly AA, is generally associated to cancer promotion (Yang, Huang et al. 2004, Xu and Qian 2014). Additionally, it seems that effects of fatty acids vary accordingly with the cancer cell line in study (Yang, Huang et al. 2004, Xu and Qian 2014).

Nevertheless, until complete evaluation of extracts' antitumor effects, it is not possible to accurately correlate it with extract composition, however, some prevision could be made based on other studies.

Results seem to indicate that both cell lines present a decrease in proliferation when in contact with HI and ethyl lactate extracts.

However, apparently, while in AGS cell line both extracts present similar results; in MKN45 cell line, HI extract seem to induce a higher decrease in cancer cell proliferation than ethyl lactate extract.

Regarding the lipidic content of the extracts, the results have shown that ethyl lactate extract presents a higher concentration of FA than HI extract, but a lower concentration of carotenoids.

Additionally, AGS and MKN45 lines show a distinct response to the compounds, indicating that different cell lines may respond differently to the same anti-proliferative compounds. This reinforces that in fact the antitumoural effect of a certain compound is not only dependent on the compound itself, but also on the intrinsic characteristics of cancer cell.

3.4. Conclusions

This study still is ongoing, but some conclusions may be attained by now. So far, *Gloeothece* sp. extracts obtained with ethanol, acetone, hexane:isopropanol (3:2) (HI) and ethyl lactate are able to modulate cell viability of both tested gastric cancer cell lines, AGS and MKN, in a concentration dependent-manner. However, HI extract exerted the best effect attaining the lowest IC_{50} for both cell lines.

Extracts' biochemical composition revealed a diverse concentration in carotenoid and PUFA present at values of IC_{50} for both gastric cell lines.

Preliminary results disclose some cancer cell death-promoting effect of HI and ethyl lactate upon AGS and MKN45 as well as anti-proliferative effects. Furthermore, AGS cell line appears to be more sensitive to the extracts' anti-proliferative effects than MKN45

Hence, further studies are needed to disclose the antitumoural capacity of microalgae extract compounds.

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PART III

OPTIMIZATION OF MICROALGAL LIPIDIC COMPOUNDS EXTRACTION AND PRODUCTION

CHAPTER 4

Carotenoid and fatty acid extraction from Gloeothece sp. via improved continuous pressurized solvent extraction system

Carotenoid and fatty acid extraction from *Gloeothece* sp. via improved continuous pressurized solvent extraction system

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THIS MANUSCRIPT HAS BEEN SUBMITTED FOR PUBLICATION IN BIORESOURCE TECHNOLOGY.

Abstract A laboratory-made continuous pressurized solvent extraction system (CPSE) was built and optimized for extraction of carotenoids and/or fatty acids (with putative antioxidant capacity) from microalgae *Gloeothece* sp. Biomass amount, solvent flow-rate/pressure, temperature and solvent volume – including extract fractioning and degree of recirculation, were sequentially addressed as operation parameters, using a food GRAS solvent – ethanol. Carotenoids and fatty acids were identified by HPLC-DAD, respectively, and antioxidant capacity was assessed by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ methods.

For lipidic compounds extraction, 60 °C and 180 bar were the best operating conditions. Collection in different volume fractions permitted carotenoid-rich extracts, particularly rich in lutein, PUFA and antioxidant compounds. Recirculation of ca. 8% of the maximum solvent volume tested as 3 cycles increased 1.7-fold the lutein and 11-fold the β-carotene contents. Using 5 cycles of recirculation, C18:2 n6 t was increased 7.4-fold. When compared to a conventional extraction method, ultrasound assisted extraction (UAE), CPSE proved superior in terms of extraction yields.

Keywords: *microalga, pressurized liquid extraction, lutein, β-carotene, antioxidant*

4.1. **Introduction**

Microalgae, including cyanobacteria, play an increasing role in science and industry due to the wide range of commercial and novel products obtainable therefrom (Pulz and Gross 2004). Some of the most important, and well established in the market, are carotenoids and fatty acids (Taucher et al. 2016). In general, applications of carotenoids can be divided into three main groups: i) natural dyes in food and feed industry, ii) feed additives in aquaculture and poultry farming, iii) and pharmaceutical and cosmetics sectors, due to the underlying capacity to inhibit several diseases associated with their antioxidative properties (Guedes et al 2011a and Taucher et al. 2016). Interest in microalgal fatty acids has also emerged in recent decades, for their potential therapeutic uses in disease prevention, nutritional applications or biofuel production (Amaro et al. 2011 and Guedes et al. 2011a).

In microalgae, carotenoids and PUFA are generally located in the intracellular space or accumulated in organelles (e.g. pigments), vesicles or in the cytoplasm itself. The presence of a cell wall surrounding the cells, and especially of an intact cytoplasmic membrane that acts as a semipermeable barrier, hampers extraction of such compounds (Vanthoor-Koopmans et al. 2013) – so this tends to become a costly step. Traditionally, extraction of microalgae bioproducts has been chiefly conducted from dried biomass with organic or aqueous solvents, depending on the polarity of the target compound (Ceron et al. 2008 and Luengo et al. 2014). Traditional solvent extraction techniques are widely applied throughout industry, but they require large quantities of organic solvents, are labor-intensive, and can expose extracts to excessive heat, light and oxygen – thus promoting isomerization and oxidation of labile compounds. Moreover, they require extra energy input to recover the solvents, and may contaminate the algal solids, thereby restricting options for their end uses (Iqbal and Theegala 2013). In this regard, such new technologies as pressurized liquid extraction (PLE) have emerged and bear a number of advantages. A typical PLE system pressurizes and accelerates passage of solvent through the matrix, thus improving speed and extraction efficiency of desired compounds. It resorts to conventional solvents at controlled temperature and pressure, requires less solvent and shorter extraction times, and keeps samples in an oxygen- and light-free environment – making it particularly useful for the nutraceutical industry (Carabias-Martinez et al. 2005 and Mustafa et al. 2012). Moreover, application of pressure permits use of temperatures above the solvent atmospheric boiling point, and reduces solvent surface tension thus enabling penetration of solvent into the matrix pores. It results in matrix disruption that enhances mass transfer of the compound from the sample to the solvent, thus improving extraction efficiency (Mustafa et al. 2012). Several studies have demonstrated the advantage of using high pressure and temperature with forced flow of solvent for extraction of natural compounds from solid and semi-solid

matrices by PLE (Castro-Puyana et al. 2016, Denery et al. 2004, Guedes et al. 2013, Herrero et al. 2006, Pieber et al. 2012 and Taucher et al. 2016). However, PLE has not been widely applied as a routine tool in natural product extraction due the high cost associated, coupled with its requirements for dedicated infrastructure and operation. Furthermore, most commercial instruments only allow static extractions, in which a fixed volume of solvent, under the desired conditions of pressure and temperature, contacts the sample for a given time; hence, equilibria between those sample components still bound to the matrix and those already solubilized in the solvent will likely be reached, but the efficiency of the extraction process cannot be increased beyond this point (Herrero et al. 2013). Another less advantageous characteristic of PLE is the use of extreme temperatures up to 135-200 °C, and pressures up to 200 bar or even higher (Luthria 2008). These conditions may prompt formation of pyropheophytins, a chlorophyll derivate not naturally present in the original sample, but possessing antimutagenic features as reported for *Chlorella vulgaris* (Herrero et al. 2013).

This study focused on a laboratory-made, continuous pressurized solvent extraction (CPSE) system, designed to improve the economics of microalgae bioactive compound extraction – while simplifying the overall extraction process, thus being useful for the nutraceutical and pharmaceutical industries. Instead of use of said extreme temperatures and pressures, this system resorts to moderate temperatures (40–70 °C) and pressures (70–260 bar), well within the range of operational temperatures and pressures employed in large-scale operation systems (Iqbal and Theegala 2013). Therefore, the aim of this study was to develop a low cost lab-made system with continuous pressurized solvent system, possessing a high versatility and permitting control of flow rate, temperature and flow operating mode – in order to maximize extraction of compounds known for their antioxidant capacity (such as carotenoids and fatty acids) from microalgal biomass, attempting to overcome the PLE constraints.

4.2. Material and methods

4.2.1. Microalga source and biomass production

Gloeothece sp. (ATCC 27152) was obtained from ATCC (American Type Culture Collection) (USA). Batch cultures thereof were cultivated in 5 L-flasks containing 4.5 L of medium, at 25 ºC, using Blue Green medium (BG11) (Stanier et al. 1971), buffered with Tri-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) 25 mM, at pH 8. Continuous illumination was provided via fluorescent BIOLUX lamps (250 $\mu mol_{photon}.m^{-2}.s^{-1}$), and air was bubbled at a flow rate of 0.5 $L.min^{-1}$. To ensure the microalga was in the exponential growth

phase, a pre-inoculum at an initial optical density of 0.1 at 680 nm was used for 10 days in 800 mL of the same medium. After 14 days of growth, biomass was dewatered, freeze-dried and stored under nitrogen in a desiccator prior to utilization.

2.2. Continuous pressurized solvent extraction

The lab-made continuous pressurized solvent extraction system (CPSE) at stake was built so as to allow a pressurized solvent to pass through a column containing microalgal biomass, at a pre-set temperature. As depicted in Fig. 1, it is mainly composed of an HPLC solvent injection pump (Hitachi L-2130, Japan) that pressurizes solvent at a set flow-rate in the range of 0.1–10 $mL.min^{-1}$ and a pressure up to 360 bar; a hollow column filled with microalgal biomass and an excipient; and a temperature-controlled chamber that allows the system be kept at the desired temperature.

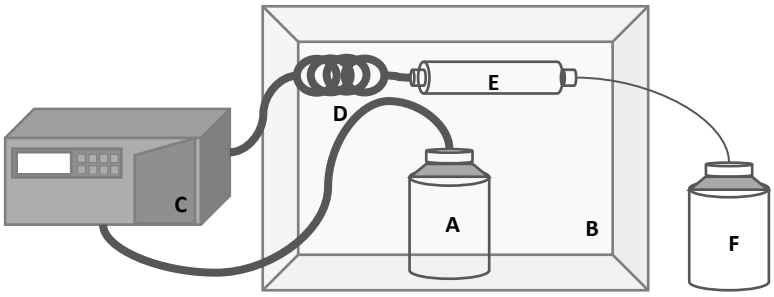

Fig. 4.1. Scheme of the lab continuous pressurized solvent extraction (CPSE) system. This system is composed by a A) solvent reservoir, B) temperature controlled chamber; C) HPLC solvent injection pump; D) pre-heating coil; E) extraction column; and F) extract reservoir.

The ethanol reservoir is kept at the desired temperature and pumped by the HPLC solvent injection pump at a pre-set flow rate, and sent to a stainless pre-heating coil 1 m-long and an internal diameter of 1 mm – which guarantees that solvent is at the desired temperature before entering the extraction column. The column placed inside the temperature-controlled chamber is 15 cm-long, with an internal diameter of 50 mm and closed with end fittings. The exit tube is 2 m-long, with an internal diameter of 50 µm, and keeps the system pressurized; it ends in the extract reservoir. The entire system was tested for leaks at the maximum operating pressures and temperatures. The system allows flushing of solvent in the tubes at the end of each assay, without the need for any inert gas (e.g. N_2); and the whole system is closed, thus avoiding contact with O_2. Between runs, the entire system was cleaned with fresh solvent to avoid any extract carryover.

In each assay, the extraction column was filled in consecutive layers of inert Ottawa sand, *Gloeothece* sp. freeze-dried biomass and another layer of Ottawa sand. It is important

to ensure that particle size is as small as possible. This has been shown to enhance extraction efficiency by shortening diffusion paths (Turner et al. 2001). *Gloeothece* sp. freeze-dried biomass was standardized by a mortar-aided previous extraction. Note that solvent, pre-heating coil and column were placed in the temperature-controlled chamber and pre-heated at the desired temperature, to assure that the whole system remains at constant temperature for at least 5 min.

4.2.2.1. Optimization of extraction conditions

The conditions of operation of this CPSE system were optimized sequentially: 1) amount of biomass in extractor; 2) solvent flow/pressure ($mL.min^{-1}$); 3) temperature (ºC); and 4) total volume of solvent used (mL). As the optimization steps were progressing, the conditions were being redefined based on the results obtained so far.

Selection of the correct solvent is one of the most crucial factors affecting pressurized extraction. The targeted compounds were fatty acids and carotenoids; based on an earlier study (Amaro et al. 2015) and similar studies (Cha et al. 2010, Jaime et al. 2010, Mustafa et al. 2012 and Pieber at al. 2012), ethanol was chosen as solvent. Besides its relatively low environmental impact, it has a positive net energy balance and a generally recognized as safe (GRAS) status – an extra advantage for its applicability in the nutraceutial industry (Mustafa et al., 2012).

To establish the biomass amount to perform extractions, 50, 100 and 150 mg of freeze-dried biomass were assayed with (at medium-low temperatures and solvent flow rates of 40 ºC and 2 $mL.min^{-1}$, respectively). The influence of solvent flow was studied at the best biomass amount, so four conditions were tested – 1, 2, 3 and 4 $mL.min^{-1}$. Once the best combination of biomass amount and solvent flow was attained, several system temperatures were tested, viz. 30, 40, 50, 60 and 70 ºC. Until this point, all solvent used was circulated in continuous flow; in order to reduce use of solvent volume, this final condition was optimized afterwards. Consequently, the extract was collected in several fractions along the extraction process, and its content in PUFA, carotenoids and antioxidant capacity were determined. Then, the minimum volume of extract that contains the major amount of the target compounds was established, to tentatively improve extraction efficiency, and its recirculation in the CPSE system was tested – via several cycles of recirculation, 2, 3, 4 and 5, that correspond to 4, 8, 12, 16 and 20 min. The system was purged at the end of each cycle, and extracts assayed for the aforementioned parameters.

All extractions were performed in triplicate, and extract dry mass was ascertained under N_2 atmosphere. Aliquots of the same extract were used to determine contents of fatty acids and carotenoids, as well as their antioxidant capacity.

4.2.3. Utrasound assisted extraction

Ultrasound-assisted extraction was performed with 50 mg of biomass, by sequential addition of 2 mL of ethanol and cell disruption by 15 min of sonication, until a final volume of 12 mL was reached. Between each addition, the extract was stirred for 20 min at 250 rpm, centrifuged at 20,000 rpm for 5 min, and supernatant (extract) collected and stored at 4ºC. To completely remove cells debris, extracts were filtered through 0.45 µm pore size, and then stored under nitrogen, at -20 °C and in darkness, prior to analysis.

4.2.4. Antioxidant scavenging capacity assessment of extracts

4.2.4.1. $ABTS^{+\bullet}$ scavenging capacity

An aliquot of extracts, obtained as described above, was evaporated and the residue re-suspended in ethanol:water, 50:50 (v/v) to a final concentration of 1 $mg.mL^{-1}$. The $ABTS^{+\bullet}$ radical-scavenging capacity of the extracts was assessed in triplicate, as described by Guedes et al. (2013). For quantification, a calibration curve using a known antioxidant – Trolox, was prepared, so antioxidant capacity of extract was expressed as $\mu g.mL^{-1}$ of Trolox with similar scavenging capacity.

4.2.4.2. $DPPH^{\bullet}$ scavenging capacity

An aliquot of each extract was likewise evaporated, and the residue resuspended in methanol to a final concentration of 5 $mg.mL^{-1}$. In this spectrophotometric assay, the scavenging reaction was measured after incubation for 30 min of 1 mL DPPH with 125 µL of sample (Amaro et al., 2015). Measures were performed at 515 nm, and quantification was performed as described above.

4.2.5. Compound identification

4.2.5.1. Determination of carotenoids profile

To identify, and then quantify carotenoids (including β-carotene and lutein, in particular), high-performance liquid chromatography (HPLC) was employed as analytical technique (Guedes et al. 2011b). An aliquot from each extract was evaporated and suspended to a concentration of 15 $mg.mL^{-1}$, and β-apo-carotenol (Sigma) was used as internal standard. The carotenoid profile was produced in a Merck-Hitachi HPLC system, equipped with a Diode Array Detector (DAD) Merck-Hitachi L-7450, to resolve, detect and identify the various chemical compounds of interest. The absorption spectra were recorded between 270 and 550 nm. The stationary-phase was a 4 x 250 mm Purospher Star RP-18e

(5µm) column (Merck). The mobile-phase was constituted by solvent A — ethyl acetate, and solvent B — acetonitrile/water at 9:1 (v/v), both from VWR, at various volumetric ratios along elution time, for an overall flow rate of 1 mL min^{-1}. The following gradient was used: 0–31 min (0–60 % A); 31–46 min (60 % A); 46–51 min (60–100 % A); 51–55 min (100 % A); 55–60 min (100-0 % A); and 60–65 min (0 % A). The elution times of the chromatographic standards were: lutein 14.4 min, and β-carotene 34.4 min. Standards were purchased in CarotNature, Lutein (No. 0133, Xanthophyll, (3R,3'R,6'R)-β,ε-Carotene-3,3'-diol with 5 % Zeaxanthin and purity of 96 %), β-carotene (No. 0003, β, β -Carotene) with 96 % purity) and β-apo-carotenol (No. 0482, 8'-Apo- β -caroten-8'-al) with 97 %, purity). Identification was by comparison of retention times and UV–visible photo-diode array spectra, following a procedure detailed by Guedes et al. (2011b).

4.2.5.2. Determination of fatty acid profile

Fatty acid methyl esters were produced from an evaporated aliquot of each extraction obtained by direct transesterification — according to the acidic method described by Lepage and Roy (1984), after modifications introduced by Cohen et al. (1988), using heptadecanoic (C17:0) acid as internal standard and acetyl chloride as catalyst. Esters were analysed in a GC Varian Chromapack CP-3800 gas chromatograph (GC), using a flame ionization detector, and quantified with the software Varian Star Chromatography WorkStation (Version 5.50). A silica CP-WAX 52 CB (Agilent) column was used, and helium was employed as carrier gas in splitless mode. Injector and detector were maintained at 260 and 280 °C, respectively, and the oven heating program was as described in Table 1. Chromatographic grade standards of fatty acids in methyl ester form CRM47885 (Supelco) were used for tentative identification, based on retention times: C13:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1 n9-cis + trans, C18:2 n6, C18:2 n6 c, C18:3 n6, C18:3 n3, C20:0, C20:1, C20:5 n3, C21:0, C22:0, C22:2, C22:1 n9. The mean of the results from the aforementioned chemical assays was used as a datum point.

Table 4.1. Oven heating program for fatty acid identification.

T (ºC)	Rate (ºC.min^{-1})	Holding time (min)	Time (min)
100	-	5	5
180	6	0	18.33
200	2	0	28.33
205	0.5	0	38.33
230	1	0	63.33
233	0.5	0	69.33

4.2.6. Statistical analysis

The experimental data were analysed using GraphPad Prism v. 5.0. A first diagnostic unfolded a non-normal distribution of the data, so 1-way ANOVA with Tukey's multi-comparison test was used to assess variances between different extract in terms of carotenoid content and antioxidant capacity, and two-way ANOVA with the same multi-comparison test in fatty acid content for extraction conditions. Since each datum point had been replicated, a representative measure of variability was available in all cases to support said statistical analyses.

