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Andrêssa Silva Fernandes

AVALIAÇÃO DE MOLÉCULAS DE CLOROFILAS E SEUS DERIVADOS EM ESPÉCIES DE MICROALGAS CULTIVADAS EM FOTOBIORREATORES

Santa Maria, RS
2017

Andrêssa Silva Fernandes

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Dissertação apresentada ao Curso de Pós-Graduação em Ciência e Tecnologia dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Mestre em Ciência e Tecnologia dos Alimentos**.

Orientadora: Profª Dra. Leila Queiroz Zepka

Santa Maria, RS
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Aprovado em 04 de agosto de 2017:

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(Charles Chaplin)

RESUMO

AVALIAÇÃO DE MOLÉCULAS DE CLOROFILAS E SEUS DERIVADOS EM ESPÉCIES DE MICROALGAS CULTIVADAS EM FOTOBIORREATORES

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Microalgas são microrganismos fotossintéticos, que por sua vez, apresentam grande potencial de inserção em processos biotecnológicos com a produção paralela de biomoléculas, tais como pigmentos que estão abundantemente presentes na biomassa microalgal. No entanto, estudos detalhados sobre a caracterização completa do perfil qualitativo e quantitativo de biomoléculas de clorofilas em espécies de microalgas são escassos na literatura. Em face disto, o trabalho teve por objetivo avaliar o perfil clorofilado em extratos de *Scenedesmus obliquus, Chlorella vulgaris e Aphanothece microscopica Nägeli* cultivadas em fotobiorreatores. O conteúdo total de clorofilas, com base no peso seco da biomassa de *Scenedesmus obliquus, Chlorella vulgaris* e *Aphanothece microscopica Nägeli* foram 7.319,0 $\mu g.g^{-1}$, 10.734,1 $\mu g.g^{-1}$ e 9.121,8 $\mu g.g^{-1}$, respectivamente. Um total de dez diferentes compostos clorofilados foram separados nos extratos, os majoritários sendo clorofila a (47,0%) e feofitina a*'* (21,8%) em *Scenedesmus;* clorofila a (57,0%) e clorofila a' (14,9%) em *Chlorella;* feofitina a' (35,8%) e clorofila a em *Aphanothece.* Além desses compostos, foi possível identificar hidroxiclorofila a', clorofila b, clorofila b', 15-Hidroxi-lactona clorofila a, hidroxifeofitina a, hidroxifeofitina a' e feofitina a. Os resultados obtidos evidenciaram que as três espécies de microalgas são alternativas promissoras para obtenção de compostos de clorofilas naturais, uma vez que apresentaram um perfil qualitativo e quantitativo relevante para exploração biotecnológica. Adicionalmente, foi publicado o capítulo de livro "Carotenoides em microalgas" (Capítulo 3), como pesquisa complementar a este trabalho.

Palavras-chave: *Scenedesmus*. *Chlorella*. *Aphanothece.* Clorofila. Fotossintético.

ABSTRACT

EVALUATION OF CHLOROPHYL MOLECULES AND THEIR DERIVATIVES IN MICROALGAE SPECIES CULTIVATED IN PHOTOBIORREATORS

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Microalgae are photosynthetic microorganisms, which, in turn, present great potential for insertion in biotechnological processes with the parallel production of biomolecules, such as chlorophylls that are abundantly present in microalgal biomass. However, detailed studies on the complete characterization of the qualitative and quantitative profile of chlorophyll biomolecules in microalgae species are scarce in the literature. The objective of this work was to evaluate the profile of chlorophylls profile in extracts of *Scenedesmus obliquus*, *Chlorella vulgaris* and *Aphanothece microscopica Nägeli* grown in photobioreactors. The total chlorophylls content, based on the dry weight of the biomass, of *Scenedesmus obliquus*, *Chlorella vulgaris* and *Aphanothece microscopica Nägeli* were 7,319.0 $\mu g.g^{-1}$, 10,734.1 $\mu g.g^{-1}$ and 9,121.8 $\mu g.g^{-1}$, respectively. A total of ten different chlorophylls compounds were separated in all the extracts, the major ones being chlorophyll a (47.0%) and pheophytin a' (21.8%) in *Scenedesmus*; chlorophyll a (57.0 %) and chlorophy a' (14.9 %) in *Chlorella*; pheophytin a' (35.8 %) and chlorophyll a (26.6 %) in *Aphanothece*. Besides these compounds, it was possible to identify hidroxychlorophyll a', chlorophyll b, chlorophyll b', 15-Hydroxy-lactone chlorophyll a, hidroxypheophytin a, hidroxypheophytin a' and pheophytin a. The results obtained evidenced that the three species of microalgae are promising alternatives for obtaining natural chlorophyll compounds, since they presented a qualitative and quantitative profile relevant for biotechnological exploration. In addition, the book chapter "Carotenoids in microalgae" (Chapter 3) was published as a complementary research to this work.

Keywords: *Scenedesmus*. *Chlorella*. *Aphanothece*. Chlorophyll. Photosynthetic.

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INTRODUÇÃO

As clorofilas são moléculas orgânicas que constituem uma grande e diversificada família de moléculas semelhantes entre si, designadas clorofila a, b, c, d, f e compostos derivados (HUMPREY, 2004; CHEN et al., 2017). São onipresentes em seres fotossintetizantes como microalgas e plantas superiores por possuírem papéis essenciais de absorção de energia luminosa no processo da fotossíntese (CHEN et al., 2015). Além disso, são relacionados a benefícios proeminentes para a saúde humana, por possuir várias atividades biológicas, incluindo ação antioxidante, antimutagênica e propriedades nutracêuticas (CHEW et al., 2017). Dada essas características, essas moléculas são amplamente empregadas em diversos setores industriais como indústria farmacêutica, cosmética, nutracêutica e alimentar. Porém, a indústria de alimentos é a que mais emprega a utilização desse pigmento como corante alimentar (HAMED, 2016).

As mudanças nas demandas do mercado e na legislação resultaram na exigência de agentes corantes naturais em substituição a corantes artificiais ou sintéticos a serem utilizados em produtos alimentares (DUFOSSÉ, 2016). Com isso, o mercado global de corantes naturais é impulsionado e deverá atingir US $ 1,77 bilhões até 2021, representando um aumento de cerca de 5,2% entre 2016 e 2021 (ZION, 2017).

Atualmente, a maior parte de clorofilas naturais comercialmente produzida é obtida a partir de fontes vegetais. No entanto, há um avanço crescente a exploração de clorofilas microalgais como fonte alternativa de obtenção desses pigmentos, devido as suas vantagens perante as fontes convencionais (BARBA, GRIMI & VOROBIEV, 2015; BEGUN et al., 2016; HAMED, 2016). As quais incluem a simplicidade de necessidades nutricionais, altas taxas de crescimento, baixas demandas por terras aráveis, simplicidade de cultivo com produção concomitante de uma diversidade de biomoléculas clorofiladas (BENAVENTE-VALDÉS et al., 2016; POOJARY et al., 2016; BRASIL et al., 2016).

Dentre as várias espécies de microalgas, destacam-se sob o aspecto de exploração biotecnológica para obtenção de clorofilas, microalgas verdes (*Chorophyta)* e as cianobácterias (*Cyanophyta)*, pois sintetizam substanciais concentrações de compostos clorofilados *(*SUGANYA et al., 2016). Contudo, estudos

detalhados sobre a caracterização completa do perfil qualitativo e quantitativo de biomoléculas de clorofilas em espécies de microalgas são escassos na literatura.

Neste sentido, visando avançar o conhecimento científico sobre fontes naturais alternativas clorofiladas, a exploração da fração de clorofilas e seus derivados em biomassa de microalgas provenientes de processos biotecnológicos faz-se necessário uma vez que, esses micro-organismos são fontes promissoras para obtenção natural dessas biomoléculas.

OBJETIVOS

Objetivo geral

Elucidar o perfil de clorofilas e seus derivados presentes na biomassa de *Scenedesmus obliquus*, *Chlorella vulgaris* (microalgas verdes) e *Aphanothece microscopica Nägeli* (cianobactéria), visando ampliar o conhecimento sobre essas biomoléculas em fontes microalgais.

Objetivos específicos

Cultivar *Scenedesmus obliquus* (CPCC 05), *Chlorella vulgaris* (CPCC 90) e *Aphanothece microscopica Nägeli* (RSMan 92) em fotobiorreatores para obtenção de biomassas.

Determinar o perfil qualitativo de clorofilas e seus derivados nas três espécies de microalgas por HPLC-PDA-MS.

Determinar o perfil quantitativo de clorofilas e seus derivados nas três espécies de microalgas por HPLC-PDA.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1. MICROALGAS

Microalgas são organismos microscópicos com taxonomia complexa, pois designa organismos muito distintos entre si quanto à origem, à composição química e à morfologia. Constituem um grupo polifilético de seres fotossintetizantes em sua grande maioria, presentes em sistemas aquáticos, com hábitos planctônicos e bentônicos (LOURENÇO, 2006; CHACÓN-LEE & GONZÁLEZ-MARIÑO, 2010).

No entanto, esses micro-organismos representam um recurso quase inexplorado, uma vez que das possíveis 20.000 a 800.000 espécies existentes, relativamente cerca de 40-50.000 espécies foram estudadas em detalhes, do ponto de vista bioquímico e fisiológico (SUGANYA et al., 2016).

Segundo Mutanda et al. (2011), sob a denominação de microalgas estão incluídos organismos com dois tipos de estrutura celular, classificados filogeneticamente em procarióticos, com representantes nos grupos *Cyanophyta* e *Prochlorophyta*; estrutura eucariótica, com representantes nos grupos *Glaucophyta, Rhodophyta, Ochrophyta, Haptophyta, Criptófitas, Dinophyta, Euglenophyta, Chlorarachniophyta e Chlorophyta*.

Destacam-se sob o aspecto de exploração biotecnológica os grupos: cianobactérias (*Cyanophyta*), clorofíceas *(Chlorophyta*) e diatomáceas (*Ochrophyta*) (MATA et al., 2010; MUTANDA et al., 2011; SUGANYA et al., 2016). As clorofíceas e cianobactérias estão entre as linhagens que vêm sendo utilizadas na produção de biomassa e compostos celulares, as quais podem ser amplamente aplicadas como insumos intermediários e produtos finais de processos relacionados à bioenergia, alimentação e farmacêuticos (JACOB-LOPES et al., 2006; QUEIROZ et al., 2007; JACOB-LOPES et al., 2007; JACOB-LOPES et al., 2008; JACOB-LOPES et al., 2009; JACOB-LOPES et al., 2010; QUEIROZ et al., 2011).

As clorofíceas, também conhecidas como microalgas verdes, compartilham o mesmo mecanismo fotossintético que as plantas superiores, de acordo com suas relações filogenéticas próximos, cerca de 90% do total de espécies (sobretudo as formas microscópicas) ocorrem em água doce, mas também podem habitar outros habitats. Na grande maioria são unicelulares, podendo também serem multicelulares. São uma fonte potencial promissora de compostos bioativos de interesse industrial como carotenoides e clorofilas (RIVIERS, 2006; VALVERDE et al., 2016).

Por outro lado, as cianobactérias são classificadas no reino das eubactérias. São seres robustos que podem utilizar até três vias metabólicas para obtenção de energia, a respiração, a fotossíntese e a fixação de nitrogênio (QUEIROZ et al., 2013; LAU, MATSUI & ABDULLAH, 2015). Nunca apresentam flagelos e por terem sua organização celular do tipo procarionte Gram-negativos, não possuem núcleo, nem organelas. Apresentam em sua estrutura a clorofila a e os fotossistemas I e II, ao contrário de outras bactérias fotossintetizantes, o que as permite realizar a fotossíntese na presença de oxigênio. Algumas espécies são estritamente fototróficas, enquanto outras, como as cianobácterias, atuam de modo facultativo, podendo crescer heterotroficamente utilizando substratos orgânicos como fonte de carbono (WIJFFELS et al., 2013).

Dentre as diversas microalgas na linhagem das clorofíceas, as espécies de microalgas dos géneros *Scenedesmus e Chlorella*, pertencentes à classe *Chlorophyceae*, são microalgas bastante comuns em ambientes de água doce, apresentam mínimos requisitos nutricionais, o que possibilita facilidade de cultivo (RIVIERS, 2006). *Scenedesmus obliquus* em termos morfológicos, possui células cilíndricas com 5-10 µm de diâmetro, unicelular e forma colônias com quatro células. Em particular, é amplamente cultivada com produtividade elevada de biomassa e relevantes concentrações de pigmentos fotossintéticos (BECKER, 2007; KIM et al., 2016).

Adicionalmente, a espécie *Chlorella vulgaris* é uma alga esférica, unicelular com um diâmetro de 2-10 µm e assim como a espécie *Scenedesmus obliquus,* possui uma taxa de crescimento rápida (BECKER, 2007; SAFI et al., 2014). É tipicamente cultivada para consumo humano devido ao seu elevado teor de proteína. Porém, o conteúdo proteico apresentado por essa microalga já não é argumento único para promover sua utilização, visto que, pesquisas direcionadas à produção de pigmentos naturais por esses micro-organismos tem se consolidado (KONG et al., 2014; DAMERGI et al., 2017; PATIAS et al., 2017).

Em contrapartida, *Aphanothece microscopica Nägeli* é taxonomicamente classificada na divisão *Cyanophyta,* classe *Cyanoficeae*, possui coloração verde azulada, células adultas elípticas acilíndricas, medindo 9,0 - 9,5 mm x 4,2 mm, cerca de 2,1 vezes mais comprida que larga. Associada a capacidade de realizar fotossíntese, está a facilidade de fixar nitrogênio atmosférico e fontes orgânicas de

carbono, o que possibilita uma maior facilidade de cultivo para sustentar seu crescimento. Essas propriedades os colocam como importante fonte alternativa de bioprodutos (ZEPKA et al., 2008; QUEIROZ et al., 2013, STREIT et al., 2017).

De fato, boa parte da ênfase que se atribui as microalgas, dá se ao fato de que esses micro-organismos podem crescer cerca de 10-50 vezes mais rápido do que as plantas terrestres conseguindo assim, uma maior taxa de fixação CO_2 (CHEN et al., 2011; HO et al., 2013; BASU et al., 2015). Assim como, esses micro-organismos podem ser cultivados sob diferentes modos para a produção de biomassa, como cultivo fotoautotrófico que envolve o processo de fotossíntese, cultivos heterotróficos, onde é necessário a inserção de fontes de carbono orgânico e o cultivo mixotrófico, no qual compreende ambas as condições autotróficas e hetetotróficas (ABINANDAN & SHANTHAKUMAR, 2015).

No entanto, esta diversidade evolutiva e filogenética significa uma grande variabilidade na composição química destes micro-organismos, no qual, podem sintetizar estruturas químicas únicas, a maioria deles com importantes propriedades bioativas, o que os torna extremamente atraentes para exploração biotecnológica como fontes comerciais de uma vasta gama de biomoléculas (HERRERO & IBÃNEZ, 2015; RODRIGUES et al., 2015; BAJHAIYA, MOREIRA & PITTMAN, 2016).