4.3. Results and discussion

4.3.1. Optimization of biomass amount

Extraction techniques are relevant not only for industrial recovery of metabolites, but also for analytical sample preparation – and the general trend is development of faster, more efficient, less expensive and greener methodologies. Sample preparation is one of the bottlenecks of analytical procedures, due the difficulty of implementation. As stated by the "Green Analytical Chemistry" (GAC) principles, it should take in consideration: 1) reduction of sample amount; 2) simultaneous extraction of multiple compounds; and 3) increase in automation processes (De La Guardia 2011). An ideal pressurized liquid extraction should use the minimum amount of food grade solvents for a selective extraction of bioactive compounds, while preserving their chemical structure. It should also exhibit great versatility and efficiency, since the physicochemical properties of solvents (e.g. density, diffusity, viscosity and dielectric constant) can be modified by changing pressure and/or temperature of solvent, thus modifying the solvating power (Herrero et al. 2013). Hence, the main purpose of our extraction system is to obtain the maximum amount of lipidic compounds, namely carotenoids and/or fatty acids, with the minimum expense of resources such as energy, solvents and feedstock.

The first step was to establish the minimum amount of biomass to be used. Three amounts of freeze-dried biomass of *Gloeothece* sp. were tested: 50, 100 and 150 mg. The selected biomass amount should maintain the proportionality between biomass and compounds in extract. Therefore, such parameters as extract mass yield, carotenoids and PUFA contents, and antioxidant capacity were determined. At this point, average values of flow and temperatures were used: 2 $mL.min^{-1}$ and 40 ºC. Furthermore, solvent volume was established following a prior assay using 150 mg of biomass (maximum biomass tested). Extraction was under continuous flow, and extract was collected in batches of 25 mL. The volume was set as 150 mL after 2 consecutive batches not presenting any visual coloration,

thus guaranteeing that solvent was in surplus to extract pigments – data not shown. Despite compounds like fatty acids not being totally extracted, volumes higher than 150 mL would increase costs with no significant extra benefits.

In terms of extract yield, and by establishing the quotient extract mass/biomass amount, there were not significant differences in the mass of extract obtained ($p < 0.05$) – ca. 22.6 ± 0.7 %, indicating that there is proportionality between biomass used and mass of extract obtained. In general, the same was observed by analysing the content in fatty acids, carotenoids and antioxidant capacity of extracts – see Table 2. Note that data are presented as a ratio of content per biomass amount tested.

Table 4.2. Average extract content in fatty acids ($\mu g_{FattyAcids}.mg_{biomass}^{-1}$), carotenoids ($mg_{carotenoids}.L^{-1}.mg_{biomass}^{-1}$) and antioxidant capacity (trolox equivalent (TE) $mg.mg_{extract}^{-1}.mg_{biomass}^{-1}$) ± standard deviation, obtained at each amount of biomass tested (50, 100 and 150 mg), at 40 ºC and under a solvent flow-rate of 2 $mL.min^{-1}$ (P=142 bar).

		Biomass (mg)		
		50	**100**	**150**
Fatty acids ($\mu g_{FA}.mg_B^{-1}$)	**14:0**	5.4 ± 0.6[a]	4.9 ± 1.0[a]	4.4 ± 0.5[a]
	16:0	24.6 ± 3.9[a]	23.0 ± 0.7[a]	23.3 ± 2.2[a]
	18:0	0.3 ± 0.0[a]	0.5 ± 0.1[a]	0.1 ± 0.0[a]
	18:1 n9	17.6 ± 3.0[a]	12.3 ± 3.7[a]	13.3 ± 2.5[a]
	18:2 n6 t	19.8 ± 3.6[a]	14.6 ± 1.0[a]	18.8 ± 2.0[a]
	18:2 n6 c	0.3 ± 0.0[a]	0.0 ± 0.0[a]	0.3 ± 0.0[a]
	18:3n6	0.9 ± 0.1[a]	0.5 ± 0.0[a]	0.4 ± 0.0[a]
	18:3 n3	36.6 ± 5.4[a,b]	27.1 ± 2.7[b]	39.3 ± 3.3[a]
Carotenoids ($mg.L^{-1}.mg_b^{-1}$)	**Lutein**	2.16 ± 0.14	1.15 ± 0.06	1.39 ± 0.18
	β-Carotene	0.16 ± 0.00[a]	0.13 ± 0.02[a]	0.12 ± 0.01[a]
Antioxidant capacity ($mg_{TE}.mg_E^{-1}.mg_B^{-1}$)	**ABTS**	129.4 ± 2.6	102.7 ± 8.0	70.1 ± 5.5
	DPPH	3.1 ± 0.1	1.9 ± 0.4[a]	1.9 ± 0.5[a]

[a–c] Means within the same row, without a common superscript, are significantly different ($p < 0.05$).

Other carotenoids besides lutein and β-carotene, and also fatty acids were extracted – yet for analysis and comparison of extraction conditions, only these were considered due their representativeness. The total antioxidant capacity of the extracts was assessed by two total scavenging assays: $ABTS^{+\bullet}$ and $DPPH^{\bullet}$. These two assays were chosen based on

earlier experience (Amaro et al. 2015), due namely to their different sensibility either for carotenoids, as $ABTS^{+\bullet}$ or to fatty acids as $DPPH^{\bullet}$.

Upon inspection of Table 2, it is clear that use of 50 mg of biomass is the most appropriate, thus avoiding unnecessary spending of biomass.

4.3.2. Optimization of solvent flow-rate

As stated before, the next stage was to optimize the solvent flow-rate, so 1 (Q1), 2 (Q2), 3 (Q3), and 4 $mL.min^{-1}$ (Q4) were tested. System pressure increases as flow increases, so pressures of 72, 210 and 260 bar were reached, respectively. Note that previous results in the biomass optimization stage were generated at 2 $mL.min^{-1}$ (Q2) under a pressure of 142 bar. Pressure facilitates transport of solvent to hard-to-reach corners, pores, surfaces and matrices (Cooney et al. 2009, Iqbal and Theegal 2013). Hence, it results in matrix disruption, and so enhances mass transfer of the compound from the sample to the solvent (Mustafa et al. 2012). However, in terms of extract yield, differences between tested flow-rates, 17.1 ± 0.95 % for Q1, were not statistically significant; 21.2 ± 2.1 % for Q2; 18.9 ± 1.3 % for Q3; and 19.5 ± 1.8 % (m_E/m_B) for Q4.

Several studies focusing on bioactive natural product extraction have pointed at the nil influence of extraction pressure; yet, the only reason they set an extraction high pressure is to maintain its liquid state due the high solvent temperatures used (100-160ºC), but its influence was not further addressed (Turner 2011, Herrero et al. 2013). However, lower temperatures were used in this study due the existence of thermolabile compounds; upon analysis of the extracts obtained (see Fig.2), it appears that flow-rate/pressure exerts a positive effect: particularly in the case of Q3 in fatty acid extraction, an average increase of 3-fold compared to Q1 was noticed; or Q4 in lutein extraction – for which an increase of 1.3-fold was apparent, as compared to Q1. Although selection of a suitable extraction solvent will probably be the most important step in optimizing PLE for microalgal metabolite extractions (Iqbal and Theegal 2013), the pressure played an important role in the case of lutein (Guedes et al. 2013) – and as observed in this study, also in fatty acid extraction. It has been shown that elevated pressures reduce the dielectric constant of immiscible solvents to values that better match the polarity of the lipids (Cooney et al. 2009; Herrero et al. 2006, Iqbal and Theegal 2013). With regard to β-carotene, the solvent flow rate (Q) did not unfold any relevant effect (Fig. 2B).

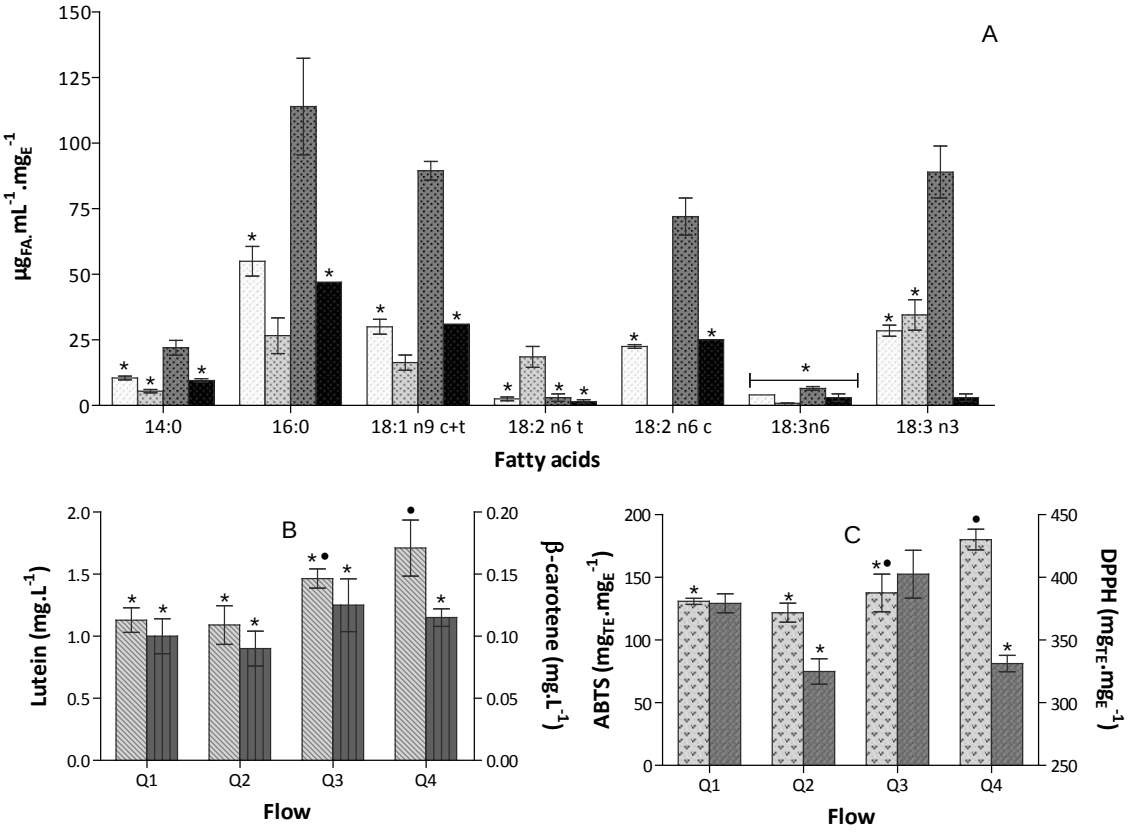

Fig. 4.2. Biochemical profile of extracts obtained from 50 mg-biomass in the various solvent flows tested at 40 ºC. **A**) Fatty acid profile expressed as μg_{FA}. $mL^{-1}.mg_{Extract}^{-1}$ obtained in ☐ Q1 (1 $mL.min^{-1}$); ▨ Q2 (2 $mL.min^{-1}$); ▩ Q3 (3 $mL.min^{-1}$) and ■ Q4 (4 $mL.min^{-1}$) at 40ºC; (bars for a common fatty acid, without a common superscript, are significantly different, $p < 0.05$); **B**) Carotenoids (equivalent of PI $mg.L^{-1}$) ▨ Lutein and ▩ β-carotene content (bars without a common superscript are significantly different, $p < 0.05$); and **C**) Antioxidant capacity expressed in trolox equivalent (TE) per extract mass, $mg.mg_{extract}^{-1}$, obtained in ▨ ABTS and ▩ DPPH assays.

Nevertheless, a decreasing trend upon PUFA extraction was observed using Q4 (P of 260 bar). A similar effect was observed by Guedes et al. (2013); when pressure increases, it may cause an increase in fluid density. This may, in turn, cause a double effect: an increase in solvent solvating power, and a reduced interaction between solvent and matrix, thus decreasing the diffusion coefficient at higher density. This phenomenon has been already described for other microalgae and metabolites (Macías-Sánchez et al. 2005, 2010 and Turner et al. 2001).

As expected, the antioxidant capacity of the extracts obtained varied according to their content in carotenoids and fatty acids – see Fig. 2. Hence, the antioxidant capacity as assessed by the $ABTS^{\bullet+}$ assay revealed no differences between extracts – except for Q4, which exhibited a higher concentration in antioxidant compounds, probably reflecting its

higher lutein content. In DPPH$^{\bullet}$ assay, Q3 and Q1 extracts attained the best results, probably due to being richer in fatty acids.

Consistent with the effect upon compound extraction, Q3 flow rate was selected to proceed with our study.

4.3.3. Optimization of temperature

Once biomass amount and solvent flow rate were defined, the next stage was testing the influence of temperature on solvent extraction system in terms of lipidic compounds.

Solvent pressure varies with increasing temperature due to a decrease in viscosity, so pressure varied as 212, 210, 195, 180, and 168 bar, at 30, 40, 50, 60 and 70 ºC respectively, despite using the same flow rate Q3 (3 mL.min^{-1}). Temperature increases the solvent potential by accelerating diffusion rates (Denery et al. 2004). Moreover, thermal energy helps overcome the cohesive (solute-solute, i.e. lipids-lipids) interactions and adhesive (solute-matrix, i.e. lipids-cell matrix) interactions (Cooney et al. 2009, Iqbal and Theegal 2013). Therefore, increasing the thermal energy increases the motion of the molecules, and so decreases the molecular interactions associated with hydrogen bonds, van der Waals forces, and dipole interactions – thus resulting in faster and easier extraction (Cooney et al. 2009).

Consequently, temperatures of 30, 40, 50, 60 and 70 ºC were employed for the extraction of compound, using 50 mg of biomass at Q3. Only 70 ºC was observed to lead to an increase of mass extract yield, ca. of 52 %, when compared to the other temperatures that reached an average of 44.4 ± 2.8 % m_E/m_B – data not shown.

In fatty acid extraction (Fig. 3A), one may conclude in general that extraction at 60 ºC produced a better yield than at other temperatures tested. When compared to the lowest yield obtained at 30 ºC, an increase of 2.6-fold was found for C16:0, 3.3-fold for C18:1n9, 3.5-fold for C18:2n6 trans, 2.7-fold for C18:3n6 cis, and 16-fold for C18:3 n3. As seen before, diffusion rates increase roughly from 2- to 10-fold when the temperature is increased from 25 ºC upwards (Iqbal and Theegal. 2013).

At 70 ºC, lipids extraction was significantly lower than at 60 ºC. Although use of higher temperatures has been claimed to enhance fatty acids extraction (Iqbal and Theegal 2013, Pieber at al. 2012), the pressures used were lower than those selected in this work. Maybe the increase of temperature reduces solvent density considerably at this pressure, thus reducing the solvent-lipids contact – and resulting in net lower lipid mass transfer rates (Halim et al. 2011). Lipids may also deteriorate by cleavage of carbon-oxygen bonds in fatty acids, due to its sensitivity to temperature at the set pressure (Fournier et al. 2006, Iqbal et al. 2013). At 50 ºC, an unexpected low extraction yield was attained. This may have occurred due to a complex interaction of non-equilibrium in mass transfer, due a change in solvent

density to set pressure, as observed before with microalgal carotenoid extraction (Guedes et al. 2013).

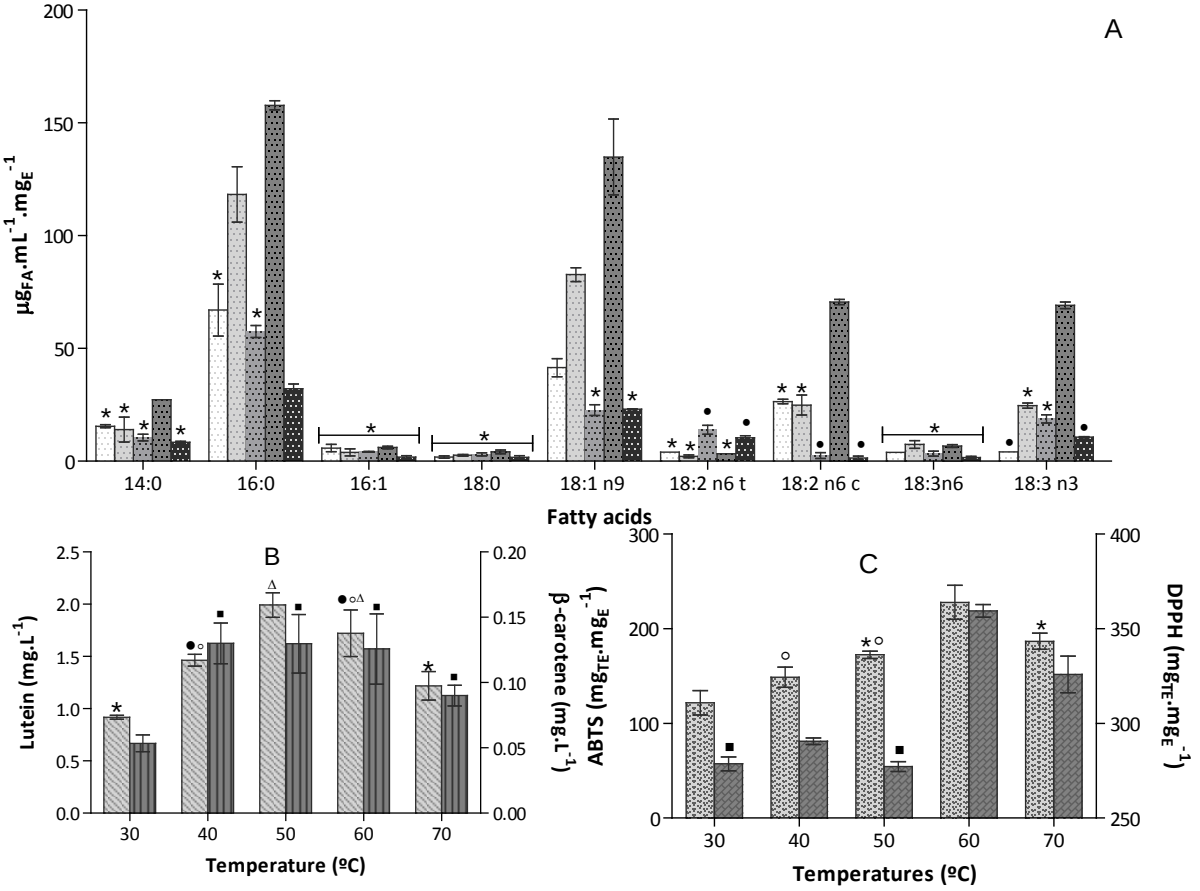

Fig. 4.3. Extracts biochemical profile obtained from 50 mg biomass, at Q3, in the various extraction temperatures tested. **A**) Fatty acid profile expressed as $\mu g_{FA}.ml^{-1}mg_{Extract}^{-1}$ obtained at □30 ºC, □40 ºC, ▒50 ºC, ■60 ºC, ▩70 ºC (bars for a common fatty acid, without a common superscript, are significantly different, $p < 0.05$); **B**) Carotenoids content ▨Lutein and ▥β-carotene expressed in equivalent of PI $mg.L^{-1}$(bars without a common superscript are significantly different, $p < 0.05$); **C**) Antioxidant capacity of extracts obtained in ▩ ABTS and ▩DPPH assays, expressed in trolox equivalent (TE) per extract mass ($mg_{TE}.mg_{extract}^{-1}$) (bars for the same assay, without a common superscript, are significantly different, $p < 0.05$).

Carotenoid recovery yield was maximum within the range 50-60 ºC for lutein, thus unfolding an increase in mass transfer rate with temperature – and indicating that 60 ºC is the most appropriate temperature, as reported previously (Jaime et al. 2010, Guedes et al. 2013, Macías-Sánchez et al. 2010). Nevertheless, temperature had no strong influence upon extraction of β-carotene, as its concentration was similar in extracts obtained within the range of 40-70 ºC, but larger when compared to those obtained at 30 ºC. Temperature affects viscosity and solubility of solvents, but it may also promote isomerization and decomposition

of labile target chemicals (Denery et al. 2004) – thus explaining the slight decrease in lutein concentration observed at 70 ºC (Fig. 3A).

In view of the above, the optimum temperature is ca. 60 °C, as indicated previously by Taucher et al. (2016).

Several pieces of evidence show that antioxidant capacity measured by $ABTS^{\bullet+}$ of such microalga extracts as *Haematococcus pluvialis* seems to be related to their free carotenoid content, mainly lutein as it dominates overall concentration (Guedes at. al. 2011b, Jaime et al. 2010 and Herrero et al. 2006); whereas a decrease in the antioxidant capacity seems to be related to the lower carotenoid content of the extracts (Jaime et al, 2010). Therefore, as expected, the antioxidant profile of $ABTS^{\bullet+}$ (Fig. 3C) is similar to that obtained for carotenoids (Fig. 3B).

On the other hand, fatty acids may also contribute to the antioxidant activity, in addition to carotenoids – as reported by Cerón et al. (2006). This fact was observed when the DPPH assay was used (Fig. 3C). Moreover, other compounds with antioxidant capacity (not identified) may have been co-extracted – like chlorophylls, phenolic compounds or other hydrophilic compounds with recognized antioxidant capacity (Cha et al. 2010).

4.3.4. Optimization of solvent total volume

The use of low solvent volumes in PLE extraction is one of its key points supporting its applicability at industrial scale (Pieber et al. 2012). As stated before, the volume used so far was in excess – so in a first attempt to reduce and find the optimum solvent volume, the 150 mL was collected in distinct and sequential fractions to ascertain compound concentration. In a preliminary assay, more than 60% of mass extract was concentrated in the first 8.3 % of solvent volume (12.5 mL). Hence, the volume was collected in 3 fractions, 1st faction of 12.5 mL (1F), 2nd fraction 12.5 mL (12.5) and the 3rd of 125 mL (3F), along the extraction routine. In the last fraction, the mass of extract was so low that precluded its subdivision in more fractions. The extract mass yields in 1F, 2F and 3F were 17.2 ± 0.1, 4.5 ± 0.4 and 6.9 ± 1.2 % m_e/m_b, obtained under the (so far) optimized conditions of temperature and solvent flow, 60 ºC and Q3 (3 $mL.min^{-1}$), respectively.

After analysing the content in fatty acids (Fig. 4A), it appears that the longer and more unsaturated the fatty acids are, the harder their extraction, thus requiring more solvent matrix contact; at these conditions, more volume is needed to extract them. Although the fatty acid concentration lies between 1.25- to 4-fold higher in 3F than in 1F, the volume in 1F represents 10% of 3F. Nonetheless, high fatty acid concentration in 3F could be useful if the extraction purpose is to obtain an extract rich in C16:0 or unsaturated fatty acids

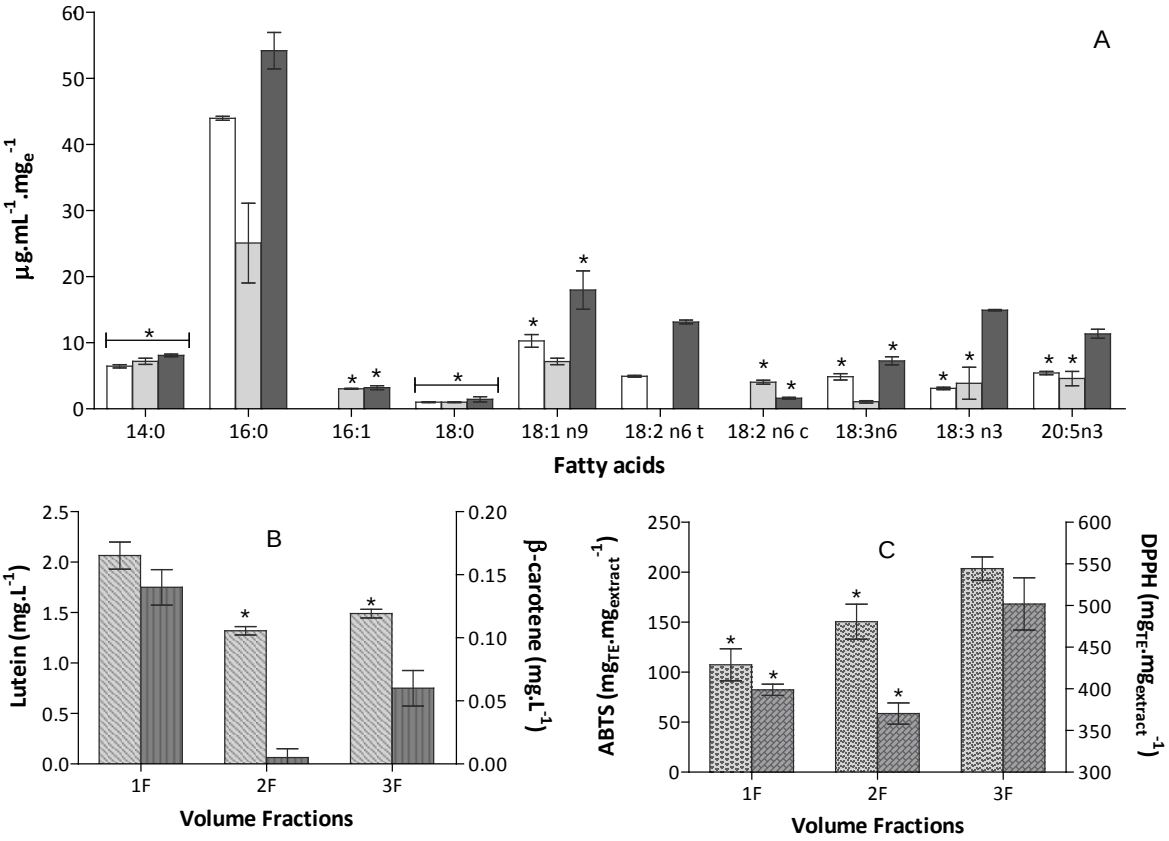

Fig. 4.4- Extracts biochemical profile obtained from 50 mg biomass, at 60 ºC and Q3, in the sequentially collected volume fractions. **A**) Fatty acid profile expressed as μg_{FA}. mL^{-1}.$mg_{Extract}$ ☐ 1F (12,5 mL), ▒ 2F (12,5 mL), ■ 3F (125 mL) (bars for a common fatty acid, without a common superscript, are significantly different, $p < 0.05$); **B**) Carotenoid content, ▨ Lutein and ▮ β-carotene expressed in equivalent of PI, $mg.L^{-1}$ (bars for a common carotenoid, without a common superscript, are significantly different, $p < 0.05$; **C**) Antioxidant capacity of the extract obtained in ⬚ ABTS and ▩ DPPH assays expressed in trolox equivalent (TE) per extract mass, $mg_{TE}.mg^{-1}$ (bars for the same assay, without a common superscript, are significantly different, $p < 0.05$).

Carotenoid content of extracts (Fig. 4B) indicates that both lutein and β-carotene are mostly concentrated in 1F. As observed with fatty acids, if the purpose is to extract solely lutein, 2F is the most appropriate fraction – even if 3F lutein is present to the same level; in terms of volume used, it represent ca. 8-fold less than in 3F. Similarly, 1F contains 28-fold more than 3F in terms of β-carotene extracted and volume used.

Based on the above results pertaining to antioxidant capacity measurements (Fig. 4C), a high antioxidant capacity would be expected particularly with $ABTS^{\bullet+}$ in 1F, due its high concentration in carotenoids (Amaro et al. 2015). Note that this antioxidant assay was performed at an extract concentration of 1 $mg.mL^{-1}$; hence, besides carotenoids, it may contain other non-antioxidant compounds that could have been co-extracted to a large scale, thus lowering carotenoid concentration in the extract (and, consequently, the overall

antioxidant capacity). For the opposite reason, a higher concentration of carotenoids and unsaturated fatty acids in F3 may explain the best antioxidant capacity as per the $ABTS^{\bullet+}$ assay. On the other hand, the higher antioxidant capacity of 3F as per the DPPH assay is consistent with its fatty acid profile.

Briefly, the volume fraction divisions may provide an extra improvement toward multiple compounds extraction. The first fraction contains the highest concentration in lutein; however, if the goal is to achieve a pure extract of these carotenoids, then 2F is the most appropriate – due to its low content in fatty acids. In other words, 3F provides a fatty acid-rich extract possessing high antioxidant capacity.

4.3.4.1. Cycles of extract recirculation

As observed before, the optimum length of an extraction process depends on the time needed to build up the equilibrium between the compound concentration in the sample matrix and the solvent (Taucher et al. 2016). Therefore, an attempt to increase the contact between solvent and matrix until solvent saturation is in order, without increasing the solvent volume – so 1F was tested in several cycles of recirculation. The time needed to obtain 1F was 4 min, so times of extract recirculation of 8, 12, 16 and 20 min were tested – corresponding to 2C, 3C, 4C and 5C, respectively. As concluded from the extract yields obtained, the volume of solvent used at 1F was not saturated, due an average increase of 21 % from 1F to 2C or 3C, and a greater one at 4C or 5C, ca. 65 % (data not shown).

As expected, the fatty acid concentration increased with the number of cycles; compared to 1F, fatty acids had the following extraction improvement using 5C: 3-fold for 18:1n9, 5-fold for C 18:2n6t, and 9-fold for C18:3n3 (Fig .5). Although PLE systems usually work in sequential cycles of static volume, this effect was observed before by Pieber et al. (2012) in fatty acid recovery – but not so far using the recirculation of extract itself for several cycles.

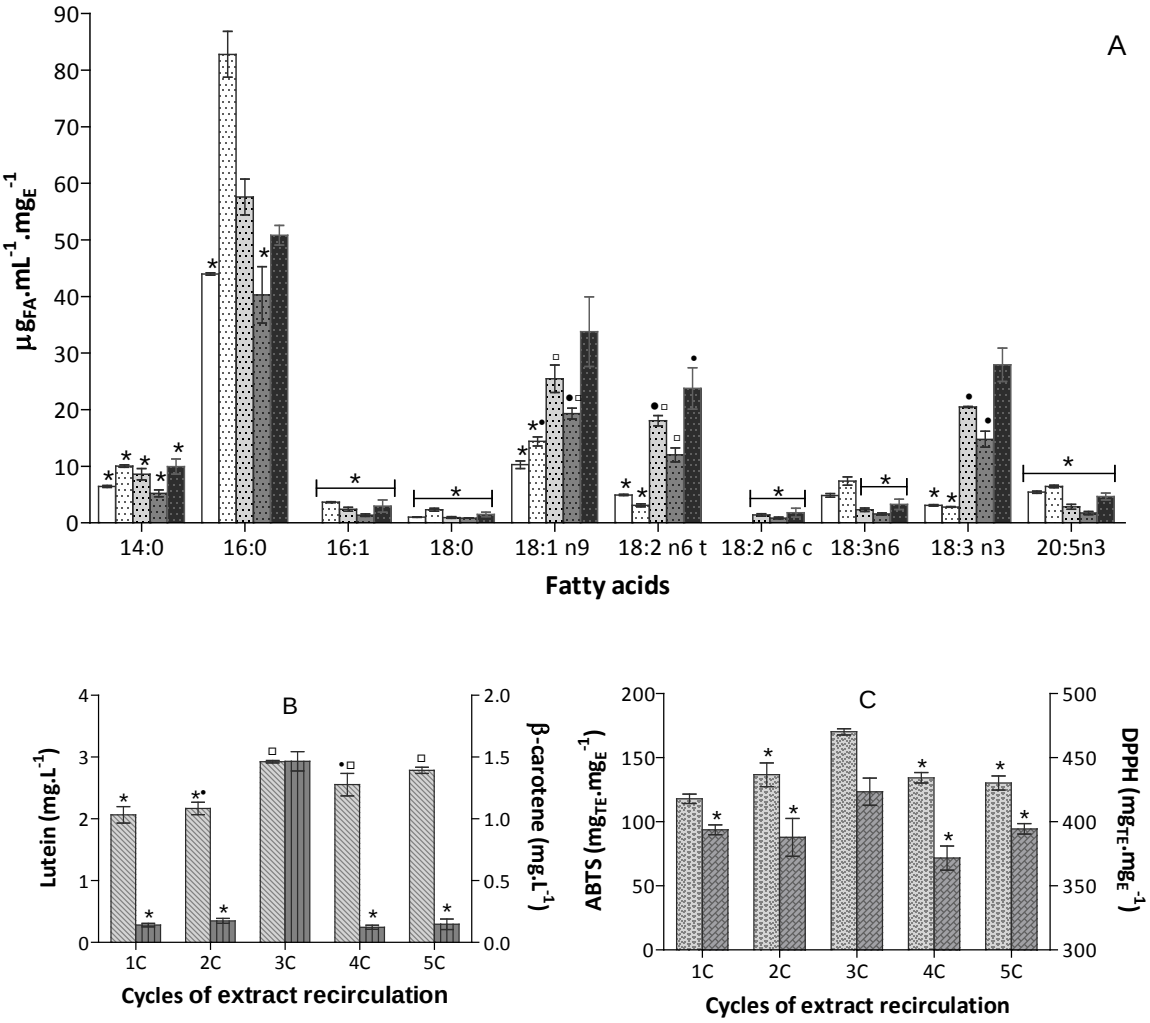

Fig. 4.5-- Extracts biochemical profile obtained from 50 mg biomass, at 60 ºC and Q3, in in tested cycles of recirculation. **A**) Fatty acid profile expressed as $\mu g_{FA}.mL^{-1}mg_{Extract}$ obtained in ☐ 1C (4 min), ⬚ 2C (8 min), ▒ 3C (12 min), ▓ 4C (16 min), ■ 5C (20 min), (bars for a common fatty acid, without a common superscript, are significantly different, $p < 0.05$); B) Carotenoid content in ▨ Lutein and ▮ β-carotene) expressed in equivalent of PI, $mg.L^{-1}$ (bars for a common carotenoid, without a common superscript, are significantly different $p < 0.05$); C) Antioxidant capacity of the extracts obtained in ▨ ABTS and ▩ DPPH assays expressed in trolox equivalent (TE) per extract mass, $mg_{TE}.mg^{-1}$.

This phenomenon was also observed in carotenoid extraction: lutein content increased 1.4-fold from 1F to 3C. In the case of β-carotene, the increase was significantly more pronounced; it had an increase of 10-fold from 1C to 3C. The yield of carotenoids starts to level out after 12 min (3C) of extraction; with additional cycles, the extraction is most likely desorption/diffusion controlled, as pointed out by Mustafa et al. (2012). A too long extraction time may also cause degradation of carotenoids if extraction is conducted in a single step

(Mustafa et al., 2012), thus explaining the decrease of β-carotene concentration in extraction cycles longer than 3C (Fig.5).

The antioxidant capacity of tested extract cycle of recirculation accordingly followed the carotenoid profile, proving that these compounds had a more pronounced effect; in fact, 3C attained the best results, either in $ABTS^{\bullet+}$ or in $DPPH^{\bullet}$, surpassing even the contribution of fatty acids in this last assay.

4.3.5. Comparison of lab-made CPSE system with ultrasound assisted extraction

Finally, the lab-made CPSE system was optimized, and the best conditions for carotenoid and/or fatty acid extraction were found toward minimum use of resources.

Common PLE has been widely compared to other extractions techniques, such as maceration, ultrasound assisted extraction (UAE) or Soxhlet extraction. Advantages of PLE arise chiefly from lower volume, shorter extraction time and potential for automation (Herrero et al. 2013). In this work, UAE was selected for comparison of results obtained with our CPSE system – and carefully conducted to avoid isomerization and degradation of compounds. The same amount of biomass was extracted with 12 mL of ethanol, as described above. As shown in Table 3, compound concentration and antioxidant capacity obtained in CPLE system are superior or equal to those obtained in the UAE, in agreement with other authors (Cha et al. 2010 and Plaza et al. 2008). Hence, this extraction system offers the advantage of permitting better extraction rates, in a shorter time and in a single step. Comparing the traditional UAE extraction method with the CPLE, one realizes that it is possible to achieve time savings in carotenoid extraction of the order of 8.7–fold, with an extraction improvement of 2.3-fold for lutein and 15-fold for β-carotene (using the same volume and 3C recirculation). The increase of the antioxidant capacity of extracts was not so significant, as a mere 1.3-fold was noticed via $ABTS^{\bullet+}$, while $DPPH^{\bullet}$ unfolded the same values as with UAE. In fatty acid extraction, the same was observed when comparing UAE with CPLE; the latter could achieve higher yields, particularly useful if the purpose is to extract individual or overall fatty acids. However, the conditions of CPLE are not the same than UAE; using the maximum volume of extraction (150 mL) at Q3 and 60 ºC, the extract obtained is particularly rich in fatty acids.

Table 4.3. Comparison of results obtained during optimization of CPLE system, with ultrasound-assisted extraction. Better results pertaining to extraction of each particular compound are marked in bold.

		Extraction methods tested			
		Ultrasound assisted extraction (12 mL)	Continuous pressurized solvent extraction system		
			Q3, 60 ºC (150 mL)	Volume fractions (1F)	Recirculation Cycles
Fatty acids	**14:0**	9.9 ± 1.4^{a,b}	**27.3 ± 0.0**	6.5 ± 0.2^{a,c}	10.0 ±1.9 (5C)^{b,c}
	16:0	38.5 ± 4.1^{a}	**157.8 ± 2.0**	44.0 ± 0.3^{a,b}	50.8±2.5 (5C)^{b}
	16:1	2.9 ± 0.2^{a}	6.1 ± 0.6^{a}	0.0 ± 0.0a	2.9±1.5 (5C)^{a}
	18:0	0.9 ± 0.2^{a}	4.1 ± 0.8^{a}	1.0 ± 0.1a	1.5 ± 0.6 (5C)^{a}
	18:1 n9	23.5 ± 0.9	**134.9 ± 16.8**	10.3 ± 1.0	33.8 ± 8.7 (5C)
	18:2 n6 t	18.8 ± 2.0^{a}	3.2 ± 0.0^{b}	4.9 ± 0.1b	**23.8 ± 5.2 (5C)**^{a}
	18:2 n6 c	4.8 ± 0.1^{a}	**70.5 ± 1.1**	0.0 ± 0.0^{a}	1.8 ± 1.1 (5C)^{a}
	18:3 n6	6.1 ± 1.1^{a}	6.7 ± 0.6^{a}	4.9 ± 0.5^{a}	3.3 ± 1.3(5C)^{a}
	18:3 n3	2.9 ± 0.1a	**69.1 ± 1.5**	3.1 ± 0.2^{a}	27.9 ± 4.2 (5C)
Carotenoids ($mg.L^{-1}$)	**Lutein**	1.22 ± 0.18	1.72 ± 0.22^{a}	2.07 ± 0.13^{a}	**2.9 ± 0.1 (3C)**
	β-Carotene	0.10 ± 0.01^{a}	0.13 ± 0.03^{a}	0.14 ± 0.01^{a}	**1.5 ± 0.1(3C)**
Antioxidant capacity($mg_{TE}.mg_{e}^{-1}$)	**ABTS**	121.6 ± 6.2^{a}	167.6 ± 21.8^{b}	117.9 ± 3.7^{a}	**168.7 ± 4.3 (3C)**^{b}
	DPPH	395.1 ± 10.9^{a}	186.1±34.1	398.9±6.7^{a}	**423.4±10.6 (3C)**^{a}

a–c Means within the same row, without a common superscript, are significantly different ($p < 0.05$).