2. METABOLISMO FOTOSSINTÉTICO DE MICROALGAS

As microalgas em condições de crescimento natural, absorvem luz solar e assimilam CO_2 como fonte de carbono do ar e nutrientes dos habitats aquáticos (PEREZ-GARCIA et al., 2011). Esse tipo de cultivo é denominado crescimento autotrófico, devido à capacidade fotossintética que esses micro-organismos possuem, no qual utilizam a energia luminosa para extrair prótons e elétrons de água para reduzir o CO_2, a fim de produzir uma biomassa rica em moléculas orgânicas (MARKOU & GEORGAKAKIS, 2011; THAWECHAI et al., 2016).

Em suma, a biomassa de cultivo de microalgas é produzida através da fotossíntese com base na seguinte reação (HOSIKIAN et al., 2010):

$$CO_2 + H_2O + \text{nutrientes} + \text{energia luminosa} \rightarrow \text{biomassa} + O_2$$

O processo fotossintético em microalgas eucarióticas, ocorre em organelas especiais, chamadas de cloroplastos. Onde, estes são envoltos por uma membrana, contendo um fluido aquoso chamado estroma, o qual contém o aparato bioquímico necessário para a fixação de CO_2, através das reações de carboxilação da fotossíntese, também conhecidas como ciclo de Calvin-Benson. O estroma contém pilhas de discos achatados delimitadas por uma membrana chamada tilacóide. Embebidos nas membranas tilacóides estão os pigmentos fotossintetizantes que promovem as reações luminosas e a síntese de ATP (FAY, 1983). Esse processo pode ser dividido em duas fases de reação, que diferem pelo fato da primeira ser dependente de luminosidade e na fase posterior, os produtos das reações com luz são subsequentemente consumidos pela redução de CO_2 a carboidratos (FAY, 1983; MARKOU & GEORGAKAKIS, 2011).

Por conseguinte, as microalgas procarióticos possuem um aparelho fotossintético semelhante em estrutura e função ao presente no cloroplasto dos seres eucarióticos, porém o mecanismo fotossintético ocorre nos tilacóides (SMITH, 1982).

Na primeira fase a qual recebe o nome de fase da luz, a energia luminosa é absorvida na forma de fótons por um complexo sistema coletor de luz ligado a pigmentos fotossintéticos e transportadores de elétrons, chamados fotossistemas. A clorofila *a*, é o principal elemento fotoquimicamente ativo que atua como receptor de energia luminosa para a efetivação do processo fotossintético, convertendo-a em energia química. Carotenoides, clorofilas b, c e d e ficobiliproteínas exibem os chamados "pigmentos acessórios de absorção de luz". Esses pigmentos absorvem a energia eletromagnética em faixas espectrais onde a clorofila a não absorve energia luminosa, principalmente em comprimentos de onda entre 400 nm e 500 nm, colaborando assim para o processo (MARKOU & GEORGAKAKIS, 2011; KOLLER, MUHR & BRAUNEGG, 2014; ZHAO & SU, 2014; D'ALESSANDRO & FILHO, 2016).

Esta energia é utilizada pelo fotossistema II na oxidação da água, liberando prótons, elétrons e moléculas de O_2. Os elétrons são transferidos através da cadeia transportadora de elétrons até o fotossistema I, e levam à redução da ferredoxina para a formação do intermediário redutor NADPH. Um gradiente eletroquímico é formado devido à liberação de prótons após a oxidação da água para o lúmen do tilacóide, o qual é utilizado para conduzir a produção de ATP via ATP sintase (BEER et al., 2009).

Os produtos fotossintéticos NADPH e ATP são os substratos para o ciclo de Calvin-Benson ou etapa independente de luz, onde o CO_2 é fixado pela microalga através da reação com um açúcar de cinco carbonos conhecido como ribulose difosfato, formando um composto instável de seis carbonos, que logo se quebra em duas moléculas de três carbonos (3-fosfoglicerato, conhecidas como PGA). O ciclo prossegue até que no final, é produzida uma molécula de glicose e é regenerada à molécula de ribulose difosfato (FAY, 1983, KAUNY & SÉTIF, 2014).

Neste processo fotossintético, as microalgas utilizam energia solar juntamente com vários nutrientes essenciais (C, N, P, S, K, Fe) que se tornam substratos para sintetizar seus compostos da biomassa e multiplicar suas células, sendo que a deficiência de um dos elementos ou a qualidade e quantidade de iluminação irá causar a redução do crescimento (SPAARGAREN, 1996).

Adicionalmente ao metabolismo fotossintético, a respiração e a fixação de nitrogênio constituem importantes rotas metabólicas das cianobactérias, passíveis de serem exploradas biotecnologicamente para diversos propósitos (TRAN et al., 2010; LAU, MATSUI & ABDULLAH, 2015).

Dentro deste contexto, algumas espécies de microalgas vêm sendo exploradas por possuírem substancial capacidade de bioconversão de matéria inorgânico (CO_2) utilizando a energia luminosa para seu metabolismo. Consequentemente, esse processo ocorre de forma relativamente rápida, com baixo custo e ecologicamente seguro. No qual, a biomassa obtida desse tipo de cultivo, torna-se uma alternativa promissora para a síntese de insumos naturais para aplicação industrial (RODRIGUES et al., 2015; SOLOVCHENKO et al., 2016).

3. BIOPRODUTOS DE MICROALGAS

Dada a sua natureza renovável e diversificada, a integração da produção de biomassa de microalgas com o passo de obtenção de bioquímicos de alto valor, como pigmentos, estabelece uma alternativa promissora para consolidar a indústria de produtos naturais que considere impactos econômicos e ecológicos (BARBA, GRIMI & VOROBIEV, 2015; GILBERT-LÓPEZ et al., 2016). Essa afirmação baseia-se no fato de que microalgas possuem vantagens perante as fontes convencionais de produção desses compostos como elevada taxa de crescimento, baixas demandas por terras

aráveis, e alta resistência a condições ambientais adversas, além da possibilidade de mitigar os problemas ambientais associados às emissões de CO_2 e efluentes (RODRIGUES et al., 2014; SANTOS et al., 2016; FERNANDES et al., 2016; MARONEZE et al., 2016; PATIAS et al., 2017).

Em paralelo, macroalgas e microalgas são, provavelmente, os dois grupos de organismos aquáticos que têm atraído maior atenção por seu potencial como fontes naturais industrialmente viáveis de compostos com estruturas diferenciadas e alguns com propriedades bioativas (HERRERO et al., 2015; RODRIGUES et al., 2015).

Dentro deste contexto, as microalgas podem agir como uma plataforma bioquímica para diversos segmentos industriais, como a indústria de cosméticos, suplementos dietéticos, produtos farmacêuticos e aplicações terapêuticas, tecnologia de alimentos, produção de "energia verde", como biogás, biodiesel, bio-hidrogênio e bioetanol (KOLLER, MUHR & BRAUNEGG, 2014; SUGANYA et al., 2016).

As principais biomoléculas de interesse comercial provenienetes de microalgas são as substâncias intracelulares (ácidos graxos, proteínas, minerais, carboidratos e pigmentos), as substâncias extracelulares (carboidratos e compostos voláteis) e os biocombustíveis. Esses produtos na biomassa microalgal apresentam características qualitativas e quantitativas que consolidam o valor biológico e nutricional destas biomassas (ZEPKA et al., 2008; ABDEL-RAOUF et al., 2012; DRAAISMA et al., 2013).

Além das biomoléculas provenientes do metabolismo microalgal, a biomassa desses micro-organismos também é utilizada a nível comercial. Nas indústrias nutracêuticas, *Spirulina* e *Chlorella* são as espécies mais importantes na comercialização como alimentos saudáveis e suplementos nutricionais com vários benefícios para a saúde, incluindo o aumento da atividade do sistema imune, os efeitos antitumorais e a promoção do crescimento animal, devido às suas abundantes proteínas, vitaminas, polissacarídeos ativos e outros compostos importantes (LIU, POHNERT & WEI, 2016).

No que diz respeito a fração lipídica, as microalgas podem acumular uma alta porcentagem desses compostos, os quais representam aproximadamente 30-50% de seu peso total (CHEW et al., 2017). O perfil de ácidos graxos desses micro-organismos está associado à presença de importantes ácidos graxos poli-insaturados, como os ácidos linolênico, linoleico, araquidônico (ARA), docosahexaenóico (DHA) e eicosapentaenóico (EPA) que são valiosos como suplementos alimentares saudáveis

(ABEDI & SAHARI, 2014; MARONEZE et al., 2016). Além disso, microalgas podem fornecer biocombustíveis de terceira geração, quando processadas através de reações químicas ou biológicas. Estes incluem biodiesel, biohidrogênio e bioetanol, os quais são considerados alternativas promissoras para substituição do combustível fóssil (AHMAD et al., 2014).

As proteínas são parte dos constituintes principais das microalgas, compreendendo 50-70% da composição da biomassa microalgal. Esses valores evidenciam seu grande potencial para ser uma fonte de proteína alternativa, uma vez que contêm todos os aminoácidos essenciais. Fato este, motivou a exploração comercial em larga escala de microalgas no século XX (SPOLAORE et al., 2006; WAGHMARE et al., 2016). Microalgas também são classificadas como uma boa fonte de minerais, como magnésio, sódio, cálcio, fósforo e potássio em maiores concentrações, assim como minerais traços (manganês, zinco, alumínio, ferro e cobre), o que torna a biomassa uma fonte susceptível a ser utilizada como suplemento alimentar na aquicultura e também como fertilizante (FABREGAS & HERRERO, 1986). Esses micro-organismos também apresentam em sua composição fontes valiosas de vitaminas essenciais como vitamina A, B_1, B_2, B_6, B_{12}, C, E, nicotinato, biotina, ácido fólico e ácido pantotênico) (MATOS et al., 2017).

Os carboidratos à base de microalgas consistem principalmente em glicose, celulose, amido e vários polissacarídeos. Entre estes, a glicose ou amido de algas é convencionalmente utilizada para a produção de biocombustíveis, como bioetanol e bio-hidrogênio, enquanto que os polissacarídeos possuem funções biológicas como armazenamento, proteção e moléculas estruturais (CHEW, et al., 2017). Adicionalmente, as microalgas sintetizam um grande número de compostos orgânicos voláteis (hidrocarbonetos, álcoois, ácidos, cetonas, ésteres e aldeídos) que poderiam ser usadas como uma fonte alternativa importante de produtos farmacêuticos, aromas e fragrâncias a baixo custo (SANTOS et al., 2016).

Alternativamente, as microalgas podem biosintetizar Substâncias Poliméricas Extracelulares (SPE) na superfície da célula, como uma forma de proteção para as mesmas. SPE’s são matrizes heterogêneas de polímeros de polissacarídeos, proteínas, ácidos nucléicos e fosfolipídos (MCSWAIN et al., 2005).

Carotenoides, ficobiliproteínas e clorofilas são as três classes de pigmentos fotossintéticos bioativos majoritários em microalgas, os quais são responsáveis pelas

cores vermelho/azul, amarelo/laranja e verde, respectivamente. (BEGUM et al., 2015). Carotenoides e clorofilas são sintetizados por todas as espécies de microalgas, porém apenas as divisões *Cyanophyta* (algas azul-verde), *Cryptophyta* (Criptomonas) e *Rhodophyta* (algas vermelhas) sintetizam ficobiliproteínas (LIN, OFFNER &TROXLER, 1990). Além do potencial de aplicação como corantes na aquicultura e nas indústrias alimentícias e farmacêuticas, esses compostos têm sido relacionados a efeitos benéficos à saúde, os quais são atribuídos principalmente às suas propriedades antioxidantes, ação anticancerígena, anti-inflamatória, anti-obesidade e atividades neuroprotetoras (GUEDES et al., 2011; RODRIGUES et al., 2015; BAJHAIYA, MOREIRA e PITTMAN, 2016; D'ALESSANDRO & FILHO, 2016).

A produção de carotenoides tendo microalgas como matéria prima tornou-se uma das atividades mais bem sucedidas na indústria de biotecnologia. A empresa química alemã - BASF é líder mundial incontestável na produção de β-caroteno de *Dunaliella salina* (BOROWITZKA, 2013). Além disso, o principal mercado de astaxantina é a produção de *Haematococcus pluvialis*, conhecida pela grande acumulação desse pigmento (LORENZ & CYSEWKI, 2004).

Assim, pigmentos naturais, são atualmente um dos produtos mais comercializados a partir de microalgas, renovando o interesse de exploração destas substâncias na biomassa microalgal (D'ALESSANDRO & FILHO, 2016).

4. CLOROFILAS

As clorofilas são pigmentos verdes que ocorrem nos plastídios da maioria das plantas, certas bactérias, abundantemente nas algas verdes conhecidas como clorofitas e também em cianobactérias. Nestes organismos, as clorofilas participam da biossíntese de biomoléculas complexas ($C_6H_{12}O_6$) a partir de moléculas mais simples (CO_2 e H_2O) pelo processo de fotossíntese (DUCAT, WAY & SILVER, 2011; SIMPSON, BENJAKUL, & KLOMKLAO, 2014). Constituem a classe de pigmentos mais abundantes e amplamente distribuídos na natureza. E devido à sua captura de energia luminosa e funções de transferência de elétrons em organismos fotossintéticos, pigmentos de clorofila são considerados os compostos essenciais do processo da fotossíntese (CHATTERJEE & KUNDU, 2015).

São moléculas orgânicas complexas, quimicamente classificadas dentro do grupo das porfirinas. Estruturalmente, as clorofilas compreendem um sistema macrociclo tetrapirrólico, ligadas por pontes metilênicas (-CH-) e com um íon Mg^{2+} no seu interior, coordenado aos anéis por 4 átomos de nitrogênio. Essa estrutura também contém no C-17, uma cadeia de ácido propiônico esterificado com o álcool fitol diterpeno (uma cadeia de hidrocarbonetos de 20 carbonos), conferindo a molécula um caráter hidrofóbico (HUMPHREY, 2004; D'ALESSANDRO & FILHO, 2016).

A síntese de clorofila em microalgas verdes e cianobactérias prossegue ao longo da via do glutamato (via C_5), na qual o primeiro percussor é o ácido 5-aminolevulênico (ALA). Duas moléculas de ALA são então condensadas formando uma molécula pirrol. Posteriormente, quatro moléculas de pirrol são polimerizada para formar um pirrole linear que sofre ciclização para produzir um tetrapirrole macrocíclico (protoporfirina IX). Na sequência, ocorre a inserção do Mg^{2+} pela enzima Mg quetalase formando o composto protoclorofilida que na presença de luz e de enzima (protoclorofilida redutase) forma a estrutura clorofilida *a.* O último passo da síntese de clorofila a é a esterificação da clorofilida *a* que é catalisado pela enzima clorofila sintase (WETTSTEIN, GOUGH & KANNANGARA, 1995; BEALE, 1999; MASUDA, 2008; ZHANG et al., 2017).