4.4. Conclusions

Our low cost, laboratory scale CPSE system proved versatile and effective in bioactive compound extraction. In general, the optimum temperature and pressure were 60 °C and 180 bar for extraction of lipidic compounds. Collecting the extract as sequential volume fractions produced separate extracts rich in carotenoids, lutein, PUFA and antioxidant compounds. An extract recirculation (12.5 mL, 8,3 % of total volume tested) for 3 cycles increased 1.7-fold lutein and 11-fold β-carotene contents, while 5 cycles permitted C 18:2 n6

t be increased 7.4-fold. When compared to ultrasound assisted extraction (UAE), our CPSE proved more efficient.

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CHAPTER 5

Fluorescent light vs. LED for Gloeothece sp. in biomass and high value-metabolite production – a promising approach from blue biotechnology?

Fluorescent light vs. LED for *Gloeothece* sp. in biomass and high value-metabolite production – a promising approach from blue biotechnology?

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THIS MANUSCRIPT IS SUBMITTED FOR PUBLICATION IN ALGAL RESEARCH.

Abstract Light plays a crucial role in photosynthetic microalgae,s and the use of specific wavelengths is a key-point for biomass production and biochemical composition.

The effects of different LED wavelengths were analysed in microalgal cultivation as an alternative of fluorescent lamps (FL).

Blue (B) and red (R) LEDs, and two combinations thereof (BR) were studied in terms of *Gloeothece* sp. biomass (X), carotenoids and fatty acids (FA) production, as well as antioxidant compounds (AC) capacity. Given the LEDs that presented better results, infrared (IR) LEDs were added, and their influence assessed on the various parameters studied.

B induces high biomass productivity (P_X) with the greatest contents in FA composition in comparison to other tested LEDs. Cultures illuminated by R attained the best AC values. BR (40:60) promoted biomass richer in carotenoids, particularly lutein and β-carotene. IR, coupled with BR (40:60) and R, enhanced P_X and FA content, while changing carotenoids and AC profile production over time.

Our results reveal, in general, that LED can be a valid alternative to FL for decreasing costs, and enhancing biomass and high value-product synthesis.

***Key Words**: cyanobacteria, infra-red, lutein, β-carotene, C18:3 n3, antioxidant capacity*

1. Introduction

Despite some species being able to undergo mixotrophic metabolism, microalgae are essentially photosynthetic organisms – so light is the essential form of energy needed for their existence. Light is composed by a large spectrum, but only the visible part apparently contains photosynthetic active radiation (PAR). Said part ranges from the violet (380 nm) to the far red at (750 nm), with a photon of blue light (about 400 nm) being more energetic than of red light (around 700 nm).

To fully understand how light affects microalga growth and biochemical composition, it is essential to understand the metabolic starting point of photoautotrophic microorganism – photosynthesis. To harvest light energy, photosynthetic organisms possess three major classes of pigments: chlorophylls (Chl), carotenoids and phycobilins – organized in light harvesting complexes (LHC). All types of LHC are composed by a core and reaction centre pigment – composed by Chl *a*, a subtype of chlorophyll present in all oxygenic photoautotrophs with absorption peaks; and light-harvesting antennae, composed by pigments such as other subtypes of Chl (*b* or *c*) and carotenoids, composing the so-called accessory (or antennae) pigments that allows the range of light absorption to be extended (Fig. 1) (Richmond 2008). Carotenoids represent a large group of biological chromophores, with an absorption range 400–550 nm. They play several roles in the photosynthetic apparatus, functioning as (i) accessory light-harvesting pigments transferring excitation to Chl *a*, (ii) structural entities within the light-harvesting and reaction centre pigment–protein complexes; and (iii) molecules required for protection against excess irradiance, chlorophyll triplets and reactive oxygen species. In prokaryotic microalga (cyanobacteria), the major antennae are composed by phycobilins (phycoerythrobilin, phycocyanobilin and phycourobilin). Hence, cyanobacteria are able to utilize red, yellow and green light – and, to a lesser extent, blue light (Schulze et al. 2014).

The thylakoid membrane, where photosynthesis occurs, contains five major complexes: light-harvesting antennae, photosystem II (PS II) and photosystem I (PS I) (both containing a reaction centre), cytochrome ($Cytb_6$ *f*) and ATP synthase. The primary function of the antenna systems is light-harvesting and energy transfer to the photosynthetic reaction centres (Richmond 2008). Two major classes of light-harvesting pigment–protein complexes can be identified there: (i) hydrophilic phycobiliproteins, found in cyanobacteria attached to the protoplasmic side of the thylakoid membrane; and (ii) hydrophobic pigment–protein complexes, such as LHC II and LHC I, containing chlorophylls and carotenoids, as depicted in Fig. 1.

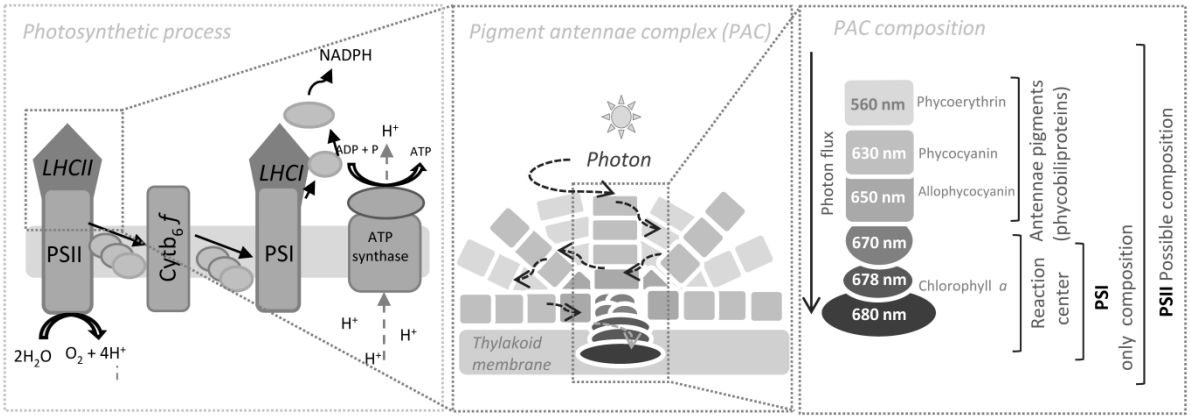

Fig. 5.1. Schematic representation of prokaryotic microalga pigment antenna complex, and its composition in terms of pigment and photosystems I (PSI) and II (PSII).

In cyanobacteria, the light-harvesting antenna serving PS I is exclusively constituted by chlorophyll *a*, while PS II is mainly composed of phycobilisome. PSII has a relatively larger optical absorption cross-section compared to PS I, which is excited by chlorophyll *a*. To balance the electron flow between PS II and PS I, cyanobacteria generally have more PS I reaction centres than PS II – a deed altered both by light intensity and spectral distribution.

Although blue and red lights are most effectively absorbed by photosynthetic pigments, photoregulation of microalgae is not limited to the photosynthetic apparatus. Many photoreceptors, i.e. pigments that absorb light and transduce light signals, are also actively involved in triggering various light responses that are independent of the photosynthetic apparatus – as is the case of those first detected in prokaryotic genes encoding phytochrome-like proteins in cyanobacteria, as depicted in Fig. 2 (Wilde et al. 1997, Dring 1988). Among all types of photoreceptors, red/far-red light absorbing phytochrome is unusual, due to its photoreversibility. This blue protein pigment can switch between two interconvertible forms, Pr (inactive form, with an absorption peak in the red region of the 650–680 nm spectrum) and Pfr (active form) (Dring 1988). When Pr is exposed to red light, it is converted to (the physiologically active) blue-green form, Pfr, thus triggering several biochemical responses.

Also, blue light has proven to influence gene expression and several metabolic pathways in algae and plants, via photoreceptors such as cryptochromes, phototropins, aureochromes, and neochromes (Beel et al. 2012); it is also responsible for endogenous breakdown of carbohydrate reserves (Kamiya and Saitoh 2002, Schulze et al. 2014). Hence, light quality can determine the other biophysical and physiological properties of microalgae, as briefly summarized in Fig. 2

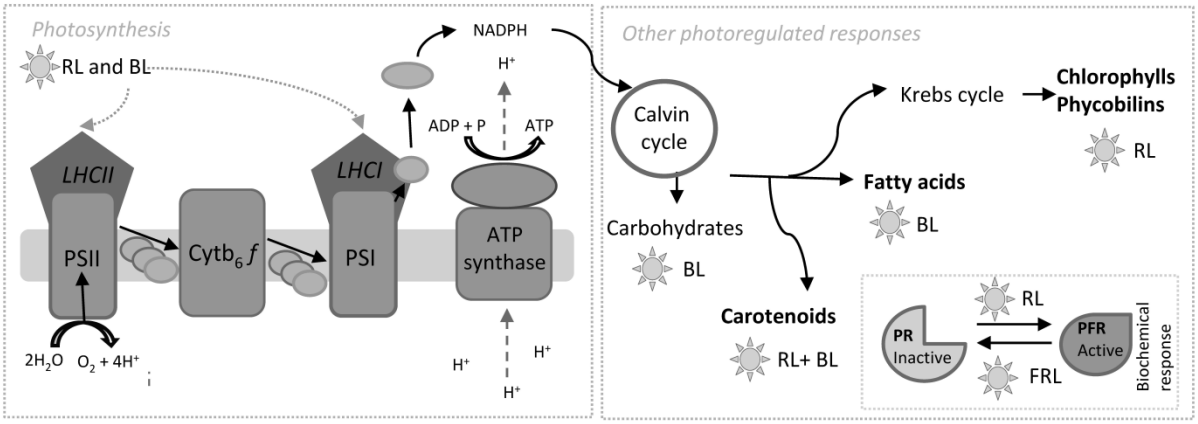

Fig. 5.2. Schematic representation of selected metabolic pathways of prokaryotic microalgae that are affected by light spectrum in BL- blue light, FRL- far-red light and RD- red light.

As found in the specialized literature, blue light promotes chlorophyll synthesis and chloroplast formation in *Chlorella* (Dring 1988), and induces nitrate and nitrite uptake in *Monoraphidium braunii* (Aparicio and Quinones 1991). Red and far-red lights showed to affect growth, cell size, and photosynthesis rate of microalgae. For example, red light emitting diode (LED) revealed to reduce cell volume of *C. vulgaris* without changing the total biomass yield when compared to fluorescent light (Lee and Palsson 1996). When supplemented to a daylight fluorescent lamp, far-red light induced much larger cell volume in *Dunaliella bardawil* cultivation than obtained with single daylight lamps, but cell population and chlorophyll concentration decreased (Sánchez-Saavedra, Jiménez and Figueroa 1996). Moreover, continuous red lighting in *C. pyrenoidosa* culture enhanced growth and ethylene production, whereas long-term far-red lighting inhibited both (Kreslavsky, Kobzar and Muzafarov 1997).

Therefore, light quality appears as a key point for microalgal growth and biochemical composition. If the most proper light source is elected, it will be possible to manipulate the microalgal biomass in terms of optimum biomass productivity, as well as content of high value metabolites for specific uses – particularly for high-end markets (Schulze et al. 2014). Fluorescent lighting is the most common light source employed in microalgae cultivation, but it possesses a width light spectrum – and (as seen before) the range of photosynthetic active radiation is more restricted, thus making them energetically inefficient (besides their energy cost and unwanted heat production). If the light source has a narrow spectral output that overlaps the photosynthetic absorption spectrum, emission of light at unusable frequencies will be eliminated, thus improving overall energy conversion(Schulze et al. 2016). Among the light sources currently available, LEDs are the only ones that meet the foregoing criteria. LEDs are light and small enough to fit into virtually any photobioreactor; other advantages include longer life-expectancy, lower heat generation and higher conversion efficiency. In

addition, LEDs have narrow light emission spectra, between 20 and 30 nm, which can be matched to photosynthetic needs (Chen et al. 2011, Yeh and Chung 2009).

Recent years have witnessed an increased interest in replacing fluorescent lighting by LED for microalga production, but still are important gaps in the knowledge of how microalgae respond to light exist. The combined use of LEDs for microalgal cultivation or general metabolic response patterns was only partly investigated to date, and very few studies have focused on prokaryotic microalgae (Schulze et al. 2014). To help fill in the gaps found in the current state of the art, the present study was aimed at understanding whether use of LED lighting is a feasible alternative to fluorescent lamps (FL). The effects of light quality upon growth rate and biochemical composition of *Gloeothece* sp., particularly blue and red LEDs and FL, were accordingly ascertained in terms of carotenoids and fatty acids. These compounds, particularly the former, have been described as potent antioxidant agents, with proved health and industrial applications – so antioxidant compound production was assessed (Guedes, Amaro and Malcata 2011, Pulz and Gross 2004, Guedes, Amaro and Malcata 2011, Guedes et al. 2011, Mazza et al. 2007). Moreover, the effect of infrared light on microalga growth and composition is essentially unknown, so this study implemented an innovative way of testing.

5.2. Material and methods

5.2.1. Microalga source and growth conditions

Gloeothece sp. (ATCC 27152) obtained from ATCC (American Type Culture Collection) (USA), was maintained at 25ºC in Blue Green (BG11) medium (Stanier et al. 1971). A pre-inoculum was cultivated for 10 days, with an initial optical density of 0.1 at 680 nm, in 800 mL of BG11, set at pH 8 buffered with Tri-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) 25 mMM. A continuous illumination with fluorescent BIOLUX lamps with intensity of 100 $\mu mol_{photon}.m^{-2}.s^{-1}$ was assured, as well as air bubbling at a flow rate of 0.5 $L.min^{-1}$.

Light conditions assays, tested in batch biological triplicates, were performed in 1800 mL of culture, also with an initial optical inoculum density of 0.1 at 680 nm. Medium, bubbling conditions and continuous illumination were the same as with the pre-inoculum , except for the light source.

5.2.2. Light emission conditions

Monochromatic blue (B) (peak at 440 nm with a range of 420–470 nm) and red (R) (peak at 660 nm with a range of 600-700 nm) were tested, as well as two dichromatic LED

percentage compositions of red and blue – RB 60:40, % and RB 50:50, %. The influence of a near-infrared LED (peak at 862 nm, with a range of 800-900 nm) was tested with the most promising LED conditions.

For a better understanding of how LED affects microalgae cultivation, a common microalga light source was used as a comparison – fluorescent BIOLUX lamps.

In all experiments, the same light intensity was used either in monochromatic or dichromatic light source – 100 $\mu mol_{photon}.m^{-2}.s^{-1}$, established by light measurement via light sensor WALZ (US-SQS/L), equipped with a logger WALZ (ULM-500).

Microalgal growth LED assay were performed in climate chambers (Aralab 600 S), equipped with removable LED panels containing blue, red and infrared LEDs. In each assay, a uniform light distribution was provided by spot lights, as well as by establishing the correct distance of cultures to the light source, as depicted in Fig. 3. The study conducted with fluorescent light was performed with BIOLUX lamps in climate chamber Aralab 750 E.

A Spectrometer OCEAN OPTICS (USB2000+) with a flat sensor was used to ascertain each light spectrum.

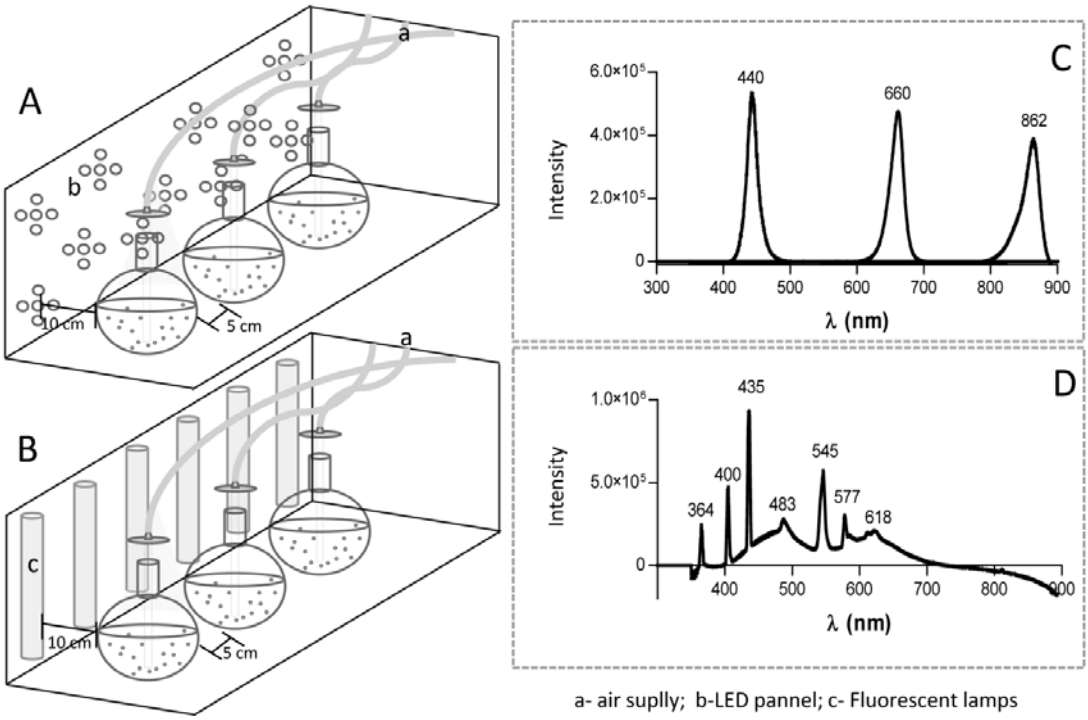

Fig. 5.3. Schematic representation of experimental set-up – one of two lighting parallel panels used for *Gloeothece* sp. cultivation under LED light (A) and fluorescent light (B), and their respective light wavelengths, LEDs (C) and Fluorescent lamps Osram Biolux (D).

5.*2.3. Biomass quantification*

For each biological triplicate, cultivated under the light conditions above, biomass growth was monitored (in duplicate) along time by optical density (OD), established spectrophotometrically at 680 nm (Schimadzu UV-1800), and by dry weight (DW). The later was determined by filtering a certain volume of culture through preconditioned GF/C glass fiber filters (Whatman, UK), and further drying at 100ºC till constant weight. The specific growth rate (μ_{max}) was also determined by a numerical regression to the experimental data, and the biomass doubling time (t_d) was calculated as $t_d = (\ln 2)/\mu_{max}$. Biomass productivity (Px) was calculated using the variation between initial and final values of DW ($g.L^{-1}$), referred to the underlying exponential phase period, according to $Px\ (t) = X_0[(\exp^{(\mu max.t)-1})/t]$, where t denotes actual sampling time and X_0 denotes initial biomass concentration.

5.2.4. Antioxidant capacity assessment

Two millilitres of each batch (in triplicate) was centrifuged, at 4000 rpm for 5 min, and the pellet was resuspended and homogenized in 2 mL of a mixture of ethanol and water (1:1, v/v). Cells were then crushed in an Ultra Turrax T 18 basic homogenizer (Ika) at 14,000 rpm for 30 s, and centrifuged at 4000 rpm for 5 min; and the supernatant (intracellular extract) was collected separately.

The radical-scavenging capacity of the microalgal intracellular extracts was assessed, in triplicate, via the ABTS radical cation ($ABTS^{\bullet+}$) assay, following the method described elsewhere (Guedes et al. 2013). For quantification, a calibration curve using a known antioxidant – Trolox, was established, so antioxidant capacity was expressed as trolox equivalents (TE) per dry weight (DW) of biomass $\mu g_{TE}.mL^{-1}.g^{-1}$.

5.2.5. Compound Identification

5.2.5.1. Carotenoids identification

To identify and quantify carotenoids (including β-carotene and lutein, in particular) produced by *Gloeothece* sp, a high-performance liquid chromatography (HPLC) system was employed as before (Guedes et al. 2011).

Under all light conditions, microalgal cell-free extracts were prepared from each biological triplicates using 20 mL of centrifuged culture sampled over culture time. The pellet was resuspended in the same volume of acetone (99.6% purity), and added with sodium sulfate (Sigma) and β-apo-carotenol (Sigma) as internal standard. Cells were then disrupted by sonication for 15 min; and the extract filtered and evaporated in a rotavapor. The residue was then resuspended in a mixture of acetone and ethyl acetate at 9:1 (v/v) prior to injection.

The carotenoids profile was obtained via a Merck-Hitachi HPLC system, equipped with a equipped Diode Array Detector (DAD) Merck-Hitachi L-7450 to resolve, detect, and identify the various chemical compounds of interest. The absorption spectra were recorded between 270 and 550 nm, and the stationary-phase was a 4 x 250 mm Purospher Star RP-18e (5µm) column (Merck). The mobile-phase was constituted by solvent A—ethyl acetate, and solvent B—acetonitrile/water at 9:1 (v/v), both from VWR, at various volumetric ratios along elution time, under an overall flow rate of 1 mL min^{-1}. The following gradient was used: 0–31 min (0–60% A); 31–46 min (60% A); 46–51 min (60–100% A); 51–55 min (100% A); 55–60 min (100-0% A); and 60–65 min (0% A). The carotenoids elution times of the chromatographic standards were: neoxanthin 7.4 min, violoxanthin 8.4 min, lutein 14.4 min, and b-carotene 34.4 min. Standards were purchased in CarotNature, Lutein (No. 0133, Xanthophyll, (3R,3'R,6'R)-β,ε-Carotene-3,3'-diol with 5% Zeaxanthin and purity of 96%), β-carotene ((No. 0003, β, β -Carotene) with 96% purity) and β-apo-carotenol ((No. 0482, 8'-Apo- β -caroten-8'-al) with 97%, purity). Identification was achieved by comparison of retention time and UV–visible photo-diode array spectra, following the procedure detailed elsewhere (Guedes et al. 2011).

5.2.5.2. Determination of fatty acid profile

By the end of each light condition experiment, biomass triplicates were harvested, by sedimentation and then centrifugation at 4000 rpm for 5 min, prior to lyophilisation.

Fatty acid methyl esters were produced from 100 mg of biomass by direct transesterification—according to the acidic method adopted previously (Guedes et al. 2011), using heptadecanoic (C17:0) acid as internal standard and acetyl chloride as catalyst. Esters were analysed in a GC Varian Chromapack CP-3800 gas chromatograph, using a flame ionization detector, and quantified with the program Varian Star Chromatography WorkStation (Version 5.50). A silica CP-WAX 52 CB (Agilent) column was used, and helium was employed as carrier gas in splitless mode. Injector and detector were maintained at 260 and 280 °C, respectively, and the oven heating program was as described in Table 1.

Table 5.1. Oven heating program for fatty acids identification.

T (ºC)	Rate (ºC.min^{-1})	Holding time (min)	Time (min)
100	-	5	5
180	6	0	18.33
200	2	0	28.33
205	0.5	0	38.33
230	1	0	63.33
233	0.5	0	69.33
240	4	14.3	90

Chromatographic grade standards of fatty acids in methyl ester form CRM47885 (Supelco) were used for tentative identification, based on comparison of retention times: C13:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1 n9-cis + trans, C18:2 n6, C18:2 n6 c, C18:3 n6, C18:3 n3, C20:0, C20:1, C20:5 n3, C21:0, C22:0, C22:2, and C22:1 n9. The mean of the results from the aforementioned chemical assays was used as a datum point.

5.2.6. Statistical analysis

The experimental data were analysed using GraphPad Prism V. 5.0. A first diagnostic unfolded a non-normal distribution of the data, so 1-way ANOVA with Tukey's multi-comparison test was used to assess variances between different light conditions on growth parameters, and two-way ANOVA with the same multi-comparison test in carotenoids, fatty acids content and antioxidant capacity for each light condition. Since each datum point had been replicated, a representative measure of variability was available in all cases to support said statistical analyses.

5.3. Results and discussion

5.3.1. Effects of light source on biomass production

Light is an essential factor for microalgae growth, and light spectral quality and intensity must be considered when choosing the right light source for their cultivation. Spectral quality is defined by the absorption spectrum of chlorophylls and other photosynthetically active pigments, such as phycobilins and carotenoids, and its energy absorption is dependent on their chemical nature (Teo et al. 2014, Carvalho et al. 2011, Lee 1999). Chlorophylls, particularly Chl *a,* that is present in all microalgae, have two major spectrum absorption bands at blue (450–475 nm) and red (630–675 nm). However, it is important to notice that

each microalga species has its particular preference on growth for a particular balance of absorption bands, so there is not a universal formula of light spectra that can be applied to all microalga towards optimum growth or metabolite synthesis (Schulze et al. 2014, Schulze et al. 2016). This discrepancy over the effect of a wavelength on microalgal growth between species has been often described in the literature, thus suggesting that the influence of light wavelength on the production of microalgal biomass is species-dependent (Chen et al., 2010; Das et al., 2011). Nevertheless, studies indicated that blue and red wavelengths are the chief responsible for different metabolic and physiological responses as those described before in *C. vulgaris* (Kim et al. 2014).

For an optimum growth, light intensity should be delivered equally over the culture surface and with adequate amount of PAR to enable photons to reach the cell in the culture (Lee 1999). An excessive intensity may lead to photooxidation and photoinhibition, while low light levels will become growth-limiting (Fu et al. 2013). Due to this, the light intensity at which culture growth becomes saturated is an important factor in determining light utilization efficiency; microalgal light saturation usually begins at an incident light intensity around 200 µmol m^{-2} s^{-1}(Carvalho et al. 2011), so a light intensity was chosen for this study that would avoid this situation, i.e. 100 µmol m^{-2} s^{-1}.

Microalgal biomass production was monitored by culture DW and OD. For a further easier and faster biomass growth evaluation, a correlation was successful applied (R^2 > 0.98) – data not shown. The selection of wavelength for OD measurement was based on the highest absorption culture peak, which may not match the optimal growth microalga wavelength. This is due the fact that cell absorption spectra include contributions of all cellular components able to absorb or scatter light, yet they may not contribute to the light-harvesting processes needed for photosynthesis – thus masking the true light requirements for growth of a specific microalga (Schulze, Barreira et al. 2014).

As expected, the prokaryotic microalga *Gloeothece* sp. exhibited different behaviours under different light conditions in terms of biomass production along time – as depicted on Fig 4A. Following inspection of the growth parameters tested (Fig. 4B), the shorter duplication time (td) – 2.54 d, higher specific growth rate (μ_{max}) – 0.2735 d^{-1}, and higher P(X) – 0.132 $g.L^{-1}d^{-1}$ indicated that blue LED is the more suitable for biomass production, even overrating the ones obtained under fluorescent light. Recall that P(X) was calculated under the exponential phase; otherwise, red LED would have yielded the best biomass productivity along the 27 d of culture. However, profitable biomass production required the fastest and highest biomass production, so P(X) was calculated and plotted as shown in Fig. 4B in order to equalize culture time.

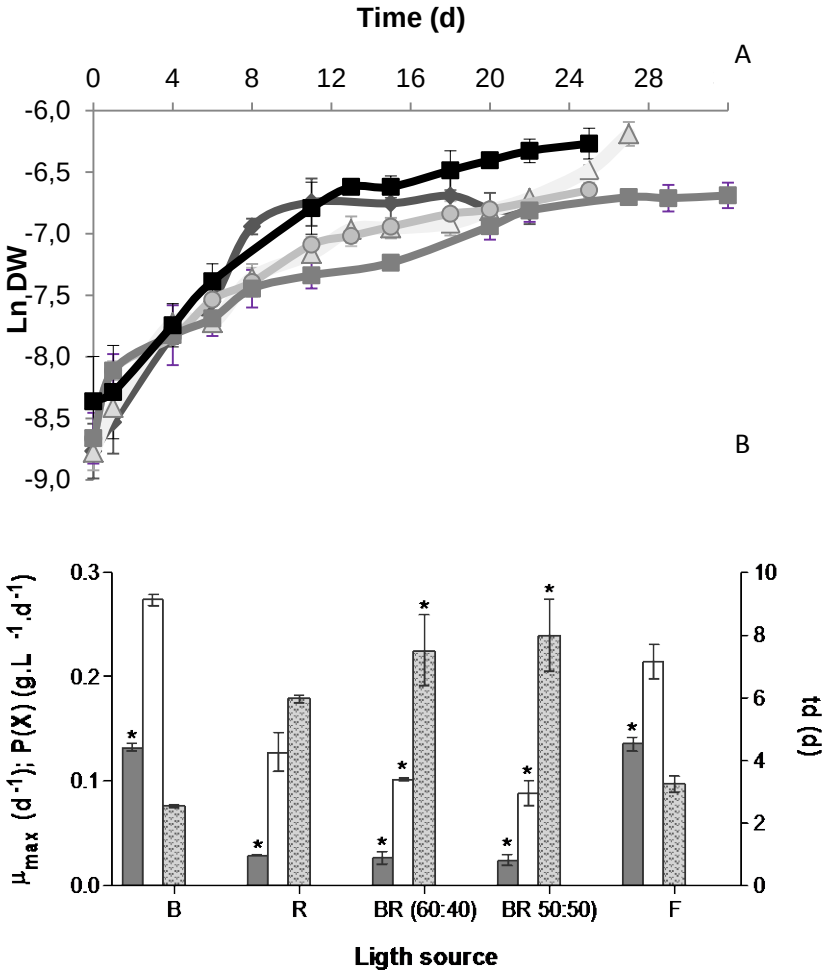

Fig 5.4. Microalgal growth curves (**A**) for each light condition B, R, BR (40:60), BR (50:50), F; and (**A**) biomass production parameters μ_{max} (d^{-1}), td (d) and P(X) biomass productivity ($mg.L^{-1}.d^{-1}$) analysed for each light condition B (blue LED), R (red LED), BR (40:60, %, LED), BR (50:50, %, LED) and F (fluorescent lamps). Bars for the same parameter without a common superscript, are significantly different ($p < 0.05$).

5.3.2. Effects of light source on microalga biochemical composition

5.3.2.1 Effects of light source on carotenoids production

Evidence has shown that some carotenoids can be overproduced by microalga in response to stressful light conditions (Fu et al. 2013); hence, a well-designed LED lighting may lead to an efficient and sustainable production of carotenoids, such as β-carotene and lutein.

Biosynthesis of carotenoids is complex and coordinated with the biogenesis of chlorophylls and proteins of the photosynthetic apparatus (Bohne and Linden 2002). As emphasized before, such carotenoids as β-carotene and lutein play a central role in PS II, harvesting blue light and transferring energy to photosystem reaction centres, while

protecting the photosynthetic apparatus against photo-oxidative damage by deactivating reactive oxygen species (ROS) and thus reducing ROS formation under excess light (Fu et al. 2013, Jahns and Holzwarth 2012).

In this study *Gloeothece* sp. carotenoids production was strongly affected by the light source, as apparent in Fig. 5. Xanthophylls such as neoxanthin, violoxanthin and lutein, and β-carotene were quantified along time under the different light spectrum conditions. All of them exhibit two peaks of production, the first in the early exponential phase (from day 1 to13 d) and another in the stationary phase (from day 13 to 25), as observed in Fig. 5. This may be due the stressful conditions that cells are submitted to in said stages. In the exponential phase, cells are at a very low density, and thus very exposed to light – so they trigger their secondary metabolism, i.e. carotenoids production, in order to stabilize the cell structure and aid in the function of photosynthetic complex. Conversely, the microalgal culture attains a state of nutrients starvation in the stationary phase, and the cell density reaches such values that cells self-shading areas inside the culture vessel increase, so cells stresses for light harvesting. Consequently, cells responded again by increasing carotenoids production in attempt to improve light harvesting (Guedes et al. 2011, Sánchez Mirón et al. 2002). This behaviour is fairly typical of microalgae, and may explains the presence of a peak in carotenoids production in the plain stationary phase – as perceived as seen in Fig. 5 around 20 –25th day of culture.

Since one of main goals of this study was to assess whether the use of LED can replace fluorescent light in microalga production, the maximum content of carotenoids at the exponential phase under fluorescent light is marked to facilitate comparison of results. Observing Fig. 5, it is possible to witness that *Gloeothece* culture – under monochromatic or dichromatic LED, attained a higher production of carotenoids than under fluorescent light. Furthermore, red light (as expected) seems to play a role upon all carotenoids production, either as monochromatic LED – particularly on violoxanthin (9.54 ± 1.24 $mg.L^{-1}.g_{DW}^{-1}$) and lutein (45.66 ± 5.98 $mg.L^{-1}.g_{DW}^{-1}$) at day 6; or in conjugation with 40% blue light in production of neoxanthin (12.5 ± 3.2 $mg.L^{-1}.g_{DW}^{-1}$ at day 6) and lutein (38.31 ± 4.92 $mg.L^{-1}.g_{DW}^{-1}$,day 6). As detected in plants (although not been fully studied), the mechanism of action of the monochromatic red light may affect terpenoid production (the basis molecule of carotenoids) in the chloroplast through phytochrome activation (Darko et al. 2014). In particular, the dichromatic conjugation of RB (60:40) seems to induce synthesis of β-carotene (from 6 to 20th day, between 9.06 ± 1.12 and 14.27 ± 0.75 $mg.L^{-1}.g_{DW}^{-1}$), as observed before in *D*. *salina* (Fu et al. 2013); and additional red or blue LED caused stress, by activating the xanthophyll cycle – although blue light is less stressful than red light. Production of violoxanthin attained values as high as those produced under red LED, but under dichromatic RB (50:50) in plain

stationary phase, at day 20 (10.63 ± 0.39 $mg.L^{-1}.g_{DW}^{-1}$) and day 25 (11.27 ± 0.35 $mg.L^{-1}.g_{DW}^{-1}$)

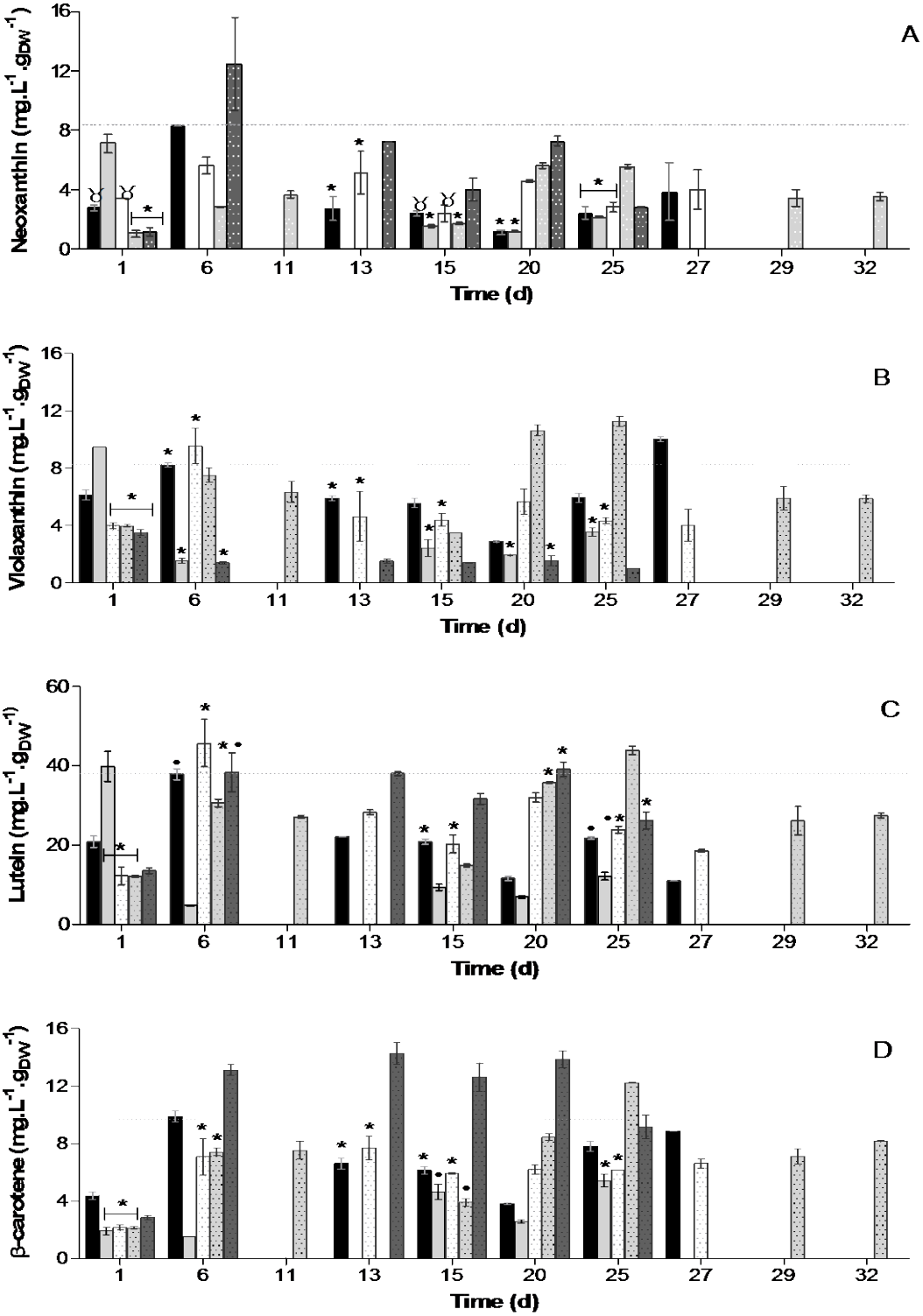

Fig. 5.5. Microalgal carotenoids production ($mg.L^{-1}.g_{DW}^{-1}$) g A) Neoxanthin, B) Violoxanthin, C) Lutein and D) β- carotene, under the different light sources ☐ B (blue LED), ☐ R (red LED), ☐ BR (50:50, %, LED), ☐ BR (40:60, %, LED) and ■ F (fluorescent lamps). Maximum carotenoids production attained in exponential phase under fluorescent lamps is

marked for comparison with use of LEDs. Bars on each day without a common superscript, are significantly different ($p < 0.05$).

As observed in microalgal growth under monochromatic LED, there is not a consensus on which light spectrum is more suitable to improve carotenoids production. Some studies claim that blue light induces production of astaxhantin in *Haematococcus pluvialis* (Katsuda et al. 2004); others show production of a larger pool of xanthophylls and higher Chl *a* content compared to red LEDs, at low light intensities, in the case of *Phaeodactylum tricornutum* (Schellenberger Costa et al. 2013); still others invoked a higher carotenoid/chlorophyll ratio under red LED when compared to blue or green LEDs in *Botryococcus braunii* Bot-144 cultures (Baba et al. 2012). Other authors refer that β-carotene and lutein accumulation is increased when red light is supplemented with blue in *Dunaliella salina* (Fu et al. 2013) – in agreement with our results. A justification of such phenomena lies on an analogy with plants – light signal transduction of blue light may be different from that of red light, and plants usually have different photoreceptors/domains (some blue light- and others red light-regulated). However, these photoreceptors could over-lapp, and thus distinct functions may explain disparate responses (Chory 2010).

5.3.2.2. Effects of light source on PUFA production

Interest in microalgal fatty acids has emerged in many fields in recent years, for their potential for therapeutic uses or nutritional applications – e.g. omega 3 and 6 like C18:3 n3 (α-linolenic acid, ALA), C18:2 n6 (Linoleic acid, LA), C18:3 n6 (ɤ -linolenic acid, GLA), or even omega 9 C 18:1 n9 (Oleic acid, OA) (Guedes et al. 2011).