No entanto, clorofilas constituem uma grande e diversificada família de moléculas semelhantes entre si, designadas clorofila a, b, c (c_1, c_2 e c_3), d e f (Figure 1) (GROSS, 1991; CHEN et al., 2010). Estruturalmente, as moléculas de clorofila diferem umas das outras, devido ao grau de saturação dos anéis pirrólicos e seus grupamentos terminais, o que altera a absorção desses pigmentos (HUMPHREY, 2004).

Essas diferenças na saturação em macrociclos de clorofila têm profundas consequências sobre a absorbância espectral desses compostos (Figure 1). Por exemplo, clorofila a, clorofila d e clorofila f têm aproximadamente iguais intensidades de absorção em azul, vermelho e verde. Por outro lado, as fitoporfirinas de clorofila c absorvem-se fracamente em vermelho e mais intensamente em torno de 450 nm (JACOB-LOPES, ZEPKA & QUEIROZ, 2017).

Clorofila a, a mais abundante e importante componente dessa família, ocorre como pigmento onipresente em todas as espécies de microalgas. Possui um grupo metila (CH_3) no carbono C-7, ao passo que a clorofila b (Figure 1) (segunda clorofila

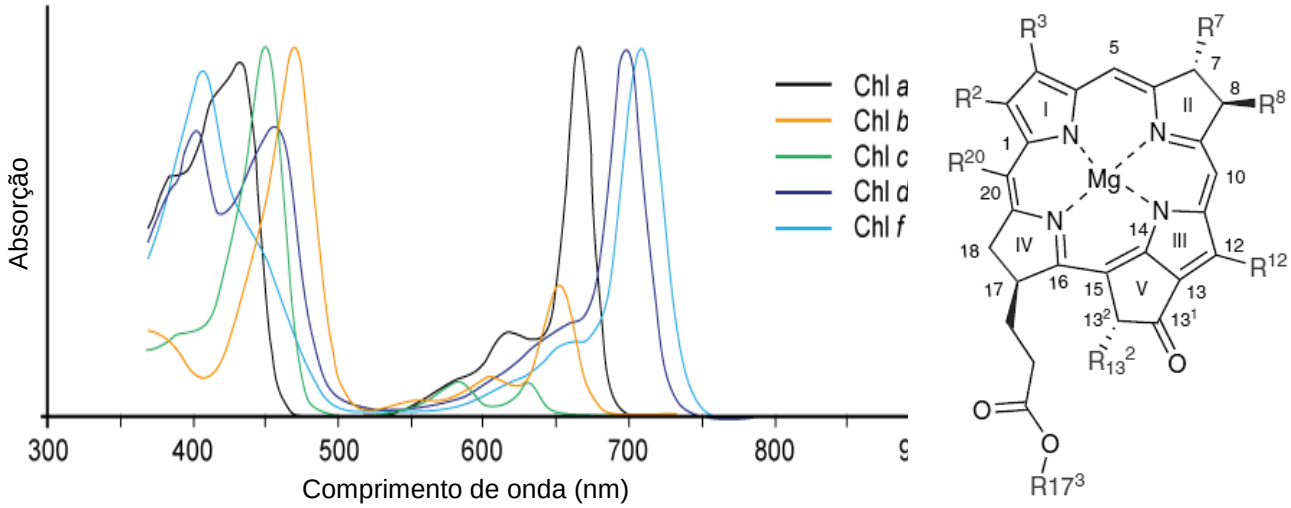
mais abundante na natureza) tem um grupo aldeído (CHO) (CHEN et al., 2010; KOLLER, MUHR & BRAUNEGG, 2014). Esta diferença estrutural resulta em a clorofila a ser um pigmento azul/verde com absorção máxima 660-665 nm e clorofila b sendo um verde/amarelo com o máximo de absorção 642-652 nm (HOSIKIAN et al., 2010). Os fatores de absorção de clorofila, evidenciam a coloração verde típica e bem conhecida das algas verdes (*Chlorophyta*) que abrigam clorofilas a e b como os pigmentos predominantes (KOLLER, MUHR & BRAUNEGG, 2014).

Adicionalmente, clorofila a é abundante em *Rhodophyta* e cianobactérias, estando localizadas nas membranas tilacóides, ao contrário da clorofila b que está presente em grandes concentrações em *Chlorophyta* e *Euglenophyta* que são semelhantes a plantas superiores e microalgas. Clorofila d está presente em diatomáceas e algumas espécies de cianobactérias. Enquanto as clorofilas c está presente em diatomáceas de água doce (HOU, RAPOSO & HOU, 2013; BEHRENDT et al., 2014; BEGUM et al., 2015). Da mesma forma, clorofila f foi encontrada em uma cianobactéria (*Acaryochloris* ssp.) (CHEN & BLANKENSHIP, 2011).

Entretanto, essas moléculas são altamente instáveis, podendo sofrer alterações em suas estruturas com a formação de compostos derivados. A maior instabilidade de moléculas de clorofila, compreende a cadeia lateral de hidrocarbonetos, o qual pode ser removida pela ação de enzimas (clorofilases) e/ou condições ácidas, alterando assim, a polaridade molecular, o que torna a molécula hidrofílica. Enquanto que Mg^{2+} pode ser removido quando exposto a tratamento ácido e térmico, o que evidencia a formação de compostos de degradação com diferentes colorações como verde claro, verde castanho e verde oliva. Condições alcalinas, exposição à luz e oxigênio, também afetam negativamente sua estabilidade induzindo mudanças em sua estrutura (SIMPSON, BENJAKUL, & KLOMKLAO, 2014). No entanto, a estabilidade das clorofilas contra a degradação pode ser aumentada pela desesterificação da clorofila e complexação com íons de cobre em substituição ao íon de magnésio (SANT'ANNA et al., 2013).

Além disso, moléculas de clorofilas podem sofrer reações de hidroxilação em suas estruturas, no qual uma série de derivados complexos hidroxilados de clorofilas são formados. Esses compostos podem ser consequência do metabolismo natural desses micro-organismos, ou devido ao método de extração aplicado para obtenção desses compostos (FERNANDES et al., 2016; CHEN et al., 2017).

Figura 1 - Estruturas de clorofilas e seus espectros de absorção.



Pigmento	R2	R3	R7	R8	Ligação 7-8	R12	$R13^2$	Ligação 17-18	R20	$R\ 17^3$
Chl a	CH_3	$CH{=}CH_2$	CH_3	C_2H_5	=	CH_3	$COOCH_3$	–	H	Fitol
Chl b	CH_3	$CH{=}CH_2$	CHO	C_2H_5	=	CH_3	$COOCH_3$	–	H	Fitol
Chl c1	CH_3	$CH{=}CH_2$	CH_3	C_2H_5	=	CH_3	$COOCH_3$	=	H	–CH = CH-COOH
Chl c2	CH_3	$CH{=}CH_2$	CH_3	$CH{=}CH_2$	=	CH_3	$COOCH_3$	=	H	–CH = CH-COOH
Chl c3	CH_3	$CH{=}CH_2$	$COOCH_3$	$CH{=}CH_2$	=	CH_3	$COOCH_3$	=	H	–CH = CH-COOH
Chl d	CH_3	CHO	CH_3	C_2H_5	=	CH_3	$COOCH_3$	–	H	Fitol
Chl f	CHO	C_2H_5	CH_3	C_2H_5	=	CH_3	$COOCH_3$	–	H	Fitol

Fonte: CROCE & AMERONGEN (2014).

Entretanto, esses derivados apresentam efeito antimutagênico e podem desempenhar um papel significativo na prevenção do câncer (CHERNOMORSKY, SEGELMAN & PORETZ, 1999).

As clorofilas também são de grande importância comercial, pois são utilizadas há muitos anos como corantes, monitoramento da produção agrícola e da

produtividade primária oceânica (JACOB-LOPES, ZEPKA & QUEIROZ, 2017). Além dessas aplicações, essas moléculas também são empregadas em diversos setores industriais como indústrias farmacêuticas e de alimentos. Porém, a área de alimentos é a que mais emprega a utilização desse pigmento como aditivo alimentar, no qual a clorofila é utilizada com o objetivo de fornecer uma coloração verde a uma variedade de alimentos e bebidas (KOLLER, MUHR & BRAUNEGG, 2014; HAMED, 2016).

Contudo, grandes avanços vem ocorrendo na compreensão das funções biológicas das clorofilas, no qual estudos comprovam que esses compostos estão relacionados a várias atividades biológicas, incluindo ação cicatrizante, anti-inflamatória, antimutagenicidade, capacidade de eliminação de radicais livres, propriedades nutracêuticas e capacidade de inibição da cristalização de oxalato de cálcio (EDWARDS, 1954; TAWASHI et al., 1980; LANFER-MARQUEZ, BARROS & SINNECKER, 2005; KAO, CHEN & CHEN, 2011; CHEW et al., 2017).

As fontes comerciais de clorofilas incluem as microalgas *Chlorella* e *Spirulina* e fontes vegetais como *Enteromorpha* (*Chlorophyta*) e *Ulva* (*Chlorophyta*) (SIMPSON, BENJAKUL & KLOMKLAO, 2014). No entanto, no Brasil, a clorofila produzida comercialmente é em parte de origem vegetal, obtida principalmente a partir de espinafres, que contém cerca de 0,06 $mg.g^{-1}$. Embora a maioria das clorofilas industriais seja extraída de fontes vegetais, há um crescente interesse na produção de clorofilas por processos biotecnológico provenientes de microalgas, visto que esses micro-organismos contém em média 1,15 mg.g-1 de clorofila (BEGUN et al., 2016, HAMED, 2016).

Assim, as microalgas verdes (*Chlorophyta*) e as demais espécies de cianobactérias (*Cyanophyta*), tornam-se uma fonte alternativa para síntese biológica destes compostos, pois apresentam em suas estruturas clorofilas como os pigmentos predominantes, podendo serem cultivadas em sistemas contínuos em contraste as fontes vegetais (KOLLER, MUHR & BRAUNEGG, 2014; D'ALESSANDRO & FILHO, 2016).

Contudo, em microalgas, os dados da literatura sobre perfil de clorofilas não são consistentes, uma vez que não se tem uma caracterização qualitativa e quantitativa completa do perfil desses compostos (CHA et al., 2010; MOHSENPOUR, RICHARDS & WILLOUGHBY, 2012; PLAZA et al., 2012; KONG et al., 2014; CHENG et al., 2015; GILBERT-LÓPEZ et al., 2017). A escassez de dados, tem sido

relacionada principalmente a deficiência de um procedimento analítico adequado para a separação e identificação desses compostos, assim como, a instabilidade química dos mesmos. A esse respeito, estudos vem sendo direcionados para o emprego de uma metodologia analítica precisa para a compreensão estrutural de clorofilas e compostos derivados em macroalgas, o que auxilia fortemente na realização de demais pesquisas em espécies de microalgas (CHEN et al., 2015a; CHEN et al., 2015b; CHEN et al., 2017).

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CAPÍTULO 2

DETERMINATION OF PROFILE OF CHLOROPHYLLS COMPOUNDS IN MICROALGAE SPECIES

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Determination of profile of chlorophylls compounds in microalgae species

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ABSTRACT

The objective of this work was to evaluate the profile of chlorophylls and their derivatives in *Scenedesmus obliquus* (CPCC05), *Chlorella vulgaris* (CPCC90), and *Aphanothece microscopica Nägeli* (RSMan92) cultivated in phototrophic conditions. The total chlorophyll content, based on dry weight of biomass, of *S. obliquus*, *C. vulgaris* and *A. microscopica Nägeli* were 7319.0 $\mu g.g^{-1}$, 10734.1 $\mu g.g^{-1}$ and 9121.8 $\mu g.g^{-1}$, respectively. A total of ten different chlorophylls compounds were separated in all the extracts, the major ones being chlorophyll a (47.0 %) and pheophytin a' (21.8 %) in *Scenedesmus*; chlorophyll a (57.0 %) and chlorophy a' (14.9 %) in *Chlorella*; pheophytin a' (35.8 %) and chlorophyll a (26.6 %) in *Aphanothece*. Besides these compounds, it was possible to identify compounds of relevance as hidroxychlorophyll a', chlorophyll b, chlorophyll b', 15-hydroxy-lactone chlorophyll a, hidroxypheophytin a, hidroxypheophytin a' and pheophytin a. Like green microalgae, cyanobacteria present great potential for producing chlorophyll compounds, notably presented in this study. Nevertheless, as far as we know, this is the first report on the compounds chlorophylls composition of cyanobacteria *Aphanothece microscopica Nägeli*.

Keywords: Chlorophyll, microalgal, HPLC-PDA-MS, photosynthetic pigments, phototrophic conditions.

1. Introduction

Chlorophylls are commercially important natural green pigments that constitute a large and diverse family of biomolecules similar to each other, representing the most abundant class of pigments. Due to their essential role of absorption and transfer of light energy, they are essential molecules in photosynthesis, being present in all oxygenated photosynthetic organisms, such as plants superior and algae (Humprey, 2004; Li & Chen, 2015). Among all species and compounds derived from chlorophylls, chlorophyll a and b are the most studied. Chlorophyll a is the most abundant pigment in all photosynthetic organisms; chlorophyll b is the second most in plants and green microalgae (da Silva Ferreira & Sant'Anna, 2017).

Given his sensitivity, the chlorophyll molecules form derived compounds, as consequence of natural metabolism, chemical or enzymatic action. Among the chlorophyll derivatives are the oxidized compounds due to a substitution of the H atom at C-13_2 by a hydroxyl group, the so-called hydroxy derivatives. By contrast, when the central magnesium atom of the tetrapyrrol ring is easily replaced by two hydrogen atoms occurs the formation of pheophytins. In addition, the rearrangement of the isocyclic ring through the formation of a lactone group, form the 15-hydroxy-lactone derivatives. On the other hand, the formation of isomers can also occur, originating from decarbomethoxylation at position C-13_2 position (Figure 1) (Chen, Ríos, Pérez-Gálvez & Roca, 2015a; Chen, Ríos, Roca & Pérez-Gálvez, 2015b; Chen, Ríos, Pérez-Gálvez & Roca, 2017). These compounds attract attention because they have higher physiological properties than the chlorophyll species (Pérez-Gálvez & Roca, 2017).

Chlorophylls have been used for monitoring agricultural production and primary ocean productivity (Jacob-Lopes, Zepka & Queiroz, 2017). Also, chlorophylls and their derivatives are widely applied in various industrial sectors such as the pharmaceutical, cosmetic, nutraceutical and food industries, due they have dye action and are associated with various

biological activities, including antioxidant, anti-inflammatory, antimutagenic, healing and nutraceutical properties (Lanfer-Marquez, Barros & Sinnecker, 2005; da Silva Ferreira & Sant'Anna, 2017). However, the area of food is the one that most uses the pigment as a food additive, in which chlorophyll is used to provide a green coloration to a variety of foods and beverages (Koller, Muhr & Braunegg, 2014; Hamed, 2016).