Light is one of the keys factor that affects fatty acids production by microalgae, so it can be used as a tool to enhance production and increase the potential of microalga exploitation (Teo et al. 2014, Guedes et al. 2010).

In an attempt to ascertain how light spectrum affects fatty acids microalgal content, in all light conditions, the biomass was collected and freeze-died when the culture reached the 4th day of the stationary phase. Fatty acid methyl esters (FAMES) were then generated and quantified by GC-FID, and the main results are depicted in Fig. 6. Under fluorescent light, *Gloeothece* sp. cultures have higher neutral lipid content. However, comparing only the LED light conditions tested under the monochromatic blue LED, this microalga produces more fatty acids relative to other LEDs tested –e.g. 1.7-fold more C16:0 (34.26 ± 3.08 $\mu g_{FA}.mL^{-1}.mg_{DW}^{-1}$) and 1.9-fold more C18:2 n6 *trans* (0.21 ± 0.01 $\mu g_{FA}.mL^{-1}.mg_{DW}^{-1}$) than dichromatic LEDs, and 1.6-fold more C18:3 n3 (24.31 ± 3.58 $\mu g_{FA}.mL^{-1}.mg_{DW}^{-1}$) than all other LEDs tested.

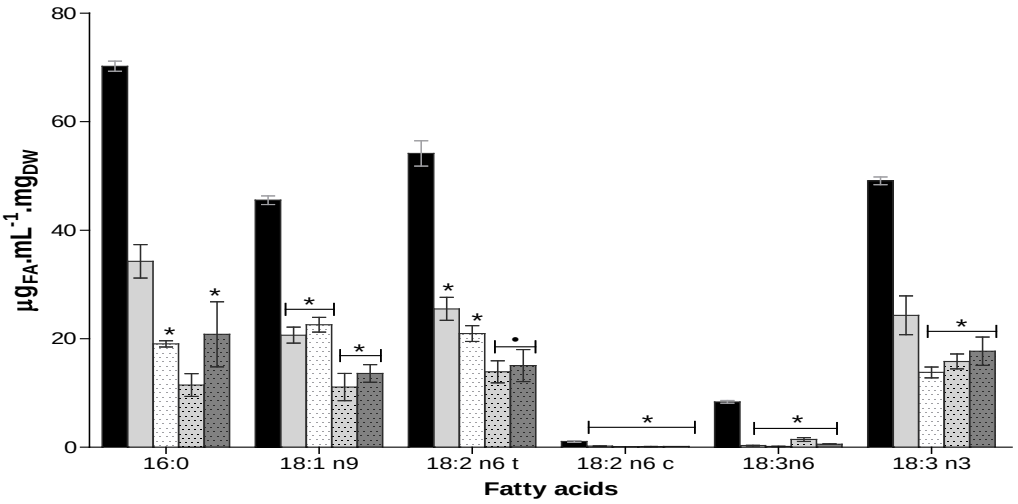

Fig 5.6. Microalgal fatty acids production ($\mu g_{FattyAcid}.mL^{-1}.mg_{DW}^{-1}$) under the different light sources B (blue LED), R (red LED), BR (50:50, %, LED), BR (40:60, %, LED) and F (fluorescent lamps). Bars for each fatty acid, without a common superscript, are significantly different ($p < 0.05$).

These effects of blue LED were observed before in *Tetraselmis* sp. and in *Nannochloropsis* sp. by Teo et al. (2014) (Teo et al. 2014). Yoshioka et al. (2012) (Yoshioka et al. 2012) also found that *Isochrysis galbana* attained maximum lipid content under blue LED, probably because the enzymes affecting the carbon dioxide rates in microalgae are basically under control of blue light. The higher the enzyme activity, the higher the accumulation of triglycerides under blue LED light (Roscher and Zetsche 1986).

5.3.2.3. Effects of light source on antioxidant capacity of intracellular extracts

Hydrogen peroxide (H_2O_2) is the most common oxidative product of photosynthesis, photorespiration, respiration and other metabolic processes in plants and microalgae. Therefore, light may contribute to the increase of microalgae oxidative stress for being a result of the photosynthetic process. Hence, production of antioxidant is triggered to scavenge free radicals, chelate catalytic metals and act as oxygen scavengers. Some microalgae contain several enzymatic and non-enzymatic antioxidant protection systems to constrain the concentration of reactive species of oxygen (ROS), in attempts to protect themselves from damage. Compounds like phenolic acids, tocopherols, terpenoids, alkaloids, phycobilin pigments and carotenoids were accordingly described to overcome said harmful effects and restore intracellular equilibrium (Guedes et al. 2011, Ho et al. 2014, Guedes et al. 2011).

To evaluated the effect of light spectrum on antioxidant compounds (AC) production, for each light condition assay, samples were taken along time as biological triplicates, and

antioxidant capacity was assed also in triplicate by $ABTS^{\bullet+}$assay. Upon inspection of the results in Fig. 7, it is possible to notice that light spectrum has an effect on AC production. Again, for easier comparison of results, the maximum production of AC by *Gloeothece* sp. in the exponential phase under fluorescent light is marked. *Gloeothece* sp. under red light attained again a higher content of AC, particularly in days 8, 18 and 20, with a maximum of 2.95 ± 0.14 $mg_{TE}.mL^{-1}.g_{DW}^{-1}$. Higher results than under fluorescent light were as well found when using dichromatic LED illumination in days 4, 18, 25 and 32 at BR (50:50) – with values between 2.27 ± 0.0784 and 2.73 ± 0.08 $mg_{TE}.mL^{-1}.g_{DW}^{-1}$, and days 8, 18 and 20 days under BR (40:60), 2.34 ± 0.11 and 2.44 ± 0.184 $mg_{TE}.mL^{-1}.g_{DW}^{-1}$, respectively. Recalling Fig. 4, it is possible to conclude that these days correspond to the exponential phase (days 4 and 8) and the stationary phase (days 18, 20, 25 and 32). As seen before in section 3.2, cells are under stress in these two growth phases, either due the excess or limitation of light energy; hence, an internal cell oxidative stress may enhance the antioxidant compound mechanism of production in attempts to restore oxidative equilibrium. Under B LED, *Gloeothece* sp. production of AC exhibited a different behaviour compared to other light conditions; besides its content being lower , it exhibited an almost constant production profile along time, with an average of 1.25 ± 0.12 $mg_{TE}.mL^{-1}.g_{DW}^{-1}$ – with the exception of the first and last day of cultivation, with 2.31 ± 0.11 and 1.72 ± 0.06 $mg_{TE}.mL^{-1}.g_{DW}^{-1}$, respectively. Another point worthy of notice is the very low values of AC content under R and BR (40:60) until the 4^{th} day of culture. The same did not happen in BR (50:50), so a higher ratio between B:R may induce more stress, and more AC with consequently be produced at startup.

Among their several functions in light harvesting, carotenoids contribute to cell structure stabilization by neutralizing reactive oxygen species and dissipating excess energy. As observed before, lutein is the major carotenoid produced by *Gloeothece* sp.; in view of early evidence with *Scenedesmus* sp. reported by Guedes et al. (2011) (Guedes et al. 2011) and in attempts to determine whether these two events are related, the profile of production of lutein and AC were compared – as depicted in Fig. 8. For most light condition (B, BR (50:50) and FL), their production profile are similar along time, so carotenoids, or lutein more specifically, may have a strong contribution to antioxidant capacity as described before in *Scenedesmus obliquus* (Guedes et al. 2011). However, under R LED (Fig. 8 C) and slightly in BR (40:60), a poorer correlation was found; despite the similarity between profiles, they present different times for peak production, thus leading to the conclusion that other compounds besides carotenoids, bearing antioxidant capacity, are synthesized by R light; this is the case of phycocyanin, as observed long ago in *Synechococcus* sp. by Tanako et al. (Takano et al. 1995), or phenolics compounds observed in lettuce leaves (Li and Kubota 2009).

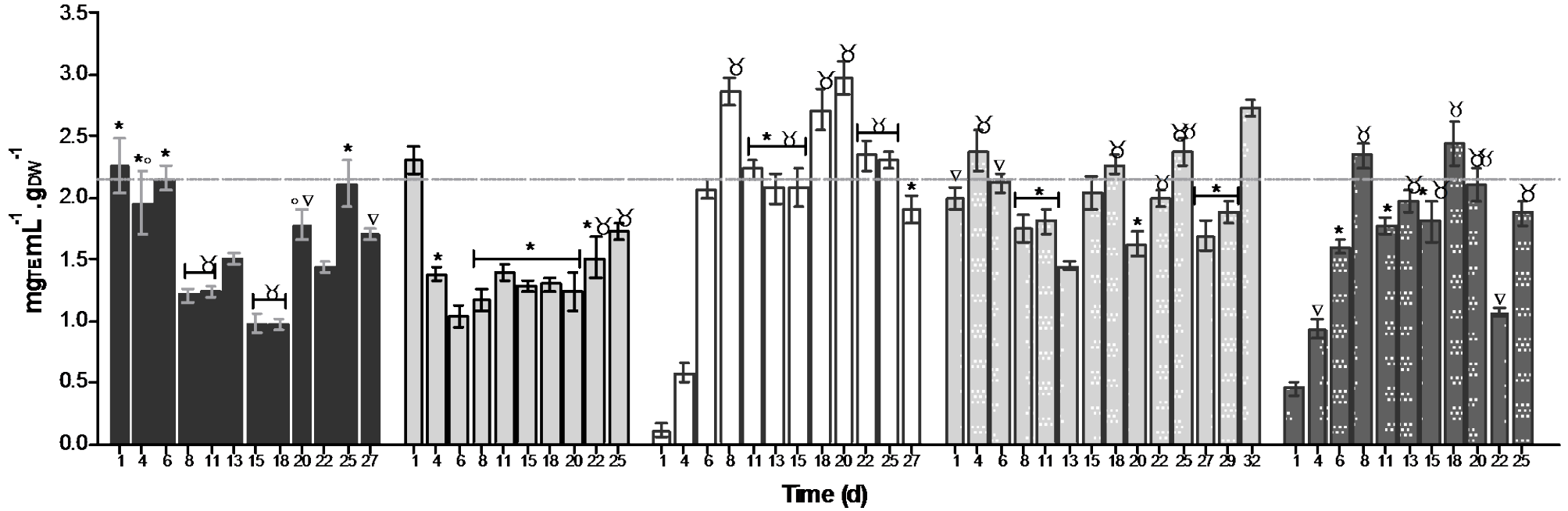

Fig 5.7. Microalgal antioxidant capacity ($mg_{TE}.mL^{-1}.mg_{DW}^{-1}$) over time and under different light sources B (blue LED), R (red LED), BR (50:50, %, LED), BR (40:60, %, LED) and F (fluorescent lamps). Bars for in each light condition without a common superscript, are significantly different ($p < 0.05$).

Antioxidant compounds have received in recent decades a growing interest in the market due to their several roles: e.g. in human health as therapeutic aids (i.e. prevention or control of several diseases), as an ingredient in functional food, or even as food preservative (Guedes, Amaro et al. 2011).

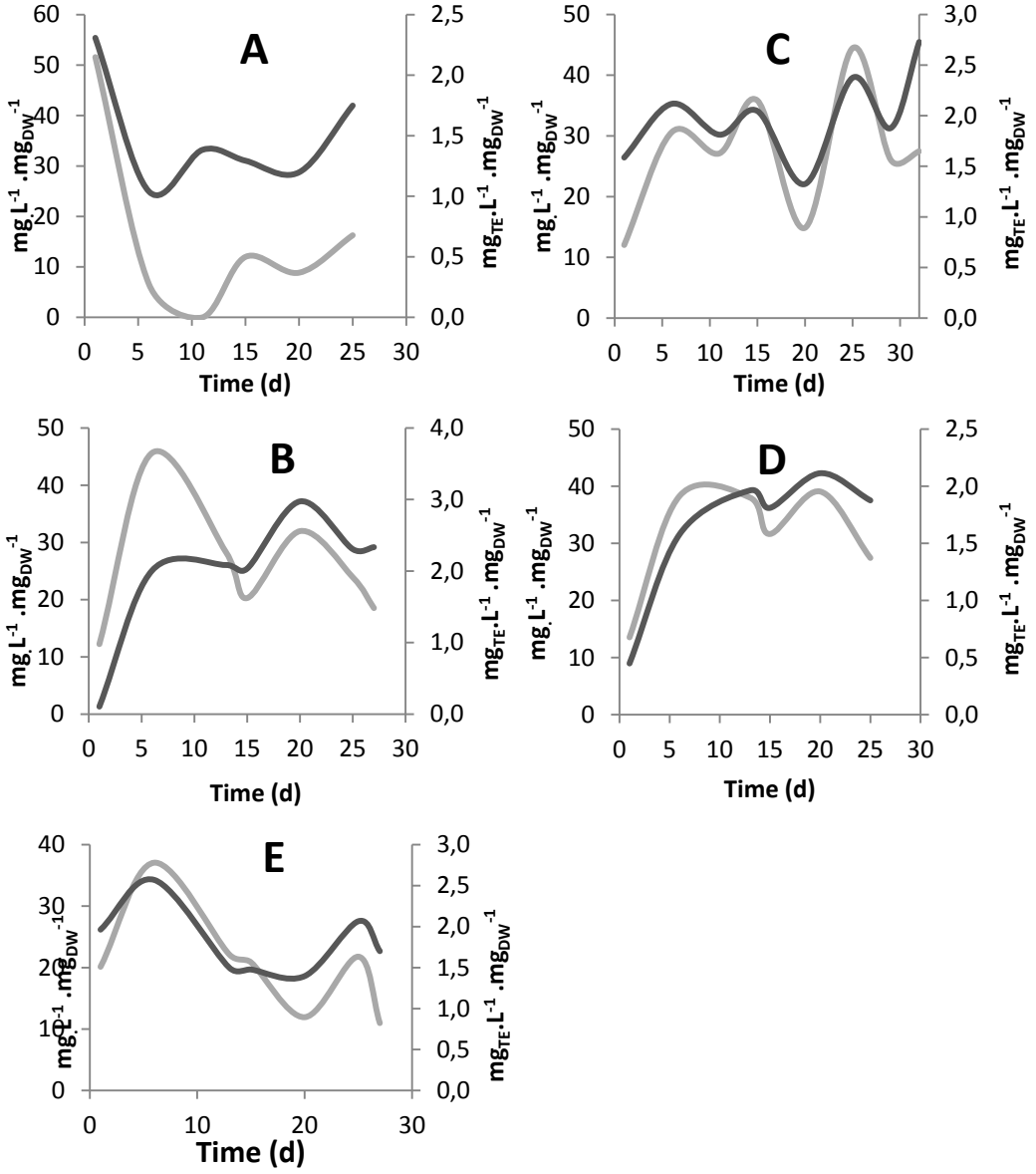

Fig 5.8. Microalgal profile of production of lutein ( ) and antioxidant ( ) capacity over time under different light conditions A) blue LED, B) red LED, C) BR 50:50, %, LED, D) BR 40:60, %, LED and E) fluorescent lamps.

5.3.3. Influence of infra-red LED on microalga growth and biochemical composition

As seen before, only the visible range of light spectrum is photosynthetically active. Up to now, the farthest wavelengths studied on photosynthetic organisms were in the range of far-red (630-750 nm); beside its low energy, it seems to exert some effects on *Dunaniella*

bardawil (Sánchez-Saavedra et al. 1996). Far red photons appeared to induce high growth rates and smaller cells, by accelerating the cell cycle in many microalgae of diverse evolutionary lines. They can suppress volumetric biomass production when supplementing a broadband light source, because they regulate light-harvesting mechanisms in microalgae (Schulze et al. 2014, Sánchez-Saavedra et al. 1996).

However, effects of infrared (IR) radiation (750-1000 nm) on photosynthetic performance have remained quite unexplored. A few studies reported on the ability of a photosynthetic bacterium, *Rhodopseudomonas capsulate,* to harvest monochromatic light at 860 nm; photons are apparently absorbed by bacteriochlorophyll, a pigment with a higher affinity for light than carotenoids (Richmond 2008). More recently, it was found that the cyanobacterium *Acaryochloris marina* is the only known prototroph harbouring chlorophyll (Chl) – which permits a good adaptation to growth under both visible and near infrared irradiance (Behrendt et al. 2012).

In this study, *Gloeothece* sp. growth under LEDs proved that this light source can be a good alternative to fluorescent lighting; it indeed promotes growth and increases carotenoids and antioxidant production, particularly under monochromatic R or dichromatic BR (40:60) LEDs. Hence, the effect of an extra near infra-red radiation (800-900 with a peak at 862 nm) was tested, when added to the aforementioned LEDs, upon *Gloeothece* sp. growth, and carotenoids, AC and fatty acids production.

5.3.3.2. Influence of infra-red LED on *Gloeothece* sp. growth

Infrared light (IR) caused different effects when coupled with different LEDS – as per observation of Fig 9. When combined with R, the P(X) decreased 4-fold and td increased 2.41 fold. When added to BR (40:60), P(X) was enhanced 1.96-fold and td decreased 2-fold with BR (40:60). However, infrared light produced an increase in μ_{max} in conjugation with R an BR (40:60), i.e. 1.3-fold and 1.9-fold, respectively, as already observed with cyanobacterium *Acaryochloris marina* (Behrendt et al. 2012). Therefore, infrared light may be used in conjugation with BR (40:60) to enhance biomass production.

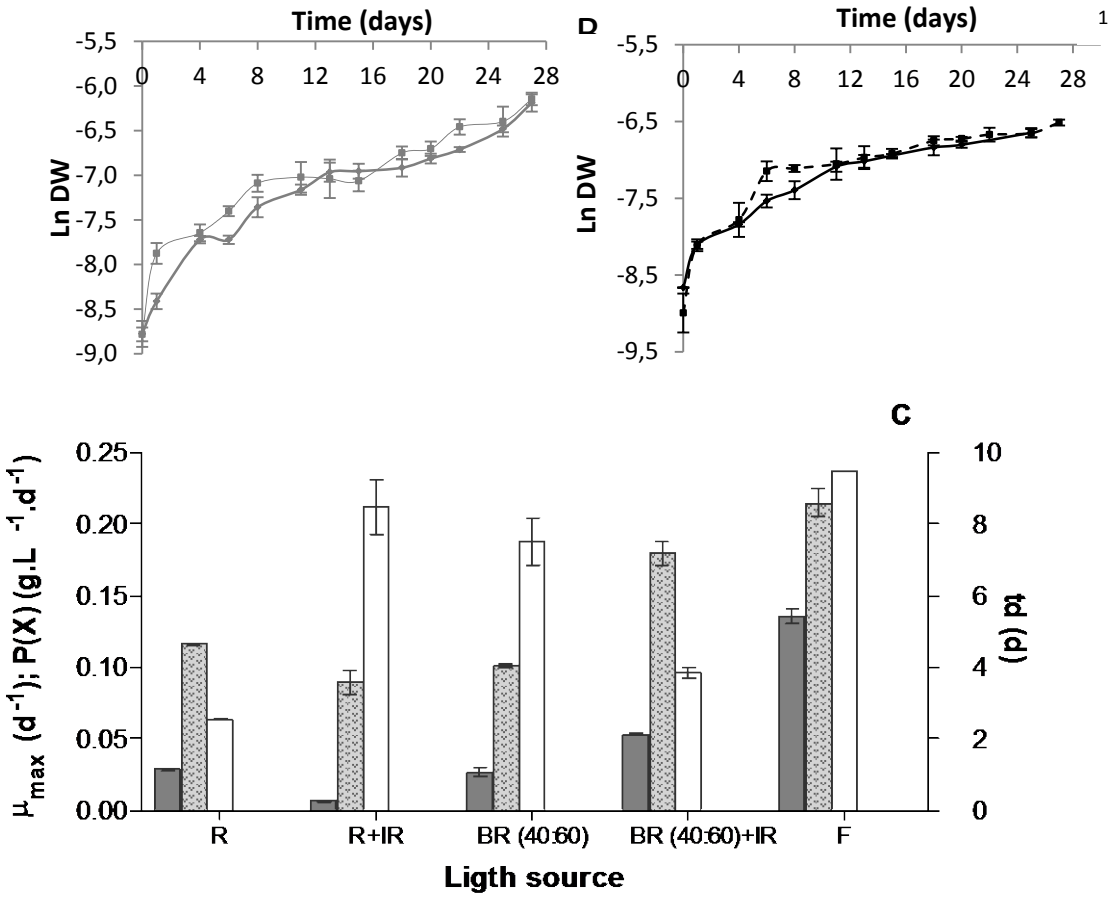

Fig 5.9. Microalgal growth curves for each light condition (**A**) R, R+IR, (**B**) BR (40:60), BR (40:60)+IR and microalgal biomass production parameters μ_{max} (d^{-1}), td (d) and P(X) biomass productivity ($mg.L^{-1}.d^{-1}$) analysed for each light condition B (blue LED), R (red LED), BR (40:60, %, LED), BR (50:50, %, LED) and F (fluorescent lamps). Bars for the same parameter and for the same light condition with and without IR without a common superscript, are significantly different ($p < 0.05$).