Currently, most commercially produced natural chlorophylls are obtained from higher plants (Begun et al., 2016). However, the global market for natural pigments is intensively growing, with prospects of reaching $ 2.50 billion by 2025 (Grand View, 2017). Due to this growth the market for natural chlorophylls has been boosted, thus seeking alternative sources to consolidate the production of these compounds. In this sense, microalgae become alternative sources of these pigments due to their advantages over conventional sources (Barba, Grimi & Vorobey, 2015; Begun et al., 2016; Hamed, 2016). These include simplicity of nutrient requirements, high growth rates, low arable land demands, simplicity of cultivation with concomitant production of a variety of chlorophyllated biomolecules (Benavente-Valdés et al., 2016; Poojary et al., 2016; Brasil et al., 2016).

Traditionally, several microalgae such as *Chlorella* spp., *Dunaliella* spp., *Nannochloropsis* spp., and *Scenedesmus* spp., *Spirulina* spp., *Haematococcus pluvialis* and *Aphanizomenon* flos-aquae are sourcesof fine chemicals (Lorenz & Cysewki, 2004; Barba, Grimi & Vorobiev, 2015). However, as commercial sources of chlorophyll, only *Chlorella* and *Spirulina* were explored (Simpson, Benjakul & Klomklao, 2014). Nevertheless, the literature lacks characterization data of the complete chlorophyll profile in these microalgae.

In this sense, the objective of this work was to identify the profile of chlorophylls and their derivatives in extracts of *Scenedesmus obliquus*, *Chlorella vulgaris* and *Aphanothece microscopica Nägeli* cultured phototrophically, aiming at a biotechnological exploration and the expansion of knowledge about these biomolecules in microalgae sources.

2. Material and Methods

2.1 Chemicals

Standards of chlorophyll a, chlorophyll b, (with purities ranging from 95.0% to 99.9%, as determined by HPLC-PDA) were purchased from Sigma-Aldrich (Missouri-MO, USA). The pheophytin a standard was obtained in our laboratory through an acid hydrolysis reaction from the standard chlorophyll a, where the Mg^{2+} ion is replaced by two hydrogen atoms (Fernandes et al., 2016). Methanol, ethanol, acetone, methyl tert-butyl ether (MTBE), ethyl acetate, petroleum ether and diethyl ether were purchassed from Sigma-Aldrich (St. Louis-MO, USA).

2.2 Microorganisms and culture media

Axenic cultures of *Aphanothece microscopica Nägeli* (RSMan92) (cyanobacteria), *Chlorella vulgaris* (CPCC90) and *Scenedesmus obliquus* (CPCC05) (green microalgae) were used in the experiments. Stock cultures were propagated and maintained in synthetic BG11 medium (Braun-Grunow medium) (Rippka, Deruelles, Waterbury, Herdman & Stanier, 1979). The incubation conditions were 30 ºC, photon flux density of 150 μmol.m^{-2}.$s^{-1}$and a photoperiod of 12 h were used.

2.3 Microalgal biomass production

The biomass production was carried out in a bubble column photobioreactor (Jacob-Lopes, Scoparo, Queiroz, & Franco, 2010) operating in intermittent regime, fed with 2.0 L of BG11 medium Rippka et al. (1979). The experimental conditions were as follows: initial concentration of inoculum of 100 mg.L^{-1}, temperature of 25 °C, aeration of 1 volume of air per volume of medium per minute, a photon flux density of 150 μmol.m^{-2}.s^{-1}, photoperiod of 12/12 hours light/dark, and a residence time of 168 h.

2.4 Biomass concentration

The biomasses were separated from the culture medium by centrifugation. It was subsequently freeze dried (Lyophilizer Liotop L101) for 24h at -50 °C above -175 μm Hg, and then stored under refrigeration until the time of analysis.

2.5 Chlorophyll extraction

The chlorophylls was exhaustively extracted from the freeze-dried samples (0.2 ± 0.02 g) with ethyl acetate and methanol in a mortar with a pestle followed by centrifugation (Hitachi, Tokyo, Japan) for 7 min at 5500 rpm (Fernandes et al., 2016). The extraction procedure was repeated until the supernatant becomes colorless. The homogenized sample suspension was filtered through a 0.22 μm polyethylene membrane, concentrated in a rotary evaporator (T<30 °C).

In order to separate carotenoids from the chlorophyll, the samples were submitted to preparatory open column chromatography. Separation of the extract was carried out on a 25 × 300 mm glass column packed to a height of about 150 mm with MgO:Hyflosupercel (1:1) activated for 4 h at 110 °C. The carotenoids were eluted with a gradient of petroleum ether with increasing concentrations of acetone and chlorophyll fraction was obtained in ethanol. The separation could be followed visually. The ethanol extract was partitioned in petroleum ether/diethyl ether [1:1 (v/v)] in a separatory funnel, and then washed with water to remove residual ethanol. The petroleum ether phase was collected and concentrated in a rotary evaporator (30 °C), flushed with N_2 and kept at -37 °C in the dark until chromatographic analysis.

2.6 HPLC-PDA-MS/MS analysis

The chlorophylls were analyzed by high performance liquid chromatography HPLC (Shimadzu, Kyoto, Japan) equipped with quaternary pumps (model LC-20AD), online degasser, and injection valve with a 20 μL loop (Rheodyne, Rohnert Park, CA, USA). The

equipment was connected in series to a PDA detector (model SPD-M20A) and a mass spectrometer with an ion-trap analyzer and atmospheric pressure chemical ionization (APCI) source (model Esquire 4000, Bruker Daltonics, Bremen, Germany). The UV-vis spectra were obtained between 450 and 660 nm, and the chromatograms were processed at 660 nm. Chlorophyll separation was carried out on a C30 YMC column (5 μm, 250 × 4.6 mm) (Waters, Wilmington, DE, USA). Prior to HPLC-PDA-MS/MS analysis, the chlorophylls extract was solubilized in MeOH:MTBE (1:1) and filtered through Millipore membranes (0.22 μm). HPLC-PDA-MS/MS parameters were set as previously described by De Rosso & Mercadante (2007). The mobile phase consisted in MeOH (solvent A) and MTBE (solvent B) mixture. A linear gradient was applied from 95:5 to 70:30 in 30 min, to 50:50 in 20 min. The flow rate was 0.9 $mL.min^{-1}$ and the column temperature set to 29 °C.

The identification was performed according to the following combined information: elution order on C30 HPLC column, co-chromatography with authentic standards, UV-Visible spectrum (λ máx, spectral fine structure), and mass spectra characteristics (protonated molecule ($[M + H]^+$) and MS/MS fragments), compared with data available in the literature (Huang, Hung, Wub & Chen, 2008; Bale, Llewellyn & Airs, 2010; Kao, Chen & Chen, 2011; Harada, Mizoguchi, Tsukatani, Noguchi & Tamiaki, 2012; Loh, Inbaraj, Liu & Chen, 2012; Wei, Li, Barrow & O'Connor, 2013; Paliwal et al., 2016; Fernandes et al., 2016; Chen, Ríos, Pérez-Gálvez & Roca, 2017).

The chlorophylls were quantified by HPLC-PDA using external calibration curves for chlorophyll a, chlorophyll b and pheophytin a with a minimum of five concentration levels. Hydroxychlorophyll a′, chlrophyll a′ and 15-hydroxy-lactone chlorophyll a where quantified using the curve of chlorophyll a; the hydroxypheophytin a, hydroxypheophytin a′ using the curve of pheophytin a; and chlorophyll b′ was quantified using the curve of chlorophyll b. Total chlorophyll content was calculated considering all identified peak areas.

2.7 Statistical analysis

Descriptive statistics, analysis of variance (one-way ANOVA) and Tukey's test ($p < 0.05$) were applied to experimental data. The analyses were performed with the software Statistica 7.0 (StatSoft, Tulsa-OK, USA).

3. Results and Discussion

Table 1 and Figure 1 shows a qualitative profile of ten chlorophyll derivatives identified in *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Aphanothece microscopica Nägeli*.

Once since a detailed description of chlorophylls identification using chromatographic information has already been reported by our research group (Fernandes et al., 2016), only chromatographic considerations on not identified chlorophyll compounds in previous reports were discussed below.

Peak 3 was identified as chlorophyll b', considering the lack of fine structure in the UV–visible spectrum (420, 657 nm), protonated molecule 907 $[M+H]^+$, fragments *m/z* as well the retention time (11.4 min). The elimination of the diterpene alcohol phytol from the C17, propionic substituent (Figure 2), resulted in the formation major fragment at *m/z* 629 $[M + H - 278]^+$. Other fragments were detected, both from the MS/MS, at *m/z* 875 $[M + H - 32]^+$, formed from the respective loss of CH_3O group. Fragment 597 $[M + H - 310]^+$, 569 $[M + H - 338]^+$, were attributed elimination of the diterpene alcohol phytol, in both fragments, together with the CH_3O group and loss of CH_3COO group, respectively (Figure 3). Similar identification data of this compound have been reported in the literature (Gauthier- Jaques, Bortlik, Hau & Fay, 2001; Huang et al., 2008; Kao et al., 2011; Gilbert-López, Barranco, Herrero, Cifuentes & Ibáñez, 2016; Chen et al., 2017).

Table 1 Characterization by HPLC-PDA-MS/MS of profile of chlorophyll compounds present in biomass of *Scenedesmus obliquus, Chlorella vulgaris*, and *Aphanothece microscopica Nägeli*.

Peak[a]	**Chlorophylls**	**t_R (min)**[b]	**$\lambda_{máx}$ (nm)**[c]	**$[M+H]^+$**	**MS/MS fragment ions (*m/z*)**
1	Hidroxychlorophyll a'	10.1-10.2	422, 663	909	891$[M+H-18]^+$; 631$[M+H-278]^+$; 613$[M+H-278-18]^+$; 553$[M+H-278-18-60]^+$
2	Chlorophyll b	10.6-10.8	428, 659	907	875$[M+H-32]^+$; 629$[M+H-278]^+$; 597$[M+H-278-32]^+$; 569$[M+H-278-60]^+$
3	Chlorophyll b'	11.4	420, 657	907	875$[M+H-32]^+$; 629$[M+H-278]^+$; 597$[M+H-278-32]^+$; 569$[M+H-278-60]^+$
4	15-hydroxy-lactone chlorophyll a	12.1-12.2	421, 660	925	nd[d]
5	Chlorophyll a	15.1-15.3	432, 665	893	615$[M+H-278]^+$; 583$[M+H-278-32]^+$; 555$[M+H-278-60]^+$
6	Chlorophyll a'	16.9-17.0	431, 665	893	615$[M+H-278]^+$; 583$[M+H-278-32]^+$; 555$[M+H-278-60]^+$
7	Hidroxypheophytin a	24.1- 24.2	409, 666	887	869$[M+H-18]^+$; 803$[M+H-63]^+$; 609$[M+H-278]^+$; 591$[M+H-278-18]^+$; 531$[M+H-278-18-60]^+$
8	Hidroxypheophytin a'	28.0	399, 663	887	869$[M+H-18]^+$; 609$[M+H-278]^+$
9	Pheophytin a	31.6-31.7	408, 666	871	593$[M+H-278]^+$; 533$[M+H-278-60]^+$
10	Pheophytin a'	33.0	408, 665	871	593$[M+H-278]^+$; 533$[M+H-278-60]^+$

[a] Numbered according to the chromatogram shown in Figure 1.

[b] t_R: Retention time on the C30 column.

[c] Linear gradient Methanol:MTBE.

[d] Not detected

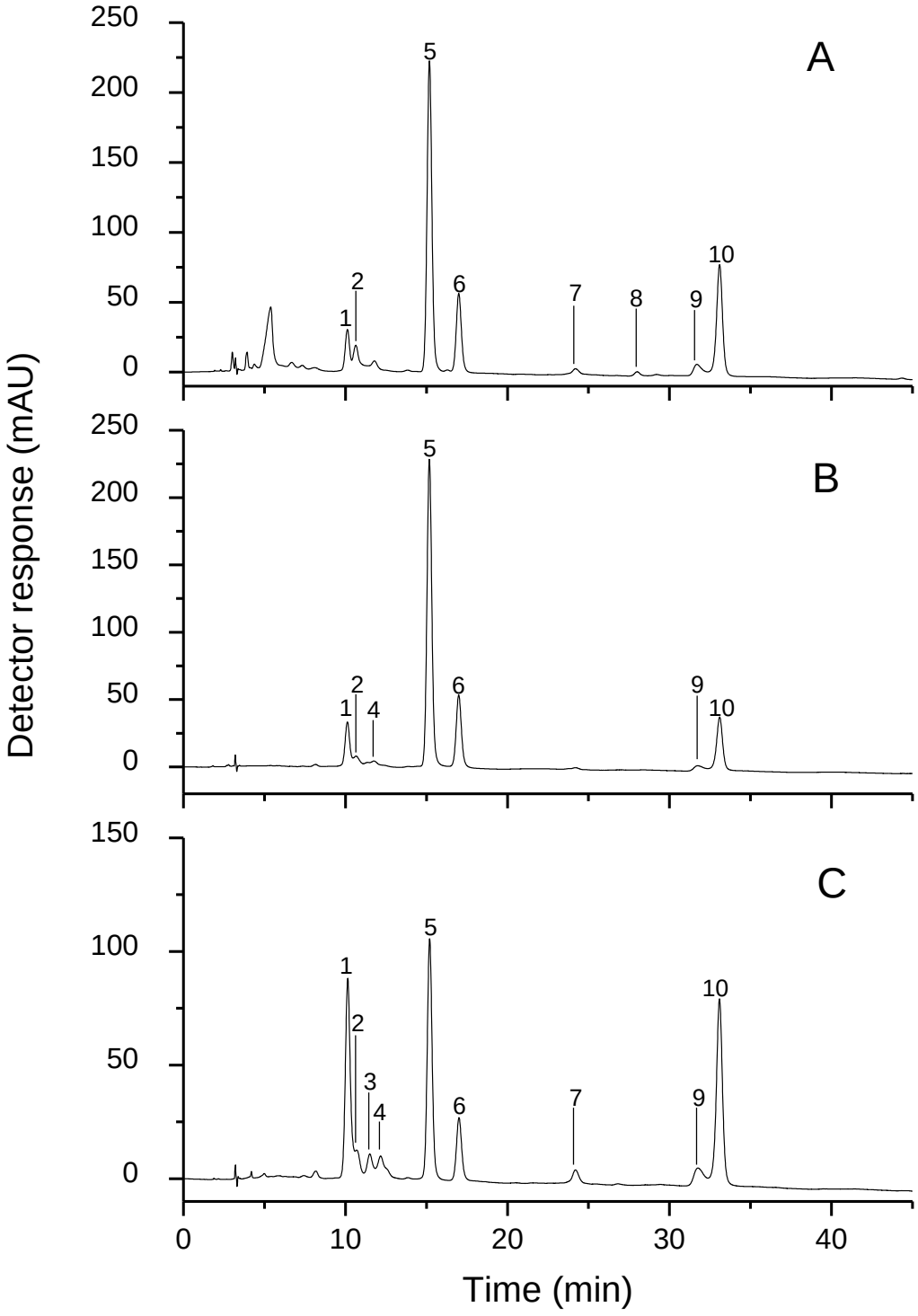
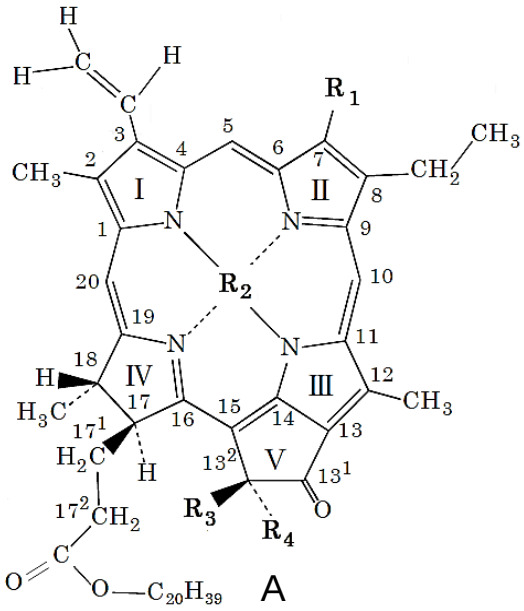
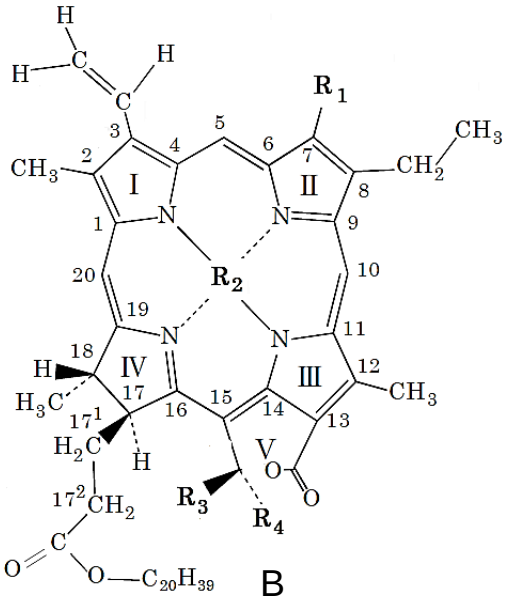


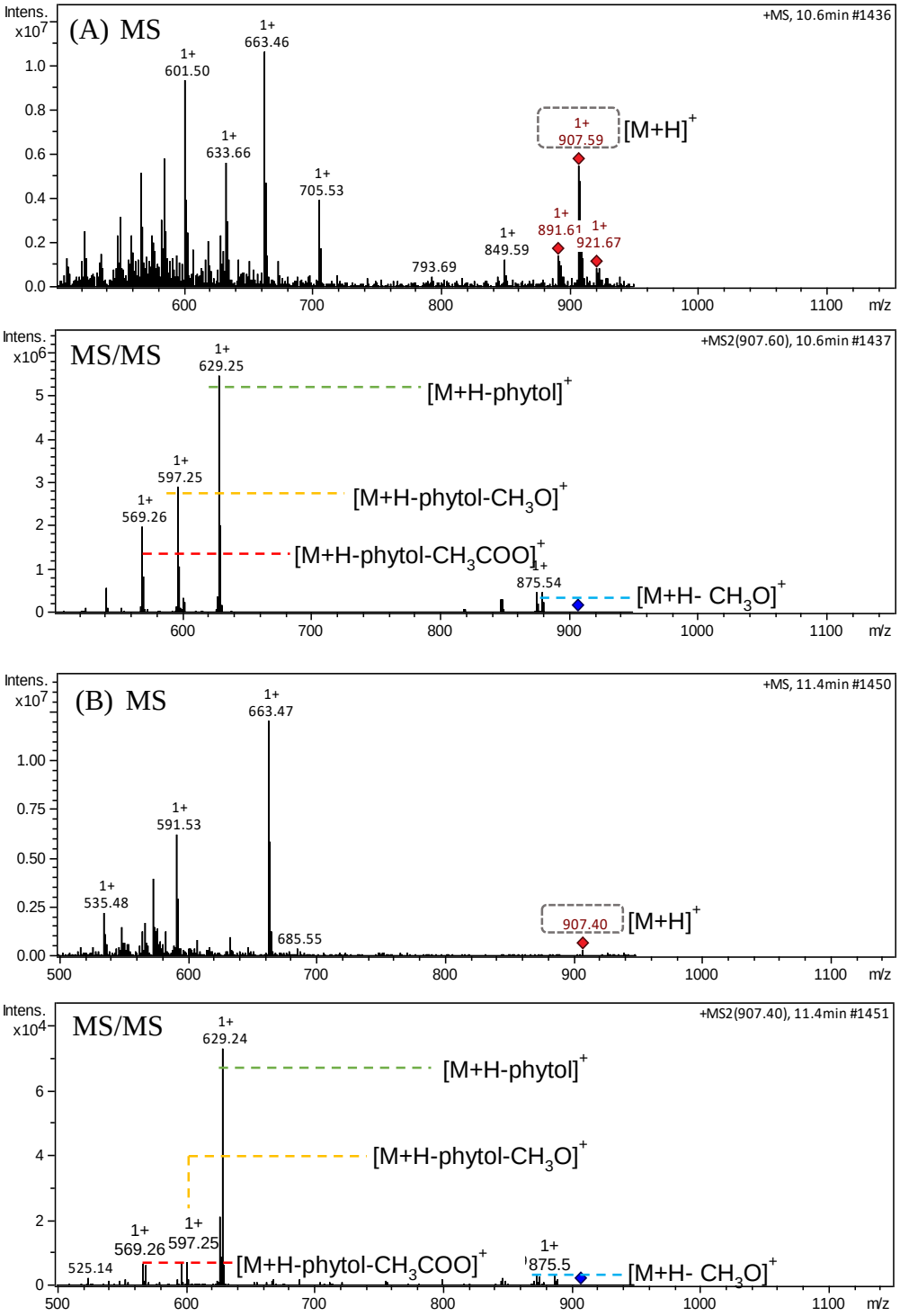
Figure 1. Chromatogram, obtained by HPLC-PDA, of the chlorophyll extract from (A) *Scenedesmus obliquus*, (B) *Chlorella vulgaris*, and (C) *Aphanothece microscopica Nägeli*. See text for chromatographic conditions. Peak identification and characterization are given in Table 1. Chromatogram was processed at 660 nm.

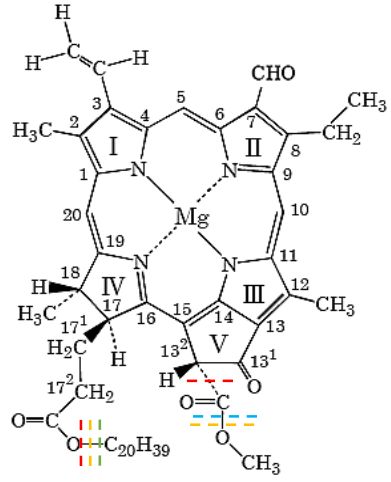




Peak	Compound	Molecular formula	Structure	R_1	R_2	R_3	R_4
1	Hidroxychlorophyll a'	$C_{55}H_{73}MgN_4O_6$	A	CH_3	Mg	OH	$COOCH_3$
2	Chlorophyll b	$C_{55}H_{70}MgN_4O_6$	A	CHO	Mg	H	$COOCH_3$
3	Chlorophyll b'	$C_{55}H_{70}MgN_4O_6$	A	CHO	Mg	$COOCH_3$	H
4	15-hydroxy-lactone chlorophyll a	$C_{55}H_{72}MgN_4O_7$	B	CH_3	Mg	$COOCH_3$	OH
5	Chlorophyll a	$C_{55}H_{72}MgN_4O_5$	A	CH_3	Mg	H	$COOCH_3$
6	Chlorophyll a'	$C_{55}H_{72}MgN_4O_5$	A	CH_3	Mg	$COOCH_3$	H
7	Hidroxypheophytin a	$C_{55}H_{75}N_4O_6$	A	CH_3	2H	OH	$COOCH_3$
8	Hidroxypheophytin a'	$C_{55}H_{75}N_4O_6$	A	CH_3	2H	$COOCH_3$	OH
9	Pheophytin a	$C_{55}H_{74}N_4O_5$	A	CH_3	2H	H	$COOCH_3$
10	Pheophytin a'	$C_{55}H_{74}N_4O_5$	A	CH_3	2H	$COOCH_3$	H

Figure 2. Structural formulas and nomenclature of chlorophylls and their derivatives identified by HPLC-PDA-MS/MS in *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Aphanothece microscopica Nägeli.*





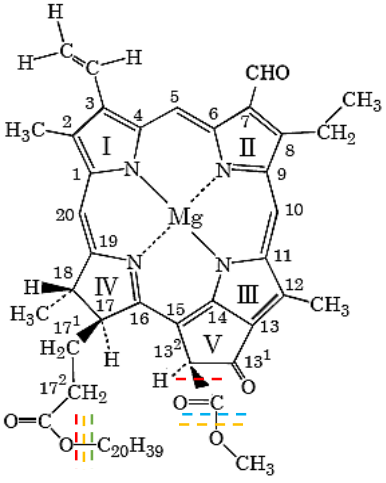


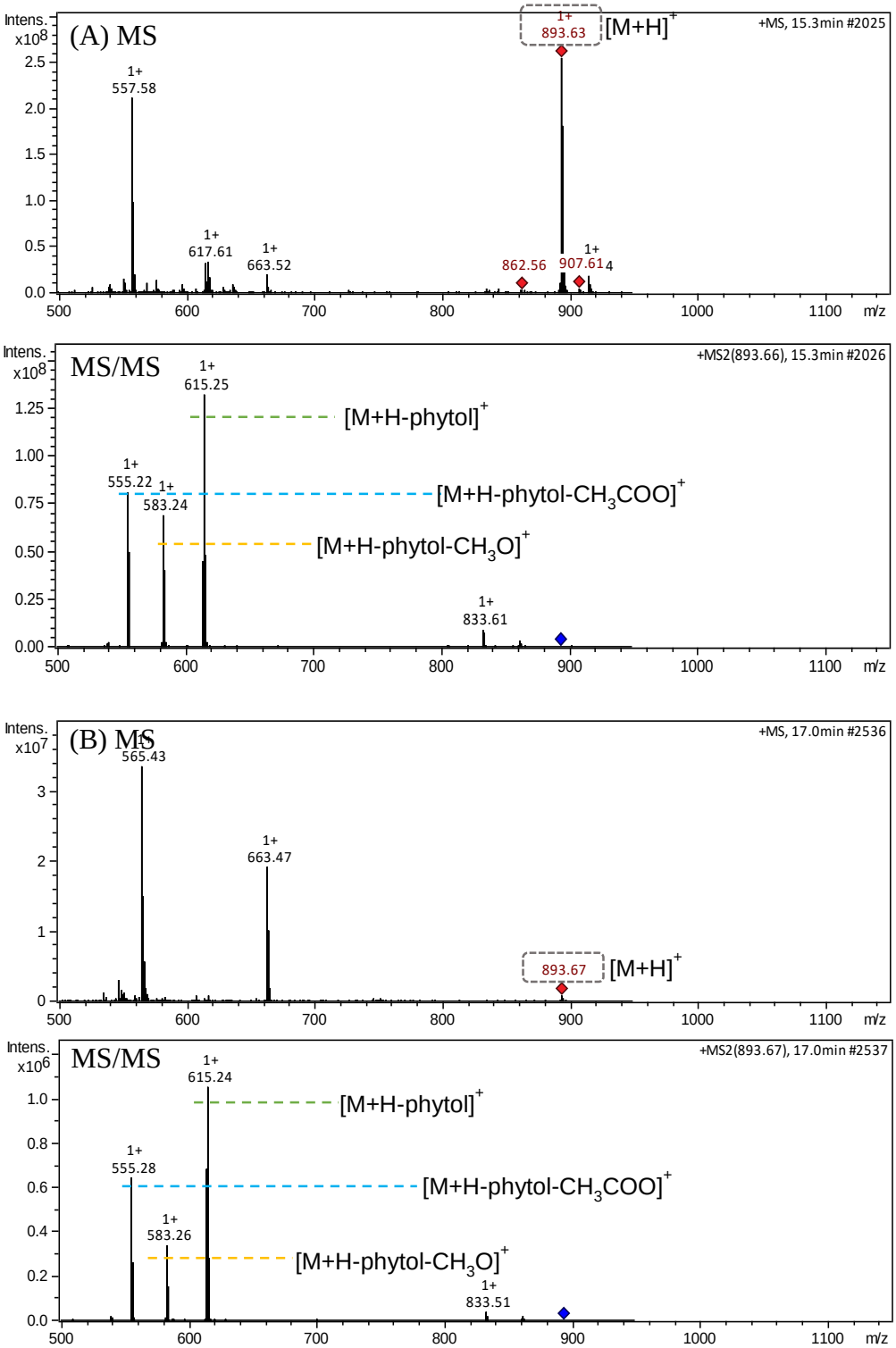
Figure 3. MS and MS/MS spectra of (A) chlorophyll b (peak 2) and (B) chlorophyll b' (peak 3).

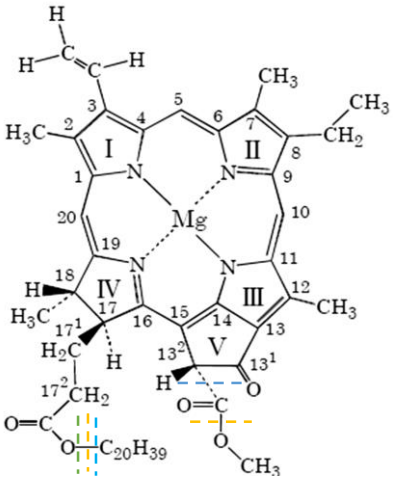
Peak 4 was identified as the hydroxylated derivative 15-hydroxy-lactone chlorophyll a (molecular formula $C_{55}H_{72}MgN_4O_7$) on the basis of the characteristic UV-visible spectra (421, 660), and protonated molecule at *m/z* 925, similar to the data from the literature (Kao et al., 2011; Chen et al., 2015a; Chen et al., 2015b; Chen et al., 2017). Accordingly, the formation of this chlorophyll derivative may be attributed to oxidation by peroxidase or strong oxidizing conditions (Kao et al., 2011).