5.3.3.3. Influence of infra-red LED on *Gloeothece* sp. biochemical composition

Recent results are scarce about the influence of infrared light on carotenoids production; and the farthest wavelength tested was far-red radiation, which proved to significantly increase carotenoids content in *Dunaniella bardawil* (Sánchez-Saavedra et al. 1996). The effect of infrared light on each carotenoids production along time was tested in conjugation with R and BR 40:60, as depicted in Fig 10. Unlike *D. bardawil* with far-red light, a wavelength of 860 nm induced a decrease of *Gloeothece* sp. carotenoids production when conjugated with both LEDs. The profile of production of all carotenoids was different when IR radiation was added; both R+IR and BR+IR only had a peak of production in the plain exponential phase, at day 25 and day 15, respectively. A single exception occurred under R+IR in β-carotene production, with two equal peaks of production at day 6 and 20.

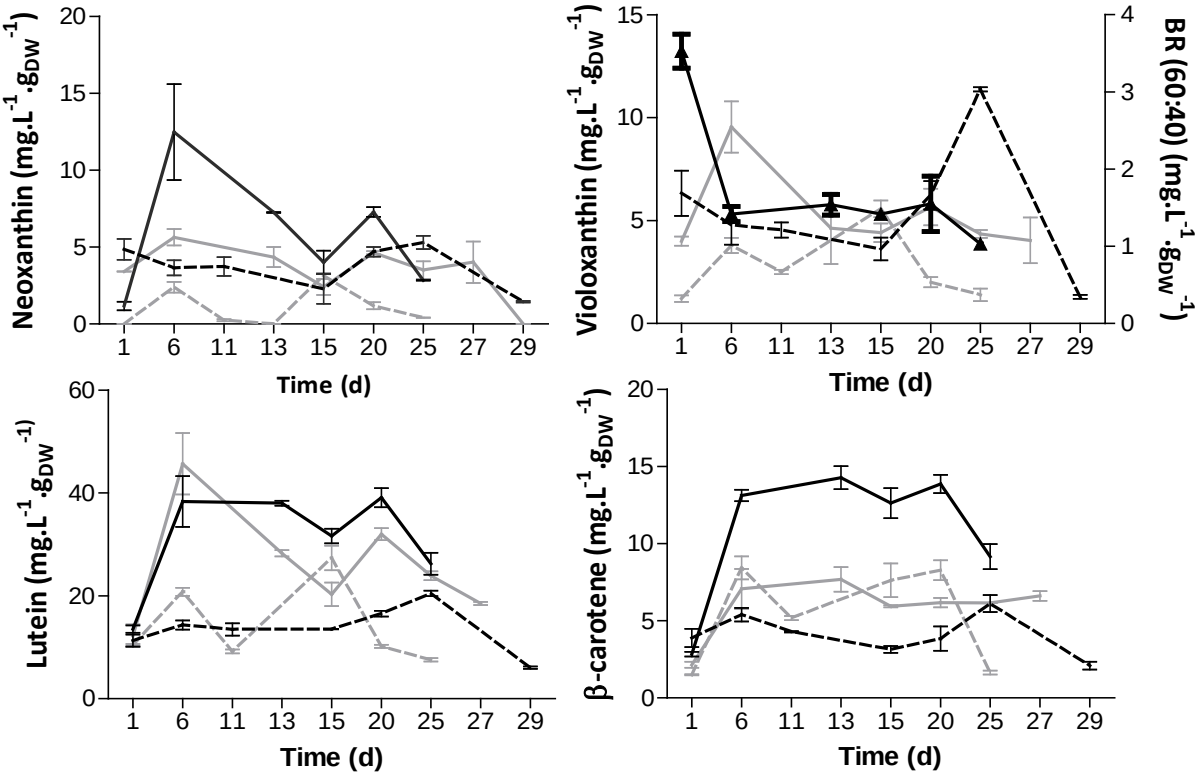

Fig 5.10. Effect of infra-red LED on microalgal carotenoids production along time, when conjugated with the R (red LED); R+IR (red LED) and BR (40:60, %, LED), BR+IR (40:60, %, LED).

However, it was observed that IR affects differently the production of fatty acids, as depicted in Fig. 11. When this radiation is added to R or BR in *Gloeothece* sp. cultures, the lipid production is increased, but this phenomenon is more pronounced in conjugation with BR – where it increases (on average) 1.7-fold each fatty acids production. On the other hand, the effect of IR when added to R LED is only statistically significant ($P<0.05$) in terms of increase on C 16:0, C18:3 n3 and C18:3 n6. Note that IR radiation had a significant role in the stimulation of production particularly of C18:3 n6.

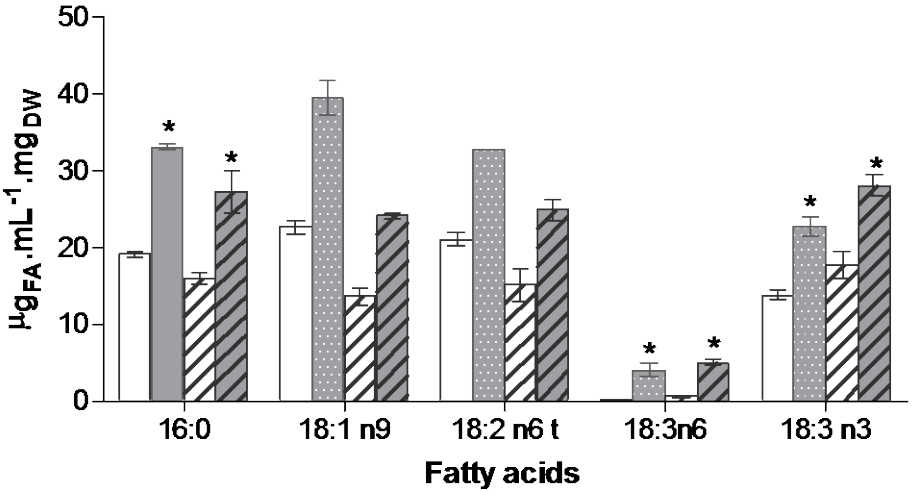

Fig 5.11. Microalgal fatty acids production ($mg_{FattyAcid}.mL^{-1}.mg_{DW}^{-1}$) under light sources R (red LED), BR (50:50, %, LED), and combined with infrared LED R+IR and BR+IR (40:60, %, LED). Bars for same fatty acid without a common superscript, are significantly different ($p < 0.05$).

In terms of AC production, R+IR and BR (60:40)+IR presented a different a profile of production along time when compared to their counterparts without IR. In conjugation with R LED, IR seems to increase their production in the stationary phase – where a peak of production occurred at day 27. Together with BR radiation, IR induced a constant production of only at day 4 and 15, corresponding to the plain exponential and stationary phases of *Gloeothece* sp. growth, respectively. Unlike previous results, encompassing single monochromatic or dichromatic B and/or R illumination, it was not possible to correlate carotenoids to AC production when IR radiation is added, although a peak of production of carotenoids and AC arose by 25th day of cultivation under BR + IR. These findings indicate that IR radiation may induce production of other AC than carotenoids.

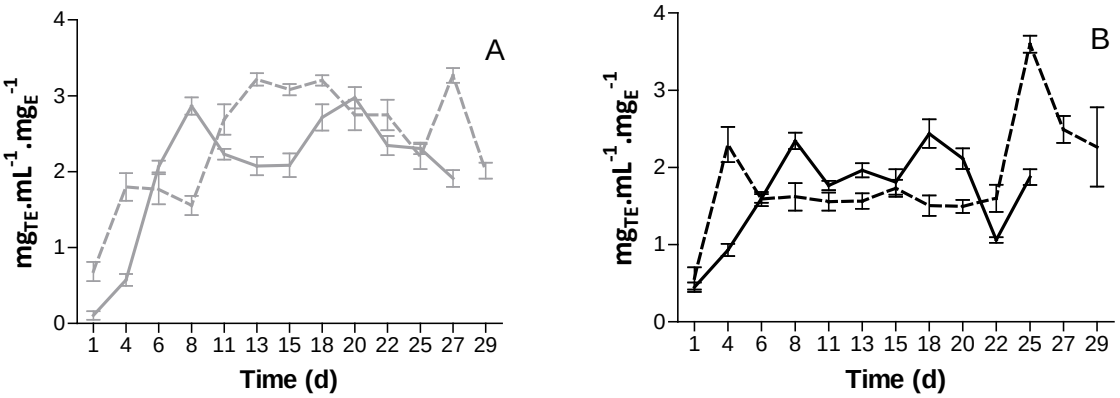

Fig 5.12. Effect of infrared LED on microalgal antioxidant capacity ($mg_{FattyAcid}.mL^{-1}.mg_{DW}^{-1}$) over time when combined with R LED (A) and BR (40:60) LED (B). R (——); R+IR (– –), BR (40:60) (▬), BR+IR (40:60) (▬ ▬).

5.4. Conclusion

LEDs appear promising as light source alternative toward biomass and metabolites production by *Gloeothece*. For biomass production, the blue LED is the most appropriate; the microalga grows faster than under FL, and accumulates a higher content in fatty acids. When using BR (40:60) LEDs, it is possible to obtain biomass rich in carotenoids, particularly lutein and β-carotene, besides AC; however, only a slightly higher content in antioxidants was obtained with R, although its P(X) was lower. When added to BR (40:60, %), IR LEDs enhanced biomass production as well as fatty acids content. They also changed carotenoids profile of production, as well as AC.

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CHAPTER 6

General Discussion

Concluding remarks

Future perspectives

6.1. General discussion

The biotechnology of microalgae has gained considerable importance in recent decades, with applications ranging from simple biomass production for food and feed to valuable products for pharmaceutical/nutraceutical uses (Pulz and Gross 2004); extracts from microalgal biomass have meanwhile gained a firm position on the market (Cohen 1999).

Microalgal PUFA and carotenoids have indeed a very promising biotechnological market for both food/feed, or for pharmacological formulation or health-promoting (Pulz and Gross 2004, Ryckebosch, Bruneel et al. 2014). Martek (USA) and Nutrinova (German) were the first companies to announce production of DHA products from microalgal biotechnology for human consumption and other applications (Pulz and Gross 2004). Furthermore, purified PUFA, as EPA and DHA obtained from the dinoflagellate *Crypthecodinium*,have been added to infant milk formulas in Europe, and as feed for hens (like heterotrophically grown *Schizochytrium* resp. *Cryptecodinium*) in order to produce ”OMEGA” eggs (Pulz and Gross 2004). Microalgae were also considered a good supplier of γ-linolenic acid, which conveyed an innovative approach to the health food market in 90’s (Radmer 1996, Apt and Behrens 1999). Microalgal lipid-based cosmetics, like creams or lotions, formulated with ethanolic microalgal supercritical CO_2-extracts, began to gain commercial importance due their provision of both nourishing and protecting effects to the skin (Muller-Feuga, Moal et al. 2003). Mostly due their antioxidant capacity, microalgal carotenoids started to be incorporated in cosmetics for preservation and protection purposes particularly as sun-screens. Furthermore, they are considered functional food/nutraceuticals; the radical-scavenging capacity of microalgal products is still gaining interest, especially in the beverage segment and in pharmaceutical applications for the therapy of such oxidation-associated diseases as inflammations (Pulz and Gross 2004, Chacón-Lee and González-Mariño 2010).

For most said applications, the market is still expanding, so the biotechnological use of microalgae will likely extend into new areas. Major contributions to bioactive compound libraries have accordingly been made, as well as assessments on their potential for cultivation at industrial scales. However, the development of innovative and efficient bioprocesses remains an obstacle to economical commercialization at large scale. There are still some constrains in microalgal biotechnology that may delay its full exploitation, thus hamering the full potential of microalga to be taken advantage

of – particularly with regard to potential pharmaceutical/nutraceutical applications. Two such bottlenecks are metabolite extraction and subsequent concentration/purification.

Extraction costs of microalgal intracellular metabolites are high; usually the downstream separation stages may account for 50%–80% of the total production costs, depending on the biochemical characteristics of the compound and the purity ratio intended. (Li, Ghasemi Naghdi et al. 2014, Cuellar-Bermudez, Aguilar-Hernandez et al. 2015). Despite the worldwide increasing interest in microalgae compounds, there is no optimum standardized method for their extraction. Remember that microalgae are characterized by a huge biodiversity – and this includes thick-walled green or red algae, silicified diatoms, cyanobacteria with multi-layered walls, red algae with wall-bound exopolysaccharides and armored dinoflagellates, which need to be broken to perform extraction (Porra 1991). Another important pre-requisite for microalgal metabolite extraction is the easy solubilisation of molecules of a wide polarity range. At laboratory scale, it is tempting to use strong solvents to extract targeted molecules. However, some of them, e.g. acetone, chloroform, dimethyl acetamide, dimethyl formamide, dimethyl sulfoxide and methanol, are unsuitable for industrial scale due to safety considerations (low lethal dose, carcinogenic, harmful, irritant or toxic features) (Jeffrey, Mantoura et al. 1997).

Therefore, there is an urgent need to combine appropriate, quick, selective, cost-effective, and environment-friendly extraction procedures to obtain bioactive compounds abiding to legal requirements, including use of food-grade solvents, and processes that allow their incorporation in food and health industries.

One method that can aid in purification of microalgal compounds is increasing their content in the microalgal cell itself by redirecting the cell metabolism towards synthesis.

As presented in the state of the art, environmental factors are a good tool to redirect microalgal metabolism towards the intended compounds, or enhance the biomass production by stimulating the growth rate of microalgae (Guedes, Meireles et al. 2010). Between the many parameters available are temperature, pH and light. Light in particular raises several issues, associated with the most common light source – fluorescent lamps. Besides high costs of maintenance, they are undesirable sources of heat, thus inducing extra costs in medium cooling (Schulze, Barreira et al. 2014).

Therefore, the work presented in this thesis consisted of an attempt to overcome the aforementioned two limiting parameters in microalgal biotechnological production of bioactive compounds – extraction and intracellular concentration. In view of the pharmacological and/or health benefits associated with antioxidant and antitumor bioactivities, this thesis was focused on microalgal metabolites possessing these

characteristics – i.e. carotenoids and PUFA. Our efforts were based on the following approaches:

I) Ascertain the solvent influence on lipidic components (carotenoids and PUFA), in terms of recovery and bioactivities therein;
II) Optimize the extraction conditions of said lipidic components, in terms of temperature and pressure, using the solvent(s) selected in I); and
III) Attempt to optimize purification of said lipidic components, by increasing their cell content via light quality as tool to redirect microalgal metabolism.

The first goal (I) was attained as described in Chapter 2. The effect of selected food GRAS solvents (acetone, ethanol, hexane:isopropanol (3:2) and ethyl lactate) on extractability of lipidic components was ascertained using two species of microalga biomass entailing different cell complexities, an eukaryote – *Scenedesmus obliquus* (M2-1), and a prokaryote– *Gloeothece* sp. Concomitantly, the extracts obtained were tested for antioxidant scavenging capacity towards 4 different radicals, two that reveal total antioxidant capacity ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) and two other related to two radicals usually produced by human natural metabolism ($O_2^{\bullet-}$ and $^{\bullet}NO$); this allowed a more comprehensive characterization of their bioactive capacity.

As expected, the chemical nature of solvents used in extraction of lipidic components appears critical upon antioxidant performance, probably due to the underlying balance between carotenoids and PUFAs.

In both microlagal species, acetone achieved in general the best performance in extraction of carotenoids, particularly lutein – in *Gloeothece* sp. as 65.3% of quantified carotenoids, and in *Sc*. *obliquus* as 47% of quantified carotenoids. *Gloeothece* sp. acetone extracts attained also good results in $ABTS^{\bullet+}$ and $^{\bullet}NO$ assays, and Sc. *obliquus* exhibited the best antioxidant capacity in $ABTS^{\bullet+}$. However, this solvent was not the best to extract PUFA, particularly in *Gloeothece* sp. In this species, ethanol extracted PUFA 5.7-fold higher than acetone, particularly with regard to ALA.

Selective extraction of specific carotenoids, such as xanthophyls (lutein and neoxanthin) by ethanol, lutein by ethyl lactate in *Sc*. *obliquus*, and PUFAs by ethyl lactate in *Gloeothece* sp., was observed in this study. The main goal was then to select a solvent able to exhibit a good performance in extraction of both carotenoids and PUFA, while exhibiting good antioxidant capacity; coupled to the fact of being easily handled in lab and susceptible to scaleup without restrictions arising from to physicochemical characteristics. Ethanol was accordingly selected for posterior studies.

Gloeothece sp. extracts showed to be active against all radicals tested, whereas *Sc. obliquus* (M2-1) ones did not seem to affect particularly $^{\bullet}NO$, with one exception (HI 3:2). *Gloeothece* sp. is a prokaryotic microalga poorly studied so far, so exploration of a new source of microalgal bioactive capacities may appear as a promising challenge toward new nutraceutical formulations. All these arguments together supported selection of *Gloeothece* sp. as model for studies thereafter.

As discussed in the state of the art, there are evidences collected from a large number of studies that confirm the positive effects of antioxidants in prevention and control of growth of certain tumours, either by acting as a chemopreventive agent when incorporated in the diet or as by inducing cell tumour apoptosis and thus inhibiting cancer cell growth/proliferation. Some of these compounds were identified as carotenoids or PUFA.

Therefore, extracts were tested for their antitumor activities (as shown in Chapter 3), departing from the positive results in antioxidant activity achieved in Chapter 2. Two gastric cancer cell lines, AGS and MNK45, served as a model to evaluate extract bioactivity.

Preliminary result revealed that all such extracts were able to interfere with cancer cell viability, although to different extents. In order to find how extracts affect the cells, assays on cancer cell death and proliferation were performed. Although the study still is on processing, preliminary results reveal that EL and HI extracts appear to be the most promising ones due their cell death and anti-proliferative effects upon the two tested gastric cancer cell lines.

Once the extractability of microalgal lipidic components via ethanol, and the bioactivities in the extracts obtained were confirmed, one proceeded to II) optimization of the extraction of said compounds via manipulation of temperature (T) and pressure (P), as described in Chapter 4. Obtained ata indicated that these two parameters were effective in acceleration and improvement of extraction in pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) systems. However, the excessively high temperatures utilized may constitute a drawback for the thermolabile compounds, further to the excessive cost incurred in equipment purchase, maintenance and operation.