Chlorophyll a' (peak 6) was identified, on the basis of retention time of peak, UV/visible ($\lambda_{máx}$), and confirmed by HPLC-MS. The protonated molecule was identified as *m/z* 893, the same value reported for chlorophyll a, because it is have the same chemical structure ($C_{55}H_{72}O_5N_4Mg$) and, therefore, also the same chromophore (Gauthier-Jaques et al., 2001; Huang et al., 2008; Bale et al., 2010). Moreover, the chlorophyll a′ isomers showed practically the same characteristic UV-visible spectra (431, 465 nm), and fragments MS/MS (615 $[M + H - 278]^+$, 583 $[M + H - 278\text{-}32]^+$, 555 $[M + H - 278 - 60]^+$) compared to the corresponding chlorophyll a identified by Fernandes et al. (2016) and this study (Figure 4). Additionally, it has been well established that in addition to degradation, chlorophylls can be susceptible to epimerization at C-13[2] for chlorophyll a' formation (Kao et al., 2011).

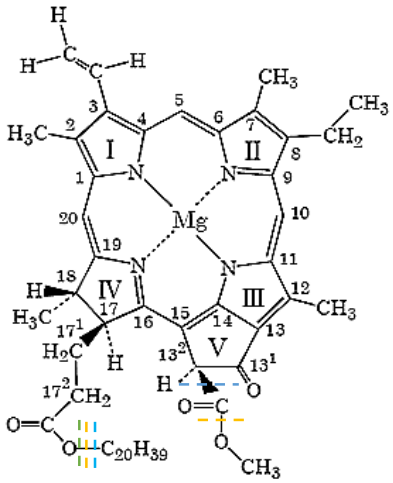
The MS/MS fragmentation patterns of a/a' isomers are basically identical in the APCI-HPLC/MS/MS conditions, with difference only in the intensity of the main fragment $[M + H]^+$, which showed higher intensity in chlorophyll a than in its isomer a' (Figure 4). The same fragmentation behavior can be observed in Figure 3 with the b/b' isomers.

In addition to the compounds described above, it was possible to identify chlorophyll b (peak 2), chlorophyll a (peak 5), its derivative pheophytin a (peak 9), as well as hydroxyl-containing derivatives were identified as hidroxychlorophyll a' (peak 1), hidroxypheophytin a (peak 7), hidroxypheophytin a' (peak 8) and the isomer pheophytin a' (peak 9).





Chlorophyll a



Chlorophyll a'

Figure 4. MS and MS/MS spectra of (A) chlorophyll a (peak 5) and (B) chlorophyll a' (peak 6).

The contents of chlorophyll and their derivatives in chlorophyll extract from *Scenedesmus obliquus, Chlorella vulgaris,* and *Aphanothece microscopica Nägeli* are shown in Table 2. The total chlorophylls contents from biomass were 7,319.0 $\mu g.g^{-1}$, 10,734.1 $\mu g.g^{-1}$, 9,121.8 $\mu g.g^{-1}$, as dry weight, respectively.

Eight chlorophylls compounds were identified in *Scenedesmus obliquus* (Figure 1 A). Chlorophyll a (peak 5) was the major compound, contributing 47.0% (3,444.1 $\mu g.g^{-1}$) to the total chlorophyll content, followed pheophytin a' (peak 10), chlorophyll a' (peak 6) and hidroxychlorophyll a', contributing around 21.8 % (1,593.0 $\mu g.g^{-1}$), 13.3 % (977.6 $\mu g.g^{-1}$) and 5.8% (428.8 $\mu g.g^{-1}$), respectively. In addition, chlorophyll b, pheophytin a, hidroxypheophytin a, hidroxypheophytin a' were detected as minor compounds. Some of the chlorophyll compounds identified in this green microalga have shown similarity to those reported by Gilbert-López et al. (2017), however, the work does not report data quantitative on each compound.

For comparasion purposes, studies have also demonstrated the presence of hydroxyled compounds in plants and photosynthetic microalgae such as *Gynostemma pentaphyllum, Rhinacanthus nasutus* (L.), *Taraxacum formosanum* and *Scenedesmus obliquus*, (Huang et al., 2008; Kao et al., 2011; Loh et al., 2012; Gilbert-López et al., 2017). At the same time, authors report that the formation of these compounds is probably caused by a hydroxylation in C10, formed by the enzyme chlorophyll oxidase (Huang et al., 2008).

A total of seven chlorophylls compounds were identified in the *Chlorella vulgaris* extract (Figure 1 B and Table 2). This microalgae presented a notably superior quantitative content (10,734.1 $ug.g^{-1}$) when compared to other microalgae under study, considering values of 1.4, 1.1 fold higher than the concentration of chlorophyll total in *Scenedesmus obliquus* and *Aphanothece microscopica Nägeli*, respectively. Also, the major chlorophylls compounds in

Table 2 Quantitative characterization of chlorophylls compounds in microalgal extracts (μg.g^{-1} dry weight).

Peak	Chlorophyll	*Scenedesmus obliquus*	*Chlorella vulgaris*	*Aphanothece m. Nägeli*
1	Hidroxychlorophyll a’	428.8^{a} ± 27.5	875.9^{b} ± 2.8	1,556.3^{c} ± 37.0
2	Chlorophyll b	382.4^{a} ± 24.5	276.1^{b} ± 0.8	150.9^{c} ± 3.5
3	Chlorophyll b'	nd	nd	127.0 ± 3.0
4	15-hydroxy-lactone chlorophyll a	nd	70.8^{a} ± 0.2	69.8^{a} ± 1.6
5	Chlorophyll a	3,444.1^{a} ± 221.1	6,124.7^{b} ± 19.6	2,434.0^{c} ± 58.0
6	Chlorophyll a'	977.6^{a} ± 62.7	1,604.6^{b} ± 5.1	696.0^{c} ± 16.5
7	Hidroxypheophytin a	160.4^{a} ± 10.2	nd	269.4^{b} ± 6.42
8	Hidroxypheophytin a'	89.7 ± 5.7	nd	nd
9	Pheophytin a	242.7^{a} ± 15.5	317.2^{b} ± 1.0	547.1^{c} ± 13.0
10	Pheophytin a'	1,593.0^{a} ± 102.2	1,464.6^{a} ± 4.6	3,270.9^{b} ± 77.9
	Total chlorophyll	7,319.0^{a} ± 469.9	10,734.1^{b} ± 34.3	9,121.8^{c} ± 217.4

nd: not detected.
Values are average and standard deviation of triplicates.
Different letters in the same line differ significantly by Tukey test (α = 0.05).

Chlorella vulgaris were chlorophyll a (57.0 %), chlorophyll a' (14.9 %), pheophytin a' (13.6 %) and hidroxychlorophyll a' (8.1 %). In addition, pheophytin a, chlorophyll b and 15-hydroxy-lactone chlorophyll a were identified as minor compounds, representing 6.4 % of the total content of chlorophylls. In contrast, the study reported by Kong, Song, Cao, Yang, Hua & Xia (2011) showed higher chlorophyll values (2,7990 $ug.g^{-1}$) for this microalgae. However, the work not a present characterization of the chlorophyll profile.

In this sense, the chromatogram of *Chlorella* showed practically the same profile qualitative as that of *Scenedesmus* extract (six compounds in common), with the exception of the compounds hydroxychlorophyll a and hydroxychlorophyll a', present only in the extract of *Senedesmus* and 15-hydroxy-lactone chlorophyll a identified only in *Chlorella* extract. Although these two species of microalgae to belong the group of green microalgae, present significant difference in their quantitative profile, with the exception the compound pheophytin a' to which did not present significant difference. Although chlorophyll b (peak 2) was identified in *Scenedesmus obliquus* and *Chlorella vulgaris*, its chlorophyll b' (peak 3) isomer was not detected in any of the extracts.

The composition of chlorophylls compounds in *Aphanothece microscopica Nägeli* can be seen in Figure 1 C and Table 2. The pheophytin a' (1,593.0 $\mu g.g^{-1}$) (35.8 %) was quantitatively dominant in chlorophyll profile of microalgae, followed by chlorophyll a (2,434.0 $\mu g.g^{-1}$) (26.6 %), hidroxychlorophyll a' (875.9 $\mu g.g^{-1}$) (17.0 %) and chlorophyll a' (696.0 $\mu g.g^{-1}$) (7.6 %). The five minor compounds (peak 2, 3, 4, 7, and 9) represented 13% of the total content. Furthermore, the 15-hydroxy-lactone chlorophyll a compound did not present significant difference when compared to *Chlorella vulgaris* microalgae. In addition, chlorophyll b' was only identified in this species of microalgae.

The contents of chlorophyll in extract from *Aphanothece microscopica Nägeli* (9,121.8 $\mu g.g^{-1}$) is higher than that found by Fernandes et al. (2016) (987.6 $\mu g.g^{-1}$) and Rodrigues, Menezes, Mercadante, Jacob-Lopes & Zepka (2015) (3,400 $\mu g.g^{-1}$).

Pheophytin a' was relatively abundant when compared to chlorophyll a in *Aphanothece microscopica Nägeli*. This can be easily explained by cell morphology, in which the synthesis and storage of chlorophylls in cyanobacteria occur dispersed in the hyaloplasm, what causes less protection of these pigments the acting of enzymes or chemistry action in the displacement of the magnesium ion, formation thus, oxidized compounds. On the other hand, smaller amounts of pheophytin a' were evidenced in green microalgae, which is probably attributed to chlorophyll being confined in chloroplasts and also protected by a hydrophobic membrane, which provides a greater stability to these compounds.

However, besides products derived from chlorophyll possibly being formed in the cell's own metabolism, formation may occur during the extraction process, due to contact with oxygen, thus forming the hydroxyl and epimers compounds. Similar information about degradation of pigments was discussed in previous studies (Huang et al., 2008 Parniakov et al., 2015a, Parniakov et al., 2015b).

Additionally, the chlorophyll a' isomer had a relatively lower quantitative profile when compared to the other compounds characterized in the three microalgae species. These results thus demonstrate a concordance with the study by Nakamura, Akai, Yoshida, Taki & Watanabe (2003) that reports low concentrations of the compound in photosynthetic microorganisms.

According to Bukata, Jerome, Kondratyev & Pozdnyakov (1995), photosynthetic microorganisms present chlorophyll a and chlorophyll b in a ratio of 3: 1, however in our study we found higher proportions of chlorophyll a, corresponding to 9:1 in *Scenedesmus obliquus*, 22:1 in *Chlorella vulgaris* and 16:1 in *Aphanothece microscopica Nägeli*, thus indicating a higher production of chlorophyll a. This high chlorophyll a/b, ratio can probably be attributed

to low enzymatic activity of oxygenase in microalgae, because this enzyme catalyzes conversion of the methyl group bound to ring II (Figure 1) to aldehyde (Xu, Tang, Wang & Chitnis 2001; Harada et al., 2012; Yen et al., 2013).

After the identification of chlorophyll profile from microalgae was possible to determine the dominant polarity of compounds as lipophilic, since they have a propionic acid esterified with diterpene phytol alcohol in C17. However, hydroxyl compounds have tendency to polar character. These compounds represent 9.2% (*S. obliquus*), 8.8% (*C. vulgaris*) and 20.7% (*A. microscopica Nägeli*).

Although the different microalgae phylum present a difference in the chlorophyll fraction, six compounds (peak 1, 2, 5, 6, 9 and 10) are common among the three microalgae investigated. When compared to the green microalga *Scenedesmus obliquus* with the cyanobacteria *Aphanothece microscopica Nägeli*, seven compounds were similar with addition of hidroxypheophytin a (peak 7). In contrast, the 15-hydroxy-lactone chlorophyll a compound (peak 4) was only identified in *Chlorella vulgaris* and *Aphanothece microscopica Nägeli*, thus showing a similarity between these microalgae.

Accordingly, the green microalgae showed compounds de chlorophyll equivalent to those of the cyanobacteria under study, when considering the qualitative profile. This is probably attributed to the route of synthesis of chlorophylls, in the two groups of microalgae, to occur along the C5 pathway, in which the first dedicated precursor of the pathway, 5-aminolevulinic acid (ALA), is synthesized from a molecule of glutamate. However, it is still a challenge to understand the specific route of these different classes of microalgae. This is, due to the fact, that these compounds are inherently unstable and reactive in the presence of oxygen and light (Beale, 1999; Lohr et al., 2005; Larkin et al., 2016).

According to Zhang et al. (2017), the chlorophylls are formated via a series of enzyme-catalyzed reactions including chelation with magnesium (Mg) by magnesium chelatase, and

attachment of the phytol side chain (from phytyl pyrophosphate by chlorophyll synthetase). At the same time, the literature not reports of these enzymes in the class of microalgae under study.

In relation to microalgal culture, Kong et al. (2011) demonstrated higher concentrations of chlorophylls in phototrophic cultures, due to the fact, that the synthesis/formation of photosynthetic pigments highly influenced by the light source (Mohsenpour, Richards & Willoughby, 2012). Most algae cultured under optimum condition were reported contained about 4 % dry weight of chlorophyll from overall cell weight (Kong et al., 2014). On the other hand, our results presented values of 3.6% (*Scenedesmus obliquus*), 5.3% (*Chlorella vulgaris*) and 4.5% (*Aphanothece microscopica Nägeli*) chlorophyll on a dry weight.

The literature reports scarce chlorophyll data on the complete characterization of these pigments in the microalgae under study (Kong et al., 2011; Plaza et al., 2012; Gilbert-López et al., 2017), which makes it difficult to compare them with data from the literature on microalgae. However, when compared to other sources, the microalgae under study showed a substantial larger quantitative profile (Burns, Fraser & Bramley, 2003; Ferruzzia & Blakeslee, 2007; Huang et al., 2008; Kao et al., 2011; Derrien, Badr, Gosselin, Desjardins & Angers, 2017). Al so, these species of microalgae becomes an alternative to the production of these metabolites by biotechnological processes with potential applications in various industrial sectors.

4. Conclusion

In the current study, the complete characterization of the profile of chlorophylls and their derivatives of *Scenedesmus obliquus*, *Chlorella vulgaris* and *Aphanothece microscopica Nägeli,* apresentation a total of ten compounds. Overall, the results strongly suggest that the species *Scenedesmus* and *Chlorella* are potential sources of chlorophyll a. In contrast, *Aphanothece* showed a substantial ability to synthesize pheophytin a. In summary, the elucidation of the presented results evidences this work as one of the most complete reports of characterization of chlorophylls and their derivatives for these three species of microalgae.

5. Acknowledgements

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CONCLUSÃO GERAL

O método analítico empregado por HPLC-PDA-MS/MS possibilitou a completa identificação e quantificação dos extratos de clorofilas de *Scenedesmus obliquus*, *Chlorella vulgaris* e *Aphanothece microscopica Nägeli*.

Considerando o perfil qualitativo, dez compostos foram identificados entre as três espécies de microalgas. Os resultados demostraram um perfil semelhante, uma vez que seis compostos foram comuns em ambas as espécies.

Em termos quantitativos, os extratos das três espécies de microalgas demostraram diferença significativa no total de clorofilas. Adicionalmente, verificou-se que a concentração de clorofilas foi maior em *Chlorella vulgaris* (1.0734,1 $\mu g.g^{-1}$). No entanto, as demais espécies também apresentaram valores substancias: 9.121,8 $\mu g.g^{-1}$ e 7.319,0 $\mu g.g^{-1}$ para *Aphanothece microscopica Nägeli* e *Scenedesmus obliquus,* respectivamente.

No que nos diz respeito, a literatura carece de dados sobre a caracterização completa das clorofilas e seus derivados nas microalgas estudadas. Neste sentido, os resultados obtidos no presente estudo contribuem significativamente para o conhecimento dessas biomoléculas em fontes microalgais.

Assim, os resultados sugerem que essas espécies de microalgas apresentam grande potencial como fonte alternativas à obtenção de clorofilas naturais por processos biotecnológicos. Contudo, faz-se necessário uma avaliação sistemática da produtividade desses compostos em escala industrial, a fim de consolidar a real aplicabilidade dessas biomoléculas.

CAPÍTULO 3

ANEXO A - CAROTENOIDS IN MICROALGAE

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Chapter

CAROTENOIDS IN MICROALGAE

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ABSTRACT

The carotenoids are isoprenoids, highly unsatured, lipophilic, featuring a variety cores from yellow to red. Appoximately 750 naturally occurring, and many of this tetraterpenes are produced from microalgae. The composition of carotenoids from microalgae can still be complex, with structural characteristics very different from those commonly found in foods, such as a greater number of carbon atoms, of c.d.b., and of hydroxyl groups, which all contribute to their great antioxidant capacity. These compounds have properties that result in biological functions beneficial to human health, such as cellular protection against free radicals, it is know that the antioxidant properties of the carotenoids are are closely related to their chemical structure. Carotenoids are a source of several bioactive compounds with great otential for applications in food, cosmect, and pharmaceutical industries. Thus, the chapter is organized in three major topics. (1) fundamental aspects of structure and biosynthesis of carotenoids from microalgae, (2) bioactive carotenoids from microalgae, (3) examples of novelty to industrial applications..

Keywords: microalgae, biosynthesis, pigments, biotechnology

INTRODUCTION

Microalgae are fast growing organisms which produce a variety of compounds which have various commercial uses them to the synthesis of bioactive compounds like pigments, and vitamins [1].

In addition, microalgae have always been considered the typical of photosynthetic microorganisms in the CO_2 fixing dependent light is the dominant mode of nutrition [2]. Also can be grown heterotrophically, without light and with addition of an exogenous carbon source using the pentose phosphate pathway. Such metabolic route serves as a source for obtaining the carotenoid biosynthetic precursors which can be divided into five main steps. The initial stages

consist of formation of isopentenyl diphosphate (IPP) and the chain elongation to geranygeranyl diphosphate (GGPP) and formation of phytoene [3].

Consuming carotenoid has been widely associated with beneficial health effects, is mainly attributed to its antioxidant properties. The imbalance between the generation of reactive oxygen species (ROS) and antioxidant status can lead to oxidative stress, which is implicated in the pathology of chronic degenerative diseases and aging processes [4].

In view of the commercial importance of these pigments the objective of the chapter this was to elucidate the fundamental aspects of structure, biosynthesis and bioactive of carotenoids from microalgae and industrial applications.

BIOSYNTHESIS OF CAROTENOIDS IN MICROALGAE

Most microalgae groups possess two cellular isoprenoid biosynthesis pathways as higher plants: the mevalonic acid (MVA) pathway for biosynthesis of the cytosolic sterols and the methylerythritol phosphate (MEP) pathway [5, 6, 7, 8, 9] present in the plastids of plants occurring in all studied microalgae species from all classes [8, 9, 10, 11].

In general, microalgae are commonly grown by fixing dissolved, inorganic carbon (CO2) and absorbing solar energy. Therefore, like most land-based plants, they perform photosynthesis and are photo-autotrophs. At the same time, some species of microalgae are also heterotrophic, using organic compounds in the growth medium as carbon and energy sources therefore, they do not need light as an energy source [12, 13].

The figure 1 demonstrate the scheme the biosynthesis direct precursors methylerythritol phosphatethe (MEP) pathway, the glyceraldehyde 3-phosphate (GAP) and pyruvate, cultivation photoautotrophic in which CO_2 and light energy are used as the carbon and energy sources, respectively [14], form Calvin cycle CO_2 is rapidly transformed to 3-phosphoglyceric acid (3-PGA) and reduced to glyceraldehyde-3-phosphate (GAP) [5, 15]. Pyruvate directly from photosynthetically fixed carbon via 3-phosphoglyceric acid (3-PGA) and phosphoenol pyruvate (PEP) [5, 16, 17].

Although pigments are traditionally thought to be the outcome of metabolisms associated with exposure to light, the capacity of some microalgae to produce some of them in the dark under specific growth conditions opens a line of research that is barely explored [18]. In cultivation heterotrophic the assimilation of one or more organic substrates as the carbon is through the Pentose Phosphate pathway has been found in microalgae [12, 19]. The glucose preferred substrate because it is utilized by a broader range of organisms as a sole carbon source and easily gives rise to the precursors for both isoprenoid biosynthetic routes [7, 20]. These glucose catabolic routes yield glyceraldehyde 3-phosphate (GAP) and pyruvate; which are the two precursors in the MEP pathway.

Synthesized by either the two pathways the geranygeranyl diphosphate (GGPP), the isopentenyl diphosphate (IPP) is isomerized to its allylic isomer, dimethylallyl diphosphate (DMAPP). Geranyl pyrophosphate (GPP) is then produced by the condensation reaction of IPP and DMAPP elongation of GPP by the addition of IPP results in the formation of farnesyl pyrophosphate (FPP) to which is further added another IPP to produce GGPP [14, 21]. In a head-to-head condensation of the two GGPP C20 compounds, the first carotene phytoene (C40).

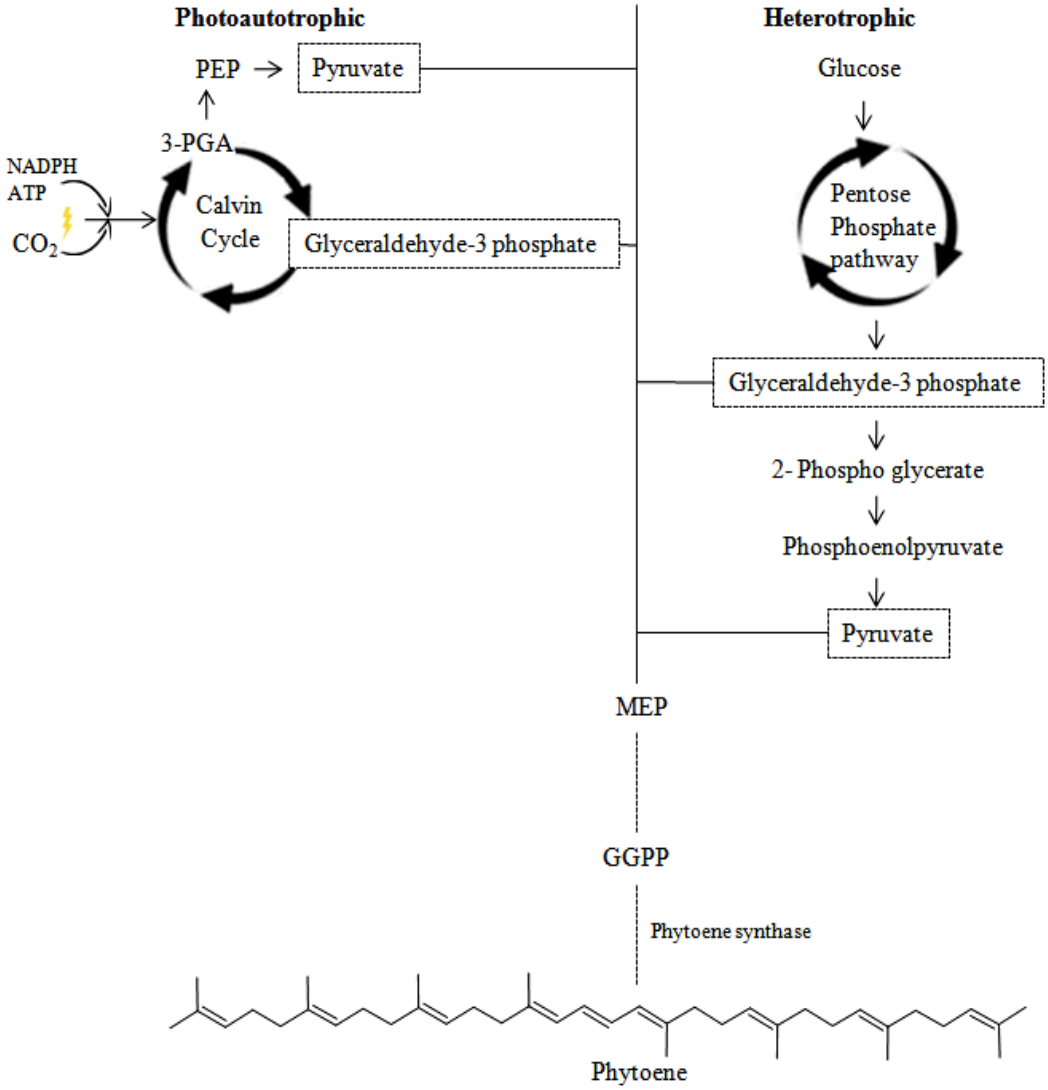

Figure 1. The biosynthesis direct precursors methylerythritol phosphate (MEP) pathway, the glyceraldehyde 3-phosphate (GAP) and pyruvate, in cultivation photoautotrophic the heterotrophic. 3-PGA= 3-phosphoglyceric acid; respectively; PEP= phosphoenolpy**;** GGPP= geranygeranyl diphosphate.

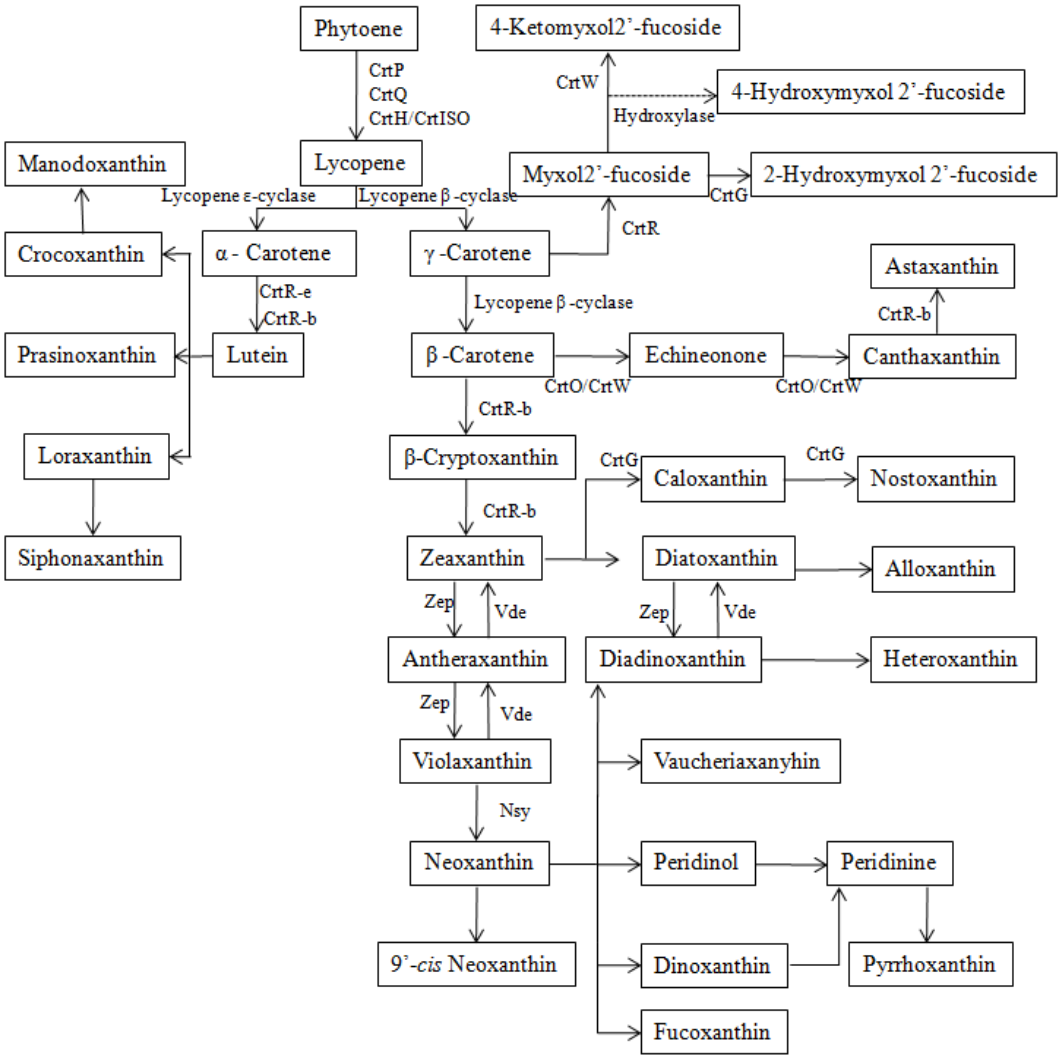

Figure 2. Diagram of pathway of carotenoids biosynthesis.

Microalgae require three enzymes in the conversion from phytoene to lycopene: phytoene desaturase (CrtP), δ-carotene desaturase (CrtQ) and cis-carotene isomerase (CrtH), all carotenoids microalgae are derived from lycopene: β-carotene, α-carotene and their derivatives [22]. Exceptionally in cyanobacteria can synthesize some unique types of carotenoids, myxol glycosides and oscillol diglycosides are monocyclic and acyclic carotenoids respectively (figure 2) [22, 23].

BIOACTIVE CAROTENOIDS AND THEIR PROPERTIES PHYSIOLOGICAL

Among the bioproducts of microalgae with possible bioactive properties, there is great interest in naturally occurring pigments, which is highlighted carotenoids due to its unique properties and its potential beneficial effects on human health, such as provitamin A, antioxidants, anti-inflammatory, anti-tumor and anti-cancer, thus showing potential exploitation as bioactive compounds [23, 24, 25, 26, 27], these classified as phytochemicals capable of modulating metabolic processes essential to the health of the cells, due to their protective action on the cellular components against oxidative damage [28, 29].