Therefore, a laboratory-made continuous pressurized solvent extraction (CPSE) system was built – which, beyond its lower cost, proved versatile and effective in bioactive compound extraction yield (and associated antioxidant properties). After a step-by-step process of optimization of all variables involved (i.e. biomass amount, flow

rate/pressure, temperature, and volume of solvent), 60 °C and 180 bar were found as optimum temperature and pressure, respectively, for extraction of lipidic compounds. Concomitantly, the total antioxidant capacity was monitored by the two methods used before, i.e. $DPPH^{\bullet}$ and $ABTS^{\bullet+}$.

The aforementioned system was developed so as to permit solvent recirculation for many cycles, as desired in attempts to maximize the solvent capacity and reduce the volume of solvent employed. To attain the correct volume of recirculation, the total volume was collected in several fractions. Their biochemical analysis revealed that each fraction was particularly rich in a specific group of compounds; for instance, while the fist extract fraction (1F) attained a content rich carotenoids (lutein and β-carotene) and PUFA, the second fraction (2F) was chiefly composed of lutein and PUFA, and the third fraction contained lutein, β-carotene and high contents of C 16:0, C 18:1 n9, C18:2 n6t, C 18:3 n3 and C20.5 n3. These findings are particularly interesting, as a single extraction process will permit pre-separation of compounds – thus alleviating the complementary need of purification.

Since the main goal here was to optimize extraction, a certain number of cycles was found to be effective without inducing degradation of compounds and keeping their antioxidant bioactivity. An extract recirculation for 3 cycles increased 1.7-fold lutein and 11-fold β-carotene content, while 5 cycles of recirculation permitted C 18:2 n6 t be increased 7.4-fold – when compared to a single cycle. Furthermore, this CPSE system proved more efficient than a conventional ultrasound assisted extraction (UAE) apparatus; CPSE with 3C extracted 4-fold more lutein and 5C extracted 14-fold more β-carotene.

At this point, it was possible to achieve extracts rich in carotenoids and characterized by high antioxidant capacity, using 12.5 mL of ethanol, 50 mg of *Gloeothece* sp. biomass, and 3C of recirculation in the CPSE system, as well as an extract rich in PUFA with 5C or recirculation; this permitted goal II) be attained.

Toward the III) goal, i.e. increasing the lipidic components of the cell in the first place, light was used as tool to redirect microalgal metabolism.

As discussed along Chapter V, light quality (spectrum) interferes with many metabolic mechanisms (including obviously photosynthesis), and may stimulate production of carotenoids and PUFA under certain narrow wavelengths of the visible spectrum. Note that the utilization of a light source that emits wavelengths beyond the range of photosynthetic active radiation harvested by microalgae means a waste of energy, and undesirable heat production when powering the most common forms of microalga culture lightning – fluorescents lamps (FL). Since light emitting diode (LED)

technology is of common use nowadays, their application in microalgal production is in order; it is small enough to fit into virtually any photobioreactor, holds a longer life-expectancy, reduces heat generation and enhances conversion efficiency. It has narrow light emission spectra, between 20 and 30 nm, which can be matched with photosynthetic and other metabolic pathway needs, as discussed in Chapter 5.

The effects of specific wavelengths were accordingy scrutinized: red, blue and two different mixtures thereof were tested upon *Gloeothece* sp. growth, as well as production of carotenoids and PUFA, and associated antioxidant capacity (AC).

Albeit several studies made available in recent years, we found (see Chapter 5), that effects of light are species-dependent; a thorough study of such effects upon synthesis of carotenoids and PUFA by ous elected species is thus essential for eventua further exploitation at large scale. For *Gloeothece* sp. biomass production, blue LED is the most appropriate – and growth is even faster than under FL. Blue light, in comparison to other LEDs tested, also provides higher cellular content of fatty acids. If the goal is to obtain *Gloeothece* sp. biomass rich in carotenoids, Blue:Red (BR) (40:60) LEDs are the most indicated; they particularly enhance the content of lutein and β-carotene, and concomitantly the AC. Under red light, biomass attained slightly higher contents in antioxidants – although biomass productivity was lower.

An attempt to better understand the role of infrared (IR) light was also pursued; this LED was tested only in addition to BR (40:60) and R, once they proved to be the most promising regarding AC. Together with BR (40:60), it enhanced biomass production, as well as fatty acids content – while it induced changes in AC profile of production along time, yet reaching similar maximum concentrations.

Besides being a promising alternative to FL, particularly B, R and BR (40:60, %), LEDs have proven a useful tool to improve (desired) metabolite concentration in *Gloeothece* sp. cells.

6.2. Concluding remarks

The work developed in this thesis was part of an effort to help overcome existing limitations upon intracellular concentration and extraction yield of microalgal bioactive metabolites (with potential pharmaceutical/nutraceutical applications).

The main conclusions drwan may be summarized as follows:

- The several food GRAS solvents tested for extraction:

    I) were able to extract lipidic components, yet to different extents in terms of carotenoids and PUFA;

II) possesses some kind of antioxidant capacity, but ethanolic extracts appeared as the most promising – due to higher amounts of carotenoids and PUFA, while exhibiting low IC_{50} antioxidant capacity against all radicals tested;
III) those obtained with ethyl lactate and hexane:isopropanol (3:2) revealed antitumor effects upon two gastric cancer cell lines, probably due to their contents in carotenoids and PUFA.

Therefore, said extracts may be considered for larger-scale nutraceutical or pharmaceutical application based on their bioactivities.

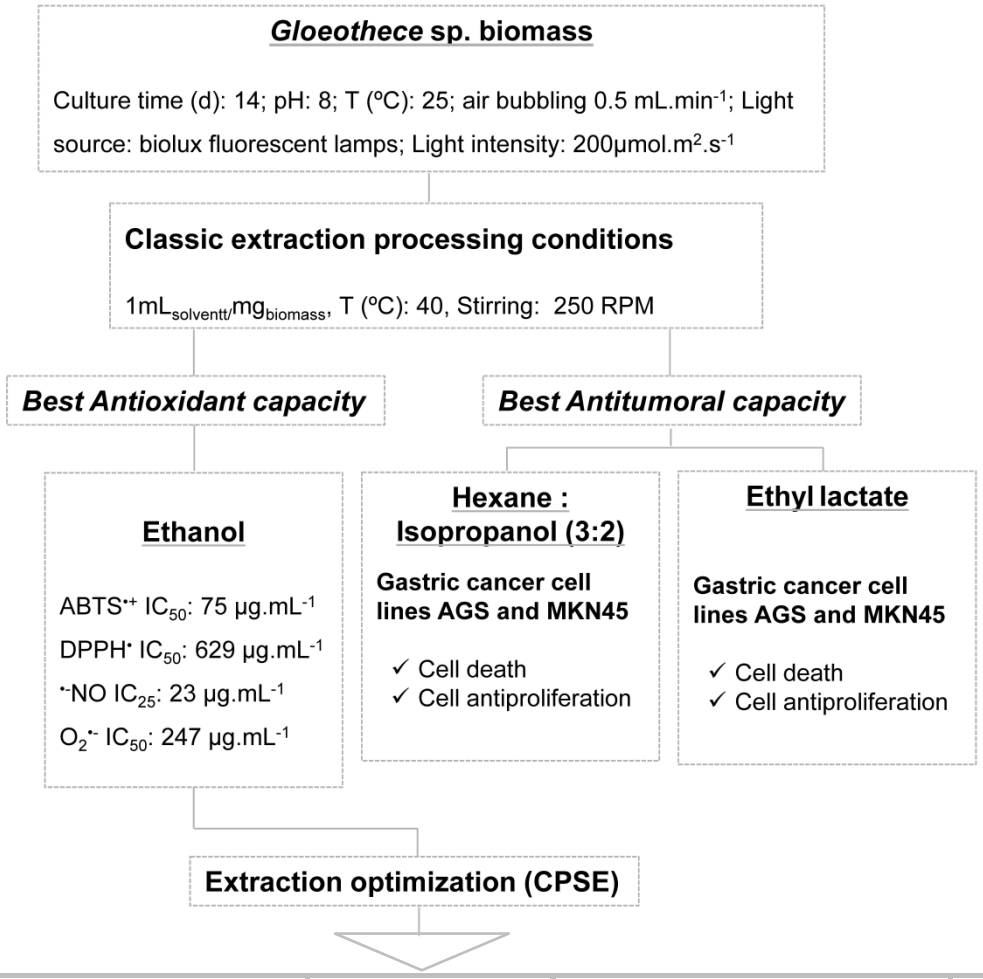
-The CPSE system developed:

I) was effective, quicker and more economical in extraction of lipidic components, when compared to a conventional extraction method;
II) temperature, pressure and solvent recirculation were optimized for extraction of carotenoids, PUFA, and compounds bearing high antioxidant capacity (which correlated with the content of lipidic compounds).
III) continuous circulation of ethanol is possible, and allowed collection of different extracts, particularly concentrated in lipidc compounds, thus avoiding complementary steps of purification.

-Use of blue (B), red (R) and/or a combination thereof BR (40:60) LEDs for *Gloeothece* sp. cultivation:

I) enhanced biomass productivity,
II) enhanced intracellular concentration of carotenoids and PUFA,
III) improved antioxidant capacity, and
IV) an extra infra-red LEDs added to R and BR 60:40 enhanced carotenoid and PUFA concentrations.

It is therefore possible to optimize conditions for *Gloeothece* sp. biomass production, either with fluorescent lamps or LEDs, towards lipidic compound eventual extraction, bearing antioxidant and antitumor potential, as depicted in Fig. 1 and Table 1.



Component concentration			Extraction operating condition
Fatty acids ($\mu g_{FA}.mL^{-1}mg_{Extract}$)	**14:0**	27.3 ± 0.0	**P** (bar): 180 (Q3) **T** (ºC): 60 $\mathbf{V_{ethanol}}$ (mL): 150
	16:0	157.8 ± 2.0	
	16:1	2.9±1.5	**P** (bar): 180 (Q3) **T** (ºC): 60 $\mathbf{V_{ethanol}}$ (mL): 12,5 **Circulation Cycles**: 5
	18:0	1.5 ± 0.6	
	18:1 n9	134.9 ± 16.8	**P** (bar): 180 (Q3) **T** (ºC): 60 $\mathbf{V_{ethanol}}$ **(mL)**: 150
	18:2 n6 t	23.8 ± 5.2	
	18:2 n6 c	70.5 ± 1.1	
	18:3n6	3.3 ± 1.3	**P** (bar): 180 (Q3) **T**(ºC): 60 $\mathbf{V_{ethanol}}$ (mL): 12,5 **Circulation Cycles**: 5
	18:3 n3	69.1 ± 1.5	**P** (bar): 180 (Q3) **T**(ºC): 60 $\mathbf{V_{ethanol}}$ (mL): 150
	Total fatty acids	*479.7*	

Component concentration			Extraction operating condition
Carotenoids ($mg.L^{-1}$)	**Lutein**	2.9 ± 0.1	**P** (bar): 180 (Q3) **T** (ºC): 60 $\mathbf{V_{ethanol}}$ (mL): 12,5 **Circulation Cycles**: 3
	β-Carotene	1.5 ± 0.1	
	Total carotenoids	*4.4*	
Antioxidant capacity ($mg_{TE}.mg_e^{-1}$)	**ABTS**	168.7 ± 4.3	**P** (bar): 180 (Q3) **T** (ºC): 60 $\mathbf{V_{ethanol}}$ (mL): 12,5 **Circulation Cycles**: 1
	DPPH	398.9 ± 6.7	

Fig. 6.1. Schematic representation of recommended growth and extraction conditions to obtain microalgal bioactive lipidic extracts of *Gloeothece* sp., either via classic extraction or continuous pressurized solvent extraction (CPSE).

Table 6.1- *Gloeothece* sp. light growth parameters at 25 ºC and pH 8, using LED as light source (R- red; BR (40:60)- blue:red (40:60), BR (40:60)+IR- blue:red (40:60) + infrared) under an intensity of 100 $\mu mol_{photon}.m^{-2}.s^{-1}$, to improve lipidic component intracellular concentration and reduce culture time.

Cell components		LEDs parameters	Maximum values attained in shortest culture	Maximum values attained under fluorescent light
Biomass production		**LED**: B $t_{exponential\ phase}$ (d):t1-t8	td:2.54 d μ_{max}: 0.2735 d^{-1} P(X): 0.132 $g.L^{-1}d^{-1}$	td: 3.24d μmax: 0.2143 d^{-1} P(X):0.1355 $g.L^{-1}d^{-1}$
Carotenoids $mg.L^{-1}.g_{DW}^{-1}$	Neoxanthin	**LED**: BR(40:60) $\mathbf{t_{culture}}$(d): 6	12,47 ± 3,12	8.34 ± 0.04
	Violoxanthin	**LED**: R $\mathbf{t_{culture}}$(d): 6	9,54 ± 1,24	8.23 ± 0.02
	Lutein	**LED**: R $\mathbf{t_{culture}}$(d):6	45,66 ± 5,98	37.85 ± 1.39
	β- carotene	**LED**: BR (40:60) $\mathbf{t_{culture}}$(d): 13	13,11 ± 0,37	9.88 ± 0.35
Fatty acids $mg_{FattyAcid}.mL^{-1}.mg_{DW}^{-1}$	C16:0	**LED**: R+IR $\mathbf{t_{culture}}$(d): 28	33,03 ± 0,55	70.21 ± 0.93
	C18:1 (n-9)	**LED**: R+IR $\mathbf{t_{culture}}$(d): 28	39,44 ± 3,16	45.52 ± 0.79
	C18:2 (n-6t)	**LED**: R+IR $\mathbf{t_{culture}}$(d): 28	32,61 ± 0,01	54.14 ± 2.34
	C18:3 (n-6)	**LED**: R+IR or BR (40:60)+IR $\mathbf{t_{culture}}$(d): 28	5,10 ± 0,32	8.36 ± 0.42
	C18:3 (n-3)	**LED**: R or BR (40:60)+IR $\mathbf{t_{culture}}$(d): 28	27,98 ± 1,59	49.09 ± 0.70
Intracellular antioxidant capacity $mg_{TE}.mL^{-1}.mg_{DW}^{-1}$	ABTS	**LED**: R $\mathbf{t_{culture}}$(d):8, 18, 20	2,85 ± 0,17	2.15 ± 0.06

6.3. Future perspectives

Several windows of opportunity for future research were open along the studies reported in this thesis, mainly in what concerns to the 3 key areas addressed under scrutiny: extraction, nature of bioactivities, and productivity of metabolites. Some suggestions of future work are described next.

6.3.1. Extraction of microalgal metabolites

In Chapter 2, a number of selected solvents were tested; however, it would be interesting to ascertain whether other solvent systems would be more effective toward extraction of specific microalgal components.

It was not fully clear how carotenoid and PUFA concentrations affect antioxidant capacity, and if there are synergistic effects between compounds. On the other hand, it is known that certain concentrations of carotenoids or PUFA may entail pro-oxidation effects. Hence, it would be helpful to fractionate the obtained extracts in several portions and test their bioactivities, and repeat this procedure with those fractions exhibiting better activity, as this might aid in identification of compound or group of compounds responsible therefor.

In order to attain a more comprehensive characterization of *Gloeothece* sp. extracts, it would be helpful to identify other compounds that could also be contributing to antioxidant and antitumor bioactivities; this includes phycobiliproteins that may attain ca. 60% of the total protein content and 20% of the dry cell weight in cyanobacteria. They have indeed been considered as a potent pharmacological and medicinal agent, due to their antioxidant capacity (Soni, Trivedi et al. 2008).

6.3.2. Bioactivities of microalgal extracts

Several pieces of evidence reviewd above indicated that the anti-inflammatory activity of carotenoids is intimately associated with antioxidant and antitumor capacity (Amaro, Barros et al. 2013, Catarina Guedes, Amaro et al. 2013). Given the antioxidant capacity of lipidic components attained in this study, it should be interesting to test the extracts as described in Chapter 2 for their anti-inflammatory and antibacterial capacity, due the reasons presented below.

In chapter 3, antitumor effects of *Gloeothece* sp. extracts against two gastric lines were ascertained. Previous evidences indicates that infection by bacterium *Helicobacter pylori* is the primary cause of gastric cancer development (Liu and Lee 2003) – such that it is classified as a type I carcinogen by the International Agency for Research on Cancer. This microorganism colonizes the stomach of half the worlwide population, and is associated not only with distal gastric cancer, but also with gastric and duodenal peptic ulcer diseases, type B gastritis, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Perez-Perez, Rothenbacher et al. 2004). In addition, the first stages of *H. pylori* infection are based on inflammatory processes that may, in the most severe cases, lead to gastric cancer. Hence, a treatment capable of reducing the rate of progression from infection by *H. pylori* to gastric cancer – through

the inflammation state induced thereby, is critical to discontinue the canonical carcinogenic pathway.

Moreover, infection by *H. pylori* has been correlated with increased oxidative stress in the gastric mucosa (Kupcinskas, Lafolie et al. 2008). Hence, diets rich in antioxidants, or their use as dietary supplements have received increasing attention toward chemoprevention. A recent study revealed that a *Chlorococcum* sp. carotenoid-based diet reduced the mucosal bacterial load associated with systemic immune response modification in *Helicobacter pylori*-infected BALB/c mice (Liu and Lee 2003).

As seen before, experimental evidence suggests that carotenoids may be able to reduce or modulate excessive ROS/RNS, with a consequent favorable impact upon inflammatory processes – and without the negative effects that usually accompany classical pharmaceutical strategies of intervention (Guedes, Amaro et al. 2011). Additionally, several studies have unfolded the anti-inflammatory effects of a few carotenoids, including violoxanthin, β-carotene and lutein, and of (phenolic) carnosic acid in inhibiting production of pro-inflammatory mediators (Hadad and Levy 2012)

In particular, violaxanthin – the major carotenoid in *Chlorella ellipsoidea*, showed to be a promising anti-inflammatory agent (Soontornchaiboon, Joo et al. 2012). Lutein and zeaxanthin have also been found to enhance the immune function (Lakshminarayana, Sathish et al. 2010) by decreasing LPS-induced NO production by 50%, in RAW 264.7 mouse macrophage cells (Rafi and Shafaie 2007). It was also able to scavenge ROS generated during the inflammatory process; inhibit pro-inflammatory mediators; and decrease the level of intracellular H_2O_2 accumulation, by scavenging superoxide (Kijlstra, Tian et al. 2012). Furthermore, β-carotene possesses anti-inflammatory activity via its function as inhibitor of redox-based processes, probably due to its antioxidant potential (Bai, Lee et al. 2005). This carotenoid has been tested *in vivo* and *in vitro*, and was demonstrated to inhibit production of inflammation in RAW 264.7 mice cell line.

6.3.3. Increasing cell metabolite concentration using light as a tool

As studied in Chapter 5, the light spectrum proved to serve as a useful tool in microalgal metabolism manipulation. Wavelengths corresponding to red (R) and blue (B) light are the major factor affecting photosynthesis and other metabolic pathways; however, only two combinations of said spectrum bands were considered. Therefore, a study of the influence of other combinations of such bands may be useful toward understanding and optimizing production of carotenoids and PUFA. Since the influence

of infra-red spectrum on said compound production was found to be favourable, further efforts should be developed to study its influence upon microalgal metabolism.

Evidence also exists that LED flashing (or pulsed) light can increase biomass production, thus allowing microalgae to exceed the proposed maximum photosynthetic efficiency by up to 17% (Schulze, Barreira et al. 2014).Hence, testing this type of light supply could contribute to better understand the light effects upon cyanobacterium metabolite production and qrowth (as happens with *Gloeothece* sp.).

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