The carotenoids consist of a symmetrical structure with a set of conjugated double bonds (c.d.b), called the chromophore absorbing light, this part of the structure responsible for the color and the properties and special features [3], such as a high antioxidant capacity [30]. Therefore, most bioactive capacity carotenoid microalgae is directly related bathochromic effect that occurs in these structures. In order that the antioxidant activity of the compounds increases directly with the increase of chromophore. However, the chain elongation also renders the molecule susceptible to geometric isomerization and oxidative degradation [3, 31].

Consequently, microalgae are able to synthesize a wide range of structural variety, where some of these compounds have diverse structures of other carotenoids found in nature. Among the carotenoids from the microalgae important role in the bioactivity level is the β-carotene, a predominant carotenoid in the group of carotenes to be composed only of carbon and hydrogen atoms. In parallel, microalgae are also able to synthesize xanthophylls, carotenoids consist of at least one oxygen atom. The major carotenoids in microalgae have reported are β-carotene, their hydroxy derivatives, zeaxanthin, its keto derivative, echinenone, lutein, canthaxanthin, antheraxanthin, violaxanthin, astaxanthin and mixo xantophil [23, 32, 33, 34, 35]. Carotenoids these, which stands out in terms of power of synthesis and have a higher bioactive capacity.

Numerous algae have shown the ability to synthesis of carotenoids, some are listed in Table 1. In recent work described by Rodrigues [23], was possible to identify the microalgae extract Phormidium showed the presence of characteristic ketocarotenoids and glycosylated carotenoids in cyanobacteria such as all-trans canthaxanthin (0.93 ug.g-1), all-trans-myxoxanthophyll (3.15 ug.g-1), all-trans-echinenone (19.87 ug.g-1) and cis-echinenone (15.70 ug.g-1) and due to their singular antioxidant properties and their potential beneficial effects on human health these carotenoids show potential of exploration like bioactive compounds. In work described by Goiris [36], emphasized that the carotenoid content from microalgae is that the upper end is obtained by conventional sources, while xanthophylls are found in higher proportion. Among these carotenoids from microalgae are the main β-carotene, lutein, violaxanthin, zeaxanthin, astaxanthin, and neoxantina to be carotenoid that stand out in terms of power of synthesizing these microorganisms and present a greater bioactive capacity [22, 23, 36, 37, 38, 39, 40]. All of these compounds in fact, fall into the class of xanthophylls by having oxygen in its structure, except the β-carotene which is absent in this compound.

It is suggested a higher antioxidant activity for microalgal carotenoids when compared to conventional sources because of the presence of unique carotenoids, which exhibit bathochromic effect, as is the case with the equinenona and cataxantina chromophore of 12 and 13 respectively (c.d.b) [41, 42], it is known that the extension of the chromophore is closely linked to increased antioxidant activity.

Table 1 - Carotenoids in microalgae

Compounds	Microalgae	Reference
β-caroteno; echinenone; cantaxanthin	*Phormidium* sp.	Rodrigues et al, 2015 [23]
β-caroteno	*Dunaliella salina*	El-Baz et al., 2002 [67]
β-caroteno, lutein	*Dunaliella salina*	García-González et al., 2005 [44]
Astaxanthin	*Chlorella zofingiensis*	Ip & Chen, 2005 [38]
Astaxanthin	*Chlorella zofingiensis*	Han & Hu, 2013 [39]
	Haematococcus pluvialis	
Astaxanthin	*Chlorella; Haematococcus*	Kim, et al., 2016 [40]
Lutein	*Chlorella sorokiniana*	Chen, et al., 2016 [37]
Zeaxanthin; neoxanthin,	*Chlorella protothecoides; Chlorella vulgaris*	Grudzinski et al., 2016 [68]
violaxanthin; lutein; β-caroteno; antheraxanthin		
Astaxanthin	*Haematococcus pluvialis*	Panis & Carreon, 2016 [69]
Lutein	*Coelastrella sp.; Desmodesmus sp*	Chiu, Soong & Chen, 2016 [70]
Canthaxanthin; echinenone;	*Scenedesmus sp.*	Abrahamsson, Rodriguez-Meizoso & Turner, 2012 [71]
β-caroteno; lutein	*Chlorella salina*	Gayathri et al., 2016 [72]
Neoxanthin; lutein; β-caroteno; astaxanthin,	*Haematococcus pluvialis*	Jaime et al., 2010 [73]
Cantaxanthin; astaxanthin; lutein	*Chlamydocapsa spp*	Leya et al., 2009 [74]
Lutein; zeaxanthin; β-caroteno	*Dunaliella salina*	Hu et al., 2008 [75]

In addition to being potent antioxidants some carotenoids activity pro vitamin A [43]. In all existing carotenoids, only 10% exhibit provitamin A activity. Among the most important, both for its high level of activity than their availability, are α- and β-carotene, and xanthophylls

However, only those with at least one ring of the β type without oxygenated functional groups with a chain polyene having at least 11 carbon atoms are potential precursors of vitamin A. Of these, β-carotene has the highest provitamin A activity since each retinal pigment molecule produces two, which is then reduced to vitamin a (retinol) [45].

In man, the conversion of beta-carotene to vitamin A is catalysed by the cleavage of the carotenoids (mainly at the intestine) by the enzymeβ-carotene 15,15′-dioxygenase .

ANTIOXIDANT ACTIVITIES

Various reactive species oxygens are generated continuously by our metabolism ($1O_2$, OH•, O_2 •- ROO•, e H_2O_2), addition also takes place exogenous sources of free radicals (for example radiation, tobacco smoke and pesticides) which human beings are exposed [46]. They are damaging biologically important molecules like lipids, DNA or proteins and are involved in the pathobiochemistry of degenerative diseases [47].

Among the various defense strategies, carotenoids are most likely involved in the scavenging of two of the reactive oxygen species, singlet molecular oxygen ($1O_2$), and peroxyl radicals [47].

Carotenoids have bioactive properties primarily due to the excellent physical Quenching singlet oxygen ($1O_2$), where the energy absorbed paragraph produce triplet oxygen ($3O_2$) is converted to rotary and vibratory energy by the chromophore system carotenoid, as well as potent scavengers of other reactive oxygen species (ROS) [46, 48, 49, 50]. The quenching rate of carotenoids increases with increasing numbers of double bounds and varies with functional groups and chain structure [3].

Several studies have reviewed antioxidant actions of carotenoids, but the role of antioxidant for carotenoid is still complex, because these compounds show a pro-oxidant effect which occurs in a high concentration of oxygen in the medium or carotenoids, although this information is still controversial and you can not distract from their health benefits [46, 51].

The antioxidant properties of carotenoids are associated with their radical scavenging properties and their exceptional ability of singlet oxygen ($1O_2$) quenching [51]. These compounds are very potent natural antioxidants because of their energy levels and their $1O_2$ quenching process, which proves to be very effective, especially for carotenoid containing 11 or more conjugated double bonds [48].

In general, the overall process oxygen deactivating singlet $1O_2$ for carotenoid is based on converting excess energy to heat through lower carotenoid [CAR] for triplet excited state [3CAR] [48, 51, 49]. This process is represented in the equation below.

$$1O_2 + [CAR] \rightarrow 3O_2 + [3CAR]$$
$$[3CAR] \rightarrow [CAR] + \text{heat}$$

According El-Agamey [51] and Rodrigues-Amaya [3], carotenoids [CAR] may scavenge radicals in an initial step that involves one or more of the following three possibilities (namely, electron transfer, allylic hydrogen abstraction) represented in the following equation:

$$[CAR] + ROO\bullet \rightarrow [CAR]^{\bullet+} + ROO^- \text{ (Electron transfer)}$$
$$[CAR] + ROO\bullet \rightarrow [CAR]\bullet + ROOH \text{ (Hydrogen abstraction)}$$

[CAR] + ROO• → [ROOCAR] • (Addition)

The factors that may influence the antioxidant activity of carotenoids in biological systems are (I) the structure and physical form of the carotenoid molecule, (II) the location or site of action of the carotenoid molecule within the cell, (III) the potential for interaction with other carotenoids or antioxidants (especially vitamins C and E), (IV) the concentration of the carotenoid, and (V) the partial pressure of oxygen [3].

Rodrigues [23], identified in the study with the microalgae Phormidium sp. 20 different carotenoids, and all-trans-β-carotene, 13-cis-zeaxanthin, all-trans-lutein, all-trans-canthaxanthin and all-trans-echinenone represent the carotenoids class (xanthophylls) having a greater number conjugated double bonds at its structure, thus causing, expansion of the chromophore which provides higher carotenoid antioxidant power.

Research is being conducted with astaxanthin from microalgae as a potent antioxidant to human health [39]. In particular, studies have reported that the antioxidant properties of astaxanthin are about 10 times higher than those of β-carotene, lutein, zeaxanthin, canthaxanthin and more than 500 times greater than α-tocopherol [52, 53]. In studies conducted by Yamashita [54], it was possible to identify this pigment in the species of microalgae and you realize that is about 40 times more potent than that of β-carotene in singlet oxygen quenching. Other works also demonstrate the presence of astaxanthin, lutein in canthaxanthin in microalgae [55].

Several studies have shown that carotenoids interact synergistically with several other antioxidants. The best known example is probably between beta-carotene and alpha-tocopherol (vitamin E) in the protection of in vivo lipid peroxidation. Another example, however, is zeaxanthin in combination with ascorbic acid (vitamin C) and vitamin E, which, together protecting human retinal pigment cells against oxidation reactions induced photoreactions [46].

BIOAVAILABILITY AND BIOACCESSIBILITY OF CAROTENOIDS

Before carotenoids can perform their health-promoting functions, they have to reach their sites of action. They must be absorbed from the intestine, transported in the circulation, and delivered to target tissues. These complicated processes are subject to many influencing factors [3].

Bioavailability refers to the portion of the carotenoid which is absorbed in the body, enters in systemic circulation and becomes available for utilization in normal physiological functions or for storage in the human body [3]. May be affected by a number of factors such as: the complexity of the matrix; chemical structure of the substance of interest; structure and amount of other compounds in the diet as well as the mass of the mucosa and intestinal transit time; the rate of gastric emptying; metabolism and the degree of conjugation and bond with carrier proteins in blood and tissues [56].

Therefore, it is known bioactive compounds that only become bioavailable in the body after being released from the food matrix and modified in the gastrointestinal tract [29]. Consequently, the bioavailable amount, which is responsible for the beneficial effects on health, antioxidant and anti-inflammatory for example, always differ from the original contents of the array, since the bioactive compounds undergo changes during the digestion process. However, it has been shown that the bioavailability of carotenoids in fruits and vegetables is significantly lower than that of algae-derived supplements [57].

According Carbonnel Capella [29], different approaches to study the bioaccessibility and bioavailability of bioactive compounds include in vitro methods (simulating gastrointestinal digestion, artificial membranes, Caco-2 cell cultures, isolation/reconstitution membranes, Ussing chambers), ex vivo techniques (gastrointestinal organs in laboratory conditions), in situ tests (intestinal perforation in animals) and in vivo models (in human and animal studies).

Bioavailability is best determined through human studies; bioaccessibility has been widely evaluated in vitro [3]. Bohn [58], describes various techniques to check the bioavailability of carotenoids.

Both in vitro tests and in vivo have advantages and disadvantages. In in vitro tests the main advantages relate to relatively inexpensive and simple techniques and uptake when followed by Caco-2 epithelial cell absorption is similar to standard conditions [59]. The main disadvantage is the extrapolation stop living in, because the conditions of the gastrointestinal tract are not fully reproduced. In in vivo tests the actual condition of digestion is the main advantage, the major challenge is the ethical constraint and lack of certificates procedure, moreover, is the extrapolation from animal studies to humans.

With reference to bioavailability studies microalgal of carotenoids, these are limited because pretreatment extract purification before the intake of these compounds is necessary in order compounds which may have toxic to human health.

INDUSTRIAL APPLICATION

The great interest in microalgae cultivation is the metabolite production capacity with high industrial value extracted from the biomass [1, 60]. Among these compounds includes the pigments, once which microalgae are recognized as an excellent source of carotenoids [25].

Bioactive pigments, such as extract carotenoids can be obtained by different extraction techniques. However, studies have been elucidated in order to develop methodologies involving non-toxic extraction methods related to green chemistry and renewable biological resources that are economically viable for production on an industrial scale.

In a process of extraction, the toxicity of the solvents used, the degradation of the compounds and the selectivity of the method are major points must be considered. When produced biologically, the pigments promote an exponential demand in the industrial market, the extent to which the availability for biotech companies provide benefits such as cost and ease of production will become an important source the competition of bioproduction with chemical synthesis. In addition, the parameters that should be considered include (1) the source of these bioactive compounds, in this case, microalgae; (2) yield obtained in a given process of extraction; (3) productivity; and (4) the selectivity [61].

There are plenty of methods to obtain carotenoid extracts. Among the most used are solvents, acids, edible oils, supercritical fluids, assisted by microwave assisted enzyme and approaches. However, when considered more efficient and compatible methods include the solvents but supercritical fluids such as carbon dioxide (SC-CO2) are the most used by industries in the economic viability regard [62].

The production of carotenoids using microalgae as feedstock has become one of the most successful activities in the biotechnology industry [63]. When industrially applied the carotenoids are widely used as color enhancers in natural foods including egg yolk, chicken meat or fish. On the face of it, the use of natural colorants has been steadily increasing primarily because of changes in consumer preference toward more natural products known to exhibit specific functional properties [64].

Currently, German chemical company - BASF is the undisputed world leader in beta-carotene production from Dunaliella salina, with over a thousand hectares of production ponds in two plants in Australia [24]. Consequently, due to this increased demand for pigments the β-carotene, the global market of carotenoids, is estimated to 2016 the value at USD 1.24 Billion. Furthermore, the prospects indicate in 2021 the value projected for USD $ 1.53 billion [65].

Additionally, the major market for astaxanthin is the production of Haematococcus spp, known by the great accumulation of astaxanthin, the pigment responsible for the pinkish-red applied to the flesh of salmonids and

other sources obtained by aquaculture. Other commercial ways of these pigments are in the form of the capsule, soft gel, tablet, powder, biomass, cream, energy drink, oil and extract. According to Cysewski [66], It is estimated that the value of astaxanthin in the world market can reach US $ 200 million, making the astaxanthin carotenoid another of the high value produced from microalgae commercial.

Some carotenoids derived specifically from microalgae as echinenone have a promising economic appeal, once 1 mg echinenone is currently sold on the market per RS 3,345.00 [42]. In addition, one of the advantages of industrial application of microalgae is that many other antioxidant compounds present in biomass are transported along with these pigments providing a synergistic effect and consequently the positive impact on human health.

Finally, the bioactive compounds synthesized by microalgae are of major importance to the industrial market. However, the use of microalgae as a source of carotenoids bioactives compounds to promote antioxidant activity, remains questionable due to many factors has not been completely clarified, and the instability of these compounds and the study of their bioavailability. In this regard, the extent to which the biosynthesis of these structures from microalgae are elucidated application will be useful in industries. In business view, efforts should be made to consolidate the technological process of producing the same.

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